Published in final edited form as:
Toxicol Appl Pharmacol. 2012 September 1; 263(2): 131–137. doi:10.1016/j.taap.2012.03.017.

Opposing Regulation of Cytochrome P450 Expression by CAR and PXR in Hypothyroid Mice

Young Joo Park[1,2], **Eun Kyung Lee**[1,4], **Yoon Kwang Lee**[3,5], **Do Joon Park**[1], **Hak Chul Jang**[1], and **David D. Moore**[3]

[1]Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea

[2]Seoul National University Bundang Hospital, Seoul, Korea

[3]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA

Abstract

Clinical hypothyroidism affects various metabolic processes including drug metabolism. CYP2B and CYP3A are important cytochrome P450 drug metabolizing enzymes that are regulated by the xenobiotic receptors constitutive androstane receptor (CAR, NR1I3) and pregnane X receptor (PXR, NR1I2). We evaluated the regulation of the hepatic expression of CYPs by CAR and PXR in the hypothyroid state induced by a low-iodine diet containing 0.15% propylthiouracil. Expression of Cyp3a11 was suppressed in hypothyroid C57BL/6 wild type (WT) mice and a further decrement was observed in hypothyroid $CAR^{-/-}$ mice, but not in hypothyroid $PXR^{-/-}$ mice. In contrast, expression of Cyp2b10 was induced in both WT and $PXR^{-/-}$ hypothyroid mice, and this induction was abolished in $CAR^{-/-}$ mice and in and $CAR^{-/-}$ $PXR^{-/-}$ double knockouts. CAR mRNA expression was increased by hypothyroidism, while PXR expression remained unchanged. Carbamazepine (CBZ) is a commonly used antiepileptic that is metabolized by CYP3A isoforms. After CBZ treatment of normal chow fed mice, serum CBZ levels were highest in $CAR^{-/-}$ mice and lowest in WT and $PXR^{-/-}$ mice. Hypothyroid WT or $PXR^{-/-}$ mice survived chronic CBZ treatment, but all hypothyroid $CAR^{-/-}$ and $CAR^{-/-}$ $PXR^{-/-}$ mice died, with $CAR^{-/-}$-$PXR^{-/-}$ mice surviving longer than $CAR^{-/-}$ mice (12.3 ±3.3 days vs. 6.3 ±2.1 days, p=0.04). All these findings suggest that hypothyroid status affects xenobiotic metabolism, with opposing responses of CAR and PXR and their CYP targets that can cancel each other out, decreasing serious metabolic derangement in response to a xenobiotic challenge.

Keywords

thyroid hormone; cytochrome P450; xenobiotic receptor

Corresponding author: David D. Moore: moore@bcm.edu, phone 713-798-3313, fax 713-798-3017.
[4]EKL current address: Center for Thyroid Cancer, National Cancer Center, Goyang-si, Korea
[5]YKL current address: Department of Integrative Medical Sciences, Northeast Ohio Medical University, Rootstown, OH 44272, USA

Y.J. Park and E.K. Lee contributed equally to the studies presented in this manuscript and should be considered co-first authors.

Conflict of Interest Statement: NONE

INTRODUCTION

Thyroid hormone is one of the most important regulators of metabolic processes, including drug metabolism (Lahtela *et al.*, 1985; Surks and Sievert, 1995). Hypothyroidism, a condition of thyroid hormone deficiency is quite prevalent, affecting about 1~2% of the general population. Among a number effects of hypothyroidism, animal data have shown that thyroid dysfunction can alter the expression of various drug-metabolizing enzymes, such as cytochrome P450s (CYPs), sulfotransferases, and uridine 5'-diphosphate-glucuronosyltransferases (Rosenberg *et al.*, 1995; Webb *et al.*, 1996; Badger *et al.*, 1998; Liddle *et al.*, 1998). CYPs are rate-limiting for drug metabolism and their induction accelerates metabolic clearance of a drug or its metabolites, while decreased CYP activity can lead to elevated accumulation of the xenobiotics, with potentially harmful effects.

CYP3A isoforms are widely recognized to be key enzymes responsible for the metabolism of 50–60% of all prescription drugs (Danielson, 2002); CYP2B enyzmes comprise another major subgroup (Xie and Evans, 2001) that is especially involved in metabolism of the anticancer drug cyclophosphamide (Roy *et al.*, 1999), the anesthetics ketamine and propofol (Court *et al.*, 2001) and the central nervous system active agents mephobarbital and bupropion (Hidestrand *et al.*, 2001). In hypothyroid patients, decreased CYP3A activity was associated with accumulation of the immunosuppressive agent Tacrolimus, resulting in renal failure (Haas *et al.*, 2000). Thus, the impact of hypothyroid status on drug metabolism has significant potential clinical significance, but the basis for the impact of hypothyroidism on CYP expression is not understood.

Pregnane X receptor (PXR, NR1I2); and constitutive androstane receptor (CAR, NR1I3) are nuclear xenobiotic receptors that are well known to mediate the hepatic regulation of various genes involved in drug metabolism, such as CYP3A4 (Kliewer *et al.*, 1999; Willson and Kliewer, 2002) and CYP2B6 (Honkakoski and Negishi, 2000), respectively, in humans. It was reported that thyroid hormone modulates expression of CAR in rat hepatocytes (Ooe *et al.*, 2009), while the effect of thyroid hormone on PXR and CYP3A has not been elucidated. We hypothesized that thyroid hormone could alter CYP expression by affecting the transcriptional activity of CAR and PXR. In this study, we investigated the impact of hypothyroid status on both basal expression of the mouse counterparts of CYP3A4 and CYP2B6, Cyp3a11 and Cyp2b10, respectively, and their regulatory responses to CAR or PXR activation.

MATERIALS AND METHODS

Animals

Animals used in all experiments were age-matched (12–14-week-old) male C57BL/6 mice (wild type; WT). To investigate the roles of the xenobiotic nuclear receptors CAR and PXR in hypothyroidism, we used age-matched congenic knockout mice model of CAR ($CAR^{-/-}$), PXR ($PXR^{-/-}$), and both CAR and PXR ($CAR^{-/-}PXR^{-/-}$) (Wei *et al.*, 2000; Staudinger *et al.*, 2001). The mice were housed in groups of four or five in plastic microisolator cages at 22°C with a 12-h light/12-h dark cycle and free access to food and water. All protocols for animal use and euthanasia were approved by the Institutional Animal Care and Use Committee at Seoul National University Bundang Hospital and all of the tissue samples were collected following an Institutional Review Board-approved protocol. All animals (n=4 or 5 in each group) were sacrificed after fasting for 6 h from 06:00 a.m. All experiments were done twice to confirm results.

All animals (WT, $CAR^{-/-}$, $PXR^{-/-}$, $CAR^{-/-}PXR^{-/-}$) were divided into two groups each: control (normal diet; ND, Purina irradiated laboratory chow 38057, Purina Korea, Seoul, Korea) and

propylthiouracil/low iodine (PTU/LI) treatment group. The PTU/LI group mice were fed a low iodine contained diet supplemented with 0.15 % PTU for four weeks to induce hypothyroidism (Mizuno *et al.*, 2004). The serum tetraiodothyronine (T4) level was 1.52 ± 0.54μg/dL in the control group and 0.99 ± 0.19μg/dL in the PTU/LI group. Serum free T4 was 0.31 ± 0.14ng/dL in the control group and 0.19 ± 0.05ng/dL in the PTU/LI group. Carbamazepine treatment (CBZ), mainly metabolized by CYP3A (Kang *et al.*, 2008), was mixed to the food at a concentration of 3.5 g/kg, and was supplied to mice for four weeks. In addition, to observe short-term effect of CBZ, mice were given liquid supplement CBZ (20mg/ml) by oral gavage (30mg/kg body weight) using a gastric sonde 2 hours before sacrifice.

Cell culture

The human hepatoblastoma cell line HepG2 was maintained in 75 cm^2 culture flasks with minimal essential medium (MEM) supplemented with 10% fetal bovine serum and incubated at 37° and 5% CO_2, and used for luciferase assay. Because HepG2 cells generally do not express endogenous CAR (Chu *et al.*, 2009), we used cotransfected CAR in the promoter studies and also used primary hepatocytes for measurement of CYP mRNA. Hepatocytes were isolated from adult C57BL/6 mice and cells were cultured as described (Galle *et al.*, 1995). Briefly, liver was perfused with Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY) containing 0.05% collagenase to disperse hepatocytes, and then isolated. Subsequently, the cell suspension was filtered through mesh filter, purified and washed by differential centrifugation with Percoll (Sigma, St.Louis, MO) and Williams medium E (WME; Gibco), respectively. Then, the cells were seeded in maintenance medium at a density of 5×10^5 viable cells/ml on 100mm collagen-coated tissue culture dishes (Nunc, Roskilde, Denmark). Viability determined by trypan blue dye exclusion. For seeding and maintenance of cells WME was used, supplemented with 10% fetal calf serum, triamcinolone (1mM), insulin (10mg/ml), penicillin(100U/ml) and streptomycin(0.1mg/ml). Cells were cultured at 37° C in a humidified atmosphere of 5% CO_2 in air.

Transient transfection of cells

One day before transfection, confluent cells were trypsinized and plated into 24-well plates with MEM supplemented with 10% charcoal-stripped serum to allow cells to reach 50-60% confluency at the time of transfection. To assess the effect of thyroid hormone receptor β (TRβ) to the CYP, HepG2 cells were transfected with receptor expression vectors (200 ng of pCMX-TRβ and 50 ng of pCDM8-RXRα (retinoid X receptor) or 250ng of empty vector) using Lipofectamine Plus (Invitrogen, Carlsbad, CA), according to the manufacturer's directions. To evaluate the transcriptional activity of CYP3A4 and CYP2B6, HepG2 cells were cotransfected with 50ng of the CYP3A4 or Cyp2b10-luciferase reporter, and an equal amount of RSV-β-galactosidase plasmid as the internal control. All expression vectors (Full-length human TRβ expression vector) and reporter vectors (CYP3A4-luc and Cyp2b10-luc) were generated as previously described (Goodwin *et al.*, 1999; Xie *et al.*, 2000). After 24 hour, the cells were treated with the pregnenolone-16α-carbonitrile (PCN, 10nM) as a PXR agonist, or 1,4-Bis[2-(3,5-dichloro-pyridyloxy)]benzene (TCPOBOP, 10nM) as a CAR agonist. Because HepG2 cells do not express human CAR, they were cotransfected with mouse CAR and treated with or without TCPOBOP. Cells were harvested and assayed for luciferase activity 24 hour after the addition of the ligands, and the reporter expression was normalized to the β-galactosidase activities using Dual luciferase reporter assay system following the manufacturer's instruction. Similar results were obtained from at least 2 independent experiments performed in triplicate.

Liver tissues for primary hepatocyte culture were collected from euthyroid WT mice. Primary hepatocytes were seeded in 6-well plates at density of 5×10^5 cells per well. After

overnight incubation, the cells transfected with expression vector (250ng of TRβ and 60ng of RXRα or 310ng of empty vector), incubated for 24 hours and harvested to extract mRNA.

Northern blotting and quantitative real-time Polymerase Chain Reaction analysis

Total RNA from livers was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and Rneasy kit(Qiagen, Valencia, CA) according to the manufacturer's instruction. Primary hepatocytes also were washed by PBS, followed by RNA extraction. Single-stranded cDNA was synthesized from 20μg of total RNA (for northern blotting) or 1ug of total RNA(for Q-PCR) in a 20μL reaction and 5uM oligo(dT) primer (synthesized by Bioneer, Seoul, Korea). RNA from each treatment group was loaded on 1% agarose gel and transferred to a nylon membrane (HybondN, Amersham Biosciences, Piscataway, NJ). Blots were hybridized using ULTRAHyb solution (Ambion, Austin, TX) with ^{32}P-labeled cDNA probes at 42 °C overnight. The membranes were washed in 2× SSC and 0.1% SDS solution for 1 h and exposed to X-ray film. Isotope probes attached in the membrane were detached with a Strip-EzDNA kit (Ambion, Austin, TX). The blots were subsequently reprobed with a radiolabeled β-actin cDNA. The levels of target gene mRNA were normalized with the levels of β-actin mRNA. Probes were used as previously described (Wei *et al.*, 2002).

Quantitative real-time PCR (Q-PCR) was carried out on single-stranded cDNA samples using TaqMan PCR kit(Applied Biosystems, Inc., Foster City, CA) in 20uL reactions containing 0.5 μmol of forward and reverse primers, 10μL of Master Mix Samples, and 4μL of cDNA. Amplification was performed using ABI PRISM 7700 sequence detection system instrument and software (Applied Biosystems, Inc., Foster City, CA). Primer sequences were as followed: mouse Cyp3a11 (mouse isoform of CYP3A4) forward AGAACTTCTCCTTCCAGCCTTGT, reverse GAGGGAGACTCATGCTCCAGTTA, probe CTAAAGGTTGTGCCACGGGATGCAGT; mouse Cyp2b10 (mouse isoform of CYP2B6) forward CAATGTTTAGTGGAGGAACTGCG, reverse CACTGGAAGAGGAACGTGGG, probe CCCAGGGAGCCCCCCTGGA ; mouse CAR forward TCAACACGTTTATGGTGCAA, reverse CTGCGTCCTCCATCTTGTAG, probe CAGATCTCCCTTCTCAAGGGAGCG ; mouse PXR forward GCCGATGTGTCAACCTACAT, reverse CTCAGGATGCACATCTCAAA, probe TGAGGACCACATCTCCCTGCTGAA; mouse glyceraldehyde-3-phosphate dehydrogenase(Gapdh) forward CCAGAACATCATCCCTGCATC, reverse GGTCCTCAGTGTAGCCCAAGAT, probe CCGCCTGGAGAAACCTGCCAAGTATG; human CYP3A4 forward TCAATAACAGTCTTTCCATTCCTCA, reverse CTTCGAGGCGACTTTCTTTCA; human CYP2B6 forward AAGCGGATTTGTCTTGGTGAA, reverse TGGAGGATGGTGGTGAAGAAG; and human GAPDH forward ATGGGGAAGGTGAAGGTCGG, reverse GACGGTGCCATGGAATTTGC. Expression was determined for the gene of interest relative to a standard curve created by serial dilution of the PCR product and was normalized against the expression of the housekeeping gene Gapdh.

Measurement of serum carbamazepine and thyroid hormone levels

Carbamazepine (CBZ; Tegretol, Novatis, East Hanover, NJ), an antiepileptic drug mainly degraded by CYP3A, was administered to mice to estimate CYP activity in vivo from measurements of serum CBZ concentration. Blood was drawn from the retroorbital sinus, to determine serum T4, free T4 and CBZ levels. The amount of obtained blood was about 500 μL. Sera were separated by centrifugation at 3,000 rpm for 20 min. Serum T4 and free T4 levels were determined using a commercial radioimmunoassay kit (Abbott, North Chicago, IL). The serum CBZ level was measured by HPLC analysis (HP1200; Agilent, Santa Clara, CA) using 100 μL of mouse serum. The limit of quantitation was 0.5 mg/L.

Statistical analysis

SPSS (version 10.0; SPSS Inc., Chicago, IL) was used for statistical analysis. A Mann-Whitney test was used as a nonparametric analysis for comparisons of mean serum CBZ levels among groups. The survival curve was drawn via a Kaplan-Meier analysis with SPSS. P values < 0.05 were considered statistically significant.

RESULTS

Nuclear receptor dependent impact of hypothyroidism on CYP expression

Since hypothyroidism alters xenobiotic metabolism in mice, we hypothesized that Cyp3a11 and Cyp2b10 expression could be affected. Thus, we measured mRNA levels of Cyp3a11 and Cyp2b10 in the hypothyroid state, which was induced by 4 weeks of treatment with a diet containing 0.15% PTU and low iodine (PTU/LI). Unexpectedly, we observed opposing effects, with Cyp3a11 repressed, whereas Cyp2b10 expression was increased (Fig. 1A). These responses are not due to hepatic effects of PTU, since treating primary hepatocytes directly with PTU did not affect Cyp gene expression. Since the nuclear receptors CAR and PXR are well known regulators of Cyp3a11 and Cyp2b10, we also evaluated the levels of PXR and CAR mRNA in the hypothyroid state. The hepatic mRNA expression of CAR was remarkably increased, whereas PXR mRNA remained unchanged in PTU/LI-treated hypothyroid mouse (Fig. 1B).

To investigate whether the altered expression of Cyp2b10 and Cyp3a11 expression in hypothyroid liver depends on CAR or PXR, we used mouse knockout models of CAR, PXR, and both receptors. In $PXR^{-/-}$ mice, the induction of Cyp2b10 by hypothyroidism was even greater than that observed in WT mice (Fig. 2A). However, this response was reversed in $CAR^{-/-}$ mice, with the low basal levels of Cyp2b10 expression hardly detectable in control and PTU/LI $CAR^{-/-}$ mice. The response of the double $CAR^{-/-}PXR^{-/-}$ mice was indistinguishable from the $CAR^{-/-}$ mice. On the other hand, the expression of Cyp3a11 was suppressed by hypothyroidism in WT mice, with a further decrement of Cyp3a11 expression observed in $CAR^{-/-}$ mice (Fig. 2B). There was no significant change Cyp3a11 expression in $PXR^{-/-}$ or $CAR^{-/-}PXR^{-/-}$ mice in the hypothyroid state (Fig. 2B). Overall, these results indicate that CAR is activated in the hypothyroid state, and that this activation drives induction of its well known target Cyp2b10, while Cyp3a11 suppression is mainly mediated by PXR. The maintenance of basal expression of Cyp3a11 in $CAR^{-/-}PXR^{-/-}$ mice, which is consistent with previous results (Zhang *et al.*, 2004), means that it must depend on additional factors.

In the hypothyroid condition induced by treatment with the PTU/LI diet, TRβ exists primarily as an unbound form without thyroid hormone (unliganded TRβ). This unliganded receptor generally represses target gene expression, but can transactivate in some cases. Thus we explored the potential effects of unliganded TRβ on CYP gene expression *in vitro*. HepG2 cells cultured in charcoal-stripped serum containing media were transiently transfected with CYP3A4 or CYP2b10 reporter plasmids, and promoter activity was determined after 24h treatment with or without appropriate xenobiotic receptor ligands, and with and without TRβ cotransfection. CYP2B promoter activity was increased by TCPOBOP treatment, as expected, and this activated CAR response was further increased by unliganded TRβ (Fig. 3A). In contrast, CYP3A4 promoter activity was suppressed by unliganded TRβ, and this effect overcame the significant but modest induction in response to the PXR agonist PCN.

We also examined endogenous CYP gene expression in similar *in vitro* conditions in both primary mouse hepatocytes (Fig. 3B) and HepG2 cells (Fig. 3C). Unliganded TRβ repressed basal mRNA expression of Cyp3a11 in mouse primary hepatocytes, and that of Cyp3A4 in

HepG2 cells. In the absence of exogenous CAR activation, which mimics the *in vivo* studies, unliganded TRβ did not affect the low basal expression of Cyp2b10 or Cyp2B6. Overall, the results of these *in vitro* studies are consistent with the responses observed in the hypothyroid mice, and suggest that unliganded endogenous TR isoforms contribute to the *in vivo* results.

Roles of CAR and PXR in the impact of hypothyroidism on CBZ metabolism in vivo

To assess the physiologic outcome of these hypothyroid effects *in vivo*, we treated mice with carbamezipine (CBZ), which is known to be metabolized by CYP3A4 in humans (Kerr *et al.*, 1994; Pearce *et al.*, 2002) and is also a well-known activator of xenobiotic responses (Oscarson *et al.*, 2006).

To investigate the effect of acute exposure of CBZ, serum CBZ levels and hepatic gene expression were measured 2 hours after oral administration of CBZ. The serum CBZ levels were not significantly different in euthyroid or hypothyroid wild type or $PXR^{-/-}$ mice. On the contrary, the serum CBZ levels were significantly elevated in $CAR^{-/-}$ and also $CAR^{-/-}PXR^{-/-}$ mice, and in both cases trended higher in the hypothyroid state (Fig. 4A), indicating a defect in CBZ clearance due to loss of the response of Cyp2b10, or potentially additional CAR targets.

To study the longer term consequences of this defective CBZ clearance, $CAR^{-/-}$ and $CAR^{-/-}PXR^{-/-}$ mice were fed CBZ-containing normal chow diet (CBZ-euthyroid mice) or CBZ-containing PTU/LI diet (CBZ-hypothyroid mice) for 4 weeks. Remarkably, CBZ treatment resulted in mortality in CAR depleted mice (Fig. 4C). This was the most severe in hypothyroid $CAR^{-/-}$ mice, all of which (six of six) died after 4~11 days of treatment with CBZ-containing PTU/LI diet. Six of eight hypothyroid $CAR^{-/-}PXR^{-/-}$ mice also died, but they survived longer than the $CAR^{-/-}$ mice, with mortality at 8~18 days after CBZ-containing PTU/LI diet treatment ($CAR^{-/-}PXR^{-/-}$ mice vs. $CAR^{-/-}$ mice, 12.3 ±3.3 days vs. 6.3 ±2.1 days, p=0.04). In euthyroid status, one of six $CAR^{-/-}$ mice and two of six $CAR^{-/-}PXR^{-/-}$ mice died. There was no mortality in WT and $PXR^{-/-}$ mice regardless of thyroid status.

Serum CBZ levels were measured after 4weeks treatment or immediately after death, although this was possible in only limited numbers of $CAR^{-/-}$ or $CAR^{-/-}PXR^{-/-}$ mice, because most of them were found dead (Fig. 4B). Serum CBZ levels were undetectable in WT and $PXR^{-/-}$ mice, which is consistent with its ability to induce drug metabolism after chronic exposure. However, CBZ was still detectable in serum of $CAR^{-/-}$ and also $CAR^{-/-}PXR^{-/-}$ mice at that time point, and appeared higher in the hypothyroid than in the euthryoid mice (Fig. 4B). Thus, CAR is essential for the normal metabolism and clearance of CBZ, and the defective clearance in the CAR null mice is worsened by hypothyroidism.

To determine whether the mortality and change of serum CBZ levels might be related to the altered CYP expression under hypothyroid condition, the expression of Cyp2b10 and Cyp3a11 was measured. As expected, CBZ treatment induced Cyp2b10 expression in euthyroid wild type and $PXR^{-/-}$ mice. PTU treatment alone was also associated with increased Cyp2b10 expression, as expected, and the combination of PTU and CBZ resulted in a much higher induction (Fig. 4D). Also as expected, $CAR^{-/-}$ and $CAR^{-/-}PXR^{-/-}$ mice failed to induce Cyp2b10 expression in response to either CBZ treatment or hypothyroid status. On the other hand, CBZ apparently does not activate mouse PXR, since Cyp3a11 expression was suppressed by CBZ treatment as well as hypothyroidism in WT mice, and even further by the combination of PTU and CBZ (Fig. 4E). These responses were increased in $CAR^{-/-}$ mice, which had elevated basal expression of Cyp3a11.

DISCUSSION

In this study, we found that the expression of Cyp2b10 and Cyp3a11 is altered in opposing directions in the hypothyroid state, which was mediated by opposing regulation of CAR and PXR. This is consistent with previous indications that hypothyroid status changes drug metabolism through the alteration of CYP activities; the activity of CYP3A4 was reduced in hypothyroid humans (Haas *et al.*, 2000), and mRNA expression of CYP2C was decreased by 70~80% in hypothyroid rats (Ram and Waxman, 1991). It was suggested that thyroid hormone regulates CYP expression through the control of the cellular levels of CYP reductase, which could inactivate CYPs by both transcriptional and post-transcriptional levels (O'Leary *et al.*, 1997). However, it has not been elucidated whether thyroid hormone can influence directly the mRNA expression of CYP2B or 3A (Brtko and Dvorak, 2011).

The thyroid hormone receptor isoforms TRα and TRβ act as ligand responsive transcription factors to initiate or block target gene expression by binding to thyroid hormone response elements (TRE) in target gene promoter regions (Wu and Koenig, 2000). In the absence of thyroid hormone, TRs generally recruit corepressors and inhibit expression of linked genes, which is consistent with the observed suppression of Cyp3A gene expression in both hypothyroid mice and in cells. The observation that the unliganded TRβ attenuates PXR-mediated CYP3A promoter activity suggests that it could bind directly to that promoter, which is in accord with a recent study demonstrating that both TRα and TRβ can bind to PXR response elements from the proximal of CYP3a4 promoter, and can suppress both basal and rifampin induced CYP3A4 promoter activity (Istrate *et al.*, 2010). However, a model in which unliganded TR inhibits Cyp3A promoter activity simply by competing for such PXR binding sites predicts that the impact of hypothyroidism would be greater in the $PXR^{-/-}$ mice, which is the opposite of what was observed (Fig. 2B). Thus, more complex mechanisms must account for the crosstalk between the unliganded TRβ and PXR in the hypothyroid state. However, the potential physiologic significance of this response is strongly supported by the increased mortality of the chronically CBZ treated mice that were both hypothyroid and CAR deficient (Fig. 4.). In these mice, the PXR dependent suppression of Cyp3a11 expression cannot be compensated for by induction of Cyp2b10, or potentially other targets.

In contrast to the suppression of Cyp3a11, hypothyroidism markedly induced Cyp2b10 gene expression and this was clearly dependent on CAR. This is consistent with a large scale study of the impact of diverse lipid lowering agents on rat liver transcriptomes, which grouped the PTU response with those of phenobarbital and other CAR activators (Omura *et al.*, 2007). While PXR acts primarily as a direct xenobiotic receptor, CAR can be activated indirectly by diverse metabolic stresses (Qatanani *et al.*, 2005), including type 1 diabetes (Dong *et al.*, 2009), fasting (Maglich *et al.*, 2004) and cholestasis (Guo *et al.*, 2003; Zhang *et al.*, 2004). Although further studies will be required to define the basis for the activation of CAR by hypothyroidism, the current results are also consistent with other previous indications of crosstalk between thyroid hormone and CAR. CAR agonist treatment accelerated thyroid hormone metabolism, which led to hypothyroid status (Qatanani *et al.*, 2005). Recently, it was reported that thyroid hormone is necessary for expression of CAR in a cryopreserved rat small hepatocyte model (Ooe *et al.*, 2009), which suggested that thyroid hormone regulates CAR expression. Interestingly, the expression of CAR mRNA was abolished under absolute depletion of thyroid hormone, whereas the CAR expression was rather more elevated under subphysiologic level of thyroid hormone (5×10^{-12}~5×10^{-10}M) than under physiologic level (5×10^{-8}M), which is consistent with our data.

In summary, our findings suggest that in hypothyroidism Cyp3a11 is suppressed by PXR-mediated mechanisms, but at the same time this suppression may be compensated to some

extent by CAR-mediated induction of Cyp2b10 and likely other targets. These reciprocal changes of Cyp3a11 and Cyp2b10 under hypothyroid conditions protect wild type mice against the toxic effects of chronic CBZ treatment observed in CAR depleted mice. We conclude that hypothyroid status has diverse effects on xenobiotic metabolism, and that opposing responses of CAR and PXR and their CYP targets can cancel each other out to decrease serious metabolic derangement in response to a xenobiotic challenge.

Acknowledgments

This work was supported by the SNUBH Research Fund [Grant no 03-2007-001]; National Institutes of Health [Grant R01 R01 DK068804]; and the R.P. Doherty , Jr. Welch Chair [Q-0022] to DDM.

List of abbreviations

CAR	constitutive androstane receptor
PXR	pregnane X receptor
PTU	propylthiouracil
WT	wild type
CYP	cytochrome P450
ND	normal diet
PTU/LI	propylthiouracil/low iodine
T4	tetraiodothyronine
CBZ	carbamazepine
MEM	minimal essential medium
WME	Williams medium E
Q-PCR	quantitative real-time PCR
TR	thyroid hormone receptor
RXR	retinoid X receptor
PCN	pregnenolone-16α-carbonitrile
TCPOBOP	1,4-Bis[2-(3,5-dichloro-pyridyloxy)]benzene

REFERENCES

Badger D, Kraner J, Fraser D, Hoglen N, Halpert J, Sipes IG. Reduction of thyroid hormone may participate in the modulation of cytochromes P450 2C11 and 3A2 by retinol. Life Sci. 1998; 63:PL367–372. [PubMed: 9870717]

Brtko J, Dvorak Z. Role of retinoids, rexinoids and thyroid hormone in the expression of cytochrome p450 enzymes. Curr Drug Metab. 2011; 12:71–88. [PubMed: 21401514]

Chu V, Einolf HJ, Evers R, Kumar G, Moore D, Ripp S, Silva J, Sinha V, Sinz M, Skerjanec A. In vitro and in vivo induction of cytochrome p450: a survey of the current practices and recommendations: a pharmaceutical research and manufacturers of america perspective. Drug Metab Dispos. 2009; 37:1339–1354. [PubMed: 19389860]

Court MH, Duan SX, Hesse LM, Venkatakrishnan K, Greenblatt DJ. Cytochrome P-450 2B6 is responsible for interindividual variability of propofol hydroxylation by human liver microsomes. Anesthesiology. 2001; 94:110–119. [PubMed: 11135730]

Danielson PB. The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans. Curr Drug Metab. 2002; 3:561–597. [PubMed: 12369887]

Dong B, Qatanani M, Moore DD. Constitutive androstane receptor mediates the induction of drug metabolism in mouse models of type 1 diabetes. Hepatology. 2009; 50:622–629. [PubMed: 19489075]

Galle PR, Hofmann WJ, Walczak H, Schaller H, Otto G, Stremmel W, Krammer PH, Runkel L. Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. J Exp Med. 1995; 182:1223–1230. [PubMed: 7595193]

Goodwin B, Hodgson E, Liddle C. The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. Mol Pharmacol. 1999; 56:1329–1339. [PubMed: 10570062]

Guo GL, Lambert G, Negishi M, Ward JM, Brewer HB Jr. Kliewer SA, Gonzalez FJ, Sinal CJ. Complementary roles of farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor in protection against bile acid toxicity. J Biol Chem. 2003; 278:45062–45071. [PubMed: 12923173]

Haas M, Kletzmayer J, Staudinger T, Bohmig G, Mayer G, Leko-Mohr Z, Druml W. Hypothyroidism as a cause of tacrolimus intoxication and acute renal failure: a case report. Wien Klin Wochenschr. 2000; 112:939–941. [PubMed: 11144010]

Hidestrand M, Oscarson M, Salonen JS, Nyman L, Pelkonen O, Turpeinen M, Ingelman-Sundberg M. CYP2B6 and CYP2C19 as the major enzymes responsible for the metabolism of selegiline, a drug used in the treatment of Parkinson's disease, as revealed from experiments with recombinant enzymes. Drug Metab Dispos. 2001; 29:1480–1484. [PubMed: 11602525]

Honkakoski P, Negishi M. Regulation of cytochrome P450 (CYP) genes by nuclear receptors. Biochem J. 2000; 347:321–337. [PubMed: 10749660]

Istrate MA, Nussler AK, Eichelbaum M, Burk O. Regulation of CYP3A4 by pregnane X receptor: The role of nuclear receptors competing for response element binding. Biochem Biophys Res Commun. 2010; 393:688–693. [PubMed: 20171174]

Kang P, Liao M, Wester MR, Leeder JS, Pearce RE, Correia MA. CYP3A4-Mediated carbamazepine (CBZ) metabolism: formation of a covalent CBZ-CYP3A4 adduct and alteration of the enzyme kinetic profile. Drug Metab Dispos. 2008; 36:490–499. [PubMed: 18096676]

Kerr BM, Thummel KE, Wurden CJ, Klein SM, Kroetz DL, Gonzalez FJ, Levy RH. Human liver carbamazepine metabolism. Role of CYP3A4 and CYP2C8 in 10,11-epoxide formation. Biochem Pharmacol. 1994; 47:1969–1979. [PubMed: 8010982]

Kliewer SA, Lehmann JM, Willson TM. Orphan nuclear receptors: shifting endocrinology into reverse. Science. 1999; 284:757–760. [PubMed: 10221899]

Lahtela JT, Arranto AJ, Sotaniemi EA. Enzyme inducers improve insulin sensitivity in non-insulin-dependent diabetic subjects. Diabetes. 1985; 34:911–916. [PubMed: 3896900]

Liddle C, Goodwin BJ, George J, Tapner M, Farrell GC. Separate and interactive regulation of cytochrome P450 3A4 by triiodothyronine, dexamethasone, and growth hormone in cultured hepatocytes. J Clin Endocrinol Metab. 1998; 83:2411–2416. [PubMed: 9661620]

Maglich JM, Watson J, McMillen PJ, Goodwin B, Willson TM, Moore JT. The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction. J Biol Chem. 2004; 279:19832–19838. [PubMed: 15004031]

Mizuno Y, Kanou Y, Rogatcheva M, Imai T, Refetoff S, Seo H, Murata Y. Genomic organization of mouse ZAKI-4 gene that encodes ZAKI-4 alpha and beta isoforms, endogenous calcineurin inhibitors, and changes in the expression of these isoforms by thyroid hormone in adult mouse brain and heart. Eur J Endocrinol. 2004; 150:371–380. [PubMed: 15012624]

O'Leary KA, Li HC, Ram PA, McQuiddy P, Waxman DJ, Kasper CB. Thyroid regulation of NADPH:cytochrome P450 oxidoreductase: identification of a thyroid-responsive element in the 5'-flank of the oxidoreductase gene. Mol Pharmacol. 1997; 52:46–53. [PubMed: 9224811]

Omura K, Kiyosawa N, Uehara T, Hirode M, Shimizu T, Miyagishima T, Ono A, Nagao T, Urushidani T. Gene expression profiling of rat liver treated with serum triglyceride-decreasing compounds. J Toxicol Sci. 2007; 32:387–399. [PubMed: 17965553]

Ooe H, Kon J, Oshima H, Mitaka T. Thyroid hormone is necessary for expression of constitutive androstane receptor in rat hepatocytes. Drug Metab Dispos. 2009; 37:1963–1969. [PubMed: 19520777]

Oscarson M, Zanger UM, Rifki OF, Klein K, Eichelbaum M, Meyer UA. Transcriptional profiling of genes induced in the livers of patients treated with carbamazepine. Clin Pharmacol Ther. 2006; 80:440–456. [PubMed: 17112801]

Pearce RE, Vakkalagadda GR, Leeder JS. Pathways of carbamazepine bioactivation in vitro I. Characterization of human cytochromes P450 responsible for the formation of 2- and 3-hydroxylated metabolites. Drug Metab Dispos. 2002; 30:1170–1179. [PubMed: 12386121]

Qatanani M, Zhang J, Moore DD. Role of the constitutive androstane receptor in xenobiotic-induced thyroid hormone metabolism. Endocrinology. 2005; 146:995–1002. [PubMed: 15564320]

Ram PA, Waxman DJ. Hepatic P450 expression in hypothyroid rats: differential responsiveness of male-specific P450 forms 2a (IIIA2), 2c (IIC11), and RLM2 (IIA2) to thyroid hormone. Mol Endocrinol. 1991; 5:13–20. [PubMed: 2017188]

Rosenberg DW, Drummond GS, Smith TJ. Depletion of cytochrome P-450 by thyroid hormone and cobalt-protoporphyrin IX in rat liver: evidence that susceptibility varies among forms of the heme protein. Pharmacology. 1995; 51:254–262. [PubMed: 8577819]

Roy P, Tretyakov O, Wright J, Waxman DJ. Stereoselective metabolism of ifosfamide by human P-450s 3A4 and 2B6. Favorable metabolic properties of R-enantiomer. Drug Metab Dispos. 1999; 27:1309–1318. [PubMed: 10534317]

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH, Kliewer SA. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc Natl Acad Sci U S A. 2001; 98:3369–3374. [PubMed: 11248085]

Surks MI, Sievert R. Drugs and thyroid function. N Engl J Med. 1995; 333:1688–1694. [PubMed: 7477223]

Webb SJ, Xiao GH, Geoghegan TE, Prough RA. Regulation of CYP4A expression in rat by dehydroepiandrosterone and thyroid hormone. Mol Pharmacol. 1996; 49:276–287. [PubMed: 8632760]

Wei P, Zhang J, Dohan D, Han Y, Moore DD. Specific and Overlapping Functions of the Nuclear Hormone Receptors CAR and PXR in Xenobiotic Response. The pharmacogenomics journal. 2002; 2:117–126. [PubMed: 12049174]

Wei P, Zhang J, Egan-Hafley M, Liang S, Moore DD. The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. Nature. 2000; 407:920–923. [PubMed: 11057673]

Willson TM, Kliewer SA. PXR, CAR and drug metabolism. Nat Rev Drug Discov. 2002; 1:259–266. [PubMed: 12120277]

Wu Y, Koenig RJ. Gene regulation by thyroid hormone. Trends Endocrinol Metab. 2000; 11:207–211. [PubMed: 10878749]

Xie W, Barwick JL, Simon CM, Pierce AM, Safe S, Blumberg B, Guzelian PS, Evans RM. Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. Genes Dev. 2000; 14:3014–3023. [PubMed: 11114890]

Xie W, Evans RM. Orphan nuclear receptors: the exotics of xenobiotics. J Biol Chem. 2001; 276:37739–37742. [PubMed: 11459851]

Zhang J, Huang W, Qatanani M, Evans RM, Moore DD. The constitutive androstane receptor and pregnane X receptor function coordinately to prevent bile acid-induced hepatotoxicity. J Biol Chem. 2004; 279:49517–49522. [PubMed: 15358766]

Highlights

- Hypothyroid status activates CAR in mice and induces Cyp2b10 expression
- Hypothyroid status suppresses PXR activity in mice and represses Cyp3a11 expression
- These responses balance each other out in normal mice
- Hypothyroidism sensitizes CAR null mice to toxic effects of carbamezipine

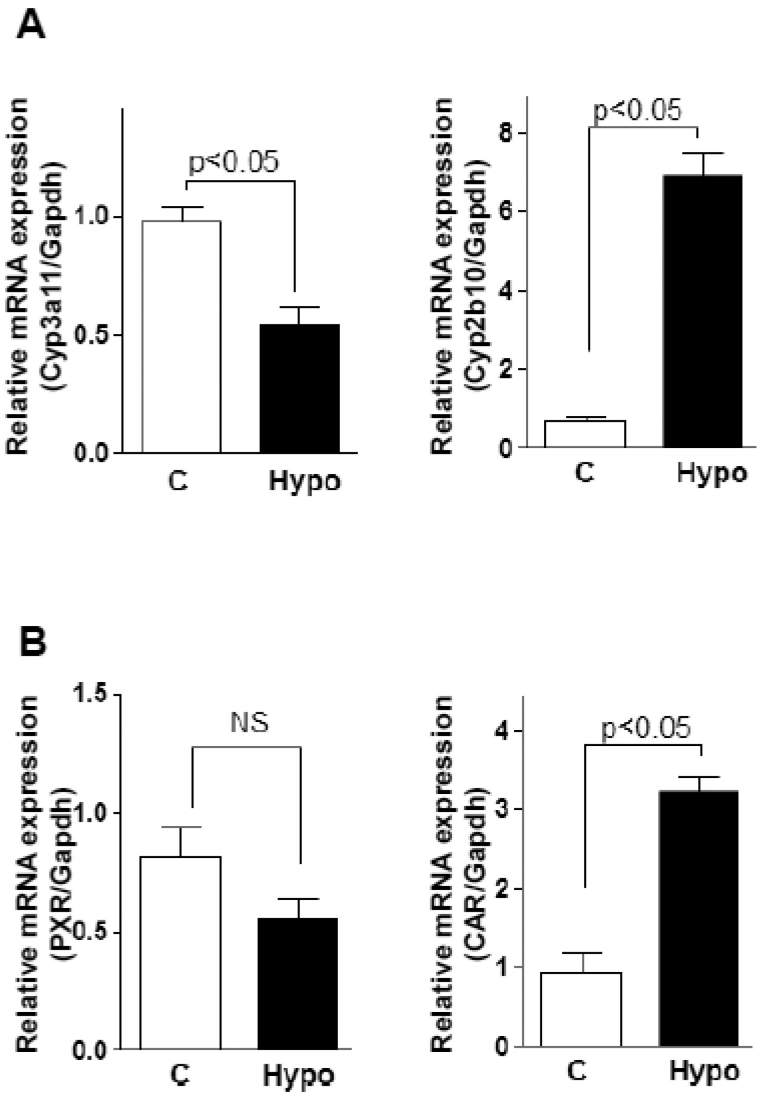


Fig. 1. Changes of the expression of CYPs and nuclear receptors in hypothyroid mice

WT mice were fed with normal chow (C: control) or PTU/LI diet (Hypo; hypothyroid), a low iodine contained diet supplemented with 0.15 % PTU to induce hypothyroid condition, for 4 weeks. Hepatic mRNA levels were measured by real time PCR. (A) Expression of Cyp2b10 or Cyp3a11. (B) Expression of CAR or PXR.

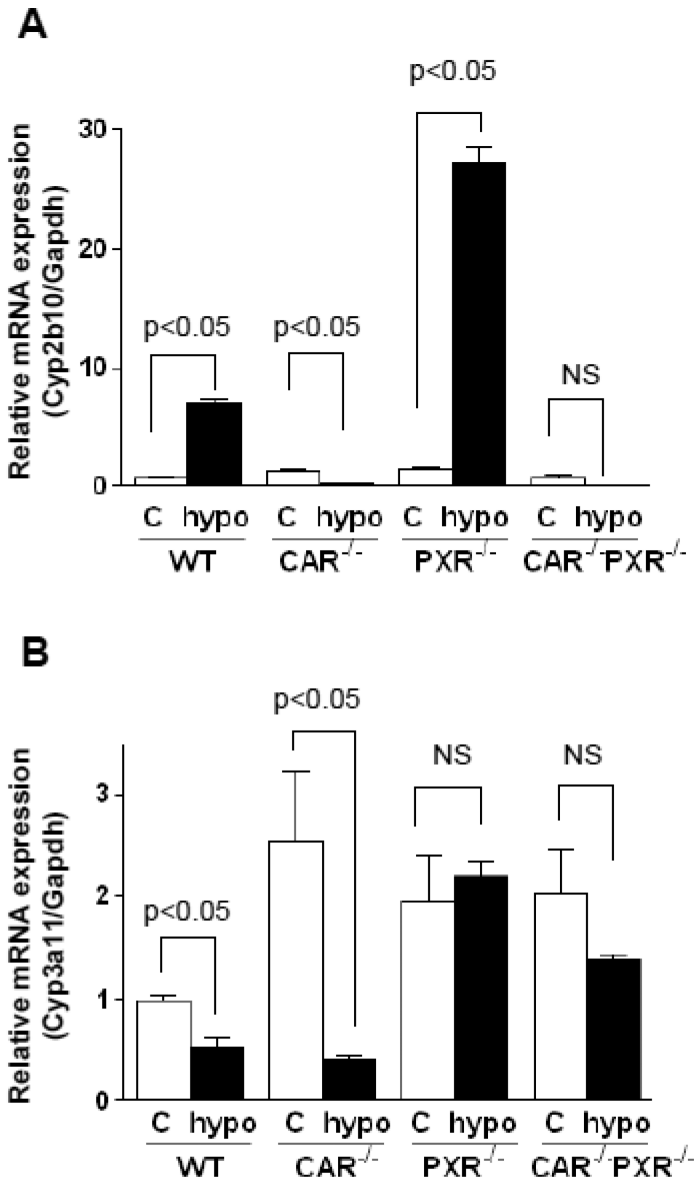


Fig. 2. CAR and PXR mediated changes of Cyp2b10 and Cyp3a11 in hypothyroid status

The WT, $PXR^{-/-}$, $CAR^{-/-}$, or $CAR^{-/-}PXR^{-/-}$ mice were fed with normal chow (C: control) or PTU/LI diet (Hypo; hypothyroid) for 4 weeks. Hepatic mRNA levels were measured by real time PCR. (A) Expression of Cyp2b10. (B) Expression of Cyp3a11.

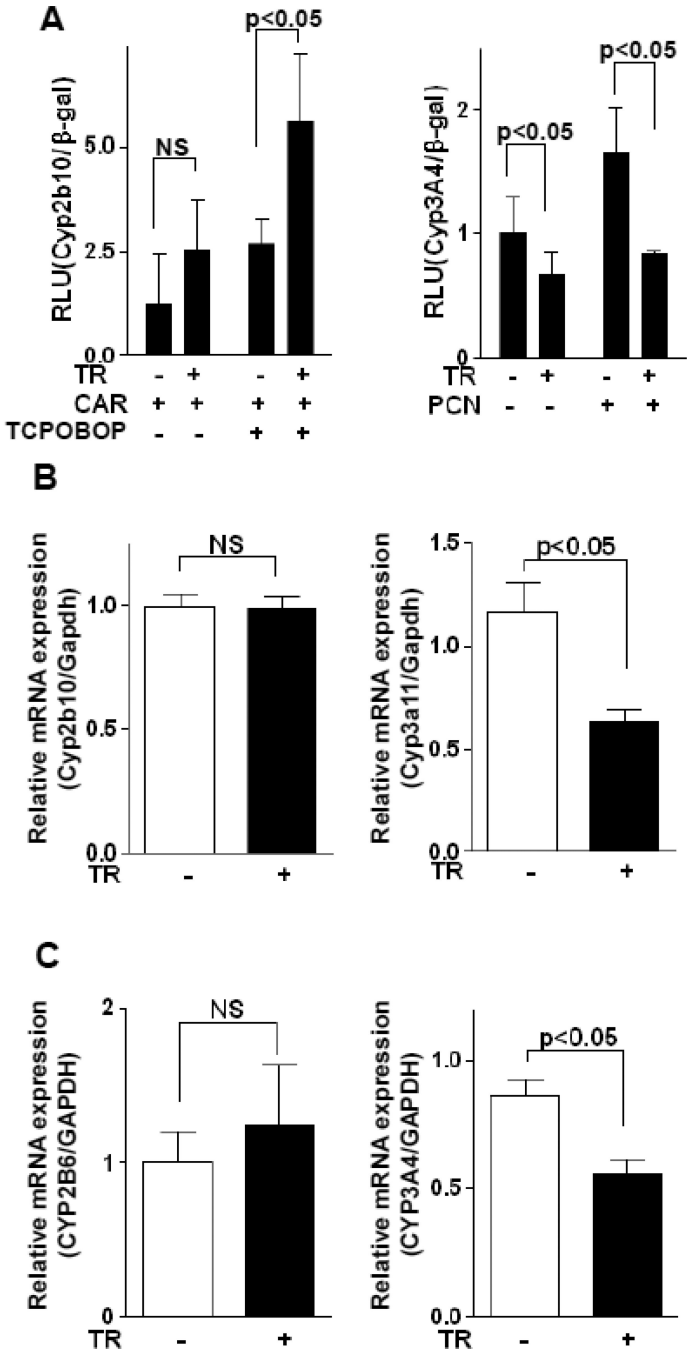
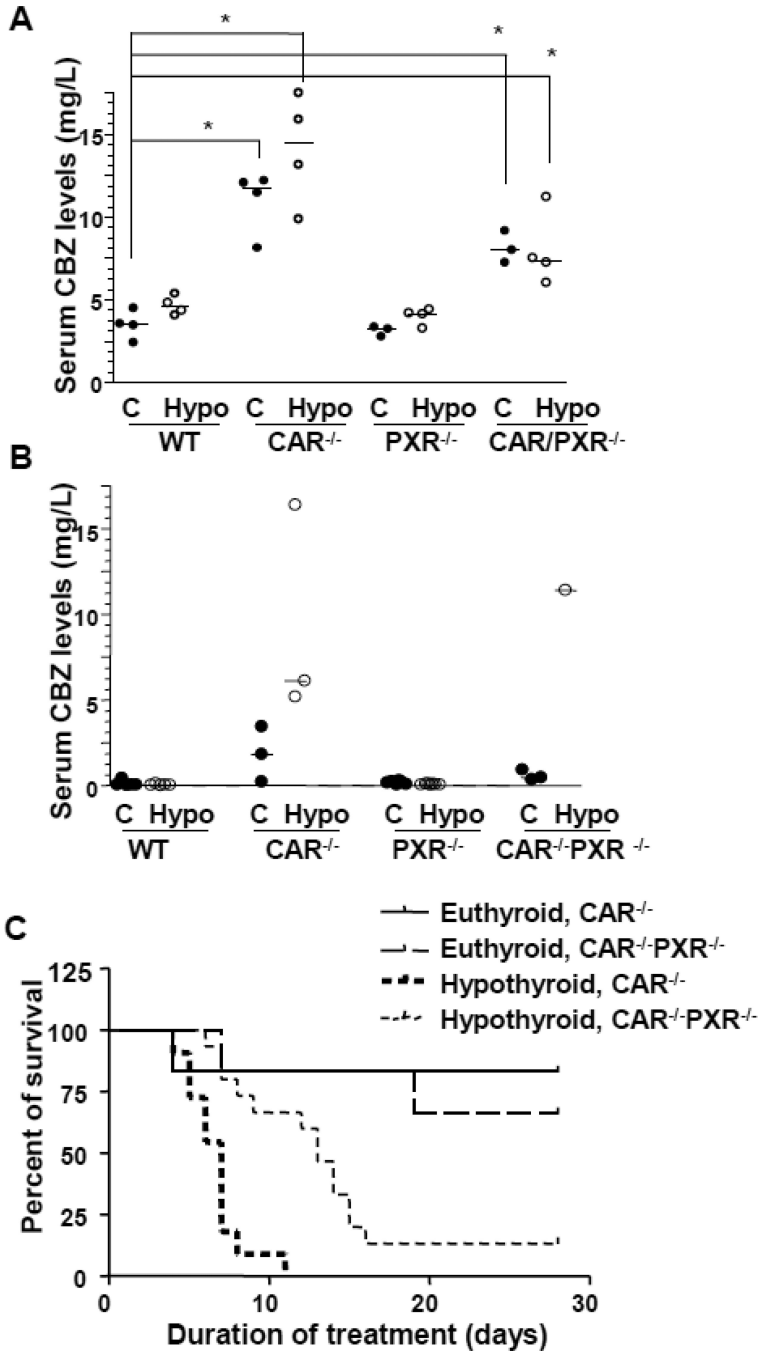


Fig. 3. The expression of CYPs in hepatocytes transfected with unliganded TRβ

To assess the effect of unliganded TRβ, cells were cultured in charcoal stripped serum containing media, and the change of relative luciferase activity (A) or mRNA expression by real time PCR (B, C) was examined after transfection of TRβ. (A) Cyp2b10 or CYP3A4 promoter activity in HepG2 cells with or without cotransfected TRβ. 10 nM of TCPOBOP or 10 nM of PCN was co-treated with TRβ in the indicated conditions. (B) Expression of Cyb2b10 or Cyp3a11in mouse primary hepatocytes. (C) Expression of CYP2B6 or CYP3A4 in human HepG2 cells.



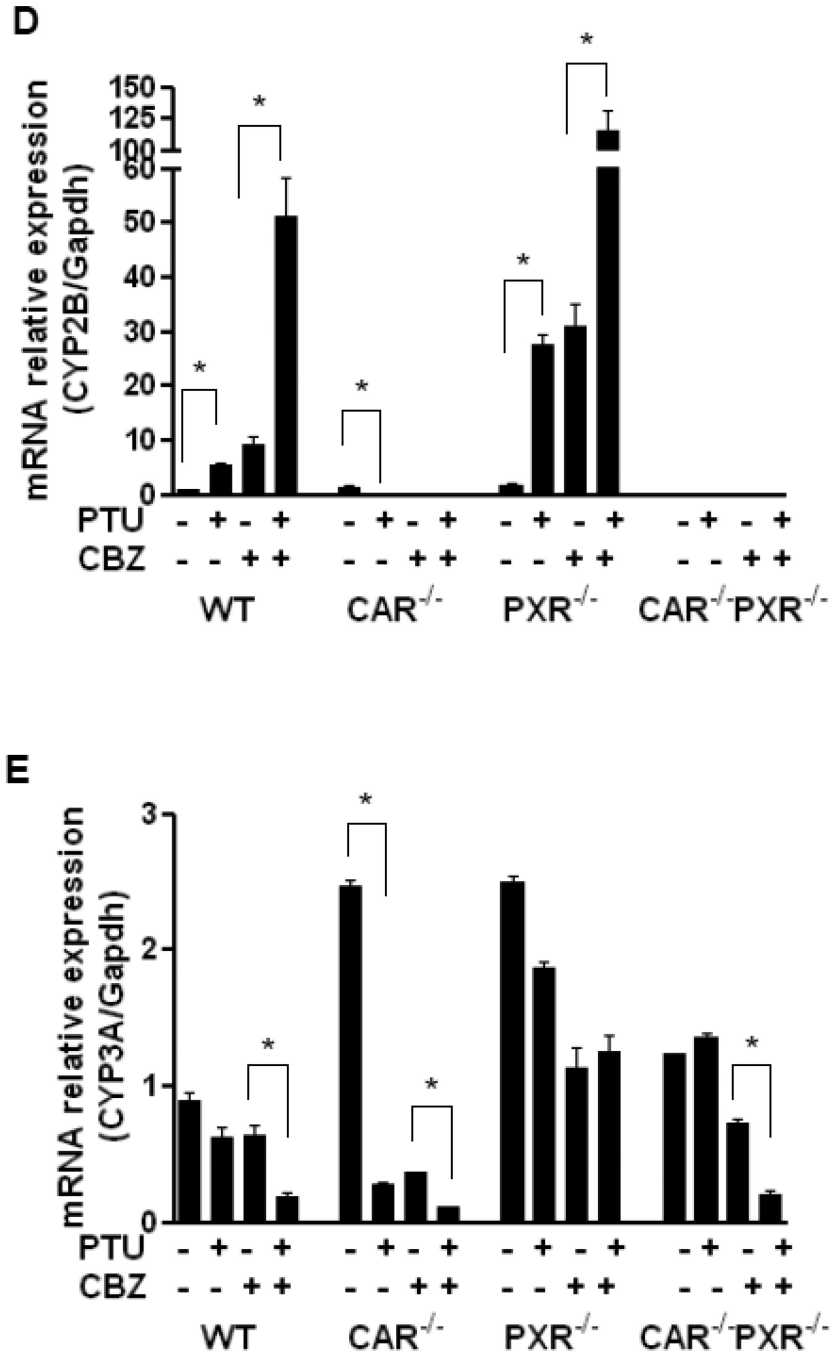


Fig. 4. Serum CBZ levels and the mortalities in CBZ treated mice

(A, B) After CBZ treatment consisting of a single oral administration (A) or 4 weeks of feeding (B), serum CBZ levels were measured by HPLC. Serum CBZ levels at 2 hours after 30mg/kg body weight of CBZ oral administration (A) or after 4weeks treatment of CBZ in diet (3.5g/kg food) or immediately after death (in case of hypothyroid $CAR^{-/-}$ or $CAR^{-/-}PXR^{-/-}$ mice) (B) in chow (C: control) or PTU/LI (Hypo: hypothyroid) diet fed mice. (C) Survival curve of 4 weeks-CBZ-treated $CAR^{-/-}$ or $CAR^{-/-}PXR^{-/-}$ mice. The survival curve of WT and $PXR^{-/-}$ mice was not represented because they showed no mortality. (D, E) The hepatic expression of Cyp2b10 (D) or Cyp3a11 (E) was measured using real time PCR

method in the 4 weeks-CBZ-treated mice.. Real time PCR was The hepatic expression of Cyp2b10 , but not Cyp3a11 (E). (*, $p<0.05$)