0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(3):984–991

doi: 10.1210/en.2004-0965

Regulation of Carbohydrate Metabolism by the Farnesoid X Receptor

Keith R. Stayrook, Kelli S. Bramlett, Rajesh S. Savkur, James Ficorilli, Todd Cook, Michael E. Christe, Laura F. Michael, and Thomas P. Burris

Lilly Research Laboratories, Lilly Corporate Center (K.R.S., K.S.B., R.S.S., J.F., T.C., M.E.C., L.F.M., T.P.B.), Indianapolis, Indiana 46285; and Department of Pharmacology and Toxicology, Indiana University School of Medicine (K.S.B., T.P.B.), Indianapolis, Indiana 46202

The farnesoid X receptor (FXR; NR1H4) is a nuclear hormone receptor that functions as the bile acid receptor. In addition to the critical role FXR plays in bile acid metabolism and transport, it regulates a variety of genes important in lipoprotein metabolism. We demonstrate that FXR also plays a role in carbohydrate metabolism via regulation of phosphoenolpyruvate carboxykinase (PEPCK) gene expression. Treatment of either H4IIE or MH1C1 rat hepatoma cell lines as well as primary rat or human hepatocytes with FXR agonists led to stimulation of PEPCK mRNA expression to levels comparable to those obtained with glucocorticoid receptor agonists. We examined the physiological significance of FXR agonist-induced enhancement of PEPCK expression in primary rat hepatocytes. In addition to inducing PEPCK expression in primary hepatocytes, FXR agonists stimulated glucose output to levels comparable to those observed with a glucocorticoid receptor agonist. Consistent with these observations, treatment of C57BL6 mice with GW4064 significantly increased hepatic PEPCK expression. Activation of FXR initiated a cascade involving induction of peroxisome proliferator-activated receptor α and TRB3 expression that is consistent with stimulation of PEPCK gene expression via interference with a pathway that may involve Akt-dependent phosphorylation of Forkhead/winged helix transcription factor (FOXO1). The FXR-peroxisome proliferator-activated receptor α-TRB3 pathway was conserved in rat hepatoma cell lines, mice, as well as primary human hepatocytes. Thus, in addition to its role in the regulation of lipid metabolism, FXR regulates carbohydrate metabolism. (*Endocrinology* 146: 984–991, 2005)

THE FARNESOID X receptor (FXR; NR1H4) is a member of the nuclear hormone receptor superfamily (1) that was recently identified as the physiological receptor for bile acids (2, 3). FXR is expressed in liver, kidneys, and intestine and plays a critical role in negative feedback regulation of bile acid synthesis by indirectly regulating cholesterol-7α-hydroxylase (Cyp7A1) (4–6). Additional roles for FXR in bile acid homeostasis have been suggested upon observation that genes such as the ileal bile acid-binding protein, bile salt export pump, and bile acid transporters are directly regulated by this nuclear receptor (7–9). FXR-null mice display additional deficiencies in lipid metabolism beyond the expected effects of bile acid metabolism, which suggested a much broader role for FXR encompassing regulation of lipoprotein metabolism (9, 10). Cholesterol and triglyceride levels were altered in these mice, and a number of genes regulating lipoprotein metabolism, including phospholipid transfer protein, apolipoprotein E (apoE), apoAI, apoCIII, apoCII, and the low density lipoprotein receptor, have been demonstrated to be regulated by FXR (11–17). In addition, when administered to rats, a synthetic FXR agonist such as GW4064 resulted in a dose-dependent decrease in plasma triglyceride levels (18).

A link between FXR and diabetes was recently suggested based upon decreased expression of this receptor in animal models of diabetes (19). These investigators also demonstrate that FXR expression is regulated by glucose via the pentose phosphate pathway and suggested that alterations of FXR expression in diabetics may contribute to dysregulation of lipid and bile acid metabolism. Other nuclear hormone receptors have long been recognized as key regulators of metabolic processes central to the pathogenesis of diabetes. One pivotal point of regulation is the expression of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme within the hepatic gluconeogenesis pathway catalyzing a critical step in gluconeogenesis, the irreversible conversion of oxaloacetate to phosphoenolpyruvate. PEPCK activity is primarily regulated by modulation of its expression at the transcriptional level by a variety of hormones, including glucagon, insulin, glucocorticoids, and thyroid hormone. The observation that variations in gluconeogenic activity in response to these hormonal influences correlate well with PEPCK expression supports the idea that PEPCK is a crucial point of regulation of this pathway (20–22). Because excessive hepatic glucose production contributes to fasting hyperglycemia in type 2 diabetics (23), PEPCK has been recognized as a potential point of pharmacological intervention.

In this study we show that FXR agonists increase the expression of PEPCK *in vitro* as well as *in vivo*. Consistent

First Published Online November 24, 2004

Abbreviations: apo, Apolipoprotein; bDNA, branched chain DNA; CDCA, chenodeoxycholic acid; CHX, cycloheximide; FOXO1, Forkhead/winged helix transcription factor; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; GSK, glycogen synthase kinase; PEPCK, phosphoenolpyruvate carboxykinase; PLTP, phospholipid transfer protein; PPAR, peroxisome proliferator-activated receptor; TRB3, mammalian homolog of *Drosophila* tribbles.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

with this observation, FXR agonists increase glucose output from primary hepatocytes. These results suggest a role for FXR in the regulation of carbohydrate metabolism and provide an additional link between FXR and diabetes.

Materials and Methods

Cell culture and reagents

H4IIE and MH1C1 (Morris) rat hepatoma cells were obtained from American Type Culture Collection (Manassas, VA) and were maintained in DMEM (Invitrogen Life Technologies, Inc., Gaithersburg, MD) containing 10% (vol/vol) fetal bovine serum (Invitrogen Life Technologies, Inc.) and 10 mM L-glutamine in a 5% CO_2 humidified atmosphere at 37 C. 4,17(20)-(*Cis*)-pregnadien-3,16-dione (*cis*-guggulsterone) was obtained from Steraloids, Inc. (Newport, RI). Prednisolone, dexamethasone, and chenodeoxycholic acid (CDCA) were obtained from Sigma-Aldrich Corp. (St. Louis, MO.). FXR agonists, GW4064 and fexaramine, were synthesized using standard organic chemistry synthetic methods.

mRNA measurement

mRNAs were measured using either branched chain DNA (bDNA; Quantigene) (Genospectra, Fremont, CA) or real time quantitative PCR technology (TaqMan). The branched chain-DNA (bDNA; Quantigene) assay for PEPCK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed according to the manufacturer's protocol. For Quantigene PEPCK mRNA measurement in the H4IIE and MH1C1 cell lines, cells were seeded in 96-well plates at 25,000 cells/well in DMEM/10% fetal bovine serum and allowed to attach overnight. The following day cells were washed once in serum-free DMEM and serum-starved for 24 h before treatment with FXR agonists, prednisolone, or guggulsterone. After a 15-min incubation at 37 C, 50 μl lysate from each well were added to capture plates (provide by Genospectra) containing either rat GAPDH or rat PEPCK-specific oligonucleotides in 50 μl lysis buffer totaling 100 μl. The capture plate was sealed and incubated overnight at 53 C in a Labline plate incubator (Fisher Scientific, Pittsburgh, PA). After overnight incubation, the bDNA and label probes were annealed as directed by the manufacturer. Finally, upon addition of luminescent alkaline phosphatase substrate, dioxitane, luminescence was quantitated using a TopCount (Packard, Downers Grove, IL). Relative PEPCK mRNA quantities were normalized using corresponding GAPDH mRNA values. For TaqMan quantitative PCR measurement of mRNA, samples were processed to isolate total RNA using the ABI PRISM nucleic acid workstation and reagents following the manufacturer's recommendations for DNA-free isolation of total RNA (Applied Biosystems, Foster City, CA). First-strand cDNA was prepared using the high capacity cDNA archive kit (Applied Biosystems) and was diluted 1:100 in water for TaqMan analysis. Quantitative PCR was performed in a 384-well format using a 10-μl total volume, performing each TaqMan reaction in quadruplicate. Specific probe sets for phospholipid transfer protein (PLTP), mammalian homolog of *Drosophila* tribbles (TRB3), PEPCK, peroxisome proliferator-activated receptor α (PPARα), and GAPDH were obtained from Applied Biosystems as assays on demand, and reactions were carried out following the manufacturer's guidelines. In experiments examining mRNA expression in primary rat hepatocytes, the hepatocytes were purchased in suspension from In Vitro Technologies (Baltimore, MD) and plated into tissue culture-treated, 96-well plates at a density of 20,000 cells/well in DMEM. After 6 h in 96-well plates, cells were treated with FXR ligands or prednisolone for 16 h. At the conclusion of treatment, mRNA was assessed by Quantigene methods as described above. In experiments examining mRNA expression from primary human hepatocytes, cryopreserved hepatocytes were obtained from Cambrex (Walkersville, MD) and grown on collagen-coated plates in hepatocyte maintenance medium. After cells were allowed to attach for 4 h, they were treated with FXR and glucocorticoid receptor (GR) ligands, as indicated, for 8 h. RNA was isolated, and PEPCK, PPARα, TRB3, and 18S were assessed as described above using TaqMan PCR.

Glucose production in primary rat hepatocytes

Primary rat hepatocytes were purchased from Cambrex (East Rutherford, NJ) on collagen-coated, 12-well plates. Upon receipt of cells, medium was immediately replaced with 1 ml hepatocyte complete medium SingleQuots containing ascorbic acid, BSA-fatty acid free, hydrocortisone, transferrin, insulin, recombinant human epidermal growth factor, and GA-1000 (Cambrex) and incubated overnight at 37 C. Upon treatment, cells were washed once with hepatocyte basal medium and treated for 24 h with prednisolone, CDCA, or GW4064 in 1 ml hepatocyte basal medium. After 24 h, cells were washed twice with glucose production buffer (820 μM $MgSO_4$, 9 mM $NaCO_3$, 20 μM HEPES, 10% BSA, 2.25 mM $CaCl_2$, 117.6 mM NaCl, 5.4 mM KCl, KH_2PO_4, 10 mM lactic acid, and 10 mM sodium pyruvate), and 400 μl glucose production buffer were added to each well and incubated at 37 C for 3 h. Glucose production was determined using the Trinder Glucose Kit (Sigma-Aldrich Corp.), where 90 μl sample were added to 45 μl Trinder reagent, incubated for 15 min at room temperature, and read at A_{505nm} using a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA).

Immunoblot analysis

After treatment of H4IIE cells for 24 h with dexamethasone (1 μM), prednisolone (1 μM), GW4064 (2.5 μM), CDCA (40 μM), or fexaramine (20 μM), total cellular protein was isolated in buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate, and 0.5% 2-mercaptoethanol. Immunoblot analysis was performed using equivalent amounts (90 μg) of total protein and electrophoresis on a 4–20% gradient SDS-PAGE gel (Invitrogen Life Technologies, Inc., Carlsbad, CA), and cells were transferred to Invitrolon polyvinylidene difluoride membranes (Invitrogen Life Technologies, Inc.). Membranes were incubated for 1 h in blocking solution (5% milk in PBS plus 0.05% Tween 20) at room temperature with shaking. Polyclonal antibodies to PPARα (SC-9000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), GAPDH (ab9485, Abcam, Cambridge, MA) were used for immunoblot analysis. Bands were visualized with SuperSignal chemiluminescent substrate (Pierce Chemical Co., Rockford, IL) on Kodak film (Eastman Kodak Co., Rochester, NY).

Analysis of the effects of GW4064 in mice

C57BL6 mice (7–9 wk of age) were treated with GW4064 (50 mg/kg twice daily, ip) or 5% acacia vehicle (four animals per group). After 7 d of treatment, the mice were killed. The expression of PEPCK, PPARα, TRB3, and PLTP was assessed by TaqMan PCR; plasma and liver trig-

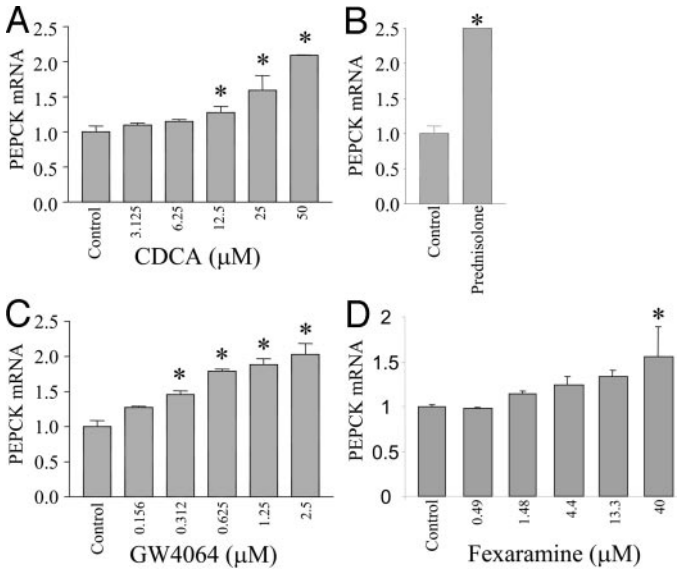

FIG. 1. Stimulation of rat PEPCK mRNA expression by FXR agonists in H4IIE rat hepatoma cells. H4IIE cells were stimulated by CDCA (A), prednisolone (B; 1 μM), GW4064 (C), or fexaramine (D) for 24 h. At the end of the treatment period, PEPCK mRNA quantity was measured using Quantigene (bDNA) technology. All values were normalized to GAPDH expression. *Asterisks* indicate values significantly different ($P < 0.05$) from control values.

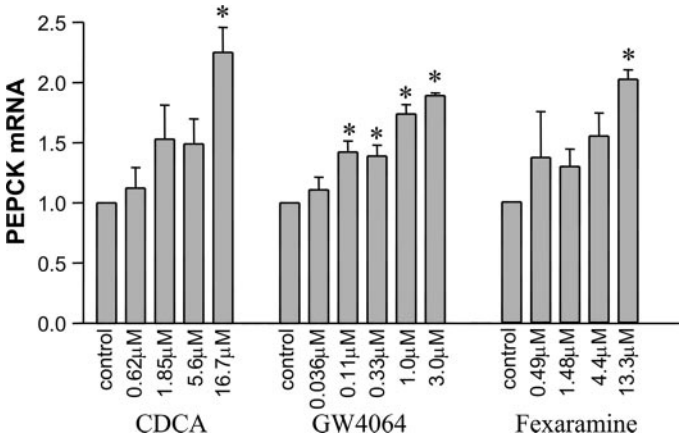

FIG. 2. Stimulation of rat PEPCK mRNA expression by FXR agonists in MH1C1 rat hepatoma cells. MH1C1 cells were stimulated by CDCA (A), GW4064 (B), or fexaramine (C) for 24 h. All values were normalized to GAPDH expression. *Asterisks* indicate values significantly different ($P < 0.05$) from control values.

lycerides as well as total plasma cholesterol and plasma glucose were assessed as previously described (24).

Statistical analysis

Statistical analysis was assessed using the *t* test or ANOVA, followed by Tukey's test. $P < 0.05$ was considered significant. All values are reported as the mean ± SEM.

Results

Using H4IIE hepatoma cells, we assessed the ability of the bile acid, CDCA, to affect the expression of PEPCK. As illustrated in Fig. 1A, we noted a dose-dependent increase in PEPCK expression (normalized to GAPDH) in response to increasing amounts of CDCA. Maximal induction was approximately 2-fold; however, this level of induction was similar to the maximal induction observed when a GR agonist, prednisolone, was tested (Fig. 1B). Next, we used specific nonsteroidal FXR agonists, GW4064 and fexaramine (18, 25), to confirm that the stimulatory effect of CDCA on PEPCK expression is mediated by FXR. As shown in Fig. 1, C and D, both GW4064 and fexaramine stimulated PEPCK expression in a dose-dependent manner and to a similar maximal level as observed for CDCA. We also examined the ability of the three FXR agonists to increase PEPCK in another hepatoma cell line, Morris MH1C1 cells. All three FXR agonists, CDCA, GW4064, and fexaramine, dose-dependently increased PEPCK mRNA expression in a manner very similar to that observed in H4IIE cells (Fig. 2).

Similar results were obtained in independent experiments when quantitative RT-PCR was used to detect PEPCK mRNA from H4IIE cells in response to FXR agonists (data not shown). In addition, we assessed the effects of FXR agonists on the expression of other key enzymes in the gluconeogenic pathway, including glucose-6-phosphatase, fructose 1,6-bisphosphatase, and pyruvate carboxylase. No significant effect on the expression of these mRNAs was observed, indicating that the FXR agonists are specifically targeting PEPCK mRNA expression within the gluconeogenic pathway (data not shown).

Guggulsterone, a plant sterol with antihyperlipidemic activity, has been reported to function as an FXR antagonist (26, 27). We tested the ability of guggulsterone to inhibit the FXR agonist-mediated increase in PEPCK expression in H4IIE cells. As shown in Fig. 3A, increasing doses of guggulsterone decreased CDCA-induced PEPCK expression. We observed very similar FXR antagonist effects of guggulsterone on GW4064-induced (Fig. 3B) and fexaramine-induced (Fig. 3C) PEPCK expression. No toxicity was observed within the range of guggulsterone examined (up to 20 μM; data not shown).

The observation that three structurally distinct FXR agonists stimulate PEPCK expression in two different hepatoma cell lines along with the ability to antagonize these effects with a FXR antagonist suggested that FXR may play a role in gluconeogenesis. To examine this hypothesis, we assessed the ability of GW4064, the most potent and selective FXR agonist along with the natural FXR ligand, CDCA, to increase PEPCK expression in primary rat hepatocytes. Figure 4A illustrates the ability of CDCA and GW4064 to increase PEPCK expression in primary hepatocytes in a dose-dependent manner. Glucose production was also monitored in primary hepatocytes in response to CDCA and GW4064. First, we compared the effects of doses of CDCA and GW4064 expected to activate FXR and PEPCK expression to the effect of a maximally efficacious dose of the glucocorticoid prednisolone. As shown in Fig. 4B, CDCA and GW4064 stimulated glucose production by hepatocytes to levels comparable to that induced by prednisolone.

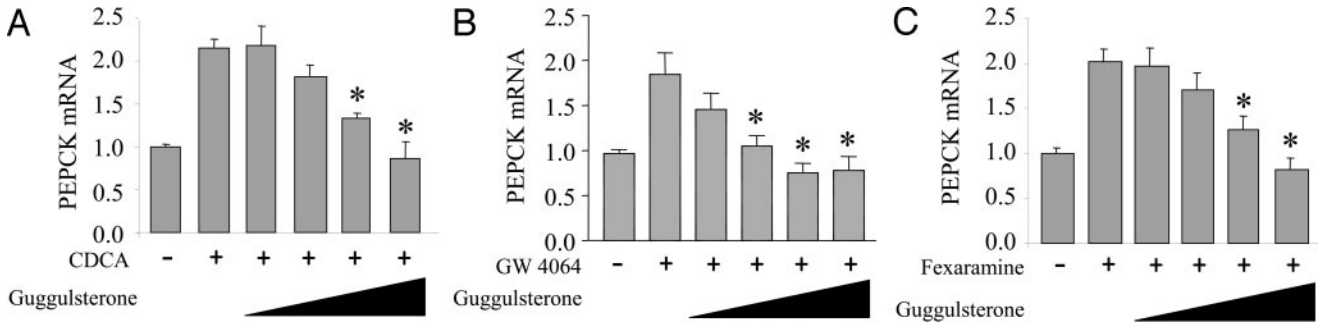

FIG. 3. Dose-dependent antagonism of FXR agonist-induced rat PEPCK expression by guggulsterone in H4IIE rat hepatoma cells. H4IIE cells were treated with one of three FXR agonists, CDCA (50 μM; A), GW4064 (2 μM; B), or fexaramine (40 μM; C) along with increasing concentrations of the FXR antagonist, guggulsterone (1.3, 3.2, 8, and 20 μM), for 24 h, followed by analysis of PEPCK expression. All values were normalized to GAPDH expression. *Asterisks* indicate values significantly different ($P < 0.05$) from FXR agonist only (no guggulsterone) values.

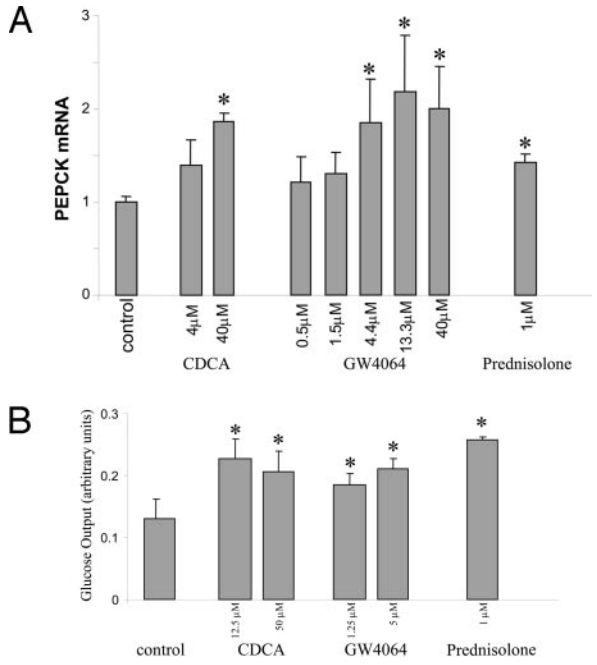

FIG. 4. FXR agonists, CDCA and GW4064, stimulate PEPCK mRNA expression and glucose output from primary rat hepatocytes. A, Primary rat hepatocytes cultured in suspension were treated for 24 h with various concentrations of CDCA or GW4064 or a single dose of prednisolone (1 μM), followed by assessment of PEPCK and GAPDH expression. B, Primary rat hepatocytes were treated with CDCA (12.5 or 50 μM), GW4064 (1.5 or 5 μM), or prednisolone (1 μM), followed by assessment of glucose output as described in *Materials and Methods*. *Asterisks* indicate values significantly different ($P < 0.05$) from control values.

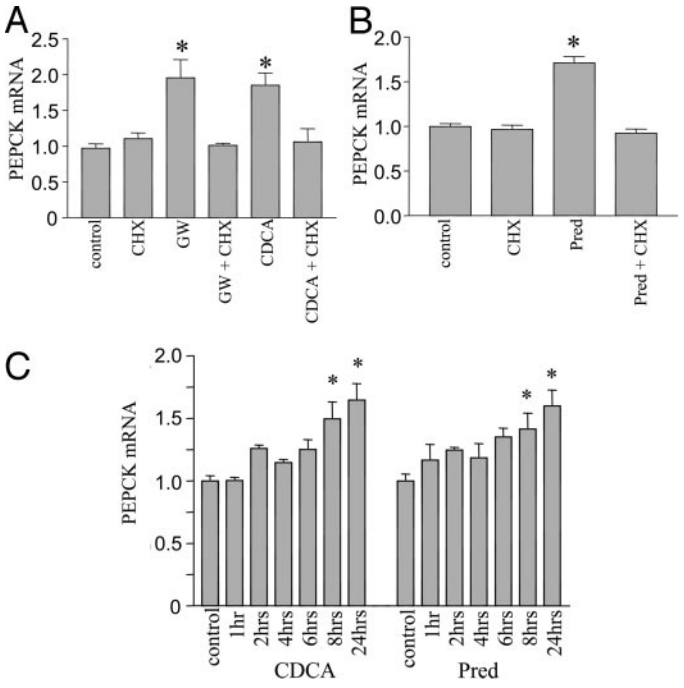

FIG. 5. Stimulation of PEPCK mRNA expression by FXR requires *de novo* protein synthesis. A, H4IIE cells were treated with CHX (10 μg/ml) along with either GW4064 (2.5 μM) or CDCA (40 μM) for 15 h, followed by assessment of PEPCK mRNA expression (bDNA method). PEPCK mRNA values are normalized relative to GAPDH expression. GAPDH mRNA levels were unaffected at 15 h of treatment. B, H4IIE cells were treated with CHX, as described above, alone or with prednisolone (Pred; 1 μM). PEPCK expression was assessed as described above. C, Time course for stimulation of PEPCK mRNA expression by either a GR agonist, prednisolone (Pred; 1 μM), or an FXR agonist, CDCA (40 μM). PEPCK expression was assessed as described above. *Asterisks* indicate values significantly different ($P < 0.05$) from control values.

To determine whether activation of FXR leads to increased PEPCK expression *in vivo*, we treated C57BL6 mice with the FXR agonist, GW4064, which is the highest affinity agonist with acceptable pharmacokinetic properties. After 7 d of treatment, the expected decreases in plasma triglyceride levels as well as total plasma cholesterol were observed (Table 1). In addition, we analyzed the level of expression of a well characterized FXR target gene, PLTP, from liver tissue and noted a 4.3-fold increase in expression in animals treated with GW4064. Consistent with the effects of activation of FXR in cell lines and hepatocytes, treatment of the mice with GW4064 induced a significant 3.6-fold increase in PEPCK expression. The increase in hepatic PEPCK expression was not accompanied by an increase in plasma glucose, which may be due to a compensatory increase in glucose utilization.

The mechanism of induction of PEPCK mRNA expression was first examined by assessing the potential requirement for *de novo* protein synthesis after treatment with FXR ligands in H4IIE cells. As shown in Fig. 5A, cycloheximide (CHX; 10 μg/ml) treatment completely prevented the induction of PEPCK mRNA expression by either FXR agonist, GW4064 or CDCA. CHX treatment alone did not affect basal PEPCK mRNA expression (Fig. 5, A and B), and GAPDH mRNA expression was also unaffected (data not shown). This suggests that activation of FXR results in the expression of an intermediate factor(s) that regulates PEPCK mRNA expression. This observation revealed additional similarities to GR-induced PEPCK mRNA expression. Several studies have shown that GR-mediated induction of PEPCK expression is also sensitive to CHX treatment (28, 29), an observation that we confirmed in H4IIE cells as illustrated in Fig. 5B. In addition, we noted that induction of PEPCK mRNA by FXR and GR agonists followed a similar time course (Fig. 5C). We next examined whether there were additional similarities between the GR and FXR signaling pathways that regulate PEPCK mRNA expression. The diabetogenic effects of the

TABLE 1. Effects of FXR agonist, GW4064, on C57BL6 mice

Treatment	Plasma TG (mg/dl)	Total plasma cholesterol (mg/dl)	Plasma glucose (mg/dl)	PLTP (fold increase)	PEPCK (fold increase)
Vehicle	107 ± 7.5	65.3 ± 6.2	174 ± 9.8	1	1
GW4064	54.5 ± 8.6[a]	36.3 ± 5.4[a]	150 ± 8.5	4.3[a]	3.6[a]

TG, Triglycerides.
[a] $P < 0.05$.

GR ligand, dexamethasone, have been recently shown to be dependent on GR induction of PPARα (30). This group also demonstrated that GR-mediated regulation of PEPCK expression is dependent on the induction of PPARα expression (30), which is consistent with the observation of CHX sensitivity (28, 29). Based on its characterization as an FXR-responsive target gene in human hepatocytes (31), we examined the role that PPARα may play as an intermediate in the FXR pathway. As illustrated in Fig. 6A, a Western blot indicated that GR ligands such as prednisolone and dexamethasone increase PPARα expression in H4IIE cells (3.5- and 3.3-fold, respectively), which is consistent with previous observations (30, 32). Interestingly, FXR ligands, CDCA, fexaramine, and GW4064, also induce PPARα expression (2.2-, 2.2-, and 2.0-fold, respectively; Fig. 6A), indicating that GR and FXR may share a pathway for the induction of PEPCK mRNA expression. GR and FXR appear to act independently of one another, because GR agonists did not increase FXR mRNA expression, and a GR antagonist, RU486, did not inhibit an FXR agonist-induced increase in PPARα expression (data not shown).

TRB3, a fasting-inducible inhibitor of the serine threonine kinase Akt/PKB (33), was recently identified as a PPARα-responsive gene responsible for PPARγ coactivator-1α-promoted insulin resistance (34). Induction of TRB3 promotes glucose output from the liver by binding and inhibiting Akt phosphorylation (33, 34). Increased TRB3 levels result in decreased insulin-dependent phosphorylation of GSK3 by Akt, resulting in increased glycogenolysis (34). In addition to the role of Akt in the regulation of GSK3 activity and glycogenolysis, Akt targets Forkhead/winged helix transcription factor (FOXO1), a critical transcription factor in the regulation of PEPCK expression (35–39). Once phosphorylated by Akt, FOXO1 is translocated out of the nucleus, leading to a decrease in PEPCK expression. Increased TRB3 levels should result in a reduction in Akt-dependent phosphorylation and translocation of FOXO1, leading to increased PEPCK expression. Thus, we predicted that activation of either GR or FXR should lead to increased PPARα and TRB3 expression, and this should be correlated with increased PEPCK mRNA expression. To examine this, we first treated H4IIE cells with either dexamethasone or GW4064 and examined the expression of all three genes. As shown in Fig. 6B, PPARα mRNA was significantly induced (~1.8-fold) by maximally efficacious doses of either agonist, consistent with our Western blot data (Fig. 6A). TRB3 mRNA was also significantly induced in response to either agonist (~1.9-fold; Fig. 6C). Furthermore, as expected, both the GR and FXR agonists significantly induced PEPCK mRNA expression (Fig. 6D).

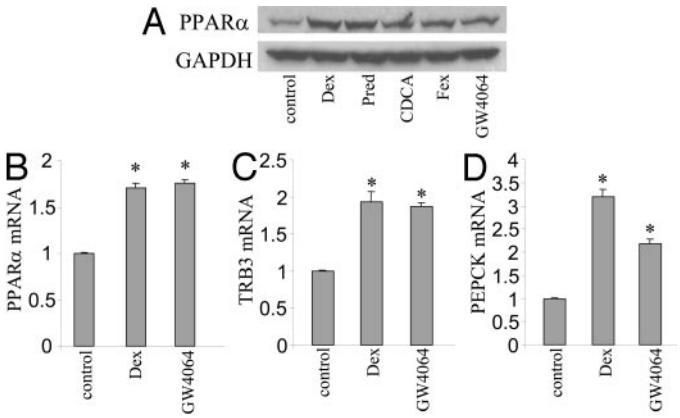

FIG. 6. Activation of GR and FXR leads to increased expression of PPARα, TRB3, and PEPCK. A, H4IIE cells were treated for 24 h with GR agonists, dexamethasone (Dex; 1 μM) and prednisolone (Pred; 1 μM), or FXR agonists, CDCA (40 μM), fexaramine (Fex; 20 μM), and GW4064 (2.5 μM), followed by Western blot for PPARα. B, H4IIE cells were treated for 24 h with the GR agonist, dexamethasone (Dex; 1 μM), or the FXR agonist, GW4064 (2.5 μM), followed by assessment of PPARα mRNA expression by quantitative RT-PCR. C, H4IIE cells were treated for 24 h with the GR agonist, dexamethasone (Dex; 1 μM), or the FXR agonist, GW4064 (2.5 μM), followed by assessment of TRB3 mRNA expression by quantitative RT-PCR. D, In experiments identical to those in B and C, PEPCK mRNA expression was monitored by quantitative RT-PCR to correlate to the increases in PPARα and TRB3 mRNA expression detected. These experiments were performed on three separate occasions, and the data were combined for statistical analysis and presentation in B–D. PPARα, TRB3, and PEPCK expression values were normalized to GAPDH expression, as determined by quantitative RT-PCR. *Asterisks* indicate values significantly different ($P < 0.05$) from control values.

Although we initially characterized this pathway in the rat H4IIE cell line, we investigated the conservation of this pathway using additional systems in two distinct species. First, we examined the expression of both PPARα and TRB3 mRNA in the livers of C57BL6 mice that were treated with GW4064 as described above. Both PPARα and TRB3 mRNA levels were significantly induced (4.1- and 5.9-fold, respectively; data not shown) along with PEPCK mRNA (3.6-fold; Table 1) in addition to the well characterized FXR target gene, PLTP (4.3-fold; Table 1). Finally, we used primary cultures of human hepatocytes, allowing us to examine this pathway in a nonrodent species. As shown in Table 2, treatment of primary human hepatocytes with a range of doses of the selective FXR agonist, GW4064, as well as the GR agonist, dexamethasone, resulted in significant induction of PEPCK mRNA expression. Consistent with observations of rat H4IIE cells and the mouse *in vivo* study, both PPARα and TRB3 mRNA levels were also significantly induced in response to treatment with GW4064 (Table 2). These data suggest that this pathway is conserved among rats, mice, and humans.

Discussion

Hepatic gluconeogenesis is significantly increased in type 2 diabetics, contributing to the observed fasting hyperglycemia. Pharmacological inhibition of this pathway has been proposed as a method to restore insulin sensitivity, which has been validated by the antidiabetic drug, metformin, that

TABLE 2. Effects of GW4064 and dexamethasone on PEPCK, PPARα, and TRB3 mRNA expression in primary human hepatocytes

Treatment	PEPCK mRNA (fold increase)	PPARα mRNA (fold increase)	TRB3 mRNA (fold increase)
Control	1 ± 0.1	1 ± 0.1	1 ± 0.1
GW4064 (100 nM)	6.6 ± 0.7[a]	7.8 ± 0.3[a]	3.4 ± 0.4[a]
GW4064 (1 μM)	15.3 ± 1[a]	10.8 ± 0.8[a]	7.2 ± 0.2[a]
Dexamethasone (1 μM)	7.3 ± 0.3[a]	7.3 ± 0.3[a]	4.6 ± 0.2[a]

All mRNA measurements were normalized to 18S rRNA levels.
[a] $P < 0.05$.

reduces hepatic glucose production via inhibition of gluconeogenesis (40). The enzyme PEPCK, which catalyzes one of the key rate-limiting steps in gluconeogenesis, has been extensively studied. PEPCK is regulated primarily at the transcriptional level in response to a variety of hormones, including insulin, glucocorticoids, glucagon, and catecholamines. In this study we demonstrate that PEPCK is also regulated by FXR, which is a nuclear hormone receptor that functions as the physiological receptor for bile acids. A natural bile acid ligand, CDCA, as well as structurally distinct, nonsteroidal, FXR-specific agonists stimulated PEPCK mRNA expression in H4IIE and MH1C1 hepatoma cells as well as in primary hepatocytes. The level of induction by the FXR agonists was comparable to maximal stimulation of expression noted upon activation with the GR agonist, prednisolone. Consistent with this observation, FXR agonists increased glucose production in primary rat hepatocytes to levels comparable to those produced by prednisolone. Activation of FXR in mice with a synthetic agonist, GW4064, also led to increased expression of hepatic PEPCK; however, we did not detect any alteration in plasma glucose levels.

Various effects of bile acids on PEPCK gene expression have been recently reported. De Fabiani *et al.* (41) recently described an FXR-independent mechanism of bile acid repression of PEPCK via induced dissociation of coactivators from hepatocyte nuclear factor-4α. Yamagata *et al.* (42) indicated that PEPCK expression is repressed in an FXR-dependent manner via induction of short heterodimer partner-mediated inhibition of hepatocyte nuclear factor-4α and FOXO1. Both of these investigators show CDCA-mediated repression of PEPCK or a PEPCK promoter-driven reporter in HepG2 cells in addition to repression of the gene in cholic acid-fed mice. In contrast to these findings, we observed that three structurally distinct FXR agonists stimulated PEPCK expression in H4IIE and M1HC1 hepatoma cells. In addition, FXR agonists increased PEPCK expression as well as glucose output from primary hepatocytes. The reason for the discrepancies in the observations is unclear; however, based on this study as well as those by De Fabiani *et al.* (41) and Yamagata *et al.* (42), it appears that there may be both FXR-dependent and -independent effects of bile acids on the regulation of PEPCK expression. It is possible that cholic acid feeding may produce promiscuous metabolites that activate other nuclear receptors, such as liver X receptor, that also regulate PEPCK (24, 43), but there is still a difference between the observation in hepatoma cell lines when treated with CDCA. It is possible that the differences may be due to use of distinct cell lines [De Fabiani *et al.* (41) and Yamagata *et al.* (42) both used HepG2 cells, whereas we used H4IIE and MH1C1 cell lines as well as primary rat and human hepatocytes]. We were unable to detect an effect of any FXR agonist on PEPCK mRNA expression in HepG2 cells (data not shown). However, the HepG2 cells also lacked a response to GR agonists (data not shown), indicating that at least some pathways regulating PEPCK gene expression were not functional. Because we show that both GR and FXR appear to use a similar PPARα-dependent mechanism of inducing PEPCK expression, the HepG2 cell model may not be appropriate. All of the cell-based systems we used, both cell lines and primary hepatocytes (rat and human), were responsive to GR agonists as well as FXR agonists. In addition, our results in hepatoma cell lines and *in vivo* agree with the results obtained in primary rat hepatocytes and correlate with glucose production from these cells.

The first suggestion about the mechanism of FXR-mediated regulation of PEPCK expression was indicated by the similarities noted between GR- and FXR-stimulated PEPCK expression. The time course for induction of PEPCK expression by either GR or FXR agonists was nearly superimposable, and both required *de novo* protein synthesis (28, 29). The diabetogenic effects of the GR ligand, dexamethasone, have been recently shown to be dependent on the induction of PPARα, which is consistent with the additional observation that GR-mediated regulation of PEPCK expression is dependent on the induction of PPARα expression (30). In addition to GR (32), FXR has been shown to regulate PPARα expression in hepatocytes (31). We found that both GR and FXR agonists induce PPARα expression in H4IIE and MH1C1 hepatoma cell lines as well as in primary hepatocytes. In addition, we observed induction of PPARα mRNA in liver from mice treated with the FXR agonist, GW4064. The initial characterization of the responsiveness of PPARα expression to FXR indicated that a farnesoid X response element was present in the human PPARα promoter, but was absent in the mouse promoter (31). Our data indicate that even in the absence of an identified farnesoid X response element in the mouse PPARα promoter, the pathway for induction of PPARα expression in response to activation of FXR is conserved in rats, mice, and humans. Additional insight into the mechanism of FXR regulation of PEPCK expression was provided by a recent study indicating that TRB3, a fasting-inducible inhibitor of the serine/threonine kinase Akt/PKB, was a PPARα-responsive gene responsible for PPARγ coactivator-1α-promoted insulin resistance (34). Indeed, we observed induction of TRB3 expression after treatment with GR and FXR agonists in several experimental models. FOXO1, a transcription factor critical for PEPCK expression, is a target of Akt. Once phosphorylated by Akt, FOXO1 is translocated out of the nucleus, leading to decreased PEPCK expression. Thus, the FXR-dependent increase in TRB3 levels we detected is consistent with a reduction in Akt-dependent phosphorylation and translocation of FOXO1 and would be ex-

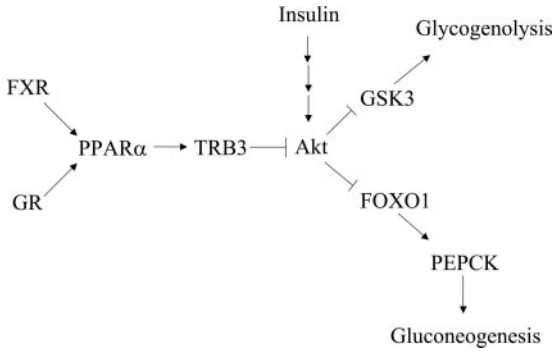

FIG. 7. Proposed model for FXR regulation of carbohydrate metabolism. Activation of FXR results in increased expression of PPARα, which leads to increased TRB3 expression. TRB3 binds to Akt and inhibits its ability to phosphorylate targets such as FOXO1 and GSK3, leading to increased gluconeogenesis and glycogenolysis.

pected to lead to increased PEPCK mRNA expression. Figure 7 illustrates a proposed mechanism for FXR-mediated regulation of PEPCK expression; however, this does not rule out other potential mechanisms that may be operating. The observation that activation of FXR leads to increased TRB3 expression suggests that FXR may also play a role in the regulation of glycogenolysis, because TRB3 has been demonstrated to inhibit insulin-dependent phosphorylation of GSK3 by Akt (33). Thus, activation of FXR may lead to coordinate stimulation of gluconeogenesis and glycogenolysis.

Duran-Sandoval *et al.* (19) recently reported a link between FXR and diabetes. Data indicating that key processes regulated by FXR, such as bile acid and triglyceride metabolism, are impaired in diabetic individuals (44–46) led these investigators to assess the potential role of FXR in animal models of type 1 and 2 diabetes. The expression of FXR was decreased in the livers of streptozotocin-induced diabetic rats as well as in diabetic Zucker rats. Furthermore, FXR expression was shown to be under the regulation of glucose via the pentose phosphate pathway, providing an unexpected link between bile acid and carbohydrate metabolism (19). Our observation that PEPCK expression and glucose production are regulated by FXR provides evidence for an additional link between carbohydrate metabolism and the well characterized lipid metabolism pathways regulated by FXR. Additionally, the finding that FXR may regulate gluconeogenesis coupled with the discovery of glucose-mediated regulation of FXR expression suggest that a feedback loop may be operating. In contrast to two recent reports suggesting that activation of FXR may be useful for inhibiting hepatic gluconeogenesis in diabetics (41, 42), our results indicate that activation of FXR may actually be unfavorable. However, FXR may still have utility as a target for antidiabetics if inhibition of its activity provides for decreased PEPCK expression. This scenario assumes that basal PEPCK expression has a significant FXR-dependent component, which remains to be investigated. Indeed, a very recent study indicates that FXR-null mice display improved glucose tolerance and insulin sensitivity (47), which is consistent with our hypothesis that inhibition of FXR activity may provide therapeutic benefit in terms of glucose homeostasis.

Acknowledgments

Received July 26, 2004. Accepted November 19, 2004.

Address all correspondence and requests for reprints to: Dr. Thomas P. Burris, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Indiana 46285. E-mail: burris@lilly.com.

K.R.S., K.S.B., and R.S.S. contributed equally to this work.

References

1. **Forman BM, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, Noonan DJ, Burka LT, McMorris T, Lamph WW** 1995 Identification of a nuclear receptor that is activated by farnesol metabolites. Cell 81:687–693
2. **Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM** 1999 Bile acids: natural ligands for an orphan nuclear receptor. Science 284:1365–1368
3. **Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B** 1999 Identification of a nuclear receptor for bile acids. Science 284:1362–1365
4. **Bramlett KS, Yao SF, Burris TP** 2000 Correlation of farnesoid X receptor coactivator recruitment and cholesterol 7α-hydroxylase gene repression by bile acids. Mol Genet Metab 71:609–615
5. **Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Willson TM, Kliewer SA** 2000 A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. Mol Cell 6:517–526
6. **Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf DJ** 2000 Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. Mol Cell 6:507–515
7. **Grober J, Zaghini I, Fujii H, Jones SA, Kliewer SA, Willson TM, Ono T, Besnard P** 1999 Identification of a bile acid-responsive element in the human ileal bile acid-binding protein gene: involvement of the farnesoid X receptor/9-*cis*-retinoic acid receptor heterodimer. J Biol Chem 274:29749–29754
8. **Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ** 2001 Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. J Biol Chem 276:28857–28865
9. **Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM, Edwards PA** 2002 Regulation of multidrug resistance-associated protein 2 (ABCC2) by nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. J Biol Chem 277:2908–2915
10. **Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ** 2000 Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell 102:731–744
11. **Cao GQ, Beyer TP, Yang XP, Schmidt RJ, Zhang YY, Bensch WR, Kauffman RF, Gao H, Ryan TP, Liang Y, Eacho PI, Jiang XC** 2002 Phospholipid transfer protein is regulated by liver X receptors in vivo. J Biol Chem 277:39561–39565
12. **Urizar NL, Dowhan DH, Moore DD** 2000 The farnesoid X-activated receptor mediates bile acid activation of phospholipid transfer protein gene expression. J Biol Chem 275:39313–39317
13. **Kast HR, Nguyen CM, Sinal CJ, Jones SA, Laffitte BA, Reue K, Gonzalez FJ, Willson TM, Edwards PA** 2001 Farnesoid X-activated receptor induces apolipoprotein C-II transcription: a molecular mechanism linking plasma triglyceride levels to bile acids. Mol Endocrinol 15:1720–1728
14. **Mak PA, Kast-Woelbern HR, Anisfeld AM, Edwards PA** 2002 Identification of PLTP as an LXR target gene and apoE as an FXR target gene reveals overlapping targets for the two nuclear receptors. J Lipid Res 43:2037–2041
15. **Claudel T, Sturm E, Duez H, Torra IP, Sirvent A, Kosykh V, Fruchart JC, Dallongeville J, Hum DW, Kuipers F, Staels B** 2002 Bile acid-activated nuclear receptor FXR suppresses apolipoprotein A-I transcription via a negative FXR response element. J Clin Invest 109:961–971
16. **Claudel T, Inoue Y, Barbier O, Duran-Sandoval D, Kosykh V, Fruchart J, Fruchart JC, Gonzalez FJ, Staels B** 2003 Farnesoid X receptor agonists suppress hepatic apolipoprotein CIII expression. Gastroenterology 125:544–555
17. **Sirvent A, Claudel T, Martin G, Brozek J, Kosykh V, Darteil R, Hum DW, Fruchart J-C, Staels B** 2004 The farnesoid X receptor induces very low density lipoprotein receptor gene expression. FEBS Lett 566:173–177
18. **Maloney PR, Parks DJ, Haffner CD, Fivush AM, Chandra G, Plunket KD, Creech KL, Moore LB, Wilson JG, Lewis MC, Jones SA, Willson TM** 2000 Identification of a chemical tool for the orphan nuclear receptor FXR. J Med Chem 43:2971–2974
19. **Duran-Sandoval D, Mautino G, Martin G, Percevault F, Barbier O, Fruchart JC, Kuipers F, Staels B** 2004 Glucose regulates the expression of the farnesoid X receptor in liver. Diabetes 53:890–898
20. **Cimbala MA, Lamers WH, Nelson K, Monahan JE, Yoo-Warren H, Hanson RW** 1982 Rapid changes in the concentration of phosphoenolpyruvate carboxykinase mRNA in rat liver and kidney. Effects of insulin and cyclic AMP. J Biol Chem 257:7629–7636
21. **Granner D, Andreone T, Sasaki K, Beale E** Inhibition of transcription of the phosphoenolpyruvate carboxykinase gene by insulin. Nature 305:549–551
22. **Magnuson MA, Quinn PG, Granner DK** 1987 Multihormonal regulation of phosphoenolpyruvate carboxykinase-chloramphenicol acetyltransferase fusion genes. Insulin's effects oppose those of cAMP and dexamethasone. J Biol Chem 262:14917–14920
23. **Magnusson I, Rothman DL, Katz LD, Shulman RG, Shulman GI** 1992 Increased rate of gluconeogenesis in type II diabetes mellitus. A ^{13}C nuclear magnetic resonance study. J Clin Invest 90:1323–1327
24. **Cao GQ, Liang Y, Broderick CL, Oldham BA, Beyer TP, Schmidt RJ, Zhang YY, Stayrook KR, Suen C, Otto KA, Miller AR, Dai JN, Foxworthy P, Gao H, Ryan TP, Jiang XC, Burris TP, Eacho PI, Etgen GJ** 2003 Antidiabetic action of a liver X receptor agonist mediated by inhibition of hepatic gluconeogenesis. J Biol Chem 278:1131–1136
25. **Downes M, Verdecia MA, Roecker AJ, Hughes R, Hogenesch JB, Kast-Woelbern HR, Bowman ME, Ferrer JL, Anisfeld AM, Edwards PA, Rosenfeld JM, Alvarez JG, Noel JP, Nicolaou KC, Evans RM** 2003 A chemical, genetic, and structural analysis of the nuclear bile acid receptor FXR. Mol Cell 11:1079–1092
26. **Wu J, Xia CS, Meier J, Li SZ, Hu X, Lala DS** 2002 The hypolipidemic natural product guggulsterone acts as an antagonist of the bile acid receptor. Mol Endocrinol 16:1590–1597
27. **Urizar NL, Liverman AB, Dodds DT, Silva FV, Ordentlich P, Yan YZ, Gonzalez FJ, Heyman RA, Mangelsdorf DJ, Moore DD** 2002 A natural product that lowers cholesterol as an antagonist ligand for FXR. Science 296:1703–1706
28. **Nebes VL, Morris SM** 1987 Induction of messenger-RNA for phosphoenol-

pyruvate carboxykinase (GTP) by dexamethasone in cultured rat hepatocytes requires on-going protein-synthesis. Biochem J 246:237–240
29. **Iynedjian PB, Jacot MM** 1980 Glucocorticoid-dependent induction of messenger-RNA coding for phosphoenolpyruvate carboxykinase (GTP) in rat-kidney: its inhibition by cycloheximide. Eur J Biochem 111:89–98
30. **Bernal-Mizrachi C, Weng S, Feng C, Finck BN, Knutsen RH, Leone TC, Coleman TY, Mecham RP, Kelly DP, Semenkovich CF** 2003 Dexamethasone induction of hypertension and diabetes is PPAR-α dependent in LDL receptor-null mice. Nat Med 9:1069–1075
31. **Torra IP, Claudel T, Duval C, Kosykh V, Fruchart JC, Staels B** 2003 Bile acids induce the expression of the human peroxisome proliferator-activated receptor α gene via activation of the farnesoid X receptor. Mol Endocrinol 17:259–272
32. **Lemberger T, Staels B, Saladin R, Desvergne B, Auwerx J, Wahli W** 1994 Regulation of the peroxisome proliferator-activated receptor-α gene by glucocorticoids. J Biol Chem 269:24527–24530
33. **Du KY, Herzig S, Kulkarni RN, Montminy M** 2003 TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. Science 300:1574–1577
34. **Koo SH, Satoh H, Herzig S, Lee CH, Hedrick S, Kulkarni R, Evans RM, Olefsky J, Montminy M** 2004 PGC-1 promotes insulin resistance in liver through PPAR-α-dependent induction of TRB-3. Nat Med 10:530–534
35. **Liao JF, Barthel A, Nakatani K, Roth RA** 1998 Activation of protein kinase B/Akt is sufficient to repress the glucocorticoid and cAMP induction of phosphoenolpyruvate carboxykinase gene. J Biol Chem 273:27320–27324
36. **Tang ED, Nunez G, Barr FG, Guan KL** 1999 Negative regulation of the Forkhead transcription factor FKHR by Akt. J Biol Chem 274:16741–16746
37. **Nakae J, Park BC, Accili D** 1999 Insulin stimulates phosphorylation of the Forkhead transcription factor FKHR on serine 253 through wortmannin-sensitive pathway. J Biol Chem 274:15982–15985
38. **Durham SK, Suwanichkul A, Scheimann AO, Yee D, Jackson JG, Barr FB, Powell DR** 1999 FKHR binds the insulin response element in the insulin-like growth factor binding protein-1 promoter. Endocrinology 140:3140–3146
39. **Nakae J, Barr V, Accili D** 2000 Differential regulation of gene expression by insulin and IGF-1 receptors correlates with phosphorylation of a single amino acid residue in the Forkhead transcription factor FKHR. EMBO J 19:989–996
40. **Barthel A, Schmoll D** 2003 Novel concepts in insulin regulation of hepatic gluconeogenesis. Am J Physiol 285:E685–E692
41. **De Fabiani E, Mitro N, Gilardi F, Caruso D, Galli G, Crestani M** 2003 Coordinated control of cholesterol catabolism to bile acids and of gluconeogenesis via a novel mechanism of transcription regulation linked to the fasted-to-fed cycle. J Biol Chem 278:39124–39132
42. **Yamagata K, Daitoku H, Shimamoto Y, Matsuzaki H, Hirota K, Ishida J, Fukamizu A** 2004 Bile acids regulate gluconeogenic gene expression via SHP-mediated repression of HNF-4 and Foxo1. J Biol Chem 279:23158–23165
43. **Laffitte BA, Chao LC, Li J, Walczak R, Hummasti S, Joseph SB, Castrillo A, Wilpitz DC, Mangelsdorf DJ, Collins JL, Saez E, Tontonoz P** 2003 Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. Proc Natl Acad Sci USA 100:5419–5424
44. **Boland LL, Folsom AR, Rosamond WD,** 2002 Hyperinsulinemia, dyslipidemia, and obesity as risk factors for hospitalized gallbladder disease. A prospective study. Ann Epidemiol 12:131–140
45. **Bennion LJ, Grundy SM** 1977 Effects of diabetes mellitus on cholesterol metabolism in man. N Engl J Med 296:1365–1371
46. **Andersen E, Hellstrom P, Hellstrom K** 1987 Cholesterol biosynthesis in nonketotic diabetics before and during insulin therapy. Diabetes Res Clin Pract 3:207–214
47. **Ma KE, Moore DD,** The role of farnesoid X receptor (FXR) in glucose metabolism. Program of the 86th Annual Meeting of The Endocrine Society, New Orleans, LA, 2004 (Abstract P2-64)

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**