The nuclear receptor CAR (NR1I3) regulates serum triglyceride levels under conditions of metabolic stress

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Abstract The nuclear receptor constitutive androstane receptor (CAR) (NR1I3) regulates hepatic genes involved in xenobiotic detoxification as well as genes involved in energy homeostasis. We provide data that extend the role of CAR to regulation of serum triglyceride levels under conditions of metabolic/nutritional stress. The typically high serum triglyceride levels of ob/ob mice were completely normalized when crossed onto a Car<sup>−/−</sup> (mice deficient for the Car gene) genetic background. Moreover, increases in serum triglycerides observed after a high-fat diet (HFD) regime were not observed in Car<sup>−/−</sup> animals. Conversely, pharmacological induction of CAR activity using the selective mouse CAR agonist TCPOBOP during HFD feeding resulted in a CAR-dependent increase in serum triglyceride levels. A major regulator of hepatic fatty oxidation is the nuclear receptor PPARα (NR1C1). The expression of peroxisome proliferator-activated receptor alpha (PPARα) target genes was inversely related to the activity of CAR. Consistent with these observations, Car<sup>−/−</sup> animals exhibited increased hepatic fatty acid oxidation. Treatment of mice with 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) significantly decreased expression of PPARα mRNA as well as Cyp4a14, CPT1α, and cytosolic Acyl-CoA thioesterase (CTE) in the liver.

These data have implications in disease therapy such as in the treatment of diabetes and nonalcoholic steatohepatitis (NASH).—Maglich, J. M., D. C. Lobe, and J. T. Moore. The nuclear receptor CAR (NR1I3) regulates serum triglyceride levels under conditions of metabolic stress. J. Lipid Res. 2009. 50: 439–445.

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Abnormally high serum triglycerides are a hallmark of metabolic syndrome/type 2 diabetes, nonalcoholic fatty liver disease (NAFLD), and nonalcoholic steatohepatitis (NASH). Research indicates that the high serum triglycerides associated with these diseases has a complex etiology. A better understanding of the molecular mechanisms that underpin dyslipidemia should facilitate improved strategies for lipid management.

Peroxisome proliferator-activated receptor alpha (PPARα) (NR1C1) agonists (e.g., fibrates) are a common treatment of diabetic dyslipidemia/primary hypertriglyceridemia. One of the major effects of PPARα activation by fibrates is decreased hypertriglyceridemia due to increased free fatty acid β-oxidation in the liver (1). The nuclear receptor PPARα stimulates gene transcription by binding to peroxisome proliferators response elements (PPRE) in the promoter of target genes (2). Many PPARα target genes are involved in pathways connected to lipid metabolism, including mitochondrial and peroxisomal fatty acid oxidation, as well as fatty acid uptake and transport. Through regulation of these target genes, PPARα has a strong influence on plasma lipid levels (3). The nuclear receptor CAR (NR1I3), on the other hand, has traditionally been associated with xenobiotic metabolism. More recently, the role of CAR has been expanded and now includes regulation of multiple metabolic processes, including pathways affecting bile acid and cholesterol/HDL metabolism (4, 5).

PPARα and CAR are induced in liver in response to fasting (6, 7). It has recently been shown that PPARα agonists lead to increased CAR expression via a PPRE (direct repeat 1, or DR1) in the CAR promoter (6). These same studies show that fasting-induced increases in free fatty acids (many of which are natural ligands for PPARα) induce CAR expression via PPARα activation. The purpose of this study was to a) look for evidence of corresponding effects of CAR on PPARα activity, and b) assess whether those effects are reflected in any changes in lipid metabolism in mice.

MATERIALS AND METHODS

Q-PCR/primer probes

Real-time quantitative PCR (RTQ-PCR) was performed using an ABI PRISM 7900 Sequence Detection System instrument

Abbreviations: CAR, constitutive androstane receptor; Car<sup>−/−</sup> mice, mice deficient for the Car gene; CTE, cytosolic Acyl-CoA thioesterase; HFD, high-fat diet; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PPARα, peroxisome proliferator-activated receptor alpha; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; WT, wild-type.

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and software (Applied Biosystems, Inc., Foster City, CA). Ten micrograms of the RNA samples were treated with 20 U/ml RNA-free DNase I for 30 min at 37°C followed by inactivation at 75°C for 5 min. Samples were quantitated by spectrophotometry and diluted to a concentration of 10 ng/μL. Samples were then converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA). Samples were assayed in duplicate or triplicate 10 μl reactions using 10 ng of cDNA per reaction. Gene-specific primers were used at 900 nM per reaction, and the gene-specific probe was used at 200 nM per reaction. Primers and probes were designed using Primer Express Version 2.2.2 (Applied Biosystems) and synthesized by Biosearch Technologies (Novato, CA). All primers and probes were entered into the NCBI Blast program to ensure specificity. Sequences are as follows: Elov15 (NM_134255, f-AGGTGTTGGAGGCGAAAATA, r-GAATTGATGAAATGGTGAAG; GAGTGTAAAGCAGTGCCCTG, p-ACAAAGGCGGATGCTGAGG); CYP4a14 (NM_007822, f-CGAGACGCGCTGAGTCTTCC, r- GAGCTCCTTGTCCTCTCAGAG, p-ATGCATGCCCCTCCATGCTT); PPARα (X57688, f-ACTGTGCTGCTCCTCTCATTATG, r-GTGTGTAATAAGGCAGTCCGCGGC, p-ACAAGAGGCGGATCTGATGGCGG); PPARGAAGTAC, p-TCCGATATGAAGATCATCGCGTCCTC); CYP2B10 (NM_009998, f-GGAGTACTTTGAAGAAGCCGTGAA, r-CCCAAGCACAGTGCTGCTT, p-ACCCTAGACACCACTGGCCGCATGT); CTE (NM_012006, f-AAGGCAGTCCCGCGCGGCAAT, r-GGATCGGCTCCTACCTCCTCC, p-ACACGTACGCTGCTGCTGCTT); CYC (NM_000998, f-CCTAAGGACCTCACCGGCAGG, r-GGATCGGCTCCTACCTCCTCC, p-ACACGTACGCTGCTGCTGCTT); Cyclophilin (NM_000807, f-CAGAAGGACCGAGCAGC, r-CGGATCGGCTCCTACCTCCTCC, p-CGGATCGGCTCCTACCTCCTCC).

Creation of Car²⁻/⁻ Lep⁻/- double knock-out mice
All procedures performed were in compliance with the Animal Welfare Act and United States Department of Agriculture regulations and approved by the Glaxo-SmithKline Institutional Animal Care and Use Committee. Car²⁻/⁻ mice were generated by Deltagen, Inc. (Redwood City, CA; www.deltagen.com) according to manufacturer directions. Targeting a vector that deletes nucleotides 38–159 of the Car open reading frame. This targeting event removes the first zinc finger of the DNA binding domain and results in a frame-shift. The resulting protein product is expected to have neither the genomic nor nongenomic properties (e.g., ability to recruit cofactor proteins to the ligand-binding domain) of wild-type CAR. Embryonic stem cells derived from the 129/Sv-P.MgSFlJ/l mouse strain were used to generate chimeric mice. F1 mice were generated by breeding with C57BL/6 females. Offspring were screened by PCR analysis of DNA obtained from tail biopsies to identify those heterozygous for the mutant Car allele. The heterozygous offspring were then intercrossed to obtain mice homozygous for the Car mutation. Car²⁻/⁻ mice were normal in terms of gross morphology and bred with normal Mendelian characteristics. The Car²⁻/⁻ mice were backcrossed on to C57BL/6 mice using Marker Assisted Backcrossing-in female to N7 and these mice were transferred to Taconic (Hudson, NY; www.taconic.com) at generation N7. From there, the mice were further backcrossed to C57BL/6 to N10, and then were mated Car⁰⁻/- x Car²⁻/- to generate the Car²⁻/- production colony.

Car⁰⁻/- mice were intercrossed with Lep⁻/- mice from the Taconic OB line. These generated Car²⁻/-/Lept⁻/- mice, which were then mated together to produce the Car²⁻/-/Lept⁻/- breeders, which is how the line was maintained. Mice were maintained on standard laboratory chow and allowed food and water ad libitum. Average Car²⁻/- animal weights (25–30 g) were different from wild-type (WT), and Car⁰⁻/- Lep⁻/- mice were not different from standard Lep⁻/- animal weights (50–60 g). In each in vivo study described, at least 4–5 animals, 10–12 weeks of age, were used.

**HFD²⁻/- TCPOBOP treatment**
Male 10–12-week-old Car²⁻/⁻ and C57BL/6 wild-type mice (4–5 mice/group) were utilized for gene expression analysis. For the pharmacological studies, C57BL/6, wild-type or Car²⁻/⁻ were treated with the CAR-selective ligand TCPOBOP by p.o. at 0.3 mg/kg once daily for 14 days, at which time blood and livers were harvested. Total RNA from liver samples was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Animals were maintained on a 60% high-fat diet (HFD) (Research Diets, D12492) or normal chow (Purina 5001) for 14 days or 8 weeks (significant changes in serum triglyceride levels required 8 weeks to manifest).

**Serum chemistry**
Serum triglyceride measurements were performed on the Olympus AU460® clinical chemistry analyzer (Olympus America Inc., Melville, NY) according to the manufacturer’s instructions.

**Determination of tissue triglyceride content**
Liver tissue was homogenized in homogenization buffer (0.1M HEPES, pH 7.4/0.1M NaCl, 1 mM EDTA) to achieve a 20% tissue homogenate with 800 μL saline in a cell homogenizer using a Polytron motor-driven homogenizer. The tissue was then extracted by combining 200 μL of 20% tissue homogenate with 800 μL saline. All procedures performed were in compliance with the Animal Welfare Act and United States Department of Agriculture regulations and approved by the Glaxo-SmithKline Institutional Animal Care and Use Committee. Car²⁻/⁻ mice were generated by Deltagen, Inc. (Redwood City, CA; www.deltagen.com) according to manufacturer directions.

**Livers from fed mice were surgically removed and a section excised from the same lobe and immediately weighed, minced with scissors, and placed on ice. Cold SET buffer (250 mM Sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4) was added at a ratio of 10 ml SET:1 g of tissue and the tissue homogenized on ice (15 s for liver; 2 × 20 s bursts for muscle) using a hand-held homogenizer (Polytron PT1200; Kinematica AG). The homogenates remained on ice until assayed.

The labeled reaction buffer was prepared by first drying 14C-oic acid (0.5uCi/reaction; PerkinElmer #NEC-317) under nitrogen. The dried fraction was resuspended in unlabeled oic acid such that the final oic acid concentration of the reaction buffer was 0.2 mM. BSA was added slowly while mixing to a final concentration of 0.5% and the mixture was incubated at 37°C for 15 min. After incubation, the labeled cocktail was added to the reaction buffer containing 100 mM sucrose, 10 mM Tris pH 7.4, 4 mM ATP, 0.05 mM CoA, 0.1 mM malic acid, 1 mM magnesium chloride, 80 mM potassium chloride, 5 mM potassium phosphate, 0.2 mM EDTA and 2 mM L-carnitine, as described previously (8, 9). Oxidation reactions were performed in tubes fitted with a stopper top, center well, and filter (Socorex #322.02) soaked with 175 μL of IN NaOH. 100 μl of homogenate was dispensed into each tube and the reactions initiated by adding 400 μl of reaction buffer. The tubes were quickly capped and incubated for 1 hour at 37°C.
with gentle shaking for 60 min in a 37°C water bath. After incubation, the filters were removed from the tubes, placed in 7 ml of scintillant, and captured dpm counted for 2 min. The oxidative activity was calculated as nmole CO₂ captured/mg tissue/hour.

**Preparation of samples for Affymetrix chip analysis**

Sample preparation for hybridization to the GeneChip arrays included isolation of total RNA from liver using the Trizol method according to manufacturer’s (Invitrogen) instructions, synthesis of double-stranded cDNA, biotin-labeling using in vitro transcription to produce labeled cRNA and fragmentation of the cRNA. Next, a hybridization cocktail was prepared containing the fragmented, biotin-labeled sample and probe array controls. The hybridization cocktail was added to the GeneChip probe array and a 16 h incubation step was carried out overnight in the hybridization oven. Following hybridization, the sample was stained with streptavidin phycoerythrin conjugate in an automated staining and washing protocol using a fluidics station. Finally the probe array was scanned and the data analyzed using PowerArray (National Institute of Statistical Sciences, Research Triangle Park, NC).

**RESULTS**

Inverse relationship observed between PPARα target gene expression and CAR activity

It has been previously established that chronic HFD (26 weeks, 45% fat) increases PPARα activity in liver (10). We assessed the expression of three PPARα target genes (Cyp4a14, CPT1α, and CTE) as surrogate markers of PPARα transcriptional activity during high-fat feeding in a WT and CAR knock-out background. We also measured the levels of PPARα mRNA itself. In our study, Car⁻/⁻ mice and their WT siblings were maintained on either regular chow or 60% fat chow (HFD) for 14 days. Consistent with previous studies, we found that PPARα mRNA and PPARα target gene mRNAs are increased after 14 days on a HFD relative to normal diet-fed mice (Fig. 1).

Two pieces of evidence indicated an inverse relationship between CAR activity and PPARα activity. First, treatment of WT animals with the potent and selective CAR agonist
were dependent on CAR activity and PPARalpha target gene levels were also increased by HFD alone. Thus, CAR activity and PPARalpha target gene responses were shown to be inversely related under the conditions tested.

Increased serum triglyceride phenotype in ob/ob mice is dependent on CAR

Diabetic rodent models, such as leptin-function deficient mouse strains (ob/ob and db/db), display multiple metabolic defects. We bred Lep2−/− (ob/ob) mice onto a Car2−/− background and assessed serum triglyceride levels (Fig. 2A). As expected, sibling Lep2−/− mice showed high levels of serum triglyceride (335 mg/dl), more than 3-fold the range normally seen in adult male mice. In dramatic contrast, serum triglyceride levels in Lep2−/− mice on a Car2−/− background were completely normalized.

Car2−/− and HFD effects on serum triglyceride levels

Maintenance of normal mice on HFD leads to decreased insulin sensitivity and increased serum triglycerides. We assessed the effects of HFD (8 weeks, 60% fat) in Car2−/− mice versus age-matched WT siblings. In the HFD feeding model, we found that increases in total serum triglyceride levels were dependent on Car (Fig. 2B). Serum triglyceride levels increased in WT mice on HFD (164 mg/dl to 218 mg/dl), whereas no significant increases were seen in Car2−/− mice.

CAR agonist increases serum triglyceride levels

To test the relationship between CAR activity and serum triglyceride levels pharmacologically, we treated WT and Car2−/− animals with the mouse CAR-selective agonist, TCPOBOP. Mice were treated daily for 14 days with i.p. injection of TCPOBOP, followed by gene expression and serum chemistry analysis. To control for TCPOBOP activity, the CAR target gene Cyp2b10 was analyzed by RTQ-PCR and shown to be induced 114-fold (P < 0.001) by TCPOBOP in these experiments. In the serum chemistry analysis, we found that treatment with TCPOBOP induced a CAR-dependent 50% increase in serum triglycerides (Fig. 2C).

Serum triglyceride decreases not due to hepatic triglyceride increases

The effect of CAR in regulating serum triglycerides could be accounted for by several different mechanisms. We first wanted to rule out that the decrease in serum triglycerides resulting from CAR deletion was not simply due
to accumulation of triglycerides in the liver. We found that the liver weight to body weight ratios remained unchanged in WT and CAR-/- after HFD for 8 weeks (Fig. 3A). We also directly assessed liver triglyceride levels and found that there were no significant increases in WT or Car-/- mice after HFD (Fig. 3B). These data indicated that the lower serum triglyceride levels observed in Car-/- animals (Fig. 2) were not due to a build up of excess triglycerides in the liver.

Hepatic fatty acid oxidation was increased in Car-/- animals

Car-/- animals (10–12 weeks) displayed increases in PPARα target genes versus WT littermates (Fig. 1). We measured hepatic fatty acid oxidation to see whether this would be consistent with the observed changes in gene expression. Indeed, when fatty acid oxidation levels were directly measured via the capture of 14C-labeled CO2 from tissue homogenates, a significant increase in Car-/- animal livers was observed (Fig. 4). These data provide one mechanism for the changes in serum triglycerides in the Car-/- seen after HFD.

Affymetrix chip analysis of Car-/- mice after TCPOBOP treatment

Liver mRNAs were subjected to Affymetrix chip analysis from WT and Car-/- mice before and after TCPOBOP treatment (see Materials and Methods). As expected, TCPOBOP up-regulation of previously described prototypical CAR target genes such as Cyp2b was observed. Two novel CAR regulated genes were also found in this study. A CAR-dependent decrease in the ELOVL5 mRNA was seen under conditions of agonist dosing in Affymetrix studies and was confirmed by real-time PCR (Fig. 5A, P = 0.001 in regular diet and 0.002 in HFD study). Also, treatment of animals with the CAR-agonist TCPOBOP resulted in a decrease in Lipin1 (Fig. 5B, P = 0.002 in regular diet and 0.03 in HFD study). No statistically significant differences were seen after TCPOBOP treatment for either Elovl5 or Lipin1 in CAR-/- animals, consistent with a CAR dependence of the TCPOBOP effects. The Lipin1 gene product selectively activates a subset of PGC-1α target pathways, including fatty acid oxidation and mitochondrial oxidative phosphorylation, while suppressing the lipogenic program and lowering circulating lipid levels (11). The Elovl5 gene product synthesizes very long chain polyunsaturated fatty acids (PUFAs) in the liver (12, 13). PUFAs have been shown to be exceptionally potent inducers of PPARα activity (14). Thus, the CAR-dependent decrease of both of these gene products would be predicted to result in decreased PPARα activity.

DISCUSSION

Increased understanding of metabolic pathway regulation in liver should aid in the identification of new targets and strategies for treatment of metabolic disorders such as diabetes, obesity, and fatty liver disease. The seemingly diverse metabolic pathways that regulate hepatic carbohydrate

Fig. 3.  A: Liver weight/body weight ratio was assessed in Car-/- relative to WT littermates after HFD (8 weeks). B: Hepatic triglyceride levels in Car-/- and WT littermates after regular chow diet and HFD (8 weeks). Nine or ten animals were used for each experimental condition.

Fig. 4. Hepatic fatty acid oxidation measurements. Livers from 10–12-week-old WT and Car-/- animals were harvested and hepatic fatty acid oxidation levels were directly measured via the capture of 14C-labeled CO2 from tissue homogenates. A mean value of 2.23 nmol CO2/mg tissue/hr were observed in Car-/- animals and a value of 1.87 in WT animals (P < 0.001). A P value was generated using a one-way Analysis of Variance (ANOVA). Four or five animals were used for each experimental condition.
The capacity for mitochondrial fatty acid β-oxidation is a critical determinant of hepatic lipid balance in the context of high levels of circulating fatty acids and triglyceride-rich lipoproteins. The expression of genes encoding enzymes involved in hepatic β-oxidation is under dynamic transcriptional control by PPARα (16). The decreased serum triglyceride phenotype of both the HFD mouse model and the ab/ob mouse model in a Car−/− background are consistent with the model that PPARα activity is increased in the absence of CAR. Furthermore, the increase in hepatic fatty acid oxidation we measured in Car−/− mice (Fig. 4) provides a mechanism of action consistent with increased PPARα activity.

The CAR-dependent regulation of the ELOVL5 and Lipin1 genes provides possible mechanisms by which CAR may regulate PPARα activity. In both cases, increased CAR activity down-regulates expression of the genes. The fact that the two gene products alter PPARα at different levels of regulation may render their combined effects more substantial than either acting alone. The ELOVL5 gene product controls the synthesis of potent PPARα ligands (12–14) while Lipin1 controls levels of expression of PPARα (11). We postulate that least part of the influence of CAR on decreasing PPARα activity and consequently PPARα target genes may be through these combined indirect mechanisms.

Other published reports also indicate possible indirect links between CAR activity and PPARα activity. For example, CAR inhibits the transcriptional activity of another nuclear receptor in liver, HNF4α (17). The ability of HNF4α to modulate PPARα activity has previously been established (18).

PPARα is regulated by interaction with PGC-1α (19), as is CAR (20). Previous work has demonstrated that mice deficient for either PPARα (21) or PGC-1α (22) exhibit fasting-induced hepatic steatosis. The fasting-induced lipid accumulation seen in these models is likely due to diminished capacity for fatty acid catabolism in the face of increased hepatic delivery of free fatty acids, illustrating the critical role of the hepatic PPARα/PGC-1α system in matching fatty acid oxidative capacity to substrate availability.

PPARα appears to be dysregulated in the liver in diseases with associated fatty liver. Studies show that an overload of free fatty acids in the liver invokes a kind of “PPARα-resistance” (i.e., PPARα activity is decreased rather than increased as would be expected) (10, 23). In fact, reduced PPARα activity has been reported to play a central role in the development of NAFLD (24, 25). Interestingly, in diabetic and NAFLD rodent models, CAR activity (as measured by up-regulated expression of its target gene, Cyp2b10) is increased (26–29). Moreover, our lab has also shown that Cyp2b10 mRNA is expressed at much higher levels in Ab/Lepob/ob (leptin-deficient mice) mice compared with their lean littermates (data not shown). According to the model presented in this paper, the over-expression of CAR may play a role in the observed “PPARα resistance” seen in these hepatic steatosis models.

Pharmacological agents that block CAR activity in these conditions may provide a therapeutic option to treat fatty liver diseases. Indeed, CAR inverse agonists have recently been proposed as a treatment for NASH because increased

and triglyceride homeostasis are inter-related. Many aspects of these are governed by nuclear receptors (examples include LXRα/β, PPARα/β/γ, FXR, and HNF4α). In this manuscript, we provide evidence that CAR is included in the list of nuclear receptors that regulate key metabolic pathway in liver and represents a potential target for metabolic disease.

A novel aspect of the proposed model is that CAR exerts some of its metabolic effects via negative regulation of PPARα activity. Interestingly, CAR expression is decreased in PPARα knock-out animals, providing more evidence for the reciprocal link between these two receptors under different physiological conditions (15). We have shown previously that, like PPARα, CAR activity is induced by fasting (7). We have not yet determined whether CAR blocks PPARα mRNA induction under these conditions.
CAR activity caused the worsening of the hepatic injury and fibrosis in a dietary model of NASH (30). It is notable that one advantage of a CAR-based therapeutic approach is that it provides the means to activate PPARα in a liver-specific manner, thus potentially limiting extra-hepatic side effects of traditional PPARα agonists.  

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REFERENCES