Published in final edited form as:

Xenobiotica. 2006 ; 36(10-11): 1152–1163.

Nuclear receptors CAR and PXR in the regulation of hepatic metabolism

ERIC S. TIEN and MASAHIKO NEGISHI

The Pharmacogenetics Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

Abstract

1. The nuclear receptors CAR and PXR were first characterized as xenosensing transcription factors regulating the induction of phase I and II xenobiotic metabolizing enzymes as well as transporters in response to exogenous stimuli.
2. It has now become clear, however, that these receptors cross talk with endogenous stimuli as well which extends their regulation to various physiological processes such as energy metabolism and cell growth. As recognition of the function of these receptors has widened, the molecular mechanism of their regulation has evolved from simple protein-DNA binding to regulation by complex protein-protein interactions.
3. Novel mechanisms as to how xenobiotic exposure alters hepatic metabolic pathways such as gluconeogenesis and β -oxidation have emerged. At the same time, the molecular mechanism of how endogenous stimuli, such as insulin, regulate xenobiotic metabolism via CAR and PXR have also become evident.

Keywords

CAR; PXR; gene regulation

1. Nuclear receptors CAR and PXR

Nuclear receptors (NRs) are a large class of ligand activated transcription factors that function to regulate a wide array of cellular processes (Alarid 2006). With few exceptions, NRs share a common domain based structure comprised of four fundamental domains: A/B domain containing the ligand independent activation function (AF-1), DNA binding domain (designated the C domain), hinge region of lesser defined function (D domain) and the E/F domain containing the ligand dependent activation function (AF-2) as well as the ligand binding domain. The most recent classification scheme for NRs is based on sequence homology in which both the constitutive active/androstane receptor (CAR) and pregnane X receptor (PXR) belong to the NR1I family (NR1I3 and NR1I2, respectively) (Baes et al. 1994, 1999). The anti-seizure drugs phenobarbital (PB) and phenytoin are the most well known activators of CAR. Xenobiotics such as 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) have also been shown to activate mouse and human CAR respectively (Tzamelis et al. 2000; Maglich et al. 2003; Wang et al. 2004). Conversely, androstanol and androstenol are inverse agonists that repress the constitutive activity of CAR (Forman et al. 1998).

Corresponding author: Masahiko Negishi, Ph.D., Tel: 919-541-2404, Fax: 919-541-0696, E-mail: negishi@niehs.nih.gov

This research was supported [in part] by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences

The first genes identified as CAR-regulated were the CYP2B family of drug metabolizing enzymes. Similar to PXR (also known as steroid and xenobiotic receptor SXR), CAR heterodimerizes with RXR and binds to the phenobarbital responsive enhancer module (PBREM) to induce the transcription of CYP2B genes (Baes et al. 1994;Choi et al. 1997;Honkakoski and Negishi 1997). Closer examination of the PBREM and the other CAR-regulated promoters revealed that CAR preferentially binds to DR4 (direct repeat spaced by 4 nucleotides) binding sites (Honkakoski et al. 1998).

Unlike CAR, PXR does not contain significant constitutive activity and must be activated by cognate ligands to elicit its effects. The most well known agonist ligands of PXR include many pharmaceutical drugs including RU486 and rifampicin (Kliewer et al. 1998;Jimenez et al. 2000). PXR has one of the largest ligand binding pockets of all NRs at over 1100 Å³ and this feature is reflected in the structural diversity of PXR ligands (Watkins et al. 2001), making this receptor quite promiscuous. PXR is most commonly associated with regulation of CYP3A genes.

2. Regulation of drug metabolism by CAR and PXR

Whereas CAR and PXR regulation of CYPs (phase I drug metabolizing enzymes) is the most characterized mechanism of gene regulation, these receptors are also involved in the regulation of other aspects of drug metabolism and excretion. CAR and PXR also regulate expression of phase II drug metabolism including UDP-glucuronosyl transferase (UGT), sulfotransferase (SULT) and glutathione-S-transferase (GST) enzymes (Saini et al. 2004;Xu et al. 2005;Zhou et al. 2005). UGTs, GSTs and SULTs function to conjugate hydrophilic groups to promote the water solubility of compounds such as exogenous drug metabolites. In the rodent liver, SULT1A1 is reported to be up-regulated by GR and CAR (Fang et al. 2003;Maglich et al. 2003). However, it should be noted that there is a species difference in regulation by nuclear receptors. SULT1A1 is regulated in humans by the transcription factors Sp1 and GABP, not any of the nuclear receptors (Hempel et al. 2004). In addition, CAR and PXR can up-regulate the expression of organic anion transporters such as BSEP, NTCP, OATP2, MRP3 and MDR2 (Staudinger et al. 2003;Wagner et al. 2005). The up-regulation of latter these proteins helps to promote the uptake of exogenous drugs into the liver to be acted upon by phase I (CYPs) and phase II (UGTs, SULTs) drug metabolizing enzymes. These transporters then move the metabolized and conjugated drug metabolites into the body's excretory pathways via the kidney or the bile.

Through the induction of the phase I and II enzymes and transporters, CAR and PXR are also able to regulate the metabolism and secretion of endogenous signaling molecules and endobiotics. The hemoglobin breakdown product bilirubin is toxic in the circulation and can cause neurological damage and jaundice (reviewed in (Shapiro 2003)). Effective removal of bilirubin from the circulation requires influx organic anion transporters to transport bilirubin into liver from the blood, UGT1A1 to glucuronidate it and efflux transporters to transport the bilirubin glucuronide into bile duct. OATP2 is located on hepatocyte membranes facing out into the blood whereas MRP2 resides on the membrane separating the hepatocyte and the bile duct (Keppler et al. 1997;Konig et al. 2000). Together, these transporters regulate bilirubin flow from blood to bile duct (reviewed in (Ito et al. 2005)). By inducing these transporters along with UGTs, CAR and PXR regulate the flow of bilirubin. The ability of CAR and PXR to promote transport of toxic endobiotics out of the body is of particular importance in some disease states of the body. Induction of liver injury through chemical or physical means can cause marked increases in circulating bile acid levels. Feeding of cholic acid to mice strongly elevates bile acid levels in the blood (Adler et al. 1975). Pretreatment of mice with PB or TCPOBOP before feeding of cholic acid resulted in significantly reduced bile acid levels (Guo et al. 2003;Wagner et al. 2005). Partial hepatectomy of mice in which two thirds of the liver

is removed causes highly elevated levels of bile acids and bilirubin in the blood. Subsequent treatment with phenobarbital reduces the levels of these toxins to more normal levels (Tien and Negishi, unpublished observations).

Increasing the metabolism and secretion of both xeno and endobiotics by regulating the genes that encode metabolizing enzymes (phases I and II) and transporters is the most well established concept of CAR and PXR function in body. However, it is now becoming apparent that these receptors also cross talk with endogenous stimuli to regulate various aspects of liver physiology, such as cell growth, tumor development, hormone homeostasis and energy metabolism. Conversely, these endogenous stimuli modulate xenobiotic metabolism via CAR and PXR. This review summarizes how CAR and PXR regulate liver physiology as well as how endogenous stimuli regulate CAR and PXR modulation of xenobiotic metabolism.

3. Cell growth

Many nuclear receptors have been implicated in the formation of tumors throughout the body (Gupta et al. 2001; Yin et al. 2001; Edwards and Bartlett 2005). The promotion of tumors is brought about by a wide variety of mechanisms such as increased cellular growth and inhibition of apoptosis (Schwartz and Shah 2005; Cheung et al. 2006). Chronic exposure to phenobarbital has been known to promote tumor formation in the liver but it was not until the discovery of CAR that a potential mechanism was identified (Peraino et al. 1971; Nishizumi 1976).

Studies have shown that CAR plays a role in the promotion of tumor formation. It is known that PB can induce liver hyperplasia (Huang et al. 2005) and tumors in mice (Ward 1983; Diwan et al. 1986). Wild type mice treated with a single injection of the tumor initiator diethylnitrosamine (DEN) followed by PB over the course of 36 weeks were shown to almost unilaterally develop hepatocellular carcinoma (HCC). In sharp contrast, CAR knockout mice showed no tumor formation after the same xenobiotic treatment (Yamamoto et al. 2004). All 31 wild type mice died of liver tumors within 52 weeks of the treatment, whereas all 30 CAR knockout mice survived. Wild type mice exhibited elevated liver weight, ALP, ALT as well as increased BrdU staining indicative of increased cellular proliferation. Conversely, CAR knockout mice did not exhibit these effects. In addition, Mdm2, an anti-apoptotic factor that represses p53, has been suggested as the mediator of CAR-dependent development of HCC (Huang et al. 2005). The region 1q21-23 of human chromosome 1 is amplified in more than half of human HCC samples (Bilger et al. 2004). Intriguingly, the CAR locus is present within this region of both mouse and human chromosomes 1. These findings point to a central role for CAR in the promotion of DEN-PB induced liver tumorigenesis and make CAR a potential target for anti-cancer therapeutics.

In addition to tumor promotion, CAR activators are known to cause liver hyperplasia and hepatomegaly. CAR is able to regulate a set of genes common to the promotion of cellular growth. After a short treatment with TCPOBOP, the mice showed increased BrdU staining as well as rapid induction of cell cycle genes including cyclin D1 and cdk2 (Ledda-Columbano et al. 2000; Ledda-Columbano et al. 2004). Gadd45 β , a gene involved in the repression of apoptosis, is also up-regulated in response to CAR activators (Columbano et al. 2005). These studies showed that whereas Gadd45 β is typically regulated by the TNF/NF κ B pathway, TCPOBOP was able to regulate Gadd45 β in the absence of TNF receptors but not in the absence of CAR suggesting that CAR is an alternate signaling pathway independent of TNF and NF κ B. CAR regulation of Gadd45 β implies that CAR plays a role in the suppression of apoptosis in the liver which may be important in the development of HCC.

4. Endocrine homeostasis

Most of the work to date concerning the role of CAR and PXR in endocrine signaling deals with the ability of endogenous hormones such as estrogens to function as activators of CAR and PXR (reviewed in (Kretschmer and Baldwin 2005)). Recent studies, however, are beginning to uncover a role for CAR and PXR in the regulation of hormone levels.

Thyroid hormone levels can be influenced by compounds such as phenytoin and phenobarbital (Rootwelt et al. 1978; Yeo et al. 1978; Ohnhaus et al. 1981). Recent works have further characterized the potential of CAR involvement in thyroid hormone signaling. Injection of mice with PB or TCPOBOP lead to decreased levels of T4 in the blood (Qatanani et al. 2005). The CAR mediated up-regulation of multiple phase II enzymes such as UGT1A1 and SULT1A1 was suggested to be the cause of increased removal of T4 from circulation (Maglich et al. 2004). However, how these enzymes preferentially conjugate T4 and remove the metabolites from the circulation remains enigmatic. In addition, the T4 level in fasting CAR knockout mice was significantly higher than those in the wild type mice, indicating that the drop in T4 after fasting may also be CAR dependent (Qatanani et al. 2005).

It was observed in our group that CAR also influences thyroid hormone activity by directly regulating synthesis of the hormone. After partial hepatectomy (PH) in which two thirds of the liver is surgically removed, wild type mice had highly elevated levels of reverse T3 (rT3), although T3 (both total and free) levels did not change. Consistent with the known function of rT3 as a competitive repressor of T3, the level of thyroid hormone target genes tyrosine amino transferase (TAT) and carnitine palmitoyl transferase 1 (CPT1) were also attenuated in the hepatectomized liver. PB treatment decreased the rT3 to the levels similar to those observed in sham operated wild type mice. CAR knockout mice did not show an increase in rT3 after PH nor a decrease in rT3 after PB. The increase of rT3 levels was correlated with the down regulation of an important thyroid hormone activating enzyme (type 1 deiodinase, Dio1). Since Dio1 catalyzes the conversion of T4 into T3, down-regulation of Dio1 results in augmenting the conversion of T4 into rT3, thus increasing rT3 levels in target tissues. Treatment with PB induced Dio1 expression and activity in a CAR dependent manner, restoring rT3 and TAT and CPT1 to their normal levels. Thus, CAR can regulate thyroid hormone activity by directly regulating the Dio1 gene in hepatectomized mice.

Metabolism of many hormones can be carried out by CYPs and UGTs. UGT1A1 and CYP3A, which can both be regulated by CAR and PXR, are known to act upon estrogen leading to excretion (Hammond et al. 1997; Sugatani et al. 2001; Sugatani et al. 2005). In addition, the CAR activator phenytoin has been found to regulate androgen and testosterone metabolism through CYP3A in the mouse brain (Rosenbrock et al. 1999; Meyer et al. 2006). In the liver, CYP3A can also metabolize testosterone and progesterone (Yamazaki and Shimada 1997). It is clear from these collected findings that regulation of drug metabolizing enzymes such as UGTs and CYPs by activation of CAR and PXR can have an effect on endocrine signaling through the increased metabolism of hormones.

The role of CAR in lipid metabolism is most often in relation to bile acid synthesis and clearance. As described earlier, CAR is central in the regulation of organic anion transporters to aid in the movement of toxic bile acids out of the blood and into the bile for excretion. The main function of bile acids is as the primary pathway of cholesterol removal from the body. Bile acids also function to solubilize fatty acids to promote further digestion. Exposure to toxic bile acids such as lithocholic acid (LCA) can lead to liver damage; therefore maintenance of proper and non-toxic levels of these compounds in the body is of extreme importance (Goodwin and Kliewer 2002)

In addition to regulation of transporters of bile acids, CAR is also involved in the regulation of enzymes that produce bile acids. Bile duct ligation is a method used to induce bile acid toxicity in mice through extrahepatic cholestasis. In wild type mice, this procedure results in decreased expression of CYP7B1 and CYP8B1. CYP7B1 catalyzes an alternative to the classical CYP7A1 regulated bile acid synthesis pathway whereas CYP8B1 is important for the maintenance of the balance between cholic acid and chenodeoxycholic acid formation (Schwarz et al. 1998; Vlahcevic et al. 2000). Deletion of CAR, however, results in a less severe repression of CYP7B1 and CYP8B1 after bile duct ligation indicating that CAR is partly responsible for the down-regulation of these genes during bile duct ligation (Stedman et al. 2005).

Much of the reported work concerning CAR and the regulation of bile acid synthesis and clearance also acknowledges a role for PXR. As mentioned earlier, CAR and PXR are inexorably linked with regard to regulation of xenobiotic metabolizing enzymes. PXR and CAR coordinately regulate a multitude of genes in bile acid metabolism including the conjugating enzyme UGT1A1 and the transporters MRP2 and OATP2 (Guo et al. 2002; Kast et al. 2002; Stedman et al. 2005). Double knockout mice that lack both CAR and PXR have provided further insight into CAR/PXR crosstalk with regard to bile acid metabolism. PXR knockout mice showed increased expression of UGT1A1, MRP2 and OATP2 suggesting that PXR may repress these genes in a healthy wild type mouse (Saini et al. 2005). Conversely, mice harboring highly active CAR showed higher expression of these genes as well as increased clearance of organic salts as shown by decreased serum bilirubin (Saini et al. 2005). Thus, while PXR appears to both positively and negatively regulate bile acid clearance, CAR only positively regulates clearance.

5. Energy metabolism

It has been known for a long time that chronic PB treatment reduces plasma glucose levels in diabetic patients (Lahtela et al. 1985). Hepatic gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase 1 (PEPCK1) are repressed in PB-treated rats and mice (Manenti et al. 1987; Argaud et al. 1991; Kiyosawa et al. 2004). Recently CAR was shown to regulate the repression of gluconeogenic enzymes (Ueda et al. 2002). The expression of PEPCK1 is repressed by PB treatment in wild type but not in CAR knockout mice (Ueda et al. 2002; Kodama et al. 2004). In addition to gluconeogenic enzymes such as carnitine palmitoyltransferase 1 (CPT1) and enoyl-CoA isomerase (ECI), -oxidation enzymes, were repressed by PB treatment in rodents in a CAR dependent manner (Ueda et al. 2002; Kiyosawa et al. 2004). The CAR-mediated repression of both gluconeogenic and -oxidation enzymes suggests that this receptor plays a critical role in regulating hepatic energy metabolism in response to drug exposures.

Kodama and colleagues investigated the molecular mechanism of CAR-mediated repression of the *PEPCK1* and *G6Pase* genes that are regulated by insulin through the IRS, insulin response sequence [(T/C)(G/A)AAACAA] (Kodama et al. 2004). The forkhead family of transcription factors directly binds to the IRS and activates the transcription of IRS-bearing genes. For example, upon the binding to IRS in their promoters, FoxO1 activates the transcription of PEPCK1 (Hall et al. 2000). In the insulin-mediated repression of gluconeogenesis, insulin activates the PI3K-Akt pathway to phosphorylate FoxO1. The phosphorylated FoxO1 is excluded from the nucleus, thus resulting in the repression of PEPCK1. In the drug-induced repression, CAR binds to FoxO1 and prevents the binding of FoxO1 to IRS, thus, acting as a corepressor, decreasing the transcription of this gene (Kodama et al. 2004) (Figure 1).

FoxA2, another forkhead family member, has been reported to regulate insulin-dependent repression of α -oxidation (Wolfrum et al. 2004). CPT1 is involved in α -oxidation of fatty acids and is thought to be repressed by CAR (Ueda et al. 2002). Recent evidence suggests that FoxA2 and CAR coordinately repress CPT1 expression though the mechanism behind this repression is still under investigation at this point in time (Nakamura et al. 2005).

Mice treated over the course of 14 days with PB exhibit regulation of a large number of genes involved in fatty acid metabolism as analyzed by microarray (Kiyosawa et al. 2004). Genes such as aldehyde dehydrogenase, lipoprotein lipase, and oxidosqualene lanosterol-cyclase were highly up-regulated and genes such as hepatic lipase, stearyl-CoA desaturases 1 and 2 and pyruvate carboxylase were all significantly down-regulated. The collective findings of these array experiments suggest that PB functions to promote cholesterol synthesis and lipolysis as well as repress glycolysis in the liver. Whereas these latter studies were not performed concurrently in the absence of CAR, the results suggest CAR as a possible mediator of these effects.

6. Regulation of drug metabolism by endocrine signaling through CAR

While it is now known that xenobiotic activation of CAR and/or PXR alters the physiological function of the liver, endogenous stimuli that regulate these functions are also involved in modulating xenobiotic metabolism via these receptors. There is mounting evidence for the modulation of CAR activity by steroid hormones in cell-based transfection assays, although work in this area *in vivo* remains scarce.

Estrogens were found to activate CAR, though generally not to the same levels as other known CAR ligands (Kawamoto et al. 2000). Ovariectomy did not alter CAR activity, however, suggesting that in female mice endogenous levels of estrogen might be too low to affect CAR. In these same studies, castration of male mice resulted in the nuclear accumulation of CAR suggesting that physiological levels of androgens can repress estrogen mediated nuclear accumulation of CAR. The activation of CAR by estrogen promotes CAR movement to the nucleus, similar to other CAR activators as well as the recruitment of coactivators (Min et al. 2002). Interestingly, estrogen activation of CAR can also promote the recruitment of corepressors to the CAR complex (Makinen et al. 2003). Considering the constitutive activity of CAR, movement of the receptor into and out of the nucleus as well as modulation of coregulator recruitment can have a significant effect on the regulation of CAR target genes. Thus, estrogen mediated effects on CAR activity represent an alternate mechanism of CAR regulation by endogenous signaling molecules.

The effect of CAR on estrogen signaling is not limited to estrogen as a CAR activator. CAR has been shown to inhibit estrogen receptor (ER) activity in cell-based transfection assays (Min et al. 2002). Overexpression of the p160 coactivator GRIP1 relieved this repression by CAR. GRIP1 is a coactivator for CAR and ER and can interact with both receptors (Norris et al. 1998; Min et al. 2002). In cell based reporter assays, GRIP1 interaction with CAR enhances CAR activity and sequesters GRIP1 away from ER repressing ER activity (Min et al. 2002). Whereas the role of GRIP1 in the overall regulation of CAR activity is not clear *in vivo*, the above findings suggest that CAR crosstalk with estrogen receptor signaling is based on the reciprocal competition for the existing coregulator pool.

Progesterone and androgen, have been shown to repress CAR activity (Kawamoto et al. 2000). Indeed, the most commonly utilized class of compounds to repress CAR are derivatives of androgen known as androstanes (Forman et al. 1998). The crystal structure of CAR showed that androstanes are able to bind in the receptor's ligand binding pocket (Shan et al. 2004). The mechanism of repression is structural in that androstane binding does not allow formation of

the salt bridge between CAR and the ligand that locks helix 12 of CAR into an active conformation. Thus, CAR can be competitively repressed by direct occupation of the ligand binding pocket. The effective concentration of androstanes to repress the CAR-mediated transactivation is at the micromolar level that is several magnitudes higher than the physiological levels in the circulation. Although androstanes are not physiological ligands of CAR, the identification of the mechanism of androstane repression of CAR could lead to the design of novel compounds to repress CAR activity and therefore has implications for the regulation of drug metabolism and treatment of diseases such as liver cancer.

Retinoids have also been shown to repress CAR activity (Kakizaki et al. 2002). The mechanism behind this repression has been attributed to increased dimerization between RXR (which is the primary heterodimer partner for CAR) and RAR in the presence of either 9-cis or all trans retinoic acid. The increased dimerization of RXR to RAR leads to less RXR available for binding to CAR and consequently less formation of a protein complex around CAR that is transcriptionally active. Given that RXR is highly expressed in the liver, it is possible that levels of RXR are not limiting. Conversely, overexpression of RAR in a transient transfection system in HepG2 cells increased the level of retinoic acid repression of CAR activity. Given the promiscuity of RXR as a heterodimer partner for nuclear receptors it is not surprising that retinoids can influence CAR activity though the *in vivo* role of retinoids remains unknown with regards to CAR.

Not only is CAR involved in the regulation of steroid signaling, CAR itself is regulated by glucocorticoids (Pascucci et al. 2000). Glucocorticoids such as dexamethasone increase CAR mRNA in the liver. This increase in CAR levels is also associated with increased sensitivity to PB stimulation upon co-treatment with dexamethasone, ultimately leading to synergistic increases in CYP2B levels.

7. Conclusions

The ability of nuclear receptors such as CAR to regulate drug metabolizing enzymes in the liver has earned these transcription factors the title of xenosensors. The mechanisms of how these receptors regulate drug metabolism is reasonably well established and attention is now turning to the role of CAR in endogenous signaling. CAR can alter endocrine signaling by promoting the clearance of molecules such as estrogen, progesterone and thyroid hormone through the regulation of the same enzymes that metabolize drugs. Alternatively, emerging evidence suggests that CAR can alter the synthesis of hormones such as thyroid hormone, also affecting endocrine signaling. Not only can CAR regulate endocrine signals, but CAR is also influenced by endocrine signals as evidenced by the ability of insulin to repress drug metabolism. The ability of CAR to function within endocrine signaling pathways also allows CAR to regulate aspects of cellular homeostasis including energy and fatty acid metabolism. CAR activators can regulate the synthesis of cholesterol and increase lipolysis in the liver while they also repress glycolysis. The involvement of CAR in endocrine signaling places CAR in the position to be a regulator of not only detoxification but also endogenous signaling. The mounting evidence of a CAR influence on endocrine signaling suggests that endocrine signals such as steroid hormones might act through CAR to regulate drug metabolism (Figure 2). Whereas there is no direct evidence to support this theory to date, CAR involvement in endogenous endocrine regulation of all phases of drug metabolism may serve as a novel mechanism for the regulation of these processes.

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Figure 1.
CAR regulation of forkhead transcription factor signaling

Figure 2.
CAR and PXR regulation of xenobiotic and endobiotic signaling