The role of chicken ovalbumin upstream promoter transcription factor II in the regulation of hepatic fatty acid oxidation and gluconeogenesis in newborn mice

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1Institut National de la Sante et de la Recherche Medicale, U1016, Institut Cochin, Paris, France; 2Centre National de la Recherche Scientifique, UMR8104, Paris, France; 3Université Paris Descartes, Sorbonne Paris Cité, France; and 4Laboratoires Fabre, Boulogne Cedex, France

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Planchais J, Boutant M, Fauveau V, Qing LD, Sabra-Makke L, Bossard P, Vasseur-Cognet M, Pégorier JP. The role of chicken ovalbumin upstream promoter transcription factor II in the regulation of hepatic fatty acid oxidation and gluconeogenesis in newborn mice. Am J Physiol Endocrinol Metab 308: E868–E878, 2015. First published March 17, 2015; doi:10.1152/ajpendo.00433.2014.—Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) is an orphan nuclear receptor involved in the control of numerous functions in various organs (organogenesis, differentiation, metabolic homeostasis, etc.). The aim of the present work was to characterize the regulation and contribution of COUP-TFII in the control of hepatic fatty acid and glucose metabolisms in newborn mice. Our data show that postnatal increase in COUP-TFII mRNA levels is enhanced by glucagon (via cAMP) and PPARα. To characterize COUP-TFII function in the liver of suckling mice, we used a functional (dominant negative form; COUP-TFII-DN) and a genetic (shRNA) approach. Adenoviral COUP-TFII-DN injection induces a profound hypoglycemia due to the inhibition of gluconeogenesis and fatty acid oxidation secondarily to reduced PEPCK, Gl-6-Pase, CPT I, and mHMG-CoA synthase gene expression. Using the crossover plot technique, we show that gluconeogenesis is inhibited at two different levels: 1) pyruvate carboxylation and 2) trioses phosphate synthesis. This could result from a decreased availability in fatty acid oxidation arising cofactors such as acetyl-CoA and reduced equivalents. Similar results are observed using the shRNA approach. Indeed, when fatty acid oxidation is rescued in response to Wy-14643-induced PPARα target genes (CPT I and mHMG-CoA synthase), blood glucose is normalized in COUP-TFII-DN mice. In conclusion, this work demonstrates that postnatal increase in hepatic COUP-TFII gene expression is involved in the regulation of liver fatty acid oxidation, which in turn sustains an active hepatic gluconeogenesis that is essential to maintain an appropriate blood glucose level required for newborn mice survival.

nuclear receptors; chicken ovalbumin upstream promoter transcription factor ii inactivation; postnatal liver metabolism; gluconeogenesis; fatty acid oxidation

CHICKEN OVALBUMIN UPSTREAM PROMOTER transcription factor II (COUP-TFII) belongs to the ligand-activated nuclear receptor superfamily and is an orphan receptor because endogenous ligand(s) have not been identified yet. It binds DNA by a zinc finger motif on a variety of hormone-responsive elements that contain direct or inverted imperfect AGGTCA repeats with various spacing (6). COUP-TFII has been implicated in the control of organogenesis, cellular differentiation, growth, and metabolic homeostasis (25). In keeping with its role in metabolic homeostasis, it was shown that COUP-TFII controls a network of genes involved in insulin biosynthesis and secretion in pancreatic β-cells (5, 9), skeletal muscle cell glucose and lipid utilization (12, 28), white adipose tissue development and energy metabolism (24, 42), ventromedial hypothalamus nucleus to regulate hypoglycemia-associated autonomic failure (35), and hepatic cholesterol homeostasis (19, 41). In addition to its role in the control of sterol metabolism, it was shown that COUP-TFII acts as a repressor of glucose-induced L-pyruvate kinase (L-PK) gene expression (26) and is an accessory factor required for induction of phosphoenolpyruvate carboxykinase (PEPCK) gene transcription by glucocorticoids (17). These results suggest that COUP-TFII is involved in the control of hepatic glucose metabolism by modulating the expression of key regulatory enzymes of glycolysis (L-PK) and gluconeogenesis (PEPCK). In keeping with this, it was shown that hepatic COUP-TFII mRNA levels are increased during fasting and conversely decreased after refeeding onto a high-carbohydrate diet (30). Both insulin through the Foxo-1 signaling pathway and glucose via carbohydrate response element-binding protein (ChREBP) transcription factor are responsible for COUP-TFII gene repression during refeeding (30). In the present study, we were interested in another nutritional transition situation associated with marked changes in hepatic metabolism, i.e., the neonatal/suckling period. Indeed, the transition from fetal to postnatal life is characterized by a rapid switch from predominant utilization of glucose as metabolic fuel to the use of a high-fat diet provided by the milk (16). Despite there being very few data available on glucose and lipid metabolisms during the early neonatal period in mice, parallels can be made with newborn rats. First, the milk triglyceride composition is quite similar in these two small rodents (38). Second, their body fat content at birth is very low (~2% in mice (21), vs. ~1% in newborn rat (16)), and a recent study has shown that plasma free fatty acid and β-hydroxybutyrate concentrations are very low in fasting newborn mice compared with suckling ones (18). These observations imply that newborn mice survival is fully dependent upon suckling to cover their energy needs not only through the development of an active oxidation of fatty acids arising from milk triglycerides hydrolysis but probably also through an active hepatic glucose production to compensate for its low availability in mice milk (32). Thus the
aim of the present work was to determine the role of postnatal nutritional and hormonal changes in the regulation of COUP-TFII gene expression and to characterize the contribution of this transcription factor in the overall regulation of glucose and fatty acid metabolisms in mouse liver during postnatal adaptation to extrauterine life.

MATERIALS AND METHODS

Animals. Mice (C57bl/6j; Charles River Laboratories) were maintained in a temperature- and moisture-controlled environment (20 ± 2°C, 45% humidity) with a 12-h light cycle. Food (Safe) and fresh water were provided ad libitum. Suckling newborn mice were kept with their natural mother until the end of experiments. PPARα null mice (23) were kindly provided by B. Staels [Institut National de la Sante et la Recherche Medicale (INSERM) U1011, Institut Pasteur, Lille, France]. All animal protocols were undertaken according to the Guidelines for Care and Use of Experimental Animals published by the European Convention for the Protection of Laboratory Animals and approved by the French Ministère de L’enseignement Supérieur et de la Recherche (permit no. A-75-836).

In utero glucagon injections. Pregnant mice (17.5 days postcoitum) were anesthetized with isoflurane (4% for induction and then 1%; Minervé). After laparotomy, fetuses from one uterine horn were injected subcutaneously through the uterus wall with 35 μg of glucagon (Novo Nordisk), with fetuses in the opposite horn receiving saline (0.25 mol/l NaCl). After 2 h, mice were euthanized, fetuses were removed, and their livers were frozen in liquid nitrogen until analysis.

Electromobility shift assay. Mutated COUP-TFII [designated as dominant negative (COUP-TFII-DN)] was designed as described previously (1) by substituting the fourth cysteine in the second zinc finger of COUP-TFII DNA binding domain by a serine (C134->S). This representation (crossover plot technique) allowed us to localize potential regulatory steps in the gluconeogenic pathway according to the fact that the product of a given enzymatic reaction becomes the substrate for the next one.

Primary cultures of hepatocytes. Six- to eight-week-old fed mice were used. Hepatocytes were isolated as described previously (13). After cell attachment (4 h), hepatocytes were cultured for 24 h in the absence or in the presence of 100 μM dibutyryl-cAMP (Sigma). For WY-14643 experiments, hepatocytes were cultured for 24 h in the absence (DMSO alone) or presence of 10 μM WY-14643 diluted in DMSO.

Gene expression. Total RNAs from liver (20 mg) or cells (2×10⁶) were purified, and mRNA was quantified as described previously (36); see Table 1 for primer sequences. Proteins were extracted and quantified by Western blotting, as described previously (36).

Lipid extraction and quantification. For hepatic measurement of triacylglycerol and nonesterified fatty acids (NEFA), total lipids were extracted in chloroform-methanol (2:1). Organic phase was evaporated and methylated with boron trifluoride-diethyl ether-acetic acid (85:15:0.5) as the mobile phase. Once separated, lipid classes (triacylglycerols, diacylglycerols, NEFA, and phospholipids) were colored with iodine vapor and extracted separately in acetone, dried, and directly quantified using NEFA FS and triglyceride FS kits (Diasys), as described previously (4). Blood triglyceride and NEFA were quantified from EDTA plasma using the same kits.

Statistical analysis. Results are means ± SE. Data were analyzed using a nonparametric Mann-Whitney test.

RESULTS

Developmental changes in hepatic COUP-TFII gene expression. The COUP-TFII mRNA concentration is low in fetal liver and during the immediate postnatal period and then increases to reach maximal levels between 3 and 13 days after birth (Fig. 1A). It is noteworthy that the peak of COUP-TFII mRNA (day 8) is threefold higher than in the liver of 24-h-fasted adult mice (Fig. 1B). Thereafter, hepatic COUP-TFII mRNA levels decrease in weaned mice to reach values similar to those found in fed adult mice (Fig. 1B). The postnatal high-fat diet is a source of lipid-derived products that are potent ligands for numerous nuclear receptors, especially PPARα (29). Interestingly, the developmental pattern of PPARα mRNA parallels those of COUP-TFII (Fig. 1A). In most mammalian species, birth is characterized by drastic changes in hormonal (fall in insulinemia, rise in glucagonemia) and nutritional environment (fetal high-carbohydrate vs. newborn high-fat diet) (16). Thus we wondered whether a postnatal rise in COUP-TFII mRNA levels could be due to the fall in plasma glucose and insulin concentrations and/or to the rise in plasma free fatty acids and glucagon levels.

Regulation of COUP-TFII gene expression. Intrauterine injections of glucagon were performed in 17.5-day-old fetal mice (6%), and neutralized. Blood and liver metabolite concentrations were determined in neutralized perchloric filtrates by enzymatic methods, as described previously (15).

Oxaloacetate was calculated according to the formula [oxaloacetate] = [pyruvate] × [malate] × kMDH/[lactate] × kLDH, where kMDH and kLDH represent the equilibrium constants of malate dehydrogenase [2.78 × 10⁻⁵, EC 1.1.1.37 (S)-malate-NAD oxidoreductase] and lactate dehydrogenase [1.1 × 10⁻⁴, EC 1.1.1.27 (S)-lactate-NAD oxidoreductase] respectively.

The gluconeogenic intermediate concentrations in the liver of COUP-TFII-DN or COUP-TFII shRNA-injected newborn mice were expressed as percentage of the concentrations found in GFP control mice. This representation (crossover plot technique) allowed us to localize potential regulatory steps in the gluconeogenic pathway according to the fact that the product of a given enzymatic reaction becomes the substrate for the next one.

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in which plasma glucagon levels were very low (16). Glucagon injection induced a 1.7-fold increase in COUP-TFII mRNA levels (Fig. 2A) and a threefold increase in PEPCk mRNA level, a well-characterized glucagon target gene. In cultured hepatocytes from adult mice, the effect of glucagon was confirmed by using its second-messenger cAMP, which markedly induced COUP-TFII gene expression (Fig. 2B). cAMP was used instead of glucagon itself since it is more efficient in 24-h-cultured hepatocytes than glucagon due to the instability of glucagon and its decreasing concentration in culture media (20, 27). On the other hand, neither palmitate nor oleate were able to induce COUP-TFII gene expression (Fig. 2C), whereas it induced carnitine palmitoyltransferase I (CPT I) gene expression is confirmed in vitro by the effect of WY-14643 (a PPARα agonist), which induces a twofold increase in COUP-TFII mRNA levels in control hepatocytes but not in PPARα-null ones (Fig. 3B).

A recent study has shown that in human hepatoma cells or other cell lines, among the five natural variants of COUP-TFII (34), the one that lacks a DNA-binding domain (COUP-TFII-DN) physically interacts with endogenous COUP-TFII and inhibits its DNA-binding activity (43), acting as a dominant negative form of COUP-TFII. Whether these mutants are present in mouse liver is unknown, but we took advantage of this observation to investigate the role of a DNA-binding-mutated COUP-TFII in the regulation of hepatic fatty acid and glucose metabolisms.

**COUP-TFII-DN characterization and effect on blood and liver metabolites in suckling newborn mice.** As shown in Fig. 4A, whereas wild-type (WT)-COUP-TFII recombinant protein binded to a DR1 probe, mutated recombinant COUP-TFII failed to do so. Moreover, increasing mutated COUP-TFII/WT-COUP-TFII molar ratio prevented WT-COUP-TFII binding, and thus the mutated COUP-TFII protein acted as a dominant negative form (COUP-TFII-DN). Twenty-four hours

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**Table 1. RT-PCR primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI Access No.</th>
<th>Sense Antisense</th>
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<td>Acyl-CoA oxidase</td>
<td>NM 015729</td>
<td>ACCGCCTATGCTTTCACCTTTT CAAGGGCTTCGAGCAGTATCAGG</td>
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<tr>
<td>CD36</td>
<td>NM 00115955</td>
<td>CAAAGCTCGCTGATGATGAGA CTGCTGAGGCTTACCTTGAG</td>
</tr>
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<td>COUP-TFII</td>
<td>NM 008907</td>
<td>AGGGATCTGGTCGGAAGGG AGAAGGGCTTCGCGAGG</td>
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<tr>
<td>Cyclophilin A</td>
<td>NM 008907</td>
<td>ATGGCACATGGTGCAAGGCAG GCCACAGGACACATAGTCAGG</td>
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<tr>
<td>FABP1</td>
<td>NM 017399</td>
<td>AGGCAAGATCTGCTGCCAGAGA TGGCACTCTCCCTCAGG</td>
</tr>
<tr>
<td>G-6-Pase</td>
<td>NM 008061</td>
<td>TTACCAAGCTTCTCTGAGG GACACATGAAAGCGTGTAG</td>
</tr>
<tr>
<td>Hepatic lipase</td>
<td>NM 008280</td>
<td>GCCAGGACTGGCTGCTTCTTC GAAAGGGCGTCGTTGGGGGA</td>
</tr>
<tr>
<td>l-CPT I</td>
<td>NM 013495</td>
<td>TCTGGAGATGACTGACCTTTC GCAAGACAGCATAAGTCCAG</td>
</tr>
<tr>
<td>LPL</td>
<td>NM 008509</td>
<td>TGAAAGGCTGGTTTCTGCAATATATG TGGACACATGGCATGAGG</td>
</tr>
<tr>
<td>mHMG-CoA synthase</td>
<td>NM 008256</td>
<td>GTCTCTGGCAAGTGGAGAACAGA GCTCACTCTCCCTCAGG</td>
</tr>
<tr>
<td>PEPCk</td>
<td>NM 011444</td>
<td>GTGCTGGAGATGCTGTCGGT GCTGGACACATGGCATGAGG</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>NM 008904</td>
<td>TAAACCTAGCCTACCTTGG CTCGACAGGGAGCTTTAAGG</td>
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<td>PPARα</td>
<td>NM 011144</td>
<td>AGGCAAGCTGCTGCTCATTTCGAA AATTGTAGGCAACCCCTTGG</td>
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**COUP-TFII, chicken ovalbumin upstream promoter transcription factor II; FABP1, fatty acid-binding protein 1; G-6-Pase, glucose-6-phosphatase; l-CPT I, l-carnitine palmitoyltransferase I; LPL, lipoprotein lipase; mHMG-CoA synthase, mitochondrial hydroxymethylglutaryl-CoA synthase; PEPCk, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PPARα, peroxisome proliferator-activated receptor-α.**

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**Fig. 1.** Hepatic chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and peroxisome proliferator-activated receptor-α (PPARα) mRNA levels. **A**: COUP-TFII (●) and PPARα (○) mRNA levels in the livers of either fetal or suckling newborn mice. **B**: COUP-TFII and PPARα mRNA levels in the livers of fed (open bars) and 24-h-fasted (black bars) adult mice. Results are expressed as %birth value (0 days) and are means ± SE of 7 animals. *P < 0.05 and **P < 0.01 compared with control.
after Ad-COUP-TFII-DN injection, 5-day-old mice presented a profound hypoglycemia, reduced ketone body concentrations, and a rise in triglyceride concentration (Table 2). The twofold increase in blood lactate concentration in COUP-TFII-DN suggested that hypoglycaemia could be due at least in part to a reduced rate of hepatic glucose production. For this purpose, we estimated hepatic gluconeogenic flux by using the cross-over plot technique. In COUP-TFII-DN suckling mice, the low

Fig. 2. Effects of glucagon, cAMP, and long-chain fatty acids in the regulation of COUP-TFII gene expression. A: 17.5-day-old fetuses from one uterine horn were injected through the uterine wall with 35 μg of glucagon (black bars), with the fetuses from the opposite uterine horn being injected with saline solution (open bars). mRNA levels were determined 4 h after injection. Results are means ± SE of 6 animals. *P < 0.05 compared with saline. B: hepatocytes from 3-mo-old mice were cultured in either the absence (control; open bars) or presence (black bars) of cAMP (10^4 M). mRNA levels were determined 24 h later. Results are means ± SE of 7 different cultures. *P < 0.05 and **P < 0.01 compared with control. C: hepatocytes from 3-mo-old mice were cultured in the absence or presence of either palmitate (0.3 mM) or oleate (0.3 mM) bound to albumin. mRNA levels were determined 24 h later. Results are means ± SE (n = 3). *P < 0.05 compared with control. PEPCK, phosphoenolpyruvate carboxykinase; L-CPT I, l-carnitine palmitoyltransferase I.

Fig. 3. Role of PPARα in the regulation of COUP-TFII gene expression. A: COUP-TFII and acyl-CoA oxidase (ACO) mRNA levels in the liver of 24-h-fasted wild-type (open bars) and PPARα-null (black bars) adult mice. Results are expressed as % wild-type value and are means ± SE of 4 animals. *P < 0.01 compared with wild-type mice. B: hepatocytes from 3-mo-old wild type (open bars) or PPARα-null (black bars) adult mice were cultured in either the absence or presence of WY-14643 (50 μM). COUP-TFII and ACO mRNA levels were determined 24 h later. Results are means ± SE of 8 different cultures. *P < 0.05 and **P < 0.01 compared with wild-type mice cultured without WY-14643.
that other rate-limiting factors were involved in the reduction in COUP-TFII-DN suckling mouse liver (Fig. 4B), suggesting pyruvate concentration was increased.

Despite reduced PEPCK mRNA expression in liver of GFP (open bars) and COUP-TFII-DN (black bars). Results expressed as %control (GFP) are means ± SE (n = 8). C: PEPCK, glucose-6-phosphatase (G-6-Pase), and hepatic lipase gene expression in liver of GFP (open bars) and COUP-TFII-DN (black bars). Results expressed as %control (GFP) are means ± SE of 8 different animals. *P < 0.05 and **P < 0.01 compared with controls.

hepatic glucose concentration (Fig. 4B) could be due to the 50–70% reduction in gene expression of PEPCK and glucose-6-phosphatase (G-6-Pase) (Fig. 4C), which are two regulatory enzymes in gluconeogenesis. Despite reduced PEPCK mRNA levels, the phosphoenolpyruvate concentration was increased in COUP-TFII-DN suckling mouse liver (Fig. 4B), suggesting that other rate-limiting factors were involved in the reduction of overall gluconeogenesis. Indeed, two reverse crossovers were observed (Fig. 4B). The first one between pyruvate and oxaloacetate suggests an inhibition of the reaction catalyzed by pyruvate carboxylase. This could result from a decrease in acetyl-CoA concentration (an obligatory cofactor for this enzyme) due to a marked inhibition in hepatic fatty acid oxidation (FAOs), as suggested by the decrease in ketone body production (Fig. 5A). This inhibition of FAOs resulted from 1) a reduced CPT I and mitochondrial hydroxymethylglutaryl-CoA (mHMG-CoA) synthase gene expression (Fig. 5B), two regulatory enzymes of FAOs and ketogenic pathways, respectively, and 2) a decrease in liver NEFA concentration (2.5 ± 0.2 vs. 3.6 ± 0.3 mmol/g for GFP, P < 0.01; n = 8). This reduced NEFA concentration could be attributed to the reduced expression of lipoprotein lipase (LPL; Fig. 5B) and fatty acid transporters CD36 and fatty acid-binding protein 1 (FABP1; Fig. 5B). Neither diacylglycerol (1.91 ± 0.37 vs. 2.14 ± 0.51 mg/g for GFP and COUP-TFII-DN, respectively; n = 6) nor triacylglycerol (23.5 ± 4.4 vs. 25.1 ± 5.0 mg/g for GFP and COUP-TFII-DN, respectively; n = 6) concentrations were affected in the livers of COUP-TFII-DN mice. Similarly, the mRNA levels of PPARγ coactivator 1α (PGC-1α) were not statistically affected in the liver of COUP-TFII-DN mice (1.0 ± 0.3 vs. 0.63 ± 0.14 for GFP and COUP-TFII-DN, respectively; n = 8).

The second crossover is located between 3-phosphoglycerate and dihydroxyacetone phosphate (Fig. 4B). The decrease in FAOs has limited the supply of reduced equivalent (NADH), as suggested by the fall in cytosolic [lactate]/[pyruvate] ratio (8.6 ± 0.4 vs. 12.6 ± 0.8), [glycerol-3-phosphate]/[dihydroxyacetone phosphate] ratio (8.6 ± 1.2 vs. 12.9 ± 0.4), and the mitochondrial [3-hydroxybutyrate]/[acetocacetate] ratio (1.10 ± 0.09 vs. 2.20 ± 0.19). Because NADH is the cofactor for the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase when the flux is oriented toward glucose production, this could explain this second crossover that, associated with reduced Glucose-6-Pase gene expression (Fig. 4C), leads to a marked fall in gluconeogenic flux.

Effect of PPARα agonist on blood metabolites and liver gene expression in COUP-TFII-DN suckling newborn mice. It has been shown that COUP-TFII is able to homodimerize and to a lesser extent heterodimerize with retinoid X receptor (RXR) (7, 31). This could suggest that part of the COUP-TFII-DN effect would be due to the titration of essential transcription factors, such as RXR (the partner of numerous nuclear receptors

Table 2. Effect of COUP-TFII-DN on blood metabolites in 5-day-old mice

<table>
<thead>
<tr>
<th>Blood metabolites, mmol/l</th>
<th>GFP</th>
<th>COUP-TFII-DN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>3.90 ± 0.27</td>
<td>1.59 ± 0.37*</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.185 ± 0.038</td>
<td>0.186 ± 0.017</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.70 ± 0.12</td>
<td>3.13 ± 0.41*</td>
</tr>
<tr>
<td>Acetocacetate</td>
<td>0.259 ± 0.015</td>
<td>0.116 ± 0.019**</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>0.800 ± 0.098</td>
<td>0.232 ± 0.050**</td>
</tr>
<tr>
<td>Total ketone bodies</td>
<td>1.059 ± 0.102</td>
<td>0.348 ± 0.064*</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2.00 ± 0.04</td>
<td>3.15 ± 0.06*</td>
</tr>
</tbody>
</table>

Suckling 4-day-old mice were injected in cheek vein with either 6.10^8 plaque-forming units (pfu) of Ad-green fluorescent protein (GFP) or the dominant negative form of Ad-COUP-TFII (Ad-COUP-TFII-DN). Blood was rapidly sampled 24 h after injections. Values are means ± SE (n = 8). *P < 0.01 and **P < 0.001 when compared with GFP animals.

Fig. 4. Effect of the dominant negative form of COUP-TFII (COUP-TFII-DN) on gluconeogenic metabolite concentrations and gene expression. A: autoradiogram of a representative EMSA using a radiolabeled double-stranded probe containing a DR1 motif. In vitro translated proteins, wild-type human COUP-TFII, or mutated COUP-TFII were incubated separately (lanes 1 and 2) or cotranslated in a molar excess ratio of 1:1 to 1:3 (lanes 3–5). B: crossover plot representation of hepatic gluconeogenic metabolite concentrations; suckling 4-day-old mice were injected in cheek vein with 6.10^8 plaque-forming units (pfu) of either Ad-GFP or Ad-COUP-TFII-DN. Livers were removed 24 h later and rapidly freeze-clamped. The concentrations of each metabolite in the liver of control GFP mice in μmol/g wet tissue were as follows: lactate, 2.52 ± 0.32; pyruvate, 0.20 ± 0.02; oxaloacetate (calculated), 0.018; malate, 0.84 ± 0.06; phosphoenolpyruvate, 0.14 ± 0.01; 2-phosphoglycerate, 0.065 ± 0.02; 3-phosphoglycerate, 0.30 ± 0.02; dihydroxyacetone phosphate, 0.024 ± 0.02; glucose-6-phosphate, 0.12 ± 0.01; glucose, 4.84 ± 0.58. Results are expressed as %control [green fluorescent protein (GFP)] and represent means ± SE (n = 8). C: PEPCK, glucose-6-phosphatase (G-6-Pase), and hepatic lipase gene expression in liver of GFP (open bars) and COUP-TFII-DN (black bars). Results expressed as %control (GFP) are means ± SE of 8 different animals. *P < 0.05 and **P < 0.01 compared with controls.
including COUP-TFII. To approach this question indirectly, we looked at whether it could be possible to rescue FAOx rates in the liver of COUP-TFII-DN mice by activating the expression of CPT I and mHMG-CoA synthase, two PPAR target genes. For this purpose, we used a specific PPAR agonist (WY-14643), assuming that to be fully active, activated PPAR also required dimerization with RXR. As shown in Table 3, CPT I and mHMG-CoA synthase mRNA return to the control level (GFP) in COUP-TFII-DN/WY-14643-injected mice, whereas neither PEPCK nor G-6-Pase mRNA levels are sta-

Table 3. Effect of WY-14643 on blood metabolites and hepatic gene expression in 5-day-old COUP-TFII-DN mice

<table>
<thead>
<tr>
<th>Blood metabolites, mmol/l</th>
<th>GFP</th>
<th>GFP + WY-14643</th>
<th>COUP-TFII-DN</th>
<th>COUP-TFII-DN + WY-14643</th>
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</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>3.70 ± 0.22</td>
<td>3.68 ± 0.33</td>
<td>1.81 ± 0.38**</td>
<td>3.57 ± 0.27##</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.246 ± 0.013</td>
<td>0.237 ± 0.032</td>
<td>0.230 ± 0.011</td>
<td>0.181 ± 0.010#</td>
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<tr>
<td>Lactate</td>
<td>1.90 ± 0.21</td>
<td>1.94 ± 0.12</td>
<td>2.76 ± 0.77**</td>
<td>2.12 ± 0.22</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>0.419 ± 0.052</td>
<td>0.528 ± 0.038</td>
<td>0.186 ± 0.015**</td>
<td>0.443 ± 0.051#</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>0.646 ± 0.063</td>
<td>0.722 ± 0.075</td>
<td>0.347 ± 0.021*</td>
<td>0.674 ± 0.072##</td>
</tr>
<tr>
<td>Total ketone bodies</td>
<td>1.03 ± 0.05</td>
<td>1.25 ± 0.07</td>
<td>0.53 ± 0.03**</td>
<td>1.09 ± 0.11##</td>
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<td>Gene expression, %GFP</td>
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<td></td>
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<tr>
<td>Acyl-CoA oxidase</td>
<td>100 ± 12</td>
<td>200 ± 26**</td>
<td>31 ± 7**</td>
<td>184 ± 50##</td>
</tr>
<tr>
<td>CPT I</td>
<td>100 ± 12</td>
<td>114 ± 15</td>
<td>28 ± 3**</td>
<td>63 ± 12#</td>
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<tr>
<td>mHMG-CoA synthase</td>
<td>100 ± 22</td>
<td>182 ± 27*</td>
<td>28 ± 3**</td>
<td>107 ± 29#</td>
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Values are means ± SE (n = 5–8). Suckling 4-day-old mice were injected in cheek vein with either 6.10⁸ pfu of Ad-GFP or Ad-COUP-TFII-DN. Intraperitoneal injections of WY-14643 (10 mg/kg) or solvent (DMSO) were performed immediately after adenofection. Blood and liver were rapidly sampled 24 h after injections. *P < 0.05 and **P < 0.01 when compared with GFP mice; #P < 0.05 and ##P < 0.01 when compared with COUP-TFII-DN mice.
COUP-TFI CONTROLS POSTNATAL LIVER ENERGETIC METABOLISM

Statistically affected by WY-16643 injection (in %GFP control: COUP-TFI-DN + WY 49 ± 10 vs. COUP-TFI-DN 23 ± 9% for PEPCK; COUP-TFI-DN + WY 49 ± 10 vs. COUP-TFI-DN 23 ± 9% for G-6-Pase). This suggests that the normalization of blood glucose level (Table 3) does not result from direct effect of WY-16643 on hepatic gluconeogenesis but probably from the rescue in hepatic FAOx rates in response to PPARα agonist.

To further support the specificity of the metabolic effects of COUP-TFI-DN, we investigated the effect of a COUP-TFI shRNA.

Effect of COUP-TFI shRNA on liver gene expression and metabolites in suckling newborn mice. Four days after shRNA injection, the level of COUP-TFI protein was reduced by 60% (Fig. 6A). COUP-TFI shRNA induced a fall in blood glucose (5.61 ± 0.22 vs. 6.65 ± 0.16 mmol/l for GFP, P < 0.01; n = 8) and total ketone body (1.23 ± 0.07 vs. 1.72 ± 0.23 mmol/l for Ad-GFP) concentrations. Key gluconeogenic (PEPCK, G-6-Pase) and ketogenic (CPT I, mHMG-CoA synthase) gene expression were slightly reduced in COUP-TFI shRNA (in %GFP control: PEPCK, 57 ± 23; G-6-Pase, 88 ± 20; CPT I, 58 ± 22; mHMG-CoA synthase, 60 ± 21). The fact that the effects of sh-COUP-TFI were less pronounced than with COUP-TFI-DN could have resulted from the persistence of a nonnegligible amount of COUP-TFI protein (40% of control GFP; Fig. 6A) at a developmental stage (7-day-old mice) where COUP-TFI mRNA levels were twofold higher than in COUP-TFI-DN experiments (4 days old; see Fig. 1). Despite such a quantitative consideration, COUP-TFI shRNA induced the same two crossovers observed previously with COUP-TFI-DN (between pyruvate and oxaloacetate and between 3-phosphoglycerate and dihydroxyacetone phosphate; Fig. 6B), suggesting that the reduction in glucose production could result from the decrease in the same regulatory gluconeogenic enzyme activity.

Fig. 6. Effect of COUP-TFI-shRNA on endogenous protein and gluconeogenic metabolite concentrations. A: representative Western blot and quantification of the effect of Ad-shCOUP-TFI on endogenous protein level. Suckling 4-day-old mice were injected in cheek vein with 15.10⁸ pfu of either Ad-GFP or Ad-shCOUP-TFI, and livers were sampled 96 h after injection. Results expressed as %control GFP are means ± SE of 8 different animals. **P < 0.01 compared with controls. B: crossovers plot representation of hepatic gluconeogenic metabolite concentrations. Livers were removed 96 h after injection and rapidly freeze-clamped. The concentrations of each metabolite in the liver of control GFP mice in μmol/g wet tissue were as follows: lactate, 2.55 ± 0.20; pyruvate, 0.23 ± 0.03, oxaloacetate (calculated), 0.017; malate, 0.74 ± 0.06; phosphoenolpyruvate, 0.16 ± 0.02; 2-phosphoglycerate, 0.081 ± 0.02; 3-phosphoglycerate, 0.28 ± 0.03; dihydroxyacetone phosphate, 0.024 ± 0.04; glucose 6-phosphate, 0.16 ± 0.02; glucose, 6.15 ± 0.36. Results expressed as %control (Ad-GFP) represent means ± SE (n = 8). *P < 0.05 compared with controls.

DISCUSSION

The perinatal period is crucial for newborns, especially small rodents such as rat and mice, because of the low amount of carbohydrate in the maternal milk (32) and the scarcity of white fat deposit (21). The successful adaptation of these neonates to the postnatal changes in nutritional environment requires important modifications of glucose and fatty acid metabolism that are orchestrated mainly by pancreatic hormones (16) and ligand-activated nuclear receptors such as PPAR, LXR, and HNF-4 (29). The present study provides the first evidence that the orphan nuclear receptor COUP-TFI contributes to the regulation of glucose and fatty acid metabolisms in suckling newborn mice liver. In fetal mouse liver, the low COUP-TFI mRNA levels are probably related to the high plasma insulin concentration that has been shown to exert an inhibitory effect on COUP-TFI gene expression in cultured adult hepatocytes (30). Indeed, injection of anti-insulin serum in 17.5-day-old mouse fetuses induced a rise in hepatic COUP-TFI mRNA level (data not shown). Conversely, in utero glucagon injection induced hepatic COUP-TFI gene expression. Taken together, these data suggest that the fall in insulin/glucagon molar ratio that occurs immediately after birth (16) triggers, at least in part, the postnatal increase in hepatic COUP-TFI gene expression. Birth is also characterized by reduced glucose availability and increased lipid-derived substrates. Because glucose exerts an inhibitory effect on COUP-TFI gene expression in adult mice hepatocytes (30), its low delivery to newborn mice liver could contribute to the postnatal induction of COUP-TFI mRNA levels. By contrast, long-chain fatty acids (LCFA) fail to induce COUP-TFI gene expression at least in adult mice hepatocytes, whereas COUP-TFI is controlled by PPARα, providing another illustration of the dissociation between PPARα and LCFA in the control of COUP-TFI gene expression (29).

To get functional relevance of the high level of COUP-TFI gene expression in liver during the suckling period, we devel-
developed two different strategies, namely a functional and a genetic invalidation of COUP-TFII protein. Both provide quite similar qualitative results and suggest that COUP-TFII contributes to the preservation of an active hepatic gluconeogenesis and FAOx, with both being crucial for the survival of newborn mice, as illustrated by the drastic (and lethal) hypoglycemia observed in COUP-TFII-DN suckling mice. Because COUP-TFII-DN and shRNA are expressed almost exclusively in hepatocytes, the decrease in blood glucose level in COUP-TFII-DN and to a lesser extent in sh-COUP-TFII newborn mice results from a reduced rate of hepatic gluconeogenesis at both genetic and metabolic levels. The decrease in PEPCK mRNA level in COUP-TFII-DN suckling mice is consistent with the role of this transcription factor in the regulation of PEPCK gene expression (17). By contrast, the fact that COUP-TFII could be involved in the regulation of G-6-Pase gene expression is yet unknown. Whether this results from a direct effect or from the titration of essential factors by COUP-TFII-DN, such as RXR (the partner of numerous nuclear receptors, including COUP-TFII), remains to be determined. This seems unlikely to explain the overall effects of COUP-TFII-DN since 1) hepatic lipase, a target gene of the farnesoid X receptor/ RXR heterodimer (37), is unaffected in COUP-TFII-DN mice and 2) activation of the PPARα-RXR heterodimer by a specific PPARα agonist partially reverses the metabolic and genetic effects of COUP-TFII-DN (Table 3).

From a metabolic point of view, the inhibition of hepatic gluconeogenesis is closely related to the reduced rates of hepatic FAOx in COUP-TFII-DN and shRNA-invalidated mice. This inhibition of mitochondrial FAOx, evidenced by the marked fall in hepatic ketone body production, could be due to 1) a decrease in substrate availability and/or 2) a decrease in regulatory gene expression. Indeed, both of these mechanisms seem to be operative. Firstly, if we assume that the sum of FAOx and esterification fluxes represents an estimation of fatty acid uptake, then it is clear that fatty acid uptake is decreased in COUP-TFII-DN mouse liver since esterification (estimated by triglyceride concentration) is unchanged, whereas FAOx is decreased. This is confirmed by the reduced expression of genes encoding regulatory proteins involved in triglyceride hydrolysis (LPL) and fatty acid transporters (CD36 and FABP-1), leading to a decrease in liver NEFA concentration in COUP-TFII-DN mouse liver. Second, inhibition of gene expression encoding regulatory enzyme of LCFA mitochondrial transfer (CPT I) and ketogenesis (mHMG-CoA synthase) markedly impaired FAOx in COUP-TFII-DN newborn mouse liver. Similar results have been found for FABP3 and the muscular isoform of CPT I gene expression in COUP-TFII-DN newborn mouse liver. Despite the fact that the regulatory steps have not been determined, similar observations have been made in adult liver of fasting PPARα- and PGC-1α-knockout mice. Invalidation of PPARα depresses mHMG-CoA synthase gene expression (22), whereas PGC-1α-knockout decreases the tricarboxylic acid cycle and oxidative phosphorylation gene expression (10), with both models leading to a reduced rate of FAOx and to

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**Fig. 7.** Schematic representation of genes controlled by COUP-TFII and PPARα and the consequences on metabolic fluxes. This scheme is based only on data reported in the present study. PC, pyruvate carboxylase; G-3-PDH, glyceraldehyde-3-phosphate dehydrogenase.
hypoglycemia due to impaired hepatic gluconeogenesis. In the present work, the 40% decrease in PGC-1α mRNA levels in COUP-TFII-DN mice (even if not statistically different from GFP mice) could be a common partner in the regulation of gluconeogenic and fatty acid oxidation genes by COUP-TFII. Indeed, the contribution of PGC-1α in the regulation of these genes expression has been clearly demonstrated (14, 44, 45).

Crossover plot studies reveal that at least two gluconeogenic steps are affected as a consequence of reduced rates of FAOx. The first crossover is located at the level of pyruvate carboxylation and could result from a decrease in pyruvate availability secondary to the release of pyruvate dehydrogenase (PDH) inhibition by LCFA and/or a reduced pyruvate transport across the mitochondrial membrane. The fact that pyruvate concentration is increased or unchanged in the livers of COUP-TFII-DN and sh-COUP-TFII mice, respectively, probably rules out an effect of LCFA on PDH as being responsible mainly for the inhibition of gluconeogenesis. This suggests that mitochondrial pyruvate uptake in exchange for acetoacetate is probably reduced and then could exert a more pronounced control of pyruvate flux through pyruvate carboxylase. This has been clearly demonstrated in isolated rat hepatocytes (3) and in high-fat diet-induced hepatic gluconeogenesis (8). Moreover, inhibition of FAOx in COUP-TFII-DN mouse liver reduces acetyl-CoA availability (Fig. 5A), which could impair pyruvate carboxylase activity, with acetyl-CoA being an obligatory cofactor of this enzyme (2). The following enzymatic reactions catalyzed by enolase and phosphoglycerate mutase appear to be near equilibrium since the mass action ratios of these two enzymes calculated from the [phosphoenolpyruvate]/[2-phosphoglycerate] and [2-phosphoglycerate]/[3-phosphoglycerate] ratios are unchanged in control (2.39 ± 0.27 and 0.20 ± 0.03 for these 2 ratios, respectively) and COUP-TFII-DN mouse liver (2.09 ± 0.15 and 0.22 ± 0.04 for the 2 ratios, respectively). Therefore, the concentrations of these three metabolites move in the same direction when gluconeogenesis is altered.

The second gluconeogenic step(s) affected by the reduction in FAOx is located at the level of glyceraldehyde-3-phosphate dehydrogenase and/or 3-phosphoglycerate kinase. The scarcity of liver samples in newborn mice does not allow us to determine the concentrations of ATP and ADP, and thus it is difficult to discriminate between these two potential regulatory steps. However, it was shown in newborn rat liver that the variation in the [ATP]/[ADP] ratio was of small magnitude upon a wide range of LCFA oxidation rates (15). If such is also true in newborn mouse liver, this would suggest that phosphoglycerate kinase does not represent a major step in the regulation of gluconeogenesis by cofactors arising from FAOx. By contrast, in both COUP-TFII-DN and to a lesser extent sh-COUP-TFII mice, the fall in mitochondrial and cytosolic redox states suggests that the activity of glyceraldehyde-3-phosphate dehydrogenase could be limited by the decreased availability in reduced equivalent (NADH) necessary to direct the flux toward glucose production. It is noteworthy that the substrate and product of glyceraldehyde-3-phosphate dehydrogenase (glyceraldehyde-3-phosphate and 1,3-phosphoglycerate, respectively) have not been determined because of their weak concentration in liver. However, as dihydroxyacetone phosphate is near equilibrium with glyceraldehyde-3-phosphate via triose phosphate isomerase, the decrease in dihydroxyacetone phosphate concentration in the liver of both COUP-TFII-DN and sh-COUP-TFII mice reduces gluconeogenesis by decreasing the availability for the subsequent reaction catalyzed by the triose phosphate isomerase. Such a decrease in dihydroxyacetone phosphate concentration has been found in the liver of very-long-chain acyl-CoA dehydrogenase (VLCAD)-invalidated mice (40). These VLCAD-knockout mice presented a severe hypoglycemia and an impaired hepatic FAOx, but unfortunately, the mechanisms coupling these two parameters had not been determined in this study. Interestingly, when FAOx rates are rescued in response to WY-14643-induced PPARα target genes (CPT I and mHMG-CoA synthase), blood glucose is normalized in COUP-TFII-DN mice.

In conclusion, this work demonstrates that postnatal changes in hormonal (fall in the insulin/glucagon ratio) and nutritional (low glucose delivery to newborn mice) environment are involved in the increase in hepatic COUP-TFII gene expression and that this transcription factor is involved in the regulation of hepatic FAOx, which in turn sustains an active hepatic gluconeogenesis that is essential to maintain an appropriate blood glucose level required for newborn mouse survival. An illustration of the relationships between FAOx and gluconeogenesis as well as the contribution of COUP-TFII and PPARα in the control of genes encoding regulatory steps of these pathways is provided in Fig. 7.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


