Are Cyp2b and Cyp3a Important in Hepatic Lipid Metabolism?

Ramiya Kumar
Clemson University, ramiya999@gmail.com

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ARE CYP2B AND CYP3A IMPORTANT IN HEPATIC LIPID METABOLISM?

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biological Sciences

by
Ramiya Kumar
May 2018

Accepted by:
Dr. William S Baldwin, Committee Chair
Dr. Lisa J Bain
Dr. Peter Van den Hurk
Dr. David M Feliciano
ABSTRACT

The Cytochrome P450s (CYP) in families 1-3 are integral to the metabolism of xenobiotics such as pharmaceuticals, pesticides and plasticizers as well as endobiotics such as hormones, bile salts and fatty acids. Recently, hepatic CYP activity was associated with fatty liver and potentially obesity. The CYPs in the 3A and 2B families are critical in xenobiotic detoxification and are regulated by transcription factors that control toxic responses in addition to energy homeostasis. Therefore, we assessed the significance of CYP3A and CYP2B in obesity and hepatic energy metabolism using Cyp3a or Cyp2b knockout mouse models. Cyp3a-null and Cyp2b-null mice were challenged with a high fat diet (HFD) for 8-10 weeks to test whether these mice were more susceptible to obesity and fatty liver disease. We determined changes in body weight, organ weight, white adipose tissue mass, hepatic lipids, glucose metabolism and sensitivity, hormone changes in these mice after 8-10 weeks of HFD treatments. We hypothesized that the lack of Cyp2b /Cyp3a will increase the susceptibility of the -null mice to diet-induced obesity. Our results indicate that Cyp2b’s are crucial in protection of HFD-induced obesity. Cyp2b-null male but not Cyp2b-null female mice gained more body weight coupled with increased white adipose tissue mass compared to their wild-type counterparts following a HFD. The response in Cyp3a-null mice were not as strong; however, gender-based differences were observed in both models. Cyp3a-null female mice were moderately protected from HFD-induced obesity. We also observed significant increase in liver triglycerides in the Cyp3a-null and Cyp2b-null mice. In conclusion, loss of Cyp2b exacerbates obesity, while loss of Cyp3a mitigates the development of HFD-induced obesity.
DEDICATION

This thesis is dedicated to my family and friends.
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CHAPTER ONE

INTRODUCTION

The purpose of this study was to determine the significance of Cytochrome P450’s (CYP) 2B and 3A in obesity and the potential for inhibition of CYPs to perturb energy and more specifically lipid homeostasis. Recent studies using hepatic cytochrome P450 oxidoreductase-null mice shows that the lack of all CYP activity in the liver leads to accumulation of unsaturated fats and these unsaturated fats activate constitutive androstane receptor (CAR) and induce the expression of Cyp2b10 to eliminate the fats (Finn et al., 2009). An earlier study from our laboratory shows that the repression of Cyp2b genes increased body weight gain, increased adipose tissue weight and accumulation of unsaturated fats in old male Cyp2b-null RNAi mediated knockdown mice (Damiri et al., 2012). To demonstrate the significance of CYP2B in lipid metabolism, we developed triple knockout mice missing hepatic Cyp2b enzymes-Cyp2b9, Cyp2b10 and Cyp2b13 using CRISPR/Cas 9 with targeted small guide RNAs. Also, we also investigated the significance of CYP3A in lipid metabolism using a seven-Cyp3a gene knockout mice model. Because CYP3A members are highly expressed in the liver making up 30-40% of hepatic CYP expression and metabolize more than 50% of pharmaceuticals available in the market, we hypothesized that this predominant hepatic CYP member would be crucial in hepatic lipid metabolism and lack of this CYP member will increase susceptibility to diet-induced obesity.
1.1 Obesity and Obesogens

Obesity is one of the fastest growing metabolic disorders in the world, and according to World Health Organization (WHO), global obesity prevalence has tripled between 1975 and 2016. WHO 2016 obesity statistics reports that 39% of adults are overweight and 13% of adults are obese (WHO, 2017). For example, the percent of obese individuals is increasing in Latin America (i.e. Panama 31.5%, Chile 21.9%), Europe (Greece 22.5%, Malta 20.7%), and the Middle East (United Arab Emirates 33.7%, Saudi Arabia 35.6%, Egypt 30.3%) (WHO, 2018). In turn, the rates of dyslipidemia, hypertension, diabetes, and other metabolic disorders have increased dramatically placing a significant burden on our healthcare systems (LeBlanc et al., 2012b; Sharp, 2009). Non-alcoholic fatty liver disease is one of the comorbidities associated with obesity, wherein there is alcohol-independent, increase in accumulation of fat in the liver exceeding >5% of total liver weight.

The primary causes of obesity are a dense calorie diet and lack of physical activity; however, recent studies show that exposure to certain environmental chemicals called obesogens can enhance the accumulation of fats, perturb lipid metabolism and lead to the development of obesity (Grun et al., 2009a). Worse yet, this may be a couple with non-alcoholic fatty liver disease (Fabbrini et al., 2010).

Obesogens increase depuration of lipids into white adipose tissue. They increase proliferation of new fat cells, increase fat content in the fat cells or by reprogramming the multipotent stem cells to generate adipocytes over bone cells (Grun et al., 2009b). Food additives such as sodium benzoate and curcumin reduce leptin levels and xenobiotics such
as tributyltin and benzyl butyl phthalates induce adipocyte differentiation through peroxisome proliferator-activated receptor gamma (PPARg) (Yin et al., 2016). These are some known obesogens that associate with obesity.

Most obesogens are thought to interact with nuclear receptors, such as the peroxisome proliferator-activated receptors (PPARs), which act as metabolic sensors (Grun et al., 2009a; Hurst et al., 2003; Li et al., 2011). PPARg is a crucial nuclear receptor in lipid homeostasis and energy metabolism (Sugii et al., 2009). PPARs bind fatty acids and eicosanoids to regulate their distribution and use (Kliewer et al., 1997). One of the standard treatment regimens for type II diabetes includes activation of PPARg using drugs such as thiazolidinediones to increase insulin sensitivity by decreasing plasma triglyceride levels; however, this leads to accumulation of lipids in white adipose tissue and weight gain in the long term (Larsen et al., 2003). Tributyltin activates PPARg: RXR to promote adipogenesis in murine 3T3-L1 cells and increases hepatic steatosis and epidydimal adipose tissue mass in adult mice exposed to tributyltin at gestational of 12-18 days (Grun et al., 2006). Similarly, male mice exposed to repeated low dosage (5µg/kg) of tributyltin for 45 days had an increase in body weight, fat mass, insulin, and leptin levels compared to control mice indicates that chronic tributyltin exposure could induce obesity (Zuo et al., 2011).

Other nuclear receptors implicated in obesity include Farnesoid-X-receptor (FXR), estrogen receptor (ER α), glucocorticoid receptor (GR), activation of the pregnane-X-receptor (PXR), and inhibition of the constitutive androstane receptor (CAR) (Paul et al., 2013; Takeshita et al., 2001). The OECD considers metabolic disorders one of three critical
areas in toxicology in addition to testicular dysgenesis and the increased incidence of autism spectral disorders (LeBlanc et al., 2012a).

**Figure 1.1: Nuclear receptors that stimulate weight gain and associated disorders.** Activation of PPARg:RXR by tributyltin induces adipocyte differentiation, increases adipose tissue weight and leads to body weight gain. The increase in body weight causes insulin resistance and glucose intolerance. Thus, leading to the development of obesity and potentially diabetes (LeBlanc et al., 2012a).

1.2 CAR (Constitutive Androstan Receptor)

CAR belongs to nuclear receptor superfamily NR113. It is responsible for the metabolism of foreign chemicals such as drugs, pesticides (Hernandez et al., 2009a) and endogenous compounds such as bilirubin (Huang et al., 2003) and fatty acids (Finn et al., 2009). CAR regulates the expression of cytochrome P450 (CYP) 2Bs and to a lesser extent CYP3As that are crucial in phase I detoxification pathways, phase II enzymes such as uridine 5’-diphospho glucuronosyltransferase (Sugatani et al., 2001) and sulfotransferase...
Inactive CAR is present in the cytoplasm bound by cytoplasmic CAR retention protein, heat shock protein (HSP90), P-23 and immunophilins (Kobayashi et al., 2003; Yoshinori et al., 2003). It can be activated by direct binding of ligands such as 1,4 bis (2-(3,5-dichloropyridyloxy)) benzene TCPOBOP (Tzameli et al., 2000). CAR can also be activated indirectly by compounds such as phenobarbital that do not bind the receptor (Kawamoto et al., 1999) but changes the phosphorylation status of Thr\textsuperscript{38} residue (Mutoh et al., 2009), which results in conformational change and allows translocation of CAR into the nucleus.

In the nucleus, CAR heterodimerizes with its partner, retinoid-X-receptor and then binds to direct repeats 4 or inverted repeats 6 in the phenobarbital response element upstream of promoter region on the DNA to induce transcription of its target genes.

**Figure 1.2: Mechanism of activation of CAR.** CAR is retained in the cytoplasm by cytoplasmic CAR retention proteins (CCRP), HSP90 and
immunophilins, while the presence of ligand represented as “x” activates CAR by causing conformational change and allowing the movement of CAR into the nucleus. CAR dimerizes in the nucleus with RXR to induce transcription of its target genes.

CAR and its target gene Cyp2b10 levels were increased in db/db mice while zucker rats, which have a mutation in leptin receptor gene, had decreased expression of CAR and Cyp2b1 demonstrates CAR’s role in regulating energy homeostasis (Xiong et al., 2002; K Yoshinari et al., 2006). CAR was activated during HFD treatment in order to get rid of the accumulating hepatic fat especially during treatment with unsaturated fats rich sunflower oil in HRN-null mice (Finn et al., 2009). Activation of CAR using mouse-specific CAR agonist TCPOBOP shows decrease in hepatic glucose production, increase insulin sensitivity and alleviate fatty liver in ob/ob mice but these changes were absent in CAR-null ob/ob double mutant mice (Dong et al., 2009). CAR also increases the production of anti-lipogenic protein, insig-1 (Roth et al., 2008) and also suppress FoxO1 to prevent it from binding insulin response sequences on gluconeogenic genes (Kodama et al., 2004). FoxO1 regulates the expression of gluconeogenic genes such as phosphoenol pyruvate carboxykinase (PEPCK1) and glucose-6 phosphate (G6P) and is also known to coregulate other nuclear receptors such as glucocorticoid receptor and thyroid receptors (Nakae et al., 2001; Zhao et al., 2001). Insulin represses FoxO1 by phosphorylating it via PI3-Akt pathway to preventing it from binding insulin response element present upstream of PEPCK1 and G6P (Matsuzaki et al., 2003). Kodama et al group demonstrated that CAR and PXR crosstalk with FoxO1 using yeast two hybrid screening and FoxO1 acts as coactivator of CAR while activated CAR binds FoxO1 to repress gluconeogenic genes such
as PEPCK1 (Kodama et al., 2004). Thus, CAR regulates energy metabolism related genes and has a protective effect against the development of obesity and diabetes.

![Diagram of CAR regulatory network]

**Figure 1.3: CAR is cross talks with transcription factors associated with anti-obesity.** FoxO1 acts as coactivator of CAR but active CAR phosphorylates FoxO1, and downregulates its target gene expression (Kodama et al., 2004). CAR activates insig-1 that downregulates sterol regulatory elemental binding protein-1(Roth et al., 2008).

FoxA2 is a hepatocyte nuclear factor that regulates lipid metabolism and ketone body formation(Wolfrum et al., 2004). FoxA2 is activated during fasting and starvation to increase triglyceride breakdown, fatty acid oxidation genes such as Carnitine palmitoyl transferase-1 a and decrease glycolysis genes such as glucokinase (Wang et al., 2001;Wolfrum et al., 2004). FoxA2 is inhibited by insulin via PI3-AKT phosphorylation.
during fed state and is excluded from the nucleus in order to downregulate its target gene expression (Wang et al., 2001). FoxA2 is involved in sexual dimorphic expression of Cyp2b9 and it is regulated by HNF4α (Hashita et al., 2008;Wiwi et al., 2004). HNF4α is an important nuclear receptor and regulates sexually dimorphic expression of key hepatic genes such as cytochrome P450 and also regulates CAR expression (Wiwi et al., 2004). Thus, FoxA2, HNF4α and CAR are important regulators of hepatic gene expression (Hashita et al., 2008;Hernandez et al., 2009b;Wiwi et al., 2004).

In our study, we characterized the compensatory changes in CYP gene expression, protein expression and cytochrome P450 enzyme activity changes in CAR-null mice. This study will help us to determine changes in xenobiotic and drug metabolism in patients with low CAR or CYP activity. In addition, CAR is important because it is the primary positive regulator of Cyp2b10 in mice and CYP2B6 in humans.

1.3 Cytochrome P450s (Cyps)

Cytochrome P450s belong to a superfamily of heme-containing monooxygenases and are crucial in phase I detoxification of exogenous compounds such as drugs and environmental pollutants (Hernandez et al., 2009a;Perloff et al., 2000) and endogenous compounds such as hormones (Waxman, 1988) and fatty acids (Bylund et al., 1998). There are 57 human CYP genes and 108 murine CYP genes classified into 18 families (Nelson, 2011). CYPs of the families 1-3 are known to play an important role in xenobiotic metabolism. But recent studies shows that the CYPs 1-3 are also important in the metabolism of fatty acids such as arachidonic acid (Capdevila et al., 1990), linoleic acid (Finn et al., 2009) and epoxyeicosatrienoic acid (Du et al., 2005).
The lack of cytochrome P450 activity in a liver specific knockdown in HRN-null mice increases fatty acid accumulation in the liver (Finn et al., 2009). Opposingly, a Cyp2e1-null shows improved glucose tolerance and insulin sensitivity coupled with increased energy expenditure and are protected from high fat diet-induced obesity (Zong et al., 2012). In the former mouse model, HRN-null mice lack CYP activity in the liver therefore treatment with high fat diet leads to accumulation of unsaturated fatty acids in the liver due to lack of functional detoxification CYPs. These hepatic lipids then activate CAR to induce Cyp2b10 expression to prevent lipid toxicity and restore lipid homeostasis but lack of cytochrome P450 oxidoreductase prevents CYP’s ability to metabolize lipids. In the latter mouse model, Cyp2e1 oxidizes foreign compounds such as alcohol and endogenous compounds such as fatty acids. Cyp2e1 generates reactive oxygen species while metabolizing its substrate in a NADH/NADH dependant pathway and also increases mitochondrial oxidative stress markers such as glutathione-s-transferase A4 (Raza et al., 2004). Increased production of these reactive oxygen species will induce inflammation and insulin resistance by activating serine/threonine kinase pathways such as nuclear factor-κB which in turn will phosphorylate insulin receptor and insulin receptor substrate 1 (IRS-1). Increase in serine phosphorylated IRS-1 decrease their tyrosine phosphorylation and also reduce their ability to bind insulin receptor or downstream targets such as PI3-kinase; thus oxidative stress and dysregulation in intracellular signaling lead to insulin resistance (Evans et al., 2005). Therefore, Cyp2e1 knockout mice did not show insulin resistance but had improved insulin sensitivity and glucose tolerance compared to their wild-type
counterparts fed high fat-diet. These studies indicate that different CYPs may perform completely different roles and outcomes when it comes to obesity and diabetes.

Cyp2b RNAi knockdown mouse model developed in our own laboratory show that older male knockdown mice had increased white adipose tissue weight and body weight compared to their wild-type counterparts. The knockdown mice also had difficulty clearing out corn oil, which is rich in 85% unsaturated fats (Damiri et al., 2012).

Thus, cytochrome P450’s has a significant role in lipid homeostasis apart from mere xenobiotic biotransformation and the development of metabolic disorders. In our study, we have used two cytochrome P450-null mice to demonstrate their role in hepatic lipid metabolism.
Fig. 1.4: Lack of Cyp2b/Cyp3a will increase susceptibility to high fat diet-induced obesity. To demonstrate significance of CYP2B/CYP3A in hepatic lipid metabolism, treatment with either chemical inhibitors such as triclopipidine and ketoconazole or targeted gene editing using Crispr/Cas9 will repress/knockout Cyp2b/Cyp3a in mice. These mice will be challenged with high fat diet for 8-10 weeks to simulate western diet-induced obesity. Mice lacking Cy2b/Cyp3a will have an increase in body weight, glucose intolerance and insulin resistance because of accumulation of unsaturated fatty acids and lack of intracellular signaling molecules such as EETs. Thus, mice lacking Cyp2b/Cyp3a will develop diet-induced obesity and non-alcoholic fatty liver disease.
1.4 CYP2B

Cytochrome P450 2B is one the least studied among the CYPs because it was considered to be expressed <1% of total hepatic CYP expression; however, recent development of sensitive probes and inhibitors have shown that it constitutes 2-10% of total hepatic CYP (Wang et al., 2008) and metabolizes approximately 25% of the drugs available in the market (Xie et al., 2001). CYP2B’s also metabolizes environmental pollutants such as nonylphenol (Lee et al., 1998), N,N-diethyl-m-toluamide (DEET) (Usmani et al., 2002), parathion (Foxenberg et al., 2007), and polychlorinated biphenyls (Warner et al., 2009) and pharmaceuticals such as efavirenz (Ward et al., 2003), bupropion (Faucette et al., 2000) and cyclophosphamide (Xie et al., 2003). CYP2B also metabolizes endobiotics such as linoleic acid (Finn et al., 2009), testosterone (Mo et al., 2009) and arachidonic acid (Capdevila et al., 1990; Keeney et al., 1998a). Cyp2b12 participates in arachidonic acid metabolism by epoxygenation to generate 11,12- and 8,9-epoxyeicosatrienoic acids (Keeney et al., 1998b), which acts as bioactive lipids and participates in intracellular signaling in renal function and blood pressure regulation (Zeldin, 2001). Cyp2b19 expressed on mouse epidermal keratinocytes metabolize arachidonic to 11, 12-epoxyeicosatrienoic acid which is crucial for regulating epidermal cornification (Du et al., 2005).

The CYP2B subfamily in humans is represented by only CYP2B6 while in mouse there are 5 isoforms Cyp2b9, Cyp2b10, Cyp2b13, Cyp2b19 and Cyp2b23. Cyp2b23 is expressed only in adolescent mice (after birth till day 20) (Peng et al., 2012) while Cyp2b19 is expressed in fetal mouse keratinocytes (Keeney et al., 1998a) and has low hepatic
expression. The hepatic CYPs are Cyp2b9, Cyp2b10, and Cyp2b13 and they show sexual dimorphism with Cyp2b9 and Cyp2b13 expressed more in the females than males (Hernandez et al., 2006; Mota et al., 2010; Wiwi et al., 2004).

CAR regulates Cyp2b10 expression (Honkakoski et al., 1998) by forming a heterodimer with RXR, binds to the phenobarbital response element present upstream of the promoter region on the DNA and induces gene expression. CAR induces Cyp2b10 expression during nutrient or metabolic stress due to changes in cAMP levels (Ding et al., 2006). FoxA2, which is female predominant in mice, induces female predominant, Cyp2b9, and is modulated by female type growth hormone secretion (Hashita et al., 2008).

Recent studies using cytochrome P450 oxidoreductase-null mice shows increase in Cyp2b expression; Cyp2b expression increased in correlation with increases in hepatic triacylglycerol levels especially during treatment with sunflower oil that is high in omega-6 polyunsaturated fatty acids. The induction of Cyp2b expression appears to be a response to eliminate the excess hepatic lipids (Finn et al., 2009). Cyp2b RNAi knockdown male mice generated in our laboratory showed increases in hepatic fatty acid accumulation during treatment with corn oil and increase in body weight coupled with an increase in white adipose tissue weight compared to their wild-type counterparts. The older knockdown mice also did not show any difference in Cyp2b expression levels during corn oil treatment when compared to wild-type mice despite the repression of Cyp2b genes in this study (Damiri, 2011). A HFD rich in soybean oil administered to mice lead to an increase in body weight, adiposity and increased deposition of fat in the liver coupled with
hepatocyte ballooning. These mice show a significant increase in Cyp2b; similar to our observations in earlier studies (Deol et al., 2015).

6-weeks old C57/BL6oja Hsd mice fed a high fat diet (40% fat) made up of fats such as 70% sunflower oil and 18% coconut oil for 12-weeks shows significant increase in body weight and white adipose tissue weight, increase in hepatic lipids and leptin levels compared to their standard-diet fed counterparts. Pearson correlation study in these mice revealed a strong correlation between Cyp2b9 expression level and weight gain, white adipose tissue gain and leptin levels (Hoek-van den Hill et al., 2015). Recent microarray analysis done to determine gene expression changes in brown adipose tissue on mice treated with (35 kcal) high fat diet shows significant downregulation of Cyp2b10 within 2-weeks of high fat diet treatment (McGregor et al., 2013). These studies show that CYP2B is highly inducible by high fat diet.

Taken together, CYP2B metabolizes fatty acids, it is induced by dietary fatty acids and its loss is associated with dysregulation of lipid homeostasis. Therefore, we hypothesize that lack of Cyp2b9, 10 and 13 in our knockout mice will increase their susceptibility to high fat diet-induced obesity.

1.5 CYP3A

Cytochrome P450 3A is the most predominant CYP in the liver (constitutes 30-40% of the CYPs in the liver) and metabolizes > 60% of the drugs available in the market (Guengerich, 2002). CYP3A plays a crucial role in the biotransformation of drugs such as valproic acid (Cerveny et al., 2007), midazolam (Perloff et al., 2000) and simvastatin (Pruksaritanont et al., 2003) and environmental pollutants such as endosulfan (Casabar et
al., 2006), chlorpyrifos (Tang et al., 2001) and phthalates (Cooper et al., 2008). CYP3A also metabolizes endogenous compounds such as lithocholic acid (Staudinger et al., 2001), arachidonic acid (Bylund et al., 1998) and steroid hormones (Waxman et al., 1983). CYP3A4 metabolizes linoleic acid to 11-hydroxyoctadecanoic acid and arachidonic acid is oxidized to generate 13- hydroxyecosatrienoic acid (HETE), 10-HETE and 7-HETE (Bylund et al., 1998). The arachidonic acid epoxides do not have any known function in the liver but inhibition assays suggest that Cyp3a-mediated metabolite is responsible for relaxation of arterial endothelium (Ayajiki et al., 1999).

Anandamide is a derivative of arachidonic acid that is oxidized by CYP3A4 to generate 5,6-,8,9-,11,12- and 14,15-epoxyeicosatrienoic acid ethanolamide (EET-EA) that can activate cannabinoid receptors (CB1/2). Anandamide and CYP3A metabolites bind CB1 that is responsible for nociception, cognition, memory and orexigenic effects in peripheral and central nervous system (Pratt-Hyatt et al., 2010;Snider et al., 2010). Cyp3a-metabolite activates cannabinoid receptor 2 (CB2) which is responsible for immunosuppression and obesity (Zhang et al., 2016). Similarly, CYP3A metabolizes linoleic acid into an oxidized metabolite which has been associated with nociception via activation of a major class of nociceptors called Transient receptor potential vanilloid 1(TRPV1) cation channel in inflamed dental pulps(Ruparel et al., 2013).Thus, Cyp3a-derived metabolites of unsaturated fatty acids are responsible for specific functions and disruption of this function by chemical inhibitors or genetic impairments may affect fatty acid metabolism, distribution and use (Wojnowski et al., 2006).
CYP3A expression is regulated by pregnane-x-receptor (PXR) and CAR. PXR is a promiscuous nuclear receptor and is activated by environmental pollutants such as methoxychlor and bisphenol-A (Jacobs et al., 2005; Takeshita et al., 2001) and endogenous compounds such as pregnanes, other steroids, and Triol (intermediate in cholesterol metabolism) (Dussault et al., 2003; Kliewer et al., 1997). Recent studies show that activation of mouse PXR increases obesity and metabolic disorders (Spruiell et al., 2014).

HRN-null mice lacking cytochrome P450 oxidoreductase shows complete loss of all CYP activity in the liver, but subsequent treatment with sunflower oil causes increase in Cyp2b10 and Cyp3a11 mRNAs in these mice. Thus, high levels of unsaturated fat induce CYP activity as a protective mechanism from fatty acid toxicity (Finn et al., 2009). Fatty acids induce CYP3A expression in HepG2 cells (Hu et al., 2014). Studies using high fat diet-induced obese guinea pigs (Patoine et al., 2013) and mice (K Yoshinari et al., 2006) show significant decrease in CYP3A. CYP3A expression is increased in both type 1 and type 2 diabetes mouse models (Patoine et al., 2014). Thus, the most predominant hepatic CYP, CYP3A that metabolizes more than 60% of drugs, is induced by dietary fatty acids and, metabolic diseases such as obesity and diabetes modulate CYP3A expression.

In our study, we used a Cyp3a-null mouse model to study the compensatory changes in other CYP gene expression, protein expression and enzyme activity in mice lacking seven Cyp3a genes Cyp3a11, Cyp3a16, Cyp3a25, Cyp3a41, Cyp3a44, Cyp3a57, and Cyp3a59. We also challenged the Cyp3a-null mice with high fat diet for 8-weeks to demonstrate the significance of CYP3A in hepatic lipid metabolism because of its high
expression, role in fatty acid metabolism, and propensity for disrupted activity and expression by anthropogenic chemicals.

1.6 Mouse model

Animal models have been used in obesity research to study the mechanisms and pathways affected during the progression of disease. Rodents are suitable for obesity studies because they are easy to handle, have a relatively short lifecycle, their genetic similarities (and differences) to humans are well studied, and it is easy to perform controlled experiments (Andrade et al., 2006). The development of leptin resistant mice increased their use in obesity research (Zhang et al., 1994). We have used C57Bl/6 (B6) background mice because its genome sequence is available, this is the most common strain of mouse used in biomedical research, and B6 are susceptibility to diet-induced obesity.

Cytochrome P450 null mouse models have been used to study the metabolism and distribution of drugs in toxicology research (Mota et al., 2011; Scheer et al., 2010; Van Herwaarden et al., 2007). We have used three different mouse models in this study; CAR-null, Cyp3a-null, and Cyp2b-null, of which Cyp3a-null and Cyp2b-null were used in our obesity studies. All three models were used in Aim 1 so that we would have a basic understanding of the compensatory changes that take place in the absence of CAR, CYP3A and CYP2B. CAR is a crucial regulator of Cyp3a and Cyp2b expression. Further, we have treated the Cyp3a-null and Cyp2b9/10/13-null mouse models with high fat diet to simulate western diet based type 2 obesity development in human and demonstrate the significance of CYP3A and CYP2B in lipid homeostasis and obesity development.
In this study, we developed novel triple knockout mice missing the hepatic Cyp2b members, Cyp2b9, Cyp2b10 and Cyp2b13 using CRISPR/Cas9 technology. We did not knock out the other two Cyp2b19 and Cyp2b23 because they are located several genes downstream. Neither Cyp2b19 nor Cyp2b23 are significantly expressed in the liver as Cyp2b19 is expressed in keratinocytes while Cyp2b23 is expressed in adolescent mice and have very low hepatic expression (Keeney et al., 1998a; Peng et al., 2012; Renaud et al., 2011). Moreover, there are five development-associated genes that are located in between Cyp2b9/10/13 cluster and Cyp2b19 and Cyp2b23 genes. We confirmed the loss of Cyp2b9, 10 and 13 using PCR in our mouse colony and loss of functional Cyp2b protein expression using western blot. This Cyp2b9/10/13-null mouse model can be used to investigate CYP2B’s role in xenobiotic and endobiotic metabolism. It can also demonstrate if lack of Cyp2b9, Cyp2b10 and Cyp2b13 will increase susceptibility to dyslipidemia, diet-induced obesity and fatty liver disease.

CRISPR/Cas9 is an adaptive immune mechanism in viruses and archae (Mojica et al., 2000). Clustered regularly interspaced short palindromic repeats (CRISPR) are a series of short repeats while Cas 9 is an endonuclease protein that has together been used to genetically engineer cells and model organisms using endogenous DNA repair mechanism. Small guide RNA is designed for target genes, which is composed of 20-nucleotide sequence complementary to target sequence at the 5’ end and double stranded structure at 3’ end to bind Cas 9. The 20-nucleotide sequence can be modified according to target as long as it includes PAM (protospacer adjacent motif). When foreign DNA are integrated into CRISPR loci that are transcribed and processed into short RNA sequences called
(CRISPR RNA) cr RNA. Now crRNA will guide RNA for Cas9 matching DNA and PAM sequences. Thus, crRNA directs cleavage of foreign DNA by Cas9 endonuclease after complementary base pairing with similar sequences to increase specificity of the deletion (Doudna et al., 2014).

1.7 Specific aims

Cyp3a constitutes 40% and Cyp2b constitutes 2-10% of total hepatic CYPs in the human liver (Zanger et al., 2013), and these contribute to the metabolism of most of the xenobiotics such as environmental pollutants (Hernandez et al., 2009a), drugs (Perloff et al., 2000) and endobiotics such as bile acids (Huang et al., 2003), hormones (Waxman et al., 1983) and fatty acids (Bylund et al., 1998). We hypothesize that lack of either CYP3A or CYP2B will increase susceptibility to diet-induced obesity and fatty liver disease: CYP3A and CYP2B contribute to metabolism of unsaturated fatty acids and most of the western diet is composed of unsaturated fats. Recent studies have also indicated altered expression levels of Cyp3a and Cyp2b during the development of metabolic disorders such as obesity, diabetes and fatty liver disease (Blouin R A et al., 1993; Hoek-van den Hill et al., 2014; Leung et al., 2016; Patoine et al., 2014). We will use mice lacking CYP2B or CYP3A genes and treat them with a high fat diet to demonstrate the significance of CYP2B and CYP3A in lipid homeostasis, obesity and fatty liver disease.

**Aim 1: Determine compensatory changes in the detoxification CYPs in CAR-null, Cyp3a-null and Cyp2b9/10/13-null mouse models**

1. Determine whether changes in hepatic CYP gene expression occurs in CAR-null, Cyp3a-null and Cyp2b9/10/13-null mouse models by qPCR.
2. Determine whether these changes in CYP gene expression manifest themselves at the protein and activity level. We will prepare RNA and microsomes from these mouse models and measure CYP expression and activity by qPCR, Western blotting, and testosterone hydroxylase assays.

**Aim 2: Test whether Cyp3a-null are mice are susceptible to HFD-induced obesity and non-alcoholic fatty liver disease (NAFLD)**

1. We will provide wild type (WT) and Cyp3a-null mice a high-fat diet for 8-weeks. Mouse weight, tissue weights, fasting serum glucose, and glucose tolerance will be monitored in each mouse model.

2. We will determine changes in key lipid metabolism genes such as Carnitine palmitoyl transferase 1a and Sterol regulatory binding protein 1 using qPCR. We will also determine compensatory changes in detoxification CYP gene and protein expression after the dietary treatment using qPCR and western blots.

3. We will also determine biochemical changes in hormone levels and hepatic lipids using Enzyme Immuno Assay and mass spectrophotometry respectively.

4. Behavioral studies will be done to determine susceptibility of Cyp3a-null mice to anxiety and fear.
Aim 3: Test whether Cyp2b9/10/13-null are mice are susceptible to HFD-induced obesity and non-alcoholic fatty liver disease (NAFLD)

1. We will provide wild type (WT) and Cyp2b9/10/13-null mice a high-fat diet for 10-weeks. Mouse weight, tissue weights, fasting serum glucose, and glucose tolerance will be monitored in each mouse model.

2. We determine changes in key lipid metabolism genes such as Carnitine palmitoyl transferase 1a and Sterol regulatory binding protein 1 using qPCR. We will also determine compensatory changes in detoxification CYP gene and protein expression after the dietary treatment using qPCR and western blots.

3. We will also determine biochemical changes in hormone levels and serum lipids using Enzyme Immuno Assay and mass spectrophotometry respectively.

4. Behavioral studies will be done to determine susceptibility of Cyp2b9/10/13-null mice to anxiety and fear.
Reference


Perloff, M. D., von Moltke, L. L., Court, M. H., Kotegawa, T., Shader, R. I., & Greenblatt, D. J. (2000). Midazolam and triazolam biotransformation in mouse


Snider, N. T., Walker, V. J., & Hollenberg, P. F. (2010). Oxidation of the endogenous cannabinoid arachidonoyl ethanolamide by the cytochrome P450


CHAPTER TWO

COMPENSATORY CHANGES IN CYP EXPRESSION IN THREE DIFFERENT TOXICOLOGY MOUSE MODELS: CAR-NUL, CYP3A-NUL, AND CYP2B9/10/13-NUL MICE

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2.1 Abstract

Targeted mutant models are common in mechanistic toxicology experiments investigating the absorption, metabolism, distribution, or elimination (ADME) of chemicals from individuals. Key models include those for xenosensing transcription factors and cytochrome P450s (CYP). Here we investigated changes in transcript levels, protein expression, and steroid hydroxylation of several xenobiotic detoxifying CYPs in constitutive androstane receptor (CAR)-null and two CYP-null mouse models that have subfamily members regulated by CAR; the Cyp3a-null and a newly described Cyp2b9/10/13-null mouse model. Compensatory changes in CYP expression that occur in these models may also occur in polymorphic humans, or may complicate interpretation of ADME studies performed using these models. The loss of CAR causes significant changes in several CYPs probably due to loss of CAR-mediated constitutive regulation of these CYPs. Expression and activity changes include significant repression of Cyp2a and Cyp2b members with corresponding drops in 6α- and 16β-testosterone hydroxylase activity. Further, the ratio of 6α-/15α-hydroxylase activity, a biomarker of sexual dimorphism in the liver, indicates masculinization of female CAR-null mice, suggesting a role for CAR in the regulation of sexually dimorphic liver CYP profiles. The loss of Cyp3a causes fewer
changes than CAR. Nevertheless, there are compensatory changes including gender-specific increases in Cyp2a and Cyp2b. Cyp2a and Cyp2b were down-regulated in CAR-null mice, suggesting activation of CAR and potentially PXR following loss of the Cyp3a members. However, the loss of Cyp2b causes few changes in hepatic CYP transcript levels and almost no significant compensatory changes in protein expression or activity with the possible exception of 6α-hydroxylase activity. This lack of a compensatory response in the Cyp2b9/10/13-null mice is probably due to low CYP2B hepatic expression, especially in male mice. Overall, compensatory and regulatory CYP changes followed the order CAR-null > Cyp3a-null > Cyp2b-null mice.

**Keywords:** metabolism, CAR; cytochrome P450, sexually dimorphic, Crispr, Cyp3a, Cyp2b
2.2 Introduction

Nullizygous mouse models have become commonplace in toxicology research (Gaytan et al., 2014; Scheer et al., 2014), especially the use of xenobiotic receptor and Cyp subfamily-null mice (Hernandez et al., 2009a; Mota et al., 2011; Scheer et al., 2010; Van Herwaarden et al., 2007). These models are widely used in the study of the metabolism and distribution of pharmaceuticals and hazardous environmental chemicals (Mota et al., 2011; Scheer et al., 2010; Van Herwaarden et al., 2007). To properly interpret the data observed, especially within absorption, distribution, metabolism, and excretion (ADME) studies, it is critical to have an understanding of the compensatory changes in cytochrome P450 (CYP) expression that occurs in these mouse models. The purpose of this study is in part to evaluate changes that occur in constitutive androstane receptor (CAR)-null, Cyp3a-null, and the newly developed Cyp2b9/10/13-null mouse models, estimate the impact that compensatory changes may have on xenobiotic metabolism, and interpret the basis for these changes.

The constitutive androstane receptor (CAR; NR1I3) is a xenobiotic sensor activated either directly by ligand binding such as 1,4-bis [2-(3,5-dichloropyridoxy)] benzene (TCPOBOP) (Tzameli et al., 2000) or indirectly in which the chemical of interest induces nuclear translocation through changes in phosphorylation status such as phenobarbital (Honkakoski et al., 1998; Mutoh et al., 2013; Sueyoshi et al., 2008). Modulators of CAR activity include environmental pollutants, pharmaceuticals, natural products, and endogenous chemicals such as steroids, bile acids, and fatty acids (Baldwin et al., 2009; Finn et al., 2009; Hernandez et al., 2009a; Tien et al., 2006). CAR activation leads to
increased transcription of genes involved in phase I-III detoxication, including the cytochrome P450s (CYP) with greater CYP2B6 induction than CYP3A4 or CYP2C9 induction (Kretschmer et al., 2005; Wilson et al., 1998). We have observed compensatory changes in CYP expression in CAR-null mice on the B6/SV129 background (Hernandez et al., 2009b). Here we take a more comprehensive look at compensatory changes in CAR-null mice, but on the B6 background.

CYP3A is the most predominant CYP in the liver encompassing 30-40% of the total hepatic CYP content and metabolizing more than 60% of the drugs available on the market (Maurel, 1996). In addition to the metabolism of numerous pharmaceuticals and environmental pollutants (Hernandez et al., 2009a; Perloff et al., 2000), CYP3A metabolizes endogenous molecules such as lithocholic acid (Staudinger et al., 2001), arachidonic acid (Bylund et al., 1998) and steroid hormones (Waxman, 1988). Recently, knocking out Cyp3a was shown to increase Cyp2c-mediated metabolism of midazolam (Van Waterschoot et al., 2008), potentially due to activation of the pregnane X receptor (PXR). Because Cyp3a is the predominant hepatic CYP and of such importance in toxicology, it is likely that loss causes compensatory mechanisms that alter the metabolism of endogenous and exogenous substances. In some cases these alterations may not be (at least in part) due to loss of Cyp3a, but instead increases in the production of other metabolites produced through the induction of CYPs in subfamilies 2a, 2b, and 2c.

There are currently two different Cyp3a knockout mouse models; not including humanized models. In the model produced by Van Herwaarden et al (Van Herwaarden et al., 2007) on an FVB background, the Cyp3a members clustered in a 0.8Mb region of
chromosome 5 were eliminated by Cre-lox, while Cyp3a13 located 7 Mb centromeric to the cluster was deleted by traditional targeting methods (Van Herwaarden et al., 2007). Another model produced on the C57Bl6 (B6) background, eliminated all of the Cyp3a members on the chromosome 5 cluster by Cre-lox, but did not eliminate Cyp3a13 (Hasegawa et al., 2011; Scheer et al., 2010). For the purposes of the present study this model is more attractive, because responses in this mouse can be compared to other mouse strains on the B6 background.

CYP2B is probably the least studied of the hepatic detoxication CYPs in families 1-3 because it was traditionally considered to have <1% of total hepatic CYP expression and in turn was called the overlooked or forgotten CYP (Wang et al., 2008). However, recent studies using more sensitive probes and inhibitors have shown that CYP2B6 constitutes 2-10% of the total CYP expressed in the liver. It is estimated that CYP2B6 metabolizes approximately 25% of drugs available on the market (Wang et al., 2008) such as efavirenz (Anakk et al., 2003), bupropion (Faucette et al., 2000) and cyclophosphamamide (Xie et al., 2003). In addition, CYP2B metabolizes environmental pollutants such as nonylphenol (Lee et al., 1998), parathion (Foxenberg et al., 2007) and polychlorinated biphenyls (Warner et al., 2009), and endogenous molecules such as testosterone (Mo et al., 2009), arachidonic acid (Capdevila et al., 1990; Keeney et al., 1998), linoleic acid (Finn et al., 2009) and epoxyeicosatrienoic acid (Du et al., 2005).

We produce and describe the first exclusive Cyp2b-null mouse model in this manuscript. There are other models that lack Cyp2b or Cyp2b activity. These include P450 oxidoreductase-null mice (HRN or POR-null) that lack all CYP activity because they lack
this crucial cofactor (Gu et al., 2003; Henderson et al., 2003), and the Cyp2a(4/5)bgs-null mouse model that lacks a 1.2 megabase region of chromosome 7 containing Cyp2a4, 2a5, 2b9, 2b10, 2b13, 2b19, 2b23, 2g1, 2s1, Nalp9a, Nalp9c, Nalp4a, Vmn1r185, and Vmn14184 (Wei et al., 2013). This mouse model lacks all of the Cyp2b members, but also lacks other Cyp2 members (2a4, 2a5, 2g1, 2s1) as well as five non-CYP genes found between the two Cyp clusters on chromosome 7 (Damiri et al., 2012). Our new mouse model lacks three of the five Cyp2b members, 2b9, 2b10, and 2b13, which are the primary hepatic Cyp2b’s found in tandem repeat (Damiri et al., 2012; Jarukamjorn et al., 2001; Peng et al., 2012). There are six genes between the Cyp2b9/10/13 cluster and Cyp2b19 and Cyp2b23 (S1 Fig). We did not delete Cyp2b19, which is primarily expressed in skin (Du et al., 2005) and testes (Renaud et al., 1995), or Cyp2b23, which until recently was not known to be expressed (Renaud et al., 1995). Recent work suggests Cyp2b23 is expressed briefly at very low levels in the livers of young mice (Cui et al., 2012; Peng et al., 2012).

Many of the xenobiotic detoxifying CYPs are expressed in a sexually dimorphic manner (Hernandez et al., 2006; Park et al., 1999; Wiwi et al., 2004a). Murine male predominant hepatic CYPs include Cyp2d9 and 4a12 (Noshiro et al., 1986; Wiwi et al., 2004a), which are not regulated by CAR. Murine female predominant hepatic CYPs include Cyps 2a4, 2b9, 3a41, 2c40 and 3a44 (Burkhart et al., 1985; Hernandez et al., 2006; Jarukamjorn et al., 2002; Noshiro et al., 1986; Wiwi et al., 2004a), of which several are regulated by CAR (Hernandez et al., 2009b). Furthermore, CAR demonstrates greater expression (Petrick et al., 2007) and activity in females than males (Ledda-Columbano et al., 2003). This may be due to increased regulation of CAR by hepatocyte nuclear factor
4α (HNF4α) in females (Kamiyama et al., 2007), estrogen activation of CAR (Kawamoto et al., 2000), androgen inhibition of CAR (Baldwin et al., 2009; Forman et al., 1998), or a combination of these factors. Taken together, this data indicates that CAR has greater activity in female mice and therefore maintains basal expression of several CYPs in a sexually dimorphic fashion. Therefore, changes in the basal expression of several CYPs, including several female predominant CYPs will be compared between wild-type and CAR/CYP-null mice.

The overall purpose of this manuscript is to compare and evaluate changes in CYP gene expression, protein expression, and enzyme activity in three toxicology knockout models; CAR, Cyp3a-null, and Cyp2b9/10/13-null mice. CAR is a key regulator of Cyp2b and Cyp3a expression and therefore these CYP models are reasonable models to investigate in order to discern how they are altered in comparison to CAR-null mice.

2.3 Materials and methods

2.3.1. 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 or Clemson University Animal Care and Use Committee. Mice are on a C57/Bl6 (B6) background, provided water and food ad libitum, and between 8-11 weeks old at the time of euthanasia. Mice were euthanized by carbon dioxide asphyxiation followed by bilateral thoracotomy. While each mouse is on a B6-background, each B6 mouse is from a different source. CAR-null mice (Dong et al., 2009; Tzameli et al., 2000) and their B6 wild-type (WT) controls
(B6) were housed at Baylor College of Medicine (BCM) on the B6 background bred at BCM. Cyp3a-null mice (Scheer et al., 2010) and their respective B6-WT controls were obtained from Taconic (Hudson, NY USA). These mice lack the Cyp3a57, Cyp3a16, Cyp3a41, Cyp3a44, Cyp3a11, Cyp3a25, and Cyp3a59 genes all located within approximately 0.8Mb of each other in a tandem repeat region on chromosome 5, but still contain the Cyp3a13 gene located 7 Mb centromeric from the cluster on chromosome 5 (Scheer et al., 2010).

Cyp2b9/10/13-null mice were produced using the Crispr/Cas9 system. Corresponding WT controls (B6) were obtained from The Jackson Laboratory (Bar Harbor, ME) and used to produce the Cyp2b9/10/13-null mice. Cyp2b9, Cyp2b10, and Cyp2b13 are the three Cyp2b members primarily expressed in the liver (Cyp2b9/10/13) and found in tandem repeat (Appendix A-1)(Damiri et al., 2012; Jarukamjorn et al., 2001; Peng et al., 2012). Each of the three hepatic genes was targeted (Fig. 2.1). Cas9 mRNA from Streptococcus pyogenes and a 20nt guide sequence that was specific to the target site with an 83nt scaffold sequence, which was common to all the sgRNAs was injected into the cytoplasm of the mouse blastocyst (Horii et al., 2014). The scaffold sequence was guuuuagagcuagaauagcaaguuuaagguuccguauucaacuugaaaguggcaccggucggugc uuuuuuu. The Cyp2b10 guide sequence was: uuggaggacggauacagg(AGG). The Cyp2b13 guide sequence was: (CCC)ugcaagaguucccaagag, and the Cyp2b9 guide sequence was: acattgatacctaccttcttg(AGG). The protospacer adjacent motif (PAM) is shown in parenthesis. The incorporation efficiency at each site in vitro was Cyp2b10, 47.6%, Cyp2b13, 33.3%, and Cyp2b9, 33.3%. The resultant injection of more than 100
embryos produced two mice with a 287kB deletion lacking all three hepatic Cyp2b members found in tandem repeat. Each mouse was genotyped to ensure the presence of the knockout using the F2/R2 primer set (F2: 5’-gcacagacctaggctggtctgt-3’ and R2: 5’-gcacagacctaggctggtctgt-3’) that produces a 1066 bp band in triple-gene knockout mice. To ensure we are not working with heterozygotes, we also genotyped for the presence of Cyp2b13 (F: 5’-cagactctgttagacggaccc-3’ and R: 5’-ccccaaggaataaatcactc-3’) (Fig. 2.1).
Fig. 2.1: Construction of Cyp2b9/10/13-null mice. (A) Cyp2b9/10/13-null mice were produced using Crispr/Cas9 with sgRNA target sites for all three genes. A 287 kb deletion mutant was produced that lacks these three Cyp2b genes found in tandem repeat on chromosome 7. (B) Two mice heterozygote for a chromosome deletion lacking the three Cyp2b genes (Cyp2b10, 2b13, 2b9) in tandem repeat were produced. PCR confirmation of the 287kb deletion from the first null mice produced is shown in lanes 4 and 21 using the F2/R2 primer combination that produces a 1066 bp fragment. (C) Subsequent breeding produced mice lacking Cyp2b9/10/13 as demonstrated by the presence of the 1066 bp PCR product. (D) Heterozygotes were discerned from homozygotes by PCR of Cyp2b13 indicating a heterozygote.

2.3.2. Sample preparation

Eight to eleven-week old B6, CAR-null, and Cyp2b9/10/13-null male and female mice (n = 4-6) were euthanized by CO₂ asphyxiation. Livers were excised and diced into several pieces and snap frozen for RNA extraction or microsome preparation and then stored at -80°C. Eight to ten-week old Cyp3a-null and corresponding B6 controls from
Taconic were euthanized at Taconic Biosciences (Hudson, NY USA), the livers were snap frozen and shipped on dry ice to Clemson. RNA was extracted from a little less than half of the liver using the Bio-Rad spin columns with DNAse (Bio-Rad, Hercules, CA USA) according to the manufacturer’s instructions. RNA concentrations were determined spectrophotometrically at 260/280 nm (Molecular Devices, Ramsey, MN USA). Reverse transcription was performed to make cDNA using 200 units MMLV-RT, a 10 mM dNTP mixture, and 0.05 mg random hexamers (Promega Corporation, Madison, WI USA). 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 et al., 1974). Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad) according to the manufacturer’s instructions.

2.3.3. 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 qPCR primers were previously published (Damiri et al., 2012; Hernandez et al., 2006). Samples were diluted 1:10 and amplifications of the standard curve performed in triplicate using a 96-well IQ™ Real-Time PCR detection system (Bio-Rad) with 0.25X RT² SybrGreen (Qiagen Frederick, MD USA) as the fluorescent double strand intercalating dye to quantify gene expression as described previously using Muller’s equation to determine relative quantities of each CYP (Hernandez et al., 2007; Muller et al., 2002).
Western Blots

Western Blots were performed on 30 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) where the blot was blocked using 1% skim milk/0.1% 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. Rabbit anti-mouse Cyp2b10 antibody was produced by our laboratory and previously characterized (Mota et al., 2011; Mota et al., 2010). Rabbit anti-rat CYP3A1 and rabbit anti-human CYP2C8/9/19 were obtained from Chemicon International (Temecula, CA USA). Mouse anti-human CYP2A6 was originally obtained from Gentest™ Corporation (San Jose, CA USA) and used with the CAR-null and Cyp3a-null mice. CYP2A6 antibody was later obtained from Thermo-Fisher (Rockford, IL) for use with the Cyp2b9/10/13-null mice when the original stock from Gentest was no longer available. Rabbit anti-mouse β-actin (Sigma Aldrich, St. Louis, MO USA) was used to ensure equal loading of samples. Goat anti-rabbit IgG (Bio-Rad) alkaline-phosphatase coupled secondary antibodies were used for recognizing CYP2A6, Cyp2b10, CYP3A1, and CYP2C8/9/19 primary antibodies. Goat anti-mouse (Bio-Rad) IgG were used to recognize the β-Actin primary antibodies. Primary antibodies were diluted 1:1000, and secondary antibodies were diluted 1:500. Bands were visualized using a chemiluminescent kit according to the manufacturer’s directions (Bio-rad). Chemiluminescence was quantified on a Chemi-Doc system with Quantity One software (Bio-Rad). Western blot results regarding specific CYP protein data are referred to as
subfamilies (i.e. CYP3A) instead of a specific protein because the antibodies most likely recognize several different subfamily members (Acevedo et al., 2005; Damiri et al., 2012).

2.3.5. Testosterone hydroxylase assays

Testosterone hydroxylase assays were used to measure CYP activity as previously described (Hernandez et al., 2006). [4-14C]Testosterone (Perkin-Elmer, Waltham, MA) was used to visualize testosterone metabolites separated by thin-layer chromatography and quantify. Testosterone metabolites with a LS5801 liquid scintillation counter (Beckman, Fullerton, CA USA).

2.3.6. Microarrays

There were 3 or 4 biological replicates used for each of the genotype-sex groups (GSE90614). Liver RNA was isolated by mechanical disruption followed by RNAzol and was further purified using silica membrane spin columns (RNeasy®, Qiagen, Valencia, CA). RNA integrity was assessed by the RNA 6000 LabChip® kit using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Gene expression in the livers of the mice was evaluated using Affymetrix mouse 430PM arrays. Procedures for labeling, hybridization, washing and scanning were carried out according to the manufacturer's recommendations. Gene expression results were analyzed in Partek Genomic Suite by standard methods. Briefly, .cel files were imported and normalized by Robust Multichip Average (RMA). Differentially expressed genes (DEGs) were determined by ANOVA with a false discovery rate of 0.05, and a fold change cutoff of +/- 1.2 fold.
2.3.7. Assembly of Cyp expression data from microarray studies carried out in CAR-null mice

Comparisons of publicly available gene expression profiles were conducted using the meta-analysis function of the Illumina BaseSpace Correlation Engine. The meta-analysis function allows for specified gene expression profiles (called “biosets” within Correlation engine) to be examined for gene expression changes. We compared biosets from CAR-null mice (GSE40120) and from 28 and 91-day old CAR/PXR-null mice (GSE60684), which most closely approximate the ages of the mice used in this study, and then filtered gene expression data to investigate changes in expression of Cyp family members.

2.3.8. Statistical Analysis

Statistical tests were performed with GraphPad Prism software 6.0 (La Jolla, CA USA). ANOVA was used to compare three or more treatment groups followed by Fisher’s PLSD as the post-hoc test, and a p-value of ≤ 0.05 was regarded as significantly different from control values.

2.4 Results and discussion

2.4.1. CAR-null mice

CAR regulates the expression of Cyp2a, Cyp2b, and Cyp3a subfamily members (Hernandez et al., 2009a). We examined the expression of these subfamily members in part based on the work of ourselves and others that indicates HNF4α regulates CAR expression with HNF4α > CAR > PXR regulation of constitutive Cyp expression (Hernandez et al., 2009b; Wortham et al., 2007). Thus, we examined the expression of
CYPs previously shown to be constitutively regulated by HNF4α (Wiwi et al., 2004a) and hypothesized that CAR-null mice would show changes in constitutive CYP gene expression, corresponding protein expression and enzyme activity. HNF4α and to a lesser extent CAR are crucial transcription factors in the sexually dimorphic expression of hepatic CYPs, including Cyp2a4, Cyp2b9, Cyp2b10, Cyp2b13, Cyp3a41, and Cyp3a44 (Hernandez et al., 2009b; Wiwi et al., 2004a). CAR-null female mice show significant down-regulation of Cyp2b9, Cyp2b10, Cyp2b13, and Cyp3a11 compared to WT-B6 mice. Interestingly, Cyp3a11 showed slight female predominance, about 1.7X, in two of our three studies in the WT (B6) mice (Tables 2.1-2.3). Previous work with B6/SV129 mice indicated that Cyp3a11 is female predominant (Hernandez et al., 2009b); however studies with FVB/NJ mice indicate that Cyp3a11 expression is gender neutral (Hernandez et al., 2006).

CAR-null females show nearly a complete loss of Cyp2b13 and 6- and 19-fold decrease in Cyp2b9 and Cyp2b10, respectively. CAR-null males show a similar trend with respect to these Cyp2b members, although the data were not significant (Table 2.1), most likely because most Cyp2b members show lower expression in males (Hernandez et al., 2009a). Cyp2c40 expression decreased nearly 2-fold in CAR-null female mice, but these mice still showed higher expression of this female predominant CYP than male mice. Of the female predominant CYPs examined only Cyp2a4 showed increased expression in CAR-null mice (Table 2.1). This result suggests that CAR negatively regulates Cyp2a4 expression. Results from HNF4a-null mice indicate that Cyp2a4 is also negatively regulated by HNF4α (Wiwi et al., 2004a). Given HNF4α’s role in regulating CAR
it is possible that HNF4α in part regulates Cyp2a4 by regulating CAR expression and activity.

**Table 2.1:** Compensatory changes in CYP gene expression in CAR-null mice.

<table>
<thead>
<tr>
<th>Gene</th>
<th>MALES</th>
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<th>FEMALES</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>CAR-null</td>
<td></td>
<td>WT</td>
<td>CAR-null</td>
<td></td>
</tr>
<tr>
<td>Cyp2a4</td>
<td>1.00 ± 0.241</td>
<td>5.276 ± 0.904</td>
<td>13.983 ± 0.762</td>
<td>15.241 ± 3.940</td>
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<tr>
<td>Cyp2b9</td>
<td>1.00 ± 0.353</td>
<td>0.424 ± 0.096</td>
<td>18.35 ± 2.896</td>
<td>0.151 ± 0.063</td>
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<tr>
<td>Cyp2b10</td>
<td>1.00 ± 0.692</td>
<td>0.196 ± 0.027</td>
<td>10.186 ± 2.249</td>
<td>0.054 ± 0.011</td>
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<tr>
<td>Cyp2b13</td>
<td>1.00 ± 0.312</td>
<td>0.021 ± 0.006</td>
<td>4.139 ± 0.798</td>
<td>0.009 ± 0.004</td>
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<tr>
<td>Cyp2c29</td>
<td>1.00 ± 0.251</td>
<td>0.395 ± 0.388</td>
<td>0.306 ± 0.091</td>
<td>0.188 ± 0.082</td>
<td></td>
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<tr>
<td>Cyp2c40</td>
<td>1.00 ± 0.950</td>
<td>0.805 ± 0.753</td>
<td>6.992 ± 0.551</td>
<td>3.174 ± 0.936</td>
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<tr>
<td>Cyp3a11</td>
<td>1.00 ± 0.341</td>
<td>0.461 ± 0.085</td>
<td>1.805 ± 0.283</td>
<td>0.904 ± 0.176</td>
<td></td>
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</tr>
<tr>
<td>Cyp3a41</td>
<td>1.00 ± 0.475</td>
<td>430.0 ± 405.8</td>
<td>81.01 ± 33.43</td>
<td>442.0 ± 178.9</td>
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</tr>
</tbody>
</table>

Data are presented as relative mean ± SEM. Statistical significance determined by ANOVA followed by Fisher’s LSD as the post-hoc test (n=5-6). ‘b’ indicates WT females different than CAR-null females ‘c’ indicates WT males different than WT females Letter with no asterisk indicates a p-value < 0.05

Protein expression and testosterone hydroxylase activity generally corresponded well to the qPCR results. Cyp2b9, 2b10, and 2b13 were all repressed in CAR-null female mice, CYP2B protein concentrations were significantly reduced as determined by Western blots (Fig. 2.2) and 16α- and 16β-hydroxylase activities (Fig. 2.3), both of which are associated with CYP2B or CYP2B induction were repressed as expected (Acevedo et al., 2005; Waxman, 1988). In addition, Cyp3a mRNA expression, CYP3A protein expression, and 6β-hydroxylase activity were all higher in WT females than WT males (Table 2.1; Fig. 2.2 and 2.3). However, 6β-hydroxylase activity in CAR-null males compared to WT
males were not consistent with protein expression, but comparable to the qPCR data. In addition, CYP2A protein was significantly down-regulated in CAR-null females (Fig. 2.2), while Cyp2a4 mRNA was increased (Table 2.1). There was no significant difference in the Cyp2a-mediated, 15α-hydroxylase activity between WT and the corresponding CAR-null mice of the same sex. Overall, these minor discrepancies may be due to preferential antibody recognition of specific Cyp3a or 2a subfamily members such as Cyp3a13, 41, and 44, or Cyp2a5.

![Western blots of male and female CAR-null mice showing significant changes in CYP expression relative to their WT counterparts. Results are expressed as relative mean of the WT compared to CAR-null mice of the same sex. Statistical differences were determined by Student’s t-tests (n = 2) with * (p < 0.05) ** (p < 0.01) indicating significant differences.](image)

**Fig. 2.2: Compensatory changes in CYP protein expression in CAR-null mice.** Western blots of male and female CAR-null mice show significant changes in CYP expression relative to their WT counterparts. Results are expressed as relative mean of the WT compared to CAR-null mice of the same sex. Statistical differences were determined by Student’s t-tests (n = 2) with * (p < 0.05) ** (p < 0.01) indicating significant differences.
Fig. 2.3: Testosterone hydroxylation is perturbed in CAR-null mice in a gender-specific manner. (A) Testosterone hydroxylation was determined in male and female WT and CAR-null mice as described in the Materials and Methods. Data are presented as mean specific activity (μmol/min/mg protein) ± SEM (n = 5). (B) Ratio of 6α/15α-hydroxytestosterone as a biomarker of CYP sexual dimorphism in the liver. An a indicates a significant difference between WT male and CAR-null male mice, b indicates a significant difference between WT female and CAR-null female mice, c indicates a significant difference between male and female WT mice and d indicates a significant difference between the male and female CAR-null mice. Statistical differences were determined by two-way
ANOVA followed by Fisher’s LSD as the post-hoc test in (A) and one-way ANOVA followed by Fisher’s LSD in (B). A letter without an asterisk indicates a significance of p < 0.05, asterisk indicate significance of *p<0.01, **p<0.001, and *** p<0.0001, respectively.

Sexual dimorphism is observed in WT mice in the production of 6β-, 6α-, and 16α-OH testosterone (Fig. 2.3). Sexual dimorphic differences in testosterone hydroxylation are also clear in the CAR-null mice. For example, we observed significant induction of 15α-OH testosterone in CAR-null females, and a drop in 6β-OH testosterone production in CAR-null males compared to WT males (Fig. 2.3). Therefore, we examined the 6α/15α-OH testosterone ratio, which is much greater in females than males, controlled by androgen status, and considered a biomarker of androgen disruption in mice (Wilson et al., 1999). The 6α/15α-OH testosterone ratio is 3.2-fold higher in WT females than WT males. However, 6α/15α-OH testosterone ratio is 5.1-fold higher in WT females than CAR-null females and in turn the 6α/15α-OH testosterone ratio is 1.2-fold higher in CAR-null males than CAR-null females; the opposite direction of what is expected. Thus, the CAR-null females have a lower 6α/15α-OH testosterone ratio than WT males because of masculinization of CYP profiles in the liver in CAR-null females. However, the CAR-null females show no differences in liver concentrations of testosterone (Appendix A-2).

Overall, the masculinization of hepatic testosterone metabolism profiles reflects the systematic loss of female predominant CYPs in the CAR-null mice (Table 2.1). CAR may regulate sexual dimorphism in the liver in mice through androgen inhibition in males as several different androgens are CAR inverse agonists (Forman et al., 1998), including androgens used as performance enhancing drugs (Baldwin et al., 2009). Interestingly,
PXR-null mice also show sexual dimorphic effects by promoting estrogenic activity due to the loss of sulfotransferase-mediated estrogen metabolism through activated PXR (Kodama et al., 2011; Wang et al., 2014). However, more likely sexually dimorphic differences or loss of sexual dimorphism is directly due to the loss of CAR and its role in regulating female predominant CYPs in conjunction with HNF4α (Hernandez et al., 2009b; Wiwi et al., 2004a; Wortham et al., 2007).

Sexual dimorphism of hepatic CYPs is primarily regulated by the periodization of growth hormone release that regulates Stat5b (Choi, 2000; Oshida et al., 2016; Park et al., 1999; Wiwi et al., 2005). Other transcription factors regulated in a sexually dimorphic fashion that in turn regulate sexually dimorphism include HNF4α, FoxA2, and CAR (Hashita et al., 2008; Hernandez et al., 2009b; Wiwi et al., 2004a; Wiwi et al., 2004b). For example, FoxA2 promotes the expression of the female specific hepatic CYP, Cyp2b9; HNF4α positively regulates the expression of several CYPs including Cyp2b10, Cyp2b13, Cyp3a41, and Cyp3a44 in females and negatively regulates Cyp2b9 and Cyp2a4 in males (Wiwi et al., 2004a); and CAR is thought to positively regulate Cyp2b13, Cyp2c29, and potentially Cyp2b10 (Hernandez et al., 2009b). Some of CAR’s sexually dimorphic activity may be direct, but some may also be due to HNF4α’s regulation of CAR (Wortham et al., 2007). Independent studies by Baldwin’s (Hernandez et al., 2006; Hernandez et al., 2009b) and Corton’s laboratories (Oshida et al., 2016) have shown chemical activation of CAR can induce feminization of the liver. Taken together, masculinization of the liver does not necessarily involve testosterone.
The presence of microarray data from CAR-null mice in GEO allowed us to perform comparisons between our data and female CAR-null and WT mice (GSE40120), and male CAR/PXR double-null and WT mice (Luisier et al., 2014)(GSE60684) from previous studies. In all comparisons, the expression of the Car gene was suppressed (~3-14-fold) in the null mice, as expected. Cyp genes increased in expression included Cyp2a5, Cyp2c38, Cyp2c39, Cyp2g1, Cyp4a14, Cyp51, and Cyp7a1. Cyp genes decreased in either CAR-null or CAR/PXR-null mice included Cyp2a12, Cyp2b10, Cyp2b9, Cyp2e29, Cyp2c37, Cyp2c50, Cyp2c54, Cyp2c70, Cyp2u1, Cyp4a12a, Cyp4v3, and Cyp7b1. Cyp2c55 was increased in male CAR-null mice and suppressed in female CAR/PXR-null mice. Genes in the Cyp2c subfamily were differentially expressed in both directions. This may account for discrepancies between the qPCR and Western blots for Cyp2c, and it is also possible that a drop in 16α-hydroxylase activity is in part due to a drop in Cyp2c expression or Cyp2b expression.

Overall, CAR is a regulator of the hepatic Cyp2b genes and loss of CAR causes a considerable drop in Cyp2b expression. Furthermore, CAR may regulate sexual dimorphism in the liver as loss of CAR activity decreases the 6α/15α-OH testosterone ratio, a biomarker of masculinization of the liver (Hernandez et al., 2009b;Wilson et al., 1999) that may also reflect an overall drop in CYP activity (Wortham et al., 2007). CAR expression is female predominant emphasizing the need to include both genders in drug trials and toxicant biotransformation studies (Meibohm et al., 2002;Petrick et al., 2007). This is important as it provides data that may help us inform translational studies or
physicians when prescribing personalized medicines. It may also reveal the mechanism behind specific chemical sensitivities in patients with low CAR or CYP activities.

2.4.2. Cyp3a-null mice

CYP3A accounts for 30-40% of hepatic CYP expression (Maurel, 1996). We hypothesized that the loss of seven Cyp3a genes would perturb the hepatic P450 profile and lead to compensatory changes. One of the compensatory changes observed is an increase in Cyp3a13 expression (Table 2.2). Cyp3a13 is the only Cyp3a member not deleted, as it is 7Mb upstream from the 7-Cyp3a gene cluster (Scheer et al., 2010). Cyp3a13 is almost certainly the basis for recognition of CYP3A in Western blots (Fig. 2.4) and the observation that 6β-hydroxylase activity only decreased 67% in males and 74% in females. Although other CYPs also produce 6β-hydroxytestosterone (Shou et al., 2000), data indicates that approximately 90% of 6β-hydroxytestosterone is produced by CYP3A enzymes in humans (Gelboin et al., 1995; Shou et al., 2000).

Table 2: Compensatory changes in CYP gene expression in Cyp3a-null mice.

<table>
<thead>
<tr>
<th>Gene</th>
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<th>FEMALES</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Cyp3a-null</td>
<td>WT</td>
<td>Cyp3a-null</td>
</tr>
<tr>
<td>Cyp2a4</td>
<td>1.00 ± 0.328</td>
<td>47.957 ± 16.267&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.326 ± 0.708</td>
<td>70.06 ± 20.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyp2b9</td>
<td>1.00 ± 0.321</td>
<td>7.743 ± 7.319</td>
<td>117.96 ± 7.899&lt;sup&gt;***&lt;/sup&gt;</td>
<td>115.134 ± 20.868&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>1.00 ± 0.310</td>
<td>6.247 ± 4.831</td>
<td>9.7 ± 1.447&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.435 ± 2.435</td>
</tr>
<tr>
<td>Cyp2c29</td>
<td>1.00 ± 0.457</td>
<td>29.431 ± 15.874</td>
<td>5.193 ± 2.213</td>
<td>23.586 ± 7.049</td>
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<tr>
<td>Cyp2c40</td>
<td>1.00 ± 0.413</td>
<td>25.913 ± 15.709</td>
<td>21.722 ± 12.568</td>
<td>38.772 ± 4.732</td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>1.00 ± 0.168</td>
<td>0.000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.68 ± 0.336&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyp3a13</td>
<td>1.00 ± 0.371</td>
<td>26.143 ± 9.424&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.294 ± 1.12</td>
<td>28.882 ± 8.366&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyp3a25</td>
<td>1.00 ± 0.247</td>
<td>0.002 ± 0.001&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.75 ± 0.121</td>
<td>0.001 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyp3a41</td>
<td>1.00 ± 0.131</td>
<td>0.000</td>
<td>14.138 ± 3.967&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data represented as mean +/- SEM (n = 4). Statistical significance determined by one-way ANOVA followed by LSD as the post-hoc test.
‘a’ indicates WT males different than Cyp3a-null males
‘b’ indicates WT females different than Cyp3a-null females

54
‘c’ indicates WT males different than WT females
‘d’ indicates Cyp3a-null males different than Cyp3a-null females
Letter with no asterisk indicates a p-value < 0.05 and * indicates a p-value < 0.01, ** indicates a p-value < 0.001, *** indicates a p-value < 0.00001

Other CYPs also went through compensatory changes in the Cyp3a-null mice. qPCR data demonstrates significant induction (48- and 70-fold) of Cyp2a4 in Cyp3a-null female and male mice, respectively compared to their corresponding WT counterparts (Table 2.2). Western blots confirm the increase in CYP2A in females but not males (Fig. 2.4). Cyp2a4 up-regulation is similar to the observations made in CAR-null mice, suggesting either a drop in CAR activity in Cyp3a-null mice or more likely an increase in PXR activity. CAR and PXR crosstalk and there is weak but insignificant induction of several other CAR/PXR regulated CYPs (Hernandez et al., 2009b; Mota et al., 2011; Xie et al., 2000) including Cyp2b10, Cyp2c29, and Cyp2c40 (Table 2.2) with increased protein levels of CYP2B (Fig. 2.4). Therefore, we consider it more likely that PXR activity is increased potentially due to a lack of metabolism of a CYP3A metabolized endobiotic such as bile acids that in turn activate PXR (Hashimoto et al., 2013; Je et al., 2015; Staudinger et al., 2001).
Fig. 2.4: Compensatory changes in CYP protein expression in Cyp3a-null mice. Western blots of male and female Cyp3a-null mice show significant changes in CYP expression relative to their WT counterparts. Results are expressed as relative mean of the WT compared to Cyp3a-null mice of the same sex. Statistical differences were determined by Student’s t-tests (n = 3) with * (p < 0.05) ** (p < 0.01) *** (p < 0.001) indicating significant differences.

Testosterone hydroxylase activity was greatly diminished at the 6β-position as expected because of the loss of CYP3A. Few other testosterone hydroxylase activities are perturbed significantly with the exception of 2α-hydroxylase activity (Fig. 2.5). Hydroxylation of testosterone in the 2α-position is primarily considered a product of CYP2C. An increase in CYP2C protein was not measured; however, Cyp2c members were
up to 29-fold higher by qPCR. This may be due to the CYP2C antibody preferentially recognizing CYP2C members not induced in the Cyp3a-null mouse model. CYP2C is increased in Cyp3a-null mice exposed to the PXR activator midazolam (Van Waterschoot et al., 2008; Vrzal et al., 2010), but CYP2C induction may be muted or lacking in untreated Cyp3a-null mice. Another possibility is that there are interactions between the hydroxylated products of testosterone. For example, 2α-hydroxylation of testosterone may be inhibited by 6β-hydroxytestosterone, and in turn the loss of CYP3A activity allows for increased 2α-hydroxylation. Ultimately, the data suggests the potential for compensatory CYP activity in the Cyp3a-null model complicating the interpretation of xenobiotic metabolism data. This observation is supported by previous work (Van Waterschoot et al., 2008; Vrzal et al., 2010).
Fig. 2.5: Changes in testosterone hydroxylation in Cyp3a-null mice. (A) Testosterone hydroxylation was determined in male and female WT and Cyp3a-null mice as described in the Materials and Methods. Data are presented as mean specific activity (μmol/min/mg protein) ± SEM (n = 4). (B) Ratio of 6α/15α-hydroxytestosterone as a biomarker of CYP sexual dimorphism in the liver. An a indicates a significant difference between WT male and Cyp3a-null male mice, b indicates a significant difference between WT female and Cyp3a-null female mice, c indicates a significant difference between male and female WT mice and d indicates a significant difference between the male and female Cyp3a-null mice. Statistical differences were
6α- and 15α-hydroxylation were increased in Cyp3a-knockout mice, but not significantly despite a significant increase in Cyp2a4 mRNA and CYP2A protein in Cyp3a-null female mice compared to WT female mice (Fig. 2.4; Table 2.2). These hydroxylase activities are mediated by sexually dimorphic CYP2A members in mice (Baldwin et al., 1992; Burkhart et al., 1985). Therefore, we examined the 6α/15α ratio. The 6α/15α-OH testosterone ratio is 2.03-fold higher in WT females than WT males; however, the ratio drops to 1.29 when comparing Cyp3a-null females to Cyp3a-null males (not significantly different). The changes are not nearly as large as observed in the CAR-null mice, but the loss of significant sexual dimorphism suggests the potential for a small to moderate increase in hepatic masculinization of the females coupled with a small amount of feminization of the males. No differences in hepatic testosterone were observed (Appendix A-2).

CYP3A metabolizes 50-60% of drugs available in the market (Maurel, 1996) and is inhibited by fatty liver disease (Patoine et al., 2013) and diabetes mellitus (Dostalek et al., 2011). Because CYP3A plays such a prominent role in drug metabolism, changes in CYP3A expression and activity are crucial during the development of pharmaceuticals. Overall, the loss of CYP3A caused minor changes in the expression of the other CYPs examined with minimal changes in activity at least under untreated (pristine) conditions. There is minimal masculinization of liver testosterone hydroxylase activities; however,
these changes are not as strong as observed in CAR-null mice. Taken together, Cyp3a-null mice may show compensatory metabolism of drugs by other CYPs that may compound the interpretation of the metabolism data; however, most of the lost CYP activity is directly attributable to the loss of CYP3A members (Fig. 2.5).

2.4.3. Cyp2b9/10/13-null mice

CYP2B metabolizes approximately 25% of drugs available in the market despite making up only 5-10% of the total CYPs expressed in the human liver (Wang et al., 2008). We hypothesized that the lack of Cyp2b9, Cyp2b10 and Cy2b13 will lead to compensatory changes by altering CYP expression levels in the liver. In addition to a decrease in Cyp2b9, 10 and 13 gene expression, we also observed significant down regulation of Cyp2a4, Cyp2c40 and Cyp3a13 mRNA in Cyp2b9/10/13-null female mice compared to WT female mice (Table 2.3). However, significant changes were not observed in Cyp2b9/10/13-null male mice potentially because these Cyp2b subtypes, especially Cyp2b9 and Cyp2b13 are primarily expressed in female liver (Hernandez et al., 2009b; Renaud et al., 2011).

Table 2.3: Compensatory changes in CYP gene expression in Cyp2b9/10/13-null mice.

<table>
<thead>
<tr>
<th>Gene</th>
<th>MALES</th>
<th></th>
<th>FEMALES</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Cyp2b9/10/13-null</td>
<td>WT</td>
<td>Cyp2b9/10/13-null</td>
</tr>
<tr>
<td>Cyp2a4</td>
<td>1.00 ± 0.187</td>
<td>5.162 ± 3.948</td>
<td>18.864±4.066&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.217 ± 2.294&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyp2b9</td>
<td>1.00 ± 0.692</td>
<td>0.054 ± 0.018</td>
<td>3.007 ± 0.897&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.047 ± 0.020&lt;sup&gt;b&lt;/sup&gt;*</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>1.00 ± 0.655</td>
<td>0.041 ± 0.014</td>
<td>2.274 ± 0.678</td>
<td>0.035 ± 0.015&lt;sup&gt;b&lt;/sup&gt;*</td>
</tr>
<tr>
<td>Cyp2b13</td>
<td>1.00 ± 0.709</td>
<td>0.059 ± 0.048</td>
<td>36.189 ± 7.478&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.082±0.078&lt;sup&gt;b&lt;/sup&gt;**</td>
</tr>
<tr>
<td>Cyp2c29</td>
<td>1.00 ± 0.091</td>
<td>2.277 ± 0.760</td>
<td>0.628 ± 0.123</td>
<td>1.472 ± 0.792</td>
</tr>
<tr>
<td>Cyp2c40</td>
<td>1.00 ± 0.123</td>
<td>0.911 ± 0.636</td>
<td>5.202 ± 2.169&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.620 ± 0.846&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>1.00 ± 0.124</td>
<td>0.790 ± 0.173</td>
<td>0.828 ± 0.101</td>
<td>0.524 ± 0.045</td>
</tr>
<tr>
<td>Cyp3a13</td>
<td>1.00 ± 0.170</td>
<td>0.551 ± 0.114</td>
<td>1.088 ± 0.121</td>
<td>0.536 ± 0.077&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Western blots confirm the null genotype of the hepatic CYP2B proteins (Fig. 2.6). Cyp2b is expressed at low levels in males and was not detectable in our western blots, but is clearly deleted in the Cyp2b9/10/13-null females. Western blots also confirm the down-regulation of Cyp2a mRNA in females shown by qPCR. Protein levels of Cyp2a genes dropped 41-46% in female Cyp2b9/10/13-null mice and protein levels of Cyp2a genes increased significantly (about 3-fold) in male Cyp2b9/10/13-null mice (Fig. 2.6). This new Cyp2a antibody recognizes two bands in our Western blots unlike first antibody used with the CAR-null and Cyp3a-null mice. B6 mice have several Cyp2a isoforms and Cyp2a22, which is primarily hepatic (Cui et al., 2012; Peng et al., 2012) is 50kDa in C57Bl6/J mice (XP_006539922.1) while the other Cyp2a isoforms (4/5/12) are 56 kDa. Messenger RNA levels of Cyp2a4 were not significantly altered in males, but Cyp2a4 mRNA increased 5-fold (Table 2.3), consistent with the increase in protein expression. Western blots did not confirm decreases in Cyp2c40 or Cyp3a13 protein expression in females; however, there are many Cyp2c and Cyp3a genes. Many of these other CYP isoforms were either not significantly changed, not tested, or in the case of the Cyp2c subtypes showed opposing trends (Table 2.3). It is also possible that the antibodies preferentially recognize specific isoforms that are not differentially expressed.

<table>
<thead>
<tr>
<th>Cyp3a25</th>
<th>1.00 ± 0.346</th>
<th>0.983 ± 0.350</th>
<th>0.991 ± 0.083</th>
<th>0.515 ± 0.126</th>
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<tr>
<td>Cyp3a41</td>
<td>1.00 ± 0.756</td>
<td>0.112 ± 0.107</td>
<td>19.459 ± 5.931</td>
<td>11.168 ± 1.627</td>
</tr>
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</table>

Data represented as mean +/- SEM (n = 4). Statistical significance determined by one-way ANOVA followed by LSD as the post-hoc test.
‘b’ indicates WT females different than Cyp2b9/10/13-null females
‘c’ indicates WT males different than WT females
‘d’ indicates Cyp2b9/10/13-null males different than Cyp2b9/10/13-null females
Letter with no asterisk indicates a p-value < 0.05 and * indicates a p-value < 0.01, ** indicates a p-value <0.001, ***indicates a p-value <0.00001
**Fig. 2.6: CYP protein expression in WT and Cyp2b9/10/13-null mice.**

Western blots of male and female Cyp2b9/10/13-null mice show significant changes in CYP expression relative to their WT counterparts. Cyp2a isoforms show two bands as Cyp2a22 is 50kDa in B6 mice and the other Cyp2a isoforms are 56 kDa. Results are expressed as relative mean of the WT compared to CAR-null mice of the same sex. Statistical differences were determined by Student’s t-tests (n = 3) with * (p < 0.05) *** (p < 0.001) indicating significant differences.

Global gene expression was measured in the livers of the null mice and compared to that in wild-type mice because there are so few compensatory changes in the Cyp2b9/10/13-null mice. Microarray data showed that in males, there are no statistically significant differentially expressed genes between the Cyp2b-null mice and wild-type mice consistent with the qPCR data. In female mice, Cyp2b9, Cyp2b10, and Cyp2b13 were all significantly down-regulated in the Cyp2b9/10/13-null strain. Cyp2a4 and Cyp2c40 were both significantly down-regulated in the female Cyp2b9/10/13-null strain compared to wild
type females by qPCR. These genes were not detected as differentially expressed by microarray, possibly because probe sets for both genes are not isoform specific. Probe ID 142230_s_at targets both Cyp2a4 and 2a5, and Probe ID 1423244_at targets Cyp2c40 and Cyp2c68. Additionally, 22 probe sets corresponding to 18 genes were also differentially expressed (Table 2.4). Importantly, the microarrays show no detectable compensatory increases in expression of other CYP genes. Overall, there are very few compensatory changes in the Cyp2b9/10/13-null mice.

**Table 2.4:** Genes differentially expressed in Cyp2b9/10/13-null female mice compared to WT female mice following microarray analysis.
Testosterone hydroxylation activity did not show any significant changes in the triple knockout mice except for the expected drop in testosterone 16α-hydroxylase activity

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>RefSeq Transcript ID</th>
<th>p-value</th>
<th>Fold-Change</th>
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</thead>
<tbody>
<tr>
<td>Cyp2b10</td>
<td>NM_0099999</td>
<td>2.38E-06</td>
<td>-13.1906</td>
</tr>
<tr>
<td>Cyp2b9</td>
<td>NM_010000</td>
<td>1.49E-10</td>
<td>-49.0951</td>
</tr>
<tr>
<td>A1132709</td>
<td>A1132709</td>
<td>2.80E-10</td>
<td>-43.2901</td>
</tr>
<tr>
<td>Dbp</td>
<td>NM_016974</td>
<td>2.27E-08</td>
<td>15.2984</td>
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<td>Rnf170</td>
<td>NM_029965</td>
<td>1.45E-06</td>
<td>-7.01792</td>
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<td>Lgalsl</td>
<td>NM_173752</td>
<td>3.46E-06</td>
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<td>Prpf38b</td>
<td>NM_025845</td>
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<td>C77080</td>
<td>NM_001033189</td>
<td>1.14E-05</td>
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<td>Dnmt3b</td>
<td>NM_001003960</td>
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<td>Sf1</td>
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<td>1.3156</td>
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<td>NM_011119</td>
<td>4.66E-06</td>
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<td>Tef</td>
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<td>Gstm3</td>
<td>NM_010359</td>
<td>9.57E-06</td>
<td>1.65659</td>
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<td>Nr1d2</td>
<td>NM_011584</td>
<td>9.22E-06</td>
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<td>Slc25a37</td>
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<td>Aldh1a7</td>
<td>NM_011921</td>
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<td>1.2603</td>
</tr>
<tr>
<td>Inpp5f</td>
<td>NM_178641</td>
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<td>Iqgap1</td>
<td>NM_016721</td>
<td>1.78E-05</td>
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</tr>
<tr>
<td>Foxq1</td>
<td>NM_008239</td>
<td>2.36E-05</td>
<td>2.43071</td>
</tr>
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</table>
in female mice compared to male mice (Fig. 2.7). Surprisingly, no significant changes were observed in 16β-hydroxytestosterone levels in Cyp2b9/10/13-null mice (Fig. 2.7). Cyp2b subfamily members are known to hydroxylate at the 16α– and 16β-positions (Acevedo et al., 2005; Smith et al., 1998; Waxman et al., 1983). Phenobarbital and TCPOBOP, powerful CAR activators are known to induce both 16α- and 16β-hydroxylase activity, especially 16β-hydroxylase activity in part because of its induction of Cyp2b10 (Hernandez et al., 2009b; Waxman et al., 1983). CYP2B, CYP2C and CYP2D members contribute to 16-position hydroxylation of testosterone (Hernandez et al., 2009b; Lee et al., 2006; Yamada et al., 2002); however, Cyp2d9 is the only male specific 16-hydroxylase indicating that this CYP is the primary 16α- and 16β-OH in males (Hernandez et al., 2009b). Therefore, our data suggests that CYP2B is not the primary 16-hydroxylase; instead it is the inducible 16-hydroxylase (Hernandez et al., 2006; Hernandez et al., 2009b; Imaoka et al., 1989).

Interestingly, we also observed a significant (p<0.01) reduction in 6α-OH testosterone activity in the Cyp2b9/10/13-null mice compared to their WT counterparts using one-way ANOVA followed by Fisher’s LSD post-hoc (p < 0.05) instead of the two-way ANOVA showed in the figure. This either suggests that Cyp2b members are involved in the constitutive metabolism of testosterone in the 6α-position or that there is a drop in Cyp2a members crucial in 6α-hydroxylation. Testosterone 6α-hydroxylase activity was also decreased in CAR-null mice, but not Cyp3a-null mice (Fig. 2.3). A drop in Cyp2a was measured in Cyp2b9/10/13-null females but not males (Fig. 2.6; Table 2.3). In turn, 6α/15α ratio was significantly higher in WT females than Cyp2b9/10/13-null females
(2.6X), suggesting weak to moderate hepatic masculinization of Cyp2b9/10/13-null females. There were no differences in serum or hepatic testosterone concentrations between WT and Cyp2b9/10/13-null mice indicating that the difference in 6α/15α ratio is directly due to the drop in Cyp2a protein expression or loss of Cyp2b’s.
Fig. 2.7: Testosterone hydroxylation determined in WT and Cyp2b9/10/13-null mice. (A) Testosterone hydroxylation was determined in male and female WT and Cyp2b9/10/13-null mice as described in the Materials and Methods. Data are presented as mean specific activity (µmol/min/mg protein) ± SEM (n = 4). (B) Ratio of 6α/15α-hydroxytestosterone as a biomarker of CYP sexual dimorphism in the liver. An ‘a’ indicates a significant difference between WT male and Cyp2b9/10/13-null male mice, ‘b’ indicates a significant difference between WT female and Cyp2b9/10/13-null female mice, ‘c’ indicates a significant difference between
male and female WT mice and * indicates a significant difference between the male and female Cyp2b9/10/13-null mice. Statistical differences were determined by two-way ANOVA followed by Fisher’s LSD as the post-hoc test in (A) and one-way ANOVA followed by Fisher’s LSD in (B). A letter without an asterisk indicates a significance of p < 0.05, asterisk indicate significance of *p<0.01, **p<0.001, and *** p<0.0001, respectively.

2.5 Conclusions

CAR-null mice show greater changes in CYP expression and activity relative to their WT counterparts than Cyp3a-null and Cyp2b-null mice. This is probably because CAR directly regulates the expression of several CYPs either constitutively or through activation by endogenous and exogenous substrates. Because CAR regulates constitutive CYP expression the use of CAR-null mice alone could cause incorrect interpretations of chemical metabolism. In addition, CAR appears to regulate sexual dimorphism of CYP expression within the liver as lack of CAR caused masculinization. Given that CAR activation is feminizing (Hernandez et al., 2006; Hernandez et al., 2009b; Oshida et al., 2016), CAR has greater transcriptional activity in females than males (Ledda-Columbano et al., 2003), CAR regulates a number of female predominant CYPs (Hernandez et al., 2009b), and CAR is inhibited by androgens (Baldwin et al., 2009; Forman et al., 1998), it may not be all that surprising that the loss of CAR causes masculinization of the liver (Fig 3). Overall, CAR-null mice may show significant changes in CYP-mediated drug metabolism following exposure because of the significant changes in CYP expression.

Cyp3a-null mice show some compensatory changes in CYP expression and testosterone metabolism. We would not expect the changes to be as broad as the CAR-null mice because the regulation of the other CYPs is not direct. However, the small changes
observed in 2α-OH and 16α-OH testosterone levels suggest that the loss of CYP3A activity alters liver substrate profiles for CAR, PXR, and potentially other nuclear receptors/transcription factors that regulate CYP expression. Alternatively, several CYPs may show changes in CYP activity because they compensate for the loss of CYP3A and the lack of competition for the substrate. Based on the changes in expression profiles such as significant increases in Cyp2a4 and Cyp3a13 (the only Cyp3a gene retained in the knockout mouse model), we would predict that the lack of CYP3A is causing the activation of PXR and potentially a drop in CAR activity. Additionally, the observed increase, though not significant, in expression of Cyp2b10, Cyp2c29 and Cyp2c40 suggest increase in PXR activity in Cyp3a-null mice.

Cyp2b-null mice show very few overall changes in CYP expression outside the loss of CYP2B (Tables 3 and 4; Figs 6 and 7). Unlike CYP3A, which is the most abundant CYP in murine livers, CYP2B shows relatively low expression, lower than CYP3A, 2C, and 2D subfamily members (Wang et al., 2008). Therefore, we would expect fewer compensatory changes. Overall, most if not all changes in drug metabolism in the Cyp2b9/10/13-null mice would reflect the loss of Cyp2b.

In conclusion, toxicology models such as CAR-null and various CYP-null mice show changes in other CYPs due to direct control of expression or compensatory changes in expression. These changes in CYP expression, especially those regulated by CAR, may alter hepatic CYP expression, CYP-mediated xenobiotic metabolism, and hepatic CYP masculinization. Perturbations in non-CYP3A/2B-mediated metabolism of xenobiotics
may occur in these models with significant changes in metabolism more likely in CAR-null compared to Cyp3a-null or Cyp2b-null mice.

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Competing Interests

The authors have no conflicts of interest to declare
References


Wortham, M., Czerwinski, M., He, L., Parkinson, A., & Wan, Y. J. (2007). Expression of constitutive androstanone receptor, hepatic nuclear factor 4a, and P450
oxidoreductase genes determines interindividual variability in basal expression and activity of a broad scope of xenobiotic metabolism genes in the human liver. *Drug Metab Dispos, 35*(9), 1700-1710.


CHAPTER THREE

HIGH FAT DIET-INDUCED OBESITY IS MITIGATED IN CYP3A-NULL FEMALE MICE

This manuscript is under revision at Chemical-biological interactions journal

3.1 Highlights:

- Cyp3a-null female mice are resistant to the effects of a high-fat diet
- Cyp3a-null females show improved metabolic responses, including glucose tolerance
- In contrast, Cyp3a-null male mice show increased liver triglycerides
- Gene expression changes indicate decreased lipid metabolism and increased uptake
3.2 Abstract

Recent studies indicate a role for the constitutive androstane receptor (CAR), pregnane X-receptor (PXR), and hepatic xenobiotic detoxifying CYPs in fatty liver disease or obesity. In addition, Cyp3a-null mice express hepatic Cpt1α at much greater levels regardless of gender (18-123X greater) than WT mice. Therefore, we examined whether Cyp3a-null mice show increased obesity and fatty liver disease following 8-weeks of exposure to a 60% high-fat diet (HFD). Surprisingly, HFD-fed Cyp3a-null females fed a HFD gained 50% less weight than wild-type (WT; B6) females fed a HFD. In contrast, Cyp3a-null males gained more weight than WT males, primarily during the first few weeks of HFD-treatment. Cyp3a-null females also recovered faster than WT females from a glucose tolerance test; males showed no difference in glucose tolerance between the groups. Serum concentrations of the anti-obesity hormone, adiponectin are 60% higher and β-hydroxybutyrate levels are nearly 50% lower in Cyp3a-null females than WT females, in agreement with reduced weight gain, faster glucose response, and reduced ketogenesis. In contrast, Cyp3a-null males have higher liver triglyceride concentrations and lipidomic analysis indicates an increase in phosphatidylinositol, phosphatidylserine and sphingomyelin. None of these changes were observed in females. Last, Pxr, Cyp2b, and IL-6 expression increased in Cyp3a-null females following HFD-treatment. Cyp2b and Fatp1 increased, while Pxr, Cpt1α, Srebp1 and Fasn decreased in Cyp3a-null males following a HFD, indicating compensatory biochemical responses in male (and to a lesser extent) female mice fed a HFD. In conclusion, lack of Cyp3a has a positive effect on
acclimation to a HFD in females as it improves weight gain, glucose response and ketoacidosis.

**Keywords:** P450, non-alcoholic fatty liver disease (NAFLD), ketosis, toxicology

**Abbreviations:**

- *Acox*  Acyl-CoA oxidase
- *ApoE*  Apolipoprotein E
- *Cpt1a*  Carnitine palmitoyl transferase 1 a
- *CYP*  Cytochrome P 450
- *EET*  Epoxideicosatrienoic acid
- *Fabp4*  Fatty acid binding protein 4
- *Fasn*  Fatty acid synthase
- *Fatp*  Fatty acid transporter protein
- *Foa2*  Forkhead box protein A2
- *HETE*  Hydroxyicosatetraenoic acid
- *HFD*  High fat diet
- *HODE*  Hydroxyoctadecadienoic acid
- *Pepck1*  Phosphoenolpyruvate carboxykinase 1
- *Ppara*  Peroxisome proliferator activated receptor alpha
- *Pparγ*  Peroxisome proliferator activated receptor gamma
- *PXR*  Pregnane X receptor
<table>
<thead>
<tr>
<th><strong>Srebp1</strong></th>
<th>Sterol regulatory element-binding protein 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>Wildtype</td>
</tr>
</tbody>
</table>
3.3 Introduction

Obesity is a complex disorder that affects nearly 1 out of 3 Americans [1] and is considered an epidemic throughout much of the developed world [2, 3]. For example, the percent of obese individuals is increasing in Latin America (i.e. Panama 35%, Chili 22%), Europe (Greece 23%, Malta 26%), and the Middle East (United Arab Emirates 34%, Saudi Arabia 36%, Egypt 30%) [2]. In turn, the rates of dyslipidemia, hypertension, diabetes, and other metabolic disorders increased dramatically placing a large burden on our healthcare systems [4, 5].

The primary problem is excess food coupled with inactivity. Therefore, healthier food choices and lifestyle changes are common treatment regimens used to control obesity. However, there are multiple internal and external factors that regulate metabolic activity and allocation of dietary lipid and carbohydrate resources [6, 7]. Understanding these forces provides targets for initiating dietary and environmental changes and tackling this disease. Many cytochrome P450s (CYPs) are crucial detoxication enzymes that metabolize drugs [8], environmental chemicals [9], steroids [10] and fatty acids [11], and changes in CYP activity and expression are observed during obesity and diabetic conditions [12-15].

The constitutive androstane receptor (CAR) [16, 17] and pregnane X-receptor (PXR) [18] are two nuclear receptors involved in chemical detoxication, and associated with metabolic diseases although possibly in opposing directions. CAR activation alleviates symptoms of obesity and fatty liver disease and murine PXR activation increases obesity [15, 19, 20], although there are conflicting reports on PXR’s effects on obesity and insulin resistance [21]. These nuclear receptors are key regulators of CYPs such as Cy2b
and Cyp3a subfamily members [18, 22, 23]. In a world in which we are exposed to numerous environmental toxicants each and every person may show different CYP activity and in turn show differential metabolism of xeno- and endobiotics, including unsaturated fatty acids because of the different diets and chemicals to which they have been exposed [24, 25]. Therefore, it is possible that chemicals that modulate CYP activity are obesogens or metabolic disruptors [5, 26].

Interestingly, complete loss of hepatic CYP activity in a conditional knockout of P450-oxidoreductase (HRN) caused enlarged livers, and hepatic steatosis, especially after the mice were fed sunflower oil (88% unsaturated fatty acids). In turn, increased hepatic concentrations of unsaturated fatty acids in the HRN mice activated CAR and induced Cyp2b10 and Cyp3a11 providing a putative new role for CAR in the recognition of unsaturated fatty acids and suggests that CYP induction may be a protective mechanism from fatty acid toxicity [20]. Subsequent studies also demonstrated CYP3A induction by fatty acids in human cells [27]. Further, Cyp3a expression and activity is decreased in obese guinea pigs and diabetic humans [28, 29], but increased in diabetic mice [15, 30]. These results suggest a role for Cyp3a in obesity given that Cyp3a expression changes with diet, loss of hepatic CYPs causes fatty liver disease, and the Cyp3a’s are the most abundant hepatic CYPs.

CYP3A4 accounts for approximately 30-40% of total CYP protein in the human liver and is responsible for the metabolism of more than 60% of the drugs on the market [31]. Cyp3a subfamily members produce a number of epoxidated products of linoleic acid and arachidonic acid in human and rodents. CYP3A4 primarily metabolizes linoleic acid
to 11-hydroxy-octadecadienoic acid (11-HODE), and the production of 11-HODE is increased 10X by the CYP3A inducer, dexamethasone, in rats [32]. CYP3A4 oxygenates arachidonic acid to a number of metabolites including 13-, 10-, and 7-hydroxyeicosatrienoic acid (13-HETE, 10-HETE, 7-HETE) [32]. The epoxides formed are stable, but also metabolized by epoxide hydrolases to diols [32]. Whether these epoxides have a specific purpose in the liver is unknown; however, inhibition assays suggest that a Cyp3a-mediated arachidonic acid metabolite is in part responsible for relaxation of arterial endothelium [33]. A Cyp3a and Cyp2j produced metabolite of linoleic acid is a key product in the activation of nociception [34], and CYP3A4 metabolizes the arachidonic acid derivative, anandamide, to a high affinity cannabinoid ligand [35-37]. Thus, Cyp3a metabolizes unsaturated fatty acids for specific functions and disruption of this metabolism by xenobiotics or genetic impairments may have consequences that perturb fatty acid metabolism, distribution, and use [38, 39].

Ultimately, we decided to investigate the role of Cyp3a in obesity because Hepatic P450 Reductase-null (HRN) mice with no P450 activity show hepatic steatosis with increased Cyp2b10 and Cyp3a11 expression, perturbations in liver size, lipid homeostasis, and increased polyunsaturated fatty acids (PUFAs) [20]. In addition, activation of PXR, the key regulator of Cyp3a members, increases obesity in females but not males[14, 19]. This implicates detoxification CYPs in obesity, and Cyp3a members are the most prominent hepatic CYPs. However, the potential role of Cyp3a on obesity and diet-induced fatty liver disease has not been investigated. We used mice lacking seven of the eight Cyp3a genes on a B6 background to monitor the effects of a high-fat diet (HFD) (diet-induced
obesity) on weight gain, glucose and insulin tolerance, fatty liver, and alterations in hepatic gene expression consistent with metabolic disorders.

3.4 Materials and methods

3.4.1. Livers from untreated WT and Cyp3a-null mice

Frozen livers from untreated 8-9 week old WT and Cyp3a-null mice were purchased from Taconic (Hudson, NY USA) and shipped on dry ice. These mice lack the Cyp3a11, Cyp3a16, Cyp3a25, Cyp3a41, Cyp3a44, Cyp3a57, and Cyp3a59 genes located within 0.8Mb from each other on chromosome 5. Cyp3a13, which is separate from the rest of the Cyp3a cluster by 7Mb, is still present within this knockout mouse model (Hasegawa et al., 2011; Scheer et al., 2010). The liver tissue was diced into three fractions such that half a liver was frozen for microsome preparation, a quarter of a liver was stored in Tri-Reagent (Sigma, St. Louis, MO USA) for RNA extraction while the remaining liver was frozen immediately and stored at -80°C.

3.4.2. High-fat Diet Treatment of WT and Cyp3a-null mice

All mice studies were done according to the National Institute of Health guidelines for humane use of research animals and were pre-approved by Clemson University’s Institutional Animal Care and Use committee. Seven - nine week old male and female, WT (C57Bl/6) and Cyp3a-null mice (n=8) were purchased from Taconic and housed for a week for acclimation prior to HFD treatment.

Eight-ten week-old acclimated WT and Cyp3a-null mice were fed pelleted adjusted calorie diet (60% kcal from fat, TD.06414; Harlan, Madison, WI USA) for eight weeks.
One male Cyp3a-null mouse was eight weeks old and all other mice were 9-10 weeks old. This mouse also lost weight early in the study and therefore was eliminated from the study. Thus, all remaining mice were 9-10 weeks old at the start of the HFD treatment. Changes in body weight, feed consumption rate, and metabolic tests such as glucose (GTT) and insulin tolerance tests (ITT) were performed. A timeline of procedures is provided (Appendix B-1). Weight gain was monitored weekly and feed consumption was determined every other day. Fasting plasma glucose levels were determined during week 2, 4 and 6. Glucose and insulin tolerance tests were performed during weeks 4 and 6, respectively. At the end of the study the mice were anesthetized, blood collected by heart puncture, euthanized and liver, kidney, white adipose tissue and testis were excised and weighed. The organs were immediately frozen on dry ice and stored at -80°C or placed in 10% formalin (Fisher, Fairlawn, NJ USA) for further studies. The liver was dissected into four fractions and frozen immediately on dry ice for microsome preparation, RNA isolation, and lipidomics. The portion of the liver used for RNA extraction was stored in Tri-Reagent (Sigma Aldrich). The last portion of the liver was placed in formalin for histopathology.

3.4.3. Glucose and Insulin tolerance tests

Mice were fasted for 4-5 hr prior to GTT or ITT during weeks four and six of the HFD study, respectively. Fasting blood glucose was determined using an Alphatrak 2 (Chicago, IL USA) blood glucose meter following a tail bleed. Glucose tolerance was determined following an intraperitoneal injection of 1 g/kg of their body weight of D-glucose (Sigma Ultra) with blood glucose readings every 20 minutes for the first hour and
every 30 minutes for the second hour. ITT was determined after intraperitoneal injection of 0.75 U/g of their body weight of Novolin N (Nova Nordisk, Bagsvaerd, DK) followed by blood glucose measurements every 30 min for 2 hours.

3.4.4. cDNA and microsome preparation

RNA was extracted using Tri-Reagent (Sigma Aldrich) according to the manufacturer’s instruction and residual genomic DNA was removed by DNase (Bio-Rad, Hercules, CA USA) treatment. RNA was quantified by determining 260/280 using a spectrophotometer (Molecular devices, Ramsey MN USA). Microsome and cytosol fractions were prepared by homogenization with a dounce homogenizer followed by differential centrifugation as previously described (Van der Hoeven et al., 1974). Protein concentrations were determined with Bradford reagent (Bio-Rad).

3.4.5. Quantitative real-time PCR (qPCR)

qPCR was used to quantify changes in gene expression. Briefly, cDNA was prepared from 2 µg RNA with 200 units of MMLV reverse transcriptase, 10mM dNTP mixture and 0.5 mg random hexamers (Promega corporation, Madison WI USA). Changes in gene expression were determined using previously published primers for CYPs (Damiri et al., 2011; Hernandez et al., 2006) and key genes related to energy homeostasis and lipid metabolism (Appendix B-2). Samples were diluted 1:5 and amplified in triplicates using a 96-well plate IQ™ Real-Time PCR detection system (Bio-Rad) with 0.25X RT² SybrGreen (Qiagen Frederick, MD USA) to quantify gene expression compared to a reference gene as previously described using Muller’s equation to determine changes in gene expression (Muller et al., 2002). PCR efficiency was determined based on a standard curve prepared
using a sample mixture containing all the cDNA samples diluted at 1:1, 1:5, 1:25, 1:125, 1:625 and 1:3125 dilutions (Kumar et al., 2017).

3.4.6. Serum concentrations of testosterone, adiponectin and β-hydroxybutyrate

Serum testosterone and β-hydroxybutyrate concentrations were detected by using Enzyme Immunoassay (EIA) and colorimetric kits, respectively, purchased from Cayman Chemical Co (Cayman Chemical, Ann Arbor, MI USA). Serum adiponectin concentrations were determined using an EIA kit from Bertin pharma (Montigny Le Bretonneux, FR).

3.4.7. Histopathological analysis using H&E and oil red O

A clean slice of liver was made following necropsy and was placed in 10% formalin (Fisher). Hematoxylin and Eosin (H&E) and oil red O staining was performed at Colorado Histoprep (Fort Collins, CO USA).

3.4.8. Quantification of liver triglycerides and polar lipids

Liver triglyceride concentrations were determined using a colorimetric kit from Cayman Chemical Co. Liver tissue (40-60 mg) was homogenized in a dounce homogenizer with 0.5 ml phosphate buffer mixed with 0.5 ml of a 3:2 mix of hexane: isopropyl alcohol. The homogenate was transferred into a fresh tube and the extraction repeated with 3:2 hexane: isopropyl alcohol. The organic layer was transferred into a fresh tube and the hexane extraction was repeated twice. The combined organic layers were then dried under nitrogen and re-constituted using standard diluent provided in the kit to determine liver triglyceride concentrations according to the manufacturer’s protocol.
Polar lipids were extracted from microsomes (2 mg/ml) by the addition of chloroform (0.1% butylated hydroxytoluene) and methanol in 1:2 parts into a glass tube. The contents of the tube were vortexed and centrifuged at 600 rpm for a minute. The lower layer was transferred into a fresh tube the chloroform extraction was repeated 3X. The combined lower layers were washed with 1M potassium chloride and then with water. Samples were dried under nitrogen and shipped on dry ice to the Kansas Lipidomics Research Center for analysis by electrospray ionization triple quadruple mass spectrometry (Applied Biosystems API 4000) as described previously (Isaac et al., 2007; Sengupta et al., 2016).

3.4.9. Statistical Analysis

Data are presented as mean ± SEM (n = 7 - 8). Statistical analyses were performed by Student’s t-test using Graphpad Prism version 6. A p-value < 0.05 was considered statistically significant.

3.5. Results

3.5.1. Changes in the expression of hepatic energy metabolism-related genes in untreated Cyp3a-null mice

Mice lacking all hepatic CYP activity have steatosis (Finn et al., 2009) and several CYPs are either regulated by transcription factors involved in energy homeostasis or metabolize crucial lipids and bile acids involved in digestion, absorption and utilization of lipids (Hafner et al., 2011). Therefore, we first investigated whether the liver of 8-9 week old Cyp3a-null mice show differences in the expression of several energy metabolism-
related genes compared to WT mice by qPCR. Most of the genes investigated were not changed. However, carnitine palmitoyl transferase (*Cpt1a*) is significantly up-regulated by 134X and 18X in Cyp3a-null male and female mice, respectively, compared to their WT counterparts. Fatty acid binding protein-4 (*Fabp4*) is up-regulated in Cyp3a-null females 13X compared to their WT counterparts (Table 3.1). *Ppara* and *Srebp1c* regulate *Cpt1a* and *Fabp4*, respectively, suggesting compensatory changes in liver lipid metabolism in Cyp3a-null mice that help metabolism of excess lipids (Ayala-Sumuano et al., 2011; Kumar et al., 2017; Rakshandehroo et al., 2009).

**Table 3.1:** Compensatory changes in energy and lipid metabolism genes in Cyp3a-null mice.
Data represented as relative mean +/- SEM (n = 4). Statistical significance determined by Student’s t-test. *Indicates a p-value ≤ 0.05.

### 3.5.2. Weight gain is mitigated in HFD-treated Cyp3a-null female mice in comparison to HFD-treated WT female mice

Weight gain was monitored in WT and Cyp3a-null male and female mice fed a HFD over eight-weeks. Female Cyp3a-null mice gained significantly less weight than their WT counterparts as early as week one and by the seventh week Cyp3a-null female mice only gained 3.13g while WT mice gained 6.13g (Fig. 3.1). In contrast, Cyp3a-null male mice did not show a significant change in weight compared to their WT counterparts over the full course of the study; however, Cyp3a-null male mice gained more weight (p-value
= 0.06) initially and weighed more than their WT counterparts over the course of the study (Fig. 3.1). Differences in weight gain are irrespective of feed consumption as there are no significant differences in feed consumption between the genotypes (Appendix B-3). Overall, gender plays a role in the Cyp3a-null mice’s response to a HFD.

**Fig. 3.1: Gender is crucial in the response of Cyp3a-null mice to HFD treatment in comparison to WT mice.** Change in body weight of female (A) and male (B) mice during eight-weeks of HFD-treatment. Changes in body weight are also represented as area under the curve to confirm results in females (A) and males (B). Data are presented as mean ± SEM. Statistical significance was determined by Student’s t-tests (n = 7 or 8) (* p<0.05, ** p<0.01).

3.5.3. *Cyp3a-null female mice, but not Cyp3a-null male mice, respond faster to a glucose challenge than their WT counterparts*

Fasting blood glucose concentrations were similar between the different genotypes after 2, 4, or 6-weeks of HFD-treatment (Appendix B-4). A GTT was performed on the
fourth week of the HFD treatment because of differences in weight gain. Interestingly, Cyp3a-null female mice recover faster than the WT mice at the 40-90 minute intervals following the initial glucose injections (p-value = 0.03 – 0.07). Area under the curve measurements confirmed the rapid recovery in the Cyp3a-null female mice (Fig. 3.2), and indicates higher lean mass in the Cyp3a-null female mice than their WT counterparts (McGuinness et al., 2009). In contrast, there was no significant difference in glucose tolerance between the male genotypes (Fig. 3.2).

An insulin tolerance test was performed on week 6 to determine insulin sensitivity (Bowe et al., 2014). There were no significant differences in insulin tolerance between the Cyp3a-null and WT mice in either gender (Fig. 3.3). We also did not find any differences in fasting insulin levels between the Cyp3a-null and WT mice in either gender (Appendix B-4). This suggests that the faster glucose recovery observed in the Cyp3a-null female mice is independent of insulin response.
Fig. 3.2: Cyp3a-null female mice, but not Cyp3a-null male mice, fed a HFD show increased sensitivity to a glucose challenge in comparison to WT mice. A GTT was performed as described in the Materials and Methods after 4-weeks of HFD-treatment in female (A) and male (B) mice. GTT results are also represented as area under the curve (AUC) to confirm results for females (C) and males (D). Data are presented as mean blood glucose levels ± SEM. Statistical significance was determined by Student’s t-tests (n = 7–8) (* p<0.05).
Fig. 3.3: HFD does not significantly perturb insulin concentrations or action in Cyp3a-null mice. An ITT was performed as described in the Materials and Methods after 6-weeks of HFD-treatment in female (A) and male (B) mice. ITT results are also represented as area under the curve (AUC) to confirm results for females (C) and males (D), and ITTs are not different between WT and Cyp3a-null mice of either gender. Data are presented as mean ± SEM. Statistical significance was determined by Student’s t-test (n = 7-8).

3.5.4 Differences in organ weights between WT and Cyp3a-null mice

Liver, kidney, spleen, abdominal and inguinal white adipose tissue (WAT), and testis were excised and weighed (Table 3.2). WAT weighed 30% more in the WT females compared to their Cyp3a-null counterparts consistent with increased weight gain in WT females; however, increased WAT was not statistically significant (p-value = 0.12). In contrast, the kidneys of Cyp3a-null females are 20% heavier than kidneys from WT females, suggesting compensation for a toxic burden such as ketoacidosis (Adrogue et al.,
In addition, testes weights are 29.2% lower in Cyp3a-null males than WT males fed a HFD although the decreased testes weights are primarily due to two males that showed very low testis weights. A drop in testis weights sometimes in only one testis is not uncommon in rodent HFD studies (Datar et al., 2017). In contrast, liver weights are 8% higher in Cyp3a-null males than WT males fed a HFD and this result is consistent (Table 3.2).

**Table 3.2:** Organ weights determined at the termination of study after 8 weeks of HFD treatment.

<table>
<thead>
<tr>
<th>Models</th>
<th>Body</th>
<th>Liver</th>
<th>Kidney</th>
<th>WAT(^a)</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-F</td>
<td>23.75±1.03</td>
<td>0.91±0.04</td>
<td>0.25±0.01</td>
<td>1.13±0.20</td>
<td></td>
</tr>
<tr>
<td>Cyp3a-null F</td>
<td>23.88±1.34</td>
<td>0.88±0.04</td>
<td>0.30±0.01***</td>
<td>0.78±0.23</td>
<td></td>
</tr>
<tr>
<td>WT-M</td>
<td>35.88±1.22</td>
<td>1.39±0.06</td>
<td>0.40±0.01</td>
<td>3.20±0.36</td>
<td>0.24±0.01</td>
</tr>
<tr>
<td>Cyp3a-null M</td>
<td>39.14±0.74*</td>
<td>1.50±0.54*</td>
<td>0.42±0.01</td>
<td>3.57±0.32</td>
<td>0.17±0.02**</td>
</tr>
</tbody>
</table>

\(^a\)White adipose tissue (WAT)

Data represented as mean +/- SEM (n = 7 or 8). Statistical significance determined by Student’s t-test. *Indicates a p-value ≤ 0.05, **indicates a p-values ≤ 0.01, and ***indicates a p-value ≤ 0.001.

3.5.5. *Serum concentrations of testosterone, adiponectin, and β-hydroxybutyrate*

Adiponectin increases insulin sensitivity and is frequently increased in lean compared to obese individuals (Nigro et al., 2014). The leaner Cyp3a-null females have 1.7X greater serum adiponectin concentrations than WT females further demonstrating that the lower weight gain and adipose levels had physiological/endocrine effects (Fig. 3.4A).
β-hydroxybutyrate (B-OHB) is produced along with acetoacetate from fatty acids as an alternative fuel source during fasting, low carbohydrate diets, or exercise. Because the kidney eliminates B-OHB we hypothesized that the increase in kidney weight in the Cyp3a-null females was due to an increase in B-OHB. Instead, Cyp3a-null female mice show significantly lower B-OHB concentrations (49% lower) than the WT females (Fig. 3.4B), indicating that the WT mice have significantly more ketoacidosis, potentially due to increased WAT and consistent with the slower response to the glucose challenge. In contrast, we did not observe any significant changes in serum adiponectin and B-OHB levels in male mice. Furthermore, we did not observe a significant change in serum testosterone concentrations in the male mice despite smaller testes (Fig. 3.4C), probably because the lower testes weights were due to two outlier individuals.
**Fig. 3.4:** Serum adiponectin, β-hydroxybutyrate and testosterone in WT and Cyp3a-null mice fed a HFD. Commercial kits were used to measure (A) adiponectin, (B) β-hydroxybutyrate and (C) testosterone in serum samples as described in the Materials and Methods. Data are presented as mean ± SEM. Statistical significance was determined by Student’s t-tests (n = 7 - 8) (* p<0.05).

3.5.6. **Liver triglyceride concentrations are higher and accompanied by macrovesicular steatosis in Cyp3a-null male mice**

Higher adiponectin and lower WAT coupled with a faster response to a glucose challenge and lower ketones in HFD-treated Cyp3a-null female mice indicate lower liver triglycerides and steatosis, and greater feedback inhibition of β-oxidation in HFD-treated WT female mice (Xu et al., 2008). Therefore, liver triglyceride concentrations were
measured. Surprisingly, there was no significant difference in liver triglycerides between the WT and Cyp3a-null female mice (Fig. 3.5A).

In contrast, Cyp3a-null males showed a (1.5X) increase in liver triglycerides compared to WT males (Fig. 3.5B) that was coupled with a 8% greater liver weight. Oil Red O and H&E staining were used to determine zonation and types of steatosis (microvesicular and macrovesicular) in the liver tissue, which is one of the significant markers for the development of metabolic syndrome (Levene et al., 2012). Histopathology results (Fig. 3.5C-I) indicate mild macrovesicular steatosis without inflammation in the midzonal regions of the liver in male mice while female mice showed diffuse microvesicular steatosis without inflammation in HFD-fed mice. These changes were more visible following Oil red O staining and indicate accumulation of lipid droplets due to HFD-treatment in both the genders although in different ways. However, histopathological analysis did not associate increased steatosis with the absence of Cyp3a genes.
Fig. 3.5: Hepatic lipid levels and histopathological changes observed in WT and Cyp3a-null mice fed a HFD. Liver triglycerides in female (A) and male (B) mice were determined as described in the Materials and Methods. Data are represented as mean ± SEM. Statistical significance was determined by Student’s t-tests (n = 7 - 8) (* p<0.05). Histopathological changes were evaluated by H&E or Oil red O staining of liver tissues following 8-weeks of HFD treatment in WT and Cyp3a-null mice. WT (C,D) and Cyp3a-null (E,F) female liver slices stained with H&E and Oil red O, respectively. WT (G,H) and Cyp3a-null (I,J) male liver slices stained with H&E and Oil Red O, respectively. Scale 0.2 mm
3.5.7. Phosphatidylserine and phosphatidylinositol are increased in Cyp3a-null male mice

Fatty acids are responsible for the formation of lipid signaling molecules. Lipidomic analysis demonstrated a few significant changes between the lipid profiles of WT and Cyp3a-null males fed a HFD; however no differences were observed between WT and Cyp3a-null females (Table 3.3). Cyp3a-null males display a two-fold increase in total polar lipids as well as an increase in the concentrations of several specific lipid groups including total phosphatidylserine (PS; 2.65X), phosphatidylinositol (PI; 2.3X), sphingomyelins (SM; 2.7X), phosphatidylglycerol (PG; 2.7X), and phosphatidic acid (PA; 3.44X) in comparison to WT males (Table 3.3). Principle component analysis (PCA) indicates lipid species are primarily clustered by gender; not genotype with the exception of large-chain PS, some sphingomyelin, and mid-chain phosphatidylcholine (PC) species (Appendix B-5). When normalizing to relative concentrations to look for changes in composition between WT and Cyp3a-null males, differences are observed only in PS, PI, and SM concentrations by 1.6X, 1.3X and 1.98X respectively (Appendix B-6). The relative increases in polar lipids is primarily due to higher concentrations of long chain lipid species containing 38:4 and 40:4 in PS and PI, respectively (Fig. 3.6).

Table 3.3: Lipidomics measured after eight weeks of HFD treatment on Cyp3a-null mice.
### Data represented as mean signal per mg of protein +/- SEM (n = 5). Statistical significance determined by Student’s t-test. *Indicates a p-value ≤ 0.05 and ** indicates a p-value ≤ 0.01.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Females WT</th>
<th>Females Cyp3a-null</th>
<th>Males WT</th>
<th>Males Cyp3a-null</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total LysoPC</td>
<td>0.775±0.240</td>
<td>0.466±0.297</td>
<td>1.083±0.239</td>
<td>1.834±0.424</td>
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<tr>
<td>Total PC</td>
<td>45.200±10.050</td>
<td>36.98±16.570</td>
<td>58.75±11.550</td>
<td>98.720±13.940</td>
</tr>
<tr>
<td>Total SM / DSM</td>
<td>3.115±0.691</td>
<td>2.272±1.217</td>
<td>2.855±0.961</td>
<td>7.739±1.398*</td>
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<tr>
<td>Total ePC</td>
<td>1.459±0.312</td>
<td>1.317±0.509</td>
<td>2.082±0.434</td>
<td>3.359±0.517</td>
</tr>
<tr>
<td>Total LysoPE</td>
<td>0.217±0.061</td>
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<td>0.358±0.087</td>
<td>0.631±0.114</td>
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<tr>
<td>Total PE</td>
<td>8.005±2.107</td>
<td>7.91±4.225</td>
<td>11.95±2.681</td>
<td>19.84±3.300</td>
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<tr>
<td>Total ePE</td>
<td>0.144±0.042</td>
<td>0.156±0.086</td>
<td>0.309±0.076</td>
<td>0.535±0.093</td>
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<tr>
<td>Total PI</td>
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<td>2.348±1.220</td>
<td>3.907±0.961</td>
<td>9.007±1.437*</td>
</tr>
<tr>
<td>Total PS</td>
<td>0.510±0.118</td>
<td>0.553±0.241</td>
<td>0.542±0.138</td>
<td>1.434±0.286*</td>
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<tr>
<td>Total ePS</td>
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<td>0.003±0.001</td>
<td>0.005±0.002</td>
<td>0.014±0.003*</td>
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<tr>
<td>Total PA</td>
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<td>0.026±0.010</td>
<td>0.025±0.004</td>
<td>0.086±0.020*</td>
</tr>
<tr>
<td>Total PG</td>
<td>0.009±0.004</td>
<td>0.013±0.007</td>
<td>0.034±0.012</td>
<td>0.092±0.020*</td>
</tr>
<tr>
<td>Total lipids</td>
<td>61.97±14.01</td>
<td>52.06±24.45</td>
<td>81.89±16.97</td>
<td>163.6±7.685**</td>
</tr>
</tbody>
</table>
Fig. 3.6: Phosphatidylinositol and phosphatidylserine are increased in Cyp3a-null male mice. Data are represented as mean ± SEM. Statistical significance was determined by Student’s t-tests (n=5) (* p<0.05, ** p<0.01).

3.5.8. Changes in the expression of hepatic energy metabolism related genes in HFD-treated Cyp3a-null mice

Following HFD-treatment for 8-weeks, Cpt1a, as well as Fasn, ApoE, Pxr and Srebp1a are down-regulated by approximately 0.5X in Cyp3a-null male mice compared to their WT counterparts (Table 3.4). In contrast, IL-6 and Pxr is upregulated about 2.0X in Cyp3a-null females, but not males. Fatp1 is up-regulated 2.1X in males and 2.25X in females; however only the up-regulation in males is significant. Overall, the expression
data suggests a decrease in lipid oxidation and synthesis and an increase in transport in the male mice, which may explain the increase in liver triglycerides. These changes in gene expression are consistent with a decrease in AMPK activity. However, we found no significant change in AMPK phosphorylation levels between the WT and Cyp3a-null mice (Appendix B-7). Because recent studies indicated that several Cyps are regulated by a HFD (Deol et al., 2015; Finn et al., 2009), we also investigated the expression of several Cyps. With the exception of the knocked out Cyp3a members, there are only a few Cyps that showed changes in gene expression and only Cyp2b protein expression in males (2.8X) was increased significantly; consistent with increases in Cyp2b9, a gene controlled by the metabolic receptors, FoxA2 and CAR (Appendix B-7) (Hashita et al., 2008; Hernandez et al., 2009; Jarukamjorn et al., 2001). The lower PXR expression in males may also help explain minor steatosis coupled with no increase in apoptosis or inflammation (Gautam et al., 2018).

Because recent studies indicated that several Cyps are regulated by a HFD (Deol et al., 2015; Finn et al., 2009), we also investigated the expression of several Cyps (and PXR). With the exception of the knocked out Cyp3a members, there are only a few Cyps that showed changes in gene expression and only Cyp2b protein expression in males (2.8X) was increased significantly; consistent with increases in Cyp2b9, a gene controlled by the metabolic receptors, FoxA2 and CAR, or potentially Cyp2b10, a gene controlled by the nuclear receptors CAR and PXR (Appendix B-7) (Hashita et al., 2008; Hernandez et al., 2009; Jarukamjorn et al., 2001; Mota et al., 2011).
**Table 3.4:** Dysregulation of energy and lipid metabolism genes in Cyp3a-null mice after eight weeks of HFD treatment.

<table>
<thead>
<tr>
<th>Genes</th>
<th>FEMALES</th>
<th>MALES</th>
<th>FEMALES</th>
<th>MALES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Cyp3a-null</td>
<td>WT</td>
<td>Cyp3a-null</td>
</tr>
<tr>
<td>Acox</td>
<td>1.00±0.111</td>
<td>0.885±0.145</td>
<td>1.00±0.140</td>
<td>0.623±0.111</td>
</tr>
<tr>
<td>ApoE</td>
<td>1.00±0.160</td>
<td>1.411±0.273</td>
<td>1.00±0.156</td>
<td>0.437±0.107*</td>
</tr>
<tr>
<td>Car</td>
<td>1.00±0.116</td>
<td>1.769±0.430</td>
<td>1.00±0.198</td>
<td>0.564±0.052</td>
</tr>
<tr>
<td>Cpt1a</td>
<td>1.00±0.093</td>
<td>1.152±0.196</td>
<td>1.00±0.129</td>
<td>0.510±0.083**</td>
</tr>
<tr>
<td>Fasn</td>
<td>1.00±0.152</td>
<td>0.871±0.164</td>
<td>1.00±0.184</td>
<td>0.450±0.067*</td>
</tr>
<tr>
<td>Fatp-1</td>
<td>1.00±0.312</td>
<td>2.259±0.637</td>
<td>1.00±0.217</td>
<td>2.088±0.416*</td>
</tr>
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<td>Foxa2</td>
<td>1.00±0.182</td>
<td>1.354±0.247</td>
<td>1.00±0.202</td>
<td>1.052±0.137</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.00±0.150</td>
<td>2.018±0.454*</td>
<td>1.00±0.222</td>
<td>0.640±0.141</td>
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<td>Pepck1</td>
<td>1.00±0.193</td>
<td>1.199±0.327</td>
<td>1.00±0.298</td>
<td>0.397±0.105</td>
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<tr>
<td>Ppara</td>
<td>1.00±0.122</td>
<td>1.201±0.164</td>
<td>1.00±0.211</td>
<td>0.970±0.168</td>
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<tr>
<td>Pparγ</td>
<td>1.00±0.053</td>
<td>1.296±0.207</td>
<td>1.00±0.327</td>
<td>0.878±0.153</td>
</tr>
<tr>
<td>Pxr</td>
<td>1.00±0.158</td>
<td>1.919±0.386*</td>
<td>1.00±0.234</td>
<td>0.407±0.075*</td>
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<td>Srebp1</td>
<td>1.00±0.127</td>
<td>0.967±0.213</td>
<td>1.00±0.145</td>
<td>0.567±0.058*</td>
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<tr>
<td>Srebp1a</td>
<td>1.00±0.254</td>
<td>1.118±0.346</td>
<td>1.00±0.188</td>
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<tr>
<td>Srebp1c</td>
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<td>1.355±0.266</td>
<td>1.00±0.160</td>
<td>0.536±0.131</td>
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<tr>
<td>Cyp2b9</td>
<td>1.00±0.137</td>
<td>1.495±0.154*</td>
<td>1.00±0.340</td>
<td>4.250±3.585</td>
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<tr>
<td>Cyp2b10</td>
<td>1.00±0.210</td>
<td>1.283±0.176</td>
<td>1.00±0.202</td>
<td>1.819±0.331</td>
</tr>
</tbody>
</table>

Data represented as relative mean +/- SEM (n = 7 or 8). Statistical significance determined by Student’s t-test. * Indicates a p-value ≤ 0.05 and ** Indicates a p-value ≤ 0.01.
3.6 Discussion

Cyp3a-null female mice gained 50% less weight than WT female mice and Cyp3a-null male mice are slightly heavier than their WT counterparts following treatment with a HFD (diet-induced obesity) (Fig. 3.1). This indicates gender specific responses to the HFD and demonstrates a role of Cyp3a in the metabolism and utilization of fatty acids. Diverse gender responses might be expected as several Cyp3a members are sexually dimorphic in the liver. For example, *Cyp3a41* and *Cyp3a44* are both female specific (Anakk et al., 2006; Hernandez et al., 2006; Hernandez et al., 2009) and human CYP3A4 is expressed slightly more in females than males (Wolbold et al., 2003; Zanger et al., 2013).

Cyp3a-null female mice reacted to a glucose challenge more rapidly than WT mice, which is consistent with their reduced weight gain and 30% lower WAT weight. In addition, plasma adiponectin concentrations are higher and serum B-OHB concentrations are lower in Cyp3a-null female mice, which is consistent with lower WAT, faster recovery from glucose, and rapid β-oxidation (Ceddia et al., 2005; Meidenbauer et al., 2014; Sweeney et al., 2004). Adiponectin plays a central role as it increases hepatic glucose and fatty acid utilization, and in turn protects the liver from fatty liver disease (Kadowaki et al., 2006).

Interestingly, resveratrol protects from alcohol-induced fatty liver disease in part by increasing fatty acid oxidation as observed through greater B-OHB coupled with enhanced adiponectin, and increased expression of *Cpt1a* as well as other genes associated fatty acid oxidation (Ajmo et al., 2008; Yao et al., 2014). Adiponectin also decreases lipid uptake by the liver in part by decreasing CD36 and this could provide a mechanism for compensating for a diet rich in fatty acids (Tarn et al., 2014). In addition, the Cyp3a
inhibitor naringin found in grapefruit and other citrus also reduces serum B-OHB concentrations (Kwatra et al., 2012; Murunga et al., 2016), is associated with reduced adiposity, weight gain in mice and humans, and increased Cptla expression through Ppara activation coupled with Srebp-1 inhibition (Alam et al., 2014; Cho et al., 2011; Murphy et al., 2014). However, Cptla expression was much greater in Cyp3a-null mice than WT mice prior to the HFD, but no significant difference in Cptla or other energy-related genes was found after a HFD in female mice. It is also possible that the liver is not the only organ in which Cyp3a plays a role in the metabolism and utilization of fatty acids in HFD-fed female mice as several of the perturbed parameters are regulated by WAT, kidney and skeletal muscle (Auguet et al., 2014; Ceddia et al., 2005; Reid et al., 2008).

In contrast to females, Cyp3a-null male mice gained weight relative to WT mice; albeit a relatively small amount. In addition, Cyp3a-null male mice have increased liver lipids including a 1.5X increase in liver triglycerides (Fig. 3.5B) and 2X increase in total polar lipids (Table 3.3). Specific lipid groups (PI, PS, SM) and long chain fatty acids that are 38-40 carbons are increased in the livers of Cyp3a-null male mice relative to WT mice (Fig. 3.6). An increase in long-chain fatty acids is consistent with increased storage and increased metabolism (Montgomery et al., 2013), perturbations in the transport of long-chain fatty acids is associated with liver disease (Odaib et al., 1998), and increased SM is associated with obesity (Choi et al., 2015; Iqbal et al., 2017). However, while SM is associated with obesity, PI is associated with anti-obesity effects by regulating hepatic lipid metabolism genes (Shimizu et al., 2010). Therefore, most but not all lipid markers are consistent with fatty liver disease and obesity.
Furthermore, down-regulation of Cpt1a, ApoE, Fasn, Pxr and Srebp-1 genes indicates a down-regulation of lipid metabolism associated genes while Fatp1 is up-regulated and indicates an increase in fatty acid transport genes involved in accumulation (Kohjima et al., 2007; Xie et al., 2010). These changes in gene expression are consistent with increased liver lipids (Anstee et al., 2006; Vallim et al., 2010). Interestingly, a drop in Srebp1 was also observed in steatotic HRN mice lacking all hepatic CYP activity (Kishi et al., 2005), probably due to the increased retention of unsaturated fatty acids in the liver (Hannah et al., 2001). Typically, increases in free fatty acids are associated with increases in Srebp1 and Fasn (Zhang et al., 2011) leading to increased Srebp1c activation that causes increased deposition of fat in the liver (Knebel et al., 2012). This suggests that there are other forces regulating the hepatic increase in triglycerides in Cyp3a-null mice or the down-regulation of Srebp is a compensatory response to the increased liver lipids. Overall, most but not all of these lipid markers are consistent with increased weight, liver triglycerides and polar lipids, and it is likely that decreased Cyp3a-mediated liver metabolism plays a role in the increased liver lipids through changes in metabolism.

In addition to xenobiotics, CYP3A isoforms are involved in the metabolism of steroids, bile acids, and unsaturated fatty acids (Guengerich, 2002; Hafner et al., 2011). CYP3A4 has been shown to metabolize fatty acids such as arachidonic acid to generate epoxyeicosatrienoic acids (EETs) that have anti-inflammatory effects (Thompson et al., 2012). High-fat diets generate an endogenous fatty acid molecule, anandamide that can be converted to arachidonic acid by fatty acid amide hydrolase that can be further epoxygenated by Cyp3a to EETs. Anandamide and Cyp3a-mediated metabolites (Pratt-
Hyatt et al., 2010) bind cannabinoid receptors such as cannabinoid receptor 1 (CB1) in order to alter nociception, cognition, memory and orexigenic effects in the peripheral and central nervous system; however, there were no differences in food consumption between WT and Cyp3a-null mice. For example, CB1 regulates adiponectin activity in obese mice, and Rimonabant, a CB1 inverse agonist causes weight loss, increases Cpt1a, while decreasing Fasn in WT male mice, but not adiponectin-null mice (Tarn et al., 2014). Cannabinoid receptor 2 (CB2) is associated with immunosuppression and agonists reduce obesity (Zhang et al., 2016). Given that the fatty acid, anandamide and its Cyp3a metabolites are potent activators of CB1/2, it is possible that the loss of Cyp3a plays a role in perturbing fatty acid metabolism and epoxidation, including the production of anandamide and other fatty acid derivatives, that typically would act as signaling molecules (Brizzi et al., 2011; Snider et al., 2009), and reduce obesity.

However, this does not explain why males and females react differently except for the possibility that the gender specific Cyp3a members in mice differentially metabolize steroids, arachidonic acid, anandamide, and other fatty acids. Murine 6β-hydroxylation of testosterone is significant higher in females than males demonstrating the higher Cyp3a activity in females than males (Kumar et al., 2017). Of course, this difference in 6β-hydroxylation is lost in Cyp3a-null mice, indicating the differences between males and females for obesity and NAFLD is not directly caused by Cyp3a metabolism of steroids, but may be due to the metabolic differences that lead to sexually dimorphic differences in Cyp3a expression. It is possible that there are distinct differences in Cyp3a-mediated fatty acid metabolism in males compared to females as females express different Cyp3a
isoforms, primarily Cyp3a41 and Cyp3a44. However, the substrate profiles for these murine isoforms are not known and that includes their metabolism of anandamide, arachidonic acid, and other PUFAs.

Gender differences in PXR regulation and feedback within Cyp3a-null mice may also play a role in the sexually dimorphism of weight gain. For example, mPXR is associated with increased weight gain and hPXR with protection from weight gain in male mice with increased Cpt1a and PPARα levels (Spruiell et al., 2014b). In contrast, hPXR activation/presence causes greater weight gain than mPXR in female mice (Spruiell et al., 2014a). Because PXR positively regulates Cyp3a, the PXR studies are consistent with our data in which loss of Cyp3a increased obesity in males and protected females. Therefore, while we don’t know the exact mechanism of the sexually dimorphic weight gain in Cyp3a-null mice, it appears that differential PXR/Cyp3a activation and subsequent regulation of gene expression plays a role.

In summary, the loss of seven Cyp3a genes lead to sexual dimorphic changes during the eight weeks of high fat diet treatment with Cyp3a-null female mice showing a healthier acclimation to a high fat diet through decreased weight gain, higher adiponectin, lower B-OHB levels, and a better response to glucose; while the Cyp3a-null males succumb to increased fatty liver including slightly increased weight, higher hepatic polar lipids and triglycerides, and greater liver weight. Overall, the changes in HFD-induced health parameters in females are consistent with other studies showing that compounds that inhibit Cyp3a or perturb Cyp3a-mediated fatty acid metabolism protect from obesity (Murunga et al., 2016; Zhang et al., 2016), and the increases in weight in males are moderate.
Interestingly, PXR activation and subsequent CYP3A induction is associated with weight gain (Spruiell et al., 2014a). Ultimately, inhibition of CYP activity by environmental compounds or drugs may alter hepatic and non-hepatic lipid levels with different effects in males than females in which males may present increased steatosis and females show a mitigation of the effects of a HFD.

Conflicts of Interest

The authors have no conflicts of interest to declare

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References


Damiri, B., & Baldwin, W. S. (2011, June 4-7, 2011). *RNAi repression of Cyp2b indicates that this subfamily of P450s is involved in fat metabolism.* Paper presented at the Endocrine Society, Boston, MA.


CHAPTER FOUR
CYP2B9/10/13-NULL MALE MICE ARE SUSCEPTIBLE TO HIGH-FAT DIET-INDUCED OBESITY

4.1 Abstract

CYP2B metabolizes pesticides, plasticizers, unsaturated fats and more than 25% of drugs available in the market. Recent studies using the cytochrome P450 oxidoreductase knockout mouse treated with unsaturated fatty acids shows activation of CAR and a profound increase of Cyp2b10 presumably to ameliorate hepatic lipid accumulation. Data from our laboratory using RNAi-mediated Cyp2b-knockdown mouse indicated that the older knockdown mice were heavier and contain greater serum lipid concentrations, especially males. To investigate the role played by CYP2B in lipid metabolism we have developed a CYP2B triple knockout lacking Cyp2b9, 10 and 13 using CRISPR/Cas9. We treated them with a 60% fat diet (HFD) for 10 weeks. We determined physiological, metabolic and molecular level changes in WT and Cyp2b-null mice. Male but not female Cyp2b-null mice weigh approximately 15% more than WT counterparts fed a HFD, primarily due to an increase in white adipose tissue. Serum parameters indicate increased b-hydroxybutyrate in Cyp2b-null HFD-fed mice, a marker of ketoacidosis. In addition, leptin, adiponectin, and cholesterol were increased in HFD-fed Cyp2b-null male mice compared to HFD-fed WT mice. Cholesterol, primarily due to increased HDL is increased in both normal diet and HFD fed male and female mice. Liver triglycerides were significantly higher than their similarly treated WT counterparts (ND and HFD), indicating
a role for Cyp2b in fatty acid metabolism regardless of diet. This probably contributed to the lower levels of triglycerides in Cyp2b-null mice. Overall our data indicates that the repression or chemical inhibition of CYP2B may exacerbate metabolic disorders and cause obesity.

**Keywords:** Non-alcoholic fatty liver disease (NAFLD), P450, Triglycerides, ketoacidosis Obesity.

**Abbreviations:**

- Cyp: Cytochrome P450
- ND: Normal diet
- HFD: High fat diet
- WAT: White adipose tissue
- B-OHB: β-hydroxybutyrate
- CAR: Constitutive androstane receptor
- EET: Epoxyeicosatrienoic acid
- ALT: Alanine aminotransferase
4.2 Introduction

The World Health Organization (WHO) defines obesity as “abnormal or excess accumulation of fat that may impair health”. According to National Center for Health Statistics (NCHS) more than 40% of adults and 18.5% youth are obese in United States as of 2015-2016 (Hales et al., 2017). Factors that cause obesity include poor diet, changes in lifestyle, genetics, and the environment (Choquet et al., 2011; Grun et al., 2009; Romieu et al., 2017). The primary problem is excess food coupled with inactivity. However, data indicates there are other anthropogenic factors, including environmental toxicants that exacerbate obesity by modulating the use and allocation of nutrient resources leading to increased white adipose tissue and obesity (Grun et al., 2009; Hatch et al., 2010; Sharp, 2009; Wahlang et al., 2013a). This led to the terms “obesogen” or “metabolic disruptor” (Grun et al., 2009; Hatch et al., 2010; Wahlang et al., 2013a; Wahlang et al., 2013b) that refers to a new subclass of endocrine disruptors that perturb metabolic signaling, energy, and lipid homeostasis (LeBlanc et al., 2012b; Riu et al., 2011). In turn, the Organization for Economic Cooperation and Development (OECD) considers metabolic disorders one of three critical areas in toxicology in addition to testicular dysgenesis and autism spectral disorders (LeBlanc et al., 2012a).

Chemical exposures shown to cause obesity (Baillie-Hamilton, 2002; Grun et al., 2009), include perinatal exposure to bisphenol A that increases lipogenic gene expression and hepatic steatosis in male rats (Marmugi et al., 2012). Tributyltin activation of PPARγ induces obesity in part through increased adipocyte differentiation (Chamorro-Garcia et al., 2013), and TCDD activation of AhR induces obesity and steatosis through increased
uptake of fatty acids (Angrish et al., 2012; Ayala et al., 2010; Chang et al., 2016). Inactivation of the xenobiotic receptor, the constitutive androstane receptor (CAR), and activation of the pregnane X-receptor (PXR) are also associated with obesity (Dong et al., 2009; Spruiell et al., 2014a; Spruiell et al., 2014b). Furthermore, perturbations in hepatic Cytochrome P450 (CYP) activity were recently associated with lipid accumulation (Finn et al., 2009; Hoek-van den Hill et al., 2015; Hoek-van den Hill et al., 2014; Wang et al., 2005; Zong et al., 2012).

CYPs, primarily in families 1-3, metabolize pharmaceuticals (Zanger et al., 2013), environmental pollutants (Foxenberg et al., 2007) and endobiotics such as fatty acids (Arnold et al., 2010), bile acids and steroids (Waxman, 1988). Several CYP1-3 members are crucial in fatty acid metabolism. For example, CYP3A4 metabolizes arachidonic acid (anandamide), CYP2J2 metabolizes arachidonic acid to epoxyecosatrienoic acid (EET) in cardiomyocytes (Wu et al., 1996), and Cyp2b members metabolize arachidonic acid with high affinity (Capdevila et al., 1990; Keeney et al., 1998). For example, Cyp2b19 expressed by mouse keratinocytes, epoxygenates arachidonic acid to 11,12 and 14,15 EET that plays a vital role in cornification of epidermal cells (Du et al., 2005; Ladd et al., 2003) and CYP3A4 metabolizes arachidonic acid to 13- hydroxyeicosatrienoic acid (HETE), 10-HETE and 7-HETE (Bylund et al., 1998).

Recent studies implicate Cyp2b in obesity. For example, CYP2Bs are inducible by anti-obesity transcription factors (CAR; FoxA2) (Hernandez et al., 2007; Hernandez et al., 2009; Mota et al., 2010; Mota et al., 2011). CAR is a key regulator of Cyp2b, including Cyp2b9 and Cyp2b10 (Hernandez et al., 2009; Mota et al., 2010; Oshida et al., 2015), and
CAR activation by TCPOBOP ameliorates obesity, diabetic activity and fatty liver disease in ob/ob mice, but not ob/ob mice null for CAR (Dong et al., 2009). FoxA2, which is activated by fasting and fatty acids, is inhibited by insulin, implicated in sporadic cases of early onset Type II diabetes, and hepatic FoxA2-null mice are age-dependent obese (Bochkis et al., 2008; Bochkis et al., 2013; Wolfrum et al., 2004). Thus, Cyp2B expression is induced by CAR and Foxa2 during nutritional or metabolic stress, potentially as a protective mechanism. Furthermore, cytochrome P450 oxidoreductase (HRN-null) conditional knockout mice that lack all the CYP activity in the liver show activation of CAR and significant increases in Cyp2B mRNA during treatment with PUFA-rich sunflower oil (Finn et al., 2009). Similarly, treatment with soybean oil (rich in 55% linoleic acid) resulted in weight gain, increased white adipose tissue and glucose intolerance in association with a significant increase in Cyp2b9 and 13 (Deol et al., 2015). Cyp2b9 is also the most highly induced gene in a couple of diet-induced obesity studies (Hoek-van den Hill et al., 2015; Leung et al., 2016). Last, studies in our lab using a Cyp2b RNAi-based knockdown mouse model (Damiri et al., 2012) shows increases in body weight and adiposity with age and a reduced propensity to eliminate corn oil that is rich in PUFAs such as linoleic acid (Damiri, 2011). Taken together, the Cyp2b enzymes are associated with obesity and we predict that lack of Cyp2b either by gene knockout or repression by inhibitors coupled with high-fat diet treatment will perturb lipid metabolism, increase body weight and lead to the development of obesity.

In our study, we have used our newly developed Cyp2b9/10/13-null mice that is missing important hepatic Cyp2B members, Cyp2b9, Cyp2b10 and Cyp2b13 to
demonstrate the significance of Cyp2B in hepatic lipid metabolism. We treated these mice with 60% fat rich diet for 10-weeks. We hypothesize that lack of Cyp2b9, Cyp2b10 and Cyp2b13 will increase susceptibility to high fat diet-induced obesity in Cyp2b9/10/13-null mice compared to wild-type mice. We determined physiological changes such as bodyweight changes and organ weight changes, metabolic changes such as ability to metabolize glucose, insulin tolerance and hormone changes and gene expression changes. Our results show that lack of Cyp2b9,10,13 in mice causes gender-based differences in response to high fat diet. Male Cyp2b9/10/13-null mice show increase in body weight, increase in white adipose tissue weight and increase in liver triglycerides. However, female Cyp2b9/10/13-null did not show increase in body weight but did show increase in liver lipids.

4.3 Materials and Methods:

4.3.1. High-fat diet treatment of Cyp2b9/10/13-null mice

All mice studies followed National Institute of Health guidelines for humane use of research animals and were pre-approved by Clemson University’s Institutional Animal Care and Use Committee (IACUC). Cyp2b9/10/13-null (Cyp2b-null) mice were developed using Crispr/cas 9 technology on C57/Bl6 background mice as described earlier (Kumar et al., 2017). Briefly, the liver predominant Cyp2b genes, Cyp2b10, 13 and 9 present as tandem repeat sequences on chromosome seven, were targeted individually using small guide RNA sequences (sg RNA). Each sgRNA sequence was made up of Cas9 mRNA from Streptococcus pyogenes, 20 nt guide sequence specific for the target gene and 83nt
scaffold sequence. The three sgRNA’s were delivered into the cytoplasm of mouse blastocyst and successful transgenic embryo had 287 kb deletion due to the lack of three Cyp2b genes. The successful homozygous knock-out mice were screened using primers published in our earlier manuscript (Kumar et al., 2017). Wildtype C57/Bl6 were purchased from The Jackson Laboratory at 4 weeks of age (Bar Harbor, ME, USA).

Mice were acclimated for 4-weeks prior to the dietary treatments. Nine-ten week old male and female mice (n=9) from WT and Cyp2b9/10/13-null mice were divided into normal diet (Harlan Madison WI USA) and high-fat diet (TD.06414 Adjusted Calorie Diet (60/Fat)) groups. ND consisted of 18.6% protein, 6.2% fat and 44.2% carbohydrates providing 3.1 Kcal/g. HFD consisted of 60.3% fat (37% saturated fat, 47% monounsaturated fat and 16% polyunsaturated fats), 21.3% carbohydrates and 18.4% protein providing 5.1 Kcal/g.

All mice were fed either the ND or HFD for ten weeks. Feed consumption was measured every other day and mice were weighed every week to determine their weight gain. Following a 4-5 hour fast, fasting plasma glucose concentrations were determined during weeks 2, 5 and 8 with an Alphatrek-2 glucometer (AlphaTRAK, Chicago IL USA). Glucose tolerance tests (GTT) were performed on weeks 5 and 8. At the end of the study mice were anesthetized, blood was collected by heart puncture and euthanized by carbon dioxide asphyxiation. Organs such as liver, kidney, white adipose tissue and brain were excised and weighed. Organs were stored on dry ice and then moved to -80°C freezers or stored in 10% formalin (Fisher, Fair Lawn NJ USA) until further analyses were performed. Livers were dissected into five fractions and snap frozen in liquid nitrogen for microsome
preparation, RNA extraction and lipidomics. Liver sections for histopathology were stored in 10% formalin (Fisher) while the remaining liver was stored on dry ice and stored in -80°C until required. A timeline of experimental procedures performed on the mice is provided (Appendix C-I).

4.3.2. Glucose tolerance and insulin tolerance tests

Glucose tolerance tests were performed during weeks 5 and 8. Briefly, the mice were fasted for 5 hr and then injected i.p. with 1g/kg of their bodyweight with D-glucose (Sigma Ultra, St. Louis MO USA). The blood glucose levels were measured using Alphatrek-2 glucometer (AlphaTRAK) in 30 min intervals for 2 hours for GTT. The mice were fasted for 5 hr and then injected i.p. with 0.75 U/kg of their bodyweight of novolin (Nova Nordisk, Bagsvaerd, DK) for ITT. The blood glucose levels were measured using Alphatrek-2 glucometer (Alpha TRAK) in 30 min intervals for 1 hour.

4.3.4. Sample preparation

RNA was extracted from the liver using BioRad spin columns and DNAse (Bio-Rad, Hercules, CA) digestion to remove genomic DNA by following the manufacturer’s instructions. RNA concentrations were determined spectrophotometrically by measuring absorbance at 260/280 nm (Molecular Devices, Ramsey, MN USA). cDNA was prepared by treating 2 µg of RNA with 200 units of MMLV, 10mM dNTP and 0.5mg of random primers (Promega corporation, Madison WI USA). Microsomes were prepared by homogenizing frozen livers in 3ml buffer followed by differential centrifugation as described previously (Van der Hoeven et al., 1974). Protein concentration was determined using Bradford reagent (Bio-Rad).
4.3.5. Histopathological analysis

A clean slice of liver was made following necropsy and was placed in 10% formalin (Fisher). Hematoxylin and Eosin (H&E) and oil red O staining was performed at Colorado Histoprep (Fort Collins, CO USA). The pathological study was done on these slides at Colorado Histoprep (Fort Collins).

4.3.6. Quantitative real time polymerase chain reaction (qPCR)

Gene expression changes were determined by qPCR using previously published primers (Hernandez et al., 2009). Samples were diluted 1:5 and amplified in triplicates using 96 well plates on the IQ™ real time system (BioRad) with 0.25X RT² Sybr Green (Qiagen Frederick, MD USA) as the intercalating dye. Relative gene expression changes were quantified by comparing the gene of interest to geometric mean of 18S and glyceraldehyde 3-phosphate dehydroygenase (GAPDH) as the reference genes and using Muller’s equation as previously described (Muller et al., 2002). qPCR efficiency was determined with a standard curve made from a sample mixture containing all cDNA samples diluted 1:1, 1:4, 1:16, 1:64, 1:256, and 1:1024 as described previously (Hernandez et al., 2009).

4.3.7. Immunoblots

Immunoblots were performed using 30 μg of microsomal protein separated on 12% SDS-polyacrylamide gels (BioRad). Protein was transferred onto 0.2μm polyvinylidene difluoride (PVDF) membrane and were recognized using polyclonal antibodies to Cyp3a (Chemicon International, Temecula CA USA), Cyp2b (previously developed in house)(Hernandez et al., 2009), Cyp4a (Thermofisher Scientific, Rockford IL USA). β -
actin was used as the reference protein. Chemiluminescent western blot detection was done using alkaline phosphatase conjugated secondary antibodies, where in anti-mouse IgG (Immunostar, Bio-Rad) was used to determine β-actin and anti-rabbit IgG (Immunostar, Bio-Rad) was used to determine CYPs. Protein was quantified by densitometry (Quality One, BioRad, Hercules, CA).

4.3.8. Serum lipid panel

Blood samples were collected by heart puncture and incubated at room temperature for 30 min followed by centrifugation at 6000 rpm for 10 minutes. Serum samples were transferred into fresh tubes and 100µl aliquots were shipped on dry ice to Baylor College of Medicines Comparative Pathology Laboratory (Houston, TX) for determination of serum cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), high density lipoprotein (HDL) and low density lipoprotein (LDL).

4.3.9. Serum concentrations of adiponectin, leptin, β-hydroxybutyrate and liver triglycerides

Serum adiponectin and leptin concentrations were determined using an EIA kit from Bertin Pharma (Montigny Le Bretonneux, FR), and serum β-hydroxybutyrate concentrations were determined by using a colorimetric kit from Cayman Chemical Co (Ann Arbor, MI). Liver triglyceride concentrations were quantified following organic extraction (Chapter three) with a colorimetric kit (Cayman Chemical, Ann Arbor, MI).
4.3.10. Statistical Significance

Data are presented as mean ± SEM (n = 8-9). Statistical analysis was performed by one-way and two-way ANOVA followed by Fisher’s LSD as the post-hoc using Graphpad Prism version 6. A p-value ≤ 0.05 was considered statistically significant.

4.4 Results

4.4.1. High-fat diet increases body and white adipose tissue weight

Cyp2b9/10/13-null male mice fed a HFD weighed 15% more than WT male mice fed a HFD (Fig. 4.1A). Cyp2b9/10/13-null male mice weighed significantly more after only 4-weeks of HFD treatment and stayed heavier for the rest of the study period compared to WT male mice fed a HFD (Fig. 4.1A). Much of this weight gain is from increased abdominal and inguinal white adipose tissue, which increased 55% more in the Cyp2b9/10/13-null HFD mice compared to the WT HFD treated mice (Table 4.1A). Exposure to a ND for 10-weeks did not cause a significant increase in weight gain in Cyp2b9/10/13-null mice compared to WT mice. Mice fed a HFD consumed more calories compared to their normal diet counterparts (Appendix C-2A); however, Cyp2b9/10/13-null mice had similar calorie intake compared to WT mice when examined by diet (HFD or ND). Therefore, alterations in dietary intake do not explain increased weight gain in Cyp2b9/10/13-null mice.

Cyp2b9/10/13-null and WT female mice fed a HFD gained significantly more weight and had higher white adipose tissue mass compared to mice fed a ND. However, genotype had no effect on body weight gain or white adipose tissue mass, indicating that
the loss of Cyp2b9/10/13 did not cause obesity in female mice; only male mice (Fig. 4.1B; Table 4.1B).

Table 4.1B: Organ weights determined in WT and Cyp2b9/10/13-null mice after 10-weeks of dietary treatment

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Body weight</th>
<th>WAT</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-ND M</td>
<td>26.48±0.57</td>
<td>0.51±0.06</td>
<td>1.23±0.03</td>
<td>0.34±0.01</td>
<td>0.48±0.03</td>
</tr>
<tr>
<td>Cyp2b-null ND M</td>
<td>25.75±0.39</td>
<td>0.69±0.06</td>
<td>1.08±0.03**</td>
<td>0.31±0.01</td>
<td>0.46±0.01</td>
</tr>
<tr>
<td>WT-HFD M</td>
<td>30.43±0.92**</td>
<td>1.96±0.22***</td>
<td>1.06±0.04**</td>
<td>0.37±0.01</td>
<td>0.44±0.00</td>
</tr>
<tr>
<td>Cyp2b-null HFD M</td>
<td>34.12±0.88***</td>
<td>3.04±0.19***</td>
<td>1.08±0.04</td>
<td>0.37±0.02**</td>
<td>0.43±0.01</td>
</tr>
</tbody>
</table>

Fig. 4.1: Changes in body weight during the 10-weeks of dietary treatments. Body weight of A) male and B) female, WT, and Cyp2b9/10/13-null mice were monitored during the 10-week feeding study. Male but not female Cyp2b-null mice show increased weight during HFD treatments. Data are represented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher’s LSD as the post-hoc test (n= 8-9). An ‘a’ indicates WT-ND are different than Cyp2b9/10/13-null-ND, ‘b’ indicates WT-ND are different than WT-HFD, ‘c’ indicates Cyp2b9/10/13-null-ND are different than Cyp2b9/10/13-null-HFD, ‘d’ indicates WT-HFD are different than Cyp2b9/10/13-null-HFD.
4.4.2. High-fat diets decrease glucose tolerance

Fasting plasma glucose levels were determined during weeks 5 and 8. Cyp2b9/10/13-null male mice fed a ND show a 20% increase in fasting glucose concentrations compared to their WT counterparts on both weeks 5 and 8. Perturbation in fasting glucose concentrations was consistent and male specific (Appendix C-3A). HFD did not significantly increase fasting glucose with the exception of Cyp2b9/10/13-null females fed a HFD compared to Cyp2b-null females fed a ND.

Glucose tolerance tests (GTT) were done to determine if the loss of hepatic Cyp2b members reduced the mouse’s ability to respond to a glucose challenge as a biomarker of metabolic disease. In males, a HFD increased response times leading to increased glucose retention in the serum. In addition, the Cyp2b9/10/13-null male mice fed HFD were not able to lower their serum glucose concentrations back to normal (at 2 hr) in comparison to the other groups such as the WT male mice fed a HFD on week 5, indicating perturbed glucose tolerance. However, this effect was not significant overall by area under the curve.

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Body weight</th>
<th>WAT</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-ND F</td>
<td>20.47±0.44</td>
<td>0.36±0.06</td>
<td>0.87±0.03</td>
<td>0.26±0.01</td>
<td>0.44±0.00</td>
</tr>
<tr>
<td>Cyp2b-null ND F</td>
<td>20.29±0.22</td>
<td>0.33±0.03</td>
<td>0.83±0.02</td>
<td>0.25±0.01</td>
<td>0.45±0.00</td>
</tr>
<tr>
<td>WT-HFD F</td>
<td>23.17±0.87ab</td>
<td>0.95±0.15ab</td>
<td>0.84±0.03</td>
<td>0.26±0.01</td>
<td>0.45±0.00</td>
</tr>
<tr>
<td>Cyp2b-null HFD F</td>
<td>24.15±0.93abc</td>
<td>1.22±0.24abc</td>
<td>0.87±0.04</td>
<td>0.28±0.01c</td>
<td>0.45±0.01</td>
</tr>
</tbody>
</table>

Data represented as mean +/- SEM (n = 8/9). Statistical significance determined by two-way ANOVA followed by Fisher’s LSD as the post-hoc test. The test for interaction between treatments and serum lipids is significant (p < 0.0001).

‘a’ indicates WT ND different than Cyp2b9/10/13-null ND
‘b’ indicates WT ND different than WT HFD
‘c’ indicates Cyp2b9/10/13-null ND different than Cyp2b9/10/13-null HFD
‘d’ indicates WT HFD different than Cyp2b9/10/13-null HFD,
No asterisk indicates a p-value ≤ 0.05, * indicates a p-value ≤ 0.01, ** indicates a p-value ≤ 0.0001 and *** indicates a p-value ≤ 0.00001.
(AUC), nor was this effect repeatable on week 8. Instead we observed that Cyp2b-null ND males showed decreased glucose tolerance relative to WT ND-fed males at 30 minutes; however, these mice responded normally after this initial time point. We also performed insulin tolerance tests on ND treated WT and Cyp2b9/10/13-null male mice due to the minor differences in the 30-minute blood glucose levels on week 8, but there were no significant differences in insulin sensitivity between WT and Cyp2b9/10/13-null males (Appendix C-4).

In females, a HFD had no significant effects on glucose clearance until week 8 and the lack of Cyp2b enzymes had no effect on glucose tolerance (Fig. 4.2B). Overall, Cyp2b9/10/13-null female mice showed no particular changes in glucose tolerance compared to WT mice. Cyp2b9/10/13-null male mice showed changes in fasting glucose and some minor differences in serum glucose clearance time in males; however, some of these measured changes were not consistent between weeks 5 and 8 (Fig. 4.2).
**Fig. 4.2:** Glucose tolerance tests shows significant changes in Cyp2b9/10/13-null male mice. Glucose tolerance tests were performed on weeks 5 (A) and 8 (B) as described in the Materials and Methods. Results are shown for males and females over the time course of the assay and as area under the curve (AUC). Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher’s
LSD as the post-hoc test (n= 8-9). ** Indicates a p-value < 0.01. An ‘a’ indicates WT-ND are different than Cyp2b9/10/13-null-ND, ‘b’ indicates WT-ND are different than WT-HFD, ‘c’ indicates Cyp2b9/10/13-null-ND are different than Cyp2b9/10/13-null-HFD, ‘d’ indicates WT-HFD are different than Cyp2b9/10/13-null-HFD.

4.4.3. Lack of hepatic Cyp2b9/10/13 increases hepatic triglycerides

Cyp2b9 and Cyp2b10 are induced during high fat diet treatment, presumably to help clear fatty acids from the liver (Finn et al., 2009; McGregor et al., 2013). The lack of Cyp2b9/10/13 in the knockout mice increased liver triglycerides by 2.3X in the ND-fed Cyp2b9/10/13-null male mice compared to ND-fed WT males (Fig. 4.3A). HFD exacerbated liver triglyceride concentrations; however, there were no differences between HFD-fed Cyp2b9/10/13-null male mice and WT male mice (Fig. 4.3A). In females, HFD-fed mice had significant increases in liver triglycerides compared to ND-fed mice; however, genotype had little effect on liver triglycerides (Fig. 4.3B). Histopathological analysis did not show significant morphological changes indicative of fat deposition either by H&E or Oil Red O overall with the exception of a few mice that showed increased Oil Red O staining indicative of increased liver lipids (Fig. 4.3CD). Surprisingly, liver weight was decreased in all male HFD groups and in the male Cyp2b9/10/13-null mice compared to WT ND-fed mice (Table 4.1A).
Fig. 4.3: Liver triglyceride concentrations are significantly increased in Cyp2b9/10/13-null mice. Liver triglycerides were extracted and determined as described in the Materials and Methods using commercial kits. Histopathology analysis using H&E staining procedure did not show any significant changes between the genotypes (C-D). Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher’s LSD as the post-hoc test (n= 8-9). * indicates a p-value < 0.05, ** indicates p-value < 0.001 and *** indicates p-value < 0.0001. An ‘a’ indicates WT-ND are different than Cyp2b9/10/13-null-ND, ‘b’ indicates WT-ND are different than WT-HFD, ‘c’ indicates Cyp2b9/10/13-null-ND are different than Cyp2b9/10/13-null-HFD, ‘d’ indicates WT-HFD different than Cyp2b9/10/13-null-HFD.
4.4.4. Serum lipids were perturbed in Cyp2b9/10/13-null mice

Serum triglyceride levels were significantly decreased in Cyp2b9/10/13-null male mice compared to WT mice following ND (28%) or HFD (25%) treatments (Table 4.2A), in contrast to the increased triglyceride levels found in the liver (Fig. 4.3A). This indicates either decreased metabolism and distribution of hepatic lipids or increased uptake of serum lipids into the liver. Female mice also showed a similar decrease (29%) in serum triglycerides in ND-Cyp2b9/10/13-null mice compared to ND-WT mice (Table 4.2); however, this was not statistically significant. In addition, HFD-fed Cyp2b9/10/13-null female mice show a 31% increase in serum triglycerides compared to HFD-fed WT mice.

In contrast, serum cholesterol and HDL increased 14% and 18%, respectively, in ND-fed Cyp2b9/10/13-null male mice compared to ND-fed WT male mice. Serum cholesterol and HDL increased 8% and 12%, respectively, in HFD-fed Cyp2b9/10/13-null male mice compared to HFD-fed WT male mice. (Table 4.2A). Despite lower weight gain and white adipose tissue mass, females showed greater changes in serum cholesterol and HDL concentrations. HFD-fed Cyp2b9/10/13-null female mice significantly increased cholesterol (19%) and HDL (106%) and cholesterol (19%) was increased though not statistically significant compared to HFD-fed WT mice (Table 4.2B). There were no significant changes in LDL or VLDL serum levels only in the relatively healthy HDL serum concentrations. In addition, ALT, a biomarker of liver damage, was increased only in Cyp2b9/10/13-null females fed a HFD, indicating that these mice suffered some liver damage such as apoptosis or necrosis. Overall, serum lipid concentrations were perturbed
in Cyp2b9/10/13-null mice often regardless of dietary treatment, indicating that Cyp2b members are crucial in normal lipid metabolism.

**Table 4.2:** Serum lipid levels in Cyp2b9/10/13-null compared to WT mice treated with normal diet (ND) or high fat diet (HFD)

<table>
<thead>
<tr>
<th>Lipid panel</th>
<th>WT ND M</th>
<th>Cyp2b-null ND M</th>
<th>WT HFD M</th>
<th>Cyp2b-null HFD M</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>17.00 ± 1.48</td>
<td>17.80 ± 0.97</td>
<td>15.00 ± 0.55</td>
<td>14.00 ± 1.00</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>103.80 ± 1.63</td>
<td>118.60 ± 6.27**</td>
<td>170.4 ± 7.00***</td>
<td>182.75 ± 3.61***d</td>
</tr>
<tr>
<td>HDL</td>
<td>97.00 ± 2.21</td>
<td>114.02 ± 4.45***</td>
<td>150.76 ± 6.24***</td>
<td>168.60 ± 4.94**<em>d</em></td>
</tr>
<tr>
<td>LDL</td>
<td>10.98 ± 0.58</td>
<td>16.34 ± 1.09</td>
<td>26.34 ± 2.46b**</td>
<td>30.20 ± 1.57c</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>80.40 ± 7.88</td>
<td>58.00 ± 5.93<em>a</em>**</td>
<td>69.60 ± 3.96b</td>
<td>52.25 ± 3.15d*</td>
</tr>
<tr>
<td>VLDL</td>
<td>16.04 ± 1.59</td>
<td>11.56 ± 1.19a</td>
<td>13.94 ± 0.80</td>
<td>10.45 ± 0.61</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lipid panel</th>
<th>WT ND F</th>
<th>Cyp2b-null ND F</th>
<th>WT HFD F</th>
<th>Cyp2b-null HFD F</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>20.20 ± 1.69</td>
<td>17.80 ± 0.80</td>
<td>37.20 ± 8.49</td>
<td>52.00 ± 27.91c</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>76.40 ± 3.70</td>
<td>77.40 ± 1.94</td>
<td>98.00 ± 12.63</td>
<td>116.60 ± 8.52c</td>
</tr>
<tr>
<td>HDL</td>
<td>72.48 ± 4.09</td>
<td>77.36 ± 2.57</td>
<td>54.18 ± 22.92</td>
<td>109.98 ± 9.00cd**</td>
</tr>
<tr>
<td>LDL</td>
<td>8.68 ± 1.82</td>
<td>10.20 ± 1.46</td>
<td>8.58 ± 3.24</td>
<td>12.48 ± 0.59</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>80.80 ± 17.74</td>
<td>57.40 ± 12.70</td>
<td>78.40 ± 18.58</td>
<td>102.40 ± 17.69c*</td>
</tr>
<tr>
<td>VLDL</td>
<td>16.16 ± 3.53</td>
<td>11.46 ± 2.52</td>
<td>15.68 ± 3.75</td>
<td>20.48 ± 3.55</td>
</tr>
</tbody>
</table>

Data represented as mean ±/− SEM (n = 5). Statistical significance determined by two-way ANOVA followed by Fisher’s LSD as the post-hoc test. The test for interaction between treatments and serum lipids is significant (p < 0.0001).

‘a’ indicates WT ND different than Cyp2b9/10/13-null ND.

‘b’ indicates WT ND different than WT HFD.

‘c’ indicates Cyp2b9/10/13-null ND different than Cyp2b9/10/13-null HFD.

‘d’ indicates WT HFD different than Cyp2b9/10/13-null HFD.

No asterisk indicates a p-value ≤ 0.05 and * indicates a p-value ≤ 0.01, ** indicates a p-value of ≤ 0.0001.

4.4.5. Serum concentrations of adiponectin, leptin and β-hydroxybutyrate:

Adiponectin was significantly increased in HFD-fed Cyp2b9/10/13-null males compared to their normal diet counterparts and HFD-fed WT males by 13% and 17% (Fig.
The significant increase in white adipose tissue weight by 55% in the HFD-fed Cyp2b9/10/13-null males (Table 4.1A) could have increased the amount of adiponectin in these mice. ND-fed Cyp2b9/10/13-null females had 16% significant increase in adiponectin levels compared to ND-fed WT females (Fig. 4.4A). But HFD-fed Cyp2b9/10/13-null females had 25% decrease in adiponectin compared to ND-fed Cyp2b9/10/13-null females and this may be due to significant increase in WAT in the HFD-fed Cyp2b9/10/13-null females compared to ND-fed Cyp2b9/10/13-null females.

Leptin regulates metabolic responses for satiety and energy expenditure during starved and fed states, but HFD consumption increases leptin levels and leads to the development of leptin resistance (Kahn et al., 2000). We observed a similar trend of significant increase in serum leptin levels in HFD fed mice compared to their ND counterparts in both the genders, however, Cyp2b9/10/13-null males on HFD had 75.5% more serum leptin compared to WT males on HFD (Fig. 4.4B).

Beta-hydroxybutyrate (B-OHB) is generated by the liver from fatty acids during prolonged exercise or starvation to meet the energy requirements and compensate for lower carbohydrate levels. B-OHB can be utilized as energy source by active tissues such as brain and muscles. Cyp2b9/10/13-null males fed a HFD had significant increase in B-OHB levels by 2.3-fold compared to WT males on HFD (Fig. 4C). The significant increase in B-OHB could be related to significant increase in WAT in the Cyp2b9/10/13-null males on HFD, which could provide fatty acids to fuel ketogenesis in the liver.
Fig. 4.4: Serum adiponectin, leptin and β-hydroxybutyrate concentrations in WT and Cyp2b9/10/13-null mice. Commercial kits were used to determine (A) adiponectin, (B) leptin and (C) β-hydroxybutyrate concentrations in serum samples. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher’s LSD as the post-hoc test (n= 8-9). * Indicates a p-value < 0.05, ** indicates a p-value < 0.01, *** indicates a p-value < 0.001 and **** indicates p-value < 0.0001. An ‘a’ indicates WT-ND are different than
Cyp2b9/10/13-null-ND, ‘b’ indicates WT-ND are different than WT-HFD, ‘c’ indicates Cyp2b9/10/13-null-ND are different than Cyp2b9/10/13-null-HFD, ‘d’ indicates WT-HFD are different than Cyp2b9/10/13-null-HFD.

4.4.6. Gene expression changes:

Cyp2a4 increased by 4.2X in ND-fed Cyp2b9/10/13-null male mice compared to ND-fed WT male mice that is consistent with our earlier studies characterizing Cyp2b9/10/13-null mice (Kumar et al., 2017). This similar comparison was not statistically significant between the HFD-fed WT and HFD-fed Cyp2b9/10/13-null male mice (Table 4.3A). However, Cyp2a4 was significantly up-regulated in ND-fed Cyp2b9/10/13-null female mice by 2.6X and HFD-fed Cyp2b9/10/13-null female mice by 1.8X compared to their corresponding WT female counterparts (Table 4.3B). We also performed western blots to determine compensatory changes in Cyp protein expression but there were no significant changes in Cyp proteins, except for the down regulation of Cyp2b protein in the Cyp2b9/10/13-null mice compared to the WT male mice (Appendix C-5).

We observed a significant increase in FoxO1 gene expression in the HFD-fed Cyp2b9/10/13-null male mice compared to HFD-fed WT male mice by more than 2.8X (Table 4.3A). FoxO1 induces adipogenesis along with regulating gluconeogenesis. Therefore, increases in FoxO1 may be associated with higher liver triglycerides and the increase in WAT in HFD-fed Cyp2b9/10/13-null male mice (Table 4.1A).

CD68 and Col27a1, biomarkers associated with innate immune response or inflammation and tissue growth and repair, respectively (Pace et al., 2003; Pietilainen et al., 2006) were perturbed in the HFD-fed Cyp2b9/10/13-null mice compared to their HFD-fed WT mice in both males and females. Col27a1 was almost exclusively induced in the
Cyp2b9/10/13-null mice while female showed induction of Col27a1 and down-regulation of CD68. (Table 4.3AB). These genes are early markers of inflammation and tissue repair and suggest that liver damage is starting to occur in the HFD-fed female mice and Cyp2b9/10/13-null HFD-fed male mice (Table 4.3AB).

Table 4.3: Changes in gene expression in (A) male and (B) female Cyp2b9/10/13-null mice compared to WT mice fed a normal diet (ND) or a high-fat diet (HFD)

<table>
<thead>
<tr>
<th>Genes</th>
<th>WT ND M</th>
<th>Cyp2b-null ND M</th>
<th>WT HFD M</th>
<th>Cyp2b-null HFD M</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68</td>
<td>1.00 ± 0.18</td>
<td>2.00 ± 1.28</td>
<td>1.39 ± 0.27</td>
<td>10.19 ± 2.50***</td>
</tr>
<tr>
<td>Col27a1</td>
<td>1.00 ± 0.20</td>
<td>1.34 ± 0.36</td>
<td>0.94 ± 0.07</td>
<td>2.83 ± 0.98</td>
</tr>
<tr>
<td>Cpt1a</td>
<td>1.00 ± 0.32</td>
<td>3.07 ± 1.13</td>
<td>4.48 ± 0.83</td>
<td>2.73 ± 1.00</td>
</tr>
<tr>
<td>Cyp2a4</td>
<td>1.00 ± 0.32</td>
<td>5.16 ± 1.99*</td>
<td>2.07 ± 0.57</td>
<td>3.92 ± 1.38</td>
</tr>
<tr>
<td>Fasn</td>
<td>1.00 ± 0.13</td>
<td>1.23 ± 0.40</td>
<td>0.94 ± 0.14</td>
<td>1.59 ± 0.59</td>
</tr>
<tr>
<td>Foxo1</td>
<td>1.00 ± 0.21</td>
<td>1.16 ± 0.16</td>
<td>0.98 ± 0.20</td>
<td>2.81 ± 0.57***</td>
</tr>
<tr>
<td>Ppara</td>
<td>1.00 ± 0.40</td>
<td>1.68 ± 0.63</td>
<td>0.81 ± 0.15</td>
<td>2.21 ± 1.17</td>
</tr>
<tr>
<td>Srebp1</td>
<td>1.00 ± 0.49</td>
<td>1.56 ± 0.59</td>
<td>1.91 ± 0.53</td>
<td>4.00 ± 1.91</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genes</th>
<th>WT ND F</th>
<th>Cyp2b-null ND F</th>
<th>WT HFD F</th>
<th>Cyp2b-null HFD F</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68</td>
<td>1.00 ± 0.26</td>
<td>0.39 ± 0.14</td>
<td>5.32 ± 1.69*</td>
<td>0.26 ± 0.04***</td>
</tr>
<tr>
<td>Col27a1</td>
<td>1.00 ± 0.09</td>
<td>1.66 ± 0.31</td>
<td>0.73 ± 0.19</td>
<td>2.72 ± 0.76**</td>
</tr>
<tr>
<td>Cpt1a</td>
<td>1.00 ± 0.34</td>
<td>1.49 ± 0.26</td>
<td>4.14 ± 2.81</td>
<td>1.18 ± 0.21</td>
</tr>
<tr>
<td>Cyp2a4</td>
<td>1.00 ± 0.11</td>
<td>2.63 ± 0.20***</td>
<td>1.14 ± 0.14</td>
<td>2.06 ± 0.29***</td>
</tr>
<tr>
<td>Fasn</td>
<td>1.00 ± 0.18</td>
<td>1.22 ± 0.10</td>
<td>1.31 ± 0.59</td>
<td>0.51 ± 0.07</td>
</tr>
<tr>
<td>Foxo1</td>
<td>1.00 ± 0.12</td>
<td>0.90 ± 0.16</td>
<td>1.34 ± 0.25</td>
<td>1.24 ± 0.22</td>
</tr>
<tr>
<td>Ppara</td>
<td>1.00 ± 0.13</td>
<td>2.97 ± 0.39</td>
<td>3.62 ± 1.80*</td>
<td>2.25 ± 0.19</td>
</tr>
<tr>
<td>Srebp1</td>
<td>1.00 ± 0.32</td>
<td>1.17 ± 0.05</td>
<td>4.29 ± 2.63</td>
<td>1.32 ± 0.19</td>
</tr>
</tbody>
</table>

Data represented as mean +/- SEM (n = 5). Statistical significance determined by one-way ANOVA followed by Fisher’s LSD as the post-hoc test.

'a' indicates WT ND different than Cyp2b9/10/13-null ND.
'b' indicates WT ND different than WT HFD.
'c' indicates Cyp2b9/10/13-null ND different than Cyp2b9/10/13-null HFD.
'd' indicates WT HFD different than Cyp2b9/10/13-null HFD.
No asterisk indicates a p-value ≤ 0.05 and * indicates a p-value ≤ 0.01, ** indicates a p-value of ≤ 0.0001, *** indicates a p-value of ≤ 0.00001.
Discussion:

HFD-fed Cyp2b9/10/13-null male mice gained 15% more body weight than HFD-fed WT male mice and most of the weight gain was contributed to a 55% increase in WAT mass in the HFD-fed Cyp2b9/10/113-null male mice (Fig. 4.1A; Table 4.1A). This demonstrates that the loss of the primarily hepatic Cyp2b members increases WAT that in turn leads to obesity and suggests that inhibition of these enzymes by environmental chemicals or pharmaceuticals may have similar obesogenic effects.

HFD-fed female mice gained significantly more body weight and WAT mass compared to the ND-fed female mice. However, the lack of Cyp2b enzymes did not exacerbate weight gain as HFD-fed Cyp2b9/10/13-null mice weighed the same as HFD-fed WT mice. In addition, ND-fed Cyp2b9/10/13-null male mice and female mice did not show significant differences in body weight gain compared to ND-fed WT mice. Interestingly, the increased body weight and WAT mass was not associated with poor glucose tolerance or the development of diabetes (Fig. 4.2).

The increased weight gain in Cyp2b9/10/13-null mice was accompanied by an perturbations in serum lipids. For example, serum triglycerides were significantly down-regulated in both ND- and HFD-fed Cyp2b9/10/13-null male mice compared to their WT male counterparts. The decrease in serum triglycerides was accompanied by a corresponding increase in liver triglycerides (Fig. 4.3; Table 4.2A). This effect on serum and liver triglycerides was not observed in HFD-treated WT mice, but only the Cyp2b9/10/13-null mice indicating a reduced capacity of the liver to metabolize fats.
Cyp2b9/10/13-null females showed a similar trend as males when provided a ND, but not when provided a HFD as instead serum triglycerides were increased along with liver triglycerides in the Cyp2b9/10/13-null female mice. The HFD-fed Cyp2b9/10/13-null mice were the only group with increased ALT, suggesting that the combination of a HFD and Cyp2b-knockout caused minor liver damage in females.

Serum cholesterol and HDL were significantly increased in the Cyp2b9/10/13-null male mice compared to WT male mice (Table 4.2A). Increased serum cholesterol coupled with decreased serum triglycerides was previously in HFD-studies performed in C57/B16 mice (Eisinger et al., 2014). The increased serum cholesterol was associated with increased liver damage. Recent studies indicate that high cholesterol is associated with progressive NAFLD and a potential marker for NASH (Kerr et al., 2012; Walenbergh et al., 2015). However, we did not observe significant changes in LDL and VLDL, the typical biomarkers of cardiovascular disease, between WT and Cyp2b9/10/13-null mice. Instead only HDL was significantly raised when comparing HFD-fed WT and Cyp2b9/10/13-null mice. Therefore, the increased liver triglycerides and serum cholesterol indicating the Cyp2b9/10/13-null male mice are progressing towards hepatic steatosis and NASH, but the typically accompanied cardiovascular disease may not be a chronic issue (Kawano et al., 2013).

However, histopathology did not show any pathological changes indicative of significant liver injury and only a few mice, WT or Cyp2b9/10/13-null, showed significant lipid accumulation as measured by oil red O (Fig. 4.3). However, the gene expression of Col27a1 and CD68 increased significantly in HFD-fed Cyp2b9/10/13-null male mice.
compared to HFD-fed WT male mice (Table 4.3B). Col27a1 and CD68 are associated with tissue repair and the presence of activated kupffer cells respectively and increase in gene expression suggests tissue damage and increased inflammatory responses in the livers of male HFD-fed Cyp2b9/10/13-null mice. We suspect that based on increased serum cholesterol, increased liver triglycerides, and increased expression of Col27a1 and CD68 that the males are at an early stage of liver damage that was not evident by H&E.

Changes in adipokines such as adiponectin and leptin contribute to the development of obesity and insulin resistance (Fig. 4.4) (Kahn et al., 2000). Serum leptin acts as a metabolic regulator of satiety and energy expenditure, and a HFD induced serum leptin in both genotypes and genders. HFD-fed Cyp2b9/10/13-null males showing the highest levels, significantly greater than HFD-fed WT males in agreement with increased WAT mass found in HFD-fed Cyp2b9/10/13-null mice relative to other HFD-fed WT mice and mice not provided a HFD. Serum adiponectin and B-OHB, biomarker of ketogenesis, were also significantly increased in HFD-fed Cyp2b9/10/13-null male mice, but not females (Fig. 4.4AB). Taken together, WAT, liver triglycerides, leptin, and B-OHB levels were all increased in Cyp2b9/10/13-null mice, primarily in males, indicating an unhealthy lipid state with increased β-oxidation.

Cyp2a4 was induced in ND-fed Cyp2b9/10/13-null and both ND- and HFD-fed Cyp2b9/10/13-null female mice (Table 4.2B). Cyp2a4 is female predominant and the lack of three hepatic Cyp2b genes leads to increased expression of Cyp2a4 gene (Kumar et al., 2017). Cyp2b9 is highly induced following obesogenic diets (Leung et al., 2016; McGregor et al., 2013), and Cyp2a4 may also be compensating for the lack of Cyp2b9 in HFD-fed
Cyp2b9/10/13-null females. HFD-fed Cyp2b9/10/13-null male mice also show increased gene expression of FoxO1 compared to HFD-fed WT male mice and ND-fed Cyp2b9/10/13-null male mice (Table 4.3AB). FoxO1 regulates gluconeogenesis via glucokinase, and adipogenesis via Pparγ. Inhibition of foxO1 using the chemical inhibitor, AS1842856 suppresses white adiposite differentiation and generates healthier white adipocytes (Zou et al., 2014). Thus, induction in FoxO1 might be associated with increased WAT mass in the HFD-fed Cyp2b9/10/13-null mice (Table 4.2A).

In summary, Cyp2b9/10/13-null mice are diet-induced obese and more susceptible to obesity than WT mice because of perturbed hepatic lipid homeostasis, indicating that these hepatic Cyp2b genes are important in fatty acid metabolism (Fig. 4.5). Cyp2b9/10/13-null mice were obese with increased WAT mass, liver triglycerids, serum cholesterol, leptin, and B-OHB. Cyp2b9/10/13-null male mice show increased accumulation of liver triglycerides and decrease in serum triglyceride suggests difficulty in allocating and utilizing the fatty acids. Therefore, it is possible that chemical inhibition of Cyp2b members by pesticides, plasticizers, and pharmaceuticals could elicit similar effects on lipid metabolism and lead to obesity.
Fig. 4.5: Schematic representation of changes in Cyp2b9/10/13-null male mice. ND-fed Cyp2b9/10/13-null males develop early stage hyperlipidemia and show that just the lack of Cyp2b9,10 and 13 causes change sin lipid homeostasis. HFD-fed Cyp2b9/10/13-null males show that lack of Cyp2b coupled with 60% fat diet leads to the development of obesity and hyperlipidemia.

Conflicts of Interest

The authors have no conflicts of interest to declare

Acknowledgements

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References


The overall purpose of this research was to demonstrate the significance of Cyp2b and Cyp3a in hepatic lipid metabolism. Cyp3a-null and Cyp2b9/10/13-null mouse models indicate that these enzymes contribute to hepatic lipid homeostasis and their absence increases susceptibility to dyslipidemia and obesity at least in the male knock out mouse models. The key findings from the Cyp3a study implied that the lack of seven Cyp3a genes in the mouse model led to gender-based differences in response to HFD-treatment. The Cyp3a-null female mice gained significantly less weight compared to WT females, while the Cyp3a-null males were slightly heavier compared to WT males. Cyp3a-null females acclimate better to a HFD diet while Cyp3a-null males had significant perturbations in lipid metabolism. The findings from the Cyp2b study suggests that the lack of three Cyp2b genes alters lipid homeostasis in the Cyp2b9/10/13-null male irrespective of their dietary treatment compared to WT male mice. Cyp2b9/10/13-null male mice fed a HFD gained significantly more weight and white adipose tissue mass compared to their HFD-fed WT counterparts. Cyp2b9/10/13-null males developed diet-induced obesity and suffered from perturbations in lipid metabolism while Cyp2b9/10/13-null females mostly did not show any changes to the lack of Cyp2b genes.

Cyp2b and Cyp3a are highly polymorphic showing inter-individual, ethnic, and gender variability (Hernandez et al., 2009; Jarukamjorn et al., 2006; Mo et al., 2009; Wiwi et al., 2004; Zanger et al., 2013). These genes are also highly inducible during HFD-treatment (Deol et al., 2015; Finn et al., 2009; Hoek-van den Hill et al., 2015) or exposure
to specific chemical inducers (Hernandez et al., 2007; Wang et al., 2008). Mice lacking all CYP activity in the liver shows increased accumulation of unsaturated fatty acids in the liver but induced Cyp2b10 via CAR to reduce lipotoxicity (Finn et al., 2009). Cyp3a genes are altered during the development of metabolic disorders such as obesity and diabetes (Dostalek et al., 2011; Patoine et al., 2013). In addition, dysregulation in Cyp3a and Cyp2b genes are observed during soybean oil diet treatment in mice that suffer from increase in adiposity, body weight and fatty liver disease (Deol et al., 2015). Therefore, dysregulation of CYP gene expression leads to endocrine disruption by increasing adiposity and alters lipid homeostasis during the development of metabolic syndromes such as obesity and diabetes. Cyp2b and Cyp3a are crucial in lipid metabolism and their roles in PUFA metabolism need further study. In addition, their substrate classification may ultimately need to be further clarified as they are more than just xenobiotic CYPs (Guengerich et al., 2011).

5.1 Aim 1

We characterized the three mouse models used in this study - CAR-null, Cyp3a-null and Cyp2b9/10/13-null mouse models. CAR regulates expression of genes such as Cyp2a, Cyp3a and Cyp2c other than its biomarker gene, Cyp2b10. CAR-null mice show downregulation of Cyp2a4, Cy2b9, Cyp2b10, and Cyp3a11 and these changes in gene expression were consistent with a significant drop in protein expression and enzyme activity studies performed using western blots and testosterone hydroxylase assays, respectively. Lack of CAR leads to masculinization of the liver in females as measured by
the increase in the 6α/15α-OH testosterone ratio (Hernandez et al., 2009; Wilson et al., 1999). This is probably because CAR predominantly regulates the expression of the female predominant CYPs, potentially because androgens are known inhibitors of CAR activity. Thus, CAR-null studies indicate that CAR is instrumental in regulating sexual dimorphic expression of CYP enzymes in the liver along with HNF4α and Foxa2 (Hashita et al., 2008; Wiwi et al., 2004) similar to earlier studies (Hernandez et al., 2009; Mota et al., 2010).

*Cyp2a4* gene expression is significantly increased in Cyp3a-null mice similar to CAR-null mice. This suggests that there is a drop in CAR activity or more likely an increase in PXR activity in the Cyp3a-null mice. Testosterone hydroxylation in the 6β-position is lower, which is generated primarily by Cyp3a members. We also observed a significant drop in 6α/15α-OH testosterone ratio in the Cyp3a-null mice suggesting some loss of sexual dimorphism in the knockout mice. Overall, Cyp3a-null shows minor compensatory changes compared to the CAR-null mouse because even though Cyp3a constitutes 30-40% of hepatic CYPs, it does not directly regulate the expression of other genes. However, lack of certain Cyp3a-metabolites could alter the liver substrate profile for nuclear receptors such as CAR and PXR that regulate CYP expression. Additionally, the observed increase, though not significant, in expression of *Cyp2b10*, *Cyp2c29* and *Cyp2c40* suggest increase in PXR activity in Cyp3a-null mice. We predict that the lack of Cyp3a increases PXR activation and potentially represses CAR activity through increased hepatic androgens based on CYP gene expression changes observed in these mice.
The Cyp2b9/10/13-null mouse model shows very few compensatory changes in CYP gene expression and activity. Global gene expression studies performed using microarray and qPCR studies shows no statistically significant differentially expressed genes in the Cyp2b9/10/13-null mice compared to WT mice. Cyp2b is expressed at lower levels than Cyp3a, Cyp2d, and Cyp2c subfamily members, and therefore the lack of Cyp2b probably has few effects and in turn induces fewer compensatory changes. We observed a weak to moderate masculinization in the Cyp2b9/10/13-null mice.

The findings from aim 1 strongly suggest the importance of estimating changes in xenobiotic metabolism using a combination of nuclear receptor and CYP mouse models to determine the contribution of specific CYP genes to xenobiotic and endobiotic metabolism.

**Fig. 5.1: Characterization of CAR-null female mice.** CAR is the regulator of Cyp2b and lack of CAR decreases Cyp2b expression. CAR also regulates sexually dimorphic genes in the liver. Thus, lack of CAR induces...
masculinization of liver CYP expression as measured by the \( 6\alpha-/15\alpha\)-OH ratio.

5.2 Aim 2

HRN-null mice missing hepatic cytochrome P450 oxidoreductase gene show lack of all the CYP activity in the liver and significant increase in accumulation of unsaturated fatty acids in their liver. These mice show increase in expression of \( Cyp2b10 \) and \( Cyp3a11 \) by activating CAR, which may be to reduce fatty acid build up and a protective mechanism to regulate lipid homeostasis (Finn et al., 2009). Cyp3a constitutes 30-40% of hepatic Cyps and metabolizes more than 60% of drugs available in the market (Guengerich, 2002). Therefore, purpose of this aim was to demonstrate the significance of Cyp3a in hepatic lipid metabolism. We treated Cyp3a-null mice with HFD for 8-weeks to determine if lack of Cyp3a genes increases susceptibility to diet-induced obesity.

Over the 8-weeks of HFD treatment, Cyp3a-null females gained 50% less body weight than WT females; however, the Cyp3a-null males were slightly heavier compared to WT males. This sexually dimorphic response was not surprising, since some Cyp3a genes show gender specific expression such as \( Cyp3a41 \) and \( Cyp3a44 \) that are female predominant (Anakk et al., 2006; Hernandez et al., 2006; Hernandez et al., 2009).

Our data indicates that the Cyp3a-null females were healthier compared to WT females, which is evident from their ability to respond faster to a glucose challenge, greater (60%) serum adiponectin concentrations and lower (50%) serum \( \beta \)-hydroxybutyrate after 8-weeks of HFD-treatment. However, we did not observe any changes in the gene expression of key lipid metabolism genes between WT and Cyp3a-null females. Hence,
the observed healthier trend in the Cyp3a-null females may be due to a 30% decrease in white adipose tissue mass that leads to an increase in adiponectin with better regulation of glucose and fatty acid utilization (Kadowaki et al., 2006); some of which may be extrahepatic. These anti-obesity responses in Cyp3a-null females are consistent with other studies using well-known Cyp3a inhibitors such as naringin found in grapefruit (Alam et al., 2014; Fujioka et al., 2006) that decreases serum β-hydroxybutyrate and is associated with increasing lipid oxidation mediated through Pparα activation (Alam et al., 2014; Cho et al., 2011).

In contrast, liver lipids increased 1.5X in Cyp3a-null males compared to WT males. Cyp3a-null male mice had significant increases in phosphatidylinositol (PI) sphingomyelin (SM) and phosphatidylserine (PS), especially long chain fatty acids of 38-40 carbons. Thus, these mice had increased storage and metabolism of lipids. PI is associated with anti-obesity and SM is associated with obesity by regulating lipid metabolism genes (Shimizu et al., 2010). Cyp3a-null males also had a significant decrease in fatty acid metabolism genes such as Cpt1a, Srebp1 and Fasn coupled with a significant increase in the lipid transporter, Fatp1. This would be consistent with the observed increased in liver lipids in the males. Thus, lipidomic and qPCR studies indicate that lack of Cyp3a increases liver lipids by increasing uptake and decreasing metabolism.

The findings from this aim propose that Cyp3a enzymes indirectly alter the gene expression through their products such as EETs can alter key lipid metabolism gene expression and contribute to the development of metabolic syndrome. High fat diets generate an endogenous fatty acid molecule, anandamide that is further metabolized by
Cyp3a to produce EETs. Anandamide and EETs activate CB1/2 receptors that regulate nociception, cognition, memory and orexigenic effects in peripheral and central nervous system. Recent studies indicate that CB1 receptor in the presence of its inverse agonist, regulates adiponectin activity leading to weight loss (Tarn et al., 2014) and CB2 receptor in the presence of its agonist reduces obesity (Zhang et al., 2016). Since Cyp gene expression shows sexual dimorphism, it is not surprising that we observe gender-based differences in response to HFD and these crucial signaling molecules may be generated by the sexually dimorphic Cyps.

5.2.1 Future studies

Our Cyp3a-null mice still have Cyp3a13 intact and may be its presence is reducing the impact of loss of the other CYP genes especially because we observe a significant increase in its expression in our qPCR data, therefore, it will be interesting to see if the loss of Cyp3a13 in this model would amplify the compensatory changes in the liver due to the loss of Cyp3a members.

Basal metabolic rate (BMR) could be determined using respirometry to test if that contributed to weight loss in Cyp3a-null females. We conducted behavioral tests to determine differences in ambulatory movement, boldness and anxiety in WT and Cyp3a-null mice on week 8. Preliminary data indicates significantly more ambulatory movements made by Cyp3a-null females compared to WT females (unpublished data). Therefore, increase in activity in Cyp3a-null females may have altered energy expenditure to induce weight loss in these mice.
50% decrease in body weight gain
30% decrease in WAT
Significant increase in glucose

60% decrease in serum adiponectin
49% decrease in serum β-hydroxybutyrate, biomarker for ketogenesis
Significant increase in gene expression for: Cyp2b9 (female predominant Cyp) and IL6 (acts as both pro-inflammatory and anti-inflammatory signal).

Cyp3a-null females are protected from diet-induced

Loss of Cyp3a downregulates Cyp3a-metabolites such as EETs and anandamide and leads to an anti-obese phenotype in Cyp3a-null females but only induces moderate weight gain in the males

Physiologic al changes

Cyp3a-null males were slightly heavier than WT males.

Cyp3a-null males show perturbations in lipid metabolism

1.5X increase in liver triglycerides

Significant increase in individual polar lipid species such as phosphatidyl inositol, phosphatidyl serine and sphingomyelin
Significant decrease in lipid oxidation genes (Cpt1a), lipid synthesis genes (Fasn, Srebp1a) but 2X increase in lipid transporter (Fatp1)

60% decrease in serum adiponectin
49% decrease in serum β-hydroxybutyrate, biomarker for ketogenesis

Biochemica l and molecular changes

Phenotypic changes

Female

Cyp3a-null mice

Male

-
Fig. 5.2: Mechanistic changes observed in Cyp3a-null mice gender-based differences is observed in responses to HFD and lack of Cyp3a genes which is shown at physiological level, molecular and biochemical changes and predictive mechanistic changes that lead to the development of sexually dimorphic phenotype.

5.3 Aim 3

Earlier studies by Finn et al. group using HRN-null mice lacking Cytochrome P450 oxidoreductase null mice shows lack of all hepatic CYP activity in the liver, which led to significant increase in accumulating fatty acids in the liver. Unsaturated fatty acids in the liver activated CAR, which induced Cyp2b10 expression in these mice in order to prevent fatty acid induced toxicity (Finn et al., 2009). In addition, Cyp2b-knockdown mouse model developed in our own lab had difficulty in clearing out corn oil rich in unsaturated fatty acids and showed increased adiposity and weight gain. This was observed in aging male knockdown mice (Damiri, 2011). Therefore, the purpose of this aim was to demonstrate the significance of Cyp2b in hepatic lipid metabolism using a Cyp2b9/10/13-null mice developed in our lab using Crispr-Cas9 and sgRNA technology (Kumar et al., 2017).

We determined whether Cyp2b9/10/13-null mice provided a HFD for 10-weeks are more susceptible to diet-induced obesity than WT mice. Key findings from this study show that HFD-fed Cyp2b9/10/13-null male mice gain 15% more body weight and have 55% more WAT mass than HFD-fed WT male mice. Cyp2b9/10/13-null male mice show significant increases in liver triglyceride, which is complemented by a significant decrease in serum triglycerides in comparison to WT male mice.

ND-fed Cyp2b9/10/13-null male mice show significant increase in fasting plasma glucose, serum cholesterol, HDL and liver triglycerides compared to ND-fed WT male
mice. An increase in liver triglycerides by 2.3-fold may have led to mild glucose intolerance in these mice that is indicated by an increase in fasting glucose levels in the ND-fed Cyp2b9/101/3-null mice. However, we did not observe any differences in glucose tolerance. Also, increases in liver triglycerides without significant changes in β-hydroxybutyrate indicates that these mice are not generating greater levels of ketones. Thus, ND-fed Cyp2b9/10/13-null mice suffer from perturbations in lipid metabolism and distribution but do not develop obesity.

The key finding of our work is that HFD-fed Cyp2b9/10/13-null male mice are obese in comparison to HFD-fed WT mice. In addition, HFD-fed Cyp2b9/10/13-null mice show significant increases in serum cholesterol, HDL, and adipokines such as leptin and adiponectin, and β-hydroxybutyrate. The increase in adipokines may be due of significant increase in WAT and the increase in β-hydroxybutyrate indicates occurrence of ketosis in HFD-Cyp2b9/10/13-null mice. This suggests that hepatic Cyp2b enzymes are anti-obesity enzymes involved in lipid signaling, the metabolism of lipids, and obesity.

There was significant increase in CD68, surface marker for macrophages and activated kupffer cells and Col27a1, a gene associated with tissue repair and growth in the HFD-fed Cyp2b9/10/13-null male mice suggesting that inflammatory responses were triggered due to liver damage, however, we did not observe any changes in ALT in these mice (Gruben et al., 2014; Pietilainen et al., 2006). FoxO1, transcription factor associated with gluconeogenesis and adipogenesis was significantly increased in the HFD-fed Cyp2b9/10/13-null mice compared to HFD-fed WT male mice. FoxO1 induces adipogenesis by activating Pparγ and increases hepatic glucose production by inducing
gluconeogenic genes such as phosphoenol pyruvate kinase-1 (Pepck-1). Recent studies done on 3T3L1 cells show that inhibition of FoxO1 by the chemical inhibitor, AS1842856, generates smaller, healthier white adipose tissue by regulating Pparγ (Zou et al., 2014). Thus, up-regulation of FoxO1 may be associated with significant increase in WAT in the HFD-fed Cyp2b9/10/13-null mice.

The female data shows treatment with HFD induces changes in body weight, WAT and liver triglycerides but did not show any genotypic changes. However, serum HDL was significantly increased in HFD-fed Cyp2b9/10/13-null females compared to HFD-fed WT females while serum cholesterol, triglyceride and ALT were increased in HFD-Cyp2b9/10/13-null females compared to ND-fed Cyp2b9/10/13-null females. Adiponectin was increased in ND-fed Cyp2b9/10/13-null females compared to HFD-fed Cyp2b9/10/13-null. Thus, females show diet-based differences in different parameters.

Overall, Cyp2b9/10/13-null male mice demonstrate perturbations in lipid metabolism, distribution and utilization compared to WT male mice and HFD treatment exacerbates these responses contributing to the development of diet-induced obesity in HFD-fed Cyp2b9/10/3-null male mice. Our data indicates that the hepatic Cyp2b subfamily are anti-obesity CYPs and the chemical inhibition of these CYPs could exacerbate obesity in males.
5.3.1 Future studies

Most of the changes observed in HFD-fed Cyp2b9/10/13-null male mice are associated with increased WAT in these mice, therefore it will be interesting to study gene expression changes in WAT and even brown adipose tissue (BAT) by RNA-sequencing. Recent time course study performed on BAT to monitor molecular changes during the development of diet-induced obesity has shown induction of Cyp2b9, thus, Cyp2bs are involved in inducing obese phenotype in the BAT (McGregor et al., 2013).

Our lab is currently determining global gene expression changes and changes in lipidomic profile in Cyp2b9/10/13-null and WT mice. The results from these studies could help us determine major metabolic pathways altered due to lack of hepatic Cyp2b9,10 and 13. Our lab is planning to perform lipidomic analysis on liver tissue to determine if there are any changes in phospholipid species and oxylipids due to lack of hepatic Cyp2b9,10 and 13 or due to HFD treatment.

Recent lipidomic studies performed on C57/Bl6 that developed diet-induced obesity shows significant changes in lipidomic profile in obese-individuals and especially, individual lipid species such as phosphatidylcholine and phosphatidylinositol(s) associated with pro-obesity were increased (Eisinger et al., 2014). In addition, intracellular location of lipids such as diacylglycerol and fatty acid composition of the liver such as monounsaturated fatty acids/saturated fatty acids have been shown to influence insulin resistance and obesity phenotype (Gruben et al., 2014). Therefore, we predict that lipidomic profiles from our study will help us identify unique lipid biomarkers that are generated by Cyp2bs.
Further studies using chemical inhibitors of Cyp2b such as endosulfan, atrazine and chlorpyrifos could be combined with HFD-treatment to demonstrate the interaction between chemical exposure and diet can be done to closely replicate a more realistic situation of human exposure.
Cyp2b regulates lipid metabolism
Lack Cyp2b metabolites such as EETs influence lipid gene expression
Liver lipids such as PC, PI and MUFA/SA induce obesity
**Fig. 5.3: Mechanistic changes observed in Cyp2b9/10/13-null mice**

Gender-based differences is observed in responses to HFD and lack of Cyp2b genes which is shown at physiological level, molecular and biochemical changes and predictive mechanistic changes that lead to the development of sexually dimorphic phenotype. The significance percentage mentioned is Cyp2b9/10/13-null compared to WT of the corresponding diet treatment.

### 5.4 Conclusion

Obesity is a world-wide problem, more than 1/3\(^\text{rd}\) of adults in US are obese. The annual medical expenses for obesity related disorders account for $147 million in 2008. The common treatment procedures involving healthy diet and active lifestyle, which have not been able to control the growing number of obese individuals. But our results from Cyp3a-null and Cyp2b9/10/13-null mouse models indicate the importance of considering environmental exposure to chemicals along with current treatment of obesity to tackle this disease.

Characterization of the Cyp3a-null and Cyp2b9/10/13 null mouse models indicate that Cyp3a and Cyp2b are involved in fatty acid metabolism. Lack of Cyp3a protects Cyp3a-null female mice from diet-induced obesity but induces moderate weight gain in Cyp3a-null males. Lack of Cyp2b9/10/13-null induces perturbations in lipid metabolism, distribution and utilization and HFD worsens the progression to obesity.

Cyp3a and Cyp2b regulate lipid metabolism via epoxide metabolites or by altering ratio of mono unsaturated/ polyunsaturated fatty acids or by altering phospholipid ratio in the liver. Further research would enable us to determine if chemical inhibitors of Cyp3a or inducers of Cyp2b could be used to design effective obesity treatment procedures. Studies
using chemical inhibitors will add some more potential environmental obesogens that induce obesity through Cyp3a or Cyp2b to the growing list of obesogens.

Overall, Cyp2b has a protective role against the development of obesity and Cyp3a has a pro-obesity role in males, but anti-obesity role in females.
References


Damiri, B. (2011). Lentiviral-mediated RNAi knockdown yields a novel mouse model for studying CYP2B function. *All Dissertations*, 782. doi: [https://tigerprints.clemson.edu/all_dissertations/782](https://tigerprints.clemson.edu/all_dissertations/782)


Appendix A

Supplementary figures from Chapter Two

Appendix A-1: A cluster of Cyp2b genes are found on chromosome 7 (26,500K – 27,630K). All five Cyp2b subfamily members are located in the 7A region of chromosome 7. However, there are six genes between two Cyp2b regions; Therefore, we knocked out the three-predominant hepatic CYPs (Cyp2b9/10/13) via partial chromosomal deletion using Crispr/Cas9 because it would not impact other genes.
Appendix A-2: Testosterone concentrations in nullizygous mice. Testosterone concentrations in the liver of CAR-null (A), Cyp3a-null (B), Cyp2b9/10/13-null (C) and serum of Cyp2b9/10/13-null mice were measured and compared to their WT counterparts. Testosterone concentrations from liver cytosol or serum were measured by EIA using a kit from Cayman Chemical Company (Ann Arbor, MI). Data are presented as mean testosterone concentrations + SEM (n = 3-4). A cindicates a significant difference between male and female WT mice and dindicates a significant difference between male and female nullizygous mice. There are no significant differences between nullizygous mice and their WT counterparts. Statistical differences were determined by one-way ANOVA followed by Fisher’s LSD as the post-hoc test A letter without an asterisk indicates a significance of p < 0.05, asterisk indicate significance of **p<0.001, and *** p<0.0001, respectively.
Appendix B

Supplementary figures from Chapter Three

Appendix B-1: Timeline showing procedures performed during the eight weeks of high fat diet treatment.
Appendix B-2: Primer Sequences used to determine changes in gene expression using qPCR.

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<td>Ppar γ</td>
<td>TGGTGAACCCTCTGGAGATTC</td>
<td>AATTTCCTTGAAAGTGTCTCATAGG</td>
<td>58</td>
</tr>
<tr>
<td>Pxr</td>
<td>GAGCTCAGATGAAACCTT</td>
<td>TCTCTGCGGGAAGCTGCA</td>
<td>64.7</td>
</tr>
<tr>
<td>Srebp1</td>
<td>AGCGAGTGCCACACAAAGCA</td>
<td>GCCAAAAGACAGGGCCGTCAC</td>
<td>57</td>
</tr>
<tr>
<td>Srebp1a</td>
<td>TAGTCCGAAAGCGGGGTGGCGGCCGCG</td>
<td>GATGTCTGTTCAAACCCGCTGTTGTCAGT</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>CCA</td>
<td>TCT</td>
<td></td>
</tr>
<tr>
<td>Srebp1c</td>
<td>ATCGGCGCGGAAGCTGCCGGGTAGCG</td>
<td>ACTGTCTGTTGTGATGAGCTGGAGCAT</td>
<td>62.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Annealing temperature

<sup>b</sup> Primer sequences (Chamorro-Garcia et al., 2013; Damiri et al., 2012; Fan et al., 2011; Hernandez et al., 2009; Ito et al., 2012; Matsebatlela et al., 2015; Sui et al., 2014; Zhang et al., 2013; Zhou et al., 2009)
Appendix B-3: Feed consumption rate of female (A) and male (B) WT and Cyp3a-null mice. Feed consumed was measured every other day during the entire eight-week period. Data are presented as mean + SEM per week. There are no differences in feed consumption between genotypes. Statistical significance was determined by Student’s t-tests (n=7 - 8).
Appendix B-4: Fasting blood glucose and insulin levels. Blood glucose concentrations were determined during weeks 2, 4 and 6 (A-C) as described in the Materials and Methods. Blood insulin concentrations were determined using ELISA (D) kit from EMD Millipore Corporation (EMD Millipore Corporation, Billerica MA). Mice were fasted 4-5 h during weeks 2, 4 and 6 and fasting plasma glucose levels determined each time by tail bleed and serum fasting insulin determined using blood collected by tail bleed during week 6. Data are presented as mean blood plasma + SEM. Statistical significance was determined by Student’s t-tests (n=7-8).
Appendix B-5: Principal component analysis (PCA) indicates gender differences in hepatic lipid species. Hepatic lipid species are primarily influenced by gender not the lack of Cyp3a with the exception of a few SM, PS, and PC species.
Appendix B-6: Relative change in lipid levels after eight weeks of HFD treatment on Cyp3a-null mice.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>FEMALES</th>
<th>MALES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Cyp3a-null</td>
</tr>
<tr>
<td>Total LysoPC</td>
<td>1.221±0.161</td>
<td>0.820±0.184</td>
</tr>
<tr>
<td>Total PC</td>
<td>73.240±0.461</td>
<td>75.900±3.329</td>
</tr>
<tr>
<td>Total SM / DSM</td>
<td>5.113±0.454</td>
<td>4.232±0.313</td>
</tr>
<tr>
<td>Total ePC</td>
<td>2.389±0.053</td>
<td>2.335±0.140</td>
</tr>
<tr>
<td>Total LysoPE</td>
<td>0.330±0.034</td>
<td>0.295±0.044</td>
</tr>
<tr>
<td>Total PE</td>
<td>12.400±0.691</td>
<td>10.930±3.064</td>
</tr>
<tr>
<td>Total ePE</td>
<td>0.216±0.019</td>
<td>0.197±0.069</td>
</tr>
<tr>
<td>Total PI</td>
<td>4.210±0.188</td>
<td>3.652±0.672</td>
</tr>
<tr>
<td>Total PS</td>
<td>0.828±0.035</td>
<td>1.489±0.447</td>
</tr>
<tr>
<td>Total ePS</td>
<td>0.003±0.002</td>
<td>0.007±0.003</td>
</tr>
<tr>
<td>Total PA</td>
<td>0.038±0.006</td>
<td>0.114±0.060</td>
</tr>
<tr>
<td>Total PG</td>
<td>0.012±0.003</td>
<td>0.031±0.009</td>
</tr>
</tbody>
</table>

Data represented as mean percentage of total signal +/- SEM (n = 5).

Statistical significance determined by Student’s T-test.

*Indicates a p-value < 0.05 and ** Indicates a p-value <0.01.
Appendix B-7: Compensatory changes in hepatic CYP gene expression after eight weeks of HFD treatment in Cyp3a-null mice. Immunoblots were performed and quantified as described previously with β-actin as the reference protein (Hernandez et al., 2009)

<table>
<thead>
<tr>
<th>Genes</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Cyp3a-null</td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>1.00 ±0.240</td>
<td>0.015±0.014**</td>
</tr>
<tr>
<td>Cyp3a13</td>
<td>1.00 ±0.081</td>
<td>1.188±0.207</td>
</tr>
<tr>
<td>Cyp3a25</td>
<td>1.00 ±0.173</td>
<td>0.017±0.016***</td>
</tr>
<tr>
<td>Cyp3a41</td>
<td>1.00 ±0.282</td>
<td>0.010±0.010**</td>
</tr>
<tr>
<td>Cyp2a4</td>
<td>1.00 ±0.264</td>
<td>2.826±0.603*</td>
</tr>
<tr>
<td>Cyp2b9</td>
<td>1.00 ±0.137</td>
<td>1.495±0.154*</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>1.00 ±0.210</td>
<td>1.283±0.176</td>
</tr>
<tr>
<td>Cyp2c29</td>
<td>1.00 ±0.135</td>
<td>1.315±0.170</td>
</tr>
<tr>
<td>Cyp2c40</td>
<td>1.00 ±0.176</td>
<td>1.046±0.108</td>
</tr>
</tbody>
</table>

Data represented as relative mean +/- SEM (n = 7 or 8). Statistical significance determined by Student’s t-test. * Indicates a p-value <0.05, ** Indicates a p-value <0.01, *** Indicates a p-value <0.001 and **** Indicates a p-value <0.0001.
WT F  Cyp3a-null F  WT M  Cyp3a-null M
Appendix B-8: Changes in serum leptin levels in Cyp3a-null mice. Serum leptin concentration was determined using EIA kit from Bertin pharma (Montigny le Bretonneux, FR). Data are presented as mean blood plasma ± SEM. Statistical significance was determined by Student’s t-tests (n=4-5).
Appendix C

Supplementary figures for Chapter four

Appendix C-1: Timeline of the different procedures performed during a 10-week treatment of 9-10 week old WT and Cyp2b-null mice with either a normal diet (ND; 6.2%) or a high-fat diet (HFD; 60% fat).
Appendix C-2: Feed consumption determined in WT and Cyp2b9/10/13-null mice during the 10-weeks of dietary treatment. Data are presented as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher’s LSD as post-hoc test (n= 8-9). ‘a’ indicates WT ND are different than Cyp2b9/10/13-null ND, ‘b’ indicates WT ND are different than WT HFD, ‘c’ indicates Cyp2b9/10/13-null ND are different than Cyp2b9/10/13-null HFD, ‘d’ indicates WT HFD different than Cyp2b9/10/13-null HFD.
Appendix C-3: Fasting blood glucose levels determined during weeks 5 and 8. Mice were fasted 4-5hr and fasting plasma glucose levels were determined using a glucometer after tail bleeding. Data are presented as mean blood plasma ± SEM. Statistical significance was determined by one-way ANOVA followed by Fisher’s LSD as the post-hoc test (n=8-9). *Indicates a p-value < 0.05; ‘a’ indicates WT-ND are different than Cyp2b9/10/13-null-ND, ‘b’ indicates WT-ND are different than WT-HFD, ‘c’ indicates Cyp2b9/10/13-null-ND are different than Cyp2b9/10/13-null-HFD ‘d’ indicates WT HFD are different than Cyp2b9/10/13-null-HFD.
Appendix C-4: Insulin Tolerance Test done on week 9. (A & B) Insulin tolerance test was performed as described in materials and methods and (C & D) results are represented as area under the curve. Data are presented as mean blood glucose level + SEM. Statistical significance was determined by Student’s t-test (n = 8 or 9). ‘a’ indicates WT ND are different than Cyp2b9/10/13-null ND, ‘b’ indicates WT ND are different than WT HFD, ‘c’ indicates Cyp2b9/10/13-null ND are different than Cyp2b9/10/13-null HFD, ‘d’ indicates WT HFD are different than Cyp2b9/10/13-null HFD.
Appendix C-5: Immunoblots show protein expression changes in WT and Cyp2b9/10/13-null mice. Immunoblot was done using microsome samples as described in materials and methods. Density of the blots was quantified and shown above the immunoblots. Data represents relative mean of WT compared to Cyp2b9/10/13-null mice of the same gender and dietary treatment. Statistical differences were determined by Student’s t-tests (n = 3) (* p < 0.05 ** p < 0.01).

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WT-ND</td>
<td>Cyp2b-null ND</td>
</tr>
<tr>
<td>Cyp3a</td>
<td>1.0</td>
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</tr>
<tr>
<td>Cyp4a</td>
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</tr>
<tr>
<td>Cyp2b</td>
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</tr>
<tr>
<td>b-actin</td>
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</tr>
<tr>
<td></td>
<td>WT-ND</td>
<td>Cyp2b-null ND</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
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</tr>
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<td></td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.0 b****</td>
</tr>
</tbody>
</table>
References


Chamorro-Garcia, R., Sahu, M., Abbey, R. J., Laude, J., Pham, N., & Blumberg, B. (2013). Transgenerational inheritance of increased fat depot size, stem cell


human hepatic peroxisome proliferator-activated receptor alpha less than that of a mouse but may activate constitutive androstane receptor in liver. Hindawi.


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Chamorro-Garcia, R., Sahu, M., Abbey, R. J., Laude, J., Pham, N., & Blumberg, B. (2013). Transgenerational inheritance of increased fat depot size, stem cell


Fan, Y., Guo, Y., Hamblin, M., Chang, L., Zhang, J., & Chen, Y. E. (2011). Inhibition of glucogenic genes by calcium-regulated heat-stabled protein 1 via repression of...


human hepatic peroxisome proliferator-activated receptor alpha less than that of a mouse but may activate constitutive androstane receptor in liver. *Hindawi.*


