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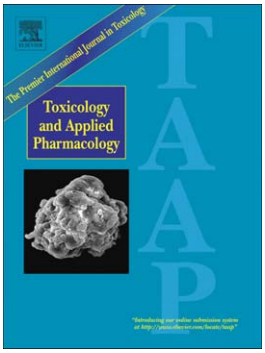
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Abbreviations: CAR, constitutive androstane receptor; ChREBP, Carbohydrate Responsive Element-binding protein; CYP2B10, cytochrome P450, family 2, subfamily B, polypeptide 10; CYP2C29, cytochrome P450, family 2, subfamily C, polypeptide 29; CYP3A11, cytochrome P450, family 3, subfamily A, polypeptide 11; ELOV6, Elongation of long-chain fatty acids family member 6; FASN, Fatty acid synthase; GCK, glucokinase; GPAT, glycerol-

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3-phosphate acyltransferase; LPK, L-type pyruvate kinase; LXR, Liver X receptor; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PEPCK, phosphoenolpyruvate carboxykinase 1; PNPLA3, patatin-like phospholipase domain-containing protein 3; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene; PB, Phenobarbital ; SCD1, Stearoyl-CoA desaturase-1; SREBP1c, sterol regulatory element binding protein1c; TBP, TATA-box binding protein; THRSP-SPOT14, Thyroid hormone responsive Spot14 homolog.

Conflict of interest: No conflict of interest.

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ABSTRACT

The Constitutive Androstane Receptor (CAR, NR1I3) has been newly described as a regulator of energy metabolism. A relevant number of studies using animal models of obesity suggest that CAR activation could be beneficial on the metabolic balance. However, this remains controversial and the underlying mechanisms are still unknown. This work aimed to investigate the effect of CAR activation on hepatic energy metabolism during physiological conditions, i.e. in mouse models not subjected to metabolic/nutritional stress. Gene expression profiling in the liver of CAR knockout and control mice on chow diet and treated with a CAR agonist highlighted CAR-mediated up-regulations of lipogenic genes, concomitant with neutral lipid accumulation. A strong CAR-mediated up-regulation of the patatin-like phospholipase domain-containing protein 3 (*Pnpla3*) was demonstrated. *Pnpla3* is a gene whose polymorphism is associated with the pathogenesis of nonalcoholic fatty liver diseases (NAFLD) development. This observation was confirmed in human hepatocytes treated with the antiepileptic drug and CAR activator, phenobarbital and in immortalized human hepatocytes treated with CITCO. Studying the molecular mechanisms controlling P*npla3* gene expression, we demonstrated that CAR does not act by a direct regulation of *Pnpla3* transcription or via the Liver X Receptor but may rather involve the transcription factor Carbohydrate Responsive Element-binding protein. These data provide new insights into the regulation by CAR of glycolytic and lipogenic genes and on pathogenesis of steatosis. This also raises the question concerning the impact of drugs and environmental contaminants in lipid-associated metabolic diseases.

Keywords: PNPLA3/Adiponutrin; Constitutive Androstane Receptor; Liver X Receptor; Lipogenesis; Non-alcoholic fatty liver disease.

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1. INTRODUCTION

The Constitutive androstane receptor (CAR, NR1I3) was initially characterized as a xenosensor playing an important role in the transcriptional activation of multiple xenochemical metabolizing enzymes in response to drugs and endocrine-disruptive chemicals (1, 2). Recent studies have also linked CAR to lipid and glucide metabolism. Clinical studies demonstrated a link between the use of CAR-activating drugs and the occurrence of metabolic disruptions in patients. Long-term use of phenobarbital (PB), a CAR activator, as an antiepileptic drug, induced a change in plasma lipid profiles of patients (3-5) and chronic treatment with other CAR activators like valproic acid or tamoxifen were associated with the occurrence of hepatic disorders (6, 7).

The role of CAR in the repression of genes involved in gluconeogenesis and β-oxidation was clearly established while its role in lipogenesis remains controversial. Hepatic gluconeogenesis regulatory enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were reported to be repressed by CAR in several studies (8-12). It was suggested that CAR inhibits gluconeogenic genes through a competition with the hepatocyte nuclear factor 4 (HNF4) for binding in direct repeat 1 (DR1) motif in the promoter of gluconeogenic genes (8). It was also proposed that this inhibition was through interaction with the forkhead box protein O1 (FOXO1) to prevent its fixation in the insulin response sequence in gluconeogenic genes (13) or by facilitating the ubiquitination and degradation of PGC1α (14). Inhibition of genes like cytochrome P450 4A14 (*Cyp4a14*), enoyl-CoA isomerase (*Eci*), carnitine palmitoyltransferase 1A (*CPT1a*) and cytosolic acyl-CoA thioesterase (*Cte*), involved in β-oxidation process is well established (15-17). It was supposed to occur through interaction with the peroxisome proliferator-activated receptor α (PPARα), which is the major regulator of fatty acid metabolism (17). The role of CAR in the regulation of lipogenesis is more controversial, some studies reporting CAR as an "anti-

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obesogenic" factor (18-20) and others as a "pro-lipogenic factor" (15, 21, 22). In leptin-deficient (ob/ob) or high fat fed mice CAR activation was shown to alleviate hepatic steatosis by repressing lipogenic genes like the stearoyl-CoA desaturase-1 (*Scd1*), sterol regulatory element-binding protein 1c (*Srebp-1c*) and the acetyl-CoA carboxylase (*Acc*) (18, 23). It was suggested that CAR inhibited lipogenesis through an interaction with Insig-1 a protein blocking the proteolytic activation of SREBP-1c (24). But some other studies reported on an up-regulation of lipogenic genes by CAR on human hepatocytes likely through a nonclassical S14 pathway (21). The effect of CAR activation on non-alcoholic steatohepatitis (NASH) pathogenesis in NASH-induced dietary mouse models was also subject to controversy (25, 26).

To our knowledge, there is no study investigating the effect of CAR activation on lipogenic gene expression and on the development of steatosis in physiological conditions, i.e. in mouse models not subjected to metabolic/nutritional stress and without pre-established disease. This is what we sought to evaluate in this study. In these conditions, CAR was identified as an enhancer of hepatic *de novo* lipogenesis and glycolysis through a Liver X Receptor (LXR)-independent pathway. Concomitantly, CAR activation resulted in hepatic lipid accumulation. We also demonstrated for the first time that CAR is a new player in the patatin-like phospholipase domain-containing protein 3 (*Pnpla3*) regulation, a gene whose polymorphism (I148M) in human represents a strong modifier of NAFLD severity and progression (27, 28).

2. MATERIAL AND METHODS

2.1. Chemicals.

Culture media and additives were purchased from Gibco-Life technologies (Saint Aubin, France). The 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), dimethyl sulfoxide

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(DMSO), phenobarbital (PB) and 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Williams E medium, fetal calf serum (FCS), insulin, glutamine and dexamethasone were purchased from Sigma-Aldrich (St. Quentin Fallavier, France).

2.2. Animals and treatment.

All *in vivo* experiments were conducted following French national and European laws and regulations relating to the housing and use of animals in research and were approved by an independent ethic committee (Toxcométique, INRA ToxAlim, Toulouse, France). This study employed CAR knockout mice (CAR-/-; 10-11-week-old) back-crossed to C57BL/6J, as described in Wei et al. (29), and aged-matched C57BL/6J wild-type (WT) counterparts (Charles River, Les Oncins, France; one week acclimatization) as well as, LXRαβ double-deficient mice (LXR-/-; 12-week-old)–from a C57BL/6J x 129/SvJ mixed genetic background and littermate controls (30). CAR-/- mice were provided by Dr. Urs A. Meyer (Biocenter, University Basel, Switzerland) and LXR-/- mice were provided by Dr David J. Mangelsdorf (Howard Hughes Medical Institute, Dallas, TX). For short-term treatment, male and/or female mice (*n*=6/group) were injected intraperitoneally (i.p.) once-daily during three days with either corn oil (vehicle) or TCPOBOP (3 mg/kg of body weight) dissolved in corn oil. For chronic treatment, male mice (*n*=6/group) were injected once-weekly with TCPOBOP (3mg/kg of body weight; i.p. injection) during seven consecutive weeks using corn oil as vehicle. Mice were housed at INRA's transgenic rodent facility (ToxAlim, Toulouse, France) in a pathogen-free room under a standard 12-h light, 12-h dark cycle and fed a standard chow diet (SAFE Diet A04, Augy, France) with *ad libitum* drinking water.

2.3. Blood and organ sampling.

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Blood was collected at the submandibular vein in EDTA-coated capillaries as described in (31). Plasma was prepared by centrifugation (2,000g; 10 minutes) and kept at -80°C until use. Following euthanasia by cervical dislocation (fed state), the liver and pWAT were removed, weighed, dissected, snap-frozen in liquid nitrogen, and stored at -80°C until use.

2.4. Biochemical Assays.

Hepatic lipid content was quantified as described in (32). Plasma levels of triglycerides and glucose were determined on a biochemical analyzer, COBAS-MIRA+ (Roche Diagnostics; Anexplo Service Phénotypage, Toulouse, France). Plasma insulin was assayed with the ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Crystal Chem, Downers Grove, IL, USA).

2.5. Histology.

Frozen liver samples were embedded in Neg50 (Fisher Scientific, Courtaboeuf, France). Sections (5 µm, Leica RM2145 microtome, Nanterre, France) were stained with Oil-Red-O and hematoxylin/eosin and visualized with a Leica DFC300 camera (Leica Microsystems, Nanterre, France).

2.6. Cell culture experiments.

HepG2 human hepatoma cells and JWZ murine hepatic cells, also known as MuSH immortalized hepatocytes (33), and kindly provided by Joshua P. Gray (Center for Molecular Toxicology and Carcinogenesis, Pennsylvania State University, University Park, Pennsylvania, USA), were grown in DMEM supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum (FBS). The culture medium of JWZ cells was supplemented with dexamethasone (1 µM). To allow CAR overexpression, HepG2 and JWZ cells were transfected with either pCR3-hCAR (HepG2) /

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pCR3-mCAR (JWZ) plasmid, or pCR3-empty plasmid using lipofectamine 2000 (Invitrogen, Cergy Pontoise, France) according to the manufacturer's protocol. Following 48 h of transfection, cells were harvested with Trizol® (Invitrogen, Cergy Pontoise, France) before RNA extraction.

Immortalized human hepatocytes (IHH, (34)) were cultured in Williams E medium containing 11 mM glucose and supplemented with 10% FCS, 100U/ml penicillin, 100µg/ml streptomycin, 120 nM insulin, 2% glutamine and 50nM dexamethasone. Cells were then exposed to DMSO 1% or CITCO 1 µM (1% DMSO) in the same cell culture media containing 4% FCS instead of 10%. Following 48h of treatment, cells were harvested with Trizol® (Invitrogen, Cergy Pontoise, France) before RNA extraction.

2.7. Luciferase reporter gene assay.

JWZ cells were co-transfected either with a 3.3 kb Pnpla3 promoter construct cloned into a pGL3-basic vector, or the $(NR1)_5$-tk-luc pGL3 construct, or a pGL3-empty vector (0.5 µg), and with the pRL-TK plasmid (25 ng), and either with pCR3-mCAR or pCR3-empty (0.5 µg), using Lipofectamine 2000. The Pnpla3 promoter construct and $(NR1)_5$-tk-luc pGL3 construct were a gift from Dr Marthe Moldes (Centre de Recherche Saint-Antoine, INSERM, UMR 938, Université Paris 6, Paris, France) (35) and Dr Masahiko Negishi (Reproductive & Developmental Biology Laboratory / Pharmacogenetics Group, Research Triangle Park, North Carolina, USA), respectively. After 48 hours exposure, cells were lysed in cell culture lysis reagent (Promega, Madison, USA). Luciferase activities were determined according to the manufacturer's instruction (Dual-Luciferase® Reporter (DLR™) Assay System, Promega). Lysate samples were assayed in triplicate from four independent experiments. The firefly luciferase activity of each sample was normalized to Renilla luciferase activities.

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2.8. Gene expression studies.

Total RNA was extracted with Trizol® reagent. Transcriptomic profiles were obtained by quantitative real-time polymerase chain reaction (RT-qPCR) as described in (32). Primers for SYBR Green assays are presented in Table 1. qPCR data were normalized by TATA-box binding protein (TBP) for mouse messenger RNA (mRNA) levels or β-actin/*PSMB6* for human messenger RNA (mRNA) levels as indicated in figure legends and raw data were analyzed with LinRegPCR (36).

2.9. Immunoblot Analysis. Nuclear protein extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific), according to the manufacturer's instructions. Following separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), nuclear protein extracts were probed with primary antibodies from Cell Signaling Technology (LAMIN A/C: 2032; Danvers, MA , USA), Novus Biological (CHREBP: nb400-135; Littleton, CO, USA), and secondary antibodies from Biotium (CF680 or CF770-labeled; Hayward, CA,USA). Band intensities were normalized to those of LAMIN A/C. The images were analyzed on the Odyssey Infrared Imaging system (Li-Cor, Lincoln, NE, USA).

2.10. Primary Culture of Human and Mouse Hepatocytes.

Liver samples were obtained from liver resections performed in adult patients for medical reasons unrelated to our research program. The use of human specimens for scientific purposes was approved by the French National Ethics Committee. Written or oral informed consent was obtained from each patient or family prior to surgery. The clinical characteristics of the liver donors are presented in Table 2. Primary human hepatocytes were prepared and cultured as described previously (37) and were either treated with 500 μM phenobarbital (PB) dissolved in 0.1% DMSO for 24 hours or untreated (0.1% DMSO). Mouse hepatocytes were

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prepared as described previously (38) and cultured in the presence of TCPOBOP (250 µM in 0.1% DMSO; 24h) or DMSO (0.1%).

2.11. Statistical analysis.

All the data were analyzed using GraphPad Prism 7.0. Significance was assessed by appropriate unpaired Two-tailed Student's t-tests or two-way ANOVA followed by Tukey's multiple comparison *post hoc* test as indicated. P*< 0.05 was considered significant.

3. RESULTS

3.1. Short-term CAR activation by TCPOBOP induces hepatic expression of genes related to lipid biosynthesis.

To investigate the effect of CAR activation on hepatic energy metabolism in healthy mice, CAR-/- and controls (WT) male mice on chow diet were treated with either corn oil (vehicle) or a CAR-specific ligand, TCPOBOP at 3 mg/kg/day, a dose previously used to investigate the effect of CAR activation on hepatic lipid metabolism (39-41). The hepatic mRNA expression of the prototypical CAR target genes (*Cyp2b10*, *Cyp2c29* and *Cyp3a11)* was induced by TCPOBOP in WT mice but not in CAR-/- mice (Figure 1A). Furthermore, CAR activation in WT mice induces a down-regulation of *Pepck* gene expression, while the expression of both glucokinase (*Gck*) and L-type pyruvate kinase (*Lpk*), which catalyse the first and the final step of glycolysis pathway respectively, was increased (Figure 1B). Moreover CAR activation induced an upregulation of lipogenic genes such as the fatty acid synthase (*Fasn*), elongation of long-chain fatty acids family member 6 (*Elovl6*), and glycerol-3-phosphate acyltransferase (*Gpat*), which incorporates newly synthesized fatty acids into triacylglycerol. The thyroid hormone responsive Spot14 homolog (*Thrsp-Spot14*), which encodes a protein regulating *de novo* lipogenesis through a molecular mechanism still undefined was also upregulated (Figure 1C). However, the hepatic mRNA expression of all

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these genes was unaffected in TCPOBOP-treated CAR-/- male mice (Figure 2), highlighting a CAR-dependent regulation of the glycolytic-lipogenic pathway. No sexual dimorphism was observed in WT mice. Indeed similar gene expression profiles were observed in the liver of TCPOBOP-treated WT females and males compared to their respective vehicle-treated counterparts (Sup Figure 1). However, some genes were slightly but significantly induced in TCPOBOP treated CAR-/- females suggesting the activation of other pathways in this particular context (Sup Figure 1).

The coordination of lipid metabolism in white adipose tissue and in liver is important for whole-body metabolic homoeostasis. Measuring expression of lipogenic genes (*Ssd1, Elovl6, Gpat*) in adipose tissue showed no induction of expression in this tissue (Sup Figure 2).

3.3. Short-term CAR activation by TCPOBOP upregulates Pnpla3 and Pnpla5 expression.

Pnpla3 gene polymorphism has emerged as a new marker of human hepatic steatosis (27). Initially discovered in the adipose tissue, PNPLA3 is highly regulated by changes in energy balance (35, 42, 43). In the present study, CAR activation by TCPOBOP induced a strong increase in *Pnpla3* expression (Figure 1D). This was fully abrogated in TCPOBOP-treated CAR-/- males and remained slightly increase in counterpart females (Figure 1D; Sup Figure 1). *Pnpla5* is located immediately upstream of the *Pnpla3* gene and exhibits both lipase and transacylase activities *in vitro* (44). In this study, *Pnpla5* gene expression pattern was similar to the one observed for *Pnpla3* (Figure 1D), whereas the expression of the triglyceride lipase *Pnpla2* did not differ between groups (Figure 1D).

3.4. Short-term CAR activation by TCPOBOP increases hepatic lipid accumulation.

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We next assessed whether the increased expression of lipogenic genes was correlated with changes in hepatic neutral content. Hepatic neutral lipids were first stained with Oil-Red-O. The representative pictures in Figure 2 illustrate that the number and size of lipid droplets were increased by TCPOBOP treatment in WT mouse liver (Figure 2A, top panels), but did not change in the liver of CAR-/- mice (Figure 2A, bottom panels). Surprisingly we observed a greater accumulation of lipids in the liver of vehicle-treated CAR-/- mice compared to vehicle-treated WT mice (Figure 2A, left panels). Quantification of the liver lipid content by gas chromatography confirmed these observations. TCPOBOP treatment in WT mice did not affect the level of total hepatic cholesterol (data not shown), but hepatic cholesterol esters and triglycerides were significantly increased compared to vehicle treatment (Figure 2B). These changes were associated with a significant increase in liver weight (Table 3). However, in CAR-/- mice hepatic lipid content and liver weight were unchanged in response to TCPOBOP (Figure 2B, Table 3). Finally, plasma triglyceride and glucose levels were decreased following TCPOBOP-treatment in WT mice but unchanged in CAR-/- mice, and basal levels of both parameters were higher in vehicle-treated CAR-/- mice compared to WT (Table 3).

3.5. Pnpla3 promoter activity is not directly regulated by CAR.

To further explore the underlying mechanisms behind CAR's actions, we performed *in silico* analysis which revealed the presence of a CAR-DR4 (Direct Repeat 4) responsive element 2740 bp upstream of the ATG start codon of *Pnpla3* mouse promoter (Sup Table 1). We next determined whether the effect of CAR activation on *Pnpla3* expression was transcriptionally mediated. As shown in Figure 3, a reporter luciferase construct containing the mouse *Pnpla3* promoter (-3300 nt upstream of the luciferase ORF) (35) was co-transfected into JWZ cells with either a vector expressing mCAR (pCR3-mCAR), or a control vector (pCR3-empty). Additionally, a construct containing five copies of the optimal CAR

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monomer binding motif (DR4-type NR1 sequence) upstream of the *tk-luciferase* promoter was used as a positive control. As expected, luciferase activity of the control ($(NR1)_5$-tk-luc-pGL3) was 22-fold increased upon mCAR overexpression (Figure 3A) while no significant change was observed for the construct containing the Pnpla3 promoter (Figure 3B). Altogether, our data suggest that CAR does not directly regulate *Pnpla3* gene transcription.

3.6. LXR is not required for TCPOBOP-induced response of lipogenic genes.

Since LXRα and LXRβ are important transcriptional regulators of lipogenesis (45), we next assessed whether LXRα/β could mediate CAR's actions. We treated LXRα/β double-deficient (LXR-/-) mice and control mice with the CAR-specific ligand, TCPOBOP, or corn oil (vehicle), as performed previously. In WT mice, TCPOBOP induces a markedly increased hepatic mRNA expression of the prototypical CAR target gene *Cyp2b10* (Figure 4A). Similarly, the expression of the glycolytic enzymes (*Gck*, *Lpk*) and lipogenic enzymes (*Fasn, Elovl6, Thrsp-Spot14* and *Pnpla3*) was upregulated (Figure 4B-4C). These results suggest that TCPOBOP-mediated activation of CAR-target genes, including *Pnpla3,* is independent of LXR pathway.

3.7. CAR activation by TCPOBOP regulates ChREBP mRNA and protein levels.

The transcription factors Carbohydrate-responsive element-binding protein (ChREBP) and sterol regulatory element-binding protein 1c (SREBP1c) play a pivotal role in the liver by regulating glycolysis and lipogenesis (46). Furthermore PNPLA3 expression is under their direct transcriptional control (35). Thus we investigated whether these factors could be involved in the CAR-mediated activation of lipogenic genes and lipid accumulation.

In adipocytes, ChREBPα isoform is likely responsible for inducing ChREBPβ which is transcribed from an alternative promoter (47). In this study, the mRNA expression of both

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isoforms was significantly induced in response to TCPOBOP in WT mice but unchanged in CAR-/- mice, although a larger variability was observed for *Chrebpβ* (Figure 5A). In addition the mRNA expression of *Srebp1c* was unchanged in response to TCPOBOP in WT and CAR-/- mice (Figure 5B).

Since the nuclear translocation of ChREBP is a critical step for activation of its target genes, the ChREBP subcellular localization in response to TCPOBOP was checked. In the liver of TCPOBOP-treated WT mice, an increase of nuclear ChREBP content was observed, but not in TCPOBOP-treated CAR-/- mice (Figure 5C).

3.8. Chronic CAR activation by TCPOBOP induces hepatic expression of genes related to lipid biosynthesis and lipid accumulation in the liver.

To determine whether the observed effects are not the result of an acute response to CAR activation, CAR-/- and controls (WT) male mice on chow diet were treated with either corn oil or TCPOBOP at 3 mg/kg/day once-weekly during seven consecutive weeks. As observed previously, CAR activation by TCPOBOP induced the up-regulation of the prototypical CAR target genes *Cyp2b10* and *Cyp3a11*, as well as a down-regulation of the *Pepck* and the overexpression of key lipogenic genes including *Pnpla3* (Figure 6A-C). However, the expression of the same genes was unchanged in TCPOBOP-treated CAR-/- male mice (Figure 6A-C). The representative pictures in Figure 6D illustrate an accumulation of lipid droplets induced by TCPOBOP treatment in the liver of WT mice compared to those of vehicle-WT mice (Figure 6D). Chronic CAR activation also resulted in a greater accumulation of lipids in the liver of vehicle-treated CAR-/- mice compared to vehicle-treated WT mice as observed previously after a short term exposure to TCPOBOP (Figure 2A).

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3.9. CAR activation induces Pnpla3 expression in human hepatocytes.

Pnpla3 gene regulation by CAR represents an original result never reported before. Thus we sought to determine whether the CAR-dependent induction of *pnpla3* was also observed *in vitro*, particularly in human hepatocytes.

We first overexpressed CAR in human hepatoma HepG2 cells and murine hepatic cell lines using pCR3-hCAR (human CAR isoform) or pCR3-mCAR (murine CAR isoform) expression vector respectively. In these cell lines, both hCAR and mCAR proteins have been shown to be constitutively active (48). CAR overexpression resulted in significant inductions of the CAR target genes *CYP2B6*/*Cyp2b10* and *PNPLA3/Pnpla3* (Figure 7A and 7C). Furthermore, the mRNA expression of these genes was significantly induced in primary mouse hepatocytes treated 24h with TCPOBOP (250µM) compared to the control condition (DMSO) (Figure 7B). Similar results were obtained using primary human hepatocytes treated with the CAR-activator phenobarbital (PB; 500 µM; 24h), a barbiturate drug widely used to treat epilepsy (49). As shown in Figure 7D, hepatocytes from three of the four donors display an increase of *PNPLA3* expression correlated with inductions of *SCD1* in response to PB treatment. It is known that PB is an indirect CAR activator and is considered to be a mixed CAR/PXR activator. Consequently, immortalized human hepatocytes were treated with CITCO which is a well-characterized specific direct activator of human CAR (50). CAR activation by CITCO resulted in significant inductions of *CYP2B6,* as well as of the lipogenic genes *Fasn*, *Pnpla3* and an increase in the mRNA levels of *ChREBP* and *SREBP1c* (Figure 7E).

Overall, these results suggest a possible translation of the results obtained in mice to humans.

4. DISCUSSION

In the present study, we showed that CAR activation by TCPOBOP positively regulates genes involved in hepatic glycolysis as well as lipogenesis and induces steatosis in mouse

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liver. We demonstrated for the first time that CAR regulates *Pnpla3* expression both in mouse liver and human hepatocytes. The subsequent study of the underpinning mechanisms highlighted that CAR does not act by a direct regulation of *Pnpla3* transcription or via the Liver X Receptor. We confirmed the previously reported inhibitory effect of CAR on gluconeogenesis through the repression of PEPCK (8-12). We measured the expression of a large panel of key genes regulating glycolysis and different steps of lipogenesis including fatty acid synthesis (*Fasn*), elongation (*Elovl6*) and esterification (*Gpat*) which had never been assayed before. We showed a clear synergic and CAR-dependent up-regulation of all these genes both in males and females. A slight induction of some genes in CAR -/- female livers suggests the activation of other pathways in this particular context (*Cyp2b10, Cyp2c29, Pnpla3*; Sup Figure 1). These results are consistent with those reported by Breuker et al., highlighting a prolipogenic effect of CAR through the up-regulation of Thrsp-Spot14, a regulator of the lipogenic process, in human primary hepatocytes (21). It is unlikely that the upregulation of lipogenic genes and lipid accumulation are a consequence of acute response to TCPOBOP since longer exposure time to this chemical indicates similar results (Figure 6).

The Pregnane X receptor (PXR) is another xenosensor closely related to CAR. It is well established that CAR and PXR exhibit similar roles in the regulation of β-oxidation and gluconeogenesis but could display opposing actions on lipid homeostasis (51). Indeed PXR is considered as a pro-lipogenic receptor and has a putative role in the pathogenesis of steatohepatitis (52), whereas CAR was originally considered as displaying anti-lipogenic properties (53, 54). These assumptions were based on data from mouse models subjected to metabolic/nutritional stress (18, 23). In the present study, healthy mice on a standard chow diet were used. In these conditions, we identified CAR as an enhancer of hepatic *de novo* lipogenesis suggesting an identical role of CAR and PXR in the regulation of lipid metabolism. This assumption is supported by the regulation by CAR of *Pnpla3*, a gene whose

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polymorphism has recently emerged as an important marker of liver steatosis (55). The rs738409 variant (encoding an amino acid substitution Ile148Met) of PNPLA3 is a strong modifier of the NAFLD etiology by increasing liver fat content and the disease severity and progression (27, 28, 56). As a lipogenic enzyme, *Pnpla3* was reported to be transcriptionally regulated by SREBP-1c, LXR and ChREBP in response to insulin and glucose respectively (35, 42, 43). Our major finding is that *Pnpla3* is also regulated by CAR. This regulation was confirmed in hepatocytes of human donors treated with the anti-epileptic and CAR activator, PB. It was also confirmed in human hepatocytes treated with CITCO which is a well-characterized specific direct activator of human CAR. These results suggest its relevance for human.

Little is known about the mechanisms underpinning the CAR-mediated regulation of lipogenic genes. Our results highlighted an indirect regulation of the mouse *Pnpla3* promoter by CAR. Since LXR is a central regulator of hepatic *de novo* lipogenesis, and could crosstalk with CAR (57), we next investigated its potential involvement. Gene-expression profiles were similar independently of the presence or absence of LXR, suggesting that the induction of lipogenic genes upon TCPOBOP treatment is not mediated by LXR. Underlying mechanisms of gene regulation by CAR of glycolytic and lipogenic genes are probably more complex and may involve one or more other transcription factors. Most of the genes we found up-regulated by CAR are known to be regulated by SREBP-1c, CHREBP or both (58). While further investigations are ongoing, the preliminary results presented in Figure 5 highlight that the transcription factor CHREBP could mediate the observed effects in mice and both factors might mediate CITCO-induced responses in immortalized human hepatocytes (Figure 7E).

CAR activation by TCPOBOP resulted in a hepatic lipid accumulation consisting in triglycerides and cholesterol esters. Thus, the results presented here are in contrast to those

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showing that CAR activation alleviates hepatic steatosis (18, 23). We showed that this activation could instead have deleterious effects on mouse liver leading to aberrant hepatic *de novo* lipogenesis. The regulation by CAR of energy metabolism is probably more complex, and depends on the metabolic context. However, these results raise the question concerning the impact of drugs and environmental contaminants in lipid-associated metabolic diseases, such as obesity, insulin resistance, and related clinical disorders (type 2 diabetes and cardiovascular diseases), since most of them are CAR regulators. These risks could be particularly important in subjects carrying the PNPLA3 rs738409 C>G variant which could be more vulnerable.

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TABLE LEGENDS

Table 1. Oligonucleotide sequences for real time PCR.

Table 2. Clinical characteristics of the liver donors. F = female; M = male; PHH = primary human hepatocyte; Age in years.

Table 3. Body weight, relative liver weight and plasma parameters of WT and CAR-/- mice (n=6 males) treated with either vehicle (corn oil) or TCPOBOP (3 mg/kg/day) for 3 days. The statistical significance was evaluated using two-way *ANOVA* followed by *Tukey's post hoc test (*a: significant treatment effect, TCPOBOP *vs* vehicle; b, significant genotype effect, CAR-/- *vs* WT*;* $p<0.05$). Values are mean ± SEM.

FIGURE LEGENDS

Figure 1. **CAR regulates the expression of key genes involved in hepatic glucose metabolism and *de novo* lipogenesis**. Hepatic mRNAs from wild-type (WT) and CAR null (CAR-/-) male mice (*n* = 6 mice per group) treated with either corn oil (vehicle) or the CAR agonist, TCPOBOP (3 mg/kg/day) for three days were used to assay by RT-qPCR the relative expression of **(A)** Cytochrome P450 enzymes (*Cyp2b10*, *Cyp2c29*, *Cyp3a11*), and genes involved in **(B)** glucose metabolism (*Pepck*, *Gck*, and, *Lpk*), **(C)** *de novo* lipogenesis (*Fasn*, *Elov6*, *Gpat*, *Thrsp-Spot14*) and **(D)** PNPLA family members (*Pnpla3*, *5*, *2*). Data were normalized to TBP mRNA expression levels and to the vehicle-treated mice for each genotype. Statistical significance was evaluated using the unpaired Two-tailed Student's t test. Values shown are the mean ± SEM. Asterisk denotes a significant difference compared with control condition of each genotype: *P<0.05; **P<0.01; ***P<0.001.

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Figure 2. **CAR activation by TCPOBOP increases hepatic triglycerides and cholesterol esters levels. (A)** Oil-red O-staining of neutral lipids performed on histological sections of livers from WT and CAR-/- mice treated for three days with either corn oil (vehicle) or the CAR agonist, TCPOBOP (3 mg/kg/day). Neutral lipids appear in red (original magnification x200). **(B)** Neutral lipids were extracted from the liver of the same animals. After extraction, lipids were analyzed by gas chromatography. The presence of internal standards enabled to quantify the neutral lipids. Statistical significance was evaluated using two-way *ANOVA* followed by *Tukey's post hoc test*. Asterisk denotes a significant difference compared with control condition for each genotype: **P<0.01; ***P<0.001.

Figure 3. Pnpla3 promoter activity is not directly regulated by CAR. Luciferase assays were performed in JWZ cells transiently co-transfected with either a pCR3-mCAR vector expressing mCAR (mCAR) or with a control pCR3-empty vector (mCAR), and with **(A)** a construct containing five copies of the optimal CAR monomer binding motif (DR4-type NR1 sequence) in front of the *tk-luciferase* promoter ($(NR1)_5$-tk-luc), or **(B)** with a reporter containing the mouse Pnpla3 promoter (-3300nt upstream of the luciferase ORF). The pGL3-empty basic vector was used as negative control. Firefly luciferase activity of each sample was normalized to Renilla luciferase activity. Results are expressed as mean ± SEM from four independent experiments. Statistical significance was evaluated using two-way *ANOVA* followed by *Tukey's post hoc test.* *P<0. 05;**P<0. 01; ***P<0. 001.

Figure 4. TCPOCOP-induced up-regulation of lipogenic genes is not mediated by LXRαβ. Hepatic mRNAs from wild-type (WT) and LXR null (LXR-/-) male mice (*n* = 6 mice per group) treated with either corn oil (vehicle) or the CAR agonist, TCPOBOP (3

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mg/kg/day) for three days were used to assay by RT-qPCR the relative expression of **(A)** the prototypical CAR target gene *Cyp2b10*, and the relative expression of genes involved in **(B)** glucose metabolism (*Gck, Lpk*), and in **(C)** *de novo* lipogenesis (*Fasn, Elov6, Thrsp-Spot14*, and *Pnpla3*). Data were normalized to TBP mRNA expression levels and to the vehicle-treated mice for each genotype. Statistical significance was evaluated using the unpaired Two-tailed Student's t test. Values shown are the mean ± SEM. Asterisk denotes a significant difference compared with control condition of each genotype: **P<0.01; ***P<0.001.

Figure 5. CAR activation by TCPOBOP increased *Chrebp* mRNA expression and nuclear ChREBP content. (A) Quantification of *Chrebp*α and β and*Srebp1c* mRNA levels by RT-qPCR from WT and CAR-/- male mice (*n* = 6 mice per group) treated with either corn oil (vehicle) or the CAR agonist, TCPOBOP (3 mg/kg/day) for three days. Values shown are the mean ± SEM. Data were normalized to TBP mRNA expression levels and to the vehicle-treated mice for each genotype. Statistical significance was evaluated using the unpaired Two-tailed Student's t test. **(C)** Representative western blot images and quantification of nuclear ChREBP (ChREBPn) content in liver extracts (*n* = 2 mice per group). LAMIN A/C antibody was used as loading controls**.** Values shown are the mean ± SEM. The Statistical significance was evaluated using two-way *ANOVA* followed by *Tukey's post hoc test*. Asterisk denotes a significant difference compared with control condition: *P<0.05; **P<0.01.

Figure 6. Chronic activation of CAR regulates the expression of key genes involved in hepatic glucose metabolism and *de novo* lipogenesis and leads to hepatic triglycerides accumulation. Hepatic mRNAs from wild-type (WT) and CAR null (CAR-/-) male mice (n = 6 mice per group) treated with either corn oil (vehicle) or the CAR agonist, TCPOBOP (single

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ip injection of 3 mg/kg/week) for seven weeks were used to assay by RT-qPCR the relative expression of **(A)** Cytochrome P450 (*Cyp2b10*, *Cyp2c29*), and genes involved in **(B)** glucose metabolism (*Pepck, Lpk*), **(C)** *de novo* lipogenesis (*Fasn, Elovl6, Thrsp-Spot 14* and *Pnpla3*). Data were normalized to TBP mRNA expression levels and to the vehicle-treated mice for each genotype. Statistical significance was evaluated using the unpaired Two-tailed Student's t test. **(D)** Oil-red O-staining of neutral lipids performed on histological sections of livers from the same animals. Neutral lipids appear in red (original magnification x200). Asterisk denotes a significant difference compared with control condition for each genotype: **P<0.01; *** P<0.001

Figure 7. CAR activation induces *Cyp2b10/CYP2B6* and *Pnpla3/PNPLA3* gene expression *in vitro*. The hepatic mRNA levels of *Cyp2b10* and *Pnpla3* were examined by RT-qPCR in **(A)** JWZ cell lines transfected either with a vector expressing mCAR or a control vector (-) and, in **(B)** primary mouse hepatocytes treated for 24 hours with TCPOBOP (0.1% DMSO) or 0.1% DMSO (control). The data were normalized to TBP mRNA expression levels. **(C)** The hepatic mRNA levels of *CYP2B6* and *PNPLA3* were examined by RT-qPCR in HepG2 cell lines transfected either with a vector expressing hCAR or a control vector (-). **(D)** Cultured hepatocytes from four donors were exposed to phenobarbital (PB; 500 µM) or DMSO (control) for 24 hrs. The hepatic mRNA levels of *CYPBB6*, *PNPLA3* and *SCD1* were examined by RT-qPCR and normalized to β-actin mRNA expression levels (HepG2/human hepatocytes). **(E)** Immortalized human hepatocytes (IHH) were treated for 48 hours with CITCO (1µM) or DMSO (control). The hepatic mRNA levels of *CYP2B6*, *LPK*, *ACC*, *PNPLA3*, *ChREBP* and *SREBP1c* were examined by RT-qPCR and normalized to *PSMB6* mRNA expression levels. All the data were analyzed by Two-tailed Student's t-test.

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Asterisk denotes a significant difference compared with the control condition: *P<0.05; **P<0.01; ***P<0.001.

Sup Figure 1. **CAR regulates the expression of key genes involved in hepatic glucose metabolism and *de novo* lipogenesis in females**. Hepatic mRNAs from wild-type (WT) and CAR null (CAR-/-) male mice (*n* = 6 mice per group) treated with either corn oil (vehicle) or the CAR agonist, TCPOBOP (3 mg/kg/day) for three days were used to assay by RT-qPCR the relative expression of **(A)** Cytochrome P450 enzymes (*Cyp2b10*, *Cyp2c29*, *Cyp3a11*), and genes involved in **(B)** glucose metabolism (*Pepck*, *Gck*, and, *Lpk*), and **(C)** *de novo* lipogenesis (*Pnpla3*, *Thrsp*-*Spot14*). Data were normalized to TBP mRNA expression levels and to the vehicle-treated mice for each genotype. Statistical significance was evaluated using the unpaired Two-tailed Student's t test. Values shown are the mean ± SEM. Asterisk denotes a significant difference compared with control condition of each genotype: *P<0.05; **P<0.01; ***P<0.001.

Sup Figure 2. **CAR activation does not regulate the expression of key genes involved in *de novo* lipogenesis in white adipose tissues**. mRNA levels in perigonadal white adipose tissues from wild-type (WT) and CAR null (CAR-/-) male mice (*n* = 6 mice per group) treated with either corn oil (vehicle) or the CAR agonist, TCPOBOP (3 mg/kg/day) for three days were used to assay by RT-qPCR the relative expression of genes encoding *de novo* lipogenesis enzymes (*Scd1*, *Elovl6*, *Gpat*) as well as the lipogenic transcription factor Peroxisome proliferator-activated receptor gamma (*Pparγ*). Data were normalized to TBP mRNA expression levels and to the vehicle-treated mice for each genotype. Statistical significance was evaluated using the unpaired Two-tailed Student's t test. Values shown are the mean ± SEM.

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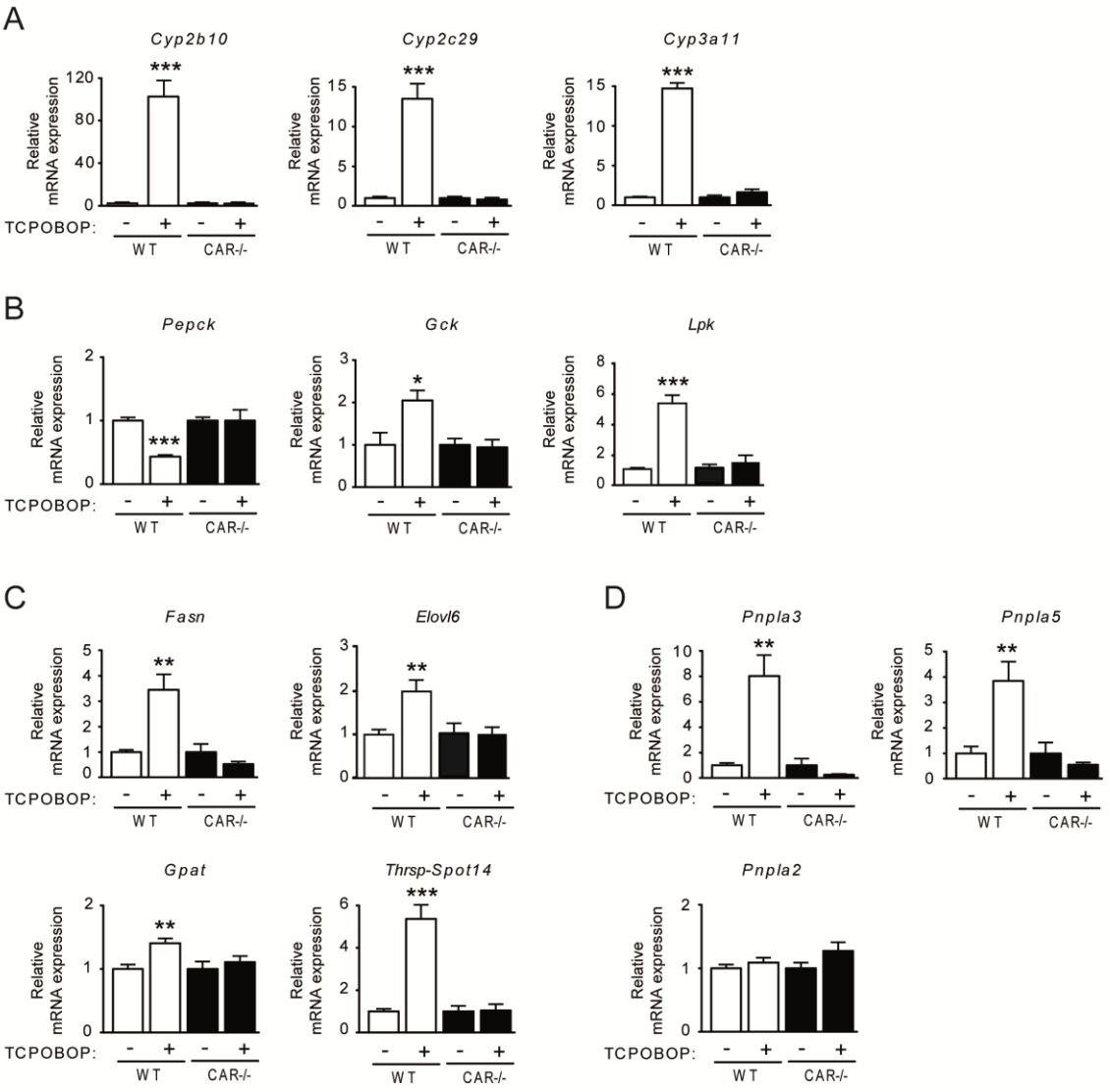


Figure 1

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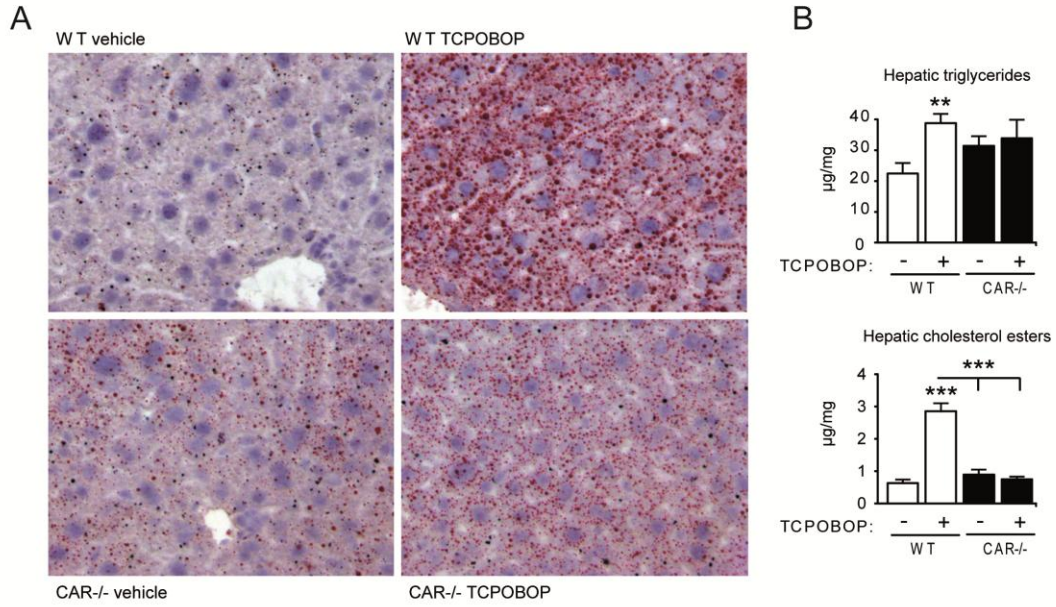


Figure 2

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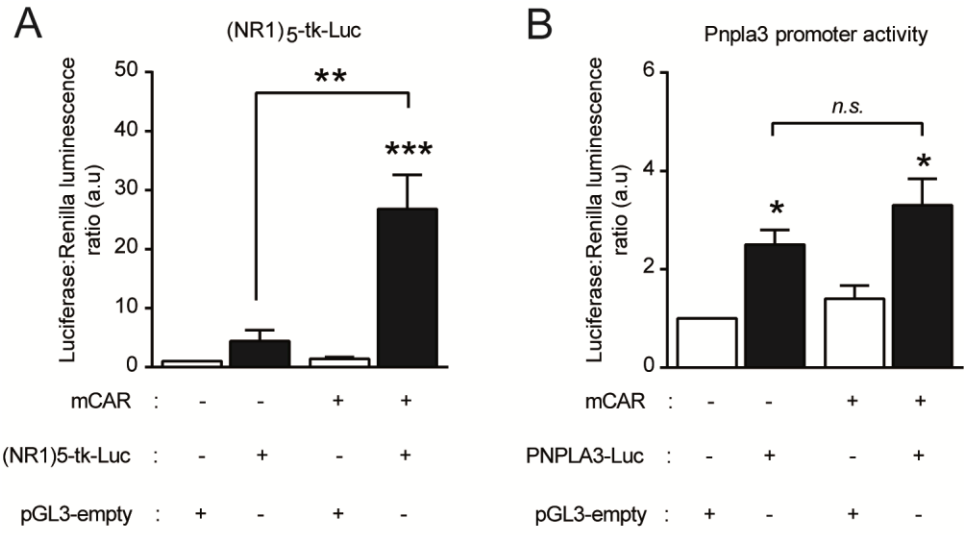


Figure 3

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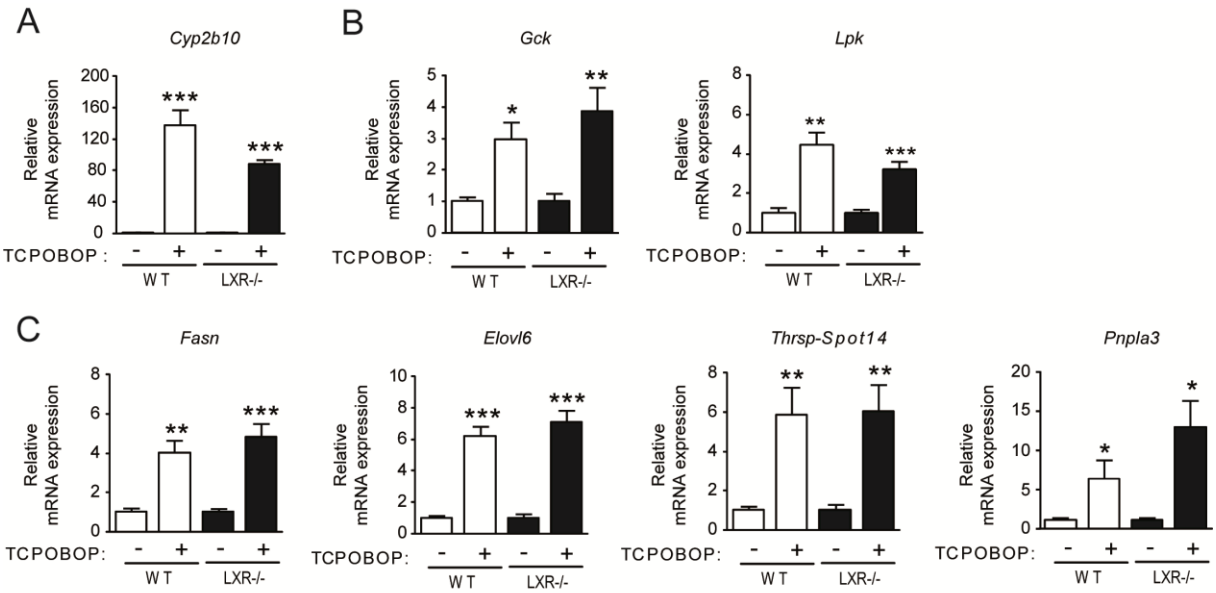


Figure 4

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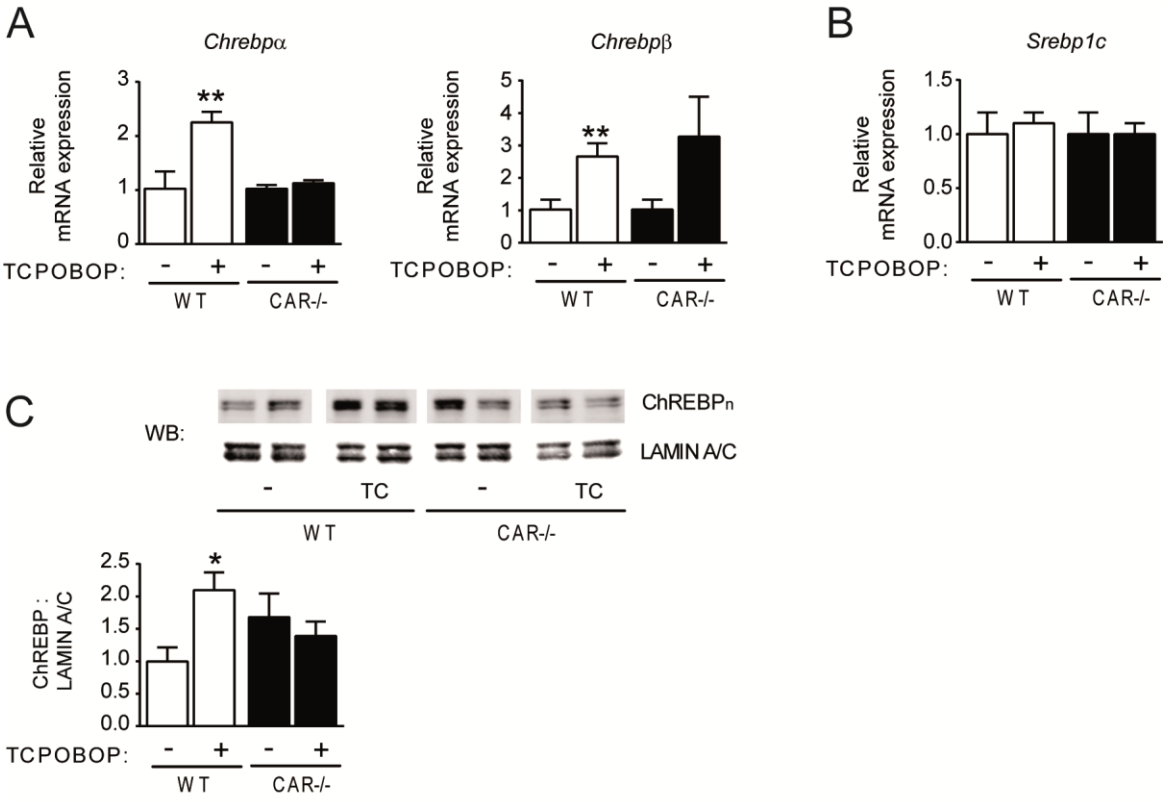


Figure 5

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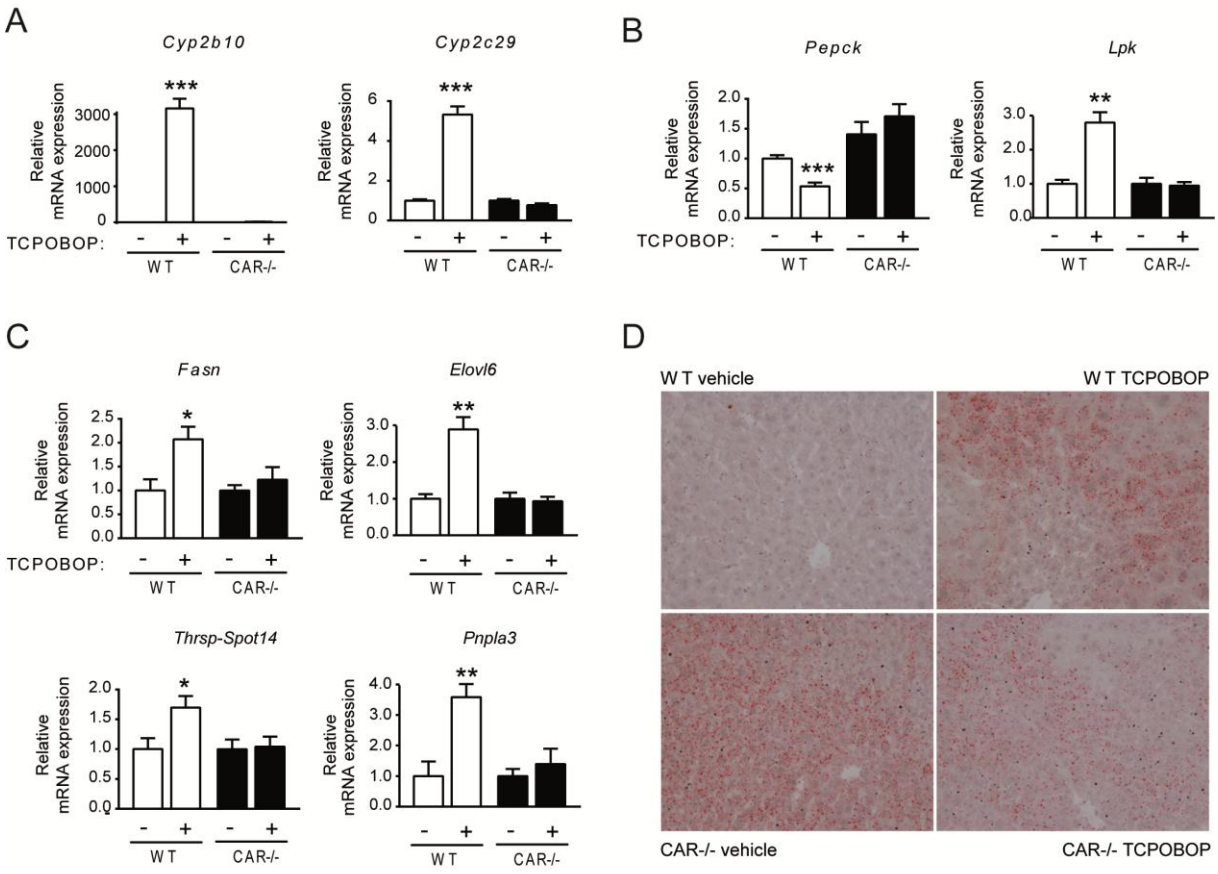


Figure 6

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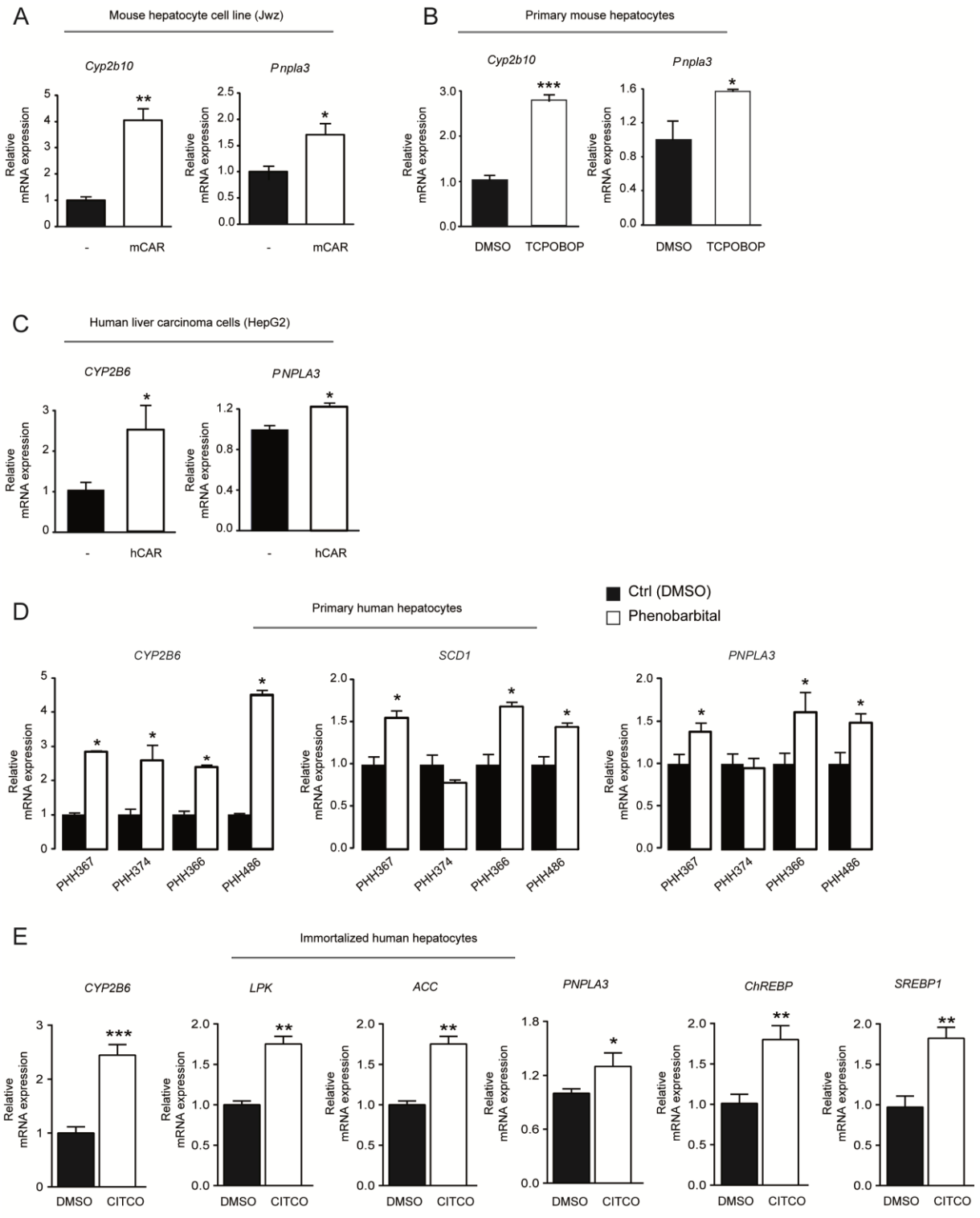


Figure 7

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Table 1: **Oligonucleotide sequences for real time PCR.**

Gene	NCBI Refseq	Forward primer (5'-3')	Reverse primer (5'-3')
Mus musculus			
Chrebpα	NM_021455	CGACACTCACCCACCTCTTC	TTGTTCAGCCGGATCTTGTC
Chrebpβ	NM_021455	TCTGCAGATCGCGTGGAG	CTTGTCCCGGCATAGCAAC
Cyp2b10	NM_009999	TTTCTGCCCTTCTCAACAGGAA	ATGGACGTGAAGAAAAGGAACAAC
Cyp2c29	NM_007815	GCTCAAAGCCTACTGTCA	CATGAGTGTAAATCGTCTCA
Cyp3a11	NM_007818	TCACACACACAGTTGTAGGCAGAA	GTTTACGAGTCCCATATCGGTAGAG
Elovl6	NM_130450	TCTGATGAACAAGCGAGCCA	TGGTCATCAGAATGTACAGCATGT
Fasn	NM_007988	AGTCAGCTATGAAGCAATTGTGGA	CACCCAGACGCCAGTGTTC
Gck	NM_010191	AGGAGTTCTATAACCTGCTGCGAT	GGTCTTCAAGCTGCTGCTGAGT
Gpat	NM_0081449	AGACGAAGCCTTCCGACGA	TGGACATGATAGCGCAGGACT
Lpk	NM_013631	TCGACTCAGAGCCTGTGGC	AGTCGTGCAATGTTCATCCCT
Pparγ	NM_001127330	GACCAACAGCCTGACGGG	TGAATATCAGTGGTTCACCGCTT
Pepck	NM_011044	GAACCCCAGCCTGCCC	GAGCAACTCCAAAAAACCCG
Pnpla2	NM_025802	AGTGTCCTTCACCATCCGCTT	GGATATCTTCAGGGACATCAGGC
Pnpla3	NM_054088	ACGCGGTCACCTTCGTGT	AGCCCGTCTCTGATGCACTT
Pnpla5	NM_029427	GCTACTGCCCTGTACACTGCC	GATGCTCCTCATCCAATCCAA
Scd1	NM_009127	CAGTGCCGCGCATCTCTAT	CAGCGGTACTCACTGGCAGA
Srebp1c	NM_011480	GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGGCCAG
Tbp	NM_013684	ATAGGATGAGGGTCTTAAGCCTGTG	GGTTTGTATATGCTTGGGCCAG
Thrsp-Spot14	NM_009381	AACGGAGGAGGCCGAAGAAG	GTTGATGCACCTCGGGGTCT
Homo Sapiens			
ACC	NM_198834	CAGGCTGGCAGCTATGTTCA	GCCGGATCCCATGGTCA

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βACTIN	NM_001101	AGGCCCCCCTGAACCC	ATCACGATGCCAGTGGTACG
ChREBP	*NM_032951*	*TTCCTCGGCCATGACCC*	*AGGAGGAAATCAGAACTCAG GAAG*
CYP2B6	*NM_000767*	*GGAGGAGCGGATTCAGGAG*	*TGGAATCGTTTTCCAAAGACG*
LPK=PKLR	NM_000298	TGCTGCTGCCATCATTGTG	GGTACCGAGACAGAAGCTGG G
PNPLA3	NM_025225	ACCCTCTCGCCCAGGCT	CACCTTTGTCTTTCATTTCTTC ACTC
PSMB6	NM_001270481	CAGAACAACCACTGGGTCCTA CA	AGCAGAAAATGCGGTCGTG
SCD1	NM_005063	CCTGCAAGTTCTACACCTGGC T	CCCAGGGCACTGACAAAATA G
SREBP1C	NM_004176.4	GGCGAGCCGTGCGAT	TGCCCCACTCCCAGCAT

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Table 2. Clinical characteristics of the liver donors.

Liver Identification	Sex	Age	Pathology
PHH367	M	59	Cholangiocarcinoma
PHH374	F	41	Adenoma
PHH366	M	62	Metastasis of colorectal cancer
PHH486	F	78	Hepatocellular carcinoma

F = female; M = male; PHH = primary human hepatocyte; Age in years.

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Table 3. Body weight, relative liver weight and plasma parameters

	WT vehicle	WT TCPOBOP	CAR-/- vehicle	CAR-/- TCPOBOP
Mean body weight (g)	23.8 ± 0.60	23.7 ± 0.63	25.6 ± 0.77	25.5 ± 0.89
Liver : body weight ratio	0.041 ± 0.002	0.069 ± 0.001[b]	0.044 ± 0.002	0.046 ± 0.001
Plasma glucose (mmol/L)	5.32 ± 0.30	4.19 ± 0.41[a]	7.72 ± 0.32[b]	7.65 ± 0.56
Plasma insulin (ng/mL)	0.39 ± 0.06	0.30 ± 0.04	0.56 ± 0.14	0.49 ± 0.10
Plasma triglycerides (mmol/L)	0.85 ± 0.07	0.61 ± 0.02[a]	1.04 ± 0.11[b]	1.02 ± 0.07

Body weight, relative liver weight and plasma parameters of WT and CAR-/- mice (n=6 males) treated with either vehicle (corn oil) or TCPOBOP (3 mg/kg/day) for 3 days. The statistical significance was evaluated using two-way *ANOVA* followed by *Tukey's post hoc test (*a: treatment effect, TCPOBOP *vs* vehicle; b, genotype effect, CAR-/- *vs* WT*;* $p<0.05$). Values are mean ± SEM.

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Highlights

- Induction of hepatic glycolytic and lipogenic genes upon CAR activation by TCPOBOP
- These effects are not mediated by the nuclear receptor LXR
- CAR activation resulted in hepatic lipid accumulation
- Pnpla3 expression is regulated by CAR in mouse liver and human hepatocytes

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