AN EXAMINATION OF THE ARYL HYDROCARBON RECEPTOR
IN LIVER METABOLISM

A Dissertation in
Biochemistry, Microbiology, and Molecular Biology

by

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor from the basic helix-loop-helix PER/ARNT/SIM family of proteins that is evolutionarily conserved in both vertebrates and invertebrates. Known as a "promiscuous" receptor, AHR can bind to several different classes of chemical compounds such as polycyclic aromatic hydrocarbons (PAH) and flavonoids. When not bound to ligand, AHR resides in a cytosolic complex containing two molecules of heat shock protein 90, one molecule of X-associated protein 2, and a molecule of p23. Upon ligand binding, this complex is transported to the nucleus via nuclear importins and AHR dissociates to form a heterodimer with aryl hydrocarbon nuclear translocator (ARNT). The AHR/ARNT heterodimer then binds to specific DNA sequences known as dioxin response elements (DRE) within the promoter region of target genes (e.g. cytochrome P450 enzyme 1A1, CYP1A1) to activate their transcription.

Previous studies have primarily examined AHR within the context of ligand-mediated transcriptional activation of its prototypical target gene, Cyp1a1. However, the AHR can also influence gene transcription in the absence of exogenous ligand and/or Cyp1a1 expression. Using a conditional AHR knockout mouse model that lacks hepatocyte-specific AHR expression (Ahr\textsuperscript{flfx} Alb\textsuperscript{Cre}), this dissertation examines how basal AHR activity in the absence of Cyp1a1 transcription can influence metabolic homeostasis. In particular, the data reveal that the loss of hepatocyte-specific AHR expression correlates with reduced body and liver mass relative to congenic AHR-expressing mice (Ahr\textsuperscript{flfx}). Additionally, Ahr\textsuperscript{flfx} Alb\textsuperscript{Cre} mice maintained on purified AIN-93M diet display impaired glucose tolerance without any perturbations of insulin sensitivity. Conversely, Ahr\textsuperscript{flfx} Alb\textsuperscript{Cre} mice challenged with a high-sucrose dietary modification of AIN-93M exhibit reduced insulin sensitivity without any difference in glucose tolerance. Most
notably, $\text{Ahr}^{fx/\text{fx}}\text{Alb}^{\text{Cre}}$ mice challenged with a high-fat/high-sucrose (HF/HS) diet exhibit significantly decreased gene/protein expression of key enzymes involved in de novo fatty acid synthesis and fatty acid import, as well as significantly increased expression of key fatty acid export genes. Furthermore, inflammatory gene expression is also significantly reduced in HF/HS-fed $\text{Ahr}^{fx/\text{fx}}\text{Alb}^{\text{Cre}}$ mice relative to $\text{Ahr}^{fx/\text{fx}}$. Together, the data suggest that basal hepatocyte-specific AHR signaling may promote diet-induced steatohepatitis in $\text{Ahr}^{fx/\text{fx}}$ mice.

Utilizing the $\text{Ahr}^{fx/\text{fx}}\text{Alb}^{\text{Cre}}$ mouse model, this dissertation also explores the role of AHR in regulating hepatic fibroblast growth factor 21 (FGF21) production. FGF21 is an important metabolic hormone and regulator of the fasting response. Notably, FGF21 can attenuate obesity-associated morbidities when administered to various genetic and diet-induced mouse models of the disease. In the absence of exogenous AHR ligand, non-fasted $\text{Ahr}^{fx/\text{fx}}\text{Alb}^{\text{Cre}}$ mice exhibit 4-fold greater hepatic $\text{Fgf21}$ expression relative to $\text{Ahr}^{fx/\text{fx}}$, along with elevated expression of the FGF21-target gene $\text{Igfbp1}$. Furthermore, in vivo agonist activation of AHR reduces hepatic $\text{Fgf21}$ expression during a fast. The $\text{Fgf21}$ promoter contains several putative dioxin response elements (DREs) and utilizing electromobility shift assays, we demonstrate that the AHR/ARNT heterodimer binds to a specific DRE which overlaps binding sequences for peroxisome proliferator-activated receptor $\alpha$ (PPAR$\alpha$), carbohydrate response element-binding protein (ChREBP), and cAMP response element-binding protein hepatocyte specific (CREBH). In addition, agonist-activated AHR impairs PPAR$\alpha$-, ChREBP-, and CREBH-mediated $\text{Fgf21}$ promoter activity in Hepa-1 cells. Similarly, treatment of Hepa-1 cells with AHR agonist ablates potent ER stress-driven $\text{Fgf21}$ expression, while pre-treatment with AHR antagonist blocks this effect. Finally, the data demonstrate that pre-treatment of primary human hepatocytes with AHR agonist attenuates PPAR$\alpha$-, glucose-, and ER stress-driven induction of $\text{FGF21}$ expression,
indicating this phenomenon is not mouse-specific. Overall, the data show that AHR contributes to hepatic energy homeostasis partly through the constitutive repression of \textit{FGF21} expression and signaling. Therefore, future studies should examine the potential use of AHR antagonists, such as the flavonoid compounds readily found within edible plants, to increase FGF21 expression and subsequently produce therapeutic outcomes.

In summary, the data from this dissertation indicate that basal hepatocyte-specific AHR activity plays a critical role in the regulation of liver metabolism and that AHR agonists are not only capable of activating DRE-dependent transcription, but can also suppress or interfere with certain metabolic gene pathways. Nevertheless, the exact mechanisms through which AHR exerts these effects remain unclear. Ultimately, this dissertation presents contrasting roles for AHR when activated with ligands and when exerting its regulation in the absence of exogenous ligand. Therefore, future research should aim to define the role of AHR within these separate contexts to gain a better understanding of the complex mechanisms through which AHR regulates transcription.
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<tr>
<td>3-MC</td>
<td>3-methylcholanthrene</td>
</tr>
<tr>
<td>ACACA</td>
<td>Acetyl Coenzyme A carboxylase alpha</td>
</tr>
<tr>
<td>AHH</td>
<td>Aryl hydrocarbon hydroxylase (CYP1A1)</td>
</tr>
<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AIN</td>
<td>American Institute of Nutrition</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>APOB100</td>
<td>Apolipoprotein B100</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
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<td>bHLH</td>
<td>Basic helix-loop-helix</td>
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<td>BaP</td>
<td>Benzo A pyrene</td>
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<tr>
<td>BNF</td>
<td>Beta-naphthoflavone</td>
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<tr>
<td>CA-AHR</td>
<td>Constitutively active aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<tr>
<td>ChREBP</td>
<td>Carbohydrate response element-binding protein</td>
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<tr>
<td>ChoRE</td>
<td>Carbohydrate response element</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
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<td>CREBH</td>
<td>cAMP response element binding protein, hepatocyte specific</td>
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<td>CPT1</td>
<td>Carnitine palmitoyltransferase 1</td>
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<td>CYP</td>
<td>Cytochrome P450</td>
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<td>DDIT3</td>
<td>DNA damage inducible transcript 3</td>
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<td>Diacylglyceride acyltransferase</td>
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<td>Dioxin response element</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>Ductus venosus</td>
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<tr>
<td>EIF2</td>
<td>Eukaryotic initiating factor 2</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>Electromobility shift assay</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ER stress response element</td>
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<td>FABP</td>
<td>Fatty acid binding protein</td>
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<td>Fatty acid synthase</td>
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<td>Free fatty acids</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FOXO1</td>
<td>Forkhead box protein other 1</td>
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<tr>
<td>G6PC</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
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<td>GNF351</td>
<td>N-(2-((1H-indol-3-yl)ethyl)-9-isopropyl-2-(5-methylpyridin-3-yl)-9H-purin-6-amine</td>
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<td>GK</td>
<td>Glucose kinase</td>
</tr>
<tr>
<td>GPDH</td>
<td>Glycerol-3-phosphate dehydrogenase</td>
</tr>
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<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GYS2</td>
<td>Glycogen synthase 2</td>
</tr>
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<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
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<td>HF/HS</td>
<td>High-fat/High-sucrose</td>
</tr>
<tr>
<td>HS</td>
<td>High-sucrose</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone sensitive lipase</td>
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<td>HSP90</td>
<td>Heat-shock protein 90</td>
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<td>I3C</td>
<td>Indole-3-carbinol</td>
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<tr>
<td>ICZ</td>
<td>Indolo[3,2b]carbazole</td>
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<td>Insulin-like growth factor binding protein 1</td>
</tr>
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<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRE1</td>
<td>Inositol requiring enzyme 1</td>
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<td>IRS1/2</td>
<td>Insulin receptor substrate 1/2</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>L-PK</td>
<td>Liver pyruvate kinase</td>
</tr>
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<td>MGAT</td>
<td>Monoacylglyceride acyltransferase</td>
</tr>
<tr>
<td>MLX</td>
<td>Max-like protein X</td>
</tr>
<tr>
<td>MTTP</td>
<td>Microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric oxide synthase, inducible</td>
</tr>
<tr>
<td>NRC</td>
<td>National Research Council</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PC</td>
<td>Pyruvate carboxylase</td>
</tr>
<tr>
<td>PCK1</td>
<td>Phosphoenol pyruvate carboxykinase</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PEMT</td>
<td>Phosphatidylethanolamine N-methyltransferase</td>
</tr>
<tr>
<td>PAS</td>
<td>Per/ARNT/Sim</td>
</tr>
<tr>
<td>PER</td>
<td>Period</td>
</tr>
<tr>
<td>PFK2/FBPs2</td>
<td>Phosphofructokinase 2/fructose-2,6-bisphosphatase 2</td>
</tr>
<tr>
<td>PGC</td>
<td>Peroxisome proliferator-activated receptor (\gamma) co-activator</td>
</tr>
<tr>
<td>PHKB</td>
<td>Phosphorylase b kinase subunit beta</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
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<td>--------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PKLR</td>
<td>Pyruvate kinase, liver and red blood cell</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PPRE</td>
<td>PPAR response element</td>
</tr>
<tr>
<td>PUR</td>
<td>Purified AIN-93M diet</td>
</tr>
<tr>
<td>PYGL</td>
<td>Glycogen phosphorylase, liver</td>
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<tr>
<td>RT-qPCR</td>
<td>Reverse-transcriptase quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SAA3</td>
<td>Serum amyloid A3</td>
</tr>
<tr>
<td>SAHRM</td>
<td>Selective aryl hydrocarbon receptor modulator</td>
</tr>
<tr>
<td>SC</td>
<td>Standard rodent chow</td>
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<tr>
<td>SCD1</td>
<td>Stearoyl-CoA desaturase 1</td>
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<tr>
<td>SGA360</td>
<td>1-allyl-3-(2,4-dimethoxy-phenyl)-7-trifluoromethyl-1H-indazole</td>
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<tr>
<td>SIM</td>
<td>Single minded protein</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol response element binding protein</td>
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<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachloro-dibenzo-p-dioxin</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UDP</td>
<td>Urine diphosphate</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<td>WAT</td>
<td>White adipose tissue</td>
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<td>X5P</td>
<td>Xylulose-5-phosphate</td>
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<td>XAP2</td>
<td>X-associated protein 2</td>
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<td>XBP1</td>
<td>X-box binding protein 1</td>
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CHAPTER 1: INTRODUCTION
1.1 GLUCOSE HOMEOSTASIS

For nearly all organisms, glucose plays a central role in energy metabolism. Glucose is not only an important precursor for various metabolic intermediates in biosynthetic reactions, it is also relatively rich in free energy (complete oxidation to CO$_2$ and H$_2$O has a standard free energy of -2.840 kJ/mol). Accordingly, many organisms maintain intracellular stockpiles of glucose in the form of high molecular weight polymer and when energy demands increase, break these stockpiles down to produce ATP.

While glucose is a quintessential nutrient, the body must nevertheless maintain a careful balance of glucose levels because low levels (hypoglycemia) can result in brain damage, while excessively high levels (hyperglycemia) can lead to conditions such as insulin resistance. In animals, this balance in glucose is maintained through the combined work of several different tissues and organs. Among these, the liver has a particularly important role in maintaining glucose levels as it is the site of short-term glucose production and storage. When glucose is in excess (e.g. upon eating a meal), the liver removes glucose from the bloodstream and stores it in the form of glycogen. Once hepatic energy stores are full, any further excess of glucose is converted into triglycerides and transported to adipose tissue for long-term energy storage. When dietary glucose is no longer available, the liver responds by inhibiting the pathways of glucose utilization and initializes glucose production via gluconeogenesis and the breakdown of glycogen. When necessary, the liver can also secrete ketone bodies for extrahepatic tissues to use as a source of energy.

1.1.1 Glucose Metabolism in Liver and Adipose

Hepatic glycogen provides the body with an immediate supply of glucose when dietary glucose is no longer available (e.g. between meals). Structurally, glycogen is a polymer of linear
α(1→4)-linked glucose chains with α(1→6)-linked side chains occurring at intervals of approximately 6-8 residues. Figure 1.1 outlines the mechanism through which glucose is converted into glycogen. When extracellular glucose levels are elevated, glucose enters into hepatocytes via glucose transporter 2 (GLUT2) and is immediately converted to glucose-6-phosphate via the enzyme glucose kinase (GK). Glycogen synthesis begins with the conversion of glucose-6-phosphate into glucose-1-phosphate and subsequent addition of uridine diphosphate (UDP) to form UDP-glucose. Glycogen synthase (GYS2) then catalyzes the transfer of glucose from UDP-glucose to the non-reducing end of a branched glycogen molecule via an α(1→4) linkage. However, this enzyme is unable to construct the α(1→6) branches. This process is instead carried out by amylo(1→4) to (1→6) transglycosylase.
**Figure 1.1.** Overview of hepatic glucose metabolism. Abbreviations used: Acaca, acetyl-CoA carboxylase alpha; Acly, ATP citrate lyase; Dgat, diacylglyceride acyltransferase; Fasn, fatty acid synthase; Glut2, glucose transporter 2; Gpat, glycerol-3-phosphate acyltransferase; GK, glucose kinase; Gpdh, glycerol-3-phosphate dehydrogenase; Gys2, glycogen synthase 2; Mgat, monoacylglycerol acyltransferase; Pap, phosphatidate phosphatase; Scd1, stearoyl-Coenzyme A desaturase 1.

When hepatic glycogen stores are full, the liver converts any further excess of glucose (and other carbohydrates) into triglycerides. The liver then packages these triglycerides onto apolipoproteins that transport it to adipose tissue for long-term storage. Unlike glycogen, triglycerides are not composed directly of glucose molecules. Rather, triglyceride synthesis utilizes metabolic intermediates from the glucose-dependent glycolysis pathway. Structurally, a
triglyceride is an ester composed of a glycerol molecule fused to three fatty acid chains. The glycerol molecule found in a triglyceride is provided through conversion of the glycolytic intermediate dihydroxyacetone phosphate into glycerol-3-phosphate, via the enzyme glycerol-3-phosphate dehydrogenase (GPDH). Pyruvate, the final product of glycolysis, serves as the source of acetyl-CoA in fatty acid synthesis. However, fatty acid synthesis occurs in the cytosol and the conversion of pyruvate to acetyl-CoA only occurs in the mitochondrion. Since the mitochondrion is impermeable to acetyl-CoA, a molecule of citrate must first be removed from the TCA cycle and exported to the cytosol, then subsequently broken down into oxaloacetate and acetyl-CoA via ATP citrate lyase (ACLY) (Figure 1.1).

To form the individual fatty acid chains of a triglyceride, acetyl-CoA is irreversibly converted to malonyl-CoA in the cytosol via acetyl-CoA carboxylase alpha (ACACA), then subsequently assembled into long carbon chains through a repeating four-step sequence of reactions catalyzed by fatty acid synthase (FASN). To form triglycerides, the two free hydroxyl groups of glycerol-3-phosphate are acylated by two fatty acyl-CoA molecules to create phosphatidic acid through the enzymes glycerol-3-phosphate acyltransferase (GPAT) and monoacylglycerol acyltransferase (MGAT). Phosphatidic acid is then converted to 1,2-diacylglycerol via phosphatidate phosphatase (PAP) and undergoes transesterification with a third fatty acyl-CoA via diacylglyceride acyltransferase (DGAT) to form the final triglyceride molecule (Figure 1.1).

1.1.2 Mobilization of Energy Stores During Fasting

Under certain circumstances, the body must be able to rapidly mobilize hepatic glycogen stores. For example, hepatic glycogen stores must be broken down to provide glucose to the brain when dietary glucose is no longer available. The breakdown of hepatic glycogen occurs
through three enzymes: glycogen phosphorylase, oligo α(1→6) to α(1→4) glucanotransferase, and phosphoglucomutase (Figure 1.2). Specifically, glycogen phosphorylase (PYGL) releases units of glucose-1-phosphate from the non-reducing end of each individual glucose chain. This activity ceases when the enzyme reaches a point four residues away from a branch. Here, oligo α(1→6) to α(1→4) glucanotransferase (AGL) releases a glucose molecule, then transfers the remaining three residues to the other branch to form a single continuous chain that PYGL can act upon. Last, phosphoglucomutase converts glucose-1-phosphate into glucose-6-phosphate, which can then be used to produce energy through glycolysis or converted to glucose for export.

While glycogen is important as an immediate source of energy and glucose, it only provides the body with a short-term supply. After a 24 hour fast, glycogen accounts for less than 20% of the body's glucose production (1). The remaining glucose is generated through hepatic gluconeogenesis (Figure 1.2). Mitochondrial pyruvate can serve as a starting material for gluconeogenesis in the cytosol, but must first be transported there in the form of malate. The two rate-limiting enzymes involved in pyruvate-dependent gluconeogenesis are pyruvate carboxylase (PC), which catalyzes the conversion of pyruvate to oxaloacetate, and phosphoenolpyruvate carboxykinase (PCK1), which catalyzes the conversion of oxaloacetate to phosphoenolpyruvate.

During prolonged fasting, the body initiates the release of triglycerides from fat for additional energy production and gluconeogenesis. Briefly, lipolytic hormone signaling (see Section 1.1.3) results in elevated adipocyte concentrations of cAMP, thereby activating protein kinase A (PKA). PKA then phosphorylates hormone sensitive lipase (HSL) and perilipin molecules on the surface of the lipid droplet, allowing HSL to enter into the lipid droplet and hydrolyze the triglycerides into free fatty acids and glycerol. The free fatty acids then enter into circulation and are taken up by various tissues to undergo β-oxidation for ATP production.
Another starting substrate for gluconeogenesis, glycerol, derives from the breakdown of triglycerides into one molecule of glycerol and three molecules of fatty acids within adipose tissue. Glycerol released into the bloodstream undergoes aquaglyceroporin 9 (AQP9)-facilitated import into the liver and is then converted to glycerol-3-phosphate, and subsequently glucose, through a series of reactions (2) (Figure 1.2). The remaining three fatty acids are meanwhile imported via membrane fatty acid transporters, such as cluster of differentiation 36 (CD36) and fatty acid binding protein (L-FABP/FABP1), then broken down in the mitochondria to acetyl-CoA through the process of β-oxidation. The resulting acetyl-CoA is subsequently used for ATP production via the citric acid cycle or is converted into ketone bodies via ketogenesis for export to other tissues as a source of energy (Figure 1.2).

**Figure 1.2.** Mobilization of energy stores. Glycogenolysis (green box), the breakdown of hepatic glycogen, provides a source of glucose in the absence of dietary glucose. Additional glucose can be provided through gluconeogenesis (yellow box), utilizing glycerol or pyruvate as the starting material. In certain situations, ketone bodies produced via ketogenesis (red box) provide a source of energy for extrahepatic tissues. Abbreviations used: AGL, oligo α(1→6) to α(1→4) glucanotransferase; AQP9, aquaglyceroporin 9; BDH, 3-hydroxybutyrate dehydrogenase; FFA, free fatty acid; G6PC, glucose-6-phosphatase; HMGCL, 3-hydroxy-3-methylglutaryl-CoA lyase; HMGCS, 3-hydroxy-3-methylglutaryl-CoA synthase; L-FABP, liver-type fatty acid binding protein; PC, pyruvate carboxylase; PCK1, phosphoenolpyruvate carboxykinase 1; PYGL, glycogen phosphorylase.
1.1.3 Insulin, Glucagon, and Glucose Homeostasis

In order to maintain steady glucose concentrations and prevent conditions of hyperglycemia or hypoglycemia, the body employs two key hormones: insulin and glucagon. Insulin, which the pancreas releases in response to elevated glucose concentrations, specifically directs the transfer of blood glucose into peripheral tissues for energy utilization or storage. Conversely, depletion of glucose stimulates pancreatic secretion of glucagon to activate pathways involved in glucose production (gluconeogenesis) and the release of glucose from energy stores (lipolysis and glycogenolysis). Simultaneously, glucagon also inhibits pathways of glucose utilization such as glycogen synthesis and glycolysis. Ultimately, through the coordinated release of these two hormones, the body is able to maintain blood glucose levels in the narrow physiological range of 4 to 7 mM.

The insulin-mediated response to glucose encompasses a complex mixture of cellular pathways within multiple tissues and initiates when insulin binds to its cognate receptor on insulin-responsive cells (e.g. adipocytes). As these receptors are tyrosine kinase receptors, they mediate the phosphorylation of insulin receptor substrate 1/2 (IRS1/2) and subsequent activation of phosphatidylinositol-3-kinase (PI3K). IRS1/2-activated PI3K further triggers activation of phosphoinositide-dependent kinase 1/2 (PDK1/2), which phosphorylates protein kinase B (PKB/AKT) at threonine 308 to mediate additional AKT-dependent downstream phosphorylations. In muscle and adipose tissues, IRS1/2 phosphorylation also leads to phosphorylation of Casitas B-lineage lymphoma (CBL), which increases glucose transporter 4 (GLUT4) recruitment to the cell membrane (3). Together with the aforementioned phosphorylation cascades, insulin thereby increases glucose uptake and glycogen synthesis within adipose and muscle. Phosphorylation of IRS1/2 also activates the mitogen-activated
protein kinase pathway; however, this pathway is not involved in metabolism and will therefore not be discussed.

In the liver, insulin-mediated AKT-dependent phosphorylation events result in the activation of several glucose-utilizing pathways (e.g., glycogen synthesis via phosphorylation-dependent inhibition of glycogen synthase kinase 3, GSK3) (Figure 1.2A). AKT-dependent phosphorylations also inhibit the transcription of genes associated with glucose production such as G6PC and PCK1. Notably, these genes share a specific recognition sequence that binds to several different transcription factors. Particular among these transcription factors, Forkhead box proteins other 1 (FOXO1) activates gluconeogenic gene expression, but is inactivated through insulin-driven, AKT-mediated phosphorylation (4–6). An additional mediator of insulin action is the transcription factor sterol response element binding protein 1c (SREBP1C). Insulin mediates both activation of SREBP1C transcription (dependent on liver X receptor, LXR), as well as PI3K-dependent proteolytic cleavage of SREBP1C protein to its mature active form (7). This active form of SREBP1C stimulates lipogenic gene transcription, particularly that of FASN and ACACA (8, 9). Another important consequence of SREBP1C activation is the induction of GK transcription (10). GK converts glucose to glucose-6-phosphate, which is a prerequisite step for any form of glucose metabolism.

When glucose levels are depleted, insulin action ceases and results in the de-repression of glucagon excretion from the pancreas. Consequently, serum levels of glucagon begin to increase and impart several metabolic changes. Specifically, binding of glucagon to its receptor activates GTP-binding protein Gsa to trigger a rise in cAMP levels among targeted cell types (11). In hepatocytes, increased cAMP levels reduce SREBP1C activation to inhibit fatty acid synthesis and activate protein kinase A (PKA) to mediate a cascade of phosphorylations that facilitate the
effects of increased circulating glucagon (Figure 1.3B). For example, PKA-dependent phosphorylation cascades induce PYGL phosphorylation to facilitate the breakdown of glycogen into glucose and stimulate GSK3-mediated phosphorylation of glycogen synthase to inhibit glycogen synthesis (12, 13). Another result of PKA activity is increased phosphorylation of the bi-functional enzyme phosphofructokinase-2/fructose-2,6-bisphosphatase (PFK-2/FBPase-2). This phosphorylation results in a reduction of hepatic fructose-2,6-bisphosphate concentrations, simultaneously activating gluconeogenesis via phosphofructokinase-1 activation and inhibiting glycolysis via fructose 1,6-bisphosphatase inhibition (14). Gluconeogenic gene expression is additionally activated through the PKA-dependent cAMP response element binding protein (CREB) pathway.

Figure 1.3. Insulin, glucagon, and insulin-independent regulation of glucose metabolism. (A) Insulin released from the pancreas in response to dietary glucose increases uptake of glucose within insulin-responsive tissues. (B) When glucose levels decrease, de-repression of pancreatic glucagon release occurs, stimulating glucogenic activities in the liver. (C) ChREBP also regulates glucose metabolism, independent of insulin action. Abbreviations used: ACACA, acetyl-CoA carboxylase; CBP, CREB-binding protein; ChREBP, carbohydrate response element binding protein; CREB, cAMP response element binding protein; FASN, fatty acid synthase; FBPase2, fructose-2,6-bisphosphatase; FOXO1, forkhead box protein other 1; GCGR, glucagon receptor; GLUT2, glucose transporter 2; GYS2, glycogen synthase 2; GSK3, glycogen synthase kinase 3; IR, insulin receptor; IRS1/2, insulin receptor substrate 1/2; L-PK, liver-type pyruvate kinase; PC, pyruvate carboxylase; PCK1, phosphoenolpyruvate carboxykinase; PDK1/2, phosphoinositide-dependent kinase 1/2; PFK2, phosphofructokinase 2; PGC1A, PPARγ coactivator 1; PHKB, phosphorylase kinase B; PI3K, phosphatidylinositol-3-kinase; PKA, protein kinase A; PKB, protein kinase B (AKT); PP2A, protein phosphatase 2A; PYGL, glycogen phosphorylase; SREBP1C, sterol response element binding protein 1 c.
1.1.4 Insulin-Independent Regulation of Glucose Homeostasis

While insulin is an essential regulator of glucose homeostasis, additional pathways that operate independent of insulin exist. Indeed, deletion of *Srebp1c* in mouse only reduces fatty acid synthesis by 50%, indicating that other factors must be involved in regulating such insulin-responsive processes (15). In 1994, Lefrançois-Martinez et al. demonstrated that glucose is able to regulate liver-type pyruvate kinase (L-PK), the enzyme involved in the final step of glycolysis, independent of insulin or cAMP action (16). Around the same time, researchers also discovered a distinct DNA sequence necessary for carbohydrate-dependent regulation of hepatic carbohydrate metabolism. The sequence, two CACGTG motifs separated by 5 nucleotides, was coined the carbohydrate response element (ChoRE) (17). However, the exact transcription factor responsible for binding to this motif and activating transcription remained elusive. In 2001, Yamashita et al. were able to purify this protein from the nuclear extracts of a carbohydrate-fed rat liver, using the L-PK DNA motif as an affinity tag (18). They subsequently named the protein carbohydrate response element-binding protein (ChREBP).

Insulin-independent ChREBP regulation of carbohydrate metabolism occurs through the allosteric regulation of protein phosphatase 2A (PP2A) by xylulose-5-phosphate (Figure 1.3C). Under physiological concentrations of extracellular glucose, ChREBP resides in a phosphorylated, inactive form bound to 14-3-3 proteins in the cytosol (19). During periods of supraphysiological glucose concentrations, increased formation of xylulose-5-phosphate results in the activation of PP2A to effect the removal of a phosphate from ChREBP. This dephosphorylated form of ChREBP is subsequently transported into the nucleus, where it forms a heterodimer with Max-like protein X (MLX) (20). The ChREBP/MLX heterodimer complex then binds to ChoRE sequences within the promoter region of target genes to activate
transcription (17, 21). Examples of important metabolic ChREBP target genes include *LPK* and *FASN* (22, 23). Demonstrating that ChREBP plays a valuable role in numerous aspects of glucose metabolism, mice that lack any *Chrebp* expression exhibit greater hepatic glycogen accumulation, smaller adipose deposition, and decreased concentrations of plasma free fatty acids relative to wild-type littermates (24).
1.2 KEY CONSIDERATIONS REGARDING RODENT DIETS

Hepatic glucose metabolism is difficult to study because numerous environmental factors can influence its activity. Unfortunately, an important, often overlooked consideration when studying glucose metabolism in rodents is the composition of the rodent diet utilized. Precise knowledge of each individual dietary component is crucial to enable accurate data interpretation. When comparing the effects of dietary challenge, one must ensure that the diets in question differ only in a single macronutrient or micronutrient attribute, otherwise data interpretation proves difficult.

All rodent diets can be roughly broken down into the following major components, or macronutrients: carbohydrates, fat, protein, dietary fiber, vitamins, and minerals. Typically, these components are quantified as grams per kilogram diet and/or kilocalories per kilogram diet when describing a diet. Section 1.2 begins with a discussion of the overall dietary needs of rodents, while subsequent sections highlight the key properties of common rodent diets used for laboratory research. In addition, the advantages/disadvantages of each diet presented are discussed.

1.2.1 Dietary Requirements of Mice

In truth, estimating the nutritional requirements of a mouse is complicated as these needs differ between strains and as of writing, thousands of mouse strains are currently available. In addition to strain-specific variations, the nutritional requirements of a mouse also change throughout its lifecycle. Most of all, very few studies have actually focused on determining the nutritional needs of mice. Instead, researchers have historically extrapolated this information from the values known for rats. Despite these limitations, the National Research Council (NRC)
has nevertheless compiled estimates of the nutritional needs of mice and these are considered adequate for most mouse strains. Furthermore, these guidelines have been in place since 1995 and researchers have not found a need to modify them since.

Table 1.1 shows the NRC-recommended values for the major nutritional components of a mouse diet (25). Since the mice utilized in this dissertation are all adult males outside the rapid growth phase, this discussion will only focus on the dietary needs during maintenance (*i.e.* non-growth, non-lactation). Fat content is recommended at 4% (50 g per kg diet) and to prevent a deficiency of essential fatty acids, diets should contain a minimum of 0.68% dietary linolenic acid. Two good sources of linolenic acid are soya oil and corn oil, thus they are often used as the source of fat. Unlike fat content, the NRC provides no recommendations on carbohydrate content, merely stating that the typical mouse diet will contain a relatively high concentration of this component. However, research shows that diets containing 60% *w/w* or greater glucose, fructose, or sucrose can rapidly induce diabetes (26). Dietary proteins provide an important source of essential amino acids and for the maintenance phase, NRC recommends a protein concentration of 12% *w/w* (25). While very little research exists concerning the levels of essential amino acids required for maintenance, data published by R.C. Theuer (1971) provide an adequate estimate of these values and are summarized in Table 1.1 (27). Last, fiber (routinely utilized to increase diet bulk) is recommended at 50 grams per kilogram of diet (28).
Table 1.1. NRC dietary recommendations for major dietary components. Table is adapted from Table 10-4 in reference (29).

Table 1.2 outlines the NRC-recommended guidelines concerning the vitamin and mineral requirements of mice. A discussion of each individual component would prove laborious to the reader and is outside the scope of this dissertation. Similarly, a discussion of dietary vitamin or mineral deficiencies in mice is not necessary as nearly all commercial diets are formulated to provide more than the NRC-recommended quantities. However, instances in which an excess of a particular vitamin or mineral is both harmful and likely to be encountered warrants mention. In particular, excessive concentrations of vitamins A and D$_3$ in the diet are associated with certain toxicities. At very high concentrations, vitamin A will cause bone defects and eye defects, while excess vitamin D$_3$ can result in hypercalcemia and calcium deposition in muscle and kidney (30–34). In addition to vitamin D$_3$, high phosphorous concentrations, and/or a low ratio of calcium to
phosphorous in the diet can also produce calcification of the kidneys, or nephrocalcinosis (35, 36).

<table>
<thead>
<tr>
<th>Mineral</th>
<th>NRC Estimate</th>
<th>Vitamin</th>
<th>NRC Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>5 g/kg</td>
<td>Vitamin A</td>
<td>2.4 iu/g</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>3 g/kg</td>
<td>Retinol</td>
<td>720 µg/kg</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.5 g/kg</td>
<td>Vitamin D₃</td>
<td>1.0 iu/kg</td>
</tr>
<tr>
<td>Potassium</td>
<td>2-8 g/kg</td>
<td>Cholecalciferol</td>
<td>25 µg/kg</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.5 g/kg</td>
<td>Vitamin E</td>
<td>32 mg/kg</td>
</tr>
<tr>
<td>Chloride</td>
<td>0.5 g/kg</td>
<td>Vitamin K₃</td>
<td>1.0 mg/kg</td>
</tr>
<tr>
<td>Iron</td>
<td>35 mg/kg</td>
<td>Vitamin B₁</td>
<td>4.0 mg/kg</td>
</tr>
<tr>
<td>Zinc</td>
<td>10-30 mg/kg</td>
<td>Vitamin B₂</td>
<td>7.0 mg/kg</td>
</tr>
<tr>
<td>Manganese</td>
<td>10 mg/kg</td>
<td>Niacin</td>
<td>15.0 mg/kg</td>
</tr>
<tr>
<td>Copper</td>
<td>6 mg/kg</td>
<td>Vitamin B₆</td>
<td>6.6 mg/kg</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.15 mg/kg</td>
<td>Pantothenate</td>
<td>16.0 mg/kg</td>
</tr>
<tr>
<td>Iodine</td>
<td>0.15 mg/kg</td>
<td>Vitamin B₁₂</td>
<td>10.0 µg/kg</td>
</tr>
<tr>
<td>Molybdenium</td>
<td>0.15 mg/kg</td>
<td>Biotin</td>
<td>200 µg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Folate</td>
<td>0.5 mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Choline</td>
<td>833 mg/kg</td>
</tr>
</tbody>
</table>

Table 1.2. NRC guidelines on vitamin and mineral requirements in mice. Adapted from Table 10-7 in reference (29).

In addition to the recommended values for nutritional content, one can also obtain a crude prediction of overall maintenance energy requirements based on the body weight of the mouse and environmental temperature, using the following equation: $\text{MEI}_{m} = -8.91(\text{ET}) + 382 \text{ kcal/W}^{0.75}/\text{day}^{\dagger}$ (29). The facilities that house the mice used in this dissertation are maintained at approximately 23°C, thus the estimated maintenance energy requirement for a 25 gram mouse

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\(\dagger\) Where $\text{MEI}_{m}$ is the metabolizable energy intake during maintenance, ET is environmental temperature, and $W^{0.75}$ is the body weight in kilograms adjusted to metabolic body size (37, 38).
would be 46.6 kcal/day in this facility. However, the number should be approached with caution because the values used to construct the aforementioned equation derive from food intake measurements and estimates of metabolizable energy density. Therefore, several potential sources of error exist in the data collection such as food spillage and variations in the moisture content of the diet used.

1.2.2 Standard Rodent Chow

The most widely utilized types of rodent diet in the scientific literature are natural ingredient diets that typically contain a variety of agricultural products (e.g. corn, wheat, alfalfa meal, soybean meal, plant by-products, etc.). As there is no standard terminology in the industry for this type of diet, manufacturers may also refer to these diets as "standard", "chow", or "grain-based" diets. In this dissertation, the term "standard rodent chow (SC)" is utilized to describe such natural ingredient diets. For the most part, SC diets are designed to support all three stages of the rodent lifecycle: growth, maintenance, and reproduction. However, some manufacturers may additionally offer separate SC diets that meet the appropriate dietary requirements for each individual stage.

In general, one can classify SC diets as either open-formula or closed-formula. For open-formula diets, all information about the diet's formulation is publically disclosed, including the list of ingredients used and their concentrations. Thus, any researcher can reproduce these diets if so desired. Furthermore, public disclosure of the formula enables researchers to identify any potential effects of dietary composition on experimental outcome via retrospective analysis. In contrast, closed-formula diets are considered proprietary and while a manufacturer may provide a list of ingredients, they frequently withhold the exact concentrations used. Instead,
manufacturers will typically publish a periodical technical sheet with laboratory test results and estimations of select macronutrients in the diet.

Numerous manufacturers of closed-formula SC diets - including the manufacturer of the standard chow diet used in this dissertation - advertise their diets as providing constant nutrition. These constant nutrition diets maintain stable concentrations of several important nutrients, but the manufacturer can choose to modify the quantity of each ingredient at any time without disclosure. For the most part, variation in measured macronutrients such as fat and protein is kept minimal. However, variations in composition can still cause fluctuations in other non-measured components, such as the levels of flavonoids or phytoestrogens. Notably, flavonoids are known to bind AHR (see Section 1.3.3) and therefore might influence AHR signaling in studies that utilize constant nutrition standard rodent chows (39).

Variations in the composition of natural ingredient diets from different manufacturers can have a significant impact on experimental outcomes. For example, researchers found that discrepancies in phytoestrogen content between two commercial rodent diets from different manufacturers influenced their experimental results in regard to uterine gene expression and reproductive function (40). In another study, researchers observed significant variations in exocrine pancreatic secretion between mice maintained on commercial diets from two different manufacturers (41).

Ultimately, using standard rodent chow for metabolic studies in rodents can confound data interpretation, especially when comparing studies from different laboratories. Although most manufacturers claim that their natural ingredient diets are designed to minimize experimental variation, until specific industry standards are set in place, commercial natural ingredient diets will continue to be a poor choice for metabolic studies because of their
variability in composition. Instead, researchers should utilize open-formula diets when investigating rodent metabolism.

1.2.3 Purified Rodent Chow

Given the variability of standard natural ingredient rodent chows, scientists early on realized the need for an open-formula purified rodent diet. That is, a rodent diet with constant and reproducible composition, easily manipulated for studying the effect of dietary changes. The American Institute of Nutrition (AIN) first published the formula for such a rodent diet in 1977, naming it AIN-76 (42). In 1980, the AIN updated this formula to address concerns such as an apparent vitamin K deficiency (43). Nonetheless, researchers continued to experience major issues when utilizing the diet, most notably the occurrence of kidney calcification in female rats. In response to this, AIN composed two new formulas in 1993, AIN-93G and AIN-93M, both of which scientists continue to use today (28). AIN-93G is typically utilized for mice in the growth phase and during mating, while AIN-93M is designed for the general maintenance of adult mice.

Compared to the AIN-76A diet, the AIN-93 formulae contain a marked reduction in sucrose, a replacement of corn oil with soybean oil, the addition of tertiary-butylhydroquinone to prevent lipid oxidation, and several modifications of trace mineral and vitamin content. The composition of diets AIN-93G and AIN-93M are shown in Table 1.3. To account for different nutritional needs during the rapid growth phase and pregnancy, the AIN-93G formula contains a higher fat and protein content than AIN-93M, with a subsequent reduction of cornstarch content.

Standard chow and purified diet deviate significantly in their compositions. Though the nutritional content of rodent chow is variable, commercially available data allows for rough
comparisons. As per manufacturer data (Lab Diet, USA) at the time of publication, chemical analysis of a widely used brand of rodent chow (Lab Diet #5001) indicates that 29.83% of calories are derived from protein, 13.43% percent from fat, and 56.74% are provided through carbohydrates. In comparison, AIN-93M provides merely 13.91% of calories through protein and 10% through fat, but 74% of calories are derived from carbohydrates.

<table>
<thead>
<tr>
<th>Ingredients (g/kg)</th>
<th>AIN-93G</th>
<th>AIN-93M</th>
<th>High-Sucrose Diet</th>
<th>High-Fat/High-Sucrose Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>140</td>
<td>140</td>
<td>143&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>397.49</td>
<td>465.69</td>
<td>240.69</td>
<td>100</td>
</tr>
<tr>
<td>Dextrin</td>
<td>132</td>
<td>155</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
<td>400</td>
<td>392.19</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>70</td>
<td>40</td>
<td>40</td>
<td>---</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>---</td>
<td>---</td>
<td>--</td>
<td>50</td>
</tr>
<tr>
<td>Vegetable Oil</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>1.8</td>
<td>1.8</td>
<td>3.7</td>
</tr>
<tr>
<td>Vitamin Mix&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mineral Mix&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Total Energy (kcal/kg)</strong></td>
<td>3760.05</td>
<td>3601.99</td>
<td>3706.99</td>
<td>4271.71</td>
</tr>
<tr>
<td>% kcal Sucrose</td>
<td>10.64</td>
<td>11.10</td>
<td>43.16</td>
<td>36.72</td>
</tr>
<tr>
<td>% kcal Carbohydrates</td>
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<td>74.00</td>
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<tr>
<td>% kcal Fat</td>
<td>16.76</td>
<td>10.00</td>
<td>9.71</td>
<td>31.60</td>
</tr>
<tr>
<td>% kcal Protein</td>
<td>19.04</td>
<td>13.91</td>
<td>13.52</td>
<td>13.45</td>
</tr>
</tbody>
</table>

<sup>a</sup> Defined L-amino acids used in place of casein  
<sup>b</sup> Products used may vary, but are equivalent  

**Table 1.3.** Composition of AIN-93 purified diets and their variants. Formulation information was obtained from Dyets, Inc. (Bethlehem, PA).

<sup>‡</sup> Data are obtained from product sheet available for Lab Diet rodent diet #5001 (accessed October 2016)
1.2.4 High-Sucrose and High-Fat Dietary Challenges

Feeding carbohydrate-rich diets to mice has long been used as a model of hepatic steatosis and/or insulin resistance. Hepatic steatosis is a condition in which the rate of fatty acid import and/or synthesis exceeds that of fatty acid catabolism, thus leading to the abnormal accumulation of fat droplets in the liver (44). In most cases, steatosis is strongly associated with insulin resistance, a state in which the body is no longer sensitive to insulin. When steatosis occurs alongside sustained hepatic inflammation (which may result from free radical production due to excess peroxisomal fatty acid oxidation), the condition is referred to as steatohepatitis. In dire cases, steatohepatitis can further develop into cirrhosis and liver failure.

Research shows that when dietary sucrose content is increased to provide 65% or more of total caloric intake, mice will begin to develop insulin resistance, increased body weight, and macrovesicular steatosis (45, 46). Similarly, male C57BL/6 mice fed a diet in which sucrose provides 48% of caloric intake exhibit increased LPS sensitivity, decreased natural killer T cell populations and increased expression of pro-inflammatory cytokines (47). Overall, the ability of HS diet to induce hepatic steatosis in mice makes this diet a suitable model for investigating the underlying pathways of steatosis and potential therapeutic approaches.

Similar to HS diets, high-fat (HF) diets are widely utilized in rodents as a model of human obesity. HF diets are especially useful for investigating the underlying genetic pathways involved in obesity because genetic models of obesity (i.e. Ob/Ob mice) can confound data interpretation in this regard. The effects of a HF diet on mice are well characterized and include hyperglycemia, glucose intolerance, insulin resistance, reduced circulating insulin levels, and increased hepatic glucose output (48). In addition, HF-feeding induces hepatic steatosis and can
result in hepatocellular inflammation via activation of nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB (49). Interestingly, recent data indicate that the gut microbiome and the metabolites they produce also play an important role in HF diet-induced obesity (50). For example, Turnbaugh et al. (2006) demonstrate that transplantation of the gut microbiota from HFD-fed conventional mice to low-fat diet (LFD)-fed germ-free mice results in greater accumulation of body fat and an enrichment of genes involved in carbohydrate uptake/utilization, relative to LFD-fed germ-free mice transplanted with gut microbiota from LFD-fed conventional mice (51).

In addition to examining the effects of fat-rich and carbohydrate-rich diets, researchers have also investigated how interactions between the levels of these two macronutrients influence hepatic lipogenesis (52). When dietary fat content is kept relatively low, the type of carbohydrate used in the diet can greatly affect hepatic lipogenesis. For example, a fructose-based diet will result in significantly higher hepatic fatty acid synthesis than a wheat- or starch-based diet when dietary fat is kept to 0-5% w/w. As dietary fat content increases though, the effect of carbohydrate type becomes less pronounced. Nevertheless, mice consuming fructose or sucrose-based diets consistently show greater hepatic lipogenesis independent from the level of carbohydrate or fat intake. In contrast, total lipogenesis decreases as dietary fat increases and the type of carbohydrate does not affect this.
1.3 THE ARYL HYDROCARBON RECEPTOR

1.3.1 Discovery of the AHR

By the end of the 1960's, researchers had clear evidence that polycyclic aromatic hydrocarbons (PAH) induced the activity of a drug-metabolizing enzyme known as aryl hydrocarbon hydroxylase (AHH) in numerous mammalian species. By 1972, researchers had also discovered that PAHs fail to induce AHH activity in certain strains of in-bred laboratory mice. Subsequent inter-crossing of responsive (e.g. C57BL/6) and "non-responsive" (e.g. DBA/2) mouse strains led to the hypothesis that PAH induction of hydroxylase activity must be inherited as a simple autosomal dominant trait through an aryl hydrocarbon (Ah) genetic locus (53). The dominant "responsive" allele was denoted as Ahrb, while the recessive, "non-responsive" allele was denoted Ahrd. However, Poland et al. (1974) later demonstrated that contrary to some PAHs, administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) can induce AHH activity in "non-responsive" mice to a similar degree as in "responsive" mice, but consistently required a higher dosage to accomplish this. Likewise, studies from the Nebert laboratory repeatedly found that PAHs in growth medium can induce hydroxylase activity in cultured fetal cells derived from "non-responsive" mice, but only when using a significantly higher dose of PAH relative to the dose necessary for AHH induction in fetal cells from "responsive" mice (54–56). Accordingly, these data implied that the term "non-responsive" was a misnomer and mice that carry the Ahrd genetic locus are merely less responsive to certain AHH-inducing compounds. Furthermore, researchers from the Poland laboratory proposed that the Ahr genetic locus must encode for an aryl hydrocarbon receptor (AHR) protein that is necessary for
downstream induction of AHH activity and that the \( Ahr^d \) genetic locus specifically encodes for a mutant form of this protein which exhibits diminished binding affinity for certain AHH-inducing compounds (57). In the years following, researchers furnished additional evidence to support the existence of such an AHR protein through experiments that utilized a radio-labeled \([^3]H\) form of TCDD (58). However, definitive proof for the existence of AHR did not occur until the synthesis of a photoaffinity ligand, 2-azido-3-iodo-7,8-dibromodibenzo-\( p \)-dioxin, capable of covalently binding to AHR when excited by ultraviolet light (59). Using this ligand, the Poland laboratory determined that AHR physically exists as an apparent dimeric protein with 95 and 70 kDa subunits. However, later experiments indicated the 70 kDa subunit was merely a proteolytic fragment of the 95 kDa subunit in Hepa-1 cells (60).

1.3.2 Structure/Mechanism of Action

Through the use of the photoaffinity ligand 2-azido-3-iodo-7,8-dibromodibenzo-\( p \)-dioxin, researchers demonstrated that the AHR exists as a 95 kDa protein (59). Subsequent sequence analysis of a cDNA clone encoding for this protein further revealed that AHR is a member of the basic helix-loop-helix PER/ARNT/SIM (bHLH/PAS) family of proteins that contains distinct PAS A/B and Q-rich regions (61). Additional experiments demonstrated that one could further divide the transactivation domain of AHR into three sub-domains: acidic, Q-rich, and proline/serine-rich (P/S) (62, 63). Figure 1.4 details the location of these domains, as well as other known functional domains discussed below.

At the same time that researchers began to uncover the functional domains of AHR, Perdew (1992) utilized a combination of chemical cross-linking and experimentation with the newly synthesized photoaffinity ligand to demonstrate that the AHR exists as a heterotetrameric complex in the cytosol and as a heterodimer in the nucleus (64). The three additional components
of the cytosolic complex were found to be two molecules of heat shock protein 90 (HSP90) and one molecule of X-associated protein 2 (XAP2) (65, 66). Later experiments revealed the participation of p23 in this complex as well (67). Meanwhile, researchers determined that the heterodimeric partner for AHR in the nucleus was aryl hydrocarbon nuclear translocator (ARNT) (68). However, the name selected for this protein is a misnomer because ARNT does not play a direct role in the translocation of AHR to the nucleus (69).

**Figure 1.4**. Structure of the mouse AHR\(^{B1}\) protein.

To date, our understanding on the mechanism of action of AHR is as follows. In the absence of ligand, AHR resides in the cytosol as a heterotetrameric complex (65). Upon ligand binding (e.g. TCDD), the cytosolic complex localizes to the nucleus and AHR dissociates from the complex to bind to its heterodimerization partner ARNT (Figure 1.5A-B). This activity is mediated in part by interactions between the nuclear localization sequence (NLS) and the nuclear pore protein β-importin (70). Within the nucleus, the AHR/ARNT heterodimer binds to a specific nucleotide sequence known as the dioxin response element (DRE), found within the 5′-UTR of various genes. This DRE-binding activity subsequently results in the activation of target gene (e.g. Cyp1a1) transcription (Figure 1.5C). Recently, AHR was also found to elicit changes
in gene expression independent of DRE-binding (71). Such activity might occur through protein-protein interactions within the cytosol or via AHR heterodimerization with partners other than ARNT in the nucleus (Fig 1.5D-E).

**Figure 1.5.** AHR mechanism of action. (A) AHR normally resides in the cytosol in a complex with XAP2, p23, and two molecules of HSP90. (B) Ligand binding induces translocation of AHR to the nucleus and dissociation from the cytosolic complex. (C) In the nucleus, AHR forms a heterodimer with ARNT and binds to DRE sequences within target genes to activate transcription. (D) AHR can also interact with other proteins in the cytosol or (E) nucleus to exert its effects.
1.3.3 AHR Ligands and Their Classifications

AHR ligands are ubiquitous in our environment. The most widely studied group of AHR ligands is halogenated aromatic hydrocarbons (HAH), due to their high affinity for AHR and the myriad of harmful effects they impart. By far, TCDD is the best characterized chemical among this group. To date, no other compound has been shown to bind to AHR with an affinity higher than that of TCDD (72). Similar to the HAHs are a group of chemicals known as polycyclic aromatic hydrocarbons (PAH). PAHs, such as 3-methylcholanthrene (3-MC) and benzo[a]pyrene (BaP), bind with much lower affinity and are readily metabolized (73). Accordingly, PAH-mediated AHR transcription tends to occur in a more temporal manner that HAH-associated transcription.

While most characterized AHR ligands are artificial HAH or PAH compounds, numerous other ligands are found naturally within the typical human diet. One prime example is indolo[3,2-b]carbazole (ICZ), the major acid condensation product of a compound found readily in cruciferous vegetables, indole-3-carbinol (I3C). Interestingly, ICZ binds to AHR with an affinity close to that of TCDD, which has led some to believe that ICZ and other similar indole-containing compounds may represent "endogenous" AHR ligands (74). However, the exact definition of endogenous AHR ligand remains an area of dispute in the literature. In addition to ICZ, researchers have reported that several other indole derivatives of tryptophan can also bind with high affinity for AHR, including indirubin and indigo (75). Separate from the indole-containing compounds, certain flavonoids (e.g. quercetin) are also known to bind AHR (39, 76). Notably, flavonoid compounds naturally occur in large quantities within certain plants and plant products.
In the context of this dissertation, AHR ligands can be placed into one of three different classifications: agonists, antagonists, and selective aryl hydrocarbon receptor modulators (SAhRM) (Figure 1.6). Specifically, AHR agonists are ligands that result in activation of the canonical AHR signaling pathway (i.e. nuclear translocation, heterodimerization, and DRE-binding), as evidenced through increased transcription of the prototypical AHR target gene CYP1A1. Simply put, an AHR antagonist is an AHR ligand that inhibits agonist-driven, DRE-dependent CYP1A1 transcription. However, certain antagonists (i.e. 1-allyl-7-trifluoromethyl-1H-indazol-3-yl]-4-methoxyphenol, SGA360) are known to also exhibit DRE-independent effects, particularly the repression of cytokine-driven acute phase gene expression (77). Such antagonists are classified as SAhRM, with the repression of acute phase gene expression representing an example of "SAhRM activity". While agonists cannot be considered SAhRM because SAhRMs are defined in this text as displaying antagonist activity, they can still exert SAhRM activity. For example, Patel et al. (2009) demonstrated that TCDD promotes repression of cytokine-driven acute phase gene expression in the absence of DRE binding by utilizing an AHR mutant incapable of binding to DRE sequences (78). Based on the previous definition of SAhRM presented here, ligands that exhibit antagonist activity in the absence of SAhRM activity will therefore considered in this text as "pure antagonists". An example of a pure antagonist is the compound N-(2-(1H-indol-3-yl)ethyl)-9-isopropyl-2-(5-methylpyridin-3-yl)-9H-purin-6-amine (GNF351) (79).
Figure 1.6. Chemical structure and classifications for key AHR ligands.
1.4 MOUSE MODELS OF AHR

1.4.1 $Ahr^d$ Mouse Models with Low-Affinity for Ligands

As previously discussed, the discovery of AHR arose through initial observations of its ability to induce AHH activity. A particularly important observation made early on was that researchers could pharmacologically induce AHH activity in C57BL/6N mice using certain compounds, but that higher doses of these compounds were necessary to do so in other common mouse strains such as DBA/2N, NZW/BLN, or NZB/BLN (53, 55–57). Eventually, this difference in responsiveness was attributed to a mutation within the genetic $Ahr$ locus that results in a mutant AHR protein with diminished affinity for binding to AHH-inducing ligands and researchers denoted the less responsive allele as $Ahr^d$ (58, 80).

The discovery of these two allelic forms of AHR implied that researchers could easily compare the consequences of AHR ligand treatment in C57BL/6N and DBA/2N mice to isolate effects specific to ligand activation. This comparison is innately flawed however, as these two strains are not otherwise genetically identical. To truly isolate any ligand-mediated effects of AHR activation, one must compare the two alleles of $Ahr$ in mice that share an identical genetic background (congenic mice). Accordingly, Alan Poland and Daniel Nebert successfully introduced the low-affinity $Ahr^d$ allele onto a C57BL/6J genetic background during the 1990's to produce such a congenic model (B6.D2N-$Ahr^d$/J). By using this model, researchers have demonstrated a wide array of ligand-dependent, AHR-mediated effects such as the TCDD-mediated reduction of estrogen receptor binding capacity or TCDD-elicited reductions in hepatic pyruvate carboxylase activity (81, 82).
1.4.2 AHR-Deficient Mice

Between 1995 and 1997, three laboratories independently generated and characterized an AHR-deficient mouse (83–85). Frank Gonzalez and his team accomplished this by replacing the first exon of Ahr with a neomycin gene, while another group led by Yoshiaki Fujii-Kuriyama in Japan, instead replaced this exon with the bacterial gene beta-galactosidase fused to a nuclear localization signal (84, 85). Similarly, researchers from the Bradfield laboratory generated an Ahr-null mouse by completely removing exon 2, which codes for the basic helix-loop-helix region necessary for interaction with ARNT (83, 86).

The development of an AHR-deficient mouse model subsequently led to the discovery that AHR is involved in numerous physiological pathways, including angiogenesis (87), cardiovascular development (88), hematopoiesis (89), development of the female reproductive organs (90), and wound healing (91). Given that AHR is involved in such an array of physiological processes, systemic AHR deficiency has also been shown to result in numerous deleterious health effects including decreased weight and fertility and the presence of liver pathology (86). As a result, the interpretation of any data obtained using these models is difficult because one cannot discern between the primary and secondary effects of AHR deficiency.

1.4.3 Ahr<sub>fx</sub>f<sub>x</sub> Alb<sub>Cre</sub>, a Targeted Deletion of AHR in Hepatocytes

During the development of the Ahr-null mouse in the Bradfield laboratory, researchers observed that their Ahr-null mice exhibited significantly smaller livers, reduced fecundity, and decreased body weight. Given these results, they hypothesized that AHR likely plays a role in the development of the liver and in 2000, published data showing that AHR is in fact required for proper closure of the ductus venosus (DV) during liver development (92). They further demonstrated that TCDD-treatment of hypomorphic AHR mice (Ahr<sub>fx</sub>f<sub>x</sub>neo/neo) is able to rescue
developmental closure of the DV. Briefly, hypomorphic AHR mice are C57BL/6 mice that carry the $Ahr^d$ allele, with a Neo cassette flanked by two $LoxP$ sites introduced at either end of exon 2 (93). However, AHR expression levels in these mice are approximately less than ten percent of the levels observed in B6.D2N-$Ahr^d$/J mice.

In 2005, researchers from the Bradfield laboratory further discovered that AHR-dependent liver development and hepatotoxicity are actually mediated by different cell types (94). To demonstrate this, they crossed $Ahr^{fx/fx}$ mice (generated from $Ahr^{fneo/fneo}$ mice by removing the Neo cassette) with mice that express the $Cre$ transgene driven by either an albumin promoter ($Alb^{cre}$, strain designation: B6.Cg-Tg(Alb-cre)21Mgn_J) or a Tie2 kinase promoter ($Tek^{cre}$, strain designation: B6.Cg-Tg(Tek-cre)12Flv_J) to remove AHR from hepatocytes and endothelial cells, respectively. While the DV failed to close in $Ahr^{fx/fx} Tek^{cre}$ mice, the livers of $Ahr^{fx/fx} Alb^{cre}$ mice exhibited proper developmental closure of the DV and thus, normal hepatic development. They also presented further evidence that hepatocytes, and not endothelial cells, are the major contributor to the AHR adaptive response in liver.

The $Ahr^{fx/fx} Alb^{cre}$ mouse model serves as an excellent tool for investigating the hepatocyte-specific effects of AHR activation. These mice are also useful for determining the physiological role of AHR in the mature liver because they exhibit normal liver development comparable to wild-type C57BL/6 mice. Indeed, we utilize the $Ahr^{fx/fx} Alb^{cre}$ mouse model in this dissertation to investigate the physiological role of AHR in the absence of exogenous ligand activation. Nevertheless, a major limitation to the $Ahr^{fx/fg} Alb^{cre}$ mouse model is that these mice carry the low-affinity $Ahr^d$ allele and therefore, likely do not represent the full extent of homeostatic AHR activity in the presence of endogenous ligand. Likewise, this model is not useful for isolating ligand-mediated, AHR-dependent effects in hepatocytes based on the low
affinity of \( Ahr^d \) for AHR ligands. However, one might alternatively view this model as representing the ligand-independent mechanisms of AHR-dependent gene regulation, given that the \( Ahr^d \) allele exhibits lower affinity for ligand binding relative to \( Ahr^b \).

1.4.4 Constitutively-Active AHR Protein

One commonly used model of AHR is the transgenic constitutively-active AHR (CA-AHR) mouse model. Andersson et al. (2002) generated such a model by deleting the minimal ligand binding domain without disrupting DNA binding function (95). The resulting mutant AHR protein is unable to bind ligands, but mediates constitutive transcription of \( Cyp1a1 \). A major limitation to this model is that these mice still express ligand-inducible AHR protein, in addition to CA-AHR. Such genetic heterogeneity can be problematic when interpreting data, especially due to the possibility for dietary AHR ligands within plant materials used for the manufacturing of rodent chows (76, 96). In addition, these mice exhibit CA-AHR expression within numerous organs, despite specific targeting of this mutation to lymphatic organs. Consequently, this model is not useful for studying the influence of AHR on metabolism because discerning which dysregulations account for the effects of CA-AHR expression, as well as the organs that they originate from, proves difficult. Despite these limitations, the CA-AHR is still a useful model to screen for potential AHR target genes.
1.5 THE ROLE OF ARYL HYDROCARBON RECEPTOR IN METABOLISM

Researchers have long known that TCDD can alter metabolism through AHR-dependent mechanisms. In fact, researchers discovered early on that AHR overexpression could alone influence metabolic gene expression in the absence of exogenous ligand, suggesting that AHR might play a role in metabolic homeostasis (97). Since those initial discoveries, much research has sought to determine what exactly that role is and the following sections outline our current understanding of how AHR might be involved in regulating metabolism. However, many of the experimental approaches used to study AHR in this regard contain major flaws and will serve as a point of discussion.

1.5.1 The Role of AHR in Adipocyte Differentiation

TCDD has been well characterized as a mediator of the wasting syndrome, a disease characterized by severe loss of body weight and the disruption of lipid and carbohydrate metabolism (98). As a highly lipophilic compound, TCDD significantly accumulates within adipose tissue where it likely mediates regulatory changes to promote this disease (99). Indeed, TCDD treatment of 3T3-L1 pre-adipocytes results in the inhibition of adipocyte differentiation, while administration of TCDD in vivo is associated with inhibition of glucose transport, lipoprotein lipase activity, and fatty acid synthesis (100–103). Similarly, AHR overexpression in 3T3-L1 fibroblast cells suppresses adipocyte differentiation, but this effect can be reversed via treatment with PPARγ ligands (97). In contrast to pre-adipocytes, TCDD has no apparent effects when administered to mature adipocytes (103). The fact that AHR overexpression can suppress adipocyte differentiation in the absence of ligand suggests that AHR might be important for homeostatic regulation of differentiation. However, research to date has overwhelmingly focused
on the role of AHR in TCDD-mediated inhibition of adipocyte differentiation, thus the ligand-independent role of AHR in adipogenesis remains poorly understood.

1.5.2 TCDD-Elicited Changes in Metabolism

While investigations into the relationship between TCDD and metabolism began decades ago, research concerning the potential role of AHR in metabolic homeostasis has only recently emerged. In 2005, two independent groups simultaneously published data from microarray analyses on hepatic tissues from TCDD-treated rodents. Specifically, TCDD was found to affect hepatic gene expression in pathways ranging from amino acid metabolism to cholesterol and bile acid biosynthesis (104, 105). However, these studies lacked any AHR-deficient models to control for possible AHR-independent effects. The first conclusive proof that AHR is involved in metabolism did not appear until 2008, when Sato et al. examined the long-term effects of low-dose TCDD exposure in C57BL/6N and Ahr-null mice (104). Their data confirm that TCDD treatment alters the hepatic expression of genes involved in cholesterol synthesis, lipogenesis, and glucose metabolism in an AHR-dependent manner.

Subsequent to the initial discoveries described above, numerous other publications have further explored how TCDD might elicit AHR-dependent effects on metabolism. In 2010, Diani-Moore et al. demonstrated that TCDD-mediated suppression of hepatic gluconeogenesis occurs through AHR regulation of TCDD-inducible poly(ADP-ribose) polymerase (Tiparp) (106). That same year, the Zacharewski group published ChIP-chip data from TCDD-treated ovariectomized mice demonstrating that thousands of DNA regions are AHR-enriched at 2 and 24 h post-treatment and linked the data to their concurrent studies on TCDD-elicited hepatic steatosis (107, 108). More recently, this group also examined TCDD-elicited effects on liver, serum, and adipose lipid content and the toxicogenomic, liver-specific effects of long-term TCDD exposure.
in ovariectomized mice (109, 110). The data from these later studies are consistent with their observations in TCDD-elicited hepatic steatosis.

Despite the wealth of information on AHR-dependent, TCDD-elicited changes in metabolism, most data is of limited use for determining the role of AHR in metabolic homeostasis. A major limitation to such studies is that TCDD can directly alter the secondary and tertiary structure of lipoprotein molecules, which might impair cellular uptake of LDL and VLDL and cause a build-up of lipoproteins in the blood serum (111). In addition, TCDD can elicit a battery of gene expression that is independent of AHR activation (112). Another major caveat to these studies is that they may not be physiologically relevant. For example, the administered TCDD dose is usually well above the typical range to which humans may be exposed. Whereas the majority of TCDD studies utilize dosages within the µg/kg range, mean estimates of human exposure are merely within the range of pg/kg/day (113). Ultimately, while experiments with TCDD can be useful for identifying potential metabolic AHR target genes, they are not suitable for examining the role of AHR in metabolic homeostasis.

1.5.3 Allelic Variations in Ahr and Their Effect on Metabolism

In contrast to using TCDD to investigate the role of AHR in metabolism, several groups have instead approached this task utilizing the various allelic variants of Ahr that are available. The first to do so was the Xie laboratory at the University of Pittsburgh in 2010. These researchers investigated fatty acid metabolism and hepatic steatosis using a mouse model that expresses a constitutively active form of AHR (CA-AHR) (114). Specifically, they found that constitutive AHR activation spontaneously induces hepatic steatosis and that the fatty acid import protein CD36 is a transcriptional target of AHR. These authors also posited that increased CD36 expression likely accounted for the AHR-mediated hepatic steatosis witnessed in CA-
AHR mice. As discussed in Section 1.4.4 however, this mouse model of AHR is inherently flawed. Furthermore, while utilizing CA-AHR may be a useful tool for identifying AHR transcriptional targets, our laboratory has since published data demonstrating that not all AHR activity is dependent upon DRE-binding (78). Therefore, much like studies performed with TCDD, data collected from CA-AHR mice are of limited use in discerning the homeostatic role of AHR in metabolic homeostasis.

In 2012, the Tischkau laboratory published the next major study to investigate the role of AHR in metabolic homeostasis and determined that Ahr-null mice exhibit enhanced insulin sensitivity and improved glucose tolerance. Their data further demonstrated that AHR deficiency disrupts the endogenous circadian rhythms of hepatic peroxisome proliferator-activated receptor alpha (Ppara), brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein (Bmal), and period circadian clock 1 (Per1) expression. The authors largely attributed these metabolic changes to attenuated PPARα activity (115). During that same year, another study on AHR and metabolism surfaced, this time from the laboratory of Craig Tomlinson, investigating how C57BL/6 mice harboring the Ahr<sup>b</sup> or Ahr<sup>d</sup> allele respond to a Western (high-fat) diet (116). While they found no gross differences between Ahr<sup>b</sup> or Ahr<sup>d</sup> mice fed a standard chow diet, they demonstrated major differences in how these mice respond to a Western diet. In general, they observed that the low-affinity Ahr<sup>d</sup> allele conferred a level of protection against diet-induced obesity.

More recently, our laboratory has published two studies showing the involvement of AHR in de novo fatty acid and cholesterol synthesis (71, 117). The first of these studies examined de novo cholesterol synthesis and demonstrated that AHR activation not only represses hepatic cholesterol synthesis, but that this occurs independent of DRE-binding. Similarly, the
second study showed that *de novo* fatty acid synthesis is also repressed by AHR activation independent of DRE-binding. Both studies employed the use of mouse model that exhibits hepatocyte-localized expression of a mutant AHR protein incapable of binding to DRE sequences. This mouse model was generated by establishing a point mutation at alanine 78, then introducing the mutant onto a $\text{Ahr}^{f/f}\text{Alb}^{cre}$ background using the hepatocyte-specific transthyretin (TTR) promoter.
1.6 FIBROBLAST GROWTH FACTOR 21 (FGF21)

1.6.1 Fibroblast Growth Factors

The fibroblast growth factor (FGF) family of proteins is a large group of 22 proteins involved in an array of biological processes including mitogenesis and wound-healing (118, 119). FGF proteins vary in molecular weight between 17-34 kDa, but share a core homologous region of 120-130 amino acid residues that form 12 antiparallel β-strands (120, 121). Using sequence homology and phylogeny, one can further separate the FGF protein family into six distinct subfamilies: FGF1, FGF2, FGF4, FGF8, FGF9, and FGF15/19 (122). With the exception of the FGF15/19 subfamily, all FGFs are considered paracrine factors secreted through the classic endoplasmic reticulum-Golgi apparatus secretory pathway (123). All FGF proteins mediate their effects through binding to fibroblast growth factor receptors (FGFRs) and in most cases, require interactions with heparin sulfate glycosaminoglycans in the extracellular matrix for efficient binding (124). FGFRs are receptor tyrosine kinases, and upon binding FGF, mediate downstream phosphorylation events to confer regulation (Figure 1.7A). Four genes encode for FGFRs, Fgfr1-4, three of which (Fgfr1-3) yield two isoforms (b and c) via alternative splicing.

1.6.2 Fibroblast Growth Factor 21

Among the members of the fibroblast growth factor family, fibroblast growth factor 21 (FGF21) has lately received much attention for its ability to reduce the obese phenotype. Researchers originally discovered FGF21 within a mouse embryo cDNA library during a screen for novel fibroblast growth factor family members (125). These authors also determined that this novel FGF family member was highly expressed in the adult mouse liver, and to a much lesser extent, in the thymus. FGF21 is an uncharacteristic member of the fibroblast growth factor
family because while it can bind to the FGF receptor, it does not exhibit strong interactions with heparin sulfate. This allows FGF21 to act in an endocrine fashion, as it can dissociate from the originating cell and enter into circulation (126). High-affinity binding of FGF21 to FGFR instead requires interactions between FGFR and the single-pass transmembrane protein, βKlotho (Figure 1.7B) (127). The asymmetric distribution of βKlotho throughout the body allows for selectivity in the site of action of FGF21. In particular, βKlotho is highly expressed in the metabolically active liver and brown/white adipose tissues (127). Most notably, FGF21 production is primarily limited to the liver and stimulated by various different physiological conditions. These conditions, as well as the transcription factors necessary for induction of Fgf21 transcription under them, are discussed in Section 1.6.3.

Figure 1.7. Mechanisms of fibroblast growth factor-dependent gene regulation. (A) With the exception of FGF15/19 and FGF21, all members of the FGF family require heparin sulfate for high-affinity binding to fibroblast growth factor receptors (FGFR) and subsequent downstream phosphorylation events. (B) FGF21 (and FGF15/19) binding to FGFR requires interactions with βKlotho protein.
1.6.3 The Complex Regulation of FGF21

Numerous transcription factors have been implicated in the regulation of hepatic FGF21 production to date, each one activating FGF21 transcription under specific physiological conditions. Although researchers are continually uncovering new pathways of FGF21 activation, only the three physiological conditions of FGF21 induction that are relevant to this dissertation are discussed: fasting, excess levels of carbohydrates, and endoplasmic reticulum (ER) stress.

1.6.3.1 FGF21 as a Mediator of the Fasting Response

Research has repeatedly shown that FGF21 is important during fasting and starvation. In 2007, three independent studies demonstrated that fasting a mouse results in elevated hepatic expression of FGF21 (128–130). In addition, all three studies showed that transcriptional Fgf21 induction required the expression of peroxisome proliferator-activated receptor alpha, Ppara. Inagaki et al. (2007) further show that PPARα forms a heterodimer with retinoid X receptor alpha (RXRα) and binds to the Fgf21 promoter to activate its transcription during fasting (128). However, data from one recent study suggest that PPARα may instead form a heterodimer with the active N-terminal fragment of cAMP response element binding protein hepatocyte specific (CREBH) to facilitate Fgf21 transcription during fasting (131).

PPARα, a member of the peroxisome proliferator-activated receptor family important to fatty acid metabolism, is primarily expressed in liver and brown adipose tissue (BAT) and plays a major role in the response to fasting (132, 133). During a prolonged fast, an increased load of FFA to the liver results in fatty acid binding to PPARα and subsequent induction of its activity (134). In particular, PPARα stimulates the expression of rate-limiting enzymes involved in peroxisomal β-oxidation (\textit{i.e.} acyl-CoA oxidase, ACO) and mitochondrial β-oxidation (\textit{i.e.}
CPT1A) (135, 136). In fact, mice that lack any PPARα expression exhibit impaired hepatic uptake and oxidation of FFAs under fasting conditions (133). Interestingly, administration of exogenous FGF21 to Ppara<sup>−/−</sup> mice can reverse the effects associated with PPARα deletion (e.g. steatosis, hypoketonemia, hypoglycemia, and hypothermia), thereby suggesting that FGF21 is involved in mediating the PPARα fasting response (128). In further support of this hypothesis, conditions that mimic a fast, such as a ketogenic diet and suckling in mouse neonates, also result in the induction of Fgf21 expression (129, 137).

Our knowledge about the consequences of FGF21 activation during prolonged fasting is as follows. In the liver, FGF21 promotes ketogenesis through increased protein, but not mRNA expression of HMGCS2 and CPT1A (128). FGF21 also increases hepatic transcription of pancreatic lipase (PNLIP), pancreatic lipase related protein 2 (PNLIPRP2), and carboxyl ester lipase (CEL) and may additionally contribute to ketogenic activity by further increasing FFA load to the liver during fasting. Additional evidence suggests that FGF21 elevates hepatic fatty acid oxidation, TCA cycle flux, and gluconeogenesis during starvation through increased expression of PPARγ coactivator protein 1 alpha (PGC1α) (138). In white adipose tissue, FGF21 might also play a role in increasing lipolytic activity given that transgenic FGF21 mice exhibit increased HSL and adipose triglyceride lipase (ATGL) mRNA/protein, as well as decreased adiposity, relative to wild-type mice (128). Last, FGF21 also appears to be involved in fasting-induced torpor, as torpor is elevated in FGF21 transgenic mice and mice infected with an FGF21-expressing adenovirus, but not in wild-type mice.

**1.6.3.2 Carbohydrate-Dependent Induction of Fgf21 Transcription**

Paradoxically, hepatic Fgf21 expression is increased during prolonged fasting, but also in mice fed a carbohydrate-rich diet (139, 140). In the liver, glucose-mediated activation of FGF21
occurs through direct binding of ChREBP to the FGF21 promoter (141). Interestingly, ChREBP modulation of Fgf21 expression was recently implicated in regulating sugar intake and sweet taste preference. Specifically, FGF21 selectively suppress sugar appetite in response to carbohydrate intake by acting on the paraventricular nucleus of the hypothalamus in a ChREBP-dependent manner (142). In the companion paper to this study, researchers further demonstrated that FGF21 can suppress sweet and alcohol preference in mice and monkeys. Such effects were associated with decreased dopamine levels, again suggesting that FGF21 might be acting through the central nervous system (143).

In cultured adipocytes, FGF21 can enhance insulin-independent glucose uptake by increasing GLUT1 mRNA and protein levels (144). The physiological relevance of this in vitro data has been confirmed in studies utilizing mice and non-human primates (144, 145). Furthermore, treatment of cultured adipocytes with PPARγ agonists, along with administration of thiazolidinedione (TZD) or feeding in mice, has been shown to induce adipocyte FGF21 levels (115, 146). In fact, FGF21 appears to be a key mediator of PPARγ-dependent adipocyte gene expression given that it potentiates the effects of TZD on adipocyte differentiation. Accordingly, loss of FGF21 function correlates with impaired PPARγ signaling and resistance to the insulin sensitizing properties of TZD.

In an attempt to explain why FGF21 is paradoxically induced by fasting and feeding, Potthoff et al. propose two hypotheses in their 2012 review of FGF21 (147). First, FGF21 might regulate the efficient capture of nutrients when they first become available after an extended fast. This particular hypothesis is supported by data showing strong Fgf21 transcriptional induction in WAT under fasting-refeeding conditions (148). Second, FGF21 simply regulates pathways important to both the fed and fasted states, for example, triglyceride synthesis. After feeding,
triglyceride synthesis is necessary for storing any excess dietary fat and carbohydrate, but is also necessary during fasting because most fatty acids are still re-esterified back to triglycerides in liver and adipose. Regardless of the explanation, one can nevertheless make clear distinctions between fasting-mediated and feeding-mediated transcriptional FGF21 activation. For example, the fasting-dependent induction of FGF21 transcription occurs exclusively in liver, where it acts in both an autocrine and endocrine fashion to activate glucogenic pathways. In contrast, carbohydrate-mediated induction of FGF21 transcription primarily occurs in adipose tissue, where it acts in an autocrine fashion to stimulate glucose uptake independent of insulin action. However, as recent studies suggest, hepatic FGF21 output may also be important when dietary carbohydrates are in excess.

1.6.3.3 ER Stress-Mediated Activation of Fgf21 Transcription

Aside from the activation of Fgf21 expression under conditions of fasting and high-carbohydrate diet feeding, ER stress, a condition in which misfolded proteins accumulate inside the ER, can also induce Fgf21 transcription. The first evidence for this was published by Schaap et al. (2013), who implicated the eukaryotic initiating factor 2, alpha (eIF2α)/activating transcription factor 4 (ATF4) phosphorylation cascade in the induction of Fgf21 expression (149). The eIF2α/ATF4 cascade represents one of the three branches of the unfolded protein response (UPR), which is the adaptive cellular response to ER stress. The other two branches of the UPR involve activating transcription factor 6 (ATF6) or inositol-requiring enzyme 1 alpha (IRE1α) and X-box binding protein 1 (XBP1). Whereas activation of ATF6 occurs via transmembrane proteolysis, efficient XBP1-activation of the UPR requires IRE1α-dependent splicing of XBP1 mRNA (150). Jiang et al. (2014) demonstrated that XBP1 can activate Fgf21 transcription in response to ER stress, but to date, Fgf21 transcriptional activation via ATF6 has
not yet been observed (151). Interestingly, XBP1-driven *Fgf21* gene expression results in negative feedback via the suppression of eIF2α/ATF4 (151).

The fact that *Fgf21* transcription is activated by multiple branches of the UPR indicates that this factor must play an important role in the response to ER stress. Indeed, Jiang et al. (2014) demonstrate that administration of recombinant FGF21 to tunicamycin-treated mice results in the alleviation of ER stress-mediated hepatic triglyceride overload (151). The authors hypothesize that *Fgf21*-mediated suppression of the eIF2α-ATF4-DDIT3 (DNA damage inducible transcript 3, DDIT3) pathway may represent a major mechanism through which FGF21 reduces ER stress-driven hepatic steatosis, especially given the role of DDIT3 in the perturbation of metabolic processes such as fatty acid oxidation and lipoprotein secretion (152). They further postulate that FGF21-directed suppression of *DDIT3* transcription might also influence ER stress-induced apoptosis, as is observed with FGF2 (153). Lastly, FGF21-dependent alleviation of steatosis is associated with the inhibition of SREBP1c protein maturation and may represent an additional mechanism to alleviate hepatic triglyceride overload.

### 1.6.4 AHR-Mediated Regulation of FGF21

Recently, two independent laboratories demonstrated that AHR is able to activate *Fgf21* transcription. The first study, published by Cheng et al. (2014), determined that daily injections of 40 μg/kg TCDD or 200 mg/kg BNF in C57BL/6J mice over four consecutive days result in increased hepatic *Fgf21* gene expression (154). Furthermore, they found that treatment of Hep3B or Hepa1c1c7 cells with 10 nM TCDD for 16 h produces a 150-fold and 230-fold induction of hepatic *Fgf21* expression, respectively, and that these effects do not occur in the absence of AHR expression. The authors also present data showing that AHR binds to the -105/+1 region of the
The second study, published by Lu et al. (2015), demonstrates that mice expressing a constitutively-active form of AHR display elevated hepatic Fgf21 mRNA expression. Like the previous study, these authors also provide data showing AHR binds to the -105/+1 region of the Fgf21 promoter. In chapter three of this dissertation, we present contradictory data that demonstrate AHR activation attenuates the induction of FGF21 gene expression by other transcription factors (155). A discussion of our results in the context of what has been previously shown in the literature is presented in Section 3.4.

1.6.5 FGF21 as a Therapeutic Target

For more than a decade, FGF21 has been viewed as a potential therapeutic target in the treatment of obesity. In 2005, Kharitonenkov et al., demonstrated that administration of FGF21 to ob/ob mice improves glucose tolerance, insulin sensitivity, and decreases the levels of serum triglycerides (156). Coskun et al. (2008) later demonstrated that administration of FGF21 to ob/ob over the course of two weeks also decreases body weight, increases energy exposure, and suppresses de novo lipid synthesis in the liver (157). Similar to these studies in ob/ob mice, Xu et al. (2009) further demonstrated that FGF21 administration reverses the effects of diet-induced obesity in mice (158). Most importantly, researchers have demonstrated that administration of exogenous FGF21 to diabetic non-human primates also produces beneficial effects, including reduced fasting levels of insulin and a reduction of serum LDL (145). Moreover, recent drug discovery efforts have revealed an FGF21 variant, LY2405319, that is physically stable and able to be expressed in yeast with equal in vivo biological potency relative to the native FGF21 molecule (159). Notably, LY2405319 has had much success in early clinical trials and when administered to obese human subjects diagnosed with type 2 diabetes, results in weight loss, improved sensitivity to insulin, and decreased serum triglycerides (160, 161).
Contradictory to the ameliorative effects of recombinant FGF21 administration, increased FGF21 protein and mRNA has been linked to obesity in mice, while elevated serum levels of FGF21 are linked to pathologies such as obesity, non-alcoholic fatty liver disease (NAFLD), and type 2 diabetes in humans (162–165). Jiang et al. (2014) provide evidence to suggest that elevated FGF21 levels in such pathologies might be related to the unfolded protein response (UPR) pathway, stimulated by the onset of ER stress. Specifically, these authors demonstrate that XBP1 is able to directly activate Fgf21 transcription under ER stress and that both of these factors are elevated during diet-induced obesity (151). An alternative hypothesis is that increased FGF21 protein during obesity might represent a state of FGF21 "desensitization" or "resistance", similar to insulin resistance. However, administration of FGF21 to hyperglycemic ob/ob mice has nevertheless been shown to normalize hyperglycemia, indicating that local metabolic changes in the liver may explain the elevated levels of serum FGF21 during obesity (166).
CHAPTER 2:

THE IMPACT OF BASAL AHR ACTIVITY WITHIN HEPATOCYTES ON THE
RESPONSE TO DIETARY CHALLENGE IN MICE
2.1 Introduction

Historically, researchers have studied the AHR within the context of its ability to regulate gene expression in response to exogenous agonist ligands. Traditionally, AHR agonists are defined as ligands that when bound to AHR, induce nuclear localization, heterodimer formation with ARNT, and subsequent activation of DRE-dependent transcription. Canonically, the presence of such agonist-mediated AHR activity is represented by the induction of Cyp1a1 transcription. However, agonist-driven AHR activity can also occur in the absence of Cyp1a1 transcription. For example, Patel et al. (2009) demonstrated that TCDD promotes the repression of cytokine-driven acute phase gene expression in the absence of DRE binding through the use of an AHR mutant that is unable to bind to DRE sequences (78). Similarly, researchers have shown that AHR can even exert regulation in the apparent absence of any exogenous ligand. In one study, AHR was found to constitutively bind to the Il6 promoter and maintain the DNA in a primed state that is readily accessible to inflammatory transcriptional activators (167). Likewise, naïve AHR receptor in unstimulated cells can be found constitutively bound to a wide range of gene clusters, and upon ligand binding, is displaced from these targets in favor of binding to the promoter regions of genes important to xenobiotic metabolism (168). Together, these studies imply that while increased Cyp1a1 transcription is a key indicator of AHR-dependent transcriptional activity, it likely does not represent all AHR activity. Ultimately, the importance of AHR activity occurring in absence of Cyp1a1 transcriptional induction and its exact physiological role remain poorly understood.

To date, AHR has been implicated in the regulation of numerous physiological pathways such as angiogenesis and female reproduction (87, 90). Only recently have investigators also demonstrated a role for AHR in hepatic metabolism. For the most part, these studies rely on data
obtained using xenobiotic-mediated activation of AHR, mice that express a constitutively active AHR mutant, or AHR-deficient mice (95, 106, 108, 114). However, these are all poor models of homeostatic AHR signaling and instead, represent conditions of aberrant AHR signaling. More recently, researchers examined hepatic gene expression in high-fat (HF) diet-fed mice harboring the \(Ahr^b\) or \(Ahr^d\) allele (116). While this study provides an improved understanding of how typical agonist-driven AHR activity influences metabolism, the role of AHR in the absence of such activity remains unknown. Furthermore, whether endogenous ligands bind with equal affinity to the \(Ahr^b\) and \(Ahr^d\) alleles is also unclear.

Although knowing the composition of a rodent diet is necessary to draw any conclusions from metabolic studies in which they're utilized, researchers often overlook this key factor in their experiments. Research studies concerning the role of AHR in metabolism utilize a wide variety of rodent diets, ranging from closed-formula standard chows or partially-disclosed diet formulations, to purified high-fat and/or high-sucrose diets (116, 169–171). In some cases, closed-formula rodent chows may even be utilized as a control for open-formula purified dietary challenges. Moreover, diet formulations can significantly vary between batches and commercial manufacturers. In fact, we postulate that within the scientific literature, instances of contradictory data concerning the role of AHR in metabolism might simply stem from differences in the rodent diet used.

This study employs a mouse model that lacks AHR protein expression within hepatocytes \((Ahr^{fx/fx}Alb^{Cre})\) to examine how the loss of hepatocyte-specific AHR signaling impacts metabolic homeostasis under several dietary conditions. Specifically, \(Ahr^{fx/fx}Alb^{Cre}\) mice and AHR-expressing mice from the parental strain \((Ahr^{fx/xf})\) were fed a common closed-formula commercial rodent chow (SC), open-formula AIN-93M purified rodent chow (PUR), or open-
formula high-sucrose (HS) and high-fat/high-sucrose (HF/HS) modifications of AIN-93M (28, 93). Importantly, we find no essential difference in the levels of Cyp1a1 transcription between Ahr\textsuperscript{fx/fx} and Ahr\textsuperscript{fx/fx Alb\textsuperscript{Cre}} mice. Therefore, any changes in metabolic homeostasis between these mice must largely reflect the impact of basal AHR activity\textsuperscript{§}. In particular, we show that the loss of basal AHR activity within hepatocytes generally correlates with decreased liver and body mass, along with alterations of white adipose accumulation and adipocyte morphology. In addition, Ahr\textsuperscript{fx/fx Alb\textsuperscript{Cre}} mice maintained on a PUR diet exhibit less efficient uptake of serum glucose under fasting conditions, without any change in insulin sensitivity relative to Ahr\textsuperscript{fx/fx} mice. Conversely, HS-fed Ahr\textsuperscript{fx/fx Alb\textsuperscript{Cre}} mice exhibit decreased insulin sensitivity during a fast without any difference in glucose tolerance or perturbations of fed-state insulin signaling.

Regardless of diet, histological analysis reveals that the loss of hepatocyte-specific AHR expression correlates with reduced hepatocyte ballooning degeneration, a hallmark of steatohepatitis (172). Therefore, we hypothesize that basal AHR activity within hepatocytes contributes to and/or predisposes these mice to diet-induced steatohepatitis. Indeed, we present evidence showing Ahr\textsuperscript{fx/fx Alb\textsuperscript{Cre}} maintained on a HF/HS diet exhibit a lower degree of hepatic lipid deposition, inflammation, and fatty acid import gene expression, as well as increased gene expression of key proteins involved in triglyceride export. Taken together, our data indicate that in the absence of exogenous ligand, basal AHR activity within hepatocytes plays an important role in regulating hepatic metabolism. From a toxicology viewpoint, the results suggest that AHR antagonism may therefore be a suitable therapeutic approach for treating diet-induced steatohepatitis.

\textsuperscript{§} "Basal AHR activity" refers to all AHR activity that occurs in the absence of exogenous ligands and Cyp1a1 transcription.
2.2 Materials and Methods

Animal Experiments – 12-week old, age-matched male B6.129SvJ-Ahr<sup>Tm3.1Bra</sup> (<sup>Ahr<sup>fx/fx</sup></sup>) and <sup>Ahr<sup>fx/fx</sup></sup><sup>Alb</sup><sup>Cre</sup> mice (n = 6-8 per group) were maintained on standard rodent chow (Purina, USA) (SC), purified diet (PUR) AIN-93M (Dyets, USA), or high-sucrose diet (HS) (Dyets, USA) for up to 16 weeks, or on high-fat/high-sucrose diet (HF/HS) (Dyets, USA) for 5 weeks. The composition of each diet is shown in Table 2.1. <sup>Ahr<sup>fx/fx</sup></sup> and <sup>Ahr<sup>fx/fx</sup></sup><sup>Alb</sup><sup>Cre</sup> mice were kindly provided by Christopher Bradfield (University of Wisconsin). Mice were housed on corncob bedding in a pathogen-free, temperature and light-controlled facility, and were given access to food ad libitum. All mouse experiments were carried out humanely with approval, and in accordance to the Animal Care and Use Committee of the Pennsylvania State University guidelines.

Serum Composition - Blood was collected via the cardiac puncture method. After 30 min incubation at room temperature to allow for clotting, samples were centrifuged at 10,000 x g for 15 min and the supernatant removed for further analysis. Triglycerides/cholesterol (Wako Chemicals, Japan), free fatty acids (Cayman Chemicals, USA), and insulin (EMD-Millipore, Germany), were measured using colorimetric assays according to the manufacturers' directions. Serum ALP was screened using a mammalian liver profile sample rotor and VetScan VS2 analyzer (Abaxis Inc.).
<table>
<thead>
<tr>
<th>Ingredients (g/kg)</th>
<th>Standard Rodent Diet</th>
<th>AIN-93M</th>
<th>High-Sucrose Diet</th>
<th>High-Fat/High-Sucrose Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>n/a*</td>
<td>140</td>
<td>140</td>
<td>143&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>210</td>
<td>465.69</td>
<td>240.69</td>
<td>100</td>
</tr>
<tr>
<td>Dextrin</td>
<td>n/a</td>
<td>155</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>Sucrose</td>
<td>38.3</td>
<td>100</td>
<td>400</td>
<td>392.19</td>
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<tr>
<td>Soybean Oil</td>
<td>n/a</td>
<td>40</td>
<td>40</td>
<td>---</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>n/a</td>
<td>---</td>
<td>--</td>
<td>50</td>
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<tr>
<td>Vegetable Oil</td>
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<td>---</td>
<td>---</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>50</td>
<td>50</td>
<td>50</td>
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<tr>
<td>L-Cystine</td>
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<td>1.8</td>
<td>1.8</td>
<td>3.7</td>
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<tr>
<td>Vitamin Mix&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>10</td>
<td>10</td>
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<tr>
<td>Mineral Mix&lt;sup&gt;b&lt;/sup&gt;</td>
<td>n/a</td>
<td>35</td>
<td>35</td>
<td>35&lt;sup&gt;b&lt;/sup&gt;</td>
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<td><strong>Total Energy (kcal/kg)</strong></td>
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<td>3601.99</td>
<td>3706.99</td>
<td>4271.71</td>
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<tr>
<td>% kcal Sucrose</td>
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<td>11.10</td>
<td>43.16</td>
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<td>% kcal Carbohydrates</td>
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<td>74.00</td>
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<td>53.65</td>
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<tr>
<td>% kcal Fat</td>
<td>13.43</td>
<td>10.00</td>
<td>9.71</td>
<td>31.60</td>
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<tr>
<td>% kcal Protein</td>
<td>29.83</td>
<td>13.91</td>
<td>13.52</td>
<td>13.45</td>
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</table>

*not available or not applicable
<sup>a</sup>Defined L-amino acids used in place of casein
<sup>b</sup>Products used may vary, but are equivalent

**Table 2.1.** Comparison of SC, PUR, HS, and HF/HS experimental diets.

*Liver Triglycerides/Cholesterol* - To extract liver triglycerides and cholesterol, 50 mg of liver tissue was homogenized into 1 mL of standard diluent assay reagent (Cayman Chemical, USA), then centrifuged at 10,000 x g for 10 min at 4° C. From the supernatant, triglycerides and cholesterol were quantified using colorimetric assays according to the manufacturer's instructions (Wako Chemicals, Japan).
Histology - For Hematoxylin and Eosin (H&E) staining, liver or adipose samples were fixed onto individual cassettes for 24 h in a 10% formaldehyde solution (Macron Chemicals, Centre Valley, PA) and transferred into 70% ethanol for long-term storage. Staining services were provided by Histoserv, Inc (Germantown, MD). Adipose size was calculated using cellSens Standard software (Olympus) under 400X magnification. All histology images were captured using an Olympus BX43 microscope equipped with an SC100 digital camera (Olympus).

Glucose/Insulin Tolerance Test - For the glucose tolerance test (GTT), mice were fasted overnight for 16 h, then administered 1 g/kg D-glucose (Sigma-Aldrich, Inc.) via i.p. injection. Blood glucose concentrations were measured prior to injection, as well as 15, 30, 60, and 120 min after injection using a blood glucose monitor (CVS-Caremark, USA). For the insulin tolerance test (ITT), mice were fasted for 5 h, then administered 0.75 U/kg human recombinant insulin (Sigma-Aldrich, Inc.) via i.p. injection. Blood glucose concentrations were measured with a blood glucose monitor (CVS-Caremark, USA) prior to injection, as well as 15, 30, 60, and 120 min post-injection.

RNA Extraction and Quantitative RT-PCR – RNA was extracted from frozen liver sections using TRI Reagent (Sigma-Aldrich) according to manufacturer's instructions. cDNA was then generated using a High-Capacity cDNA Archive Kit (Applied Biosystems, USA). Gene expression was measured using PerfeCTa™ SYBR® Green SuperMix for iQ (Quanta Biosciences, USA) with the primers listed in Table 2.2. Quantitative analysis was conducted using MyIQ software (Bio-Rad Laboratories, USA) and the data were normalized to ribosomal protein L13a (Rpl13a) or β-actin (Bactin) mRNA expression.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5' → 3')</th>
<th>Gene</th>
<th>Sequence (5' → 3')</th>
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<td>Acaca F</td>
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<td>Il16 F</td>
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<td>ATGCTGTTTCCCTAGGCTAACATCT</td>
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<td>Apob100 F</td>
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<td>Il1b F</td>
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<td>TGGGGAAGGAAACAACTGAGA</td>
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<td>ATACTGCGTGGTCTAGAAGTCCCTTG</td>
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<tr>
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<td>Mttp F</td>
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<td></td>
<td>CTGGCTCCGAAAAGGACATCC</td>
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<td>AGCGTGTTGAAAGGACCTTAT</td>
</tr>
<tr>
<td>Bactin F</td>
<td>GAGGCCCAAGACAGAAGAG</td>
<td>Nos2 F</td>
<td>CAACGTCTCCGTTCTCTTG</td>
</tr>
<tr>
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<td>GTGTGAGTCTGTCGAGG</td>
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<td>CCTTGAGTGAGGACTGAGA</td>
</tr>
<tr>
<td>Cpt1a F</td>
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<td>Pemt F</td>
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<td></td>
<td>GTGTGAGTCTGTCGAGG</td>
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<td>GGTTACATGGACCCCACAGA</td>
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<tr>
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<td>Ppara F</td>
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<tr>
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<td>Rpl13a F</td>
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<td>CTGCCGACCTTCTCGCTCAGGG</td>
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<td>GCATCTGGCTTCCTCTCCT</td>
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<tr>
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<td>Xbp1t F</td>
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<td>GGTGTCGCTAGGGACCTACA</td>
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<td>GCCATGGGAGAGTGCTTCTG</td>
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<tr>
<td>Fasn F</td>
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<td></td>
<td>R</td>
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<tr>
<td></td>
<td>ATGCAGGTCATGCTTCTTAGCACC</td>
<td></td>
<td>R</td>
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Table 2.2. List of primers used for RT-qPCR.

*Protein Expression Analysis* - Total protein was extracted from liver sections using Tissue Protein Extraction Reagent (TPER) according to manufacturer's instructions (Thermo Fisher Scientific Inc.). Protein concentration was assayed using a Bradford colorimetric assay kit (Thermo Fisher Scientific Inc.) and 40 µg of protein was resolved on either a 6% or 8% Tricine-
SDS-polyacrylamide electrophoresis gel. Proteins were then transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA) and probed with the following antibodies: rabbit anti-ACC1 (Protein Tech), rabbit anti-AKT (Cell Signaling Technology), rabbit anti-phospho-AKT (Thr308) (Cell Signaling Technology), mouse anti-β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), mouse anti-CD36 (BD Pharmigen), mouse anti-FASN (BD Pharmigen), mouse anti-HSP70 (Thermo Fisher Scientific Inc.), mouse anti-P23 JJ3 (Thermo Fisher Scientific Inc.), or rabbit anti-SCD1 M38 (Cell Signaling Technology). Bands were quantified using ImageJ software (173).

Statistical Analysis – All data are presented as mean ± SEM. Statistical analyses were performed using a two-tailed Student’s t test or one-way ANOVA.

2.3 Results

$Ahr^{f/f}$ and $Ahr^{f/f} Alb^{Cre}$ mice exhibit no significant differences in Cyp1a1 transcriptional activity in the absence of exogenous ligand. $Ahr^{f/f}$ mice harbor the $Ahr^d$ allele, which exhibits an apparent lower affinity for ligands relative to the $Ahr^b$ allele. Nevertheless, treatment with 10 µg/kg TCDD (using corn oil as a vehicle) via gavage markedly induces hepatic Cyp1a1 in these mice (Figure 2.1A). Thus, the data indicate that this allelic form of AHR can still facilitate agonist-driven AHR activity associated with the induction of Cyp1a1 transcription. Whereas purified diet contains minimal plant material according to its formulation, closed-formula commercial chows typically include several plant species that may harbor AHR agonists (e.g. alfalfa or soybean meal). Therefore, we examined whether Cyp1a1 transcription might be higher in SC-fed $Ahr^{f/f}$ mice due to the presence of plant material. As shown in Figure 2.1B, hepatic Cyp1a1 transcription is approximately 3-fold greater in SC-fed $Ahr^{f/f}$ mice than in $Ahr^{f/f}$ mice maintained on any purified diet. Regardless of diet however, $Ahr^{f/f}$ and $Ahr^{f/f} Alb^{Cre}$ mice
exhibit similar levels of Cyp1a1 transcription. Ultimately, the data suggest that in the absence of exogenous ligand, any changes in metabolic homeostasis due to the loss of hepatocyte-specific AHR expression do not primarily stem from AHR activity associated with the induction of Cyp1a1 transcription. Instead, these differences largely represent the loss of basal hepatocyte-specific AHR activity occurring in the absence of Cyp1a1 transcription. Two examples of such AHR activity include the interaction of AHR with cytosolic proteins (in addition to, or separate from the HSP90-XAP2-P23 complex) and the constitutive binding of AHR to various gene promoters.

Figure 2.1. In the absence of exogenous ligand, Ahr^{fx/fx} and Ahr^{fx/fx}Alb^{Cre} mice exhibit no significant difference in Cyp1a1 transcription. (A) Treatment with 10 µg/kg TCDD via gavage is able to induce Cyp1a1 transcription in Ahr^{fx/fx} mice harboring the low-affinity Ahr^{d} allele. (B) Cyp1a1 expression is greater in SC-fed Ahr^{fx/fx} and Ahr^{fx/fx}Alb^{Cre} mice relative to mice maintained on purified diets. Data are presented as mean ± SEM. Statistical analyses were performed using a two-tailed Students t test. Letters indicate statistical significance, * P < 0.05 or ** P < 0.01, relative to the sample group represented by the corresponding parenthetical letter.
Loss of hepatocyte AHR signaling is associated with reduced body mass and relative liver mass. To explore how basal AHR activity influences metabolism, we first compared body mass between SC, PUR, HS, and HF/HS-fed Ahr^flox/flox and Ahr^flox/flox Alb^Cre mice (Table 2.3). While the loss of AHR expression in hepatocytes does not affect body mass in SC-fed mice, PUR-fed Ahr^flox/flox Alb^Cre mice exhibit a significant 14% loss in body mass relative to Ahr^flox/flox mice. Similarly, Ahr^flox/flox Alb^Cre fed a HS or HF/HS diet exhibit a modest 6.4% or 11.3% decrease in respective body mass relative to Ahr^flox/flox mice. In mice maintained on a PUR, HS, or HF/HS diet, the loss of hepatocyte-specific AHR signaling also correlates with decreased relative liver mass. Specifically, relative liver mass in Ahr^flox/flox Alb^Cre mice is significantly reduced 14% relative to Ahr^flox/flox mice when maintained on PUR diet, 6% on a HS diet, and 11% under HF/HS challenge (Table 2.3). In contrast, relative liver mass is comparable between SC-fed Ahr^flox/flox and Ahr^flox/flox Alb^Cre mice. Consistent with these observations, hepatic triglyceride content is also similar in SC-fed Ahr^flox/flox and Ahr^flox/flox Alb^Cre, but significantly 3-fold lower in HS-fed Ahr^flox/flox Alb^Cre mice and modestly 1.7-fold lower in HF/HS-fed Ahr^flox/flox Alb^Cre mice relative to Ahr^flox/flox. Despite our observations that relative liver mass is decreased in Ahr^flox/flox Alb^Cre mice relative to Ahr^flox/flox, PUR-fed Ahr^flox/flox and Ahr^flox/flox Alb^Cre mice exhibit similar hepatic triglyceride concentrations. Last, we observe that liver cholesterol concentrations are similar in Ahr^flox/flox and Ahr^flox/flox Alb^Cre mice fed a SC, PUR, or HS diet. However, HF/HS-fed Ahr^flox/flox Alb^Cre mice exhibit a significant 43% decrease in hepatic cholesterol concentrations relative to Ahr^flox/flox mice.
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<th>SC</th>
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<td>$Alb^{Cre}$</td>
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<td>$Alb^{Cre}$</td>
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<td><strong>Body Mass (g)</strong></td>
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<td>25.57 ± 0.294</td>
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<td><strong>Relative Liver Mass$^a$</strong></td>
<td>0.436 ± 0.020</td>
<td>0.471 ± 0.004</td>
<td>0.471 ± 0.0041*</td>
<td>0.399 ± 0.011*</td>
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<td><strong>Liver Triglycerides (mg/100 mg Liver)</strong></td>
<td>0.42 ± 0.044</td>
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<td><strong>Liver Cholesterol (mg/100 mg Liver)</strong></td>
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<td>0.208 ± 0.040</td>
<td>0.278 ± 0.036</td>
<td>0.314 ± 0.024</td>
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$^a$Values shown are grams per 10 grams body mass

**Table 2.3.** Gross physiology of $Ahr^{fx/fx}$ and $Ahr^{fx/fx} Alb^{Cre}$ mice maintained on different rodent diets. All data are presented as mean ± SEM. Statistical analyses were performed using a Student's t test with significance denoted as follows: *P < 0.05, **P < 0.01, ***P < 0.001 relative to $Ahr^{fx/fx}$.

**Hepatocyte-targeted deletion of AHR influences adipose tissue mass and adipocyte morphology.**

To further investigate how the loss of basal hepatocyte-specific AHR activity can influence metabolic homeostasis, we quantified the relative accumulation of adipose in $Ahr^{fx/fx}$ and $Ahr^{fx/fx} Alb^{Cre}$ mice. When maintained on a SC or PUR diet, $Ahr^{fx/fx}$ and $Ahr^{fx/fx} Alb^{Cre}$ mice exhibit similar accumulations of WAT. However, HS-fed $Ahr^{fx/fx} Alb^{Cre}$ mice exhibit significant 1.5-fold less accumulation of WAT in comparison to $Ahr^{fx/fx}$ mice. While not statistically significant, $Ahr^{fx/fx} Alb^{Cre}$ challenged with HF/HS diet also exhibit a 26% reduction in relative WAT mass compared to $Ahr^{fx/fx}$ (Figure 2.2A). To further investigate adipose metabolism in $Ahr^{fx/fx}$ and $Ahr^{fx/fx} Alb^{Cre}$ mice, we performed histological analyses on WAT sections to compare adipocyte morphology. Shown in Figure 2.2B-E are H&E stains of WAT tissue samples at 400X magnification. Although we find no difference in adipose tissue mass between SC-fed $Ahr^{fx/fx}$ and $Ahr^{fx/fx} Alb^{Cre}$ mice, adipocytes appear smaller in H&E-stained samples from $Ahr^{fx/fx} Alb^{Cre}$ mice (Figure 2.2B). To quantify this potential difference, we measured adipocyte area in tissue...
samples from three mice, then averaged the results. Indeed, we find that average adipocyte size is significantly reduced in SC-fed $Ahr^{fs/fx}Alb^{Cre}$ mice (12% vs. $Ahr^{fs/fx}$, $P = 0.04$). Consistent with previous data, we observe that adipocytes are comparable in size between PUR or HF/HS-fed $Ahr^{fs/fx}$ and $Ahr^{fs/fx}Alb^{Cre}$ mice, but appear smaller in HS-fed $Ahr^{fs/fx}Alb^{Cre}$ mice relative to $Ahr^{fs/fx}$ (Figure 2.2C-E). In support, quantitative analysis shows that average adipocyte size is reduced 17% in HS-fed $Ahr^{fs/fx}Alb^{Cre}$ mice relative to $Ahr^{fs/fx}$, though this difference falls short of statistical significance ($P = 0.058$).
Figure 2.2. H&E staining of adipose tissue from $Ahr^{fx/fx}$ and $Ahr^{fx/fx} Alb^{Cre}$ mice under different dietary challenges. (A) Relative adipose mass. (B) H&E staining of adipose tissue from $Ahr^{fx/fx}$ and $Ahr^{fx/fx} Alb^{Cre}$ mice maintained on standard rodent chow, (C) purified rodent chow, (D) high-sucrose, and (E) high-sucrose/high-fat diet. All images were produced at 400X magnification. Statistical analyses were performed using a Student's t test. Letters indicate statistical significance, * P < 0.05 or ** P < 0.01, relative to the sample group represented by the corresponding parenthetical letter.
Fasting Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice maintained on PUR diet demonstrate diminished rates of glucose uptake from serum without any aberrations in insulin signaling. To investigate the influence of basal hepatocyte-specific AHR activity on glucose metabolism and insulin signaling, we performed glucose and insulin tolerance tests in mice maintained on PUR diet for an extended feeding period of 12 weeks. We specifically chose this diet because the formulation is publicly available and as we previously show, is associated with no significant level of agonist-driven AHR activity. The results of the GTT are shown in Figure 2.3A and demonstrate that after a bolus 1 g/kg \textit{i.p.} dose of glucose, fasting Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice display slower glucose uptake relative to Ahr\textsuperscript{fx/fx} mice. Utilizing area under the curve analysis (AUC) to quantify the difference, we specifically find that Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice exhibit a significant 1.6-fold decrease in glucose tolerance relative to Ahr\textsuperscript{fx/fx} mice. Next, to investigate whether decreased glucose uptake in Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice is associated with enhanced insulin action, we administered an insulin tolerance test. As shown in Figure 2.3B, Ahr\textsuperscript{fx/fx} and Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice maintained on PUR diet exhibit similar rates of glucose uptake from serum after \textit{i.p.} injection of 0.75 U/kg insulin, thus indicating no significant difference in insulin responsiveness. Supporting this, we observe no difference in the ratio of P-AKT to AKT protein expression between non-fasted PUR-fed Ahr\textsuperscript{fx/fx} and Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice in response to basal insulin signaling (Figure 2.3C). Furthermore, mRNA and protein expression of genes involved with fatty acid synthesis are also not significantly different between Ahr\textsuperscript{fx/fx} and Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice maintained on PUR diet (Figure 2.3D-E).
Figure 2.3. Fasting PUR-fed $Ahr^{fx/fx} Alb^{Cre}$ mice exhibit decreased rates of glucose uptake without any apparent differences in insulin signaling. (A, left) Serum glucose concentrations after i.p. injection with 1 g/kg glucose. (A, right) Area under the curve calculations. (B, left) Serum glucose concentrations after i.p. injection of 0.75 U/kg recombinant insulin. (B, right) Area under the curve calculations. (C) Ratio of pAKT to AKT protein in PUR-fed $Ahr^{fx/fx}$ and $Ahr^{fx/fx} Alb^{Cre}$ mice. (D) Relative ACACA mRNA and protein expression. (E) Relative FASN mRNA and protein expression. All data are presented as mean ± SEM. Statistical analyses were performed using a Student's t test. Letters indicate statistical significance, * P < 0.05, relative to the sample group represented by the corresponding parenthetical letter.
Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} maintained on a HS diet exhibit decreased sensitivity to insulin under fasting conditions, without any signs of glucose intolerance or disruptions of basal insulin pathway signaling. We next examined hepatic glucose metabolism in HS-fed mice to determine whether loss of hepatocyte-specific AHR expression might alter the response to a challenge of increased dietary carbohydrates. When maintained on a high-sucrose diet, Ahr\textsuperscript{fx/fx} mice exhibit no difference in glucose tolerance relative to Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice (Figure 2.4A). Despite a similar tolerance to glucose, HS-fed Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice are less sensitive to insulin and when given a bolus dose of insulin, exhibit decreased clearance of glucose from the blood relative to Ahr\textsuperscript{fx/fx} (Figure 2.4B). When the data are quantified as AUC, Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice specifically show a significant 1.4-fold decrease in insulin sensitivity. Although insulin sensitivity differs between fasting Ahr\textsuperscript{fx/fx} and Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice, we observe no difference in the ratio of P-AKT/AKT in response to basal insulin action, thereby suggesting there is no significant state of insulin resistance in Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice (Figure 2.4C). While we observe no change in mRNA levels of Acaca between the two genotypes, ACACA protein levels are nevertheless modestly reduced 40% (P = 0.14) in Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice relative to Ahr\textsuperscript{fx/fx} (Figure 2.4D). Similarly, gene and protein expression of FASN are also moderately decreased 32% (P = 0.25) and 24% (P = 0.16), respectively, in Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice relative to Ahr\textsuperscript{fx/fx} (Figure 2.4E).
Figure 2.4. HS-fed \(Ahr^{+/+}\)\(Alb^{Cre}\) mice exhibit decreased insulin sensitivity during a fasted state. (A, left) Serum glucose concentrations after i.p. injection with 1 g/kg glucose. (A, right) Area under the curve calculations. (B, left) Serum glucose concentrations after i.p. injection of 0.75 U/kg recombinant insulin. (B, right) Area under the curve calculations. (C) Ratio of pAKT to AKT protein in HS-fed \(Ahr^{+/+}\) and \(Ahr^{+/+}\)\(Alb^{Cre}\) mice. (D) Relative ACACA mRNA and protein expression. (E) Relative FASN mRNA and protein expression. All data are presented as mean ± SEM. Statistical analyses were performed using a Student’s t test. Letters indicate statistical significance, * \(P < 0.05\), relative to the sample group represented by the corresponding parenthetical letter.
In the absence of exogenous ligand, basal hepatocyte-specific AHR expression correlates with increased hepatocyte ballooning degeneration under HS challenge and additionally with increased immune cell infiltration under HF/HS challenge. To investigate how hepatocyte-specific AHR activity in the absence of Cyp1a1 transcription might influence hepatic morphology, we performed histological analyses on liver tissue samples from Ahr\textsuperscript{fx/fx} and Ahr\textsuperscript{fx/fx}Alb\textsuperscript{Cre} mice. As shown in Figure 2.5A, Ahr\textsuperscript{fx/fx} mice fed a SC diet exhibit no aberrant liver pathologies relative to Ahr\textsuperscript{fx/fx}Alb\textsuperscript{Cre} mice. In contrast, Ahr\textsuperscript{fx/fx} mice maintained on PUR diet exhibit a greater degree of hepatocyte ballooning degeneration compared to Ahr\textsuperscript{fx/fx}Alb\textsuperscript{Cre} mice (Figure 2.5B), while HS challenge further augments this difference (Figure 2.5C). In addition, Ahr\textsuperscript{fx/fx} and Ahr\textsuperscript{fx/fx}Alb\textsuperscript{Cre} mice also exhibit signs of immune cell infiltration under HS challenge.

In trend with our observation of modestly increased hepatic triglyceride content, Ahr\textsuperscript{fx/fx} mice maintained on a HF/HS diet appear to exhibit modestly increased fat accumulation relative to Ahr\textsuperscript{fx/fx}Alb\textsuperscript{Cre} mice as evidenced by the increased size of macrovesicular fat droplets (Figure 2.5D). In contrast to modestly elevated lipid deposition, Ahr\textsuperscript{fx/fx} mice display significantly greater immune cell infiltration relative to Ahr\textsuperscript{fx/fx}Alb\textsuperscript{Cre} mice maintained on HF/HS diet.
Figure 2.5. H&E staining of livers from $Ahr^{fx/fx}$ and $Ahr^{fx/fx} Alb^{Cre}$ mice maintained on various rodent diets. Diets shown include (A) standard rodent chow, (B) purified rodent chow, (C) high-sucrose, and (D) high-sucrose/high-fat diet. Macrovesicular steatosis due to HF/HS feeding is readily visible in both genotypes, though occurs to a modestly greater extent in $Ahr^{fx/fx}$ mice. Inflammatory foci are present in HS- and HF/HS-fed $Ahr^{fx/fx}$ mice, as well as HS-fed $Ahr^{fx/fx} Alb^{Cre}$ mice. All images shown were produced at 200X magnification.
Under HF/HS challenge, Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice exhibit diminished de novo lipogenesis and fatty acid import, along with increased transcription of key genes involved in triglyceride export. Based on histological analysis and hepatic triglyceride quantification, HF/HS-challenged Ahr<sup>fx/fx</sup> mice display modestly elevated hepatic lipid accumulation relative to Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup>. Therefore, we investigated whether de novo lipogenesis might be diminished in Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice. Indeed, we observe that hepatic gene/protein expression of key enzymes involved in de novo lipogenesis is significantly reduced in Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice relative to Ahr<sup>fx/fx</sup> (Figure 2.6). Specifically, Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice exhibit a significant 50% loss in hepatic Acaca gene expression and a significant 27% decrease in relative ACACA protein concentrations compared to Ahr<sup>fx/fx</sup> mice (Figure 2.6A). Similarly, hepatic Fasn expression is significantly 69% lower in Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice compared to Ahr<sup>fx/fx</sup>, while relative hepatic FASN protein is reduced 27% (Figure 2.6B). In addition, Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice exhibit a significant 78% decrease in hepatic Scd1 gene transcription and a 54% reduction in relative SCD1 protein levels (Figure 2.6C). Though slightly reduced in Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice, Srebp1c gene expression is not significantly different between the two genotypes (Ahr<sup>fx/fx</sup>, 2.256 ± 0.36 vs. Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup>, 1.686 ± 0.35).

In addition to de novo lipogenesis, we also examined key proteins involved in the import and export of fatty acids from the liver. Notably, we observe that hepatic expression of Cd36, an AHR target gene involved in fatty acid import, is significantly reduced 2-fold in Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice relative to Ahr<sup>fx/fx</sup> and corresponds with a significant 1.4-fold decrease in CD36 protein expression (Figure 2.6D) (114). Though not significantly different, the expression of two other genes involved in hepatic fatty acid uptake, fatty acid binding protein 1 (Fabp1) and fatty acid transporter member 5 (Fatp5), are also modestly reduced in Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice relative to Ahr<sup>fx/fx</sup> (Figure 2.6E). Among the key proteins involved in fatty acid export, we demonstrate that...
Ahr^{fx/fx} Alb^{Cre} mice exhibit a significant 1.7-fold increase in the transcription of apolipoprotein B100 (Apob100), a protein utilized to package hepatic triglycerides for export (Figure 2.6F). Likewise, transcription of microsomal triglyceride transfer protein (Mttp), the rate-limiting enzyme in the transfer of triglycerides to APOB100, is increased 1.3-fold in Ahr^{fx/fx} Alb^{Cre} mice relative to Ahr^{fx/fx}, but falls short of statistical significance (P = 0.07) (174). Last, we demonstrate that Ahr^{fx/fx} Alb^{Cre} mice exhibit a significant 1.5-fold increase in phosphatidylethanolamine N-methyltransferase (Pemt) transcription relative to Ahr^{fx/fx}. Notably, reduced expression of Pemt, the enzyme that converts phosphatidylethanolamine to phosphatidyl choline, has been associated with the development of NAFLD on a HF diet (175).
Figure 2.6. HF/HS diet-fed $Ahr^{fx/fs}$ $Alb^{Cre}$ mice exhibit decreased de novo fatty acid synthesis and fatty acid import, along with elevated expression of key triglyceride export genes relative to $Ahr^{fx/fs}$. Decreased hepatic gene expression and protein levels of (A) ACACA, (B) FASN, and (C) SCD1 in $Ahr^{fx/fs}$ $Alb^{Cre}$ mice. (D) $Ahr^{fx/fs}$ $Alb^{Cre}$ mice display decreased hepatic gene and protein expression of Cd36. (E) Fabp1 and Fatp5 gene expression is modestly lower in $Ahr^{fx/fs}$ $Alb^{Cre}$ mice. (F) Increased expression of genes involved with fatty acid export in $Ahr^{fx/fs}$ $Alb^{Cre}$ mice. Abbreviations used: Acaca, acetyl CoA carboxylase alpha; Apob100, apolipoprotein B100; Cd36, cluster of differentiation 36; Fabp1, fatty acid binding protein 1; Fasn, fatty acid synthase; Fatp5, fatty acid transporter member 5; Mttp, microsomal triglyceride transfer protein; Pemt, phosphatidylethanolamine N-methyltransferase; Scd1, stearoyl-CoA desaturase 1. All data are presented as mean ± SEM from 5-6 mice per group. Statistical analyses were performed using a Student’s t test. Letters indicate statistical significance, * P < 0.05 or ** P < 0.01, relative to the sample group represented by the corresponding parenthetical letter.
Acute phase gene expression, but not ER stress, is decreased in HF/HS-fed Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice relative to Ahr<sup>fx/fx</sup>. Congruent with an apparent increase in hepatic immune cell infiltration in HF/HS-fed Ahr<sup>fx/fx</sup> mice, we observe that serum levels of alkaline phosphatase (ALP), a marker of hepatic injury, are significantly elevated in Ahr<sup>fx/fx</sup> mice relative to Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> (Figure 2.7A). Therefore, we investigated whether the loss of basal hepatocyte-specific AHR activity might associate with reduced inflammatory gene expression under HF/HS challenge. Indeed, we find that Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice exhibit a significant 61% decrease in the expression of tumor necrosis factor alpha (Tnfa) and a significant 54% reduction in interleukin-6 (Il6) mRNA, along with a significant 55% loss of interleukin-1 beta (Il1b) expression relative to Ahr<sup>fx/fx</sup> mice (Figure 2.7B-D). In addition, Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice exhibit modest reductions in serum amyloid A3 (Saa3) and C-reactive protein (Crp) transcript levels, though these differences are not statistically significant (Figure 2.7E-F). In contrast, hepatic nitric oxide synthase (Nos2) expression is markedly reduced 87% in Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice relative to Ahr<sup>fx/fx</sup> (Figure 2.7G). To determine the presence of ER stress, we measured the ratio of spliced Xbp1 mRNA (Xbp1s) to native Xbp1 mRNA (Xbp1n), as this ratio increases significantly under ER stress. Figure 2.7H shows that this ratio is similar between HF/HS-fed Ahr<sup>fx/fx</sup> and Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice and therefore indicates that the loss of hepatocyte-specific AHR does not influence ER stress. In addition, there is no difference in Atf4 or Ddit3 gene transcription, two indicators of ER stress, between these strains of mice (Figure 2.7I-J).
Figure 2.7. Ahr<sup>fx/fx</sup> Alb<sup>Cre<sup> mice fed a HF/HS diet exhibit reduced hepatic inflammation relative to Ahr<sup>fx/fx</sup>. (A) Serum concentration of ALP in HF/HS-fed Ahr<sup>fx/fx</sup> and Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice. Hepatic expression of (B) Tnfa, (C) Il1b, and (D) Il6. (E) Expression of Saa3 (F) Crp and (G) Nos2. (H) Ratio of spliced Xbp1 transcript relative to native transcript. (I) Expression levels of Atf4 and (J) Ddit3, two markers of ER stress. Data are presented as mean ± SEM. Statistical analyses were performed using a Student's t test. Letters indicate statistical significance, * P < 0.05 or ** P < 0.01, relative to the sample group represented by the corresponding parenthetical letter. Abbreviations used: ALP, alkaline phosphatase; Atf4, activating transcription factor 4; Crp, C-reactive protein; Ddit3, DNA damage-inducible transcript 3; Il6, interleukin-6; Il1b, interleukin-1 beta; Nos2, nitric oxide synthase 2; Saa3, serum amyloid A3; Tnfa, tumor necrosis factor alpha; Xbp1n, X-box binding protein 1 native mRNA; Xbp1s, X-box binding protein 1 spliced mRNA.
2.4 Discussion

Previous research studies have typically employed mouse models of aberrant AHR expression to define the role of AHR in metabolism and from these reports, scientists have gained a better understanding of how potent, agonist-driven AHR activity associated with the induction of \textit{Cyp1a1} can influence metabolism (108, 114, 169). Nevertheless, how AHR may alter metabolic homeostasis in the absence of such activity remains largely unknown. In this study, we utilize a conditional hepatocyte-targeted deletion of AHR in mice to investigate the role of AHR in hepatic metabolism. Our data notably demonstrate that levels of \textit{Cyp1a1} gene transcription are no different between \textit{Ahr}^{fx/fx} and \textit{Ahr}^{fx/fx}Alb^{Cre} mice. Therefore, any alterations in metabolic homeostasis resulting from the loss of hepatocyte-specific AHR expression largely reflect the impact of basal AHR activity on liver metabolism, rather than the AHR activity associated with transcriptional activation of \textit{Cyp1a1}. Interestingly, hepatic \textit{Cyp1a1} transcription is greater in SC-fed \textit{Ahr}^{fx/fx}Alb^{Cre} mice than in \textit{Ahr}^{fx/fx}Alb^{Cre} mice maintained on any of the purified diets. This may represent the contributions of AHR activity within non-hepatocyte type liver cells to overall hepatic \textit{Cyp1a1} transcription. Ultimately, the lack of any significant difference in \textit{Cyp1a1} transcriptional activity between \textit{Ahr}^{fx/fx} and \textit{Ahr}^{fx/fx}Alb^{Cre} mice validates the \textit{Ahr}^{fx/fx}Alb^{Cre} mouse model as a useful tool for identifying the influence of basal hepatocyte-specific AHR activity on metabolism in the absence of exogenous ligand.

In general, we observe that the loss of hepatocyte-specific AHR signaling reduces relative liver mass and can influence body mass, adipose tissue mass, and adipocyte morphology. In mice maintained on SC, HS, or HF/HS diet, these differences occur without any change in food intake (data not shown). However, we observe that loss of hepatocyte AHR expression in PUR-fed mice correlates with a significant 20% (P = 0.007) reduction of food intake relative to
Ahr<sup>fx/fx</sup> mice. Our data also show that glucose tolerance is diminished in PUR-fed Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice relative to Ahr<sup>fx/fx</sup> mice, without any apparent differences in insulin sensitivity. Likewise, we observe no perturbations in the ratio of phosphorylated AKT protein, a major mediator of the insulin response, nor any change in hepatic lipogenesis during a fed state. Furthermore, circulating insulin is also comparable in PUR-fed Ahr<sup>fx/fx</sup> and Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice (Table 2.4). Therefore, the ability of fasting Ahr<sup>fx/fx</sup> mice to absorb glucose from the bloodstream at a faster rate than Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice likely derives from differences within the insulin-independent pathways of glucose metabolism. For example, AHR may influence the glucose-regulated ChREBP pathway involved in hepatic glucose uptake or the liver-directed uptake of glucose into other tissues through metabolic hormones such as FGF21. In fact, given the recent implication of AHR in regulating FGF21 transcription and the newly discovered role of FGF21 in suppressing sugar intake/sweet taste preference, we are currently investigating the possibility that AHR-mediated regulation of FGF21 could account for the decreased food intake we observe in PUR-fed Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice relative to Ahr<sup>fx/fx</sup> (142, 154).

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<td>1.09 ± 0.10</td>
</tr>
</tbody>
</table>

**Table 2.4.** Serum chemistry profile of Ahr<sup>fx/fx</sup> and Ahr<sup>fx/fx</sup> Alb<sup>Cre</sup> mice maintained on various diets. All data are presented as mean ± SEM. Statistical analyses were performed using a Student's t test with significance denoted by the following: * p < 0.05 relative to Ahr<sup>fx/fx</sup>.
Whereas PUR-fed $Ahr^{fx/fx}Alb^{Cre}$ mice exhibit decreased glucose tolerance without any change in insulin sensitivity, HS-fed $Ahr^{fx/fx}Alb^{Cre}$ mice exhibit decreased insulin sensitivity without any change in glucose tolerance. Interestingly, the data indicate that although fasted $Ahr^{fx/fx}Alb^{Cre}$ mice exhibit diminished sensitivity to insulin during fasting, there are no signs of insulin resistance in HS-fed $Ahr^{fx/fx}Alb^{Cre}$ mice relative to $Ahr^{fx/fx}$ as evidenced by a comparable responsiveness to basal insulin action. In agreement, fed-state serum insulin concentrations are also similar in $Ahr^{fx/fx}$ and $Ahr^{fx/fx}Alb^{Cre}$ mice (Table 2.4). Nevertheless, we observe modestly lower protein and mRNA expression of key fatty acid synthesis genes in $Ahr^{fx/fx}Alb^{Cre}$ mice during the fed state. Therefore, basal hepatocyte AHR signaling in HS-fed mice appears to influence insulin-mediated glucose uptake under fasting conditions, but may potentially play an entirely separate, insulin-independent role in regulating fatty acid synthesis during a fed state. Ultimately, the role of AHR in the response to HS is highly complex and remains an area of ongoing investigation in our laboratory.

H&E staining of hepatic tissues reveals that $Ahr^{fx/fx}$ mice generally exhibit a greater degree of hepatocyte ballooning degeneration relative to $Ahr^{fx/fx}Alb^{Cre}$ mice. Given that increased hepatocyte ballooning is a major hallmark of steatohepatitis in mice, basal hepatocyte-specific AHR signaling appears to promote the development of this disease (172). In fact, we speculate that increased hepatocyte ballooning degeneration in $Ahr^{fx/fx}$ mice may directly relate to the greater degree of lipid deposition and inflammation (two other steatohepatitis-associated pathologies) observed in HF/HS-fed $Ahr^{fx/fx}$ mice. This hypothesis is the subject of ongoing research in the laboratory.

When given a HF/HS challenge, $Ahr^{fx/fx}$ mice exhibit modestly greater hepatic lipid accumulation than $Ahr^{fx/fx}Alb^{Cre}$ mice as evidenced through quantification of hepatic triglycerides
and cholesterol, as well as histological analysis. Our data suggest that this outcome derives partly from the decreased expression of genes involved in \textit{de novo} fatty acid synthesis in $Ahr^{fx/fx} Alb^{Cre}$ mice. Indeed, our data agree with previous studies indicating that $Ahr^{-/-}$ mice exhibit reduced expression of \textit{Fasn}, \textit{Acaca}, and \textit{Scd1} when maintained on a high-fat diet (169). Another factor likely involved with the modestly increased accumulation of hepatic lipids in HF/HS-fed $Ahr^{fx/fx}$ mice is greater AHR-dependent gene/protein expression of hepatic fatty acid importers such as CD36. Increased \textit{Cd36} mRNA has been previously implicated in the spontaneous development of hepatic steatosis in mice that express a constitutively active form of AHR (114). Furthermore, AHR agonist 3-methylcholanthrene (3MC) is known to induce hepatic steatosis through AHR-dependent \textit{Cd36} transcription, while TCDD treatment increases hepatic lipid deposition and expression of lipid transport genes in mice maintained on HF diet (108, 176). Our observations that $Ahr^{fx/fx}$ mice accumulate greater quantities of lipid in the liver and exhibit elevated \textit{Cd36} expression relative to $Ahr^{fx/fx} Alb^{Cre}$ mice support the ongoing hypothesis that AHR plays an important role in hepatic fatty acid import and in particular, the modulation of \textit{Cd36} expression.

In addition to decreased \textit{de novo} fatty acid synthesis and import, elevated transcription of key fatty acid export genes may also be involved with the apparent decrease in hepatic lipid deposition among HF/HS-fed $Ahr^{fx/fx} Alb^{Cre}$ mice. Interestingly, our data indicate HF/HS-fed $Ahr^{fx/fx}$ mice exhibit diminished \textit{Apob100} expression relative to $Ahr^{fx/fx} Alb^{Cre}$ mice, without any evidence of ER stress. During steatohepatitis, ER stress can result in increased turnover of APOB100 protein, resulting in a diminished capacity to export hepatic triglycerides (177). Our observations that HF/HS-fed $Ahr^{fx/fx}$ mice do not exhibit signs of ER stress in the liver, yet display reduced transcription of \textit{Apob100} therefore suggest that basal AHR signaling within
hepatocytes might reduce the overall capacity for hepatic triglyceride export. However, additional research is necessary to test this hypothesis.

An important defect of fatty acid metabolism that could also contribute to increased hepatic lipid deposition in HF/HS-fed $Ahr^{fx/fx}$ is the impairment of fatty acid oxidation. However, we found that gene expression of the rate-limiting enzyme in mitochondrial β-oxidation, carnitine palmitoyltransferase 1 alpha ($Cpt1a$), is similar in $Ahr^{fx/fx}$ and $Ahr^{fx/fx}Alb^{Cre}$ mice (Figure 2.8A). In agreement, expression of $Ppara$, a transcription factor important in the regulation of $Cpt1a$ expression, is also comparable between these two genotypes (Figure 2.8B). Last, we also found no change in the transcription of cytochrome P450 enzyme 4A10 ($Cyp4a10$), an enzyme involved in peroxisomal β-oxidation (Figure 2.8C). Together, the data suggest that β-oxidation likely occurs at similar rates in HF/HS-fed $Ahr^{fx/fx}$ and $Ahr^{fx/fx}Alb^{Cre}$ mice. Therefore, we conclude that impaired fatty acid oxidation does not contribute to the modestly decreased lipid deposition observed in $Ahr^{fx/fx}Alb^{Cre}$ relative to $Ahr^{fx/fx}$. Instead, decreased lipid deposition results from the combination of decreased de novo fatty acid synthesis, reduced fatty acid uptake, and increased triglyceride export in HF/HS-fed $Ahr^{fx/fx}Alb^{Cre}$ mice.
Figure 2.8. Fatty acid oxidation gene expression is comparable in HF/HS-fed $Ahr^{f/fx}$ and $Ahr^{f/fx} Alb^{cre}$ mice. (A) $Cpt1a$, (B) $Ppara$, and (C) $Cyp4a10$ gene expression in HF/HS-fed mice. Abbreviations used: $Cpt1a$, carnitine palmitoyltransferase 1 alpha; $Ppara$, peroxisome proliferator-activated receptor alpha; $Cyp4a10$, cytochrome P450 enzyme 4A10. All data are presented as mean ± SEM. Statistical analyses were performed using a Student's t test.

In mice maintained on HF/HS diet, we show that basal AHR signaling within hepatocytes is associated with increased inflammatory gene expression and immune cell infiltration. In addition, $Ahr^{f/fx}$ mice exhibit greater serum concentrations of ALP, a marker of hepatic injury, relative to $Ahr^{f/fx} Alb^{cre}$ mice under HF/HS challenge. Together with the observation that hepatic lipid deposition is modestly greater in HF/HS-fed $Ahr^{f/fx}$ mice, our data suggest that basal AHR activity within hepatocytes contributes to greater development of steatohepatitis under HF/HS challenge. Such results are consistent with previous studies indicating that $Ahr^{-/-}$ mice are "protected" from diet-induced hepatic inflammation and steatosis (169). However, $Ahr^{-/-}$ mice exhibit impaired immunity and display developmental defects in the liver that can complicate the interpretation of results when using this model (84, 89). Given that the AHR-deficient mouse model we utilize in this study exhibits normal liver and immune development, our data provide clear evidence that loss of basal hepatocyte AHR activity may be involved with the development of steatohepatitis (94).
Although the data presented here are consistent with previous research concerning the influence of AHR on hepatic steatosis, another recent study in $Ahr^{fx/fx} Alb^{Cre}$ mice demonstrated the opposite of what we have shown (171). Specifically, these authors found that loss of hepatocyte AHR signaling exacerbates HF diet-induced hepatic steatosis. The reason for the discrepancy in results is unclear. A likely possibility is that the contradicting data reflect differences in the composition of the rodent diets utilized. For example, the proportion of calories provided through protein in their diet is 46% higher and the proportion provided from fat is approximately 82% higher than in the diet utilized here. In addition, while our diet features a relatively high concentration of sucrose, the concentration of sucrose in their diet is not provided. Furthermore, the dietary source of carbohydrates is not listed in their manuscript either. Therefore, differences in the type of carbohydrate used and its dietary content might also explain discrepancies between the data.

In summary, we demonstrate that in the absence of exogenous ligand, basal AHR signaling within hepatocytes plays a crucial role in regulating liver metabolism. For example, loss of this AHR activity can alter insulin sensitivity and glucose tolerance in PUR or HS-fed mice. Our data also indicate that the basal hepatocyte AHR activity present in $Ahr^{fx/fx}$ mice may make them more prone to the development of steatohepatitis. Accordingly, when these mice are challenged with a HF/HS diet, they exhibit greater accumulation of hepatic lipids through the combination of elevated de novo lipogenesis, increased fatty acid import, and a diminished capacity for fatty acid export. In addition, HF/HS-fed $Ahr^{fx/fx}$ mice exhibit greater immune cell infiltration and increased pro-inflammatory gene transcription relative to HF/HS-fed $Ahr^{fx/fx} Alb^{Cre}$ mice. Most importantly, our data demonstrate that these outcomes occur in the absence of any significant difference in Cyp1a1 transcription between $Ahr^{fx/fx}$ and $Ahr^{fx/fx} Alb^{Cre}$.
mice. Therefore, our data illustrate an important, novel role for basal hepatocyte AHR activity in regulating liver metabolism and in the progression of hepatic disease. Future work in the laboratory will aim to identify the alternative pathways through which AHR acts and how they relate to and/or influence AHR activity associated with the induction of Cyp1a1 transcription. In addition, we are investigating the potential use of AHR antagonist to treat disorders of hepatic metabolism such as hepatic steatosis and steatohepatitis.
CHAPTER 3:
HEPATIC ARYL HYDROCARBON RECEPTOR ATTENUATES FIBROBLAST GROWTH FACTOR 21 EXPRESSION

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3.1 Introduction

Several lines of evidence indicate that AHR is involved in the regulation of liver metabolism. However, most of this evidence derives from studies that examine the effects of AHR activation when bound to exogenous ligand or from mouse models of AHR that exhibit a wide spectrum of aberrant AHR signaling (83, 95, 110). Therefore, how AHR influences metabolic homeostasis within the liver in the absence of exogenous ligand remains poorly understood. Fibroblast growth factor 21 (FGF21) is an important metabolic growth factor involved in the response to fasting and research shows that exogenous administration of FGF21 can attenuate obesity in various mouse models of the disease (130, 157). One recent study demonstrated that TCDD activation of AHR can increase hepatic Fgf21 expression and in another independent experiment, researchers observed elevated Fgf21 expression in mice that express a constitutively active form of AHR in the liver (154, 170). However, data from the former study indicate that hepatic Fgf21 expression is greater in Ahr−/− mice relative to Ahr+/+ mice. Further, their data demonstrate that activation of AHR with relatively low doses of TCDD represses hepatic Fgf21 expression over time (154). Such contradicting results warrant further investigation into the role of AHR in regulating Fgf21 mRNA.

In this study, we examined the physiological role of AHR in hepatic Fgf21 expression using a mouse model that lacks functional AHR protein in hepatocytes (Ahrfx/AlbCre) (94). Compared to the parental strain (Ahrfx), AhrfxAlbCre mice exhibit increased hepatic expression of Fgf21 during a non-fasting state, along with elevated serum FGF21 levels. Therefore, we hypothesize that AHR may constitutively, or through endogenous ligand binding, interfere with the activation of hepatic Fgf21 expression.
The Fgf21 promoter region contains several putative DREs, one of which overlaps a peroxisome proliferator-activated receptor response element (PPRE) and a carbohydrate response element (ChoRE). Furthermore, this DRE is found adjacent to a cAMP response element (CRE). Using EMSA, we demonstrate that AHR is able to bind to this specific DRE within the Fgf21 promoter region, while ligand-activated AHR in vitro impairs PPARα-, ChREBP-, and CREBH-mediated increases in promoter activity. In addition, AHR agonist treatment in Hepa-1 cells ablates potent, ER stress-driven activation of Fgf21 expression. Finally, we present evidence that ligand activation of AHR in human primary hepatocytes similarly attenuates PPARα-, glucose-, and ER stress-driven FGF21 expression.

3.2 Materials and Methods

Animal Experiments – 6 week-old, age-matched male C57BL6/J, C57BL6/J-Ahrfx/fx, as well as congenic B6.129SvJ-AhrTm3.1Br -fx/fx and Ahrfx/fxAlbCre mice (n = 3-6 per group), were maintained on purified AIN-93G diet (Dyets, NJ) for 3 weeks. Ahrfx/fx and Ahrfx/fxAlbCre mice were kindly provided by Christopher Bradfield (University of Wisconsin). Mice were housed on corncob bedding in a pathogen-free, temperature- and light-controlled facility, and were given access to food ad libitum. Upon sacrifice, serum samples were collected and FGF21 concentration measured using an ELISA kit, according to manufacturer's instructions (R&D Systems, MN). All mouse experiments were carried out humanely with approval, and in accordance to the Animal Care and Use Committee of the Pennsylvania State University guidelines.

Cell Culture – Hepa-1 cells were obtained from the American Type Culture Collection and maintained as previously described (178). Enriched normal primary human hepatocytes were obtained through the Liver Tissue Cell Distribution System (University of Pittsburgh, PA),
funded by NIH Contract #HHSN276201200017C. The isolated hepatocytes were seeded at >90% confluency in 24-well collagen-coated dishes and cultured as previously described, with minor modifications (179). Briefly, the cells were overlaid with 225 µg/mL BD Matrigel™ Basement Membrane Matrix (Corning, NY) and cultured in serum-free Leibovitz's L-15 media (Life Technologies, USA), supplemented with 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 nM dexamethasone, 10 nM insulin, 5 ng/ml selenium, 5 µg/ml transferrin, 1% linoleic acid, 1% albumin and 1% sodium bicarbonate. For ER stress experiments, we replaced the above-stated medium with Williams E Medium containing GlutaMAX™ (Thermo Fisher Scientific, USA) and 5 mg/mL BSA, immediately prior to addition of compounds. Due to possible human variability, primary hepatocyte experiments were repeated using two or more donors.

RNA extraction and quantitative RT-PCR – RNA was isolated as previously described (117). Gene expression was measured using quantitative RT-PCR as previously described (117), with the primers described in Table 3.1. The relative level of expression was normalized to ribosomal protein L13a mRNA (Rpl13a).

Plasmid Constructs – For the luciferase reporter assay, the -1906 to +52 upstream regulatory region of Fgf21 was cloned into pGL3-basic vector (Promega, CA). The resulting plasmid is referred to as pGL3-FGF21.

Transfections – Hepa-1 cells were seeded onto 6-well plates and grown overnight, then transfected using LipofectAMINE3000 reagent (Promega, CA) with pGL3-FGF21, pSV-β-galactosidase, and pcDNA3, along with either pCMVTNT-PPARα, pChREBP, or pcDNA3-hCREBH(N), according to manufacturer’s instructions. pChREBP was a gift from Isabelle Leclerc (Addgene plasmid # 39235) (180). pcDNA3-hCREBH(N) was a gift from Dr. Laurie
Glimcher at Weill Cornell Medical College. After 24 h recovery, cells were treated overnight with DMSO vehicle or 500 nM indolo[3,2b]carbazole (ICZ) (SigmaAldrich, MO).

Electromobility Shift Assay – EMSA was performed as previously described (181), using in vitro translated (Promega, CA) mouse AHR, ARNT, ChREBP, and MLX. As a positive control for AHR, we used a $[^{32}P]$-labeled Cyp1a1 oligonucleotide as previously described (182). In addition to the oligonucleotide described in Figure 2A, the following $[^{32}P]$-labeled oligonucleotides were used: ChoRE, 5’ GCGACATGTGATCAAGCCATGAACCC; competitor Fgf21, 5’ ACTCCTGACGCGTGATATTTGACACAC TTG; mutated competitor Fgf21, 5’ ACTCCTGACGCGCAATATTTGACACACTTG.

Statistical Analysis – All experiments were performed at least twice. All data are presented as mean ± SEM. Statistical analyses were performed using a two-tailed Student’s t test or one-way ANOVA. * p < 0.05, ** p < 0.01 or *** p < 0.001.
<table>
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<th>Gene Name</th>
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<tr>
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**Table 3.1.** List of primer sets utilized for RT-qPCR analysis
3.3 Results

*Ah*<sup>rf</sup>*fx Alb<sup>CRE</sup> mice exhibit increased expression of the fasting-induced hormone Fgf21 during a non-fasted state.* FGF21 is a key regulator of the fasting response; therefore, hepatic *Fgf21* expression occurs at a low basal level during a non-fasting state. However, non-fasting *Ah*<sup>rf</sup>*fx Alb<sup>CRE</sup> mice exhibit a significant 4-fold increase in hepatic *Fgf21* expression compared to *Ah*<sup>rf</sup>*fx mice (Fig 3.1A). In addition, circulating FGF21 concentrations in non-fasted *Ah*<sup>rf</sup>*fx Alb<sup>CRE</sup> mice are 2-fold higher than the levels observed in *Ah*<sup>rf</sup>*fx mice. Similarly, non-fasting *Ah*<sup>r<sup>-</sup></sup>* mice exhibit a significant 3.8-fold increase in hepatic *Fgf21* expression compared to wild-type mice (Figure 3.1B). Consistent with the known effects of elevated *Fgf21* expression, we observe increased expression of the downstream target gene, insulin-like growth factor-binding protein 1 (*Igfbp1*), in conjunction with the down-regulation of genes involved in fatty acid synthesis (Figure 3.1C-D). Specifically, the expression of fatty acid synthase (*Fasn*) and sterol response element-binding protein 1c (*Srebp1c*) are reduced ~2-fold in *Ah*<sup>rf</sup>*fx Alb<sup>CRE</sup> mice. In contrast, the *Fgf21* expression levels in adipose tissue are comparable between *Ah*<sup>rf</sup>*fx and *Ah*<sup>rf</sup>*fx Alb<sup>CRE</sup> mice (Figure 3.1E). These results are consistent with the targeted deletion of AHR to hepatocytes and not adipocytes. Interestingly, the repression of fatty acid synthesis still occurs within white adipose tissue despite no differences in *Fgf21* expression (Figure 3.1F). Whereas hepatic *Fasn* expression was reduced without a significant reduction in stearoyl-CoA desaturase 1 (*Scd1*) expression, we observe the opposite in white adipose tissue.
Figure 3.1. Hepatocyte-targeted deletion of Ahr results in elevated Fgf21 expression. (A) Hepatic Fgf21 expression and serum concentrations in Ahr<sup>lox<sup>lox</sup></sup> and Ahr<sup>lox<sup>lox</sup></sup>Alb<sup>Cre</sup> mice. (B) Hepatic Fgf21 expression in C57BL6/J and AHR-null mice. (C) Expression of Fgf21 target-gene Igfbp1. (D) Hepatic expression of genes involved in de novo lipogenesis. (E) Fgf21 expression and (F) de novo lipogenesis gene expression in adipose tissue from Ahr<sup>lox<sup>lox</sup></sup> and Ahr<sup>lox<sup>lox</sup></sup>Alb<sup>Cre</sup> mice. (G) Hepatic Fgf21 mRNA and serum FGF21 levels in fasted Ahr<sup>lox<sup>lox</sup></sup> mice, exposed to vehicle or 10 µg/kg TCDD by gavage. Fasn, fatty acid synthase; Scd1, stearoyl-CoA desaturase 1; Srebp1c, sterol response element-binding protein 1c. All data are presented as mean ± SEM from three or more mice. Statistical analyses were performed using either a two-tailed Student’s t test or one-way ANOVA. The latter analysis was performed when there were more than two treatment groups. Letters indicate statistical significance, * P < 0.05 or ** P < 0.01, relative to the sample group represented by the corresponding parenthetical letter.
Next, we investigated the effects of ligand-mediated AHR activation in fasting mice, given that FGF21 regulates the fasting response. For this experiment, 6-week old male Ahr<sup>fx/fx</sup> mice were exposed for 24 h to 10 μg/kg TCDD or vehicle (corn oil) by gavage, then fasted overnight. We observe that TCDD treatment significantly reduces hepatic Fgf21 expression by 50% compared to vehicle-treated mice (Figure 3.1G). However, circulating levels of FGF21 are not altered with TCDD treatment.

The ChREBP/MLX and AHR/ARNT heterodimer complexes bind to the Fgf21 promoter at a composite DRE/PPRE/CRE/ChoRE regulatory region. As depicted in Fig 3.2A, the mouse Fgf21 promoter region contains four overlapping response elements (designated DRE, PPRE, CRE, and ChoRE). Previous studies have already shown direct binding of PPARα to the Fgf21 promoter at this site of DRE/PPRE/CRE/ChoRE overlap (128). However, direct binding of ChREBP and its heterodimeric partner, MLX, to this region has not been demonstrated. Utilizing EMSA, we show that the ChREBP/MLX complex does bind to the proximal Fgf21 ChoRE (Fig 3.2B, lanes 5-8). Also shown is ChREBP/MLX complex formation with a positive control oligonucleotide containing a consensus ChoRE (Fig 3.2B, lanes 1-4). Next, to demonstrate that the AHR/ARNT heterodimer binds to the putative DRE in this region, we incubated in vitro translated AHR and ARNT proteins in the presence of a [<sup>32</sup>P]-oligonucleotide that contains the region of response element overlap (-88 to -54), with or without the addition of AHR ligand. For a positive control, we utilized [<sup>32</sup>P]-Cyp1a1 oligonucleotide. As shown in Figure 3.2C, TCDD results in AHR/ARNT heterodimerization and subsequent binding to the positive control oligonucleotide. We demonstrate that AHR agonists TCDD (Figure 3.2D, lanes 1-5) and indolo[3,2b]carbazole (ICZ) (Fig 3.2D, lanes 6-10) also result in AHR/ARNT heterodimerization and DRE binding to the [<sup>32</sup>P]-Fgf21 oligonucleotide. To confirm that this binding is specific, we incubated AHR and
ARNT in an excess of unlabeled competitor *Fgf21* oligonucleotide, with or without a mutation in the DRE, followed by incubation in the presence of labeled *Fgf21* oligonucleotide. As shown in Figure 3.2E, we observed that pre-incubating in an excess of unlabeled, non-mutated oligonucleotide prevents AHR/ARNT binding to the labeled *Fgf21* oligonucleotide (lanes 1-2), while addition of an unlabeled *Fgf21* mutant oligonucleotide does not (lanes 3-4).
Figure 3.2. The AHR/ARNT and ChREBP/MLX heterodimers bind to the Fgf21 promoter. (A) Diagram of overlapping response elements within the Fgf21 promoter region. The boxed (dashed line) region of the promoter was utilized for EMSA (B) Formation of the ChREBP/MLX complex with a control labeled oligonucleotide (lanes 1-4) and at the Fgf21 ChoRE (lanes 5-8). (C) TCDD induces AHR/ARNT heterodimerization and binding to a control [32P]-oligonucleotide. (D) TCDD results in formation of the AHR/ARNT complex at the Fgf21 DRE. (E) Addition of unlabeled oligonucleotide interrupts AHR/ARNT binding to the Fgf21 DRE (lanes 1-2). Introducing a mutation into the DRE of the unlabeled oligonucleotide eliminates this competitive binding (lanes 3-4). DRE, dioxin response element; PPRE, peroxisome proliferator-activated receptor alpha response element; CRE, cAMP response element; ChoRE, carbohydrate response element.
**Ligand-activated AHR attenuates PPARα-, ChREBP-, and ER stress-dependent Fgf21 expression in Hepa-1 cells.** Having demonstrated that the AHR/ARNT heterodimer can bind to the composite DRE/PPRE/CRE/ChoRE, we next explored the possibility of cross-talk between AHR and promoter-driven PPARα, ChREBP, or CREBH signaling. To do so, we transfected a luciferase reporter construct containing the -1906 to +52 Fgf21 promoter region into Hepa-1 cells, along with expression vectors for the different transcription factors of interest. In the absence of AHR ligands, ectopic PPARα-, ChREBP-, and CREBH-expression results in significant Fgf21 promoter-dependent expression. However, treatment with AHR agonist ICZ ablates PPARα-, ChREBP-, and CREBH-dependent induction (Figure 3.3A), suggesting that ligand-activated AHR can interfere with the promoter-driven activation of Fgf21 expression by these transcription factors. We chose to utilize ICZ in our cell culture experiments as it represents a common dietary AHR ligand. ICZ is formed from the acid condensation of its parent compound, indole-3-carbinol, a breakdown product of glucobrassicin, which naturally occurs at high concentrations within vegetables of the Brassica genus (74).

To further investigate AHR-mediated repression of Fgf21 expression in the presence of ligand, we examined the ability of AHR to attenuate ER stress-mediated activation of Fgf21 in Hepa-1 cells. To stimulate ER stress, we incubated cells overnight in serum-free medium supplemented with 0.2% BSA, then refreshed the medium and added 1 mM dithiothreitol (DTT) or 1 µM thapsigargin for 4 h. Confirming activation of ER stress, we observe significantly elevated expression of Ddit3 and Atf4 in DTT- and thapsigargin-treated cells (Figure 3.3B). In the absence of AHR ligand, DTT and thapsigargin both markedly increase Fgf21 expression >150-fold. However, 1 h pre-treatment with 500 nM ICZ reduces thapsigargin-and DTT-driven expression by 74% and 75%, respectively (Figure 3.3C). Notably, significant ICZ-mediated
repression is evident at a five-fold lower ICZ concentration (Figure 3.3D). Consistent with AHR activation, we also observe a statistically significant increase in Cyp1a1 transcriptional levels in ICZ-treated cells (Figure 3.3E). To confirm that the repressive action of AHR agonists occurs specifically through AHR, we attempted to suppress agonist-mediated activity by pre-treating cells with 1 µM N-(2-(1H-indol-3-yl)ethyl)-9-isopropyl-2-(5-methylpyridin-3-yl)-9H-purin-6-amine (GNF351), a known AHR antagonist (79). Surprisingly, GNF351 was unable to antagonize 100 nM ICZ-mediated AHR activity (Figure 3.3F). In contrast, GNF351 does successfully antagonize 2 nM TCDD-mediated AHR activity, as evidenced by the significant suppression of TCDD-driven Cyp1a1 expression (Figure 3.3G). Shown in Figure 3.3H, treatment with 1 µM thapsigargin results in a 32.6-fold induction of Fgf21 mRNA. Similar to pre-treatment with ICZ, 1 h pre-treatment with 2 nM TCDD reduces this effect by approximately 70%. However, the addition of 1 µM GNF351 for 1 h prior to TCDD-treatment reverses TCDD suppression of Fgf21 expression (Figure 3.3H).

Comparable to treatment with DTT or thapsigargin, incubating Hepa-1 cells for 24 h in glucose-free medium also activates ER stress and Fgf21 expression (Figure 3.4A). Exposing glucose-starved cells to various doses of ICZ, ranging from 250 nM to 1000 nM, results in a dose-dependent increase in the suppression of Fgf21 expression (Figure 3.4B). Furthermore, we observe the same dose-dependent suppression of Fgf21 expression upon exposure to various doses of TCDD, ranging from 0.1 nM to 5 nM (Figure 3.4C).
Figure 3.3. Ligand-activated AHR attenuates ER stress-mediated Fgf21 expression. (A) ICZ-treatment of Hepa-1 cells that transiently express PPARα, ChREBP, or CREBH suppresses promoter-driven expression. (B) Treatment of Hepa-1 cells with 1 mM DTT or 1 µM thapsigargin leads to the activation of ER stress. (C) Ligand activation of AHR in Hepa-1 cells diminishes thapsigargin and DTT-mediated induction of Fgf21. (D) 1 h pre-incubation with only 100 nM ICZ ablates thapsigargin- and DTT-mediated induction of Fgf21. (E) ICZ activates Cyp1a1 expression in Hepa-1 cells. (F) GNF351 fails to antagonize ICZ-mediated activation of AHR. (G) GNF351 successfully antagonizes TCDD-dependent Cyp1a1 expression. (H) Activation of AHR with 2 nM TCDD inhibits thapsigargin-driven Fgf21 expression. However, 1 h pre-incubation with AHR antagonist GNF351 blocks this effect. All data are representative of two or more experiments. Statistical analyses were performed using one-way ANOVA. Letters indicate statistical significance, * P < 0.05, ** P < 0.01, or *** p < 0.001, relative to the sample group represented by the corresponding parenthetical letter.
Figure 3.4. ICZ and TCDD exposure result in a dose-dependent suppression of Fgf21 in glucose-starved Hepa-1 cells. (A) 24 h glucose-starvation activates the expression of ER stress-response genes Ddit3 and Atf4, and increases Fgf21 mRNA in Hepa-1 cells. (B) ICZ exposure results in a dose-dependent suppression of Fgf21 expression in glucose-starved Hepa-1 cells. For this experiment, cells were re-treated with ICZ after 12 h. (C) In glucose-starved Hepa-1 cells, 24 h TCDD exposure also suppresses Fgf21 expression in a dose-dependent manner. Statistical analyses were performed using one-way ANOVA. Letters indicate statistical significance, * P < 0.05, ** P < 0.01, or *** P < 0.001 relative to the sample group represented by the corresponding parenthetical letter.
AHR activation suppresses PPARα-, ChREBP-, and ER stress-mediated induction of FGF21 expression in primary human hepatocytes. To further demonstrate that AHR activity attenuates PPARα-agonist, glucose-mediated, and ER stress-driven FGF21 expression, we examined the effects of AHR activation in primary human hepatocytes. As shown in Figure 3.5A, treatment with PPARα-ligand GW7647 activates CPT1A expression and significantly increases FGF21 expression >3-fold, while pre-treatment of cells with AHR agonist significantly inhibits this response. Similarly, incubation of primary human hepatocytes in medium supplemented with 30 mM glucose activates FGF21 expression after 6 h and 24 h (Figure 3.5B-C). However 10 nM TCDD or 500 nM ICZ treatment significantly suppresses Fgf21 expression by 58% and 46%, respectively. Confirming ChREBP-activation, we observe that incubating primary cells in 30 mM glucose increases the expression of liver and RBC pyruvate kinase (PKLR) (Figure 3.5D). Finally, to activate ER stress, we incubated primary human hepatocytes in the presence of 1 mM DTT for 4 h, with or without 500 nM ICZ pre-treatment for 1 h. In the presence of DTT, we observe an increase in ATF4 and DDIT3 gene expression, thereby confirming the onset of ER stress (Figure 3.5E). Consistent with previous experiments, DTT increases Fgf21 expression 69-fold, while 1 h pre-treatment with 500 nM ICZ significantly impairs DTT-induced Fgf21 expression by 46% (Figure 3.5F).
Figure 3.5. Ligand-mediated AHR activity suppresses PPARα-, ChREBP-, and ER stress-mediated induction of FGF21 expression in primary human hepatocytes. (A) ICZ-mediated AHR activation attenuates GW7647-driven FGF21 expression after 24 h co-incubation, without any effect on the expression of PPARα-target gene CPT1. (B) Liganded AHR diminishes glucose-activation of FGF21 expression in primary human hepatocytes at 6 h and (C) 24 h. (D) Incubation of primary human hepatocytes in medium containing 30 mM glucose activates ChREBP-target gene PKLR. (E) Treating primary human hepatocytes with 1 mM DTT for 4 h activates gene pathways involved in ER stress. (F) DTT treatment induces FGF21 expression in primary human hepatocytes, while 1 h pre-incubation with 500 nM ICZ attenuates this effect. Treatment groups were performed in triplicate and the data presented are representative of experiments from at least two individual donors. Statistical analyses were performed using one-way ANOVA. Letters indicate statistical significance, * P < 0.05, ** P < 0.01, or *** p < 0.001 relative to the sample group represented by the corresponding parenthetical letter.
3.4 Discussion

To date, pharmacological administration of FGF21 is well-characterized and known to exert beneficial effects in various animal models of obesity (157, 183). Additionally, FGF21 overexpression is linked to increased longevity in mice (184). However, the regulation of this metabolic hormone remains poorly understood. Our data reveal that AHR is an important regulator of Fgf21 expression during the non-fasting state. When fed a purified diet, Ahr^{fx/fx} Alb^{Cre} mice display elevated hepatic Fgf21 expression and serum FGF21 concentrations. Importantly, the Ahr^{fx/fx} background used to generate Ahr^{fx/fx} Alb^{Cre} mice carries the Ahr^d allele, which encodes a form of AHR exhibiting reduced ligand affinity relative to the Ahr^b allele (80). Therefore, a study that utilized Ahr^b conditional knockout mice (which are not currently available) would likely yield a greater increase of Fgf21 expression relative to hepatocyte-targeted AHR knockout mice. Consistent with elevated hepatic Fgf21 expression, we observed a repression of fatty acid synthesis genes in the liver. Interestingly, we observed a similar repression of fatty acid synthesis in adipose tissue collected from Ahr^{fx/fx} Alb^{Cre} mice, without any increase in adipose Fgf21 expression. Such results are consistent with the ongoing hypothesis in the literature that liver-excreted FGF21 acts in an endocrine fashion (147).

In agreement with recent data (154), our results suggest that AHR can bind directly to the Fgf21 promoter at the same location to which the PPARα/RXRα and PPARα/CREBH heterodimers bind (128, 131). Evidence implicates the PPARα/RXRα heterodimer in regulating lipid metabolism (185), while CREBH, independent of PPARα, induces a systemic inflammatory response upon ER stress (186). CREBH also modulates lipid metabolism in response to metabolic stress (187). However, the exact function of the PPARα/CREBH complex is not known. To date, formation of this complex has only been observed at the -62 to -93 region of the
Furthermore, whether CREBH can participate with other transcription factors (e.g. activating transcription factor 6) to activate Fgf21 expression upon ER stress remains unknown. Also unclear is whether AHR can compete with the PPARα/CREBH complex for DNA-binding at these sites.

Our study presents novel data indicating the ChREBP/MLX heterodimer, which mediates the insulin-independent response to glucose (22), is able to bind a ChoRE that overlaps the binding sites for the AHR/ARNT, PPARα/RXRα, and PPARα/CREBH heterodimers. Recently, investigators determined that ChREBP plays a crucial role in the FGF21-dependent control of simple sugar intake and sweet taste preference (142). By extension, the ability of AHR to attenuate ChREBP-dependent Fgf21 expression therefore presents the possibility that dietary AHR ligands, or the direct administration of AHR ligands can influence simple sugar intake and/or sweet taste preference. We are currently investigating the validity of this hypothesis through the modulation of AHR activity with different classes of ligand.

Importantly, 31 bp upstream from the site of overlapping response elements lies a characterized ER stress-response element (ERSE). XBP1, which facilitates the mammalian unfolded protein response (150), binds to this site to activate Fgf21 expression in response to ER stress (151). Given the close proximity of this site to the DRE, this element likely competes with AHR for binding to the Fgf21 promoter. In fact, we hypothesize that mutually antagonistic interactions must inherently exist between all the transcription factors that bind to the proximate binding elements (i.e. DRE, ChoRE, PPRE, CRE, ERSE) in this region of the Fgf21 promoter.

Our data indicate that ligand-activated AHR successfully ablates Fgf21 induction within Hepa-1 cells. Specifically, our reporter transfection experiments demonstrate that ICZ-stimulated AHR can compete with transcriptional activators of Fgf21 to repress promoter-driven activity.
Using a Hepa-1 cell line, we demonstrate that ICZ-mediated AHR activation ablates potent, ER stress-mediated Fgf21 induction. Importantly, this effect is specific to AHR because AHR antagonists successfully block agonist-driven repression. Unexpectedly, our data indicate that thapsigargin, in combination with AHR agonist, synergistically increases Cyp1a1 expression. We hypothesize that this increase likely represents a cellular mechanism for preventing further toxicity, after the onset of ER stress. Lastly, we demonstrate that ligand activation of AHR in primary human hepatocytes suppresses PPARα-ligand, glucose, or ER stress mediated induction of Fgf21 expression, indicating that the ability of the AHR to attenuate Fgf21 expression is conserved between mice and humans.

Throughout all of our cell culture experiments, AHR activation solely modulated Fgf21 expression, and not the expression of known PPARα, ChREBP, or XBP1 target genes. This indicates that the AHR does not affect the underlying pathways that each transcription factor regulates, and instead impacts Fgf21 expression directly. However, whether AHR-mediated repression of Fgf21 expression occurs directly through the interruption of transcription factor binding to the proximal Fgf21 promoter in vivo will require additional studies.

We provide in vitro and in vivo data that suggest that the AHR plays a role in the constitutive repression of hepatic Fgf21 expression. Data presented in a recent publication implicating Fgf21 as an AHR target gene supports our conclusions (154). The authors observed long-term, time-dependent repression of hepatic Fgf21 expression at 24 h and beyond with low dose (0.1, 1, 10 μg/kg) TCDD treatments in C57BL/6J mice. Also consistent with our results, their data demonstrate elevated hepatic Fgf21 expression in Ahr-null mice. However, the authors demonstrated a marked increase in hepatic Fgf21 expression in C57BL/6J mice given daily i.p. injections of TCDD (40 μg/kg) or β-naphthoflavone (100 mg/kg) for four days. In addition, they
reported >100-fold increase of \( Fgf21 \) expression in human and mouse hepatoma cell lines treated with 10 nM TCDD. Although we repeated their mouse hepatoma cell line experiment, we failed to observe this TCDD-mediated induction of \( Fgf21 \) (Figure 3.6). In a related, separate study involving mice that express a constitutively active form of AHR in the liver, investigators also observed increased hepatic \( Fgf21 \) expression (170). However, this constitutively active form of AHR was previously determined to exhibit AHR activity similar to high-dose TCDD exposure (188). Data obtained using high-dose treatments are often hard to interpret due to acute toxicity and AHR-independent effects on metabolism (69, 189). In addition, ER stress resulting from high-dose TCDD treatments might also contribute to the activation of \( Fgf21 \), further complicating data interpretation (190). Despite these complications, we maintain that high-dose TCDD-mediated induction of \( Fgf21 \) represents a new marker for TCDD exposure.

![Figure 3.6](image)

**Figure 3.6.** \( Fgf21 \) expression in Hepa-1 cells treated with or without 10 nM TCDD for 24 h. Data are presented as mean ± SEM. Statistical analysis was performed using a Student's t test.
We believe our data illustrate a homeostatic role of AHR in the liver, when activated by dietary ligands. We speculate that ligand-activated AHR may play a previously unrecognized role in maintaining low basal $Fgf21$ expression, given its ability to attenuate $Fgf21$ activation by PPARα, a key regulator of the fasting response, and ChREBP, a glucose-sensing transcription factor. We further speculate that AHR may modulate the cellular response to ER stress, given the ability of agonist-stimulated AHR to attenuate potent ER stress-driven $Fgf21$ expression in cell culture experiments. Ultimately, the physiological role of AHR in attenuating $Fgf21$ expression presented here contrasts with the ability of high-dose TCDD exposure to increase $Fgf21$, as others have observed (154, 170). Therefore, additional research is required to fully understand the intricate role of AHR in $Fgf21$ expression. Nonetheless, our data firmly supports a physiological role for AHR in metabolic homeostasis.

To conclude, we show that AHR is involved in the homeostatic regulation of $Fgf21$, possibly through attenuation of ChREBP-, PPARα-, and/or ER stress-dependent activation of $FGF21$ expression. Although we demonstrate that AHR binds to the $Fgf21$ promoter at a DRE that overlaps other response elements, the exact mechanism of AHR repression remains unclear. Current work in our laboratory aims to identify whether or not AHR impairs $Fgf21$ activation via direct binding to its core promoter region, or through an alternative DRE-dependent or -independent (e.g. sequestration of transcription factors in the cytoplasm) mechanism. Most importantly, the AHR may represent a useful target to modulate $FGF21$ expression levels. The fact that many non-nutritive dietary components, such as the flavonoids present in a variety of plants, can repress or activate AHR highlights the potential for regulating $FGF21$ expression through dietary modulation of AHR activity.
CHAPTER 4:
FINAL CONCLUSIONS
4.1 Final Summary

Since the initial discovery of AHR more than 30 years ago, research has primarily focused on the ability of this ligand-activated transcription factor to mediate the toxic effects of environmental contaminant exposure. Early on, scientists also postulated that the AHR might influence hepatic metabolism and while many studies explored this hypothesis, they primarily demonstrate how AHR impacts metabolism when activated with exogenous ligand and reveal very little about the influence of AHR on metabolism in the absence of ligand. Other attempts to define a role for AHR in metabolism have alternatively employed mice that lack any AHR protein expression (Ahr\textsuperscript{-/-}). However, the poor health status of Ahr\textsuperscript{-/-} mice complicates data interpretation and therefore, these studies are of limited use. In this dissertation, we instead utilized a conditional AHR knockout mouse model that lacks AHR expression within hepatocytes, but still exhibits normal liver development (94). Using this model, we investigated the influence of AHR on metabolism in the absence of exogenous ligand.

The data from chapter two demonstrated that a loss of AHR expression within hepatocytes affects various aspects of metabolism such as the insulin response and the rate of glucose uptake from serum. Our data also showed that under HF/HS challenge, the AHR plays a crucial role in promoting steatosis in Ahr\textsuperscript{fx/fx} mice. In particular, these mice exhibit modestly increased hepatic lipid deposition relative to Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} mice as a consequence of elevated de novo fatty acid synthesis, increased fatty acid import, and impaired triglyceride export gene expression. Interestingly, our histological analyses revealed that hepatocyte-associated AHR signaling promotes hepatocyte ballooning, a hallmark of steatohepatitis, in Ahr\textsuperscript{fx/fx} mice relative to Ahr\textsuperscript{fx/fx} Alb\textsuperscript{Cre} (172). We hypothesize that this activity may relate to the role of AHR in the transition from hepatic steatosis to steatohepatitis, given our observations that Ahr\textsuperscript{fx/fx} mice, but
not \( \text{Ahr}^{fx/fx} \text{Alb}^{Cre} \) mice, develop hepatic inflammation characteristic of steatohepatitis under HF/HS challenge. Most importantly, we demonstrated that there is no difference in agonist-driven AHR activity associated with \( Cyp1a1 \) transcription between \( \text{Ahr}^{fx/fx} \) and \( \text{Ahr}^{fx/fx} \text{Alb}^{Cre} \) mice, thereby indicating that the changes we observed in these mice largely stem from the loss of basal hepatocyte-specific AHR activity. Collectively, the data from chapter two highlight the importance of such AHR activity in the regulation of hepatic metabolism.

In chapter three, we provided another concrete example of how AHR impacts metabolism in the absence of ligand activation. Specifically, we demonstrated that loss of hepatocyte-specific AHR signaling, in the absence of exogenous ligand, results in elevated hepatic \( Fgf21 \) expression. Our subsequent experiments showed that the addition of AHR agonist can ablate induction of \( Fgf21 \) transcription under various conditions and that this phenomenon is conserved in primary human hepatocytes. While the exact mechanism through which AHR exerts this effect remains unknown, the data from this chapter are nevertheless significant in two regards. First, the data show that treatment with AHR agonists can not only activate DRE-dependent transcription, but also inhibit gene expression, thereby implying that our current understanding on the complete spectrum of agonist activity is likely incomplete. Second, the data are significant in that they support the notion that AHR is capable of modulating \( Fgf21 \) transcription. FGF21 is an important growth factor and can influence glucose tolerance, insulin sensitivity, sweet taste preference/intake, and energy expenditure (143, 157, 161). Therefore, our data set a precedent for determining how other classes of AHR ligand can be utilized to modulate \( Fgf21 \) transcription and whether through modulating FGF21, AHR can be used as a therapeutic target for the treatment of obesity and its related pathologies.
To conclude, this dissertation provides valuable insight into the role of AHR in metabolism. We have demonstrated that AHR appears to regulate key aspects of hepatic metabolism, largely through basal hepatocyte-specific AHR activity. In addition, our data have shown that AHR agonist-driven activity appears to extend beyond just the induction of Cyp1a1 gene transcription and likely includes a wide spectrum of activity. Together, the results suggest that the AHR has very unique and contrasting roles when activated by ligands and when exerting its regulation in the absence of ligand. Ultimately, researchers in the field of AHR must recognize these contrasting modes of AHR regulation and seek to define the role of AHR within each separate context. In doing so, we may gain a more complete understanding of the complex role of AHR in regulating not only metabolism, but also other physiological pathways with known connections to AHR as well.
4.2 Future Studies

The data from this dissertation provide valuable insights into the role of AHR in regulating metabolism. However, numerous questions and hypotheses remain unanswered. In chapter two, we demonstrated that typical agonist-driven AHR activity is comparable between \(Ahr^{fx/fx}\) and \(Ahr^{fx/fx}Alb^{Cre}\) mice as evidenced by similar levels of \(Cyp1a1\) transcription. This result likely reflects the fact that \(Ahr^{fx/fx}\) mice harbor the \(Ahr^d\) allele, which exhibits a lower affinity for ligand binding relative to \(Ahr^b\). We posit that this characteristic of \(Ahr^{fx/fx}\) mice may also indicate the data do not capture the true level of AHR agonist activity that may be present in SC due to the presence of dietary AHR ligands. To truly examine this hypothesis requires an \(Ahr^{fx/fx}Alb^{Cre}\) mouse model that is congenic to \(Ahr^{fx/fx}\) mice harboring the \(Ahr^b\) allele. Unfortunately though, this model is not yet available at the time of writing. Once this mouse model becomes available, one can also repeat the experiments from chapter two in these mice, and compare that data to the data presented here. In doing so, one may differentiate gene pathways regulated primarily through typical agonist-driven AHR activity from those regulated through basal AHR activity within hepatocytes.

In chapter two, we demonstrate that basal hepatocyte-specific AHR activity plays an important role in regulating metabolism and that AHR influences the response to dietary challenge. The majority of data from this chapter derive from mice that are not in a fasting state and therefore, primarily represent the influence of basal hepatocyte AHR signaling during the fed state. Accordingly, future research should aim to characterize how the loss of AHR expression in hepatocytes might also influence the fasting state of a mouse. Indeed, our observations that fasting \(Ahr^{fx/fx}Alb^{Cre}\) mice exhibit decreased glucose tolerance, but comparable sensitivity to insulin relative to PUR-fed \(Ahr^{fx/fx}\) mice, while the exact opposite phenomenon (i.e. decreased
insulin sensitivity, but comparable glucose tolerance relative to $Ahr^{fx/fx}$ occurs in HS-fed mice, sets a precedent for further investigation into the role of AHR in fasting.

Another important characteristic of the data from chapter two is that they primarily focus on mice fed AIN-93M purified diet and HS or HF/HS variations of it. While we examined some metabolic parameters in SC-fed mice, we largely chose not to focus on this experimental group because the formulation of this closed-formula diet may vary between batch and/or manufacturers. Therefore, how AHR influences metabolism in SC-fed mice remains largely unknown. One particularly important observation we did not expand upon is that SC-fed $Ahr^{fx/fx}$ mice exhibit significantly higher serum insulin levels during a fed state relative to $Ahr^{fx/fx} Alb^{Cre}$ mice (Table 2.4). To determine whether this result relates to decreased insulin sensitivity in SC-fed $Ahr^{fx/fx} Alb^{Cre}$ mice requires additional glucose and insulin tolerance tests. Furthermore, future studies should also examine the same metabolic gene pathways investigated in chapter two for additional insight on the reasons behind this observation.

One important aspect of glucose metabolism not examined in chapter two is the metabolism of amino acids. While the majority of energy demands are met through glycolysis and fatty acid oxidation, amino acid catabolism can account for as much as 15% of the body's energy production. In fact, many amino acids are ultimately converted to glucose or ketone bodies within the liver. However, this activity is mainly regulated transiently and influenced by the availability of certain amino acids in the diet. Nonetheless, the ability of AHR to modulate energy homeostasis through alterations in amino acid metabolism remains unexplored. A simple way to pursue this research area is with the utilization of techniques such as mass spectrometry and/or nuclear magnetic resonance (NMR) analysis. In addition to amino acid metabolism, how hepatocyte-specific AHR activity can influence the cholesterol and bile acid synthesis pathways
remains another important research area for further exploration. While the data from chapter two show that loss of hepatocyte-specific AHR activity can influence hepatic and serum cholesterol levels, the reasons for this and the mechanisms involved are not clear.

In chapter three, we demonstrate that AHR constitutively represses hepatic *Fgf21* expression in the absence of ligand. However, the exact mechanism through which AHR mediates this effect remains unknown. Two pieces of evidence suggest that the mechanism likely involves direct competition for DNA binding at the proximal *Fgf21* promoter. First, AHR, PPARα, ChREBP, CREBH, and XBP1 all bind to response elements within 50 bp of one another at the proximal *Fgf21* promoter. Second, agonist-driven AHR activation attenuates the induction of *Fgf21* through these factors without influencing the expression of other known target genes for each transcription factor. Ultimately, additional experimentation is necessary to fully demonstrate that DNA binding competition accounts for AHR-mediated repression of *Fgf21*. For example, one can utilize chromatin immunoprecipitation (ChIP) assays to quantify the ratio of AHR bound to the *Fgf21* promoter, with or without the administration of AHR agonist, under conditions in which one expects maximal binding of other transcription factors to the *Fgf21* promoter to occur (e.g. fasting or ER stress). An example of an alternative hypothesis that may explain how AHR activation suppresses *Fgf21* activity is that the receptor acts through epigenetic mechanisms (*i.e.* chromatin remodeling).

The data from chapter three indicate that ligand activation of AHR is able to suppress ChREBP-mediated *Fgf21* expression in the presence of supraphysiological glucose concentrations. Recent research has implicated ChREBP in the control of sugar intake/sweet taste preference (142). Therefore, future studies should evaluate whether ligand modulation of AHR can also directly influence these taste preferences, particularly in the context of elevated
sugar intake (*i.e.* after ingestion of a sugary meal). Given that increased FGF21 levels are associated with the suppression of sugar intake and sweet preference, an interesting experiment would be to administer pure antagonist or SAhRM (AHR ligands that may prevent nuclear localization of AHR) to mice to determine whether interrupting constitutive AHR repression of *Fgf21* expression can also result in the suppression of sugar intake/sweet taste preference.

Notably, AHR and ChREBP are both members of the bHLH family of proteins. Interestingly, the recognition sequence for ChREBP, CACGTGN5CACGTG, contains a majority of the core DRE sequence, GCGTG. Thus, AHR signaling might exhibit cross-talk with ChREBP. Supporting this theory, we demonstrate that AHR and ChREBP both bind to a composite DRE/ChoRE in the *Fgf21* promoter region, presenting the possibility that they compete for binding at these recognition sequences. Yet, whether this cross-talk between AHR and ChREBP occurs within the promoter region of other ChREBP-target genes (*e.g.* *L-PK* and *FASN*) remains unknown. Through the use of EMSA and ChIP assays, one could explore this hypothesis further in future experiments.

The ability of AHR to regulate *Fgf21* expression provides a unique opportunity to explore the potential use of AHR ligands in modulating metabolism. The scientific literature thoroughly demonstrates that administration of FGF21 in various models of obesity can attenuate disease pathology. Given that we demonstrate agonist activation of AHR represses *Fgf21*, an important hypothesis to explore is whether antagonism of AHR can be used as a therapeutic means for increasing endogenous hepatic FGF21 production during a state of obesity. In fact, certain flavonoid compounds have been shown to exhibit AHR antagonist activity (76). Importantly, flavonoid compounds are readily found in edible plants and human dietary exposure has been estimated as high as 1 g/day (191). Therefore, flavonoid-driven antagonism of AHR
might be a suitable approach to not only increase FGF21 production in obese humans through the AHR, but may also be a suitable approach for preventing and/or treating steatohepatitis as well, given our observations from chapter two.

In summary, the data presented in this dissertation reveal three primary research area for further investigation. First and foremost, while our data provide key insights into how basal AHR activity within hepatocytes can influence metabolism in mice fed a purified diet, we still lack a clear understanding of how this activity can influence metabolism in mice maintained on widely-utilized standard commercial rodent chows. Similarly, we also lack knowledge of the degree to which AHR ligands that may be present in standard commercial rodent chows can alter metabolic homeostasis via agonist-driven AHR activity associated with the induction of Cyp1a1. Second, we provide evidence that suggests AHR might exhibit cross-talk with the ChREBP gene pathway and therefore, the potential for AHR to influence sugar intake and/or sweet taste preference through cross-talk with ChREBP remains an important area for future research. The third and last important area of future research is to investigate the suitability of flavonoid-dependent AHR antagonism as a therapeutic target for 1) modulating FGF21 expression to reduce the symptoms of obesity and 2) treating and/or preventing the development of steatohepatitis.
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