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THE ROLE OF CAR AND PXR IN TOXICANT SENSITIVITY

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THE ROLE OF CAR AND PXR IN TOXICANT SENSITIVITY.

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

by
Linda Cristal Mota Bátiz
August 2010

Accepted by:
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ABSTRACT

The Constitutive Androstane Receptor (CAR) and the Pregnane X Receptor (PXR) are nuclear receptors of significant importance in the regulation of enzymes that metabolize, detoxify and eliminate compounds from the body. In this study we assessed the protective role of CAR and PXR in the basal and inducible regulation of Cytchrome P450s (CYPs), and the potential of CAR and PXR to help protect individuals from the organophosphate, parathion and the plasticizer, nonylphenol, putatively due to improved metabolism and elimination. Knockout models of these receptors were used to model susceptible populations such as children that are known to have lower CAR and PXR expression during the first six months of age. A humanized model was used to extrapolate findings to human populations. Overall, the data suggests that individuals with low CAR or PXR (newborn children), or low CAR/PXR activation (elderly) may be more susceptible to xenobiotic toxicity putatively because of the lower expression of CAR and PXR resulting in a lower expression of CYPs which leads to the inability to metabolize, detoxify and eliminate toxic compounds.
DEDICATION

A mi abuelita, mi Tico y mi familia

To my grandmother, my husband and my family
ACKNOWLEDGMENTS

I would like to thank Dr. William Baldwin for giving me the opportunity to work in his lab in the University of Texas at El Paso as well as Clemson University. I would like to thank my committee, Dr. Lisa Bain, Dr. Paula Watt, Dr. Charles Rice and Dr. Steve Klaine for their input and support throughout my studies. I would like to show my appreciation to Dr. Juan Hernandez who is currently at Baylor College of Medicine who trained me in all the lab techniques and helped me in the troubleshooting of my experiments. I want to thank Dr. Kristen Gaworecki and Dr. van den Hurk who provided their HPLC expertise. I would like to acknowledge my lab group Gautam, Yang, Omaima, Elliott, Christina and special thanks to Basma for their continuous support and help. I want to thank Dr. Bain’s lab, Amanda, Gia-Ming and Ben for their help in troubleshooting.
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CHAPTER ONE
INTRODUCTION

This study was conducted to elucidate the involvement of CAR and PXR in protecting the body from nonylphenol and organophosphate toxicity by the direct regulation of CYPs that are significantly important in the metabolism and detoxification of compounds from the body.

1.1 Nuclear Receptors

Nuclear receptors are one class of transcription factors found inside cells that respond to ligands leading to the regulation and transcription of target genes. Typically, ligand binding results in a conformational change that alters the function and activity of the receptors and allows them to bind DNA and regulate transcription (Tsai M-J and O'Malley BW 1994).

Nuclear receptor-mediated regulation of gene expression is involved in development, reproduction, metabolism and homeostasis of organisms.

<table>
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<tr>
<td>Glucocorticoid</td>
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<tr>
<td>Vitamin D</td>
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<tr>
<td>Retinoic Acid</td>
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<td>Thyroid Hormone</td>
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<tr>
<td>Pregnane X</td>
<td>Pregnane</td>
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<tr>
<td>Constitutive Androstane</td>
<td>Phenobarbital</td>
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<td>Farnesoid X</td>
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<td>Liver X</td>
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The superfamily is composed of steroid hormones (estrogens, corticosteroids, progestins,
androgens), non-steroid (thyroid hormone and retinoids), orphan receptors that do not have an identified ligand (phenobarbital, rifampicin, pregnanes, androstanol), or adopted orphans in which a ligand or set of low affinity ligands have been recently determined (CAR, PXR, HNF4α) (Handschin C and Meyer UA 2003; Evans RM 2005).

Figure 1.1 Structure of nuclear receptors. The A/B domain is important in the recruitment of coactivators and activation of the basal transcription complex. The C domain binds to the DNA at target enhancer sequences. The D domain allows flexibility and contains a nuclear translocation signal. The E domain binds the ligands, important for dimerization, and recruits coactivators at AF-2 following ligand activation. The purpose of the F domain is unknown or controversial.

<table>
<thead>
<tr>
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<th>C DNA-binding</th>
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<th>E Ligand-binding Dimerization AF-2</th>
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Nuclear receptors are structurally organized in five distinct domains. The A/B domain is in the N-terminal region, it is highly variable in size and sequence between nuclear receptors and contains a ligand independent Activation Function-1 (AF-1). A/B domain is very important in the recruitment of coactivators and is essential in bridging the gap to activate the basal transcription complex consisting of RNA polymerase II, TFII-D, TFII-B, TFII-E, TFII-F and TFII-H. AF-1 is structurally disordered, it has several phosphorylation sites that may play a role in folding, and a secondary structure formation is required for activation (Wärnmark A, Treuter E et al. 2003). The C domain is the DNA binding domain (DBD) and is highly conserved between receptors. The DBD contains two zinc fingers bound by four cysteines residues. The first zinc finger determines the specific target sequence in DNA to be bound and the second finger is
important in dimerization and controls the spacing between the specific sequences by each subunit in the dimer (Lewin B 2006). Some receptors function as homodimers and others as heterodimers. Each receptor recognizes specifically a response element (RE) in the DNA composed of two short repeats, suggestive of the ability of the receptor to bind as a dimer (Lewin B 2006). The D domain is the hinge region between the DBD and the LBD that allows flexibility to the receptor and may have a nuclear localization signal. The additional amino acids composing the hinge region can be used as an additional dimerization surface (Tsai M-J and O'Malley BW 1994). The E domain contains the ligand binding domain (LBD) localized in the C-terminal of the receptor and is functionally independent. The LBD is where the ligand binds and the receptor recruits coactivators and corepressors and nuclear localization signal occurs. Ligand binding at the E-domain significantly alters the receptor confirmation allowing for co-activator recruitment to the AF-1 and AF-2 sites on the receptor.

Corepressors may recruit histone deacetylases (HDAC) to target specific genes and repress their transcription. Repression of transcription occurs by binding corepressors protein complexes (Lewin B 2006). Following ligand interactions with the E-domain, the co-repressors are lost and coactivators are recruited. Coactivators such as histone acetylases (HAT) increase the rate of transcription by acetyllating DNA resulting in the unwinding for transcription to occur. Coactivators in the p160 family can acetylate DNA or aid in the recruitment of other coactivators such as CBP/p300 (Lewin B 2006). The AF-2 is also found in the LBD and is ligand dependent, highly conserved between receptors, and highly structured. AF-2 plays a major role in the recruitment of
coactivators that have leucine rich motifs which have been found to be of importance in the receptor and coactivator interaction (Wärnmark A, Treuter E et al. 2003). The F domain is variable in size and sequence between receptors, and is absent in some nuclear receptors.

The types of ligands that can bind and activate nuclear receptors are not restricted to endogenous compounds. Extensive research has found a wide variety of pharmaceuticals and chemical compounds that can activate or alter the function of these receptors. As a response of the body to metabolize and eliminate these compounds some orphan receptors such as CAR and PXR have been found to be very important and are key players involved in the metabolism, detoxification and elimination of chemicals.

1.2 CAR/PXR

The constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are of great importance in endogenous detoxification of compounds such as bile acids, hormones, bilirubin (Huang W, Zhang J et al. 2003) as well as the detoxification of foreign compounds like drugs such as dexamethasone, rifampicin, phenobarbital (Sueyoshi T, Kawamoto T et al. 1999) were CAR and PXR loss increases sensitivity to these compounds (Sueyoshi T, Kawamoto T et al. 1999; Hernandez JP, Chapman LM et al. 2006)

CAR and PXR are both mediators of the cellular response with different activation mechanisms (Handschin C and Meyer UA 2003). CAR’s constitutive activity is attributed to its unique structure that only contains three domains (Suino K, Peng L et al. 2004): a highly conserved DNA-binding domain; a hinge region; and a divergent
ligand binding/dimerization/transcriptional activation domain (Pascussi JM, Gerbal-Chaloin S et al. 2003). CAR’s high constitutive activity is due to shortness and rigidity of the AF2 helix, which allows the formation of additional hydrogen bonds and permits AF2 to remain in its active conformation (Suino K, Peng L et al. 2004). It is hypothesized that CAR is less promiscuous than PXR (2, (Pascussi JM, Gerbal-Chaloin S et al. 2003) because of its smaller and less flexible ligand-binding domain (Watkins RE, Wisely GB et al. 2001; Suino K, Peng L et al. 2004). CAR is sequestered in the cytoplasm by a retention protein complex composed of heat shock proteins (Hsp), immunophilins, and CAR cytosolic retention proteins (CCRP). CAR may be activated by direct and indirect mechanisms (Sueyoshi T, Kawamoto T et al. 1999; Shindo S, Numazawa S et al. 2007).

Figure 1.2 Mechanism of CAR activation. CAR is held in the cytoplasm by several retention proteins, ligand binding changes CAR’s conformation leading to retention protein dissociation and translocation into the nucleus where it binds the response element of the gene that would be transcribed.

Direct activation of CAR has been shown in very few compounds such as 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) that the ligand directly binds CAR (Tzameli I, Pissios P et al. 2000) causing a conformational change that results in the dissociation of
the retention protein complex and translocation into the nucleus. Indirect activation of CAR can be observed on the majority of compounds such as phenobarbital (PB) that do not require the ligand binding for CAR activation (Kawamoto T, Sueyoshi T et al. 1999). Protein kinase C phosphorylates threonine 38 resulting in destabilization of this helix and inactivation of CAR (Mutoh S, Osabe M et al. 2009). Dephosphorylation by a compound at this site induces conformational change, dissociation of the retention protein complex and translocation of CAR into the nucleus (Kawamoto T, Sueyoshi T et al. 1999; Sueyoshi T, Kawamoto T et al. 1999). Once CAR is inside the nucleus, it binds the IR-6 and DR-4 in the Phenobarbital Response Enhancement Module (PBREM) in DNA, heterodimerizes with the Retinoid X Receptor (RXR), and induces transcription (Honkakoski P, Zelko I et al. 1998; Sueyoshi T and Negishi M 2001). It has been hypothesized that the majority of CAR activators work through an indirect pathway instead of direct binding because of the smaller size of CAR’s ligand binding domain (Shindo S, Numazawa S et al. 2007).

PXR has evolved several features that allows a wide variety of xenobiotics to activate it, such as a small number of polar residues spaced through a smooth, hydrophobic and significantly large ligand-binding domain (Watkins RE, Wisely GB et al. 2001). PXR’s LBD possesses a flexible loop that has the ability to contract or expand and can accommodate different size chemicals. Studies found PXR to have a unique Tryptophan-Zipper homodimer in the LBD forming a unique quadruplex and is important in the recruitment of the coactivator SRC-1 (Noble SM, Carnahan VE et al. 2006).
PXR is thought to be primarily inactive in the nucleus bound to its heterodimer partner RXRα forming a repressor complex with SMRT and/or SHP, and histone deacetylases that inhibit transcription (Takeshita A, Taguchi M et al. 2002; Ourlin JC, Lasserre F et al. 2003). PXR is bound to the IR-6, ER-6 and DR-3 xenobiotic response elements (XRE) localized in the promoter regions of target genes (Blumberg B, Sabbagh W Jr et al. 1998; Kliewer SA, Moore JT et al. 1998; Sueyoshi T and Negishi M 2001).

Ligand binding and activation changes PXR conformation resulting in the dissociation of the co-repressors and initiates the recruitment of co-activators such as steroid receptor co-activator 1 (SRC-1) and histone acetylases that promote transcription (Takeshita A, Taguchi M et al. 2002; Ourlin JC, Lasserre F et al. 2003; Watkins RE, Davis-Searles PR et al. 2003).
1.3 CYPs and CYP regulation by PXR and CAR

CAR and the PXR are orphan nuclear receptors that mediate the induction of several phase I-III enzymes that are involved in the detoxification and elimination of compounds thus preventing the accumulation of toxic chemicals in the body (Staudinger JL, Madan A et al. 2003). In our research we studied the CYPs, which are in the Phase I superfamily of enzymes. CYPs are heme proteins divided into families denoted by a number e.g. CYP3, subfamilies denoted by a letter e.g. CYP3A and specific isoforms denoted by a number for specific proteins e.g. CYP3A4. Of the CYPs families, CAR and PXR are key regulators of CYP2A, 2B, 2C, and 3A (Slatter JG, Templeton IE et al. 2006; Wortham M, Czerwinski M et al. 2007).

CYP families 1-3 are mainly regulated by CAR and PXR and are involved in the metabolism of foreign chemicals. The most sensitive biomarker for CAR and PXR activation are CYP2B and CYP3A respectively, critical in metabolizing endogenous compounds and xenobiotics (Waxman DJ 1999). Studies suggest that CAR and PXR bind the same response elements in liver cells and therefore show overlapping regulation of CYP2B and CYP3A (Xie W, Barwick JL et al. 2000; Goodwin B, Moore LB et al. 2001; Smirlis D, Muangmoonchai R et al. 2001). The majority of the studies conducted have investigated the induction of CYPs regulated through CAR and PXR activation, surprisingly research is very limited in the basal regulation of CYPs by CAR and PXR.

Interestingly studies have shown sexual differences between the expression of several CYPs regulated through HNF4α (Wiwi CA, Gupte M et al. 2004), a receptor known to regulate CAR and PXR expression (Wortham M, Czerwinski M et al. 2007).
Sexual dimorphisms have been observed in Cyp2a, 2b, 2c and 3a expression regulated by HNF4α (Wiwi CA, Gupte M et al. 2004; Holloway MG, Miles GD et al. 2008) as well as gender specific CYP induction by compounds regulated through CAR activation (Ledda-Columbano GM, Pibiri M et al. 2003; Hernandez JP, Chapman LM et al. 2006).

The purpose of the research is to investigate the role of CAR and PXR in protecting organisms from toxicants. Specifically the role of CYP-regulation by CAR and PXR in xenobiotic protection will be examined with an interest in susceptible populations such as children that may have a higher sensitivity to the toxicity of these compounds due to limited metabolic capacity. Chemicals that we will examine include the organophosphate pesticide, parathion and the plasticizer, nonylphenol.

1.4 Organophosphates

Overpopulation has led to a higher demand of crop yield resulting in a significantly overuse of pesticides to achieve the necessary demand. One of the types of pesticides that are vastly used worldwide are organophosphates that are mainly used for agricultural purposes and control of pests in residences (Post A 1998; Singh BK and Walker A 2006). In 2001 the Environmental Protection Agency reported the use of organophosphates in the United States to be 73 million pounds, 70% of all insecticides use in all market sectors (United States Environmental Protection Agency 2000-2001). These pesticides have been the cause of 3 million poisonings and 200,000 deaths annually worldwide (Karalliedde L and Senanayake N 1999; Sogorb MA, Vilanova E et al. 2004).
Some of the health effects of organophosphates poisoning include several nerve, muscular and immunotoxic diseases (Colborn T, Dumanoski D et al. 1996; Ragnarsdottir KV 2000; Galloway T and Handy R 2003). The mechanism of how most organophosphates work is to inhibit the breakdown of the neurotransmitter acetylcholine by changing its structure and function, the nerves are then overstimulated causing convulsion, paralysis and death for insects and mammals (Manahan SE 1992; Ragnarsdottir KV 2000). Organophosphate toxicity is of great importance especially to those susceptible populations that might have poor metabolic capabilities. This includes young children that appear to have limited metabolic capacity (Padilla S, Buzzard J et al. 2000; Sheets LP 2000).

1.5 Nonylphenol

Nonylphenol is one of the major degradation byproducts from alkylphenol ethoxylates that have been widely used in the production of detergents, paint, herbicides and other formulated products (Nimrod AC and Benson WH 1996). Furthermore, nonylphenol is nearly ubiquitous in the environment. For example, significant concentrations of nonylphenol have been found in air at up to 81 ng/m³, in the water up to 644 ug/l, and up to 13700 ug/kg in the soil (Ying GG, Williams B et al. 2002; Vazquez-Duhalt R, Marquez-Rocha F et al. 2006). Nonylphenol was found to be one of the 30 most frequently detected compounds in U.S. streams and when found is often found at higher concentrations than most if not all of the other chemicals examined (Kolpin DW, Furlong ET et al. 2002).
Studies have found that nonylphenol not only causes acute toxicity but also has the capacity to act as an endocrine disruptor (Vazquez-Duhalt R, Marquez-Rocha F et al. 2006) by mimicking estradiol in inducing proliferation and activation of the progesterone receptor in human estrogen-sensitive breast tumor cells (Soto AM, Justica J et al. 1991). Numerous studies have demonstrated the potential endocrine disrupting effects of nonylphenol, and include blocking the Androgen Receptor (AR) (Lee HJ, Chattopadhyay S et al. 2003; Krüger T, Long M et al. 2008) activating the Estrogen Receptor (ER) (White R, Jobling S et al. 1994), Aryl Hydrocarbon Receptor (AhR) (Krüger T, Long M et al. 2008), Constitutive Androstane Receptor (CAR) (Hernandez JP, Huang W et al. 2007), and Pregnane X Receptor (Masuyama H, Hiramatsu Y et al. 2000; Hernandez JP, Huang W et al. 2007).

Recent data has demonstrated nonylphenol as a moderately potent CAR activator (Hernandez JP, Huang W et al. 2007). Studies have also shown the induction of CYP3A gene expression by nonylphenol, putatively through the activation of PXR, although the mechanism of induction was only examined in vitro (Masuyama H, Hiramatsu Y et al. 2000). Due to nonylphenol’s ubiquitous presence in the environment and capabilities in activating CAR and PXR it may cause toxicity and endocrine disruption in sensitive populations with polymorphisms or life stages, or perturb the regulation of detoxification enzymes and transporters.
1.6 Specific Aims

The purpose of this study is to characterize CYPs that are basally regulated by the constitutive androstane receptor (CAR) and the pregnane X-receptor (PXR), as well to assess the involvement of these nuclear receptors in an individual organism’s sensitivity to parathion and nonylphenol. Interestingly, while CAR and PXR are considered xenobiotic receptors, there is little to no research indicating that their presence protects us from toxic chemicals.

**Objective 1:** Investigate the role of CAR in the basal regulation of CYPs, and the sexually dimorphic regulation of CYPs following nonylphenol and TCPOBOP treatment. This study will test whether CAR regulates basal (constitutive) levels of CYPs in the liver. In addition, it will investigate possible induction in various sexually dimorphic CYPs regulated by CAR to understand whether nonylphenol, a partial agonist, and TCPOBOP, a full agonist have differing effects on CAR-mediated CYP regulation in males and females.

**Objective 2:** Assess the protective role of CAR in parathion-mediated toxicity. Determine if CAR-mediated regulation of CYPs is associated with CAR’s role in protecting individuals from parathion toxicity. Recently, our laboratory demonstrated that parathion is a CAR activator in an in vitro transaction assay (Baldwin WS and Roling JA 2009). Therefore, we hypothesize that CAR may be protecting individuals from parathion by inducing detoxification enzymes. CAR’s role in protecting organisms from parathion will be assessed by comparing parathion toxicity in WT and CAR-null mice. In addition, we will investigate whether parathion induces CYPs in vivo in a
CAR-dependent manner. Next, we will test whether CAR and WT mice show differential metabolism of parathion to its toxic (paraoxon) and non-toxic (p-nitrophenol) metabolites. The findings of this study will be used to elucidate whether there may be susceptible human populations that have low CAR expression resulting in a limited metabolic capacity and increased sensitivity to parathion.

**Objective 3: Determine the role of PXR in mediating CYP induction to protect organisms from nonylphenol toxicity.** First, we will investigate the basal regulation of CYPs by PXR as we did in the first objective with CAR. Then, we will determine if nonylphenol, a known PXR agonist activates PXR in vivo as this has not been elucidated. PXR-null and WT mice will be used to investigate whether nonylphenol induction of CYPs is PXR-dependent. Furthermore, we will test whether mice that lack PXR are susceptible to nonylphenol’s hepatic toxicity and show reduced clearance of nonylphenol from the body. Liver slices will be H&E stained and serum will be nonylphenol extracted for histopathology and GC-MS analysis to determine if nonylphenol clearance and toxicity are related to PXR status. Furthermore, hPXR mice and human primary hepatocytes will be used to better extrapolate mouse data to humans. Interestingly, while PXR is considered a xenobiotic sensor, it has never been shown to protect individuals from a toxic chemical. Our primary goal is to determine if PXR is involved in the protection of mice from nonylphenol.
RERERENCES


CHAPTER TWO

SEXUALLY DIMORPHIC REGULATION AND INDUCTION OF P450S BY THE
CONSTITUTIVE ANDROSTANE RECEPTOR (CAR)

Toxicology Volume 256, Issues 1-2, 4 February 2009, Pages 53-64

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Received 18 August 2008;
revised 31 October 2008;
accepted 3 November 2008.
Available online 11 November 2008.
2.1 Abstract
The constitutive androstane receptor (CAR) is a xenosensing nuclear receptor and regulator of cytochrome P450s (CYPs). However, the role of CAR as a basal regulator of CYP expression nor its role in sexually dimorphic responses have been thoroughly studied. We investigated basal regulation and sexually dimorphic regulation and induction by the potent CAR activator TCPOBOP and the moderate CAR activator Nonylphenol (NP). NP is an environmental estrogen and one of the most commonly found environmental toxicants in Europe and the United States. Previous studies have demonstrated that NP induces several CYPs in a sexually dimorphic manner, however the role of CAR in regulating NP-mediated sexually dimorphic P450 expression and induction has not been elucidated. Therefore, wild-type and CAR-null male and female mice were treated with honey as a carrier, NP, or TCPOBOP and CYP expression monitored by QPCR and Western blotting. CAR basally regulates the expression of Cyp2c29, Cyp2b13, and potentially Cyp2b10 as demonstrated by QPCR. Furthermore, we observed a shift in the testosterone 6α/15α-hydroxylase ratio in untreated CAR-null female mice to the male pattern, which indicates an alteration in androgen status and suggests a role for androgens as CAR inverse agonists. Xenobiotic-treatments with NP and TCPOBOP induced Cyp2b10, Cyp2c29, and Cyp3a11 in a CAR-mediated fashion; however NP only induced these CYPs in females and TCPOBOP induced these CYPs in both males and females. Interestingly, Cyp2a4, was only induced in wild-type male mice by TCPOBOP suggesting Cyp2a4 induction is not sensitive to CAR-mediated induction in females. Overall, TCPOBOP and NP show similar CYP induction profiles in females,
but widely different profiles in males potentially related to lower sensitivity of males to either indirect or moderate CAR activators such as NP. In summary, CAR regulates the basal and chemically inducible expression of several sexually dimorphic xenobiotic metabolizing P450s in a manner that varies depending on the ligand.

**Keywords:** Constitutive androstane receptor (CAR); Nonylphenol; Cytochrome P450 (CYP); Sexually dimorphic; Liver

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2.2 Introduction

The constitutive androstane receptor (CAR) is a nuclear receptor that mediates the hepatic regulation and expression of a wide variety of genes involved in endobiotic and xenobiotic clearance (Kretschmer and Baldwin, 2005). CAR regulates Phase I genes, such as CYP2B6, CYP2C8/9 and CYP3A4, phase II conjugation enzymes such as UDP-glucoronosyltranferase, and phase III transporters such as multidrug resistance-associated proteins 2 and 3 ([Goodwin et al., 2002], [Kats et al., 2002], [Sueyoshi et al., 1999], [Sugatani et al., 2001] and [Xiong et al., 2002]). In addition, CAR is involved in the regulation of gluconeogenesis, fatty acid oxidation and the metabolism of steroid hormones, bile acids, and bilirubin ([Huang et al., 2003], [Sugatani et al., 2001], [Ueda et al., 2002], [Wei et al., 2000]).

In contrast to other nuclear receptors that contain five domains, CAR contains only three: a highly conserved DNA-binding domain; a hinge region; and a divergent ligand binding/dimerization/transcriptional activation domain (Pascussi et al., 2003). This in part may explain some of the unique features of CAR including its constitutive activity (Suino et al., 2004).

Inside the cell, CAR is retained in the cytoplasm forming a complex with heat shock proteins (Hsp90), immunophilins, P-23, and cytoplasmic CAR retention protein (CCRP), a bifunctional linker protein ([Kobayashi et al., 2003] and [Yoshinori et al., 2003]). Upon activation CAR is translocated to the nucleus in response to stress by the recruitment of protein phophatase 2A (PP2A) leading to dephosphorylation of a Ser-202 near the C terminus of the ligand binding domain ([Hosseinpour et al., 2006] and
(Yoshinori et al., 2003). Interestingly, it has been hypothesized that the majority of CAR activators work through an indirect dephosphorylation pathway similar to phenobarbital instead of binding directly to CAR (Shindo et al., 2007).

CAR and its relative, the pregnane X receptor (PXR), cross talk by sharing response elements and showing overlapping affinities for some ligands (Handschin and Meyer, 2003); providing each other a backup system for responding to toxicants, but also increasing nuclear receptor interactions and making it difficult to interpret some data. The heterodimerization of CAR or PXR with RXR ([Baes et al., 1994] and [Kliewer et al., 1998]) and subsequent interaction with the phenobarbital responsive enhancer module (PBREM) or xenobiotic responsive enhancer module (XREM) induces the expression of classical biomarkers such as Cyp2b10 (CAR) and Cyp3a11 (PXR) as well as other CYP genes involved in detoxification ([Ferguson et al., 2002], [Jackson et al., 2006] and [Wang et al., 2003]). The primary CYP families involved in detoxification of foreign chemicals are found in families 1–3, and several of these are inducible by CAR or PXR (Kretschmer and Baldwin, 2005).

Many of the xenobiotic detoxifying P450s are gender specific or gender predominant ([Hernandez et al., 2006] and [Wiwi et al., 2004]). Male specific or male predominant liver P450s include Cyp2d9 and 4a12 in the mouse ([Noshiro and Negishi, 1986] and [Wiwi et al., 2004]). Female predominant liver P450s include Cyps 2a4, 2b9, 3a41 and 3a44 in the mouse ([Burkhart et al., 1985], [Noshiro and Negishi, 1986], [Sakuma et al., 2002] and [Wiwi et al., 2004]). Gender predominance has been primarily attributed to the frequency of growth hormone (GH) pulse secreted by the pituitary with
pulses more frequent in female rats and episodic bursts in male rats (Waxman et al., 1991). However, other factors including the nuclear receptors RXRα and HNF4α may in part play a role in the gender predominant expression of several P450s in the CYP2-4 families ([Cai et al., 2003] and [Wiwi et al., 2004]).

CAR demonstrates greater activity in females than males. For example, it has been reported that TCPOBOP increases liver proliferation in females more than males (Ledda-Columbano et al., 2003). Estrogens activate CAR and this may increase CAR activity in females relative to males (Kawamoto et al., 2000). Furthermore, androgens also inhibit CAR activity in mice (Forman et al., 1998), and this may reduce CAR activity in males relative to females. Lastly, CAR demonstrates female predominant mRNA expression (Petrick and Klaassen, 2007). Taken together, this data indicates that CAR may have greater activity in female mice and therefore help control and maintain basal and inductive expression of several sexually dimorphic CYPs.

Nonylphenol (NP) is a plasticizer and common environmental estrogen ([Acevedo et al., 2005] and [Soto et al., 1991]) that is also a moderately potent activator of CAR (Hernandez et al., 2007). Previous research has demonstrated sexually dimorphic regulation of Cyp2a, 2b, and 3a-subfamily members by nonylphenol ([Hernandez et al., 2006] and [Laurenzana et al., 2002]). Therefore, we hypothesized that nonylphenol may mediate its sexually dimorphic CYP induction through CAR. In addition, the CAR activators phenobarbital and TCPOBOP demonstrate gender predominant induction, as they induce the female-predominant P450, Cyp2a4, only in males (Wei et al., 2002). Therefore, we decided to compare the sexually dimorphic induction of CYPs by
TCPOBOP and NP using wild-type and CAR-null mice to determine the role of CAR in mediating gender predominant induction of CYPs.

Lastly, there is limited research regarding the possible regulatory role of CAR in basal CYP expression. Currently no data has directly monitored or linked CAR to basal CYP expression or gender predominant CYP expression. Therefore, this study will also examine the basal expression of CYPs in male and female wild-type and CAR-null mice in order to assess the role of CAR in regulating basal CYP expression. Gender differences in CYP expression mediated by CAR may cause gender-specific outcomes following exposure to pharmaceuticals or environmental chemicals. The overall purpose of this study is to determine whether CAR regulates basal CYP expression in a sexually dimorphic manner, and test whether the moderate (NP) and potent (TCPOBOP) CAR activators demonstrate significant differences in CYP induction in a gender predominant fashion in wild-type or CAR-null adult mice.

2.3 Materials and methods

2.3.1 Chemicals

Technical grade 4-NP (≈85% para-isomers) was obtained from Fluka Chemical Co. (Seelze, Germany). Absolute ethanol, Zoxazolamine (2-amino-5-chlorobenzoxazole 97% purity), 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), and ketamine HCl/ xylazine HCl (800/120 mg) were obtained from Sigma–Aldrich (St. Louis, MO).

2.3.2 Mice

All studies were carried out according to NIH guidelines for the humane use of research animals and were pre-approved by the Baylor College of Medicine, University
of Texas at El Paso, or Clemson University Animal Care and Use Committee. CAR-null mice, on a B6129 background, were previously described (Wei et al., 2000), and are housed at both the Baylor College of Medicine and the University of Texas at El Paso. Strain (B6129PF1/J), obtained from The Jackson Laboratory (Bar Harbor, ME) at 3–5 weeks old, was used as a control (wild-type; WT) to provide an approximate genetic match to the CAR-null mice. WT mice were held for 5 weeks prior to treatment, so that all treatments were performed with 8–10 week old mice. Mice were provided water, and fed ad libitum prior to and during treatments.

2.3.3 Nonylphenol and TCPOBOP treatment of wild-type and CAR-null mice

Eight to ten-week old B6129PF1/J male and female mice were randomly split into five treatment groups each (n = 5–6; 3–4 respectively). The mice were fed 0, 50, or 75 mg/kg/day NP mixed in 100 μl honey for 7 days, or injected with 0 or 3 mg/kg TCPOBOP mixed in 100 μl of corn oil 1 day prior to necropsy. Age matched male and female CAR-null mice were split into similar groups and treated the same as the wild-type mice. All mice were anesthetized by ketamine injection, and euthanized by CO₂ asphyxiation. Livers were excised, diced into several pieces; half of the liver was used for microsome preparation, and the other half was placed in TRI-Reagent® (Sigma–Aldrich) for RNA extraction. All samples were stored at −80 °C.

2.3.4 Sample preparations

RNA was extracted from approximately half of the liver with TRI-reagent® according to the manufacturer's instructions followed by DNAse (Promega Corporation, Madison, WI) digestion to remove residual genomic DNA. RNA concentrations were determined
spectrophotometrically at 260/280 nm (Molecular Devices, Ramsey, MN). Reverse transcription was performed to make cDNA using 200 units Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT), a 10 mM dNTP mixture, and 0.05 mg random hexamers (Promega Corporation, Madison, WI).

For microsome and cytosol preparation, approximately half of the liver was individually homogenized with a Dounce Homogenizer and protein fractions were prepared as described previously (Van der Hoeven and Coon, 1974). Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA) according to manufacturer's instructions.

2.3.5 Testosterone hydroxylase assays

Testosterone hydroxylase assays were used to measure CYP activity as previously described using thin-layer chromatography to separate the testosterone metabolites (Hernandez et al., 2006). Testosterone metabolites were quantified with a LS5801 liquid scintillation counter (Beckman, Fullerton, CA).

2.3.6 Quantitative real-time polymerase chain reaction (QPCR)

Quantitative real-time PCR (QPCR) was performed using primers for specific isoforms to Cyp2a, Cyp2b, Cyp2c, and Cyp3a subfamily members, or 18S as the housekeeping gene. All the primers have been previously published (Hernandez et al., 2006), except Cyp2c37. The sequences and annealing temperatures for Cyp2c37 primers are 5'-ATACTCTATATTTGGGCAGG-3' for the forward primer and 5'-GTTCCCTCCACAAGGCAGC-3'(52.5 °C) for the reverse primer. Amplifications of the samples and the standard curve were performed in triplicate using a 96-well MyiQ™
Single Color Reverse Transcription Real-Time PCR detection system (Bio-Rad) with 0.25× SybrGreen (SuperArray Biosciences Co., Frederick, MD) as the fluorescent double strand-intercalating agent to quantify gene expression as described previously ([Hernandez et al., 2006] and [Hernandez et al., 2007]) using Muller's equation to determine relative quantities of each CYP (Muller et al., 2002).

2.3.7 Immunoprecipitation

Liver cytosol from untreated male and female B6129PF1/J mice was used for immunoprecipitation of CAR. Briefly, 10 μl of CAR primary antibody rabbit polyclonal IgG (sc-13065, Santa Cruz Biotechnology, Santa Cruz, CA) was added to 200 μg cytosol from each sample and incubated for 1 h at 4 °C. Resuspended Protein A/G PLUS-Agarose (20 μl) (Santa Cruz Biotechnology) was added and incubated overnight at 4 °C on a rocker platform. The next day samples were centrifuged at 1000 × g for 5 min at 4 °C, and the supernatant was carefully aspirated and discarded. The Pellet was washed one time with 1.0 ml RIPA Buffer (Pierce Biotechnology, Rockford, IL) and three times with 1 ml PBS, each time repeating the centrifugation step mentioned above. Following the final wash, aspiration and discarding of supernatant, the pellet was resuspended in 10 μl 3× electrophoresis sample buffer and 20 μl of water for Western blotting.

2.3.8 Western blots

Western blots were performed on 30–50 μg of microsomal protein to measure CYP levels. Proteins were separated by polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gel, and transferred to 0.45 μm nitrocellulose (Bio-Rad Laboratories, Hercules, CA) where the blot was blocked using 5% skim milk/0.3% Tween 20 dissolved in phosphate
buffered saline. Pre-stained protein standards (Bio-Rad) were used as molecular weight markers. Primary antibodies were obtained from a variety of sources. Rat anti-mouse Cyp2b10 antibody is a generous gift from Dr. Randy Rose at North Carolina State University, Raleigh, NC, rabbit anti-mouse CAR was obtained from Santa Cruz Biotechnology, rabbit anti-rat CYP3A1 and CYP2C8/9/19 were obtained from Chemicon International (Temecula, CA), mouse anti-human CYP2A6 was obtained from Gentest™ Corporation (San Jose, CA), and rabbit anti-mouse β-actin (Sigma–Aldrich, St. Louis, MO) was used as a housekeeper to ensure equal loading of samples. Goat anti-rabbit IgG (Bio-Rad) alkaline-phosphatase coupled secondary antibodies were used for recognizing CYP3A1 and CYP2C8/9/19 primary antibodies. Goat anti-rat (Bio-Rad), rabbit anti-mouse (Gentest™ Corporation), and goat anti-mouse (Bio-Rad) IgG were used to recognize the Cyp2b10, CYP2A6 and β-actin primary antibodies, respectively. Primary antibodies were diluted 1:1000, and secondary antibodies were diluted 1:500. Bands were either visualized colorimetrically with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates (Bio-Rad Laboratories, Hercules, CA), or with a chemilluminescent kit according to the manufacturer's directions (Bio-rad). Colorimetric blots were scanned on a GS-710 densitometer (Bio-rad, Hercules, CA), and bands were quantified with LabWorks™ image analysis software (UVP laboratory products, Upland, CA). Chemiluminescence was quantified on a Chemi-Doc system with Quantity One software (Bio-Rad).
2.3.9 Zoxazolamine-induced paralysis in NP-treated mice

Wild-type male and female mice were randomly split into treatment groups. Sixteen wild-type and fourteen female CAR-null mice were also split into untreated, 50 mg/kg/day NP, and 3 mg/kg/day TCPOBOP treated groups as described above. Seventeen wild-type and twenty male CAR-null mice were split into untreated, 50, 75 mg/kg/day NP, and 3 mg/kg/day TCPOBPOP and treated concurrently with the female mice. Mice were treated with honey, NP mixed in honey for 7 days, or given one injection of 3 mg/kg TCPOBOP dissolved in corn oil a day prior to the ZOX-challenge. On day 8, mice were injected i.p. with 300 mg/kg Zoxazolamine (ZOX) in corn oil. After injection, initial paralysis was noted, and paralysis time was measured by placing the mice on their backs and measuring the time until they were able to consistently right themselves. Mice that did not right themselves after 11 h were euthanized.

2.3.10 Statistical analysis

Statistical tests were performed with StatView software (SAS Institute Inc., Cary, NC). ANOVA was used to compare three or more treatment groups followed by Fisher's PLSD as the post hoc test, and Student's \( t \)-tests were used to compare two groups. A \( p \)-value of \( \leq 0.05 \) was regarded as significantly different from control values and is shown in figures with an asterisk. Fisher's exact test was used to compare the survival rates of the Zoxazolamine-treated mice, and ANOVA followed by Fisher's PLSD was used to compare the paralysis times between groups. A \( p \)-value \( \leq 0.05 \) was regarded as significantly different from controls and is shown with an asterisk in the figures.
2.4 Results

2.4.1. Testosterone hydroxylase activity

We compared testosterone hydroxylase activity in male and female wild-type and CAR-null mice as testosterone hydroxylation is often used as a biomarker of P450 enzyme activity, such as Cyp2a, 2b, 2c, and 3a subfamily members. Most testosterone hydroxylase activities were unaffected by gender with the exception of the female predominant 6α-OH activity (2.5× higher in females) and the male predominant testosterone 16α-OH activity (4× higher in males) (Fig. 1). 16β-OH activity showed a trend towards male predominance, but it was not statistically significant. Hydroxylation of testosterone in the 6α-position is performed primarily by the female predominant Cyp2a4 (Baldwin and LeBlanc, 1992). Hydroxylation of testosterone in the 16-position is performed by several enzymes including Cyp2b, 2c, and 2d members ([Harada and Negishi, 1984], [Lee et al., 2006], [Ohmori et al., 1993] and [Yamada et al., 2002]). However, Cyp2d9 is the only male predominant enzyme of the three CYPs suggesting Cyp2d9 is the primary 16α- and 16β-OH in males.

CAR-null male and female mice demonstrated an increase in testosterone 15α-hydroxylase activity, but this data was only significant in male mice (Fig. 1). The female predominant 6α-hydroxylase activity also decreased in CAR-null females, but was not statistically significant. However, the 6α/15α-OH ratio, which is generally much greater in females, controlled by androgen status, and considered a biomarker of androgen disruption in mice (Wilson et al., 1999), was decreased in CAR-null female mice. Because androgens are CAR inverse agonists, this suggests that CAR is involved in
Fig. 2.1. Gender differences in testosterone hydroxylation in wild-type and CAR-null mice. Testosterone hydroxylation in the 6β-, 16α-, 16β-, and 6α-positions were determined and compared between male and female wild-type and CAR-null mice. The results are shown as mean specific activity (μmol/min/mg protein) ± SD (n = 5–6). An (a) indicates a significant difference between wild-type male (WT-M) and wild-type female (WT-F) mice, a (b) indicates a significant difference between WT-M and CAR-null male (KO-M) mice, and a (c) indicates a significant difference between wild-type female (WT-F) and CAR-null female (KO-F) mice by ANOVA followed by Fisher's PLSD test as the post hoc test (p < 0.05).
the androgen regulation of the gender predominant CYPs involved in 6α or 15α-OH activity.

2.4.2. Regulation of basal P450 expression by CAR

To further characterize the basal regulation of CYPs by CAR in male and female mice, we performed QPCR on several Cyp2 and Cyp3 family members. Seven of the eleven P450s measured by QPCR were female predominant including Cyp2a4, Cyp2b9, Cyp2b13, Cyp3a11, Cyp3a41, and Cyp3a44 (Fig. 2). Of the CYPs we examined by QPCR, only Cyp2c37 was male predominant. Cyp2b13 and Cyp3a44 showed greater than 40-fold higher levels in females, and Cyp2b9, Cyp2c40, and Cyp2a4 showed approximately 9–12.5-fold higher levels in females (Fig. 2). Cyp3a25 was gender neutral as previously published (Hernandez et al., 2006); however, Cyp3a11 was 4.2-fold higher in B6129 female mice than male mice. Our previous studies with FVB/NJ mice showed that Cyp3a11 expression was gender neutral (Hernandez et al., 2006).

Untreated CAR-null mice also show perturbed regulation of a couple of P450s relative to their wild-type counterparts. Cyp2c29 is down-regulated greater than 4-fold in both male and female CAR-null mice when compared to wild-type mice; however, only the down-regulation in males was significant. A couple of Cyp2b family members showed trends indicative to gender specific effects of CAR on the basal regulation of CYPs. Cyp2b13 expression in CAR-null male mice was increased nearly 9-fold higher, though its expression in males was still considerably lower than its expression in females. Lastly, Cyp2b10 showed a trend towards down-regulation in CAR-null female mice.
2.3.3. Sexually dimorphic induction of CYPs by NP and TCPOBOP in a CAR-dependent manner

QPCR was performed with male and female wild-type and CAR-null mice following treatment with the partial agonist NP at 0, 50, 75 mg/kg, and the full agonist and classical CAR activator TCPOBOP at 3 mg/kg. Of the eleven P450s
measured, six P450s consistently responded to NP or TCPOBOP treatment (Fig. 3 and Fig. 4). Three of the eleven P450s measured by Q-CPR (Cyp2b10, Cyp2c29, Cyp3a11) showed a similar trend following treatment with NP or TCPOBOP. The partial agonist NP induced Cyp2b10 (Fig. 3A), Cyp2c29 (Fig. 3B), and Cyp3a11 (Fig. 3C) in a CAR-dependent, female specific manner, but the full agonist TCPOBOP induced these CYPs in a CAR-dependent manner in both males and females (Fig. 3). Interestingly, Cyp3a11 was induced at 75 mg/kg/day NP in CAR-null mice, suggesting activation of PXR by NP ([Hernandez et al., 2007] and [Masuyama et al., 2000]). Overall, this data indicates that the induction of these three CYPs by CAR agonists is not gender specific. However, females are more sensitive to the effects of CAR agonists as only females responded to the partial agonist, NP (Fig. 3) ([Baldwin and Roling, 2009] and [Hernandez et al., 2007]).

Three other CYPs did not exhibit diverse changes in their gene expression patterns following treatment with NP or TCPOBOP. Cyp2a4 was significantly induced by NP only in female CAR-null mice, suggesting the activation of PXR (Fig. 4A) similar to that observed for Cyp3a11. Wild-type mice showed trends indicating NP-mediated Cyp2a4 increases in both male in female mice. Cyp2a4 was induced by TCPOBOP in male, but not female mice, suggesting that the induction of the female predominant Cyp2a4 is not very sensitive to CAR activation. Male specific induction of Cyp2a4 by CAR activators has been observed previously, presumably due to low constitutive expression of Cyp2a4 in males ([Hernandez et al., 2006] and [Wei et al., 2002]).
Alterations in the gene expression of Cyp2c40 and Cyp3a41 do not show similar patterns or easily explicable patterns following treatment with TCPOBOP or NP (Fig. 4). Both of these CYPs are highly female predominant with Cyp2c40 showing 12.4× greater
expression and Cyp3a41 showing 12.1× greater expression in females. In turn, we observed much greater variability in the gene expression from male mice. For example,

Fig. 2.4. Cyp2a4, Cyp2c40, and Cyp3a41 show differential patterns of regulation following treatment with NP or TCPOBOP in wild-type or CAR-null male and female mice. QPCR was performed as described in Section 2. On the X-axis, 0, 50, 75 refers to treatment with NP and TC refers to treatment with TCPOBOP. Data are expressed as mean ± SD (n = 5–6) for each of the different P450s in males and females. An asterisk indicates a significant difference from the untreated wild-type or CAR-null mice (p < 0.05). Statistical significance was determined by ANOVA followed with Fisher's PLSD as the post hoc test in NP-treated mice compared to their corresponding wild-type or CAR-null controls. Statistical significance was determined by Student's t-tests in TC-treated mice compared to their corresponding wild-type or CAR-null controls.

in females Cyp2c40 was induced significantly by TCPOBOP and showed an increase in expression following treatment with NP that was not statistically significant (Fig. 4b).
Thus, changes in Cyp2c40 expression in female mice followed a similar pattern to Cyp2b10, Cyp2c29, and Cyp3a11. However, in males, TCPOBOP did not induce Cyp2c40, and NP actually reduced the expression of Cyp2c40 in a CAR-dependent manner. Cyp3a41 was induced by NP and TCPOBOP in CAR-null mice but not wild-type mice. In FVB/NJ mice we had previously observed that NP down-regulated Cyp3a41 in females (Hernandez et al., 2006); however, the slight drop in levels observed in this study were not significant. TCPOBOP nor NP altered Cyp3a41 expression in male mice. Overall, gene expression of these female predominant CYPs (Cyp2a4, 3a41) was highly variable in male mice, but typically showed recognizable changes in gene expression in females. Data for several of the CYPs is not shown (i.e. Cyp2b9, 2b13, 3a25, and 3a44) because there is little or no statistically significant changes in expression following either NP or TCPOBOP treatment (Supplementary Data, Fig. 1S).

2.4.4. Western blots of hepatic CYPs following TCPOBOP and NP treatment

Western blots were performed on male and female, wild-type and CAR-null mice to investigate the effects of NP or TCPOBOP on hepatic CYP subfamily levels, determine whether changes in one or more isoform caused significant changes in a CYP subfamily, and to confirm the QPCR data by investigating CYP protein levels. Because more than one isoform in a subfamily may be recognized by an antibody, we refer only to the subfamily. Western blotting confirmed that TCPOBOP and NP induced Cyp2b and Cyp2c subfamily members in wild-type females (Fig. 5A) in a CAR-dependent fashion as treated CAR-null mice failed to demonstrate an increase in these CYPs (Fig. 5A and B). Interestingly, Cyp3a subfamily members were up-regulated by TCPOBOP but down-
regulated by NP in wild-type female mice (Fig. 5A) and this occurred in a CAR-dependent fashion (Fig. 5B). However, Cyp3a11 mRNA expression was significantly

induced by both TCPOBOP and NP in wild-type mice (Fig. 3C). The discrepancy is interesting as previous studies have demonstrated NP-mediated down-regulation of

Cyp3a protein ([Arukwe et al., 1997], [Hernandez et al., 2006] and [Laurenzana et al., 2002]) and mRNA consistent with Cyp3a41 and Cyp3a44 down-regulation (Hernandez et

Fig. 2.5. Western blots of hepatic microsomes from NP and TCPOBOP-treated wild-type and CAR-null male and female mice. Western blots were performed and visualized as described in Section 2. Blots were quantified densitometrically and the relative mean differential expression as compared to the controls is reported above the blots. An asterisk indicates a significant difference from the corresponding untreated mice by ANOVA followed by Fisher's PLSD for NP-treated mice, and Student's t-test for TC-treated mice ($p < 0.05$).
However, our current study did not show down-regulation of either Cyp3a41 or Cyp3a44 by QPCR (Fig. 4C, Supplementary Data).

In male mice, Western blotting demonstrated that NP caused no significant changes in CYP protein levels in wild-type or CAR-null mice consistent with QPCR. However, QPCR indicated TCPOBOP induced Cyp2a4, Cyp2b10, Cyp2c29, and Cyp3a11, but only the Cyp2b subfamily showed a significant increase in protein levels. In contrast, Cyp2c protein levels were decreased significantly in the TCPOBOP-treated wild-type male mice. Furthermore, several P450s (Cyp2b, 3a) were down-regulated in the TCPOBOP-treated CAR-null male mice, suggesting that CAR-null mice are sensitive to the toxic effects of TCPOBOP at the dose we provided (Fig. 5B).

2.4.5. Immunoprecipitation, Western blots, and comparison of CAR protein expression between male and female mice

Several studies have indicated that females are more sensitive to CAR agonists than males (Ledda-Columbano et al., 2003). Reasons suggested indicate repression of CAR activity by male androgens, increased activity in females because of the presence of estrogens as CAR agonists (Kawamoto et al., 2000), and greater mRNA expression of CAR in female mice (Petrick and Klaassen, 2007). However, protein expression of CAR has not been investigated. Therefore, we examined differences in cytosolic protein expression between male and female B6129 mice. Cytosol was examined because CAR is primarily found in the cytosol compared to the nucleus, and even in phenobarbital-treated animals most CAR remains in the cytosol (Dail et al., 2008). We consider the
CAR Western blots semi-quantitative because of the immunoprecipitations, which also eliminates all of the housekeeping genes on the blot. Semi-quantification of Western blots from immunoprecipitated CAR by densitometry found no significant difference between male and female CAR protein expression (Fig. 6). The immunoprecipitations and Western blots were performed a total of three times with different samples and we observed similar results (28, 43, 53% more CAR in females; \( n = 3–4 \)). In all of the cases, the results were not statistically significant, indicating that differences in CAR protein expression between males and females is not the reason for enhanced CAR activity in female mice.

Fig. 2.6. Western blots of immunoprecipitated CAR protein from untreated wild-type male and female mice. Immunoprecipitation and Western blotting was performed as described in Section 2. Blots were quantified densitometrically and the relative mean expression compared to the male mice is reported. \( M = \) male (solid lines); \( F = \) female (dashed lines). There were no significant differences as determined by Student's \( t \)-test (\( p < 0.05 \)).
2.4.6. Pharmacological effects of Zoxazolamine; CAR status, sex, and agonist treatment

Zoxazolamine, a classical CYP substrate, is a muscle relaxant often used to determine the in vivo pharmacological effects of CYP inducers or inhibitors on xenobiotic metabolism. Increased paralysis time following ZOX-treatment indicates inhibition of CYPs and decreased paralysis time indicates induction of CYPs following xenobiotic treatment or genetic alteration (wild-type compared to CAR-null mice). We used ZOX to elucidate the pharmacological effects of sex, CAR status, and agonist treatment (TCPOBOP, NP). Groups of treated and untreated male and female wild-type and CAR-null mice were injected with ZOX and paralysis time measured. This allowed us to compare differences between untreated male and female mice, wild-type and CAR-null mice of each sex, and untreated and treated mice of each sex.

Female B6129 mice were clearly more resistant to the paralyzing effects of ZOX than male B6129 mice (Table 1). None of the female mice died during ZOX-treatment, including CAR-null female mice; several male mice died including some of the wild-type mice and nearly all of the CAR-null male mice. The difference in paralysis time between male and female mice is consistent with the greater number of female predominant CYPs in the Cyp2 and Cyp3 families as these are important families in ZOX metabolism. In addition, comparing untreated wild-type female mice to untreated CAR-null female mice following ZOX-treatment demonstrated that female CAR-null mice are more susceptible to ZOX paralysis than female wild-type mice (Table 1). However, paralysis times were
not statistically different when untreated wild-type and CAR-null male mice were compared.

Table 2.1. Sex, CAR-status, and xenobiotic exposure influence Zoxazolamine-induced paralysis time.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival</th>
<th>Mean paralysis time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-UT</td>
<td>8/8</td>
<td>55 + 15</td>
</tr>
<tr>
<td>WT-NP (50)</td>
<td>6/6</td>
<td>1 + 2(^a)</td>
</tr>
<tr>
<td>WT-TC</td>
<td>2/2</td>
<td>0 + 0(^a)</td>
</tr>
<tr>
<td>CAR-null UT</td>
<td>6/6</td>
<td>105 + 38(^b)</td>
</tr>
<tr>
<td>CAR-null NP (50)</td>
<td>6/6</td>
<td>64 + 15(^b,c)</td>
</tr>
<tr>
<td>CAR-null TC</td>
<td>2/2</td>
<td>80 + 10(^b)</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-UT</td>
<td>2/5</td>
<td>203 + 80(^e)</td>
</tr>
<tr>
<td>WT-NP (50)</td>
<td>5/5</td>
<td>246 + 107(^e)</td>
</tr>
<tr>
<td>WT-NP (75)</td>
<td>4/5</td>
<td>127 + 89(^e)</td>
</tr>
<tr>
<td>WT-TC</td>
<td>2/2</td>
<td>305 + 175(^e)</td>
</tr>
<tr>
<td>CAR-null UT</td>
<td>1/6</td>
<td>600(^e)</td>
</tr>
<tr>
<td>CAR-null NP (50)</td>
<td>0/5(^d)</td>
<td>n.a.</td>
</tr>
<tr>
<td>CAR-null NP (75)</td>
<td>0/5(^d)</td>
<td>n.a.</td>
</tr>
<tr>
<td>CAR-null TC</td>
<td>1/4</td>
<td>163(^e)</td>
</tr>
</tbody>
</table>

n.a. = not applicable.

\(^a\) Different by ANOVA followed by Fisher's PLSD as compared to WT control.

\(^b\) Different by ANOVA followed by Fisher's PLSD as compared to similarly treated WT mouse.

\(^c\) Different by ANOVA followed by Fisher's PLSD as compared to CAR-null control.

\(^d\) Different by Fisher's exact test compared to the similarly treated WT mouse.

\(^e\) Mean paralysis time does not include mice that did not survive Zoxazolamine challenge.

Treatment of mice with NP or TCPOBOP significantly altered ZOX paralysis of wild-type mice. NP and TCPOBOP markedly decreased ZOX paralysis time in a similar
fashion in wild-type female mice despite TCPOBOP’s greater potency towards CAR. ZOX paralysis was unaffected by TCPOBOP-treatment in CAR-null mice demonstrating that the TCPOBOP-mediated decrease in ZOX paralysis is CAR-dependent. Similarly, the NP-mediated decrease in ZOX paralysis is in part CAR-dependent in female mice. However, NP-treated CAR-null mice show a small but significant decrease in paralysis time, suggesting activation by PXR in addition to CAR (Table 1) ([Hernandez et al., 2007] and [Masuyama et al., 2000]).

Wild-type male mice treated with NP showed a significantly greater survival rate than CAR-null male mice treated with NP because none of the CAR-null mice survived. This suggests that NP activates CAR and induces CYPs, and/or CAR-mediates basal regulation of CYPs. There were no significant differences between untreated and NP-treated male wild-type mice; however, there was a slight trend suggesting greater survival in wild-type NP-treated mice compared to untreated mice. Untreated and NP-treated mice that survived the ZOX-challenge showed no difference in paralysis time (Table 1) reflecting the poor CYP induction in male wild-type mice by NP. All of the TCPOBOP treated wild-type male mice survived but many of the untreated wild-type, CAR-null, and TCPOBOOP-treated CAR-null mice did not. Overall, TCPOBOP showed similar data to the NP treated male mice as significant changes in paralysis time and survival were not observed potentially due to small sample size (Table 1). Overall, the difference between males and females following treatment is indicative of the increased sensitivity of female mice to CAR agonists (Fig. 3, Fig. 4 and Fig. 5).
2.5 Discussion

CAR is a xenobiotic responsive transcription factor critical in the regulation of CYPs and other detoxification genes (Pascussi et al., 2008). CAR is also constitutively active (Baes et al., 1994), and CAR expression is associated with CYP expression in human liver (Wortham et al., 2007). Therefore, we hypothesized CAR may basally regulate some CYP enzymes. The basal regulation of CYPs by CAR is highly apparent pharmacologically in ZOX-treated mice. Untreated CAR-null mice were significantly more sensitive to ZOX-induced paralysis than untreated wild-type mice, especially in females (Table 1). The increased sensitivity in female CAR-null mice to ZOX is associated with slightly reduced Cyp2b10 and Cyp2c29 levels, and a shift to the male pattern testosterone 6α/15α-OH ratio (Fig. 1 and Fig. 2).

Untreated male CAR-null mice did not show a significant increase in sensitivity to ZOX based on survival and paralysis time (Table 1). However, CAR-null male mice showed significantly lower basal expression of Cyp2c29 coupled with 9-fold higher expression of Cyp2b13, and greater 15α-OH activity (Fig. 1 and Fig. 2), a biomarker of Cyp2a activity (Burkhart et al., 1985). Overall, ZOX and QPCR data suggest that CAR is moderately involved in the basal regulation of some CYPs in males and females including CYPs in the Cyp2b and Cyp2c subfamilies.

Corroborating evidence for basal regulation of CYPs by CAR exists in humans as recent data based on associations between CYP expression levels and nuclear receptors indicates that several nuclear receptors (HNF4α, CAR, PXR) are involved in the basal regulation of CYPs ([Slatter et al., 2006], [Vyhlidal et al., 2006] and [Wortham et al.,...
Recent data investigating the expression and activity of a broad scope of xenobiotic detoxification genes indicates that the basal regulation of several CYPs are controlled by HNF4α > CAR > PXR (Wortham et al., 2007). The dominance of HNF4α is not surprising because it directly controls the expression of CAR (Ding et al., 2006) and the glucocorticoid receptor (GR), which controls the expression of PXR (Pascussi et al., 2000). Colinearity among expression levels indicates that HNF4α directly controls CYP2C9, while HNF4α’s regulation of CAR controls CYP2A6, CYP2B6, CYP2C8, and CYP2C19 (Wortham et al., 2007). In summary, CAR expression is associated with the expression of CYP2A6, 2B6, 2C8, and 2C19 suggesting CAR basally regulates these genes in humans. Our data indicates that Cyp2b13, Cyp2c29, and potentially Cyp2b10 are controlled in part by CAR in mice.

Furthermore, HNF4α is important in the sexually dimorphic expression of hepatic CYPs (Wiwi et al., 2004). Several CYP members, including Cyp2a4, Cyp2b9, Cyp2b13, Cyp3a11, Cyp3a41, and Cyp3a44, are female predominant, which in part explains the significantly diminished paralysis time in female mice compared to male mice. Thus, lower basal levels of several drug metabolizing CYPs in male B6129 mice appears to have a pharmacological consequences (Table 1). HNF4α negatively controls Cyp2a4 and Cyp2b9 in male mice (Wiwi et al., 2004). We did not observe a significant change in either of these enzymes; although Cyp2b9 was increased slightly (2.1×) in CAR-null mice relative to wild-type male mice (Fig. 2). HNF4α positively regulates Cyp2b10, Cyp2b13, Cyp3a41, and Cyp3a44 (Wiwi et al., 2004), but the involvement of CAR had not been tested. We observed a significant increase in Cyp2b13 in CAR-null male mice.
relative to wild-type mice. Interestingly, microarray data from HFN4α-null mice also show a significant increase in Cyp2b13 and Cyp2b9 expression exclusively in males (Holloway et al., 2008). However, only the Cyp2b13 data is significant in CAR-null males (Fig. 2) indicating that HNF4α is the primary factor repressing the regulation of Cyp2b9 in males, while CAR is the primary factor repressing the regulation Cyp2b13 in males.

We also observed a nearly 4-fold drop in Cyp2b10 in CAR-null female mice relative to wild-type mice, and a 3.5-fold increase in Cyp3a44 in the CAR-null male mice compared to the wild-type mice. However, the expression changes in both of these CYPs was not statistically significant, which is consistent with the reduced basal regulatory control of CYPs by CAR compared to HNF4α (Wortham et al., 2007). The basal regulation of Cyp2c subfamily members in mice by HNF4α has not been investigated (Wiwi et al., 2004). Interestingly, CAR-null mice recently backcrossed to the B6 background show significant repression of Cyp2b10 in both sexes (Cho et al., 2008). This suggests that similar to rats (Yoshinari et al., 2001), mice may demonstrate strain specific sexually dimorphic effects related to CAR.

Other sex and/or strain differences may be observed as further work is performed with B6 and B6-CAR-null mice instead of B6129 and B6129-CAR-null mice. However, current data suggests that strain will not have much effect on CAR activity because other than the sex difference in basal Cyp2b10 expression in CAR-null males; most differences between genders are related to the overall repressed sensitivity of CAR activity especially after treatment with NP, a weaker, partial agonist with a shorter half-life and an unknown
mechanism of activation (indirect or direct activation of CAR). Other groups have also observed overall repressed sensitivity of CAR activity in male mice ([Petrick and Klaassen, 2007] and [Ledda-Columbano et al., 2003]) and human males ([Lamba et al., 2003]). Furthermore, similar sex differences in CYP2a, 2b, 2c, and 3a basal subfamily expression have been observed in other strains ([Wiwi et al., 2004], [Hernandez et al., 2006] and [Holloway et al., 2008]) as well as repressed induction in males compared to females following NP treatment ([Hernandez et al., 2006]). Taken together, this suggests that future research investigating CAR activity with pure inbred strains will reveal similar results as with B6129 mice.

Testosterone hydroxylase assays were also performed to assess P450 activity in various mouse models to determine basal regulation of CYP activity by CAR, and determine differences between males and females. CAR represses 15α-OH activity, especially in male mice. 15α-OH activity is associated with Cyp2a4 activity in female mice (Burkhart et al., 1985), but this activity has not been fully characterized in male mice. Cyp2a4 is expressed at much greater levels in females than males, and while 6α-OH activity declines in males relative to females, 15α-OH activity does not. Thus, the CYP that makes up the difference in 15α-OH activity in males needs further characterization, but may be another Cyp2a subfamily member such as Cyp2a5.

Because both 6α- and 15α-OH activities were perturbed by either sex or CAR-status, we examined the 6α/15α-OH ratio. The 6α/15α-OH ratio was decreased greater than 2.5-fold in female CAR-null mice compared to their wild-type counterparts (Fig. 1). Therefore, CAR-null female mice had a similar 6α/15α–OH ratio to male mice. The
6α/15α–OH ratio, which is typically much greater in females, is in part controlled by androgens and considered a biomarker of androgen disruption or androgen status (Wilson et al., 1999). This suggests that CAR is a key player in the recognition of androgen status in the liver, consistent with its repression by androstanes and other androgens (Forman et al., 1998). It is interesting to speculate that perturbations in steroid metabolism and testosterone 6α/15α–OH ratio associated with exogenous estradiol or endocrine disrupting xenobiotics such as indole-3-carbinol, DDT and vinclozolin ([Sierra-Santoyo et al., 2005] and [Wilson et al., 1999]) may be in part be mediated by the activation of CAR (Kawamoto et al., 2000). This is consistent with our theory that CAR is a steroid sensor and protector of the endocrine system (Kretschmer and Baldwin, 2005).

In addition, we investigated differential responses of male and female wild-type and CAR-null, mice to the moderately potent CAR agonist NP and the highly potent CAR agonist TCPOBOP. A robust and significant decrease in paralysis time was observed in wild-type female mice treated with NP or TCPOBOP, as paralysis was nearly lost in both treatments. This is consistent with an increase in several CYPs as measured by QPCR (Fig. 3 and Fig. 4) and Western blotting (Fig. 5) following NP or TCPOBOP treatment. In turn, CAR-null NP- and TCPOBOP-treated mice responded poorly to ZOX-treatment and lacked demonstrative CYP induction demonstrating that NP and TCPOBOP are mediating their effects through CAR. Interestingly, CAR-null females treated with NP but not TC showed a significant albeit diminished decrease in paralysis time compared to untreated CAR-null females, suggesting activation of PXR ([Baldwin et al., 2005], [Hernandez et al., 2007], [Masuyama et al., 2000] and [Mikamo et al.,
and induction of Cyp3a subfamily members in a CAR-independent fashion as observed by QPCR (Figs. 3C, 4A and C).

NP or TCPOBOP treated wild-type male mice did not respond as well as the NP or TCPOBOP treated wild-type female mice to ZOX. However, survival was increased in NP- and TCPOBOP-treated wild-type males compared to the untreated wild-type males and treated and untreated CAR-null males, suggesting that CYP levels were increased slightly in a CAR-mediated fashion. This is consistent with the CAR-dependent increase in CYP levels measured by QPCR (Fig. 3 and Fig. 4) and Western blotting (Fig. 5) in TCPOBOP-treated mice. Previously induction of Cyp2b subfamily members by TCPOBOP (Wei et al., 2000) and NP in FVB/NJ mice (Hernandez et al., 2006) was associated with ZOX clearance and indicates the importance of Cyp2b in ZOX metabolism (Hernandez et al., 2006). However, we observed no significant increases in CYP RNA (Fig. 3 and Fig. 4) or protein (Fig. 5) levels in NP-treated B6129 male mice, but did observe significantly greater survival compared to untreated mice. The lack of CYP induction by NP in B6129 male mice compared to FVB/NJ male mice may be related to differences in the mouse strain. FVB/NJ male mice show lower expression levels of several CYPs (Hernandez et al., 2006) and thus induction of these CYPs may be more sensitive markers than in the B6129 mice. This suggests that there were increases in CYPs that we did not measure by QPCR, or slight induction of several CYPs in a non-statistically significant manner made a biological impact. It should be noted that paralysis time was not changed between surviving untreated and NP-treated male mice, and this
indicates weak or no induction of CYPs in the male mice (Fig. 3, Fig. 4 and Fig. 5, Table 1).

In general, significantly greater responses to NP and TCPOBOP were observed in female mice than male mice in a CAR-dependent fashion. For example, reductions in ZOX paralysis time were greater in treated female mice than treated male mice. Furthermore, QPCR and Western blots of NP-treated mice showed induction of CYPs almost exclusively in females (Fig. 3, Fig. 4 and Fig. 5) (summarized in Table 2). Previously, we had observed significantly greater induction of CYPs in NP-treated females than males (Hernandez et al., 2006) in a study with FVB/NJ mice that led us to investigate the role of CAR in NP-mediated sexually dimorphic induction. However we did not observe the near abrogation of CYP induction by NP in males we observed in this study. This large sexually dimorphic difference was especially apparent in the female specific CAR-mediated induction of Cyp2b10, Cyp2c29, and Cyp3a11 by NP measured by QPCR. Western blots also showed significant dose-dependent changes in Cyp2a, Cyp2b, Cyp2c, and Cyp3a levels only in wild-type females, but not wild-type males treated with NP. However, TCPOBOP did not typically show sexually dimorphic induction. TCPOBOP-mediated CYP induction occurred in both sexes as measured by QPCR (Fig. 3 and Fig. 4), but showed a slight (about 0.5×) attenuated response as measured by Western blotting (Fig. 5, summarized in Table 2).

Several other studies have demonstrated sexually dimorphic differences in response to CAR ligands. For example, only male WKY rats, but not female WKY rats, respond to phenobarbital-treatment because of low CAR levels in the WKY female rats.

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Cyp2a4 is only induced by TCPOBOP in male mice potentially due to the low Cyp2a4 expression in females (Fig. 4) (Wei et al., 2002). In contrast, female mice are more sensitive to the hepatic proliferative effects of TCPOBOP than male mice (Ledda-Columbano et al., 2003), and we have previously observed greater induction of several P450s in females compared to males following NP-treatment (Hernandez et al., 2006). There is precedence for a NR1I nuclear receptor regulating basal CYP expression in a gender specific fashion. PXR-null male mice demonstrate greater Cyp3a activity than there wild-type counterparts, but PXR-null female mice demonstrate reduced Cyp3a activity compared to their wild-type counterparts (Anakk et al., 2004). There are several putative explanations for the greater response in females to CAR activation, including higher expression of CAR in female liver than male liver (Petrick and Klaassen, 2007). However, we did not measure increased CAR protein expression when comparing wild-type male and female mice (Fig. 6). There are other potential explanations for increased CAR activity in females. For example, estradiol is a CAR agonist in mice (Kawamoto et al., 2000), and additional xenobiotic activators may act in an additive fashion within the mixture of chemicals found in the liver. Lastly, androgens and their metabolites repress CAR activity, which may decrease the efficaciousness of CAR in males (Forman et al., 1998).
Table 2.2. Summary of the effects of TCPOBOP and NP on CYP expression in male and female, wild-type and CAR-null mice.

<table>
<thead>
<tr>
<th>CYP</th>
<th>Type</th>
<th>Female</th>
<th></th>
<th></th>
<th>Male</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Q-PCR</td>
<td>Western</td>
<td>Q-PCR</td>
<td>Western</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>75</td>
<td>TC</td>
<td>NP</td>
<td>TC</td>
<td>50</td>
</tr>
<tr>
<td>Cyp2a4</td>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>N.C.</td>
<td>+*</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>N.C.</td>
<td>+*</td>
<td>N.C.</td>
<td>N.C.</td>
<td>+*</td>
<td></td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>WT</td>
<td>+*</td>
<td>+*</td>
<td>+*</td>
<td>+*</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td>Cyp2c29</td>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td>Cyp2c40</td>
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<td>+*</td>
<td>+*</td>
<td>+*</td>
<td>+*</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>WT</td>
<td>+*</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>N.C.</td>
<td>+*</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td>Cyp3a41</td>
<td>WT</td>
<td>N.C.</td>
<td>-</td>
<td>N.C.</td>
<td>-</td>
<td>+*</td>
<td>N.C.</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>N.C.</td>
<td>+*</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
</tbody>
</table>

N.C.; no change,
+; trend indicating increasing levels of isoform or subfamily.
−; trend indicating decreasing levels of isoform or subfamily.
Asterisk indicates statistically significant trend.
Taken together, one or all three of these mechanisms in combination may explain the increased CAR activity and induction of CYPs in adult NP-treated female B6129 mice relative to male mice. The ability of TCPOBOP to overcome reduced CAR activity in male mice and show similar increases in CYPs in both sexes may be related to its high potency and saturation of CAR. However, CAR activity is considerably greater in treated females than males, especially when mice are treated with the weaker partial activator (and potential indirect activator), NP, instead of a potent direct acting agonist such as TCPOBOP. Because environmentally we are primarily exposed to a large number of weak agonists, sexually dimorphic differences in CAR activity may cause significant sex-dependent differences in our detoxification rates, especially CYP2B6. It is interesting to hypothesize that sex differences in CAR activity may in part explain the female predominance of human CYP2B6 (Lamba et al., 2003). Further, PCBs which activate CAR, have greater effects on thyroid hormones and thyroid hyperplasia in females than males (Hagmar et al., 2001). This is presumably due to the greater expression of CAR in human females (Lamba et al., 2003) and therefore faster CAR activated thyroid hormone clearance (Qatanani et al., 2005). Lastly, most drugs are cleared faster in human females than males ([Meibohm et al., 2002] and [Wolbold et al., 2003]). Exposure to an increasing number of pharmaceuticals and environmental chemicals such as NP may help explain increased clearance of drugs in females and lead to potential disparities in clearance rates, or increased drug–drug/drug–toxicant interactions.
2.6 Conflicts of interest

The authors have no conflicts of interest to declare.

2.7 Acknowledgements

Research support for this study was provided by start-up funds from Clemson University and NIH grants GM008012 (wsb), CA127415 (wh), and DK46546 (ddm). Juan P. Hernandez was supported in part by a NSF-AGEP award and an NIH Kirchstein fellowship, 1F31ES014113-01A1.
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CHAPTER THREE

CAR-NULL MICE ARE SENSITIVE TO THE TOXIC EFFECTS OF PARATHION: ASSOCIATION WITH REDUCED CYP-MEDIATED PARATHION METABOLISM

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Accepted for publication 6/22/2010 Drug Metabolism and Disposition

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Text pages: 15
Tables: 4
Figures: 5
Words in Abstract: 222 (Max. 250).
Words in Introduction: 764 (Max. 750).
Words in Discussion: 1309 (Max. 1500).
References: 40 (Max. 40).
Abbreviations: Constitutive Androstane Receptor: CAR; wild-type: WT; cytochrome P450: CYP; p-nitrophenol: PNP; 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene: TCPOBOP.
3.1 Abstract:

CAR is activated by several chemicals and in turn regulates multiple detoxification genes. Our research demonstrates that parathion is one of the most potent, environmentally relevant CAR activators with an EC\textsubscript{50} of 1.43 µM. Therefore, animal studies were conducted to determine if CAR was activated by parathion in vivo. Surprisingly, CAR-null mice, but not WT mice, showed significant parathion-induced toxicity. However parathion did not induce Cyp2b expression, suggesting that parathion is not a CAR activator in vivo, presumably due to its short half-life. CAR expression is also associated with the expression of several drug metabolizing CYPs. CAR-null mice demonstrate lower expression of Cyp2b9, Cyp2b10, Cyp2c29, and Cyp3a11, primarily but not exclusively in males. Therefore, we incubated microsomes from untreated WT and CAR-null mice with parathion in the presence of esterase inhibitors to determine if CAR-null mice show perturbed CYP-mediated parathion metabolism compared to WT mice. The metabolism of parathion to paraoxon and p-nitrophenol (PNP) was reduced in CAR-null mice with male CAR-null mice showing reduced production of both paraoxon and PNP, and female CAR-null showing reduced production of only PNP. Overall, the data indicates that CAR-null mice metabolize parathion slower than WT mice. These results provide a potential mechanism for increased sensitivity of individuals with lower CAR activity such as newborns to parathion and potentially other chemicals due to decreased metabolic capacity.
3.2 Introduction:

Due to a continuously growing human population, modern agriculture has relied heavily on pesticides to produce high crop yields, prevent diseases, and control pests. Organophosphates are some of the most widely used pesticides (Singh and Walker, 2006) accounting for 38% of total worldwide pesticide use for agricultural purposes and household pest control (Post, 1998). In the United States these pesticides accounted for 70% of the total insecticides used as of 2001 (Kiely et al., 2004). The Food Quality Protection Act severely restricts organophosphate use in the United States (Environmental Protection Agency, 2002); however, we are increasingly consuming imported fruits and vegetables that may come from countries that presently use organophosphates such as parathion. A total of 36% of fresh fruits are imported into the Unites States from countries such as Ecuador, Honduras, Panama, Costa Rica, Colombia, and Guatemala; while Canada and Mexico supply 83% of the fresh vegetables imported (Huang and Huang, 2007). A study in Mexico found organophosphate residues in 87% of the broccoli analyzed (Perez et al., 2009). In Colombia every sample of several crops contained residues of at least two different organophosphates (Murcia and Stashenko, 2008). Interestingly, recent studies have reported the indoor and outdoor application of the methyl ortholog of parathion in southern Texas (Saller et al., 2007). Thus, organophosphates are still routinely used worldwide and are still entering the U.S. marketplace.
Some of the health effects of organophosphate poisoning include several nerve and muscular effects, primarily caused by inhibiting the breakdown of the neurotransmitter acetylcholine (Ragnarsdottir, 2000). Of the organophosphates, parathion has been classified by the Environmental Protection Agency as pesticide Toxicity Category 1 (highly toxic) and Group C (possible human carcinogen) as well as being labeled as one of the most acutely toxic pesticides registered.

Parathion toxicity is caused by its bioactivation into a toxic metabolite, paraoxon, by cytochrome P450s (CYPs) through an oxidative desulfuration event (Gallow and Lawryk, 1991) (Fig. 1). The thionosulfur atom of the paraoxon metabolite covalently binds to the active site of acetylcholinesterase resulting in inactivation of the enzyme, and leading to the overstimulation of cholinergic neurons (Gallow and Lawryk, 1991). Esterases, such as paraoxonases and carboxylesterases, are important detoxifiers of the toxic -oxon metabolite into the non-toxic p-nitrophenol (PNP) metabolite. CYPs can also mediate the formation of PNP and are therefore critical in the detoxification of parathion (Fig. 1). CYP2B6, a key enzyme regulated by CAR (NR1I3) in humans, is considered crucial in the metabolism of parathion as are CYP2D6, and CYP2C and CYP3A isoforms, but to a lesser degree than CYP2B6 (Mutch and Williams, 2006; Foxenberg et al., 2007).

Recently, we demonstrated that a number of pesticides, including several organophosphate insecticides, activated CAR in transactivation assays. Pesticides that activate CAR include fenitrothion, SSS-tributylphosphorotrithioate, chlorpyrifos,
endosulfan, monosodium acid methane arsenate, cypermethrin, butylate, methoxychlor, and parathion. Interestingly most chemicals are partial activators, but the organophosphates parathion, SSS-tributylphosphorotrithioate, and chlorpyrifos are full activators. We hypothesized that these organophosphates are CAR ligands (Baldwin and Roling, 2009). Research by (Küblbeck et al., 2008) in part provides further evidence that organophosphates may actually be ligands, as human CAR is ligand activated by a number of sulfur-containing chemicals such as substituted sulfonamides and thiazolidin-4-one derivatives, indicating a potential preference of CAR for thiols.

CAR is a key regulator of several detoxification enzymes, including CYP2B6 in humans, Cyp2b10 in mouse, and several other CYPs in families 1-3 (Sueyoshi and Negishi, 2001; Hernandez et al., 2009a). CAR also basally regulates several CYPs and loss of CAR from mice causes subsequent alterations in the expression of Cyp2c29, Cyp2b13, and maybe Cyp2b10 (Hernandez et al., 2009b). Furthermore, CAR expression is associated with the expression of several detoxification enzymes and transporters in human liver, including CYP2A6, CYP2B6, CYP2C8, P450 oxidoreductase, and MRP2, indicating basal regulation of CYPs by CAR in humans (Wortham et al., 2007). The expression of CAR and PXR is also significantly lower in children under six months of age and in turn they show lower expression of several CYPs involved in xenobiotic detoxification (Vyhlidal et al., 2005), suggesting potential susceptibility of children to chemical toxicity due to limited metabolic capacity (Padilla et al., 2000; Sheets, 2000).
We confirmed that parathion is a CAR activator in transactivation assays and determined its EC\textsubscript{50}. Furthermore, we tested whether CYPs are induced by parathion in a CAR-dependent manner in vivo, and investigated changes in CYP-mediated parathion metabolism in untreated CAR-null mice and WT mice. Our data indicates that CAR is important in the basal regulation of CYPs, and parathion is metabolized slower and is more toxic to CAR-null mice than WT mice.

3.3 Materials and Methods

3.3.1 Chemicals

Parathion\textsuperscript{TM} (C\textsubscript{10}H\textsubscript{14}NO\textsubscript{5}PS; CAS# 56-38-2), paraoxon (C\textsubscript{10}H\textsubscript{14}NO\textsubscript{6}P; CAS# 311-45-5), PNP (C\textsubscript{6}H\textsubscript{5}NO\textsubscript{3}; CAS# 100-02-7), and chlorpyrifos (C\textsubscript{9}H\textsubscript{11}Cl\textsubscript{3}NO\textsubscript{3}PS; CAS# 2921088-2) were obtained from Chem Service (West Chester, PA). Dihydroandrosterone (DHA) (C\textsubscript{19}H\textsubscript{32}O\textsubscript{2}; CAS# 1852-53-5), Tetraisopropyl pyrophosphoramide (iso-OMPA) (C\textsubscript{12}H\textsubscript{32}N\textsubscript{4}O\textsubscript{3}P\textsubscript{2}; CAS# 513-00-8) and 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) (C\textsubscript{16}H\textsubscript{8}Cl\textsubscript{4}N\textsubscript{2}O\textsubscript{2}; CAS# 76150-91-9) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). 4-nonylphenol 85\% \textit{p}-isomers (C\textsubscript{15}H\textsubscript{24}O; CAS# 84852-15-3) were obtained from Fluka Chemical Co (Seelze, Germany). Acetonitrile (C\textsubscript{2}H\textsubscript{3}N; CAS# 75-05-8) and methanol (CH\textsubscript{4}O; CAS# 67-56-1) were obtained from B&J Brand\textsuperscript{®} (Honeywell Burdick & Jackson\textsuperscript{®}), and ethylenediamine tetra acetic acid (EDTA) (C\textsubscript{10}H\textsubscript{16}N\textsubscript{2}O\textsubscript{8}; CAS# 60-00-4) and Tween 20 (C\textsubscript{58}H\textsubscript{114}O\textsubscript{26}; CAS# 9005-64-5) were obtained from Fisher Scientific (Houston, TX).
3.3.2 Transactivation Assay

A detailed protocol for our mCAR transactivation assay with HepG2 cells has been previously published (Baldwin andRoling, 2009). The Steady-Glo luciferase assay system (Promega, Madison, WI) was used to measure reporter activity 24 h after pesticide or TCPOBOP treatments, and data was normalized to treatments with the inverse agonist, DHA as 0% activity and TCPOBOP-treated cells as 100% activity with GraphPad Prizm 4.0 (La Jolla, CA). EC50 values, and 95% confidence intervals, were also determined with the GraphPad Prizm 4.0 software package.

3.3.3 Animals

All studies were performed in agreement with NIH guidelines for the humane use of research animals and approved by The University of Texas at El Paso or Clemson University’s Animal Care and Use Committee. Mice were provided with food and water ad libitum. Male and female 8-10 week old wild-type (WT) and CAR-null mice (Wei et al., 2000) were randomly split into six groups each (n=4-6). The mice were injected with 100 μl of 5 mg/kg or 20 mg/kg of parathion mixed with 50/50 DMSO/corn oil based on previously published studies (Weitman SD et al., 1983; Sobarzo and Bustos-Obregón, 2000; Kim et al., 2005). Mice were euthanized and livers were excised 6 hours following the last treatment. As a positive control male and female, WT and CAR-null mice were injected with 100 μl of 3 mg/kg of TCPOBOP mixed with DMSO and corn oil for two days and euthanized 6 hours following the last injection. All samples were stored at -80°C.
3.3.4 Liver sample preparation

Livers from euthanized mice were excised, snap frozen, weighed, and diced into several pieces for RNA extraction and microsome preparation. Total RNA was isolated using a modified guanidinium thiocyanate-phenol-chloroform extraction protocol with TRI-reagent (Sigma, St. Louis, MO) according to the manufacturer’s specifications followed by DNase (Promega Corporation, Madison WI) treatment to remove residual genomic DNA. RNA was quantified spectrophotometrically at 260/280 nm and stored at –80°C. Reverse transcription was performed to make cDNA using MMLV-RT, a dNTP mixture, and random hexamers. cDNA was stored at –20°C. Microsomes were isolated by differential centrifugation in the presence of aprotonin, leupeptin, and PMSF as protease inhibitors. Protein concentrations were quantified using commercially available reagents and microsomes were stored at –80°C (Bio-Rad, Hercules, CA).

3.3.5 Quantification of CYP expression

QPCR was performed as described previously by us (Hernandez et al., 2007; Hernandez et al., 2009b) and others (Muller et al., 2002) using previously published primers (Wiwi CA et al., 2004a; Hernandez et al., 2009b). PCR efficiency was determined from standard curves developed from 1:1, 1:10, 1:100, and 1:1000 dilutions of a cDNA composite of all samples. Amplifications were performed in triplicate using a 96-well iQ5™ multicolor Real-Time PCR Detection System (Bio-Rad) with 0.25X SybrGreen to quantify gene expression (SABiosciences, Frederick, MD). All samples were diluted
1:10. Q-PCR results were normalized to the expression of a housekeeping gene, 18S rRNA. A minimum of forty cycles were run on all real time samples to ensure a log based growth curve. Quantification was done by taking the efficiency curve of the Q-PCR reaction to the power of the threshold cycle (Ct) over the housekeeping gene (Muller et al., 2002).

CYP protein levels were quantified by immunoblotting. Because each antibody probably recognizes more than one mouse isoform in a subfamily, we refer to the quantification of mouse CYPs only by subfamily. Mouse 2b, 2c, and 3a subfamilies were quantified using antibodies from several sources. Cyp2c and Cyp3a were quantified using a human CYP2C8/9/19 or rat CYP3A1 antibodies respectively from Chemicon, (Billerica, MA). Induction of Cyp2b by parathion was performed using a Cyp2b10 antibody from Randy Rose (North Carolina State University)(Hernandez et al., 2007). In addition, we quantified constitutive Cyp2b levels using a new and more sensitive antibody that we developed. Briefly, the GenScript (Piscataway, NJ) antigen design tool was used to design a basic peptide with high antigenicity. A cysteine was added to the C-terminus of the designed peptide \( \text{LHDPQYFEQPSFNC-C} \), and conjugated to Keyhole Limpet Hemocyanin (KLH). This peptide is poorly conserved between Cyp2b and other P450s, and therefore should be specific for only Cyp2b subfamily members. Two rabbits were injected 4X each and approximately 160 ml of blood was collected and pooled from the rabbits. The antibody was purified by affinity purification through a column containing
conjugated peptide and is specific for Cyp2b. The Cyp2b antibody was diluted 1:500 for immunoblotting.

Immunoblots were run with 30-50 μg of microsomal protein separated electrophoretically on a 10% polyacrylamide gel (SDS-PAGE), transferred to nitrocellulose membrane (Bio-Rad), blocked in TBST (Tris Buffer Saline pH=7.4 0.1%Tween 20) containing 2.5-5% dried milk for 30min-1h, incubated with antibodies at room temperature for 2h and overnight at 4°C. The nitrocellulose was then incubated at room temperature for 2h with goat anti-mouse or goat anti-rabbit (Bio-Rad; 1:500) secondary antibody depending on the source of the primary antibody. β-Actin (Sigma, St. Louis, MO) was used as a housekeeper. The protein on the blots was detected by chemiluminescence using the Immun-Star AP Chemiluminescent Protein Detection System and quantified by Chemi Doc XRS HQ using Quantity One 4.6.5 software (Bio-Rad, Inc).

### 3.3.6 Parathion metabolism

CYP-mediated parathion metabolism was examined in vitro with microsomes from untreated WT and CAR-null mice as described previously (Foxenberg et al., 2007). 250 μg of protein from mouse liver microsomes were incubated in 0.1M Tris-HCl and 5mM MgCl₂ at pH 7.4 with a final concentration of 20 μM parathion, and in the presence of the esterase inhibitors 1mM EDTA (A esterase inhibitor) and 50 uM iso-OMPA (B esterase inhibitor) (Reiner et al., 1993). Samples were incubated at 37°C in a shaking water bath; the reaction was initiated with 1 mM NADPH, and stopped after 60 minutes with 500 μl
of methanol and 0.1% phosphoric acid. Samples were filtered (0.22 μm PTFE filter; Fisher Scientific) and metabolite concentrations were measured using Reverse Phase-HPLC. Standards for all compounds were prepared in methanol and stored at -20°C prior to analysis. Parathion’s metabolites, paraoxon and PNP, were analyzed using a Waters 1525 binary reverse phase-high phase liquid chromatography pump (HyperSelect Gold C18, 5 μm, 120 Å particle size, 250 mm X 4.6 mm with guard column; ES Industries Chromega Columns, West Berlin, NJ). The mobile phase consisted of 60% acetonitrile and 40% water over 18 minutes at a flow rate of 1 ml/min. Chemical detection was determined at 275 nm for parathion and paraoxon and 310 nm for PNP. The detection limits of paraoxon and PNP were 0.0275 μg/ml and 0.0139 μg/ml, respectively.

### 3.3.7 Statistical analysis

Mann-Whitney ranked sum test was performed to test significance of toxicity observations where 1= not toxic, 2= eye leakage, 3= slow or tremors, 4= death. One-way ANOVA was performed on immunoblot data when comparing parathion treated mice to untreated groups. Student’s two-tailed t-tests were used to compare differences in CYP expression between males and females, or WT and CAR-null mice. Differences in parathion metabolism were also determined by Student’s two-tailed t-tests. Values of p < 0.05 were considered to be significant. All statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA).
3.4 Results:

3.4.1 Transactivation Assay

Previous research in our laboratory demonstrated that mCAR is activated by a variety of organophosphate insecticides. Parathion and chlorpyrifos were the most efficacious CAR activators of the environmental chemicals tested (Baldwin and Roling, 2009). Furthermore, computational models as well as hCAR assays showed that thiol-containing compounds can activate mouse and human CAR through ligand binding (Küblbeck et al., 2008). Therefore, we compared mCAR activation by parathion, chlorpyrifos, nonylphenol (a partial activator) (Hernandez et al., 2007), and TCPOBOP (positive control) (Tzameli I et al., 2000a) in dose-response transactivation assays and determined their EC\textsubscript{50}’s. With the exception of TCPOBOP, the organophosphates, parathion and chlorpyrifos were the most efficacious and potent full CAR activators we have tested to date with EC\textsubscript{50} of 1.426 μM and 1.288 μM respectively (Fig. 2). In comparison, TCPOBOP has an EC\textsubscript{50} of 0.024 μM and the partial agonist, nonylphenol, has an EC\textsubscript{50} of 2.386 μM (Table 1).

3.4.2 CAR-null mice are sensitive to parathion:

WT and CAR-null male mice were treated with 5 or 20 mg/kg/day parathion based on previously published studies (Weitman SD et al., 1983; Sobarzo and Bustos-Obregón, 2000; Kim et al., 2005) to determine if parathion is a CAR activator in vivo in addition to activating CAR in vitro. Surprisingly, the CAR-null mice but not the WT showed toxicity at 5 mg/kg/day (Table 2). All of the mice treated at 20 mg/kg/day died.
Therefore in the subsequent study with female mice the 20 mg/kg/day dose was eliminated. The parathion treated male and female CAR-null mice that showed only minor symptoms such as increased lacrimination (tears, mucus eye leakage) recovered, but the other mice were euthanized or died.

The severity of toxicity was quantified based on these symptoms; no toxicity = 1, eye leakage = 2, slow, lethargic, weak tremors, = 3, and death = 4 (Fig. 3). WT male mice showed no toxicity after parathion treatment (Fig. 3A), in contrast to CAR-null male mice that showed a significantly higher degree of toxicity (assessed as lethargic or slow with weak tremors) (Fig. 3B). WT female mice showed no toxicity or a significantly lower severity of toxicity (assessed as eye leakage) (Fig. 3C) compared to CAR-null female mice that showed eye leakage, lethargy, tremors, and even death (Fig. 3D). Overall, both male and female CAR-null mice showed significantly (p-value=0.0079) greater sensitivity to parathion than WT mice, indicating a protective role for CAR in parathion toxicity.

Interestingly, immunoblots of hepatic microsomes from parathion-treated mice showed no induction of Cyp2b (Fig. 4), 2c, or 3a (data not shown) in WT or CAR-null mice. This suggests that parathion did not activate CAR in vivo. In contrast, Cyp2b was decreased by parathion and TCPOBOP treatment in CAR-null males probably because of toxicity. We have observed a decrease in Cyp2b following TCPOBOP-treatment performed over 2-days in CAR-null mice previously (Hernandez et al., 2007). Overall,
this data suggests that parathion does not activate CAR in vivo, which might be attributed to parathion’s short half-life (Sultatos LG and LD, 1986); therefore parathion may not have reached the required hepatic concentrations to activate CAR.

We also observed weak induction of Cyp2b protein in CAR-null female mice after TCPOBOP-treatment. This was not expected, and we consider that it is probably an anomaly caused by one sample. Alternatively, it could be TCPOBOP activation of PXR, although other groups have not observed this previously. More likely, it may be caused by reduced food intake as FoxA2 (HNF3β) positively regulates Cyp2b9, especially in females, and insulin reduces Cyp2b9 expression by deactivating FoxA2 (Hashita et al., 2008).

3.4.3 Basal regulation of CYPs by CAR:
CAR has been shown to basally regulate several drug metabolizing CYPs in mice (Hernandez et al., 2009b) and humans (Wortham et al., 2007), including CYP2B6, CYP2C19, POR, MRP2, Cyp2c29, and potentially Cyp2b10. Furthermore, some of these CYPs such as CYP2B6 in humans are critical in the metabolism of parathion (Mutch and Williams, 2006; Foxenberg et al., 2007). Therefore, we hypothesized that CAR-null mice may be sensitive to parathion because of differences in constitutive expression of CYPs, especially Cyp2b subfamily members, between CAR-null and WT mice. QPCR comparisons of WT and CAR-null mice demonstrated that Cyp2b9, Cyp2b10, Cyp2c29, and Cyp3a11 were all reduced in male CAR-null mice relative to WT mice (Table 3).
Female mice showed similar trends, but the effects were smaller and only Cyp3a11 was significant (Table 4). Overall, this data confirms that CAR is important in the basal regulation of several CYPs, and CAR-null mice may show lower CYP-mediated parathion metabolism as a result.

### 3.4.4 Differential parathion metabolism in CAR-null mice

Differences in parathion metabolism between untreated WT and CAR-null mice were assessed by measuring the formation of the parathion metabolites, paraoxon and PNP, via RP-HPLC in the presence of carboxylesterase inhibitors so that CYPs would be the primary if not only enzymes responsible for the metabolism of parathion. The standard peaks for each metabolite, their retention times, and a RP-HPLC sample run at 275 and 310 nm are shown in Additional File 1. Microsomes from CAR-null male mice showed significant reductions in paraoxon (34%) and PNP (45%) production compared to WT male mice (Fig. 5A and Fig. 5B), indicating their reduced capacity for metabolizing parathion. CAR-null female mice also showed reduced metabolic capacity; however, paraoxon formation was not significantly reduced (Fig. 5C). PNP formation was reduced 41% (Fig. 5D) in female CAR-null mice compared to WT mice. Therefore, the ratio of PNP/paraoxon production in female mice was changed from 0.86 in WT mice to 0.61 in CAR-null mice, a significant difference (p-value = 0.03; Student’s t-test), indicating lower production of the non-toxic PNP in the CAR-null female mice. A greater production of PNP compared to paraoxon is consistent with decreased toxicity (Mutch and Williams, 2006). Males did not show a significant difference in PNP/paraoxon ratio.
WT males had a ratio of 0.68 and CAR-null mice had a ratio of 0.58 (p-value = 0.41). Overall, CYP-mediated parathion metabolism was reduced in both male and female CAR-null mice relative to WT mice.

3.5 Discussion:

CAR is an important transcription factor that regulates a number of key detoxification genes, including CYPs. CAR activation induces several CYP1-3 family members (Sueyoshi and Negishi, 2001; Hernandez et al., 2009a), and CAR exhibits some basal activity as untreated CAR-null mice show differential CYP expression compared to untreated WT mice (Hernandez et al., 2009b). This may leave CAR-null mice sensitive to some toxicants. In this study, a clear difference in toxicity was exhibited between CAR-null and WT mice after parathion treatment. Both male and female CAR-null mice treated with parathion showed greater sensitivity to parathion than WT mice, as observed by symptoms consistent with organophosphate poisoning and activation of the parasympathetic nervous system such as mucus discharge from the eyes, lacrimination, slow stiff, tremored movements, lethargy, and in some cases death. This research demonstrates a protective role of CAR in parathion toxicity.

Studies have shown that low CAR and PXR expression and in particular low CAR expression, is associated with reduced CYP expression in humans (Wortham et al., 2007) and mice (Hernandez et al., 2009b). Because there was no significant induction of CYPs by parathion, we conclude that the basal regulation of CYPs by CAR is crucial in the
CYP-mediated metabolism of parathion and potentially in the sensitivity of CAR-null mice to parathion. Therefore, individuals with low CAR expression may be sensitive to parathion. Newborn children under the age of 6 months have low CAR and PXR expression (Vyhlidal et al., 2005). They also show lower metabolic capacity, including reduced CYP activity and are more sensitive to a number of chemicals including organophosphate insecticides (Padilla et al., 2000; Sheets, 2000).

Data indicates that CYP2B6 is a high affinity enzyme for parathion (Foxenberg et al., 2007). CAR is a key regulator of CYP2B6 in humans and Cyp2b10 in mice (Wei et al., 2000; Sueyoshi and Negishi, 2001) so changes in parathion metabolism and parathion toxicity are consistent with CAR’s regulation of Cyp2b subfamily members. For example, phenobarbital pre-treatment increases the production of paraoxon and PNP from parathion (Kim et al., 2005), confirming the importance of CYPs induced by CAR activation such as Cyp2b10 in parathion metabolism in vivo. However, CYP2B6 exhibits two times higher production of the toxic metabolite paraoxon than the detoxification product PNP, and a higher intrinsic clearance for paraoxon formation than for PNP (Foxenberg et al., 2007). In contrast, another study found that CYP2B6 preferentially produced PNP and little paraoxon (Mutch and Williams, 2006). Both studies indicate that CYP2B6 is a key enzyme in parathion detoxification. Several CYPs including Cyp2b9, Cyp2b10, Cyp3a11, and Cyp2c29, were repressed in CAR-null males, and in turn both paraoxon and PNP production were reduced. However, Cyp2b (and Cyp2c) subfamily members were not repressed in CAR-null female mice, and in turn only PNP
production was reduced. Taken together, our data suggests that Cyp2b’s and potentially Cyp2c29 preferentially produce paraoxon in mice. Overall, CYP2B6 appears crucial CYP in the activation and detoxification of parathion in mice and humans (Kim et al., 2005; Mutch and Williams, 2006; Foxenberg et al., 2007).

Other CYPs are also important in parathion metabolism. Human CYP3A members metabolize parathion to paraoxon and PNP, and both CYP3A4 and CYP3A5 show a preference for producing the nontoxic PNP over the toxic metabolite, paraoxon (Mutch and Williams, 2006). Cyp3a11 is the only Cyp that we examined that is significantly decreased in CAR-null female mice; therefore, the decreased PNP production in CAR-null females is consistent with Cyp3a11 playing a role in parathion detoxification. Given the high expression levels of CYP3A enzymes in rodent and human liver, and the reduced PNP production in association with reduced Cyp3a11 expression in this study, CYP3A members in mice and humans are almost certainly key CYPs in parathion detoxification.

A higher PNP/paraoxon ratio indicates increased production of PNP relative to the production of paraoxon and should indicate greater detoxification. The PNP/paraoxon ratio was 41% higher in WT female mice than CAR-null female mice, suggesting a reduced capacity to produce the non-toxic parathion metabolite, PNP in the CAR-null female mice. This may play a role in the greater toxicity observed in the CAR-null mice.
However, males did not show a significant difference in PNP/paraoxon ratio, but still demonstrated greater toxicity in the CAR-null mice.

In addition, paraoxonases and carboxylesterases are involved in the metabolism of and protection from organophosphate insecticides (Costa et al., 1990; Maxwell, 1992). They are key enzymes that can detoxify the toxic metabolite of parathion, paraoxon, into the non-toxic metabolite, PNP. Recent reports indicate that TCPOBOP activation of CAR, but not phenobarbital activation of CAR regulates mouse carboxylesterase 6 (Xu et al., 2009). This suggests that repression of carboxylesterase expression in CAR-null mice may also be associated with increased sensitivity to parathion.

Current in vitro research, as well as our data found parathion to be one of the most potent and efficacious CAR activators when compared to several environmental pollutants (Baldwin and Roling, 2009) (Fig. 2). However, parathion was not a CAR activator in vivo; although significant toxicity was observed in CAR-null male and female mice. Some sublethal toxicity was also observed in WT female mice. No toxicity was observed in WT male mice; however it is possible that undetected toxicity may have perturbed the ability of WT mice to respond to parathion.

The lack of in vivo CAR activation by parathion could also be attributed to rapid metabolism of parathion and paraoxon. Therefore, oral dosing of parathion may lead to a sufficiently short half-life in mice primarily due to a first pass effect (Sultatos LG and
LD, 1986). However, low bioavailability due to parathion binding to serum proteins cannot be excluded. Taken together, parathion may not have reached the necessary concentrations to activate CAR in vivo. Lower doses provided over a longer period of time may bioconcentrate and activate CAR. However, parathion’s half-life is reported to be 6.2 min (Sultatos LG and LD, 1986). Other chemicals known to activate CAR in vivo such as phenobarbital and nonylphenol, have significantly longer half-lives of about 7.5h for phenobarbital (Iven and Feldbusch, 1983), and 2-3 h for nonylphenol in humans (Muller et al., 1998), and 3-13 h in rodents depending on the dose and sex (Green et al., 2003). Other organophosphates have longer half-lives such as chlorpyrifos where the half-life through oral treatment was 15.5h and the half-life through dermal treatment was 30h (Griffin et al., 1999). Interestingly, the methyl ortholog of parathion was found to have a half-life in rats in the liver of 19.3h (Abu-Qare et al., 2000), therefore these organophosphates may be more likely to activate CAR in vivo.

A clear connection was shown for the protective role of CAR in parathion toxicity, where CAR-null mice demonstrated an increased degree of toxicity compared to WT mice. Parathion is detoxified by CYPs, paraoxonases, and carboxylesterases. We examined changes in CYP expression in CAR-null mice relative to WT mice because of increased toxicity of CAR-null mice to parathion. Based on the lower expression of several CYPs including Cyp2b and Cyp3a subfamily members in CAR-null mice, we in part attribute the increased parathion toxicity in CAR-null mice to decreased CYP-mediated parathion metabolism because CAR is important in the basal regulation of CYPs that metabolize
parathion. However, we cannot fully discount the role of paraoxonases or carboxylesterases in the sensitivity of CAR-null mice to parathion. It is interesting to consider the potential human implications of low CAR expression to parathion toxicity, especially in susceptible populations such as children. Research has shown an increased sensitivity to pesticides in children caused by limited metabolic capacity and newborns are very susceptible (Padilla et al., 2000; Sheets, 2000). Because CAR expression is associated with CYP expression in humans (Wortham et al., 2007), and children below six months of age have low CAR expression (Vyhlidal et al., 2005), our data may indicate a potential mechanism for increased toxicity to parathion and other pesticides in newborn children.
3.6 Acknowledgements: The authors would like to thank Lisa Bain, Kristen Gaworecki, and Peter van den Hurk for their help and technical expertise.
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3.8 **Footnotes:** JPH is currently at Baylor College of Medicine, Molecular and Cellular Biology, Houston, TX 77030. This research was funded by NIH grants R15 ES017321 and S06 GM008012, and start-up funds from Clemson University.
3.9 Figure Legends:

**Figure 3.1:** Parathion is metabolized by CYPs into the toxic metabolite paraoxon and detoxified by CYPs, paraoxonases, and carboxylesterases from paraoxon to PNP or directly from parathion to PNP.

**Figure 3.2:** Dose-response curves of organophosphate insecticides in transactivation assays. HepG2 cells were co-transfected with a mCAR expression plasmid and a luciferase reporter plasmid containing the CYP2B6 PBREM, and then co-treated with the inverse agonist, DHA, plus the mCAR activators. Data was normalized to cells treated with only DHA (0% activity) and TCPOBOP (100% full agonist activity), and sigmoidal dose-response curves and EC\textsubscript{50} values were determined using GraphPad Prizm 4.0 (n = 3).

**Figure 3.3:** Increased toxicity of parathion in CAR-null mice compared to WT mice. (a) WT male mice treated with parathion. (b) CAR-null male mice treated with parathion. (c) WT female mice treated with parathion. (d) CAR-null female mice treated with parathion. A significant increase in toxicity to parathion was observed in the CAR-null male and female mice as assessed by degrees of toxicity using the Mann-Whitney ranked sum test; 1 = not toxic, 2 = eye leakage, 3 = lethargic (slow) or tremors, 4 = death (males p-value = 0.0079; females p-value = 0.0079).

**Figure 3.4:** Immunoblots of Cyp2b as a biomarker of CAR activation in vivo. Cyp2b and β-actin (housekeeper) immunoblots from hepatic microsomes of untreated,
TCPOBOP, and parathion treated WT and CAR-null male and female mice were performed and quantified.  (a) WT male mice treated with TCPOBOP (b) CAR-null male mice treated with TCPOBOP (c) WT male mice treated with parathion (d) CAR-null male mice treated with parathion (e) WT female mice treated with TCPOBOP (f) CAR-null female mice treated with TCPOBOP (g) WT female mice treated with parathion (h) CAR-null female mice treated with parathion.  An asterisk indicates statistically significant difference compared to untreated mice as performed by Student’s t-test.

**Figure 3.5:** Parathion metabolites from male and female WT and CAR-null mice. HPLC was performed to quantify the formation of the toxic metabolite, paraoxon, and the nontoxic metabolite, PNP.  (a) Paraoxon formation in male mice. (b) PNP formation in male mice. (c) Paraoxon formation in female mice. (d) PNP formation in female mice. An asterisk indicates a significant difference between the two genotypes by Student’s t-test p < 0.05.
Table 3.1: EC<sub>50</sub> and 95% Confidence Intervals (CI) for parathion compared to other mCAR activators.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>95% CI (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Agonist&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parathion</td>
<td>1.426</td>
<td>0.481 – 4.233</td>
<td>Full</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>1.288</td>
<td>0.809 – 2.049</td>
<td>Full</td>
</tr>
<tr>
<td>Nonylphenol</td>
<td>2.386</td>
<td>1.357 – 4.195</td>
<td>Partial</td>
</tr>
<tr>
<td>TCPOBOP</td>
<td>0.0248</td>
<td>0.0147 – 0.040</td>
<td>Full</td>
</tr>
</tbody>
</table>

<sup>a</sup> EC<sub>50</sub>s and 95% CI were determined using GraphPad Prizm 4.0 as described in Materials and Methods.

<sup>b</sup> Chemicals that show reduced efficacy compared to TCPOBOP are considered partial agonists.
Table 3.2: Differential toxicity of CAR-null mice in comparison to wild-type treated with 5 mg/kg/day parathion for two consecutive days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WT(M)⁺</th>
<th>CAR-null(M)⁺</th>
<th>WT(F)⁺</th>
<th>CAR-null(F)⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/kg/day</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>3 mg/kg TCPOBOP</td>
<td>0/4</td>
<td>0/5</td>
<td>0/4</td>
<td>0/5</td>
</tr>
<tr>
<td>5 mg/kg/day PTN</td>
<td>0/5*</td>
<td>4/4*</td>
<td>2/5*</td>
<td>5/5*</td>
</tr>
<tr>
<td>20 mg/kg/day PTN</td>
<td>4/4</td>
<td>4/4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁺ Number of mice showing toxicity/number of mice treated.

* Statistical significance was assessed by Mann-Whitney ranked sum test using toxicity rankings depending on the severity: 1 = not toxic, 2 = eye leakage, 3 = lethargy, slow movements, or tremors, 4 = death. Male and female data, p-value = 0.0079.
**Table 3.3:** Basal regulation of CYPs by CAR in male mice.

<table>
<thead>
<tr>
<th></th>
<th>WT Male(^a)</th>
<th>CAR-null Male(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp2b9</td>
<td>1.00 + 0.52</td>
<td>0.03 + 0.02*</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>1.00 ± 0.50</td>
<td>0.08 ± 0.07*</td>
</tr>
<tr>
<td>Cyp2b13</td>
<td>1.00 ± 0.93</td>
<td>3.99 ± 3.57</td>
</tr>
<tr>
<td>Cyp2c29</td>
<td>1.00 ± 0.41</td>
<td>0.03 ± 0.02*</td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>1.00 ± 0.26</td>
<td>0.37 ± 0.13*</td>
</tr>
</tbody>
</table>

* Indicates statistical significance using Student’s t-test (p-value < 0.05).

\(^a\) The results are shown as mean relative activity ± SEM (n= 5-6).
Table 3.4: Basal regulation of CYPs by CAR in female mice.

<table>
<thead>
<tr>
<th></th>
<th>WT Female $^a$</th>
<th>CAR-null Female $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp2b9</td>
<td>1.00 ± 0.30</td>
<td>2.07 ± 0.23</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>1.00 ± 0.31</td>
<td>0.69 ± 0.27</td>
</tr>
<tr>
<td>Cyp2b13</td>
<td>1.00 ± 0.12</td>
<td>1.14 ± 0.34</td>
</tr>
<tr>
<td>Cyp2c29</td>
<td>1.00 ± 0.27</td>
<td>0.53 ± 0.28</td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>1.00 ± 0.24</td>
<td>0.47 ± 0.16$^*$</td>
</tr>
</tbody>
</table>

* Indicates statistical significance using Student’s t-test (p-value < 0.05).

$^a$ The results are shown as mean relative activity ± SEM (n= 5-6).
Figure 2

![Graph showing relative activity of chemicals at various concentrations.](image-url)
Figure 3

A. **WT male**

B. **CAR-null male**

C. **WT female**

D. **CAR-null female**
Figure 5

A  POXON male

B  PNP male

C  POXON female

D  PNP female

nM/min/mg protein

Wildtype  CAR-null

Wildtype  CAR-null

Wildtype  CAR-null

Wildtype  CAR-null

*  **
Additional Figure 1: HPLC chromatogram showing the elution of parathion and its metabolites, paraxoxon and PNP. (A) Metabolite peaks from standards. The blue plot represents the wavelength of 310 nm that is optimal for recognition and quantification of PNP. The black plot represents the wavelength of 275 nm that is optimal for recognition and quantification of paraxoxon and parathion. Standards eluted at: parathion 16.41 min; paraxoxon 5.79 min; PNP 4.38 min. (B) Metabolite peaks from a representative sample. The black plot represents the wavelength of 310 nm that is optimal for recognition and quantification of PNP. The red plot represents the wavelength of 275 nm that is optimal for recognition and quantification of paraxoxon and parathion.

CAR-null mice are sensitive to the toxic effects of parathion: Association with reduced CYP-mediated parathion metabolism in CAR-null mice. Linda C Mota, Juan P. Hernandez, and William S. Baldwin. Drug Metabolism and Disposition
CHAPTER FOUR

PXR STATUS IS ASSOCIATED WITH CYP INDUCTION, HISTOPATHOLOGICAL EFFECTS, AND CLEARANCE OF NONYLPHENOL

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4.1 Abstract:

The pregnane X-receptor (PXR) is a xenobiotic sensing nuclear receptor that regulates the transcription of a several detoxification enzymes and transporters. Nonylphenol (NP), a byproduct of alkylphenol ethoxylates, is a pervasive environmental estrogen that activates PXR in transactivation assays in vitro. Therefore, we are interested in determining if PXR is activated in vivo by NP and investigating the role of PXR in protecting an individual from NP. Wild-type (WT), PXR-null, and humanized PXR (hPXR) mice were treated with NP at 0, 50 or 75 mg/kg/day for one week, and cytochrome P450 (CYP) induction, liver histopathology, and serum NP concentrations examined. WT mice treated with NP showed induction of Cyp2b, and male-specific induction of Cyp2c and Cyp3a. CYPs were not induced in PXR-null mice, demonstrating that PXR is necessary for NP-mediated CYP induction. Because NP also activates the constitutive androstane receptor (CAR), we expected CAR-mediated CYP induction in the PXR-null mice, but this was not observed. Furthermore, hPXR mice only showed moderate Cyp2b induction, suggesting that hPXR is not as sensitive to NP as mPXR in vivo. Cyp3a induction from three human hepatocyte donors was not significant, confirming that hPXR is not highly sensitive to NP-mediated CYP induction. Livers from NP-treated WT mice exhibited significant hepatocyte hypertrophy and eosinophilic staining in periportal regions, whereas untreated WT mice did not, indicating that NP elicits a compensatory or toxic reaction. The role of PXR in this response is difficult to discern because many of the PXR-null and hPXR mice show some hypertrophy and weak eosinophilic staining around the periportal regions regardless of...
treatment. However, mice with PXR (mPXR and hPXR) showed lower NP serum concentrations than PXR-null mice treated with NP indicating that PXR plays a role in decreasing liver toxicity possibly by basally regulating Phase I-III detoxification enzymes that promote the metabolism and elimination of NP. Overall, PXR is required for NP-mediated CYP-induction, mPXR mediates greater CYP induction than hPXR, and the presence of PXR is associated with increased clearance of NP.
4.2 Introduction:

Nonylphenol (NP) is a biological degradation product of the alkylphenol ethoxylates that are widely used in the United States as intermediates for the production of a significant number of industrial products such as detergents, lubricants, agrichemicals, rubber manufacturing, and personal care products (Reed, 1978). Commercial NP is a mixture of various isomers with para-substituted branched NP predominating in the mixture (United States Environmental Protection Agency, 2005). A large body of research has demonstrated that NP is an environmental estrogen (Soto et al., 1991; White et al., 1994; Lech et al., 1996; Wilson et al., 2004; Isidori et al., 2010) and is one of the few anthropogenic environmental estrogens shown to induce mammary cancer incidence in a rodent model (Acevedo et al., 2005).

Furthermore, NP is one of the most prevalent chemicals in United State’s waterways and was found in more than 50% of 139 rivers and streams tested in a United States Geological Survey study. In addition, when found, it is often the chemical present at the highest concentration because of its ability to adsorp strongly to soils and sediments (Kolpin et al., 2002). Furthermore, NP is quite stable and remains in the sludge even after wastewater treatment (Abad et al., 2005). Due to intensive risk assessment based on NP’s endocrine disrupting activity and it’s stability in the environment, there is a comprehensive ban of NP ethoxylate surfactants in the European Union (Quednow and Püttmann, 2009).
The Pregnane X Receptor (PXR; NR1I2) is a transcription factor involved in the regulation of several genes crucial in the detoxification of xenobiotics and endobiotics. PXR is activated by a variety of steroids, herbal medicines, pharmaceuticals, and environmental chemicals (Kliewer et al., 1998; Hernandez et al., 2009a), including several environmental estrogens such as DDT, endosulfan, dieldrin and NP (Mikamo et al., 2003; Kretschmer and Baldwin, 2005; Lemaire et al., 2006). PXR’s promiscuity is attributed to its flexible ligand binding domain allowing it to accommodate ligands that vary greatly in size, shape, and polarity (Watkins et al., 2001; Xue et al., 2007). Following activation, PXR heterodimerizes with RXRα (NR2B1), binds its response elements, and induces phase I-III enzymes (Hernandez et al., 2009a), such as MRP3 (Maher et al., 2005), GSTA4, UGT1A1 (Hartley et al., 2004), carboxylesterase 6 (Xu et al., 2009), and several CYPs in families 2 and 3 (Waxman, 1999). CYP enzymes induced by mouse PXR include Cyp2b10 and the classical biomarker, Cyp3a11 (Hernandez et al., 2009a).

NP also activates rodent and human PXR in transactivation assays performed in vitro (Masuyama et al., 2000; Hernandez et al., 2006). In addition, NP activates PXR’s relative, the constitutive androstane receptor (CAR; NR1I3) in vitro using a mouse CAR transactivation assay (Hernandez et al., 2007; Baldwin and Roling, 2009). In vivo studies with CAR +/-, and CAR -/- mice demonstrated that Cyp2b10 is induced in a CAR-dependent manner. Furthermore, human hepatocytes and humanized CAR mice treated with NP show Cyp2b10 induction, demonstrating that both mouse and human
CAR are activated by NP in vivo (Hernandez et al., 2007). However, similar studies that test whether PXR is required for CYP induction by NP has not been demonstrated in vivo.

There is circumstantial evidence that NP activates PXR in vivo. NP has been shown to induce Cyp3a subfamily members in rat liver (Lee et al., 1996) as well as mouse liver; (Masuyama et al., 2001). Furthermore, while the NP-mediated induction of Cyp2b in WT mice was lost in CAR-null mice; the induction of several other Cyps including Cyp2a4 and Cyp3a11 was observed in CAR-null female mice suggesting NP activation of PXR (Hernandez et al., 2009b). Therefore, one of the goals of this paper is to determine whether NP activates mouse and human PXR in vivo.

Furthermore, we are interested in determining if PXR protects individuals from NP exposure. Even though PXR is crucial in the induction of detoxification enzymes, the presence or activation of PXR has rarely been shown to protect individuals from xenobiotics. In contrast, PXR-null mice show fewer adverse hepatotoxic effects after co-treatment with pregnenolone 16α-carbonitrile (PCN) and acetaminophen (APAP), demonstrating the role of PXR in the bioactivation of acetaminophen to N-acetyl-p-benzoquinone imines (NAPQI) (Guo et al., 2004). However, PXR is important in protecting individuals from bile acids, and PXR-null mice treated with lithocholic acid exhibit significantly higher hepatotoxicity than WT mice (Xie et al., 2001). This
hepatotoxicity is attributed to the inability of PXR-null mice to respond and induce enzymes involved in the detoxification and excretion of this toxic bile acid.

In this study, we investigated PXR’s role in the basal regulation of drug metabolizing CYPs as basal regulation may be important in protecting the liver from xenobiotics (Mota et al., 2010). Then we investigated the role of PXR in NP-mediated CYP induction in WT, PXR-null, and hPXR mice to determine if NP activates mPXR and hPXR in vivo. Cyp3a11 induction was also assessed in primary human hepatocytes. Liver histopathology and serum levels of NP were compared between treatment groups to test whether PXR increased clearance and protected the liver from NP. Overall, the data indicates that mPXR is necessary for CYP induction, hPXR is not as sensitive as mPXR in vivo, and PXR is important in the clearance of NP from mice.

4.3 Materials and Methods:

4.3.1 Animals

All studies were performed in agreement with NIH guidelines for the humane use of research animals and approved by Clemson University Animal Care and Use Committee. Mice were provided with food and water ad libitum. Male and female 8-10 week old B6129 (WT), PXR-null (Staudinger et al., 2001) and hPXR (Lichti-Kaiser and Staudinger, 2008) mice were randomly split into groups (n = 4-6). The mice were given 100 μl of honey orally as a control, or NP (technical grade with approximately 85% p-isomers; Fluka Chemical Co., Seelze, Germany) at 50 mg/kg/day NP, or 75 mg/kg/day
NP dissolved in 100 µl honey for seven consecutive days. The positive control, dexamethasone (technical grade 98%; Sigma St. Louis MO) was dissolved in 100 µl of corn oil and injected at 75 mg/kg/day once per day for three days. All mice were anesthetized 6 hours following the last treatment, blood was collected from the mice by heart puncture, and livers excised.

4.3.2 Liver sample preparation

Livers from euthanized mice were excised, weighed, snap frozen, and cut into several pieces for RNA extraction, and microsome preparation. Total RNA was isolated using a modified TRI-reagent protocol of guanidinium thiocyanate-phenol-chloroform extraction following manufacturer’s specifications (Sigma, St. Louis, MO). To remove residual DNA from RNA isolated a DNAse digestion was performed (Promega Corporation, Madison WI). RNA was quantified at 260 nm and 280 nm wavelengths using a spectrophotometer and samples were immediately stored at –80°C. For cDNA preparation reverse transcription was performed using MMLV-RT, dNTP mixture, and random hexamers followed by immediate storage at –20°C. Microsomes were homogenized and isolated by differential centrifugation; aprotonin, leupeptin, and PMSF were used as protease inhibitors. After protein quantification (Bio-Rad, Hercules, CA), microsomes were stored at –80°C.
4.3.3 Quantification of CYP expression

QPCR was performed using previously published protocols and primers (Muller et al., 2002; Wiwi et al., 2004; Hernandez et al., 2009b). All samples were diluted 1:10 and standard curves were performed using a composite of samples diluted from 1:1 to 1:1000. To quantify gene expression amplifications were performed in triplicates using a 96-well iQ5™ multicolor Real-Time PCR Detection System (Bio-Rad) with 0.25X SybrGreen. Data normalization of Q-PCR results was performed using the expression of 18S rRNA as the housekeeping gene. Quantification was done by taking the efficiency curve of the Q-PCR reaction to the power of the threshold cycle (Ct) divided by 18S (Muller et al., 2002).

Immunoblotting was used to quantify CYP protein levels. Quantification of mouse CYP is referred to by subfamily because each antibody may recognize more than one mouse isoform in a subfamily. Antibodies from different sources were used to quantify mouse Cyp2b, 2c, and 3a. Cyp2c and Cyp3a were quantified using a human CYP2C8/9/19 or rat CYP3A1 antibodies respectively from Chemicon, (Billerica, MA). A newly developed polyclonal antibody was used to quantify Cyp2b (Mota et al., 2010). Proteins were separated electrophoretically on a 10% polyacrylamide gel (SDS-PAGE), and transferred to nitrocellulose membrane (Bio-Rad) using 30-50 µg of microsomal protein. After protein transfer the nitrocellulose was blocked in 2.5-5% dried milk for 30 min to 1 hour and washed in Tris Buffer Saline pH=7.4 0.1%Tween 20. Primary antibodies were incubated at room temperature for 2h and overnight at 4°C prior to secondary antibody
incubation at room temperature for 2h (Mota et al., 2010). β-Actin (Sigma, St. Louis, MO) was used as a housekeeping gene. Secondary antibodies used were based on the source of the primary antibodies mainly goat anti-mouse or goat anti-rabbit (Bio-Rad; 1:500) Protein detection was performed by chemiluminescence using Immun-Star AP Chemiluminescent Protein Detection Systems and quantified by Chemi Doc XRS HQ using Quantity One 4.6.5 (Bio-Rad, Inc).

4.3.4 Primary human hepatocytes

Human hepatocytes were obtained from Cellz Direct (Pittsboro, NC) and maintained as previously described (Hernandez et al., 2007). The hepatocytes were extracted from three different donors that were Caucasian females with ages of 52, 29 and 77. Cells were treated with DMSO (UT), NP, or phenobarbital (PB) as a positive control (Sigma, St. Louis, MO) for 24h. After treatments, the cells were harvested, RNA extracted, cDNA prepared and Q-PCR performed using CYP3A4 and 18s primers as previously described (Hernandez et al., 2007).

4.3.5 Histology samples

Liver samples from untreated and NP-treated WT, PXR-null, and hPXR mice were fixed in 10% formalin. Samples were trimmed, processed, embedded, sectioned, and stained with hemotoxylin and eosin at Colorado Histo-Prep (Fort Collins, CO) for blind histopathological evaluation. Standard mouse toxicologic pathology criteria and
nomenclature were used to evaluate microscopic tissue changes in each of the different treatment groups.

4.3.6 Nonylphenol Extraction and GC quantification

Nonylphenol was extracted from mice serum using a modified protocol (Danzo et al., 2002). In brief, 150 µl of mouse serum was vigorously vortexed with 1 mL saturated NaCl in glass tubes, afterwards 1 mL of ethyl acetate was added and vortexed. Mixture was allowed to settle for 10 min at room temperature, or until mixture turned biphasic. Supernatant was transferred to new glass tubes, where 1 mL of water was added, vortexed and supernatant was again transferred to a new glass tube. Solvent was evaporated with nitrogen gas, and reconstituted with 400 µl of ethyl acetate.

Standards of NP were prepared at a concentration of 0.01, 0.05, 0.1, 0.5, 1.00 µg/mL in ethyl acetate. Spectra were recorded using Agilent 7890 A gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA) using 70eV electron ionization at an ion source temperature of 230°C. Chromatographic separations were performed using 30m X 0.25mm, 0.25 µm film DB-5ms column (Agilent Technologies, Inc.) GC analyses were done using a splitless injection at 250°C, followed by a column temperature program change of 100 to 300°C over 10°/min. Quantification was done by selected ion monitoring with an ion dwell time of 25 msec. The ions used for NP were, m/z 135, 149, and 220. Quantification of all samples were performed by the external standard technique in which a known amount of a reference standard was analyzed; areas of
selected ion monitoring chromatographic peaks were integrated using GC ChemStation© software (Agilent Technologies, Inc.) Areas under the peaks were converted to plasma concentrations using the standard curve of NP. The detection limit was 0.01 µg/mL.

4.3.7 Statistical analysis

One-way ANOVA followed by Dunnett’s multiple comparison test was performed on Q-PCR data when comparing the CYP expression from the different mouse genotypes. Student’s two-tailed t-tests were used to compare differences in protein CYP expression between males and females in WT, PXR-null, and hPXR mice in immunoblots. Student’s two-tailed t-tests were used to compare CYP3A4 relative expression between UT and treated human hepatocytes. Fisher’s 2X2 was performed to determine a significant difference between untreated and NP treated WT, PXR-null and hPXR mice that exhibited hepatocyte hypertrophy. One-way ANOVA followed by Dunnett’s multiple comparison tests were performed to determine statistically significant differences in NP serum concentrations between untreated and different doses of NP treated mice of different genotypes. Values of p < 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA).

4.4 Results:

4.4.1 Comparing basal CYP expression in mPXR, PXR-null, and hPXR mice
Q-PCR was performed to determine basal regulation of CYPs by mouse and human PXR. Different isoforms of CYPs were tested by Q-PCR to compare CYP expression in WT and PXR-null male and female mice, as well as hPXR and PXR-null mice. The loss of PXR caused a significantly lower expression of Cyp3a11 in male and female PXR-null mice compared to WT mice. Interestingly, PXR-null mice showed significantly higher expression of Cyp2b10 and Cyp3a41 than WT mice (Table 1). Negative regulation of Cyp3a41 by PXR has been previously demonstrated (Anakk et al., 2004); however to our knowledge, this is the first time that Cyp2b10 has been shown to be negatively regulated by PXR. Surprisingly, mouse CYPs did not respond the same to human PXR (Table 2). For example, hPXR did not repress Cyp2b10 in the same manner as mPXR, nor did was Cyp3a11 repressed in male hPXR mice. Overall, mPXR showed stronger basal regulation of mouse CYPs than hPXR when compared to PXR-null mice.

4.4.2 CYP induction by NP in vivo is PXR-dependent

Immunoblots were performed to determine NP-mediated induction of CYPs in WT, PXR-null, and hPXR mice. Cyp2b was significantly induced by NP in male and female WT mice (Fig. 1A). Cyp3a and Cyp2c (data not shown) were significantly induced in male mice treated with 50 or 75 mg/kg/day of NP (Fig. 1A). Female WT mice did not demonstrate Cyp3a induction by NP. We have previously observed this (Acevedo et al., 2005; Hernandez et al., 2009b), presumably because of NP-mediated repression of Cyp3a41, a female specific CYP negatively regulated by PXR (Anakk et al., 2004)(Table 1). WT mice treated with the positive control, dexamethasone, also exhibit significant
induction of Cyp2b and Cyp3a (Suppl. Data Fig. 1). Cyp induction may be mediated by PXR or CAR (especially Cyp2b) activation; however, PXR-null mice did not demonstrate CYP induction following NP treatment (Fig. 1B), indicating that CYP induction by NP is PXR-dependent.

4.4.3 hPXR activation by NP:

Humanized PXR mice lack mouse PXR and instead contain human PXR. They are used to reduce uncertainty when extrapolating data from rodents to humans, and demonstrate the key role of hPXR in mediating chemical-mediated CYP induction (Lichti-Kaiser and Staudinger, 2008). The hPXR mice did not appear to respond to NP treatment as robustly as mPXR (WT) mice. Female hPXR mice had no significant CYP induction from either of the NP treatments (50 or 75 mg/kg/day) (Fig. 2A). Male hPXR mice treated with NP showed induction of Cyp2b and Cyp3a (Fig. 2A), albeit weaker induction than the WT mice. In comparison, hPXR mice treated with dexamethasone, as a positive control, showed Cyp2b induction in both male and female mice, but Cyp3a induction was not significant (Fig. 2B). Overall, CYP induction was more robust in mPXR mice than hPXR mice suggesting that NP is a weaker hPXR activator than mPXR activator in vivo.

4.4.4 CYP3A4 expression in human hepatocytes

However, weak induction of CYPs in hPXR mice could be caused by poor interactions between the necessary mouse coactivators in human PXR. Therefore, we treated fresh human hepatocytes from three donors with NP and phenobarbital (known to activate
human CAR and PXR) (Moore et al., 2000) for 24 hours and then measured CYP3A4 expression. While most patients showed weak CYP3A4 induction, there was no statistically significant difference in CYP3A4 mRNA expression between UT and NP treated hepatocytes in any of the three donors (Fig. 3A-3C). A statistically significant higher expression of CYP3A4 was observed in all of the three patients treated with phenobarbital (Fig. 3D-3F). Thus, NP does not significantly increase CYP3A4 expression in human hepatocytes. This result coincides with the hPXR result and indicates that NP is a weaker hPXR activator than mPXR activator.

4.4.5 Histopathology

Given that NP activates PXR and PXR regulates several detoxification genes both basally and when activated, we wanted to test whether PXR protects the liver from NP-mediated damage. Therefore, liver histopathology was assessed in formalin fixed H&E stained liver slides from the different mouse genotypes, and NP treatments at 0 and 50 mg/kg/day. Untreated WT mice exhibited normal liver tissue (Fig. 4A); whereas NP-treated WT mice (Fig. 4B) showed significant hepatocyte hypertrophy in the periportal region (Table 3). Increased eosin staining was also observed in NP-treated livers, which may be a sign of increased protein synthesis. In the PXR-null (Fig. 4C-4D) and hPXR (not shown) UT and NP-treated mice the liver showed minimal hepatocyte hypertrophy in the periportal regions regardless of the treatment (Table 3), and increased eosin staining was observed in both PXR-null and hPXR mice. Therefore, there was no statistically significant difference in the hypertrophy scoring between UT and NP-treated PXR-null mice or hPXR mice (Table 3). However, some of the NP-treated PXR-null
mice did show a subjectively larger increase in eosin staining (Fig. 4D). Overall, the histopathology data for the PXR-null and hPXR mice is difficult to interpret because of hypertrophy in the untreated mice. Overall, NP appears to cause some damage or an acute response as observed through increased eosin staining and hypertrophy in the WT mice, which may be mediated through PXR.

4.4.6 Nonylphenol serum concentrations

Because increased damage was observed in the NP-treated WT mice and increased eosin staining was observed in the periportal regions of some PXR-null mice, GC-MS was used to quantify serum NP concentrations and assess the ability of WT, PXR-null, and hPXR mice to clear NP. In general, there was a trend indicating greater NP in the serum of treated mice than untreated mice, especially at 50 mg/kg/day NP. Differences in serum NP concentrations between treatment groups were not significantly different in WT and hPXR mice (Table 4A,B). Male and female PXR-null mice treated with 50 mg/kg/day NP have greater amounts of NP in their serum than untreated PXR-null mice (Table 4A, B), suggesting that the lack of PXR caused perturbed clearance of NP.

There was no trend in NP serum concentrations in female hPXR mice (Table 4) because the female untreated hPXR mice showed high concentrations of NP. The basis for the high NP concentration in hPXR female mice is unknown and may be related to contamination of the plastic tubes the serum was initially stored in. Furthermore, the 75 mg/kg/day NP treatments consistently had lower serum NP concentrations that the 50
mg/kg/day group. The mice treated with 75 mg/kg/day were harder to feed, and showed less inclination for the NP-tainted honey. It is possible that the dose they ultimately received was less than the 50 mg/kg/day NP group because they did not fully ingest their share. This would also explain why the 75 mg/kg/day NP-treated groups often showed less CYP induction than the 50 mg/kg/day NP-treated groups (Fig. 1).

Data from male and female mice was combined because there were no significant differences in serum concentration between males and females (Table 4C). NP-treated WT mice showed a significant increase in serum NP concentrations (Table 4C) when the data was combined because of the increased statistical power. The p-values comparing the untreated and 50 mg/kg/day groups from PXR-null mice also increased from 0.01 to 0.001. A trend was observed in the male, female, and combined data where 50 mg/kg/day treated WT mice had lower NP serum concentrations than 50 mg/kg/day treated PXR-null mice, though the data was not statistically significant (Table 4). However, when the data from WT and hPXR mice (all PXR-positive mice) was combined and compared to PXR-null mice, there was significantly more NP in the serum of PXR-null mice than PXR-positive mice (Fig. 5). This further suggests that the presence of PXR is protective from NP potentially by regulating enzymes and transporters important in metabolizing or clearing NP.
4.5 Discussion:

PXR is important in the regulation of detoxification enzymes that are required to metabolize and eliminate compounds that may have deleterious effects such as NP (Waxman, 1999; Xie et al., 2001; Kretschmer and Baldwin, 2005; Hernandez et al., 2009a). PXR appears to protect mice from NP. In this study, only PXR-null mice showed significantly increases in NP serum concentrations (Table 4A,B). Furthermore, PXR-positive mice (mPXR or hPXR) had lower serum concentrations of NP after treatment with 50 mg/kg/day than PXR-null mice treated with 50 mg/kg/day NP. Because CYP induction occurred in hPXR and mPXR mice, but not PXR-null mice; the data demonstrates that PXR positive mice can respond to NP and induce detoxification enzymes, which is crucial in helping eliminate a toxic insult (Fig. 1-2). However, the role that mouse and human PXR plays in the basal regulation of CYPs and potentially other enzymes and transporters cannot be discounted when considering PXR’s protective role (Table 1-2).

PXR has been shown to induce many detoxification enzymes in response to many different chemicals (Hernandez et al., 2009a). However, the presence or activation of PXR has rarely been shown to protect individuals from anthropogenic xenobiotics. This study indicates that the presence of PXR (either mouse or human) reduces serum NP concentrations in mice treated with 50 mg/kg/day NP compared to mice that lack PXR. Further, the reduced NP serum concentrations in PXR-positive mice are associated with PXR-dependent CYP induction. However, PXR-status and reduced serum concentrations
were not associated in mice treated with 75 mg/kg/day, probably because mice treated with the high dose of NP did not fully ingest their food as described in the Results. This lack of a dose response, NP background contamination in the untreated female hPXR mice, and the equivocal histopathology results in the transgenic mice partially temper our enthusiasm with the positive results demonstrating PXR protects mice from NP. Some but not all of the confounding factors have apparent explanations, ultimately indicating that PXR-mediates CYP induction by NP, hPXR is not as sensitive as mPXR to NP exposure in vivo, and PXR plays a crucial role in the clearance and protection of mice from NP by increasing clearance probably due to its role in mediating basal and inducible expression of drug metabolizing enzymes.

Untreated female hPXR mice showed NP background contamination in their serum. NP contamination has been observed in previous studies (Soto et al., 1991; Danzo et al., 2002) where measurable concentrations of NP were found in untreated samples. These NP concentrations have been attributed to NP leaching from the plastic tubes during storage and centrifugation steps. Another possible source of contamination, in the case of this study, might come from the plastic water bottles, as well from the honey fed to the mice that was also stored in plastic bottle. One study showed significant concentrations of NP found in bottled water in all commercial bottles tested (Li et al., 2010). Why the contamination occurred in several untreated hPXR female mice to a greater degree than other treatments is unknown. If the contamination occurred prior to treatment of the mice then this may explain the poor induction in female hPXR mice relative to mPXR mice.
However, this does not explain why male hPXR mice also showed much less CYP induction than mPXR mice. Therefore, most likely the contamination of the samples occurred during the serum storage and NP extraction. Overall, NP contamination is an important factor that must be considered in future work because background contamination can have an impact on the results.

Histopathology demonstrated that NP caused hypertrophy in the periportal regions of WT mice. A previous study with rats also demonstrated increased hypertrophy following oral NP-treatment (Woo et al., 2007). The hepatocyte hypertrophy coincides with CYP induction and increased eosin staining around periportal regions, which is thought to be attributed to increased protein synthesis. Therefore, this data is indicative of an acute compensatory response to NP exposure in WT mice.

However, it is difficult to establish that PXR-null mice are more sensitive to the toxic or hypertrophic effects of NP on the liver than WT mice. A significant difference in hypertrophy was not measured between treated and untreated mice that lacked PXR. This is because PXR-null and hPXR mice show hypertrophy regardless of treatment (Table 3). The observed hypertrophy in treatments of PXR-null and hPXR mice (Fig. 4C-4D; hPXR data is not shown) indicates that mouse PXR is important for the normal physiology of the liver. There have been other studies that have observed hepatocyte hypertrophy in knockout mouse models, including those that lack peroxisomes and HNF4α-null mice (Hayhurst et al., 2001; Dirkx et al., 2005). Interestingly, there is more
eosin staining in some of the NP-treated PXR-null mice around periportal regions than in untreated PXR-null mice (Fig. 4C-4D), which suggests increased protein synthesis. Whether this is indicative of minor cellular damage caused by NP is not known, but suggests some sensitivity in treated PXR-null mice relative to untreated PXR-null mice. We hypothesize that the putative increased sensitivity in PXR-null mice treated with NP is due to decreased clearance of NP (Table 4A-4C).

Past studies have shown NP-mediated induction of CYP3A (Lee et al., 1996), and NP activation of PXR has been demonstrated in transactivation assays using mouse (Masuyama et al., 2000), rat, and human PXR (Hernandez et al., 2007). It is presumed that PXR is activated by NP; however, activation of PXR in vivo has not been demonstrated using knockout models. Instead only activation of CAR by NP has been definitively demonstrated in vitro and in vivo (Hernandez et al., 2007; Hernandez et al., 2009b). The data from this study showed that NP-mediated induction of Cyp2b and Cyp3a in male and female mice is PXR-dependent as induction was completely lost in PXR-null mice.

This is somewhat surprising because NP also activates CAR and we hypothesized that some CYP induction, such as the induction of Cyp2b, would be observed in PXR-null mice due to CAR activation. Studies have shown that NP activates CAR in vivo (Hernandez et al., 2009b) and in this study it was demonstrated that NP activates PXR in vivo as well. Interestingly, research has shown weak CYP induction by Q-PCR in CAR-null mice (Hernandez et al., 2009b); however, in this study there was no CYP induction observed via immunoblotting in NP-treated PXR-null mice. Overall, this suggest that CAR and PXR work together to regulate NP-mediated CYP induction. Most studies indicate that nuclear receptors often compete for resources (Yan et al.,
1998; Miao *et al*., 2006; Lee *et al*., 2008); however, in this case CAR and PXR appear to work together and increase NP-mediated CYP induction.

Male mice showed significantly higher NP-mediated CYP induction than females. Cyp2b and Cyp3a induction in males was observed at 50 and 75mg/kg/day NP. The 50mg/kg/day NP treatment showed a higher induction than 75mg/kg/day in male mice indicative of the poor feeding rate of the NP-treated mice at 75 mg/kg/day. In contrast to male mice, female mice only showed a significant induction of Cyp2b by the 50mg/kg/day NP treatment. WT female mice did not show induction of Cyp3a protein concentrations following NP-treatment, which is common and even NP-mediated down-regulation of Cyp3a protein expression has been observed (Laurenzana *et al*., 2002; Acevedo *et al*., 2005; Hernandez *et al*., 2006; Hernandez *et al*., 2009b), presumably due to the repression of Cyp3a41 and potentially Cyp3a44 (Anakk *et al*., 2004; Hernandez *et al*., 2006; Hernandez *et al*., 2009b).

Interestingly, NP is a weak activator of hPXR relative to mPXR in vivo. For example, hPXR mice treated with NP showed no significant induction of CYPs in females and much weaker induction of Cyp2b and Cyp3a in males than WT (mPXR) mice. Dexamethasone, the positive control, showed significant induction of only Cyp2b in male and female mice (Fig. 2B), potentially due to a drop in Cyp3a41 (Anakk *et al*., 2004; Hernandez *et al*., 2009b). Taken together, this data suggests that hPXR may not work as well as mPXR because of poor interactions with mPXR co-activators. However, it is also possible that the female hPXR controls were contaminated, or NP is not a strong hPXR activator in vivo.
Therefore, we obtained hepatocytes from human donors to determine NP’s ability to induce CYP3A4 expression in a model relevant to human health. NP-treated human hepatocytes have hPXR and all the necessary human co-activators for proper CYP induction. Q-PCR results showed that NP did not induce CYP3A4 significantly, but the positive control phenobarbital showed significant induction. A previous study in our laboratory demonstrated CYP2B6 induction by NP in hepatocytes, and attributed the induction to CAR, but could not rule out PXR (Hernandez et al., 2007). CYP2B6 induction in the human hepatocytes and hCAR mice was also weaker than WT mice and PB-mediated induction. Overall, the results suggest that NP is most likely a weaker human PXR activator than mouse PXR activator, and this is in part the reason for the weak CYP induction in hPXR mice and human hepatocytes.

PXR was also found to be important in the basal regulation of some CYPs. For example, male and female mice showed significantly lower expression of Cyp3a11 in PXR-null mice than WT mice, indicative of a direct role of PXR in Cyp3a11 basal regulation. Cyp2b10 and Cyp3a41 were found to be negatively regulated by PXR, whereas a higher expression of these genes is observed in PXR-null mice. Our results confirm earlier work that Cyp3a41 is negatively regulated by PXR (Anakk et al., 2004). To our knowledge this is the first time that Cyp2b10 has been shown to be negatively regulated by PXR. hPXR did not always demonstrate similar basal regulation as mPXR, primarily the regulation of the mouse Cyp2b members, Cyp2b9 and Cyp2b10, as they did not respond to human PXR regulation in the same manner as mPXR.
In summary, results suggest that the presence of PXR is important in the clearance of NP from mice as PXR-positive mice treated with 50 mg/kg/day NP have lower serum NP concentrations than PXR-null mice. This may be due to PXR-dependent induction of drug metabolizing enzymes such as CYPs, or may be because of PXR’s role in the basal regulation of CYPs and presumably other drug metabolizing enzymes. However, whether PXR provides actual protection from hepatotoxicity is ambivalent. NP-induced liver pathology in PXR-null mice was only subjectively increased over untreated PXR-null mice. Furthermore, hPXR appears to be less sensitive to the activating effects of NP than mPXR. Thus, the role hPXR in inducing drug metabolizing enzymes compared to mPXR in NP-exposed individuals. Given the role of PXR in basal and inducible regulation of CYPs, and the lower level of serum NP in PXR-positive mice, PXR probably provides some level of protection from NP. Furthermore, susceptible populations that may have lower expression or activity of PXR (Lim et al., 2005; Vyhlidal et al., 2006) may exhibit greater sensitivity to plasticizers such as NP due to their limited detoxification and elimination capacity.

Acknowledgement: The authors would like to thank Dr. Jeff Staudinger for donating his PXR-null and hPXR mouse models, and Dr. Melissa Riley for her help with the GC-MS analysis. This work was supported by NIH grant R15-ES017321 and Clemson University start-up funds.
4.6 REFERENCES


**Supplemental Data.** Dexamethasone activates mouse PXR. Western blots of Cyp2b and Cyp3a were performed using hepatic microsomes from untreated, and 75 mg/kg/day dexamethasone treated male and female mice previously described in materials and methods. An asterisk indicates statistically significant difference compared to the untreated group performed by Student’s t-test (p < 0.05).
FIGURE LEGENDS

Figure 1: Nonylphenol activates mouse PXR. Western blots of hepatic microsomes from untreated, 50 and 75 mg/kg/day nonylphenol treated previously described in materials and methods A) wild-type male and female mice B) PXR-null male and female mice. Western blots were performed for Cyp2b and Cyp3a to determine induction of CYPs by nonylphenol. An asterisk indicates statistically significant difference compared to the untreated group performed by Student’s t-test (p < 0.05).

Figure 2: Nonylphenol is a weak activator of human PXR. Western blots of hepatic microsomes were performed for Cyp2b and Cyp3a in male and female humanized mice. A) Male and female untreated and nonylphenol treated previously described in materials and methods B) Male and female untreated and dexamethasone treated used as a positive control. An asterisk indicates statistically significant difference compared to the untreated group performed by Student’s t-test (p < 0.05).

Figure 3: CYP3A4 expression in human hepatocytes treated with nonylphenol. Q-PCR was performed for CYP3A4 primers using human hepatocyte cells treated with NP and PB extracted from three different donors. A) Donor 1 NP treated hepatocytes. B) Donor 2 NP treated hepatocytes C) Donor 3 NP treated hepatocytes D) Donor 1 PB treated hepatocytes E) Donor 2 PB treated hepatocytes F) Donor 3 PB treated hepatocytes. An asterisk indicates statistically significant difference compared to the untreated group performed by Student’s t-test (p < 0.05).
Figure 4: Liver histopathology in untreated and 50 mg/kg/day NP treated mice. Mouse liver fragments were stained using hematoxylin and eosin stain previously described in materials and methods from A) Untreated wild-type B) NP treated wild-type C) Untreated PXR-null D) NP treated PXR-null.

Figure 5: Nonylphenol serum concentrations in PXR-positive (hPXR + mPXR) and PXR-negative mice. Data are expressed as mean ± SEM (n = 4–5) for each gender. Male and female data was combined. An asterisk indicates statistically significant difference compared to the wild-type group performed by Student’s t-test (p < 0.05).
Table 1. Comparison of CYP expression between WT and PXR-null mice

<table>
<thead>
<tr>
<th>CYP</th>
<th>WT Female</th>
<th>PXR-null Female</th>
<th>WT Male</th>
<th>PXR-null Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp2b9</td>
<td>14.97 ± 4.96</td>
<td>6.47 ± 1.27</td>
<td>1.00 ± 0.81</td>
<td>0.69 ± 0.46</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>2.42 ± 0.53</td>
<td>23.64 ± 5.53</td>
<td>1.00 ± 0.41</td>
<td>6.74 ± 2.28</td>
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<tr>
<td>Cyp2c29</td>
<td>0.82 ± 0.28</td>
<td>1.42 ± 0.85</td>
<td>1.00 ± 0.28</td>
<td>1.87 ± 0.44</td>
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<tr>
<td>Cyp3a11</td>
<td>1.19 ± 0.24</td>
<td>0.31 ± 0.06</td>
<td>1.00 ± 0.24</td>
<td>0.26 ± 0.08</td>
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<tr>
<td>Cyp3a41</td>
<td>60.98 ± 15.31</td>
<td>125.37 ± 23.26</td>
<td>1.00 ± 0.76</td>
<td>0.85 ± 0.31</td>
</tr>
</tbody>
</table>

Data is expressed as relative data ± SEM

* P-value < 0.05 and ** p-value < 0.01; indicate statistical significance by one-way ANOVA followed by Dunnett’s multiple comparison test.

a Indicates significant difference between WT female and WT male.
b Indicates significant difference WT female and PXR-null female mice.
c Indicates significant difference between WT male and PXR-null male mice.
d Indicates significant difference between PXR-null female and PXR-null male mice.
Table 2. Comparison of CYP expression between PXR-null and humanized PXR mice

<table>
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<tr>
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<td>Cyp2b9</td>
<td>2.20 ± 0.53</td>
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<td>0.05 ± 0.03 d**</td>
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<td>Cyp2b10</td>
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<td>Cyp3a41</td>
<td>9.71 ± 1.75</td>
<td>22.78 ± 4.78 b*</td>
<td>1.00 ± 0.87</td>
<td>0.34 ± 0.24 d**</td>
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Data is expressed as relative data ± SEM

* P-value < 0.05 and ** p-value < 0.01; indicate statistical significance by one-way ANOVA followed by Dunnett’s multiple comparison test.

a Indicates significant difference between hPXR female and hPXR male.

b Indicates significant difference hPXR female and PXR-null female mice.

c Indicates significant difference between hPXR male and PXR-null male mice .

d Indicates significant difference between PXR-null female and PXR-null male mice.
Table 3. Association of PXR status with hepatocyte hypertrophy in untreated and NP treated mice.

<table>
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<td>UT</td>
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<tr>
<td>NP</td>
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</table>

Data shown represents the number of mice that exhibited hepatocyte hypertrophy/total number of mice.

Statistical analysis performed (one-tailed Fisher’s 2X2) indicates a significant difference between untreated and nonylphenol treated WT mice (p < 0.05).

There were no differences between male and females, thus data was combined for analysis.
Table 4. Nonylphenol serum concentrations, as measured by GC-MS in mice treated with 0, 50, or 75 mg/kg/day NP.

**A. Female**

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<tr>
<td>0</td>
<td>0.0285 ± 0.0179</td>
<td>0.0221 ± 0.0122&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>50</td>
<td>0.0926 ± 0.0322</td>
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<tr>
<td>75</td>
<td>0.0791 ± 0.0100</td>
<td>0.0574 ± 0.0279</td>
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**B. Male**

<table>
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**C. Male and Female**

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<sup>#</sup> Data is expressed as mean (μg/ml) ± SEM (n = 4-6 or n = 8-12 for combined male and female data). Detection limit was of 0.01μg/ml. Values calculated below detection limit but higher than 0 were assigned a value of ½ detection limit.

<sup>a</sup> Indicates significant difference between untreated and NP-treated mice (p-value < 0.05)
b Indicates significant difference between untreated and NP-treated mice (p-value < 0.01)

c Indicates significant difference between untreated and NP-treated mice (p-value < 0.0001)

d Indicates significant difference between WT and hPXR mice (p-value < 0.05)

Statistical significance determined by ANOVA followed by Dunnett’s multiple comparison test.
Figure 1

A

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Cyp2b
Cyp3a
β-actin
Figure 4
Figure 5
Supplemental Data

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Cyp2b

Cyp3a

β-actin
CHAPTER FIVE
DISCUSSION

These studies focused in assessing the protective role of CAR and PXR in the toxicity from a pesticide, parathion, and a plasticizer, nonylphenol, in part by characterizing the basal and inductive regulation of CYPs by these receptors. The Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR) are well known to be detoxification receptors due to their role in regulating enzymes and transporters involved in the metabolism and elimination of toxic compounds from the body (Staudinger JL, Madan A et al. 2003). Some endogenous compounds that are known to be metabolized through CAR and PXR regulation are bile acids, hormones, bilirubin (Huang W, Zhang J et al. 2003) and xenobiotics like dexamethasone, rifampicin, phenobarbital, nonylphenol (Sueyoshi T, Kawamoto T et al. 1999; Hernandez JP, Chapman LM et al. 2006).

CAR and PXR are key regulators of very important detoxification enzymes such as the phase I, CYPs. These detoxification receptors primarily regulate the induction of CYP families 1-3 that are involved in drug metabolism. The most sensitive biomarker for CAR and PXR activation are CYP2B and CYP3A, respectively (Waxman DJ 1999), but CAR and PXR regulate other CYPs and show cross regulation (Pascussi JM, Gerbal-Chaloin S et al. 2003). Very few xenobiotic chemicals have been shown to be more toxic in animals lacking CAR and PXR than those with them. Studies showed that PXR/CAR CYP regulation was involved in the bioactivation of acetaminophen (APAP) into N-
acetyl-p-benzoquinone imines (NAPQi) that resulted in hepatotoxicity, where PXR-null mice showed less toxicity after cotreatment with a PXR full agonist (Guo GL, Moffit JS et al. 2004) and WT mice showed less toxicity after cotreatment with CAR inverse agonist (Zhang J, Huang W et al. 2002). In addition CAR-null mice treated with nonylphenol and/or TCPOBOP cotreated with a paralyzer zoxazolamine showed an increase in paralysis time attributed to the lack of CAR regulated CYP induction to metabolize the paralyzer thus increasing paralysis time (Hernandez JP, Chapman LM et al. 2006; Hernandez JP, Huang W et al. 2007; Hernandez JP, Mota LC et al. 2009). However, studies have found in vivo evidence that the loss of CAR and PXR increases sensitivity to endogenous compounds such as bile acids like lithocholic acid (Xie W, Radominska-Pandya A et al. 2001; Uppal H, Toma D et al. 2005). Interestingly research has demonstrated that low expression of CAR and/or PXR leads to a lower expression of CYPs in humans (Wortham, Czerwinski et al. 2007), which may lead to an increased sensitivity to toxic compounds and provides a mechanism for toxicity.

Our studies demonstrated that CAR basally regulates CYPs in mice in a similar manner to humans (Objective 1), and in turn some chemicals are not metabolized (parathion) or cleared (nonylphenol) properly (Objectives 2 and 3). Thus, parathion caused significantly increased toxicity in CAR-null mice with some CAR-null mice dying while WT mice were unaffected (Objective 2). Nonylphenol was cleared more slowly in PXR-null mice than WT mice and this may have led to a slight increase in liver damage (Objective 3).
Because CAR and PXR expression is lower in newborn humans during their first six months (Vyhlidal, Gaedigk et al. 2005), and therefore have a lower expression of CYPs this group may be sensitive to a number of chemicals. The elderly often do not show as strong as inductive response to CYP inducers and they may also be more sensitive to some drugs and environmental chemicals. Our studies provide new insight into the importance of CAR and PXR in protecting us from endo- and xenobiotics, especially as they pertain to sensitive populations.

5.1 Objective 1

The sexual dimorphic regulation of CYPs after TCPOBOP and nonylphenol treatments was studied to determine the role of CAR in CYP induction. The basal regulation of CYPs by CAR was also investigated. Results showed that the expression of Cyp2b10 in females and Cyp2c29 in male was significantly lower in CAR-null mice. This indicates that CAR is important in the regulation of these CYPs isoforms. Sexual dimorphism was observed in the expression of several CYPs where Cyp2a4, Cyp2b9, Cyp2b13, Cyp3a11, Cyp3a41, and Cyp3a44 were female predominant and only Cyp2c37 was male predominant. We also investigated sexually dimorphic induction of CYPs in a CAR-dependent manner by a potent full agonist (TCPOBOP) and a moderately strong partial agonist (nonylphenol). Cyp2b10, Cyp2c29 and Cyp3a11 were induced by nonylphenol treatment, a partial CAR activator, in a CAR-dependent manner, although it was only significant in females. TCPOBOP, a full CAR activator, induced the same CYP isoforms in both genders. The results indicate that the CYP induction by CAR agonists is
not gender specific, however, females are more sensitive to the effects of CAR agonists since only females responded to nonylphenol treatment. The Q-PCR data previously mentioned was corroborated with Western Blot data examining protein levels. Protein Cyp2b and Cyp2c were induced by nonylphenol only in females in a CAR-dependent manner. Interestingly, Cyp3a showed induction by TCPOBOP and downregulation by nonylphenol treatment in females in a CAR-dependent manner. There was no significant induction of any CYPs in males. We hypothesized that males show lower levels of CAR mRNA and protein. Immunoprecipitations followed by Western Blots did show 43% lower CAR in males, but it was not statistically significant. Overall CAR is important in the regulation of basal and induction of CYPs, especially in females. Furthermore, female mice appeared to show greater sensitivity to CAR-mediated nonylphenol (partial agonist) induction, but they did not show greater sensitivity to TCPOBOP (full agonist) mediated CYP induction. This indicates that xenobiotic induction of CYPs is not sexually dimorphic, but sensitivity of the inductive process may be sexually dimorphic with females showing greater sensitivity.

5.2 Objective 2

Data has shown that one of the most potent and efficacious environmentally-relevant CAR activators in vitro is the organophosphate, parathion (Baldwin WS and Roling JA 2009). In this study, we investigated the protective role of CAR in mediating the regulation of CYPs involved in parathion metabolism. We treated CAR-null and WT mice with parathion to discern if parathion also activated CAR and induced CYPs in
vivo. Surprisingly, we observed significant differential toxicity was observed, where a significantly higher degree of toxicity symptoms were observed in CAR-null male and female mice than WT mice. This data indicates of a significant role of CAR in protecting mice against parathion toxicity. CYP induction was assessed to determine if CAR was activated by parathion in vivo. Results showed a lack of CYP induction in male and female mice, suggesting that parathion does not activate CAR in vivo, presumably because of parathion’s short half-life. Therefore, we hypothesized that reduced CYP expression in CAR-null mice may be involved in the protective role of CAR in parathion toxicity. CAR basally regulates Cyp2b9, Cyp2b10, Cyp2c29 and Cyp3a11, where a lack of CAR leads to a lower expression of these CYP isoforms. Thus, even though parathion does not activate CAR in vivo, CAR is crucial in basally regulating CYPs that metabolize parathion. Differential parathion metabolism was tested by comparing parathion metabolite formation in WT and CAR-null male and female mice. Data showed differential metabolite formation between CAR-null and WT mice, where both the formation of paraoxon (toxic metabolite) and p-nitrophenol (nontoxic metabolite) were lower in CAR-null male mice and only the nontoxic metabolite (PNP) was significantly reduced in CAR-null female mice. This indicates slower parathion metabolism in the CAR-null mice compared to WT, suggesting the increased parathion toxicity may be putatively explained by reduced parathion metabolism by CYPs; however, other enzymes such as carboxylesterases or paraoxonases have not been thoroughly examined. In summary, CAR is crucial in protecting the body from parathion toxicity. This may be due to CAR’s role in basally regulated CYPs. Our data also provides a potential
mechanism as to why susceptible populations, such as newborns, may be at a higher risk of parathion toxicity due to their limited metabolic capacity (Padilla S, Buzzard J et al. 2000; Sheets LP 2000) caused by lower CAR expression (Wortham M, Czerwinski M et al. 2007).

Fig. 5.1 Putative mechanism for children increased sensitivity to toxicants. During the first six months of age the expression of CAR and PXR are low, leading to a lower CYP expression which results in decreased metabolism, higher sensitivity and toxicity to compounds.

5.3 Objective 3

Previously, we investigated the basal regulation of CYPs by CAR. In objective 3, we started by investigating the basal regulation of CYPs by PXR and took it one step further by examining the basal regulation of mouse CYPs by mouse PXR and human PXR (hPXR humanized mice that contain human PXR, but not mouse PXR). These results showed PXR to be important in basal regulation of CYPs, especially Cyp3a11, Cyp3a41, and Cyp2b10. PXR-mediated repression of Cyp3a41 has been previous shown (Anakk S, Kalsotra A et al. 2004); however the fact that Cyp2b10 is also repressed by PXR is new knowledge and further demonstrates PXR’s role as a transcriptional
repressor in the absence of ligand. Mice with a humanized PXR were used to elucidate the possible effects of a human population; however mouse CYPs did not seem to respond as strongly to human PXR regulation although for the most part they showed similar trends. This indicates that people with low PXR levels such as children may show reduced responses to toxicants and therefore be more susceptible to their effects.

In addition, previous work demonstrated that nonylphenol activates CAR and PXR in vitro (Masuyama H, Hiramatsu Y et al. 2000; Hernandez JP, Huang W et al. 2007), and activates CAR in vivo (Hernandez JP, Huang W et al. 2007). Therefore, we examined whether nonylphenol activates PXR in vivo. To determine the role of PXR in the induction of CYPs, different mouse genotypes were treated with nonylphenol. In vivo results showed that nonylphenol activates PXR demonstrated by significant CYP induction in WT mice, and this induction was lost in PXR-null mice. Our results demonstrate the CYP induction following nonylphenol treatment is PXR-dependent. We also expected some CYP induction in PXR-null mice because CAR may take over and mediate the induction of CYPs; however, this was not observed. Interestingly, CAR-null mice also do not show PXR-mediated induction of CYPs by nonylphenol. This indicates that in some cases CAR and PXR must work together to cause significant transcriptional activity. To our knowledge this is the first time this has been shown in vivo (Ding X and Staudinger JL 2005). Primarily people considered the opposite that nuclear receptors would compete for similar resources such as cofactors and therefore reduce each other activity (Yoshikawa T, Ide T et al. 2003).
Human PXR responded very weakly to nonylphenol suggested by a weak CYP induction seen only in male mice. We questioned whether the weak CYP induction was caused by poor interaction of human PXR with mouse transcriptional co-activators or maybe human PXR is not as responsive to nonylphenol in vivo. Therefore, human hepatocytes were treated with nonylphenol and the positive control phenobarbital, to quantify CYP3A4 expression in a human in vitro model. Results demonstrated significant CYP3A4 induction by phenobarbital, but not in the nonylphenol treated cells. This corroborates our in vivo data that human PXR does not respond as well to nonylphenol in comparison to mouse PXR.

Finally, we investigated the protective role of PXR in regulating CYPs potentially involved in preventing hepatotoxicity and clearance of nonylphenol from the body. The livers from nonylphenol treated mice of different genotypes were histologically examined to determine hepatotoxicity. Data indicated a significant hypertrophy as well as increased eosin staining in NP treated WT mice and normal physiology in UT WT. This data coincides with our in vivo results that showed CYP induction in WT nonylphenol treated mice. The observed PXR-dependent CYP induction caused by nonylphenol suggests that mouse PXR is important in the acute response to nonylphenol hepatotoxicity by inducing CYPs that in turn will help detoxify nonylphenol from the body. Interestingly hypertrophy and eosin staining was observed in hPXR and PXR-null mice regardless of the treatment, indicating that mouse PXR is important for normal hepatocyte physiology. Therefore, there was no significant difference in the number of mice showing eosin-type staining of hypertrophy in PXR-null treated mice compared to
untreated PXR-null mice. However, the amount of eosin staining was greater in some PXR-null individuals treated with nonylphenol.

Nonylphenol clearance and elimination was assessed by quantifying NP in serum of treated mice. Results indicate that NP concentrations are significantly higher in PXR-null mice compared to PXR +/+ (mouse + human). This suggests that PXR plays an important role in eliminating NP from mice serum. Overall PXR is not only important in mediating CYPs to help detoxify compounds from the body but also is important in NP clearance and elimination. These results are of great relevance especially to susceptible populations that may have lower expression of PXR and CYPs at specific life stages, posing a greater risk to nonylphenol toxicity due to limited metabolism and detoxification.

5.4 Summary

CAR and PXR have great significance in protecting individual from toxic compounds by regulating CYP induction or constitutively aiding in the detoxification of these compounds from the body. Susceptible populations are at greater risk to compounds toxicity due to the limited metabolic capacity (Padilla S, Buzzard J et al. 2000; Sheets LP 2000), since at early life stages these detoxification receptors have low expression (Vyhlidal CA, Gaedigk R et al. 2005) that leads to CYP low expression (Wortham M, Czerwinski M et al. 2007) and inability to metabolize and detoxify compounds from the body. These populations have an increased sensitivity to toxicity as a result of the lower expression or lack of CAR and PXR. This study showed that CAR
and PXR protect individuals from parathion and nonylphenol toxicity, and individuals lacking these receptors have an increased sensitivity to these compounds. Data has indicated that higher expression of CYPs especially CYP2b9, 2b10 and 2b13, which are known to be regulated by CAR and PXR, are positively correlated with longevity (Boylston WH, DeFord JH et al. 2006). CAR’s protection from everyday chemical exposure may increase our longevity by having the essential regulation of enzymes to metabolize, detoxify and clear chemicals from the body suggestive that a strong detoxification system is important in our long-term health.

Numerous studies have found children to be very sensitive to the toxic effects of chemicals such as organophosphates. It has been shown that prenatal exposure to organophosphates can lead to impaired fetal growth demonstrated by low birth weight and length (Whyatt RM, Rauh V et al. 2004). A review of epidemiological studies showed that in newborn organophosphate exposure led to increased abnormal reflexes (Young JG, Eskenazi B et al. 2005; Jurewicz J and Hanke W 2008). Research demonstrated a significant correlation between higher organophosphate concentrations in children of 24 months of age and pervasive developmental disorder i.e.g. avoiding eye contact, unresponsive to affection (Eskenazi B, Marks AR et al. 2007). Other studies have seen lower activity of paraoxonase-1 in newborns compared to adults, which is an enzyme important in the detoxification of organophosphates, (Chen J, Kumar M et al. 2003) suggestive of an increased sensitivity due to impaired metabolism.

Other chemicals that may pose a significant risk to susceptible populations are plasticizers such as nonylphenol (NP) and di(2-ethylhexyl) phthalate (DEHP) that are
heavily used for a wide variety of products and applications. Research has found measurable concentrations of various plasticizers in the umbilical cord blood of low birth weight newborns (Lin L, Zheng LX et al. 2008). Infants were reported to have the highest levels of dietary intake of DEHP (Lyche JL, Gutleb AC et al. 2009) and premature neonate that undergo intensive therapeutic medical interventions showed high measurable concentrations of DEHP because of leaching of medical devices (Calafat AM, Needham LL et al. 2004). Polyvinylchloride (PVC) infusion systems may contain up to 60% of DEHP, which can be leached from the tubing during total parenteral nutrition and studies have showed that it may lead to an increased risk of cholestasis in premature and newborn infants (von Rettberg H, Hannman T et al. 2009). Potential feminization effects by prenatal phthalates exposure have been observed in research where anogenital distance (AGD) has been decreased and incomplete testicular descent in male infants between 2 to 6 months of age (Swan SH, Main KM et al. 2005).

The detoxification receptors CAR and PXR are necessary to the body because of the vital protective role in regulating the necessary genes to metabolize toxic compounds. This research demonstrated that the lack of these receptors results in increased sensitivity due to a perturbed detoxification process. Toxic compounds pose a greater danger to susceptible populations that have low expression of these receptors and in turn are unable to protect their body of toxicity.
5.5 REFERENCES


