THE ROLE OF PXR AND IKKβ SIGNALING IN CARDIOMETABOLIC DISEASE

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THE ROLE OF PXR AND IKKβ SIGNALING IN CARDIOMETABOLIC DISEASE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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Lexington, KY

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Lexington, KY

2016

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THE ROLE OF PXR AND IKKβ SIGNALING IN CARDIOMETABOLIC DISEASE

Cardiovascular disease (CVD) is the leading cause of death worldwide and is partially attributed to perturbations in lipid metabolism. Xenobiotics, such as pharmaceutical drugs and environmental chemicals, have been associated with increased risk of CVD in multiple large-scale human population studies, but the underlying mechanisms remain poorly defined. We and others have identified several xenobiotics as potent agonists for the pregnane X receptor (PXR), a nuclear receptor that can be activated by numerous drugs as well as environmental and dietary chemicals. However, the role of PXR in mediating the pathophysiological effects of xenobiotic exposure in humans and animals remains elusive.

The work herein identified several widely used pharmaceutical agents and endocrine disrupting chemicals as PXR-selective agonists such as drugs involved in HIV therapy and phthalates/phthalate substitutes, respectively. We investigated the role of amprenavir, an HIV protease inhibitor, and tributyl citrate, a phthalate substitute, on PXR-dependent alterations in lipoprotein metabolism. Acute exposure with either xenobiotic in mice elicited increases in the proatherogenic LDL-cholesterol levels in a PXR-dependent manner. PXR activation significantly induced expression of genes involved in intestinal lipid metabolism. Further, we went on to identify the intestinal cholesterol transporter, Niemann-Pick C1-Like 1 (NPC1L1), as a direct PXR-target gene. PXR activation also stimulated cholesterol uptake in both murine and human intestinal cells. Moreover, we provide evidence that the microsomal triglyceride transfer protein (MTP) may be a direct PXR-target gene. Taken together, these findings provide critical mechanistic insight into the role of xenobiotic-mediated PXR activation on lipid homeostasis and demonstrate a potential role of PXR in mediating adverse effects of xenobiotics on CVD risk in humans.

In addition to PXR signaling, we investigated the role of IκB kinase β (IKKβ), a central coordinator of inflammation, in adipocyte progenitor cells. Targeting IKKβ in adipose progenitor cells resulted in decreased high fat diet (HFD)-elicited adipogenesis, while protecting mice from inflammation and
associated insulin resistance. Consistently, we discovered that IKKβ inhibition by antisense oligonucleotides ablated HFD-induced adiposity, while protecting mice against associated metabolic disorders. In conclusion, targeting IKKβ with antisense therapy may present as a novel therapeutic approach to combat obesity and metabolic dysfunctions.

KEYWORDS: HIV drugs, Phthalates, Pregnane X Receptor, IκB Kinase β, Adipogenesis, Insulin Resistance
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Chapter 1: Introduction

1.1 Cholesterol Metabolism – an Overview

Atherosclerotic cardiovascular disease (CVD; otherwise known as coronary artery disease, or CAD) is a leading cause of mortality and morbidity worldwide [1]. Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of lipids and inflammatory molecules in the subendothelium of the artery, which can restrict blood flow and ultimately lead to myocardial infarction and stroke. Although atherosclerosis occurs in the general population, some people are at greater risk for developing CAD and these risk factors can be classified into either modifiable or non-modifiable. Non-modifiable risk factors for developing CAD are age, gender, and genetic heritability [2]. Moreover, modifiable CAD risk factors include smoking, obesity, nutrition, hypertension, sedentary lifestyle, stress, and hyperlipidemia [2]. Elevated plasma lipid levels, particularly low density lipoprotein (LDL)-cholesterol, have been strongly associated with atherosclerosis development [3, 4]. Lipid-lowering therapy has produced up to a 30% reduction in a major coronary event, validating the cholesterol hypothesis [3-5]. Mechanisms governing cholesterol homeostasis are still being evaluated and there is an urgent need to identify new targets for cholesterol-lowering therapy to alleviate the morbidity and mortality associated with CAD.

Cholesterol is a key structural component of cellular membranes to maintain integrity, permeability, and fluidity, but also serves as precursors for bile acid and steroid hormone production. The conversion of cholesterol into bile acids is absolutely essential for solubilization of dietary fats into micelles. Although cholesterol can be synthesized by the body, it requires a substantial energy requirement; thus, intestinal cholesterol absorption has evolved to take up cholesterol from the gut lumen [6]. Cholesterol derived from de novo synthesis and absorption is carried in the circulation in the form of lipoproteins (lipid-protein complexes) which are delivered to cells for utilization (Fig. 1.1).

Efficient cholesterol transport relies heavily on the expression of specific
proteins and perturbation of these proteins may disturb whole-body cholesterol homeostasis resulting in diseases. The principle plasma lipoproteins are chylomicrons, very low-density-lipoproteins (VLDL), LDL, and high-density lipoproteins (HDL). It is well established that the proatherogenic lipoproteins are chylomicrons, VLDL, and LDL, and the antiatherogenic lipoproteins are HDL. I will briefly discuss the basic pathway of lipoprotein formation, secretion, and uptake, as well as perturbations in these processes (see Fig. 1.1 for an overview).

Chylomicrons are secreted by enterocytes into the lacteals of the intestine and they ultimately reach the circulation via the thoracic duct. Chylomicrons are comprised primarily of triglycerides and cholesterol esters. Short chain fatty acids (less than 8-10 carbons) passively diffuse into the enterocyte and enter the portal blood directly without being packaged into chylomicrons. Long-chain fatty acids are internalized by enterocytes via passive diffusion and protein-facilitated fatty acid transfer. One protein that has been reported to facilitate fatty acid uptake is the fatty acid translocase (FAT/CD36). It is worth noting that many groups have investigated the role of CD36 in atherosclerosis and the studies are currently controversial. Once in the enterocyte, long chain fatty acids are incorporated into triglycerides via the acyl CoA:diacylglycerol acyltransferase (DGAT) enzymes. These triglycerides are complexed with apolipoprotein B 48, a process involving the microsomal triglyceride transfer protein (MTP; a more thorough review below) to ultimately form chylomicrons. ApoB is a hydrophobic protein that is present in plasma as apoB-48 or apoB-100. In humans, apoB48 and apoB100 are primarily associated with chylomicrons and VLDL, respectively [7]. MTP expression is absolutely essential for generation of apoB-containing lipoproteins. For example, intestinal MTP-deficiency impairs the ability to secrete chylomicrons and, as a result, there is a robust accumulation of neutral lipids in the enterocytes [8].

Free cholesterol is also absorbed from mixed micelles by the enterocytes from the gut lumen. This process is mediated by Niemann-Pick C1-Like 1 (NPC1L1; a more thorough review below). Free cholesterol is then re-esterified
by the Acyl-CoA:cholesterol acyltransferase-2 (ACAT2) enzyme in the endoplasmic reticulum (ER). The cholesterol ester is packaged along with triglycerides to form the core of chylomicrons. Cholesterol ester packaging into chylomicrons is also regulated by MTP [9]. It is worth noting that remaining unesterified cholesterol may be effluxed back out of the enterocyte by the apically localized heterodimeric sterol transporters ATP-binding cassette (ABC) transporters G5 and G8 (ABCG5/8) (Fig. 1.1). In addition, some unesterified cholesterol may be transported out of the enterocyte into HDL via ABCA1, which is located at the basolateral membrane of enterocytes. In mice, approximately 30% of the steady-state plasma HDL-cholesterol levels are derived from intestinal ABCA1 [10].

Once the chylomicrons enter the circulation, they are lipolyzed by lipoprotein lipase, which is located on the vascular endothelium of tissues such as skeletal muscle and adipose tissue [11]. LPL hydrolyzes the triglycerides of chylomicrons and, as a result, produces cholesterol-rich chylomicron remnants [11]. The chylomicron remnants are then taken up by the liver via LDL receptor-like protein (LRP) (Fig. 1.1).

Hepatic cholesterol can be derived de novo or can be taken up by hepatocytes from circulating lipoproteins such as LDL, HDL, and chylomicron remnants via LDL-receptor (LDLR), HDL receptor scavenger receptor class B type 1 (SR-B1), and LRP, all of which are localized at the basolateral membrane of hepatocytes [6]. Free cholesterol may be converted to bile acids by multi-enzymatic reactions that convert hydrophobic cholesterol into more water-soluble amphipathic compounds [12]. Analogous to intestinal cholesterol metabolism, free cholesterol may be esterified to fatty acids via ACAT2 and be packaged into VLDL particles with triglycerides in a MTP-dependent mechanism. Free cholesterol may be transported to ABCA1 on the sinusoidal membrane for HDL biogenesis or to ABCG5/ABCG8 on the canalicular membrane for direct secretion into the bile. Moreover, in humans and nonhuman primates, NPC1L1 can counterbalance the function of ABCG5/ABCG8 by reabsorbing biliary cholesterol into hepatocytes [6].
Whether synthesized de novo or taken up from the circulation, intracellular cholesterol is very tightly controlled by a variety of mechanisms. A major mechanism for sensing intracellular sterol levels involves the sterol-response element binding proteins (SREBPs). SREBPs comprise a subclass of the helix-loop-helix leucine zipper transcription factors [13]. In mammals, there are two SREBP genes that encode SREBP1 and SREBP2, which give rise to 3 proteins [14, 15]. SREBP1a and SREBP1c are encoded from alternative promoters that are expressed at varying levels in different tissues and cultured cells. These transcripts are thought to primarily play a role in fatty acid metabolism [14, 15]. SREBP2, however, is a major regulator of cholesterol metabolism [14, 15].

SREBPs are synthesized as ER transmembrane proteins and their activation depends on a polytopic ER membrane protein known as the SREBP cleavage-activating protein (Scap). Scap acts as both an escort protein for SREBP ER-Golgi transport and as a sterol sensor. The sterol-sensing property of Scap is mediated by the polytopic membrane domain, which possesses a motif that is shared with other proteins that are postulated to interact with sterols [16]. Mutations within this sterol sensing domain (SSD) of Scap prevent sterol repression of SREBP cleavage and lead to an excess cholesterol production [16-18]. When ER cholesterol falls less than 5% of total lipids, the cholesterol-free Scap/SREBP complex translocate to the Golgi for processing by the Site-1 and Site-2 proteases [16]. Transcriptionally active SREBP then enters the nucleus to regulate genes involved in lipid metabolism. The SREBP2 isoform, for example, transcriptionally regulates LDLR, HMG CoA reductase, NPC1L1, and other genes required for cholesterol synthesis and uptake to maintain cellular cholesterol homeostasis [19].

Given the consequences of excess plasma cholesterol on CAD, there have been extensive efforts to understand the molecular mechanisms regulating cholesterol homeostasis. The pregnane X receptor (PXR) is a xenobiotic receptor that has been implicated in modulating cholesterol homeostasis and atherosclerosis. The primary focus of this dissertation was to identify specific mechanisms by which ligand activated PXR-mediates hypercholesterolemia. In
Chapter 3, I provide conclusive evidence that PXR transcriptionally regulates NPC1L1 and promotes cholesterol uptake in mouse and human intestinal cells. Further, in Chapter 5, I demonstrate that PXR may transcriptionally regulate MTP and promote chylomicron/VLDL secretion. Taken together, this work herein implicates PXR as a transcriptional regulator linking xenobiotic and cholesterol metabolism.

1.1.1 Niemann-Pick C1-Like 1 (NPC1L1)

NPC1L1 was first identified by Altmann and colleagues utilizing a bioinformatics approach that identified genes based on the following criteria: presence of a SSD, extracellular signal peptide, and enriched expression in absorptive enterocytes [20, 21]. Mice deficient in NPC1L1 had a significant reduction in sterol absorption (approximately 70%), which is similar to the reduction produced in mice treated with the potent intestinal cholesterol absorption inhibitor Ezetimibe [20]. Moreover, NPC1L1-deficient mice were resistant to Ezetimibe treatment, indicating the inhibition of cholesterol absorption by Ezetimibe was mediated through NPC1L1 [20]. Shortly thereafter, it was discovered that the target of Ezetimibe is, indeed, NPC1L1 [22]. Ezetimibe lowers circulating plasma cholesterol levels in humans by 15-20%, and when co-administered with statins can synergistically decrease plasma cholesterol levels [23-25].

NPC1L1 is a polytopic transmembrane protein comprised of 1332 amino acids. It is a homolog of Niemann-Pick C1 (NPC1), a protein when mutated results in a genetic disorder characterized by lysosomal accumulation of cholesterol and other lipids. NPC1L1 has a conserved N-terminal NPC1 domain, a signal peptide, 13 transmembrane domains, and extensive glycosylation sites. It has been demonstrated that the conserved NPC1 domain contains a sterol-binding pocket, thus it is likely that this region also binds sterols. Five of the 13 transmembrane domains constitute the SSD. The SSD is conserved in many transmembrane proteins involved in cholesterol metabolism, including NPC1, HMG-CoA reductase, SCAP, and 7-dehydrocholesterol reductase (7DHCR) [26]. The functional significance of the SSD in NPC1L1 remains to be determined.
In humans and nonhuman primates, NPC1L1 is predominantly expressed on the apical surface of absorptive enterocytes [20, 27, 28] and on the canalicular surface of hepatocytes [29]. In rodents, however, NPC1L1 is primarily expressed in the intestine, with little to no expression in the liver [20, 21]. However, the underlying mechanism for this species-specific expression is still unclear.

Mechanisms governing NPC1L1 transcriptional regulation have not been well established. Several nuclear receptors have been previously reported to regulate intestinal cholesterol absorption, including LXR, PPARα, and RXR; however, these effects may not be due to direct transcriptional regulation of NPC1L1. SREBP-2, however, is a major transcriptional regulator of NPC1L1 [30, 31] and overexpression of a constitutive nuclear form of SREBP2 enhanced NPC1L1 promoter activity quite dramatically (approximately 8 fold; R.N. Helsley unpublished). Moreover, other groups have identified hepatocyte nuclear factor 4α (HNF4α) and HNF1α as important regulators of SREBP-2 dependent regulation of NPC1L1 [31, 32]. More work is required to identify novel regulators of NPC1L1. In Chapter 3 I identified PXR as a novel transcriptional regulator of NPC1L1, which links xenobiotic and cholesterol metabolism in mice and humans.

1.1.2 Microsomal Triglyceride Transfer Protein (MTP)

MTP was first identified as a protein that associated with triglyceride and cholesterol ester transfer activity in liver microsomes [33, 34]. It was later identified that individuals with abetalipoproteinemia lack apoB-lipoproteins in their plasma and have mutations in the MTTP gene, which result in the loss of lipid transfer activity in the liver and intestine [35]. Abetalipoproteinemia is an autosomal recessive disease that is characterized by fat malabsorption, defective lipid transport, accumulation of fat-laden enterocytes, decreased plasma triglyceride and cholesterol levels, and fat-soluble vitamin deficiencies [7, 36]. MTP contains both an apoB binding domain and a lipid transfer domain. While the two domains are thought to act independent of one another, both are required for efficient secretion of apoB-containing lipoproteins [37-39]. Consistently, independent groups identified a physical interaction between apoB
and MTP [40, 41]. MTP has been demonstrated to be pivotal for apoB-containing lipoprotein assembly in two ways: (1) by acting as a chaperone mediating translocation of newly synthesized apoB across the ER membrane and (2) by facilitating the lipidation of apoB during its translocation into the ER [42].

Expression of MTP is enriched in the intestine and liver; however, MTP expression has been detected in other tissues but to a much lesser extent. MTP expression decreases from the proximal to distal end of the intestine, with negligible expression in the colon [43]. Moreover, MTP expression also varies vertically within the crypt-villus axis, with the highest expression along the villi [7]. Isolated crypts are devoid of MTP mRNA and protein [7, 44]. In Caco2 cells, MTP induction expression increases throughout differentiation into polarized enterocyte-like cells [44]. Hepatocytes also express high levels of MTP [7]. The consequence of altering MTP expression in these tissues will be discussed briefly.

Homozygous deficiency in mice results in embryonic lethality. Heterozygote deletion of MTP results in decreased LDL plasma cholesterol levels in mice fed chow, and decreased chylomicron/VLDL and LDL cholesterol levels under high-fat diet (HFD)-feeding conditions [45]. Homozygous deletion of MTP in the visceral endoderm of yolk sacs produces a marked accumulation of cytosolic fat droplets, indicative of impaired secretion of lipids [45]. To circumvent embryonic lethality in global MTP deficiency, Raabe and colleagues generated liver-specific MTP knockout mice and studied the effects on lipoprotein metabolism [46]. Suppression of hepatic MTP reduces VLDL triglycerides, as well as VLDL/LDL and HDL cholesterol levels [46]. In addition, apoB-100 is decreased in plasma by more than 95 % [46]. The liver-specific knockout mice possess profound hepatic steatosis, which is associated with elevated cytosolic lipid droplets in the hepatocytes [46]. Reduced MTP expression via antisense oligonucleotides (ASO) decreases hepatic VLDL triglycerides and cholesterol secretion in mice [47]. In contrast, hepatic overexpression of MTP results in elevated secretion of VLDL triglycerides [48].
Xie and colleagues set out to investigate the overall contribution of intestinal MTP expression on lipoprotein metabolism. Intestinal-specific knockout mice exhibit increases in the number of lipid droplets in absorptive enterocytes and the mice display steatorrhea, growth arrest, and decreases in cholesterol absorption [49]. The decreases in cholesterol absorption most likely result from reduced expression of NPC1L1 in intestinal-specific MTP KO mice. Chylomicron secretion is impaired by 80% and plasma lipids, as well as hepatic triglyceride and cholesterol content, are reduced in mice with intestinal MTP-deficiency [49]. Intestine-specific KO mice have an increase in both hepatic lipogenesis and VLDL secretion, which is attributed to the liver compensating for the lack of intestinally-derived lipid [49]. Collectively, alterations in MTP expression clearly alter apoB-lipoprotein secretion. In Chapter 5, we provide evidence that PXR may transcriptionally regulate MTP expression, but whether PXR mediates apoB secretion remains to be determined.

1.2 Highly Active Antiretroviral Therapy (HAART) – an Overview

The human immunodeficiency virus (HIV) is the cause of the acquired immune deficiency syndrome (AIDS) and is currently considered a pandemic, with approximately 37 million people globally living with HIV (UNAIDS 2014). Since the first class of antivirals were developed and used as a monotherapy in the early 1990’s, the standard of care has evolved to include a combination of antiretroviral drugs (ARVs) targeting different stages of the HIV life cycle [50]. The combination of ARVs is defined as highly active antiretroviral therapy (HAART) and has significantly reduced the morbidity and mortality associated with HIV infection and AIDS [50]. HAART is a customized combination of different classes of ARVs, including: fusion inhibitors, CCR5 antagonists, reverse transcriptase inhibitors (comprised of nucleoside [NRTIs] and nonnucleoside [NNRTIs]), integrase inhibitors, and protease inhibitors (PIs). Approximately 37% of all people living with HIV are currently on ARV drug therapy (UNAIDS 2014). Consequently, AIDS-related deaths have fallen by 35% since 2005, which is partially attributed to the development of HAART (UNAIDS 2014). As longevity increases in HIV-infected individuals, chronic
illnesses, such as accelerated atherosclerosis and cardiovascular disease, are emerging as leading health issues in this population [51].

1.2.1 Epidemiological Studies Linking HAART to CVD

Despite reductions in the morbidity and mortality associated with HIV infection, HAART has been associated with dyslipidemia and increased risk of CVD [52-62]. Findings from the Data Collection on Adverse Events of Anti-HIV Drugs study suggested that the incidence of CVD increased with longer exposure to HAART [63]. Another large-scale study including more than 23,000 HIV patients also concluded that ARV drugs such as PIs are associated with an increased risk of CVD, which is partly explained by dyslipidemia [55]. A Swiss HIV Cohort Study found that PIs can cause dyslipidemia in patients [58] and several other studies have reached the same conclusion [64, 65]. For example, Lang et al. conducted a case-control study nested within a large HIV patient database and found that the risk of myocardial infarction was increased by cumulative exposure to almost all of the studied ARV drugs [66]. Many currently recommended first-line ARV drugs including several PIs and NNRTIs have been shown to cause hyperlipidemia [67]. Despite the strong evidence linking HAART with dyslipidemia and CVD, the underlying mechanisms responsible for the adverse effects of ARV drugs remain elusive.

1.2.2 Mechanisms of HAART-Elicited Hyperlipidemia and CVD

Mounting evidence has linked HAART to alterations in plasma lipid levels in humans. Many of these studies are conducted in HIV-positive individuals; however, other studies confirmed a direct association between HAART exposure and hyperlipidemia in normal healthy subjects [68]. Liang and colleagues first identified a possible mechanism linking PI therapy to hyperlipidemia [69]. HIV PI treatment inhibited the proteasomal degradation of nascent apoB [69]. ApoB secretion was also impaired with HIV PI treatment, which associated with decreased MTP activity [69]. In the presence of oleic acid, PI treatment increased secretion of apoB-containing lipoproteins above controls [69]. The authors concluded that the PI-mediated hindrance to apoB secretion can be overwhelmed by the excess supply of core lipids [69].
Independent groups also demonstrated the ability of certain PIs to induce hyperlipidemia in animal models [70, 71]. Treatment of ritonavir for 10 days in WT mice on either a low fat or high fat diet produced pronounced increases in plasma cholesterol and triglyceride levels [70]. Ritonavir treatment also resulted in significant hepatic steatosis and hepatomegaly, which were attributable to the accumulation of transcriptionally active SREBP1 and SREBP2 [70]. In an independent study, the same group went on to demonstrate that ritonavir treatment enhanced VLDL secretion without altering lipoprotein clearance [71]. Collectively, these adverse effects with HIV PI treatment associated with increased expression of genes involved in lipid synthesis and enhanced VLDL secretion [70, 71].

The activation of the SREBP signaling pathway with HIV PI treatment stimulated the work by other groups. PI treatment in murine macrophages enhanced transcriptionally active nuclear SREBP expression, which resulted in an accumulation of free cholesterol in intracellular membranes and elevated apoptosis [72]. The authors also observed that PI treatment induced endoplasmic reticulum stress and activated the unfolded protein response (UPR) in these cells [72]. Since intracellular cholesterol can activate ER stress and the UPR [73], it is likely the adverse effects associated with PI treatment may be linked to the initial nuclear SREBP accumulation; however, this warrants further investigation. The same group went on to demonstrate that treatment with various PIs resulted in a variety of responses in mouse primary hepatocytes [74]. For example, atazanavir and ritonavir increased nuclear SREBP levels and activated the unfolded protein response, while amprenavir had no effect [74]. Further, atazanavir and ritonavir treatment suppressed CYP7A1 protein levels and bile acid synthesis, while amprenavir had no significant effect [74].

These results clearly demonstrate that individual HIV PIs may have different effects on lipid metabolism in mice, which is what is observed in humans [75]. There is an urgent need to identify new HAART-elicited off-target effects that influence lipid metabolism. In Chapter 2, I provide evidence that HIV
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Pls activate PXR and induce hyperlipidemia in mice. This new signaling pathway linking HAART to xenobiotic and lipid metabolism may partially explain the adverse side effects associated with HAART.

1.3 Endocrine Disrupting Chemicals (EDCs) – an Overview

There is growing concern about the possible health threat posed by endocrine-disrupting chemicals (EDCs), substances found in our food, environment, and consumer products that ultimately disrupt normal homeostatic processes and have the capability to cause diseases [76]. The U.S. Environmental Protection Agency define an EDC as, “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process” [76]. A few examples of compounds that have been identified as EDCs are polychlorinated biphenyls (PCBs), plasticizers (phthalates and phthalate substitutes), and certain pesticides [76]. It was originally thought that EDCs act primarily through nuclear hormone receptors; however, it is now widely accepted that EDCs act through a variety of signaling mechanisms, which include but are not limited to, nonnuclear steroid receptors, nonsteroid receptors, orphan receptors, and enzymatic pathways ultimately responsible for maintaining endocrine homeostasis [76]. EDCs have been linked to obesity and CAD; however, the mechanisms governing this phenomenon remain poorly understood.

1.3.1 Epidemiological Studies Linking EDCs to CVD

Bisphenol A (BPA) is a base compound widely used in the production of foods and plastics, and has been recently studied in humans to correlate exposure to disease risk. Using a cross-sectional analysis to measure urinary BPA and health status, Lang and colleagues recently demonstrated that higher urinary BPA concentrations were associated with cardiovascular disease, including CAD [77]. This finding was further supported by an independent group [78, 79]. Further, high BPA exposure in adolescents and young adults has been associated with significantly increased carotid intima media thickness,
a measure of atherosclerosis progression [80].

Aside from BPA, other phthalates and phthalate metabolites have been associated with atherosclerosis. Phthalate metabolites are associated with increased atherosclerosis in the elderly [81]. Interestingly, Sergeev and colleagues demonstrated that people living within close proximity to sites contaminated with organic pollutants compared to sites not containing any identified hazardous waste had a 20% increase in acute myocardial infarction hospital discharge rates [82]. Overall, there have been many studies associating environmental chemicals with disease; however, whether these compounds are causative and the mechanisms involved remain elusive.

1.3.2 Mechanisms of EDC-Elicited Hyperlipidemia and CVD

Mechanisms linking EDC exposure to cholesterol metabolism are largely unknown; however, a few groups have examined exposure to atherosclerosis and obesity, which will briefly be discussed. For example, various EDCs were screened for their ability to activate the glucocorticoid receptor (GR), and four were identified to induce GR-promoter activity [83]. This activity was correlated with enhanced adipogenesis in 3T3-L1 murine preadipocytes [83]. Surprisingly, the effect of EDCs on promoting lipid accumulation during adipogenesis was not contributed to changes in PPARγ expression [83].

Consistent with the notion that EDCs play a role in adipocyte stem cell differentiation, an independent group demonstrated that PCBs, specifically PCB-77, increased adipocyte differentiation in low doses [84]. This effect on 3T3L1 differentiation was ablated by an aryl hydrocarbon receptor (AhR) antagonist [84]. Furthermore, administration of PCB-77 increased body weight gain in WT mice, but not in AhR-deficient mice [84]. PCB-77 treatment also enhanced the obesity phenotype, as well as augmenting dyslipidemia and atherosclerosis in ApoE-deficient mice [84]. The effect on dyslipidemia with PCB-77 treatment was pronounced with approximately a 133% increase in plasma cholesterol levels [84]. The specific mechanism linking PCB exposure to hypercholesterolemia was not investigated. Our group, as well as others, discovered that BPA is proatherogenic in animal models [85, 86] (see section
1.5.1); however, the precise mechanisms linking EDC-exposure to alterations in lipid metabolism and atherogenesis are not well characterized and need to be investigated further. One important area of research in our laboratory is to understand the mechanisms linking EDC-exposure and sterol metabolism. In Chapter 3, I identified a phthalate substitute known as tributyl citrate (TBC), as an intestinal-specific PXR agonist. Further, acute TBC exposure stimulated hypercholesterolemia which may be mediated through PXR transcriptional regulation of NPC1L1.

1.4 Identification of the Pregnane X Receptor (PXR)

Pharmaceutical drugs and environmental chemicals are metabolized through a series of enzymatic reactions to generate more polar derivatives that are ultimately excreted by the body. Many of these compounds are first metabolized by a member of the cytochrome (CYP) P450 monooxygenase gene family. The CYP enzymes are comprised of four gene families (CYP1, CYP2, CYP3, and CYP4) and are referred to as phase 1 enzymes because they catalyze the first step in the detoxification process. The CYP enzymes are highly expressed in the liver and intestine. Among the family of CYP enzymes, the CYP3A family is recognized to metabolize up to 50% of clinically used drugs as well as other xenobiotics [87, 88]. Additionally, the CYP2B enzymes metabolize another 25-30% of these compounds [88]. The ability of organisms to adapt to their surroundings and possess a host defense mechanism against toxic chemicals is essential to maintain life.

The pregnane X receptor (PXR; otherwise termed the steroid and xenobiotic sensor, or SXR) was initially discovered in 1998 by multiple groups. PXR was designated NR1I2 (nuclear receptor subfamily 1, group I, member 2) using standard nomenclature and was named based on the observation that pregnanes (21-carbon steroids) activate the receptor [89]. Like other nuclear receptors, PXR contains both an N’-terminal DNA binding domain (DBD) and a C’-terminal ligand-binding domain (LBD), which are responsible for DNA binding and ligand binding as well as interaction with co-regulators, respectively. PXR is activated by numerous endogenous hormones, dietary steroids,
pharmaceutical agents, and xenobiotic chemicals [90, 91]. It functions as a heterodimer with the retinoid X receptor (RXR) to modulate gene transcription [92, 93]. PXR was initially characterized based on the observation that ligands activating the receptor induced CYP3A4 expression. Consequently, it was further identified that PXR transcriptionally regulates CYP3A4 [89, 94-96]. Similar to the CYP enzymes, PXR is highly expressed in the liver and intestine [89, 94-96], two sites of first line defense against toxic xenobiotics. The discovery of PXR led to identification of many novel target genes involved in detoxification and transport of lipophilic compounds, such as genes that encode phase I (e.g., CYP450s) and phase II (e.g., glutathione transferase) detoxification enzymes, as well as the ABC family transporters (e.g., multidrug resistance 1 [MDR1]). Since its initial discovery, PXR has been shown to be a critical regulator for other processes, such as modulating lipid levels, atherogenesis, bone homeostasis, and inflammation.

1.4.1 PXR Gene Structure
The human PXR (hPXR) gene consists of nine exons on chromosome 3. Despite only one PXR gene discovered in the human genome, three PXR variants resulting from alternative splicing have been characterized [97-100]. PXR variant 1 encodes two products (isoforms hPXR.1 and hPXR.2), through the use of alternative translational codons. PXR variant 3 (hPXR.3) has a unique 5' untranslated region and start codon, which encodes the longest isoform with the addition of 39 amino acids on its N' terminus relative to hPXR.1 [97-100]. PXR variant 4 (hPXR.4) is similar to hPXR.1 but is lacking 111 base pairs due to an in-frame deletion in the LBD at exon 5 [97-100] (Fig. 1.2). Particularly, hPXR.4 lacks ligand binding pocket resides $^{206}$Leu, $^{208}$Ser, and $^{209}$Leu and has been demonstrated to lack the ability to undergo ligand-elicited transactivation in reporter assays [100] (Fig. 1.2). Interestingly, however, hPXR.4 has been demonstrated to act as a dominant-negative protein interfering with hPXR.1 transactivation [100]. This is most likely due to competing occupation for DNA binding, thus alleviating maximal ligand activation of PXR. Further, ligands can bind to the LBD of hPXR.4, however
with weak associations and thereby corepressors remain tightly bound and coactivators are not recruited for PXR transactivation [100]. Likewise, UGT1A1, 1A3, and 1A4 gene expression were all induced by hPXR.1 and hPXR.3 but not by hPXR.4 [98]. All four variants were found in human liver and intestinal tissue; however, a great deal of interindividual variation was found [98]. This may partially explain the great deal of interindividual variation in drug metabolism associated with CYP expression.

The mouse PXR (mPXR) gene is located on chromosome 16. The mPXR gene generates two variants that are quite similar with hPXR.1 and hPXR.4 [89]. Mouse PXR variant 1 (mPXR.1) is 431 amino acids in length and mouse PXR variant 2 (mPXR.2) is a splicing variant that lacks 41 amino acids within the LBD [89] (Fig. 1.2). Similar to the hPXR.4, mPXR.2 lacks the ability to be activated by numerous ligands, such as the synthetic steroids dexamethasone and pregnenolone 16α-carbonitrile (PCN) [89]. Although mPXR.2 was expressed in a similar tissue-specific pattern as mPXR.1, it was expressed at a much lesser extent indicating mPXR.1 is the major isoform in mice [89]. Although other transcript variants may exist through the use of alternative promoters, alternative splicing, and/or alternative polyadenylation, they have not been completely characterized [91, 97, 99].

1.4.2 Species-Specific PXR Activity and Animal Models

Since the initial discovery of PXR in 1998, many orthologues have been cloned from a wide variety of organisms, including mammals [89, 94-96, 101-104], Drosophila [105], and C. elegans [101]. In Drosophila, the PXR orthologue was termed DHR96 [105]. Furthermore, three orthologues of PXR are represented in C. elegans as DAF12, NHR-8, and NHR-48 [101]. Despite acting as orthologous receptors, the pharmacology of PXR differs substantially between species. Mouse and human PXR only exhibit approximately 70-80% homology within the LBD but share approximately 95% homology in their DNA binding domains (DBD) [106, 107]. The observation for species differences in CYP3A induction observed in vivo appears to be partially reliant on sequence differences in the LBD of PXR. For example, PCN, a known CYP3A inducer in
rodents, acts as a potent agonist of mouse and rat PXR, but does not activate human PXR [106]. Further, rifampicin, a known inducer of CYP3A in humans and rabbits, does not activate mouse PXR. Thus, by binding to wide array of structurally diverse compounds, it has been suggested that PXR exhibits directed promiscuity [106, 107].

Since the homology of PXR differs quite substantially between species, mouse models were generated to understand human xenobiotic metabolism in vivo. Xie et al. generated transgenic mice expressing wild-type (WT) human PXR driven by the albumin promoter on a PXR-null background [108]. These mice express hPXR specifically in the liver, while maintaining mouse PXR-deficiency [108]. Later in the 2000s, Ma and colleagues generated another ‘humanized’ mouse model utilizing a bacterial artificial chromosome clone containing the complete hPXR gene and the corresponding 5’ and 3 flanking sequences [109]. This humanized model displays the same tissue-specific patterns as human PXR and responds to human-specific PXR agonists, such as rifampicin, but not mouse-specific PXR agonists, such as PCN [109]. Although these models are essential to understand the PXR-mediated effects on metabolic homeostasis, it raises the question about the ability of human PXR to drive expression of mouse PXR target genes. For example, the human PXR response element within the CYP3A4 promoter is not found in mouse CYP3A11 [110]. To address this issue, CYP3A4/hPXR double transgenic mice were generated [111]. Consequently, rifampicin is a robust inducer of CYP3A4 in the double transgenic model [111], compared with only slight induction of CYP3A11 [112]. Since many CYP3A4 substrates are also PXR ligands, it is essential to utilize the double transgenic model to assess the pharmacokinetics of pharmaceuticals and other xenobiotics in vivo. Two more models were generated to investigate constitutive activation of human PXR in the mouse liver and intestine. The first constitutively active mouse model was generated by utilizing the albumin promoter to drive human PXR, which was fused at the 5’ end to the herpes simplex virus activation domain (VP16) [108]. Subsequently, another model was generated utilizing the fatty acid binding protein promoter to
drive VP16-hPXR to overexpress PXR in both the liver and gastrointestinal tract [113]. Collectively, all the hPXR mouse models show a normal phenotype, except for the albumin-driven VP16hPXR mouse model, which displays growth retardation, hepatomegaly, and histological liver toxicity [108, 114].

### 1.4.3 Post-Transcriptional Regulation of PXR

Micro-RNAs (miRNAs) are short noncoding regulatory RNAs that bind to complementary regions in the 3’ UTR of mRNA target sequences and suppress their translation and/or facilitate degradation [115, 116]. Aside from alternative splicing, PXR has been demonstrated to undergo another level of gene regulation from microRNAs. PXR mRNA and protein levels were measured in 25 human livers and their relative expression was compared [117]. Interestingly, the transcript and protein levels were not associated, suggesting post-transcriptional regulation of PXR [117]. In an independent study, 19 single nucleotide polymorphisms (SNPs) correlated with plasma cholesterol in humans and many of these SNPs were found in the 3’ UTR [118]. Further, 16 microRNAs were predicted to bind PXR mRNA in human liver samples [117]. MiR-148a was later identified as a critical regulator of human PXR expression [117]. Overexpression of miR-148a suppressed PXR protein levels and, in contrast, inhibition of miR-148a elicited increases in PXR protein expression [117]. A similar binding site was discovered in CYP3A4; however, CYP3A4 was unresponsive to mir-148a overexpression [117]. Although the 3’UTR of PXR is not well conserved between species, the miR-148a element has been discovered in both mice and rat, indicating that this specific miRNA may control PXR gene expression in other mammals [117].

In another human cohort, genetic variants in the 3’ UTR were identified in specific genes involved in xenobiotic metabolism [116]. For example, bioinformatics analysis revealed 1,025 unique microRNA’s that were predicted to bind to the hPXR 3’ UTR [116]. The CYP3A4 gene was also predicted to be regulated by 889 unique microRNAs [116]. The variant rs1054190C in hPXR was predicted to result in the presence of a binding site for miR1250-5p [116]. In contrast, the variant rs1054191G was predicted to result in the loss of a
binding site predicted to bind miR-371b-3p, miR-4258, and miR-4707 [116]. Although there has been evidence of other regulatory miRNAs targeting PXR [116], the physiological consequence on PXR stability and xenobiotic and/or lipid metabolism has not been characterized.

### 1.4.4 Post-Translational Regulation of PXR

It is well known that transcriptional regulation of PXR target genes is governed by ligand-activation; however, PXR has been established to undergo a host of post-translational modifications including phosphorylation, SUMOylation, ubiquitination, and acetylation [119]. This section will provide a brief overview of post-translational modifiers in altering PXR subcellular localization, dimerization, DNA binding, coregulator activity, and transcriptional regulation.

**Phosphorylation**

Recently, there has been a host of evidence demonstrating site-specific phosphorylation of PXR, which provides a mechanism for PXR-mediated regulation of CYP expression. It should be noted that phosphorylation of PXR is mostly correlated with an inhibitory response in its transcriptional activity. Many kinases have been demonstrated to phosphorylate PXR including p70 S6K [119-122], protein kinase A (PKA) [122-124], protein kinase C (PKC) [122, 125], cyclin-dependent kinase (Cdk2) 2 [126-128] and 5 [129], thereby regulating the activity of PXR. Metabolic labeling studies indicated that immunopurified hPXR is phosphorylated by numerous kinases such as glycogen synthase kinase 3 (GSK3), casein kinase II (CK2), and Cdk1 [119, 120, 123].

It was been well documented that CYP expression is greatly reduced during hepatocyte proliferation. For example, hepatocyte exposure to human growth factors such as hepatocyte growth factor potently induces hepatocyte proliferation, yet significantly suppresses the CYP isoenzymes [119, 130, 131]. In recent work led by Lin and colleagues, it was initially hypothesized that hPXR changes expression throughout various phases of the cell cycle. This study provided compelling evidence that hPXR is phosphorylated by Cdk2, a key
regulator of the cell cycle, and suppresses its transcriptional activity [128]. This group went on to identify two small molecule inhibitors that were Cdk2 inhibitors, which ultimately increased hPXR activity. This increase in hPXR activity was thought to be ligand-independent, as these two molecules bound to hPXR with low affinity. The serine 350 (Ser350) was discovered to be the site at which Cdk2 phosphorylates PXR. Consistently, a phosphomimetic mutation (S350D) of the putative Cdk2 phosphorylation site on PXR mimicked the inhibitor effect of Cdk2. In contrast, a phosphorylation-deficient mutation (S350A) conferred resistance to Cdk2 suppression. Furthermore, PXR transcriptional activity was significantly reduced in the S-phase of the cell cycle, which is when Cdk2 activity increases, and this was not due to simply reduced hPXR expression. Since neither the phosphomimetic mutation nor the phosphorylation-deficient mutation rendered 100% resistance to the inhibitor effect of Cdk2, it is likely that Cdk2 phosphorylates multiple sites on hPXR [128]. This work is the first to identify a possible mechanism linking Cdk2 to CYP3A4 expression in hepatocytes, and implicates the importance of considering cell cycle status when investigating PXR activity and CYP expression [128].

Consistent with the notion that PXR phosphorylation suppresses transcriptional activity, another kinase downstream of the PI3K-Akt signaling pathway was demonstrated to phosphorylate PXR and suppress its activity. A study by Pondugula and colleagues demonstrated that p70 S6K acts as an upstream kinase of PXR and phosphorylates threonine 57 (Thr57), which suppresses PXR transactivation. This Thr57 residue is a highly conserved phosphorylation site within the DBD of human nuclear receptors, including numerous PXR orthologous, indicative of its importance in PXR physiology [119]. In this study, a phosphorylation-deficient (T57A) and a phosphomimetic (T57D) were generated to assess PXR activity and subcellular localization. As expected, the T57D but not the T57A mutation abolished its PXR transcriptional activity. Interestingly, however, neither mutation altered hPXRs interaction with the coactivator SRC-1. Both WT and the T57A mutant exhibited a homogenous distribution in the nuclei of cells whereas the T57D phosphomimetic mutant
exhibited a punctate pattern, suggesting that phosphorylation at T57 could alter subcellular localization. However, it is still unclear how the punctate nuclear localization pattern affects PXR signaling. Since this T57 residue is highly conserved, it raises the possibility that this amino acid is pivotal for DNA binding across many nuclear receptors. In conjunction with this notion, the T57D mutation, but not the T57A mutation, impaired hPXR's ability to bind to the CYP3A4 promoter. The p70 S6K was later described to phosphorylate T57 on PXR and suppress its transcriptional activation through loss of binding at the CYP3A4 promoter [121].

A further study was conducted to investigate the mechanism by which phosphomimetic mutations at various sites within PXR result in changes in subcellular localization and activity. Phosphomimetic mutations at threonine-290 and threonine-408 demonstrated similar subcellular localization properties, with suppressed translocation of hPXR into the nucleus [132]. In another study, the same group demonstrated the ability of Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) to phosphorylate hPXR at threonine-290, leading to its retention in the cytoplasm [133]. They went on to further illustrate that both WT and the T408D mutant immunoprecipitated with chaperone proteins to a similar extent in the cytoplasm [132]. When they treated cells with autophagy inhibitors, the T408D mutant led to higher cytoplasmic accumulation than WT and co-localized with the autophagic cargo [132]. Collectively, these data support the notion that PXR is phosphorylated at threonine 408 and undergoes autophagy regulated degradation.

There has been mounting evidence in support of additional amino acid residues on PXR that have been predicted to be phosphorylated by numerous kinases [119, 134]. Although more work is required to functionally validate the importance of each one of these kinases, PXR clearly has other levels of regulation aside from ligand-dependent transcriptional activation that modulate PXR transcriptional activity, stability and subcellular localization [119, 134].

*Ubiquitination*
Ubiquitination is a multi-step process that occurs at several levels of signal transduction. Ubiquitin attaches to the substrate by an isopeptide bond between the C’ terminus of ubiquitin and a lysine residue on the substrate [135]. The placement of ubiquitin on the substrate is governed by three enzymes: E1 (ubiquitin activating), E2 (ubiquitin conjugating), and E3 (ubiquitin ligases) [135]. The degradation of proteins by the proteasome relies on ubiquitin tagged substrates. The mechanisms governing degradation of PXR by ubiquitination have not been well investigated; however, there are a few studies suggesting PXR stability being altered by ubiquitination. In 2000, Masuyama and colleagues demonstrated that ubiquitin may regulate PXR stability. This group revealed that the suppressor for gal-1 (SUG1) interacted with PXR [136]. SUG1 is a known subunit of the 26S proteasome [137] and was first demonstrated to bind to a host of other nuclear receptors [138], but PXR was not initially described in this data. This interaction, however, only occurred in the presence of progesterone, but not with other PXR activators, such as phthalic acid or nonylphenol [136], which highlights the fact that various PXR activators may alter PXR degradation differently [119]. Subsequently, the same group went on to illustrate that PXR protein levels were increased in the presence of proteasome inhibitors [139]. Overexpression of SUG1 elicited increases in PXR degradation, as evident by enhanced proteolytic PXR fragments, which was blocked by proteasomal inhibition. Interestingly, the interaction between PXR and SUG1 depended on the presence of progesterone because proteolytic fragments were not generated in the absence of this steroid. Overexpression of SUG1 suppressed progesterone-mediated PXR transactivation on the CYP3A1 promoter in vitro [119, 136, 139]. It has been noted that the interaction with SUG1 may result from conformational changes of the receptor upon distinct ligand binding [119], such as progesterone, however more work will be required to elucidate the precise mechanism by which this occurs.

More recently, another group identified an E3 ubiquitin ligase as an hPXR-interacting protein. The ring-B-box-coiled protein interacting with protein kinase C-1 (RBCK1) was discovered by utilizing a yeast-two hybrid screening approach.
Overexpression of RBCK1 in AD-293 cells decreased PXR levels, which was inhibited by the proteasomal inhibitor MG-132. Most importantly, overexpression and silencing of RBCK1 resulted in inhibition and induction of endogenous PXR, respectively, in human primary hepatocytes. Silencing RBCK1 enhanced rifampicin-elicited induction of human PXR target genes [140].

Collectively, these results provide compelling evidence that PXR stability can be directly linked to conformational changes elicited by a specific ligand (e.g., progesterone), in part by disrupting the association between PXR and SUG1 [136, 139]. Furthermore, other groups have demonstrated that PXR is directly ubiquitinated by protein kinase A and RBCK1, both of which regulate PXR stability and ultimately, PXR transcriptional activity. However, the interaction between PXR and the ubiquitin-signaling pathway warrants further investigation [119].

**SUMOylation**

Another post-translation mechanism altering PXR protein-protein interactions and stability, ultimately fine-tuning gene transcription is SUMOylation. SUMOylation is characterized by the addition of a small ubiquitin-related modifier (SUMO) to its substrate. SUMOylation shares many common characteristics with ubiquitination [141, 142]. For instance, SUMO-specific enzymes attach SUMO proteins in an enzymatic cascade completely reliant on ATP and some SUMO proteins can serve as direct substrates for SUMOylation, thereby generating a protein that is polysumoylated [141, 142]. To date, four SUMO proteins (SUMO1, SUMO2/3, and SUMO4) have been identified in mammalian cells [141, 143, 144]. The attachment of a SUMO moiety to a target lysine residue on a specific substrate is governed by three enzymes: E1 (SUMO activating), E2 (SUMO conjugating), and E3 (SUMO ligases) [141]. SUMO modification to a specific substrate is reversible due to SUMO-specific proteases that cleave the isopeptide bond between the SUMO protein and its substrate, thereby releasing SUMO for further cycles [141].

The signaling consequences of SUMOylation are substrate-specific.
Interestingly, SUMOylation of a number of nuclear receptors have been linked to inflammation [145-147]. For example, ligand-dependent SUMOylation of PPARγ results in recruitment to inflammatory gene promoters where it inhibits transcription by suppressing clearance of corepressor complexes in mouse macrophages [147]. Moreover, ligand-dependent SUMOylation of LXR represses inflammatory gene expression in brain astrocytes [146]. Consistently, inflammatory signaling in hepatocytes increased SUMOylation of liganded hPXR [145]. The SUMOylated form of PXR represses NF-κB target gene expression but had little effect on CYP3A4 gene expression, indicating a promoter-specific response upon exposure to RIF [145]. Utilizing a bioinformatics approach, Hu and colleagues were the first to identify four potential sites of SUMOylation within hPXR; however, the contribution of each predicted lysine in global PXR SUMOylation was not investigated and warrants further investigation [145].

The same group recently confirmed their previous work demonstrating that TNFα and the hPXR ligand RIF increased both global SUMOylation and ubiquitination in primary murine hepatocytes [148]. Interestingly, however, they discovered that PXR SUMOylation driven by two different E3 ligases actually elicited different responses in PXR activity [148]. For example, the protein inhibitor of activated signal transducer and activator of transcription-1 (PIAS1), a SUMO-E3 ligase, increased SUMOylation, as well as PXR-mediated induction of the xenobiotic response [148]. In contrast, the protein inhibitor of activated STAT Y (PIASy), another SUMO-E3 ligase, increased SUMOylation and alleviated interaction of PXR with the coactivator peroxisome proliferator-activated receptor gamma coactivator-1-alpha (PGC-1α) [148]. Further, PIASy-mediated SUMOylation of PXR suppressed ubiquitin-mediated degradation by the 26S proteasome [148]. Their latest work identified a possible mechanism by which SUMOylation can alter the xenobiotic response versus the inflammatory response in hepatocytes; however, how the cell senses the environment to regulate these two processes by differential expression of different SUMO-E3 ligases and how this specifically alters the PXR response warrants further investigation.
More recently, an independent group confirmed the four predicted SUMOylation sites and identified a Negative charge amino acid Dependent SUMOylation Motif (NDSM) in hPXR [149]. Further, they demonstrated that PXR SUMOylation in the presence of ligand induces target gene expression and this was due to enhanced interaction between PXR and its coactivator, steroid receptor coactivator-1 (SRC-1) [149]. SUMOylation did not, however, alter PXRs ability to bind to the PXR response element within CYP3A4 [149]. They went on to demonstrate that site-directed mutagenesis of all four putative lysine residues along with the NDSM abolished SUMO-1 mediated transactivation [149]. Also, they identified a protein-protein interaction with an E2 conjugating enzyme known as UbCh9, and this interaction was slightly reduced with a NDSM mutant [149]. Collectively, these studies identified novel putative lysine residues involved in hPXR SUMOylation and this may contribute to ligand-elicited promoter-specific transsuppression and/or transactivation connecting inflammation to xenobiotic metabolism, respectively (For more information, see PXR in inflammation, section 1.5.4).

Acetylation

The last post-translational modifier that will be discussed in regards to PXR function is acetylation. As discussed in the sections of ubiquitination and SUMOylation, acetylation also targets the lysine side chain, which generates a great potential for crossregulation between the post-translational modifiers [150]. Lysine acetylation was first identified in histones and is now known to occur in more than 80 transcription factors and various cytoplasmic proteins [150, 151]. The acetylation pattern and impact vary from one protein to another, and the effects can be multifaceted for one protein [150, 151]. Two independent groups first described the physiological consequence of PXR acetylation and both will be discussed below.

Biswas and colleagues first demonstrated that endogenous PXR, as well as exogenous PXR, was acetylated in human cells [152]. Moreover, treatment with a PXR or RXR agonist drove deacetylation (and/or suppressed acetylation)
of PXR. Since the deacetylase sirtuin-1 (SIRT1) has been demonstrated to deacetylate other nuclear receptors [153-155], they investigated the potential involvement of SIRT1 on PXR acetylation [152]. Overexpressing SIRT1 had a modest effect on PXR deacetylation but the effect was much more pronounced with the combination of RXR/PXR ligands [152]. They did go on to determine that SIRT1 could interact with PXR both in the presence and absence of RXR/PXR ligands, suggesting a ligand-independent interaction [152]. Further, resveratrol treatment (a SIRT1 activator) in WT murine primary hepatocytes promoted lipogenesis and phenocopied the effect of PCN treatment (mouse-specific PXR agonist) and this effect was alleviated with treatment of a HDAC inhibitor (nicotinamide), suggesting deacetylation of PXR may promote lipogenesis independent of ligands [152]. Neither resveratrol nor nicotinamide are PXR agonists. The effect of resveratrol treatment in lipogenesis was alleviated in primary hepatocytes from PXR KO mice, suggestive of a PXR-dependent effect. This work provides evidence that HDACs play a role in PXR acetylation; however, SIRT1 seems to only act as a modest regulator thus more work is required to elucidate which specific HDACs are involved in PXR deacetylation. Moreover, it would be of interest to investigate possible crossregulation at specific lysine residues with SUMOylation and ubiquitination and assess the impact on PXR function. It is well known that PXR activation promotes lipogenesis; more studies are required to investigate the possible involvement of HDAC regulation on PXR activity in the context of lipogenesis.

An independent group examined the impact of energy sensing factors PGC-1α and SIRT1 on PXR expression and function. Results from a mammalian two hybrid assay indicated that SIRT1 interfered with the PXR and PGC-1α interaction. Moreover, SIRT1 interacted directly with PXR. SIRT1 may disrupt the PXR/PGC-1α interaction by two possible mechanisms that are not mutually exclusive: the well-known deacetylation of PGC-1α [156] or the deacetylation of PXR (summarized above). Collectively, both of these studies implicate acetylation as a post-translational mechanism that modulates PXR function. More work is required to elucidate the specific enzymes mediating
acetylation/deacetylation and the global effect this has on PXR activity.

1.5 The Role of PXR in Sterol Metabolism

The role of PXR in xenobiotic metabolism has been thoroughly investigated; however, its involvement in cardiometabolic diseases remains elusive. The primary focus of this dissertation is to investigate the role of PXR in cardiometabolic diseases, specifically hyperlipidemia. Accordingly, this section will provide a comprehensive review of the current literature linking PXR to lipid metabolism, atherosclerosis, diet-induced obesity, and inflammation.

1.5.1 PXR, Cholesterol Metabolism, and Atherogenesis

PXR has been implicated as a mediator of alterations in cholesterol homeostasis in mice and humans. It has been well documented that PXR transcriptionally regulates many genes involved in cholesterol catabolism, specifically in the bile acid pathway (see below). However, the role of PXR in sterol biosynthesis and secretion has been less defined. The first evidence of PXR regulating sterol metabolism was demonstrated by Bachmann and colleagues, who discovered that PXR agonist treatment in WT mice elicited increases in serum HDL-C and hepatic apoA-1 levels [157]. This effect was alleviated in PXR-deficient mice, indicating PXR is mediating this response [157].

In contrast, an independent group demonstrated that hPXR activation with rifampicin and lithocholic acid (LCA) treatment inhibited expression of ABCA1 and SR-B1 in human hepatic cells. This was further confirmed in rat primary hepatocytes treated with rPXR agonists PCN and LCA [158]. Although cellular cholesterol was unchanged with PXR agonists, cholesterol uptake or secretion into apoA1-containing lipoproteins was not investigated. The effect of PXR activation on ABCA1 and apoA1 gene expression and ultimately HDL-cholesterol levels warrants further investigation.

The role of PXR in response to bile acids was investigated in the context of altering lipoprotein metabolism. ApoA1 gene expression is negatively regulated by FXR [159, 160]; however, other nuclear receptors have been reported to positively regulate apoA1, such as HNF4α [160, 161]. Moreover,
several PXR ligands were reported to increase HDL cholesterol and ApoA1 gene expression; thus, Masson and colleagues set out to investigate the role of PXR in lipoprotein metabolism in response to dietary cholic acid (CA) [160]. The authors concluded that the expression of PXR antagonizes the CA-mediated downregulation of plasma HDL cholesterol and apoA1 gene expression [160]. The expression of a human PXR transgene in PXR-null mice completely reversed the deleterious effects of CA on plasma HDL [160]. Collectively, these data support previous claims that PXR activation increases hepatic apoA1, circulating apoA1, and HDL cholesterol in rats, mice, and humans [96, 157, 162-167].

Recently, a more thorough investigation of intestinal PXR activation on ABCA1 and HDL metabolism was published. Treatment with the human PXR ligand rifampicin significantly increased CYP27A1 expression in human intestinal Caco2 cells but not in human hepatic cells (both primary cells and the immortal HepG2 cells); however, rifampicin treatment did stimulate expression of CYP3A4 in both cell types, indicative of tissue-specific differences in gene expression [168]. This was further confirmed utilizing a CYP27A1 promoter luciferase construct overexpressed with hPXR. hPXR overexpression only stimulated CYP27A1 promoter activity in human intestinal Caco2 cells but not human hepatic HepG2 cells [168]. Further, 3-PXR binding sites were identified in the CYP27A1 promoter and gel-shift analysis revealed PXR was competent at binding to each element [168]. ChIP analysis revealed rifampicin treatment promoted SRC-1 coactivator association at each PXR-response element. It would have been interesting to utilize HepG2 nuclear extracts to repeat the ChIP experiments to determine if the recruitment of SRC-1 with rifampicin treatment is specific to human intestinal cells [168]. The authors went on to demonstrate that the direct-repeat-5 (DR-5) element approximately 1 kB upstream from the transcriptional start site is the most important by transactivation assays [168]. CYP27A1 catalyzes the oxidation of cholesterol to generate 27-hydroxycholesterol, a potent LXRα ligand [169-173]. The consequence of PXR-mediated CYP27A1 induction was elevated intracellular
27-hydroxycholesterol levels, which ultimately drove ABCA1 levels and increased cholesterol efflux from intestinal cells to ApoA1 and HDL, most likely through increased LXRα activity [168].

According to the work by Masson and colleagues, PXR antagonizes the CA-mediated decrease in plasma HDL levels. So Hoekstra and colleagues hypothesized that PXR activation may decrease atherosclerosis by promoting reverse cholesterol transport via enhancing HDL-cholesterol. They utilized both atherosclerosis mouse models (LDLR and ApoE-deficient mice) to assess the role of PXR activation on plasma lipid levels [174]. LDLR- knockout mice fed PCN for 3 days had a significant reduction in LDL-cholesterol levels, which was not attributed to decreases in VLDL-secretion [174]. The authors went on to demonstrate that lipolysis decreased with PCN treatment and this effect was primarily due to a decrease in hepatic lipase activity [174]. Hepatic lipase and its cofactor apolipoprotein A4 were both downregulated in the liver of PCN-treated mice [174]. Consistent with a decrease in lipolysis, VLDL triglycerides were increased with PCN treatment. The authors concluded that the change in the plasma lipoprotein profile was primarily due to impaired metabolism of VLDL [174]. Lastly, they examined the role of PXR in ApoE-deficient mice fed chow with and without PCN. In both hyperlipidemic models, hepatic steatosis was enhanced with PCN treatment [174]. These data support the notion that PXR activation promotes hepatic steatosis by a variety of mechanisms (Fig. 1.3); however, the effect on plasma lipid levels needs to be further investigated.

A recent study revealed that PXR transcriptionally regulates Lipin-1, an enzyme involved in triglyceride production [182]. Further, PXR-deficient mice exhibit impaired VLDL secretion compared to WT mice; however, VLDL secretion was not measured in mice treated with PXR agonists. Taken together, the current data suggest that PXR may play some role in altering VLDL secretion.

To further assess the role of PXR in lipid metabolism, Zhou and colleagues fed WT and PXR-deficient mice a low-cholesterol diet with and without PCN and examined the lipoprotein profiles [177]. After 2 weeks of
feeding, WT but not PXR KO mice fed PCN had significantly elevated proatherogenic VLDL and LDL cholesterol levels [177]. Moreover, PCN treatment for 8 weeks in ApoE-deficient mice accelerated atherosclerosis defined by neutral lipid staining in the brachiocephalic artery (+54%) and the aortic root (+116%) [177]. Plasma lipids were similar in ApoE-deficient mice fed control and PCN, however there was a slight reduction in HDL-cholesterol with PCN treatment [177]. These data suggest that the atherosclerosis phenotype is most likely independent of lipid levels in these ApoE-deficient mice. Accordingly, PXR activation increased CD36 expression and lipid accumulation in peritoneal macrophages [177]. This data implicates macrophage PXR as a modulator of atherosclerosis. Furthermore, this was the first study identifying PXR activation elicits proatherogenic hypercholesterolemia. The precise mechanisms regulating cholesterol metabolism is still unclear.

Consistent with the notion that PXR plays a role in macrophage function in atherosclerosis, our group demonstrated that PXR-deficiency on an ApoE-deficient background decreased atherosclerosis [178]. Our laboratory generated PXR and ApoE double knockout mice (PXR−/−ApoE−/−) and deficiency of PXR did not alter plasma triglycerides or cholesterol levels compared to ApoE−/− mice [178]. However, PXR−/−ApoE−/− mice had decreased atherosclerosis quantified by Oil-Red O staining in the aortic root (-40%) and brachiocephalic artery (-60%) [178]. Consistent with the previous study [177], deficiency of PXR reduced expression of CD36, lipid accumulation, and oxidized LDL uptake in peritoneal macrophages [178]. CD36 expression was also decreased in the atherosclerotic lesions of PXR−/−ApoE−/− mice [178]. Collectively, this data demonstrates that basal PXR is essential for foam cell formation and atherosclerosis.

More recently, our group demonstrated that chronic exposure to BPA enhanced atherosclerosis in PXR-humanized ApoE-deficient mice (huPXR•ApoE−/−) [86]. We previously reported that BPA and its analogs activate PXR [179]. Interestingly, we found that BPA is a potent agonist for human PXR but not for mouse or rat PXR [179]. Since BPA is a human PXR-specific ligand,
we set out to generate a model to study the effects of BPA-mediated PXR activation on atherosclerosis in mice. Thus, we utilized the previously characterized huPXR mice [109, 180], which express the human PXR gene in place of the mouse PXR gene, and crossed these mice with atherosclerosis-prone ApoE−/− mice to generate huPXR•ApoE−/− and PXR−/−ApoE−/− mice [86]. The huPXR•ApoE−/− and PXR−/−ApoE−/− mice have the same genetic background (mPXR and ApoE null alleles) except for one allele of huPXR•ApoE−/− carrying the human PXR transgene [86]. Chronic 12-week BPA treatment did not alter body weight or plasma lipid levels in either huPXR•ApoE−/− or PXR−/−ApoE−/− mice; however, BPA exposure significantly enhanced atherosclerotic lesion area in both the aortic root and the brachiocephalic artery from huPXR•ApoE−/− mice, but not from PXR−/−ApoE−/− mice [86]. Furthermore, BPA increased foam cell formation and CD36 expression in macrophages and atherosclerotic lesions of huPXR•ApoE−/−, but not in PXR−/−ApoE−/− mice [86]. These findings demonstrate that BPA exposure increases atherosclerosis development in an animal model expressing only human PXR.

Consistent with the notion that PXR agonists have been reported to alter cholesterol levels in humans, 13 SNPs were identified in PXR that correlated with aberrant LDL-cholesterol levels [181]. Interestingly, many of these SNPs were located in the 3’ UTR of PXR [181], indicating possible post-transcriptional modifications in PXR. Although the data did not demonstrate whether these SNPs correlated with elevated or decreased LDL-cholesterol levels, it would be worth investigating.

The main emphasis of the work in this dissertation was to identify new PXR ligands, as well as novel PXR transcriptional targets involved in cholesterol metabolism. We identified NPC1L1 as a direct PXR target gene in Chapter 3; however the in vivo physiological consequence of PXR activation on intestinal cholesterol absorption remains to be determined.

1.5.2 PXR in Cholesterol Catabolism – Emphasis on Bile Acids

Bile acids act as signaling molecules through a variety of mechanisms, including acting as ligands for the nuclear receptors FXR and PXR. For example,
utilizing a mammalian two-hybrid system, Xie and colleagues revealed that LCA, chenodeoxycholic acid, and deoxycholic acid are PXR agonists [183]. The major mouse metabolite of LCA, 3-keto-LCA, is a more potent PXR agonist than its parent [183, 184]. The hierarchy of potency is as follows: 3-keto-LCA>LCA>DCA=CA [88, 184, 185]. They confirmed that both these primary and secondary bile acids could activate both mouse and human PXR and induce downstream CYP3A4 expression [183]. LCA has been shown to cause cholestasis and associated hepatotoxicity [183]. When WT, PXR-null, or Alb-VPSXR transgenic animals were treated with control or LCA for 4 d, 58% of WT and 100% of PXR-null exhibited clear hepatic necrosis when treated with LCA [183]. In response to LCA treatment, compared with WT mice, PXR-null mice exhibited more hepatic inflammatory infiltration [183]. In comparison, the Alb-VPSXR transgenic mice showed no histological changes with LCA treatment [183]. These data demonstrate that constitutive activation of hepatic PXR protects against LCA-induced liver damage. To further investigate whether the induction of the CYP enzymes such as CYP3A provide a protective effect against LCA-induced hepatotoxicity, WT and PXR-null mice were pretreated with PCN prior to LCA treatment. As anticipated, the incidence of LCA-induced histological liver damage after PCN treatment was decreased approximately 60%; however, PXR-null mice remained sensitive to LCA after PCN treatment [183]. This is consistent with data demonstrating that transgenic overexpression of PXR and the sustained induction of CYP3A enhanced protection against xenobiotic compounds [108, 183]. Further, PXR-null mice exhibit loss of protection to a wide range of xenobiotics, not just LCA [183]. The authors went on to demonstrate that the CYP3A enzymes in human liver microsomes primarily catalyze LCA hydroxylation [183].

Is PXR simply a sensor that is activated by bile acids to induce expression of CYP enzymes to promote hydroxylation and excretion of bile-acid derivatives? Although that is a major function of PXR in cholesterol catabolism, independent groups have observed and identified a feedback mechanism, much like FXR, where PXR also inhibits bile-acid synthesis by suppressing the rate-limiting
enzyme, CYP7A1. Interestingly, rifampicin, a potent human PXR activator, has been used to treat cholestasis [186]. It was later identified that rifampicin is required for PXR and HNF4α binding, which blocked PGC-1α binding to HNF4α ultimately suppressing CYP7A1 gene transcription [187]. Thus, rifampicin inhibition of bile acid synthesis may be a protective mechanism against xenobiotic and bile-acid induced cholestasis [187].

Aside from transcriptionally repressing CYP7A1 to inhibit subsequent bile acid synthesis, PXR also transcriptionally activates genes involved in bile acid uptake, as well as bile acid elimination. For instance, rat PXR was identified to transcriptionally regulate the organic anion transporting polypeptide 2 (OATP2), a liver transporter that mediates uptake of a variety of compounds, including conjugated steroids, thyroid hormones, and bile salts [188]. PXR has also been demonstrated to transcriptionally activate the multi-drug resistance associated proteins 2 (MRP2) [189], MRP3 [190], and the bile salt export pump (BSEP) [191] facilitating bile acid elimination [192]. Interestingly, PXR itself is a direct transcriptional target of ligand-activated FXR [185, 193]. Taken together, PXR acts as a bile acid sensor that can mitigate the harmful effects of toxic bile acids by driving expression of genes involved in hepatic detoxification [185].

1.5.3 PXR and Energy Metabolism

Perturbations in lipid homeostasis are an underlying cause of many disease states, such as obesity and diabetes. Hepatic lipid homeostasis is tightly controlled by a variety of mechanisms including lipogenesis, β-oxidation, lipid uptake, and secretion. Many clinical observations led to the hypothesis that PXR may play a role in regulating lipid metabolism. Notably, patients treated with rifampicin develop hepatic steatosis [194], which is also observed in many animal models [114, 194-197]. This section will provide insight into the role of PXR in modulating lipid homeostasis, with an emphasis on fatty acid and triglyceride metabolism.

Recent work by independent groups reported that PXR activation causes hepatic steatosis characterized by accumulation of hepatic triglycerides [114, 194-197]. Mice expressing constitutively active hPXR in the liver [108] had
severe steatosis characterized by significant accumulation of triglycerides [114]. Further analysis revealed the lipogenic gene expression signature was vastly altered. Interestingly, however, expression of SREBP-1c, a major regulator of de novo hepatic lipid biosynthesis, was unaltered, as well as its major target genes fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) [114]. These results suggest that the effect of PXR on hepatic steatosis was SREBP-independent. Further, CD36 was induced in a PXR-dependent manner [114]. Mice lacking CD36 exhibit increased plasma free fatty acids and triglyceride levels, indicative of its role in fatty acid uptake [198]. PXR activation resulted in a decrease in plasma free fatty acids [114]; however, it is still unclear whether this is a CD36-dependent mechanism. The authors went on to identify a functional PXR DR3-type response element within the CD36 promoter. These results confirm that CD36 is indeed a PXR-target gene and may partially contribute to the hepatic steatosis phenotype observed in mice expressing constitutively active PXR [114].

PXR activation was also associated with upregulation of many genes involved in lipogenesis, such as PPARγ, stearyl CoA desaturase 1 (SCD1), and fatty acid elongase (FAE) [114, 195]. It was later confirmed that PPARγ is a PXR target gene [199, 200]. Interestingly, PPARγ is also a positive regulator of CD36 [201], indicating PXR activation may drive CD36 expression directly or through crosstalk with PPARγ (Fig. 1.3). The consequence of PXR transcriptionally regulating PPARγ in the context of hepatic steatosis remains to be determined; however, two reports have demonstrated that overexpression of PPARγ in mice induced hepatic steatosis [194, 202, 203]. The marked steatosis associated with increased levels of lipogenesis-related genes, including CD36 and SCD1, as well as others [202, 203].

Aside from PPARγ, other genes involved in lipogenesis were discovered as direct PXR targets. The thyroid hormone-responsive SPOT14 homolog (S14) plays a critical role in inducing lipogenic enzyme gene expression, notably FAS and ATP citrate lyase (ACLY) [194]. It was later identified that S14 is a PXR target gene in human hepatocytes and mouse liver [204]. S14
overexpression in human HepaRG cells provoked increases in fatty acid accumulation and lipogenesis [204]. It was previously reported that PXR activation did not alter mRNA levels of FAS and ACLY [114]. Thus, more work is required to completely understand the complex mechanisms associated with PXR and its target genes in the context of hepatic steatosis.

Several observations made by independent groups suggest that PXR activation suppresses expression of genes involved in β-oxidation. The constitutively active hPXR mice displayed a significant reduction in mRNA levels of several genes involved in fatty acid β-oxidation, such as PPARα and thiolase [114, 199]. Furthermore, an independent group revealed murine PXR activation down-regulates the mRNA level of mitochondrial carnitine palmitoyltransferase 1 (Cpt1) and mitochondrial 3-hydroxy-3-methyl-glutarate CoA synthase 2 (Hmgcs2) [195]. Cpt1α and Hmgcs2 are the rate-limiting enzymes of β-oxidation and ketogenesis, respectively. CPT1α expression is tightly controlled by a variety of transcription factors, such as the insulin-responsive forkhead factor A2 (FoxA2). FoxA2 has been shown to positively regulate genes controlling β-oxidation [205]. Interestingly, it was demonstrated that PXR physically interacts with FoxA2 through their ligand and DNA binding domains, respectively [194, 195, 199]. This interaction prevents the binding of FoxA2 to its DNA response elements and represses its activation of Cpt1α and Hmgcs2 gene promoters [194, 195, 199]. Consistent with the hypothesis that PXR affects both fatty acid β-oxidation and ketogenesis, PXR-deficient mice display elevated levels of oxygen consumption, liver mitochondrial β-oxidation, and are completely protected against HFD-induced obesity [182]. In contrast, transgenic expression of PXR suppressed hepatic β-oxidation and exacerbated hepatic steatosis [182]. Moreover, PXR activation decreased ketone body (3-hydroxybutylate) levels in mice [182]. Collectively, PXR regulates hepatic lipid metabolism by driving expression of genes controlling hepatic lipid uptake and formation, while suppressing fatty acid catabolism by turning off genes involved in β-oxidation and ketogenesis (Fig. 1.3).

Hepatic gluconeogenesis is essential for survival during fasting or
starving because glucose is necessary as a fuel source in central and peripheral tissues. Pyruvate is a precursor for gluconeogenesis, which undergoes several enzymatic steps to ultimately generate glucose. Hepatic gluconeogenesis is positively regulated by glucocorticoids, cAMP, and glucagon, and negatively regulated by insulin and glucose [194]. Major enzymes involved in this process include notably phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [194]. These genes are significantly repressed in mice expressing a constitutively active form of PXR [114]. Consistently, murine PXR activation with PCN decreased fasting blood glucose levels in WT but not PXR-deficient mice [195]. Other groups have demonstrated that PXR can bind to transcription factors and inhibit their ability to drive expression of target genes involved in gluconeogenesis. For example, PXR represses glucagon-activated transcription of both G6Pase and PEPCK by directly binding to the cAMP-response element binding protein (CREB) and inhibiting its DNA-binding ability [206]. Moreover, ligand-activated PXR can bind to the forkhead box protein O1 (FoxO1), another positive regulator of gluconeogenesis, subsequently suppressing FoxO1-mediated activation of gluconeogenic gene expression [207]. However, this mechanism does seem to be gene-specific because FoxO1 acts as a positive regulator involved in PXR transactivation of its xenobiotic target genes [207].

Although reports have demonstrated that PXR activation suppresses gluconeogenesis in mice, studies in humans have suggested that PXR agonists increase blood glucose levels [208-213]. For example, rifampicin treatment increases blood glucose levels during oral glucose tolerance tests in tuberculosis patients and healthy subjects [210, 213]. Statins, which are weak PXR agonists, increase fasting blood glucose levels in patients with and without diabetes [209, 211, 212]. Recent studies by Gotoh and Negishi have elucidated the mechanism responsible for the species-specific differences in PXR-regulated gluconeogenesis [208, 209]. They discovered that rifampicin treatment increased G6Pase and PEPCK mRNA levels in HepG2 cells stably expressing human PXR [208]. Further, they identified the serum- and
glucocorticoid-regulated kinsase 2 (SGK2) as a direct PXR target gene that is essential for PXR-induced gluconeogenic gene expression and increased glucose production [208]. This pathway is insulin-independent, as siRNA targeting FoxO1 did not affect rifampicin induction of G6Pase [208]. In human primary hepatocytes, PXR induced G6Pase in the presence of high levels of SGK2, whereas PXR repressed G6Pase in its absence [208]. These results suggest that differential expression of SGK2 may be responsible for the species-specific differences in PXR-mediated regulation of gluconeogenesis.

Gotoh and Negishi went on to demonstrate that PXR activation with statins increases binding between PXR, SGK2, and the protein phosphatase 2C (PP2C). This increase in binding stimulates PP2C dephosphorylation of SGK2 at Thr193 [209]. Nonphosphorylated SGK2 co-activates PXR transcription of gluconeogenic genes in human liver cells, thereby enhancing gluconeogenesis [209]. Interestingly, statin treatment increased SGK2 phosphorylation and decreased PEPCK1 levels in mice [209]. Thus, elevated SGK2 phosphorylation might be the underlying basis for the attenuated hepatic gluconeogenesis in mice after statin treatment [209].

Taken together, these results clearly demonstrate that PXR has a complex role in energy metabolism by promoting lipogenesis and suppressing fatty acid β-oxidation and ketogenesis (Fig. 1.3). Murine and human PXR, however, have opposing effects on gluconeogenesis possibly due to alterations in SGK2 phosphorylation and its ability to act as a PXR coactivator.

1.5.4 PXR and Inflammation

Inflammation is a biological response that recruits immune cells and tissue repair processes to sites of infection and injury [214]. Nuclear receptor ligands have been used extensively to treat inflammatory diseases [214]. Work pioneered by Hench and colleagues identified the use of cortisone for the treatment of rheumatoid arthritis [215]. It was later identified that cortisone is a ligand for the glucocorticoid receptor (GR) [214]. Constitutive activation of LXRα, for example, has been shown to suppress LPS-induced inflammatory gene expression in macrophages [216]. Moreover, ligand-activated LXR also
inhibits the induction of inflammatory genes [217-219]. The GR, as well as the
PPARs and LXRs, have been shown to suppress inflammation by a
transrepression mechanism, where the nuclear receptor tethers to the
transcription factors nuclear factor-kappa B (NF-κB) or activator protein-1,
preventing coactivator association necessary for gene activation [214]. Aside
from directly interacting with pro-inflammatory transcription factors, nuclear
receptors may also modulate inflammation by functions of their target genes.
For example, LXR activation inhibits signaling from the toll-like receptors (TLRs)
through ABCA1-dependent changes in membrane lipid composition, which
disrupt the recruitment of adaptor proteins involved in the inflammatory
response [220]. The involvement of nuclear receptors and their target genes in
inflammation is complex and warrants further investigation. Herein, I will
describe some mechanisms by which PXR undergoes reciprocal crosstalk with
NF-κB signaling.

Rifampicin, a macrocyclic antibiotic and potent PXR ligand, was first used
as an antituberculosis agent and now is used for a wide variety of bacterial and
fungal diseases [221, 222]. Aside from rifampicin, other PXR ligands have been
shown to exert immunosuppressive side-effects [222, 223]. For this reason,
PXR has recently been shown to be an attractive therapeutic target for the
treatment of inflammatory bowel disease (IBD) [224, 225]. IBD is a chronic
inflammatory disease of the gastrointestinal tract. IBD primarily includes
Crohn’s disease (CD) and ulcerative colitis (UC) [224, 225]. UC is an
inflammatory disease that affects the lining of the intestine, whereas CD can
affect any part of the digestive tract [224, 225]. While the etiology of IBD
remains to be determined, it is thought to involve increased intestinal
permeability and dysregulation of the mucosal immune system [226]. Both UC
and CD result in abdominal pain, diarrhea, rectal bleeding, and malnutrition
[224, 225]. Patients with either UC or CD exhibit a much higher incidence of
colon cancer compared to the general population [225].

The role of PXR in IBD has recently been investigated experimentally in
mouse models. Dextran sulfate sodium (DSS) is used experimentally to induce
IBD in animal models. WT mice treated with the mouse PXR ligand PCN were protected from DSS-induced IBD compared with vehicle control-treated mice [226]. However, this phenotype was not observed in PXR-null mice [226]. PCN treatment in WT mice did not enhance intestinal barrier function, but did significantly decrease NF-κB target gene expression in a PXR-dependent manner [226]. A similar phenotype was observed in human colon epithelial cells [227], as well as in PXR-humanized mice with treatment of rifaximin [228], a FDA-approved intestine-specific human PXR agonist used for the treatment of traveler’s diarrhea [224]. It was then confirmed that PXR activation inhibits LPS-induced NF-κB DNA-binding activity [227]. Interestingly, treatment with PXR ligands increases the physical association between PXR and the NF-κB subunit p65 [227]. These results demonstrate that the suppressive effect of PXR may be due to the physical association between PXR and p65, thereby decreasing the overall promoter occupancy of NF-κB on its target genes [227].

Independent groups have reached a similar conclusion. It was further confirmed that PXR agonists inhibit the expression of multiple NF-κB target genes in human liver samples [222]. PXR inhibited NF-κB activity in the absence of exogenous ligand and treatment with RIF further enhanced this repression mediated by PXR [222]. Consistently, PXR activation in mice inhibits NF-κB signaling in vivo and NF-κB target gene expression is upregulated in PXR-deficient mice, which associated with enhanced small bowel inflammation [222].

Targeting PXR for inflammatory diseases display some therapeutic promise. The underlying mechanism how PXR disrupts NF-κB signaling and ultimately inflammation needs to be dissected further. It should be noted that activation of NF-κB has also been shown to suppress PXR target gene expression [91], therefore implicating reciprocal regulation between PXR/NF-κB signaling. Since others groups demonstrated that p65 and RXR physically interact [229], the binding between p65 and RXR may interfere with the formation of the p65-p50 complex, as well as the PXR/RXR complex upon an inflammatory stimulus; however, this needs to be investigated further [224, 229].
1.5.5 IKKβ and NF-κB Signaling – an Overview

Pattern recognition receptors (PRRs) are essential to sense and respond to stimuli for early detection of invading pathogens and initiating an immune response. PRRs are activated by a host of pathogen-associated molecular patterns which are present in microbes or nucleic acids of viruses or bacteria. Chronic activation of these receptors, such as the Toll-like receptors (TLRs), has been linked to a variety of disease states, including: cancer, obesity, type-2 diabetes, and atherosclerosis. There are 10 recognized TLR genes in humans and 12 in mice [230]. TLRs are activated by a variety of pathogen-associated molecules and endogenous signals, such as proatherogenic forms of LDL [231], saturated fatty acids [232, 233], and cleavage products of amyloid precursor protein [234], which can activate TLR4 and/or TLR2 [214]. After recognition of their ligands, TLRs recruit a set of adaptor proteins, such as MyD88, which result in triggering downstream signaling cascades that culminates in the activation of the transcription factor NF-κB [235].

NF-κB belongs to the Rel-homology domain-containing protein family, which includes p65/RelA, p50/NF-κB1, p52/NF-κB2, Relb, and c-Rel [236, 237]. The prototypical NF-κB is thought to be a heterodimer consisting of the p65 and p50 subunits [236, 237]. In unstimulated cells, the heterodimer is sequestered in the cytoplasm by interacting with the inhibitor of NF-κB (IκB) proteins. Upon stimulation of TLR signaling, IκBs are phosphorylated by the IKK complex consisting of the IKKα and IKKβ protein kinases and a regulator subunit, IKKγ/Nemo. IKKβ is the predominant catalytic subunit involved in NF-κB activation [238, 239]. The phosphorylation targets IκBs for ubiquitination and degradation, allowing NF-κB to translocate to the nucleus to modulate gene transcription [237].

NF-κB signaling has been extensively studied in a variety of cell-types and disease states. The work provided within Chapter 4 of this dissertation investigates the role of IKKβ in adipose stem cell differentiation in HFD-induced obesity. The next section will provide an overview of some recent advances made in the field of inflammation and obesity, with an emphasis on IKKβ and
The Role of IKKβ Signaling in Diet-Induced Obesity and Insulin Resistance

Diet-induced obesity and associated insulin resistance is becoming increasingly prevalent, with approximately 35% of U.S. adults being obese [240]. Thus, there is an urgent need to identify therapeutic targets to treat obesity and its related conditions, including heart disease, stroke, type 2 diabetes, and cancer.

High doses of salicylates (4-10 g/day), which include sodium salicylate and aspirin, have been used to treat inflammatory conditions such as rheumatoid arthritis, but they have also been demonstrated to lower blood glucose levels [241]. It was later demonstrated that salicylates can inhibit IKKβ [242], as opposed to working through the cyclooxygenases [241]. Treatment of genetically engineered obese animal models with high doses of salicylates reversed hyperglycemia, hyperinsulinemia, and dyslipidemia by sensitizing insulin action [241]. Further, heterozygote deletion of IKKβ protected mice against insulin resistance associated with HFD-feeding [241]. It should be noted that mice with homozygous deletion of IKKβ die in utero due to enhanced liver apoptosis [243]. To further understand the tissue-specific role of IKKβ in obesity-induced insulin resistance, Arkan and colleagues generated hepatocyte and myeloid-specific IKKβ knockout mice and assessed the contribution of each tissue in insulin tolerance and glucose disposal [244]. Interestingly, hepatocyte-specific KO mice retained insulin sensitivity in the liver, but developed insulin resistance in muscle and fat [244]. Myeloid-specific KO mice, however, retained global insulin sensitivity and were protected from insulin resistance [244]. Acute LPS injection induced acute inflammation and hyperglycemia, however both hepatocyte and myeloid-specific KO mice were more glucose tolerant. Conclusions from this data suggest that IKKβ activation is deleterious to insulin signaling and inhibition in specific cell types may be beneficial against type-2 diabetes [242].

A follow-up study by an independent group tested the hypothesis that acutely elevated FFA (as opposed to chronically elevated FFA as seen above)
would stimulate hepatic insulin resistance, which would be completely reversed by sodium salicylate [245]. Seven hour infusion of intralipid suppressed insulin-induced stimulation of peripheral glucose utilization and endogenous glucose production; however, salicylate co-infusion completely prevented this phenotype [245]. The authors demonstrated that salicylate treatment reversed lipid-induced phosphorylation of insulin-receptor substrate (IRS) and protein kinase B (Akt) [245]. Years later, salicylates were also identified to activate adenosine monophosphate-activated protein kinase (AMPK) [246]. Aspirin also activates AMPK, but at high doses, so the results seen in both of these studies, as well as humans, may partially be due to AMPK activation [246].

IKKβ and NF-κB activity are enhanced in response to excess FFA in a variety of tissues, including liver, adipose tissue, and the hypothalamus [241, 244, 247-249]. Many other inducers of insulin resistance activate IKKβ, including sphingomyelinase and ceramide [250]. IKKβ has been demonstrated to phosphorylate IRS-1 promoting its degradation and thereby contributing to insulin resistance [250]. Further, mutations at these specific sites enable the mutated IRS proteins to better propagate insulin signaling [250]. Thus, IKKβ may contribute to insulin resistance by directly suppressing the insulin signaling pathway.

We and others have recently published reports investigating the role of IKKβ in adipocyte hyperplasia. Ligands that activate the IKKβ signaling pathway, such as FFA and LPS, stimulate adipocyte hyperplasia from surrounding progenitor cells [251-254]. Likewise, we demonstrated that deletion of IKKβ in smooth-muscle cells impaired adipocyte differentiation [255]. These mice had elevated energy expenditure, decreased inflammation, and were completely protected against HFD-induced obesity [255]. The main focus of the work within Chapter 4 of this dissertation was to identify the mechanism of HFD-elicited adipocyte hyperplasia by investigating the role of IKKβ specifically in the adipose progenitor population in vivo.
1.6 Scope of Dissertation

1.6.1 The Role of PXR in Xenobiotic-Induced Dyslipidemia

The general hypothesis of the research herein is that xenobiotic-elicited PXR activation promotes cardiovascular disease by modulating plasma lipid levels. We also hypothesized that intestinal PXR contributes to xenobiotic-elicited hyperlipidemia by enhancing cholesterol absorption and chylomicron secretion. To test these hypotheses, the following specific aims were proposed:

Specific Aim 1: To test the hypothesis that specific xenobiotics, such as HIV drugs and phthalates/phthalate substitutes, are PXR-specific agonists and that acute exposure to these xenobiotics induces hyperlipidemia in a PXR-dependent manner.

Specific Aim 2: To test the hypothesis that intestinal PXR is necessary for xenobiotic-induced hyperlipidemia through transcriptional regulation of NPC1L1 and MTP, which facilitates cholesterol absorption and chylomicron secretion, respectively.

1.6.2 Targeting IKKβ in Adipocyte Lineage Cells for Treatment of Obesity and Metabolic Dysfunctions

The general hypothesis of the research herein is that IKKβ signaling in adipose progenitor cells promotes adipocyte hyperplasia thereby contributing to HFD-induced obesity and insulin resistance. To test this hypothesis, the following specific aims were proposed:

Specific Aim 1: To test the hypothesis that IKKβ is essential for adipose stem cell differentiation in vitro and targeting adipose progenitor IKKβ in vivo will alleviate diet-induced obesity, adipose tissue inflammation, and insulin resistance.

Specific Aim 2: To test the hypothesis that pharmacological inhibition of IKKβ
with antisense oligonucleotides (ASOs) will protect mice from diet-induced obesity and associated metabolic disorders.
Figure 1.1 Schematic illustration of lipoprotein metabolism.
This figure depicting lipoprotein metabolism was reprinted by permission from Macmillan Publishers Ltd: Nature Reviews: Genetics [256], 2009. This illustration coincides with Chapter 1.1 (Cholesterol Metabolism- an Overview). Abbreviations: cholesterol (C), triglyceride (TG), fatty acid (FA), Niemann-Pick C1-like 1 (NPC1L1), heterodimeric ATP-binding cassette transporter G5/G8 (ABCG5/G8), microsomal triglyceride transfer protein (MTTP), chylomicron (CM), apolipoprotein B (APOB) isoform 48 and 100 (B48/B100), apolipoprotein A5 (A5), apolipoprotein C2 and C3 (C2/C3), chylomicron remnant (CMR), low-density lipoprotein (LDL), low-density lipoprotein receptor (LDLR), LDLR-related protein-1 (LRP1), very low-density lipoprotein (VLDL), VLDL remnant (IDL), hepatic lipase (HL), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), adaptor protein (AP), proprotein convertase subtilisin/kexin type 9 (PCSK9), apolipoprotein A1 (A1), ATP-binding cassette A1 and G1 (ABCA1/ABCG1), lecithin cholesterol acyltransferase (LCAT), cholesterol ester transfer protein (CETP), endothelial lipase (LIPG), and scavenger receptor class B type 1 (SRB1).
Cartoon depiction of human and mouse PXR variants, as previously illustrated [89, 98]. In humans, variant 1 (V1) corresponds to PXR wild type. Variant 2 differs from V1 by the use of alternative translational start codons. Variant 3 differs from V1 at the N-terminus, resulting in an open reading frame 39 amino acids longer (denoted in red). Variant 4 contains an in-frame deletion of 111 nucleotides (denoted in grey). In mice, V1 is analogous to human V1. Mouse V2 contains an in-frame deletion of 123 nucleotides in a similar region of the LBD and is analogous to human V4.
Figure 1: Schematic representation of the role of PXR in energy metabolism, xenobiotic metabolism, sterol metabolism, and inflammation.

A. FA uptake and lipogenesis
- Fatty acid uptake
- Lipogenesis
- Lipin1
- Spot14
- SCD-1
- MTP?
- Hepatic steatosis and VLDL secretion?

B. Inflammation
- NF-κB
- Inflammation

C. Xenobiotic metabolism
- Phase 1 enzymes: CYP3A, CYP2B, CYP2C
- Phase 2 enzymes: UGTs, SULTs, GSTs
- Transporters: MRP2, OATP2, MDR1

D. β-oxidation
- Fatty acid β-oxidation and ketogenesis
- PPARα
- Cpt1α
- Hmgcs2

E. Gluconeogenesis
- SGK (Thr193)
- PEPCK GTPase
- Gluconeogenesis (mice)
- PEPCK GDPase
- Gluconeogenesis (humans)

F. Sterol metabolism
- ABCA1
- NPC1L1
- CYP27A1
- CYP7A1
- Plasma cholesterol
Figure 1.3 Schematic representation of the role of PXR in energy metabolism, xenobiotic metabolism, sterol metabolism, and inflammation.

The information within this cartoon was compiled from previous review articles [194, 199]. (A) PXR activation induces fatty acid uptake, lipogenesis, and may promote VLDL secretion through induction of CD36, PPARγ, Lipin1, Spot14, SCD-1, and possibly MTP. PXR may directly induce CD36 expression or indirectly through PPARγ. (B) PXR inhibits NF-κB-mediated inflammation. It should be noted that mutual repression exists between these two pathways. (C) Activation of PXR results in induction of Phase 1 and Phase II drug-metabolizing enzymes and drug transporters. (D) PXR inhibits fatty acid β-oxidation and ketogenesis through its suppression of PPARα gene expression and by disrupting FoxA2-mediated activation of Cpt1α and Hmgcs2. (E) In mice, PXR inhibits PEPCK and G6Pase gene expression by interacting with the positive regulators of gluconeogenesis, CREB and FoxO1, and disrupting their ability to activate these genes in mice. In addition, mouse SGK2 remains phosphorylated at Thr193, thus it can no longer act as a PXR coactivator on gluconeogenic promoters. In humans, ligand-activated PXR promotes PP2C dephosphorylation of SGK2 at Thr193 leading to SGK2s ability to act as a coactivator for PXR on gluconeogenic promoters, thus inducing gluconeogenesis. (F) PXR activation promotes hypercholesterolemia through regulation of NPC1L1 and CYP27A1, while suppressing CYP7A1, the rate-limiting enzyme involved in bile acid synthesis. It has also been proposed that PXR may regulate ABCA1 gene expression, but these studies are currently controversial. The focus of this dissertation is to identify novel PXR target genes involved in lipid metabolism. Regulation of NPC1L1 and MTP have been described herein, thus they have larger font size in the current figure.
Chapter 2: HIV Protease Inhibitors Activate PXR and Induce Hyperlipidemia in WT Mice

2.1 Introduction

The introduction of highly active anti-retroviral therapy (HAART) has led to a marked increase in survival for people infected with human immunodeficiency virus (HIV) [257]. However, HAART has also been associated with dyslipidemia and an increased risk of cardiovascular disease [52, 53, 55, 58, 63, 64, 66]. For example, findings from the Data Collection on Adverse Events of Anti-HIV Drugs (DAD) study suggested that the incidence of cardiovascular disease increased with longer exposure to HAART [63]. Another large-scale study including more than 23,000 HIV patients analyzed the combination of drugs used in HAART and concluded that protease inhibitors (PIs) are associated with an increased risk of cardiovascular disease, which is partly explained by dyslipidemia [55]. A Swiss HIV Cohort Study found that PIs are the cause of dyslipidemia in patients [58], and several other studies have reached the same conclusion [64, 66]. Despite the strong evidence linking PIs with dyslipidemia and cardiovascular disease, the underlying mechanisms responsible for the adverse effects of PIs remain elusive.

Several PIs such as ritonavir have been implicated to activate the pregnane X receptor (PXR, also known as steroid and xenobiotic receptor) [91, 258]. PXR is a nuclear receptor activated by numerous endogenous hormones, dietary steroids, pharmaceutical agents, and xenobiotic chemicals [89, 91, 95]. PXR functions as a xenobiotic sensor that induces expression of genes required for xenobiotic metabolism in the liver and intestine, including cytochrome P450s,
conjugating enzymes (e.g., glutathione transferase), and ABC family transporters (e.g., multidrug resistance 1 [MDR1]) [90, 91]. In the past decade, the role of PXR as a xenobiotic sensor has been well established [91].

Recent evidence indicates that PXR may also play an important role in the regulation of lipid homeostasis [114, 177, 259, 260]. It is well known that many clinically relevant PXR ligands can elevate plasma lipid levels in patients and may increase their risk of cardiovascular disease [177, 261-264]. A recent meta-analysis of seven genome-wide association studies indicated that common genetic variants in PXR can affect plasma low-density lipoprotein (LDL) cholesterol levels in humans [118]. Modulation of PXR activity has been found to alter lipid homeostasis and affect plasma lipid levels in several animal models. We recently reported that chronic activation of PXR led to increased levels of plasma total cholesterol and atherogenic LDL and very-low-density lipoprotein (VLDL) in wild-type (WT) mice, but not in PXR-deficient (PXR−/−) mice [177]. Activation of PXR also increased plasma total cholesterol and VLDL levels in apolipoprotein E (ApoE)*3-Leiden mice which exhibit a human-like lipoprotein distribution on a cholesterol-rich diet [260]. Another report showed that acute activation of PXR increased plasma triglyceride levels in both LDL receptor-deficient (LDLR−/−) and ApoE- deficient (ApoE−/−) mice but decreased LDL cholesterol levels in LDLR−/− mice [174]. Activation of PXR can regulate many genes involved in lipid homeostasis including CD36, Stearoyl-CoA desaturase-1, 7-dehydrocholesterol reductase, S14, and lipin-1 in the liver, intestine, or macrophages of several animal models [91, 114, 174, 182, 204, 260, 265]. These studies indicate that PXR can mediate cholesterol and lipid homeostasis at multiple levels.

We report that amprenavir, a widely used HIV PI, is a potent PXR-selective agonist. Computational docking studies together with site-directed mutagenesis identified several key residues within PXR’s ligand-binding pocket that constitute points of interaction with amprenavir. Amprenavir efficiently activated PXR and induced PXR target gene expression in vitro and in vivo. Short-term exposure to amprenavir significantly increased plasma total
cholesterol and atherogenic lipoprotein LDL cholesterol levels in WT mice, but not in PXR$^{-/-}$ mice. Amprenavir-mediated PXR activation significantly regulated several key intestinal genes involved in lipid homeostasis. These findings provide critical mechanistic insight for understanding the impact of PIs on cardiovascular disease and demonstrate a potential role of PXR in mediating the adverse effects of PIs in humans.

2.2 Materials and Methods

2.2.1 Reagents and Plasmids
Pregnenolone 16α-carbonitrile (PCN) and rifampicin (RIF) were purchased from Sigma-Aldrich (St. Louis, MO). Amprenavir, ritonavir, nelfinavir, saquinavir, and indinavir sulfate were obtained from NIH AIDS Research and Reference Reagent Program. All chemicals were dissolved in dimethyl sulfoxide (DMSO). Human (h) and mouse (m) PXR expression vectors; GAL4 DNA-binding domain (DBD)-linked nuclear receptor ligand-binding domain (LBD) vectors (GAL4-hPXR, GAL4-mPXR, GAL4-rPXR, GAL4-RARα, GAL4-RXR, GAL4-FXR, GAL4-LXR, GAL4-PPARα, GAL4-PPARγ and GAL4-VDR); and CMX-β-gal expression vectors have been described before [179, 222, 266]. VP16-PXR, GAL4-nuclear receptor corepressor (NCoR), GAL4-silencing mediator of retinoid and thyroid hormone (SMRT), GAL4-steroid receptor coactivator (SRC1), GAL4-PPAR binding protein (PBP), PXR-dependent CYP3A4 promoter reporter (CYP3A4XREM-Luciferase); CYP3A2 promoter reporter [(CYP3A2)$_3$-luciferase]; and GAL4 reporter (MH100-Luciferase) have also been described previously elsewhere [179, 222, 266, 267].

2.2.2 Cell Culture and Transfections
The human hepatic cell line HepG2 and intestine epithelial cell line LS180 were obtained from the American Type Culture Collection (Manassas, VA). The human hepatoma HepaRG cells were purchased from Life Technologies (Carlsbad, CA). Transfection assays were performed as described previously [179, 266]. The cells were transfected with various expression plasmids or hPXR mutants as well as the corresponding luciferase reporter plasmids,
together with cytomegalovirus X-β-galactosidase control plasmids using FuGENE6 (Roche Diagnostics, Indianapolis, IN). The cells were then incubated with the corresponding ligands as indicated in the figure legends for 24 h, and β-gal and luciferase assays were performed as described elsewhere [179, 266]. Fold activation was calculated relative to solvent controls.

Each data point represents the average of triplicate experiments ± SD and was replicated in 3-5 independent experiments. EC50 values were calculated by curve fitting of data, using Prism software (GraphPad Software, San Diego, CA). For the mammalian two-hybrid assays, HepG2 cells were transfected with GAL4 reporter, VP16-hPXR, and GAL-SRC1, GAL-PBP, GAL-NCoR, and GAL-SMRT [179, 266]. The cells were then treated with compounds at the indicated concentrations.

2.2.3 Computational Docking Studies
Preparation of the protein structure for subsequent ligand-receptor docking studies was conducted as previously described [179]. Briefly, the structural coordinates of the tethered hPXR linker PXR/SRC-1 were retrieved from the RSCB Protein Data Bank entry 3HVL [268]. The larger PXR fragment of chain A, Gly142-Glu458, was extracted for molecular modeling using MOE 2010 software (Chemical Computing Group, Montreal, Quebec, Canada), and for ligand-receptor docking studies using GOLD software (version 5.0) [269]. Water molecules, salt ions, ligand [SR12813, tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate], and co-receptor fragments were deleted. After the addition of hydrogen atoms and assigning of the AMBER99 force-field charges to the protein, the hydrogen atomic positions were allowed to relax [268]. The resulting protein structural coordinates were saved in Tripos mol2 format and used later for GOLD docking.

The ligands were docked to the 3HVL [268] chain A using semiflexible docking whereby the ligand has full conformational flexibility and the hydroxyl groups of designated protein side chains in the binding pocket can rotate to optimize hydrogen bond contacts. Each ligand was docked 50 independent times. The binding pocket was defined as all atoms within an 8-Å radius around
the bound ligand, SR12813.

2.2.4 Site-Directed Mutagenesis
The hPXR full-length plasmid was used as a wild-type template to generate a series of mutant plasmids using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer-supplied protocol as described before [179]. The primers used for mutant generation are listed in Table 2.1.

2.2.5 RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction Analysis
Total RNA was isolated from mouse tissues, intestinal LS180, and HepaRG cells using TRIzol Reagent (Life Technologies) per the manufacturer-supplied protocol. Quantitative real-time polymerase chain reaction (QPCR) was performed using gene-specific primers and the SYBR green PCR kit (Life Technologies) as described previously [179, 266]. The primer sets used in this study are listed in Table 2.2.

2.2.6 Animals
C57BL/6 WT mice were purchased from The Jackson Laboratory (Bar Harbor, ME). PXR- deficient mice (PXR-/-) on C57BL/6 background were employed as described previously elsewhere [177]. All the animals were housed in a specific pathogen-free environment with a light-dark cycle maintained by the Division of Laboratory Animal Resources, University of Kentucky, and the protocol was approved by the Institutional Animal Care and Use Committee. We fed 8-week-old male WT or PXR-/- mice a semisynthetic low-fat AIN76 diet containing 0.02% cholesterol (Research Diet; New Brunswick, NJ) [177, 178, 270] and treated them by oral gavage with vehicle (corn oil) or 10 mg/kg body weight amprenavir daily for 7 days. On the day of euthanization, mice were anesthetized by intraperitoneal injection with Ketamine (Fort Dodge Animal Health, Fort Dodge, Iowa). The mice were exsanguinated by left ventricular puncture, and the blood was collected into EDTA-containing syringes. Plasma was prepared by spinning at 16,000 g for 10 min. The circulation was flushed with phosphate-buffered saline, and the intestinal and liver tissues were collected and stored in RNAlater solution (Life Technologies).
2.2.7 Plasma Analysis

Plasma total cholesterol and triglyceride concentrations were determined enzymatically by colorimetric methods as described previously (Roche Diagnostics) [177, 271]. Plasma from multiple mice (n=4-7) was pooled, and the plasma lipoprotein cholesterol distributions were determined by fast-performance liquid chromatography (FPLC) [178]. The lipoprotein fractions were isolated by spinning 60 μl of plasma in a TL-100 ultracentrifuge (Beckman Coulter, Brea, CA) at its own density (1.006 g/ml) at 70,000g for 3 hours to harvest the supernatant, and then after adjusting the infranatant with solid KBr to a density of 1.063 g/ml spinning it for 70,000g for 18 hours to harvest the supernatant [272]. The cholesterol content of each supernatant and the final infranatant were measured and taken to be very-low-density lipoprotein (VLDL) (<1.006 g/ml), low-density lipoprotein (LDL) (1.006 ≤ d ≤ 1.063 g/ml), and high-density lipoprotein (HDL) (d >1.063 g/ml) cholesterol, respectively. Cholesterol concentrations in all three fractions were then determined enzymatically by a colorimetric method (Roche Diagnostics). Plasma triglyceride levels were determined enzymatically in the original plasma sample.

2.2.8 Statistical Analysis

All data are expressed as means ± SD unless otherwise noted. Statistical analysis was performed using a two-sample, two-tailed Student t-test unless otherwise noted, with P<0.05 regarded as significant. One-way analysis of variance was used when multiple comparisons were made, followed by Dunnett’s t-test for multiple comparisons to a control. Statistical analysis was performed using SigmaPlot 13.0.

2.3 Results

2.3.1 Amprenavir Is a Potent PXR-Selective Agonist

We tested several widely used HIV PIs, including amprenavir, ritonavir, nelfinavir, saquinavir, and indinavir sulfate, for PXR activation by use of transfection assays. Because PXR exhibits considerable differences in its pharmacology across species (namely, mouse versus human) [91, 95], the potent PXR ligands RIF and PCN were used as the positive control for human
Consistent with a previous report [258], ritonavir activated PXR and induced PXR-mediated reporter activity (Fig. 2.1, A and B). Interestingly, amprenavir was a more potent PXR agonist than any of the other tested PIs (Fig. 2.1, A and B). Amprenavir can activate both human and mouse PXR and induced reporter gene activity in a dose-dependent manner (Fig. 2.1, A and B). Dose-response analysis indicated that amprenavir was able to activate PXR at concentrations as low as 1 μM, reaching peak activation at 30 μM (Fig. 2.1, C). The EC_{50} for amprenavir activation of PXR-mediated CYP3A4 promoter activity was 8.6 μM (Fig. 2.1, C). We next tested the ability of amprenavir to activate a panel of other nuclear receptors, including human retinoid acid receptor, retinoid X receptor, farnesoid X receptor, liver X receptor α, PPARα, PPARγ, vitamin D receptor, estrogen receptor α, and estrogen receptor β (Fig. 2.1, D). Amprenavir was unable to activate any of these other nuclear receptors. Results from this panel emphasize the importance of studying the effects of amprenavir-mediated PXR agonism.

### 2.3.2 Amprenavir Promotes PXR Coactivator Recruitment and Corepressor Disassociation

Nuclear receptor co-regulators play critical roles in nuclear receptor signaling [90]. We used a mammalian two-hybrid assay [179, 266] to evaluate the effects of amprenavir on PXR coregulator interaction. Similar to the potent PXR ligand RIF, amprenavir promoted the specific interactions between PXR and the coactivators SRC-1 and PBP (Fig. 2.2, A). Consistent with our previous reports [179, 266], unliganded PXR interacted with corepressors, NCoR and SMRT. Amprenavir disrupted this interaction, as did RIF (Fig. 2.2, B). Binding of amprenavir to PXR inhibits PXR/corepressor interaction and promotes PXR/coactivator recruitment, thereby inducing PXR transcriptional activation in a concentration-dependent manner.

### 2.3.3 Computational Docking and Modeling Studies

To investigate the potential interaction pattern between amprenavir and PXR, a structure-based approach was employed. Amprenavir was docked into the high-resolution crystal structure of human PXR in complex with SR12813 [268]. The results from GOLD docking are shown in Figure 2.3A. The binding
pose of amprenavir in the PXR ligand-binding domain (LBD) is similar to SR12813. The PXR LBD appears to possess four hydrophobic subpockets and a connection region in the middle. Amprenavir occupies all four subpockets, and its hydroxyl group forms a hydrogen bond with Ser247, which is located in the connection region of PXR, to help to position the drug in the optimal orientation inside the receptor. Another two hydrogen bonds were formed between amprenavir and PXR (Gln285 and His407) that provide additional electrostatic interactions.

Nonpolar contacts also play a key role in stabilizing amprenavir within the ligand binding pocket of PXR. As displayed in Fig. 2.3B, the interaction map of amprenavir and PXR revealed an important π-π stacking between ring A of amprenavir and Phe288 in subpocket 1 of PXR. Tyr306 forms a 3.5-Å edge-to-face contact to the same phenol ring of the drug that also engages in hydrophobic interactions with Trp299, the other hydrophobic residue in subpocket 1. In subpocket 2, amprenavir forms direct contacts with one residue on αAF of the PXR activation function-2 (AF-2) surface, Phe429, which may stabilize the active AF-2 conformation of the receptor and contribute to the agonist activity of amprenavir on PXR. Subpockets 3 and 4 of PXR are located on the bottom of the cavity, which corresponds to the most structurally flexible region of the PXR LBD. This region is dominated by hydrophobic residues (e.g., Met323 and Leu209) that form van der Waals contacts with amprenavir.

### 2.3.4 Key LBD Residues of PXR Are Required for Amprenavir’s Agonistic Activity

To validate the results of the docking analysis, we mutated the key amino acids responsible for amprenavir’s agonist activity including Ser247, Gln285, Phe288, and Tyr306. We also included Thr248, a key amino acid known to be important for PXR/coactivator interaction [179, 273], and Leu411, an amino acid predicted by the docking analysis not to interact with amprenavir, as the putative positive and negative controls for our mutagenesis study, respectively. As shown in Fig. 2.3C, Ser247Leu and Tyr306Leu mutations completely blocked amprenavir’s agonist activity at all tested drug concentrations. Gln285Leu and His407Ala exerted little or no effects on the drug at low concentration (5 μM). At
higher concentrations (10 and 20 μM), these mutations showed inhibitory effects on amprenavir’s activity. Phe288Ala showed some inhibitory effects on amprenavir’s activity. As expected, the Thr248Leu mutation abolished the activity of amprenavir; Leu411Phe did not inhibit but rather slightly promoted amprenavir as an agonist. In summary, our site-directed mutagenesis analysis confirmed the docking model and revealed the key residues within PXR’s binding pocket responsible for amprenavir’s agonist effects.

2.3.5 Amprenavir Induces PXR Target Gene Expression in Human Cells

We next used human hepatoma HepaRG cells [274] and intestinal LS180 cells [222, 266] to test the effects of amprenavir exposure on PXR activity and target gene expression. Similar to the known human PXR ligand RIF, amprenavir induced the expression of bona fide PXR target genes involved in phase I (CYP3A4), phase II (UGT1A1), and phase III (MDR1) metabolism in both HepaRG cells (Fig. 2.4, A) and LS180 cells (Fig. 2.4, B). These results suggest that amprenavir can activate PXR and induce PXR target gene expression in human cells.

2.3.6 Amprenavir Elevates Plasma Lipid Levels in WT Mice, but Not in PXR−/− Mice

To further investigate the effects of amprenavir on PXR activity in vivo, WT and PXR−/− mice were treated with vehicle (corn oil) or 10 mg/kg body weight of amprenavir daily by oral gavage for 1 week. Amprenavir-treated WT mice had significantly increased total cholesterol levels compared with control WT mice (Fig. 2.5, A). By contrast, amprenavir did not affect plasma cholesterol levels in PXR−/− mice (Fig. 2.5, B). The FPLC analysis of the cholesterol distribution pattern revealed that amprenavir increased atherogenic LDL cholesterol fractions in WT mice, but not in PXR−/− mice (Fig. 2.5, C and D). The lipoprotein fractions (VLDL, LDL, and HDL) were isolated by ultracentrifugation [272], and the cholesterol concentrations in all three fractions were then measured. Consistent with the FPLC results (Fig. 2.5, C and D), amprenavir treatment did not affect HDL or VLDL cholesterol levels but significantly increased LDL cholesterol levels in WT mice (Fig. 2.6, A). By contrast, amprenavir did not
affect any of the lipoprotein levels in PXR\(^{+/}\) mice (Fig. 2.6, B). These results suggest that the adverse effects of amprenavir on plasma cholesterol levels are mediated, at least in part, through PXR in mice.

### 2.3.7 Amprenavir Affects Genes Involved in Lipid Homeostasis

To determine whether amprenavir can activate PXR in these mice, the expression levels of known PXR target genes were measured in the intestine and liver. Amprenavir stimulated expression of known PXR target genes, including CYP3A11, glutathione transferase A1, and MDR1a, in the intestine (Fig. 2.7, A) of WT mice but not in PXR\(^{+/}\) mice. Interestingly, amprenavir did not affect the expression of hepatic PXR target genes (Fig. 2.7, B), which might be due to the insufficient accumulation of amprenavir in the liver. To elucidate the possible molecular mechanisms through which amprenavir-mediated intestinal PXR activation might induce dyslipidemia, the expression levels of genes involved in lipid homeostasis were measured (Fig. 2.7, C). Of the genes surveyed, CD36 showed significant change, with its expression levels increased more than 10-fold. CD36, a class B scavenger receptor, plays an important role in intestinal lipid absorption [275].

Consistent with a recent report [265], the expression levels of diacylglycerol acyltransferase 1 and 2 (DGAT1 and 2), two enzymes involved in intestinal lipid transportation and chylomicron secretion [276], were also induced by amprenavir-mediated PXR activation in WT mice. It was recently reported that a *Drosophila* PXR ortholog DHR96 regulates expression of the intestine lipase Magro (CG5932) which mediates cholesterol and triglyceride homeostasis in *Drosophila*. Magro protein is most similar to mammalian gastric lipase (LipF) (56% similarity) and lysosomal lipase (LipA) (50% similarity) [277]. Interestingly, amprenavir-mediated PXR activation stimulated the expression of both LipF and LipA in the intestine of WT mice, but not in PXR\(^{+/}\) mice (Fig. 2.7, C), indicating a possible role of intestinal PXR in mediating dietary lipid breakdown and absorption in mammals. Taken together, upregulation of those genes by PXR activation may contribute to amprenavir-elicited hyperlipidemia.
2.4 Discussion

PIs have been associated with dyslipidemia and an increased risk of cardiovascular disease [55, 58, 66], but the underlying mechanisms are still unknown. Several PIs have been shown to activate PXR which functions as a xenobiotic sensor to regulate genes required for xenobiotic metabolism in the liver and intestine [90, 91]. We identified a widely used PI amprenavir as a potent and selective PXR agonist. Amprenavir is usually taken by patients with or without ritonavir. Interestingly, a case-control study suggested that the risk of cardiovascular disease was increased by exposure to all PIs and, in particular, to amprenavir with or without ritonavir [66]. We found that amprenavir activated both human and mouse PXR but did not affect the activity of other nuclear receptors. Short-term exposure to amprenavir significantly increased plasma total cholesterol and LDL cholesterol levels in WT mice, but not in PXR−/− mice. Amprenavir-mediated PXR activation regulated several key intestinal genes involved in lipid homeostasis. These findings provide critical mechanistic insight for understanding the impact of PIs on cardiovascular disease and demonstrate a potential role of PXR in mediating adverse effects of HIV PIs in humans.

Using in silico ligand-PXR docking studies combined with site-directed mutagenesis, we identified Tyr306, Ser247, His407, and Phe288 as participating in the amprenavir-PXR interaction (Fig. 2.3). A critical polar interaction with Ser247 helps to orient amprenavir in the ligand-binding cavity that enables formation of nonpolar interactions with a highly hydrophobic region of PXR’s ligand binding pocket lined by Phe288, Trp299 and Tyr306. These three residues are highly conserved among different species and interact with structurally diverse PXR agonists as shown in multiple published PXR X-ray crystal structures. This Ser247-directed orientation of amprenavir also guided the selection of the most plausible binding alignment among the multiple poses generated by our docking simulations. Based on our previous modeling results on diverse ligands with PXR, we conclude that two essential features need to be satisfied for ligand binding to PXR, namely, (1) one key hydrogen bond with Ser247 or His407 and (2) van der Waals contacts with subpocket 1. Besides
the interactions formed by these two essential features, numerous hydrophobic contacts between amprenavir and PXR further stabilize the ligand binding and indirectly promote contact with the AF-2 region, thus forming the optimal orientation of αAF helix to interact with coactivators. Our observations regarding the key pharmacophoric features for PXR binding will hopefully provide guidance useful for PXR ligand screening in the future.

The role of PXR in xenobiotic metabolism has been well established; however, recent studies have revealed the role of PXR in dyslipidemia and atherosclerosis. We recently demonstrated the pro-atherogenic effects of PXR in animals [177, 178]. We previously reported that chronic activation of PXR elicited by feeding mice the mouse PXR ligand PCN led to increased levels of plasma total cholesterol and VLDL and LDL in WT mice, but not in PXR−/− mice [177]. In our present study, mice were treated with 10 mg/kg per day amprenavir to investigate its potential effects on lipid levels. Patients usually take a daily dose of 1,400 mg or 2,800 mg of the amprenavir prodrug fosamprenavir, which is rapidly and extensively converted to amprenavir after oral administration [278]. Pharmacokinetic studies have demonstrated that the mean peak plasma concentration of amprenavir is approximately 18 μM after a 1,200-mg single dose [279, 280], which is sufficient to activate PXR based on our results (Fig. 2.1). Therefore, we believe that amprenavir can activate PXR and regulate PXR target genes in patients taking this drug routinely.

The dose of 10 mg/kg per day amprenavir used in the present study to treat animals is considered low or modest. Nevertheless, after 1 week of treatment, amprenavir significantly increased plasma total cholesterol and LDL cholesterol levels in WT mice, but not in PXR−/− mice, indicating the significant role of PXR in mediating the adverse effects of amprenavir (Figs. 2.5 and 2.6). Interestingly, amprenavir regulated the PXR target genes in the intestine but not in the liver (Fig. 2.7). This could be explained by the relative low dose of amprenavir and short-term treatment in this study. In addition, amprenavir is metabolized by CYP3A4 enzyme in the liver [281]. Therefore, the relative low dose of amprenavir used in the present study may be insufficient for amprenavir
to reach the high concentrations required to activate PXR in the liver.

Intestinal lipid transportation plays a central role in lipid homeostasis. In addition to prototypic PXR target genes (e.g., CYP3A11, MDR1a), amprenavir significantly stimulated expression of several key genes involved in intestinal lipid homeostasis in WT mice, including CD36, DGAT1, and DGAT2. We and others have previously reported that activation of PXR induces CD36 expression and increases lipid accumulation in the liver [114], intestine [265], and macrophages [177]. The role of CD36 in mediating intestinal fatty acid uptake has been well established [275, 282, 283]; several studies have also indicated that CD36 mediates cholesterol uptake in the intestine [282, 283]. For example, cholesterol uptake was significantly decreased in the enterocytes isolated from CD36-deficient (CD36−/−) mice [283]. In a lipid infusion study, CD36−/− mice exhibited accumulation of dietary cholesterol in the intestinal lumen and reduction of cholesterol transport into the lymph [282]. Therefore, the PXR-mediated CD36 upregulation may contribute to amprenavir-stimulated elevation of cholesterol levels.

In addition, Liang et al. [284] previously reported that PIs such as ritonavir inhibit degradation and secretion of apolipoprotein B (ApoB), the primary apolipoprotein of chylomicrons and LDL. Interestingly, the inhibitory effects of PI on ApoB secretion were reversed by oleic acid, a fatty acid that stimulates neutral-lipid biosynthesis [284]. In the presence of oleic acid, PI treatment even increased ApoB secretion above controls [284]. Because CD36 plays an important role in the absorption of oleic acid [283], it is plausible that PXR-mediated CD36 expression increases oleic acid absorption and contributes to PI-induced ApoB secretion and hyperlipidemia.

The precise mechanisms through which PXR modulates lipid homeostasis and cholesterol levels in various animal models as well as in humans remain to be determined. By contrast, the *Drosophila* PXR ortholog DHR96 has been established to be a key regulator in mediating cholesterol and triglyceride homeostasis [277, 285, 286]. Recent studies have shown that DHR96 regulates expression of the intestine lipase Magro, which is most similar to mammalian
LipA and LipF [277]. Both LipA and LipF were upregulated by amprenavir-mediated PXR activation in the intestine of WT mice but not in PXR-/- mice (Fig. 2.7, C).

LipA is an enzyme responsible for hydrolysis of cholesterol esters and triglycerides within lipoprotein particles internalized by receptor-mediated endocytosis [287, 288]. Previous studies have demonstrated an important role of LipA in maintaining cholesterol levels in mice [289], and mutations in LipA result in cholesterol ester storage disease, which is associated with hyperlipidemia in humans [290]. LipF also contributes to lipid catabolism by hydrolysis of dietary triglycerides in the stomach and intestine, sequentially producing free fatty acids and diacylglycerol [291, 292]. It would be interesting to further investigate the detailed mechanisms by which PXR transcriptionally regulates LipA and LipF expression and mediates intestinal lipid homeostasis. Upregulation of those key genes by PXR activation contribute to amprenavir-elicited hyperlipidemia.

In summary, we demonstrated that amprenavir as a selectively potent PXR agonist. Amprenavir efficiently activated PXR and stimulated PXR target gene expression in vitro and in vivo. Computational docking studies and site-directed mutagenesis analysis identified key residues within PXR's ligand binding pocket that interact with amprenavir. Our study provides critical insight for understanding the mechanism by which clinically relevant PXR ligands interact and activate PXR. We also showed, for the first time, that amprenavir-mediated activation of PXR elevated lipid levels in an animal model, which has direct clinical consequence for patients under long-term treatment with amprenavir and other PIs. Activation of PXR may present a key mechanism for adverse effects of PIs in humans.
Table 2.1 Primer sequences for site-directed mutagenesis.

<table>
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<th>Mutant</th>
<th>Primer sequences</th>
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<tr>
<td>PXR-S247L</td>
<td>5'-CTGCTGCCCCACATGGGCTGACAGTTACCTACATGTTCAAAAGGC-3'</td>
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<td>5'-GCCTTTGAAACATGTAGGTTAACATGTCGACACATGGTGCTGAGATTTCAACACAGTG-3'</td>
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<tr>
<td>PXR-Q285L</td>
<td>5'-GGGCCGGCTTTGAGCTGCTGTACCTGAGATTTCAACACAGTG-3'</td>
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<td>PXR-F288A</td>
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<td>5'-CCGCTTTGAAACATGTAGGTTAACATGTCGACACATGGTGCTGAGATTTCAACACAGTG-3'</td>
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Table 2.2 Primer sequences for QPCR.

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<th>Primer sequences</th>
<th>Genes</th>
<th>Primer sequences</th>
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<tr>
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<td>mGAPDH</td>
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<tr>
<td>mLipA</td>
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<tr>
<td>mDGAT1</td>
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<td>mGAPDH</td>
<td>5'-TCTGTGGGCTCATGAG-3'</td>
</tr>
</tbody>
</table>

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Amprenavir is a potent PXR-selective agonist.
Figure 2.1 Amprenavir is a potent PXR-selective agonist.

(A and B) HepG2 cells were transfected with (A) full-length hPXR together with a hPXR reporter (CYP3A4-luc) or (B) full-length mPXR together with a mPXR reporter ((CYP3A2)3-luc) and CMX-β-galactosidase control plasmid. Cells were then treated with DMSO control, amprenavir, ritonavir, nelfinavir, saquinavir, indinavir sulfate, RIF (a hPXR ligand) or PCN (a mPXR ligand) at the indicated concentrations for 24 h. (C) HepG2 cells were co-transfected with hPXR together with CYP3A4-luc reporter and CMX-β-galactosidase plasmid. Cells were then treated with amprenavir or RIF at the indicated concentrations for 24 h. (D) HepG2 cells were co-transfected with a GAL4 reporter and a series of GAL4 constructs in which the GAL4 DBD is linked to the indicated nuclear receptor LBD. Cells were treated with DMSO control or 10 μM amprenavir for 24 h. Data are shown as fold induction of normalized luciferase activity compared with DMSO treatment and represent the mean of triplicate experiments. Results are presented as mean ± SEM.
Figure 2.2 Amprenavir promotes PXR co-activator recruitment and co-repressor disassociation.

(A and B) HepG2 cells were transfected with a GAL4 reporter and VP16-hPXR as well as expression vector for GAL4 DBD or GAL4 DBD linked to the receptor interaction domains of the indicated PXR co-activators (GAL-SRC1 and GAL-PBP) (A) or co-repressor (GAL-SMRT and GAL- NCoR) (B). Cells were then treated with DMSO control, amprenavir, or RIF at the indicated concentrations for 24 h. Results are presented as mean ± SEM.
Figure 2.3 Key residues of PXR LBD are required for amprenavir’s agonistic activity.

(A) Overlay of docked amprenavir and SR12813 (green) inside the LBD of PXR. Atoms in amprenavir were colored by atom type (oxygen in red, phosphor in yellow, nitrogen in blue and carbon in grey), while all the atoms in SR12813 were colored in green for clear comparison. Four subpockets were presented by black circles. (B) Interaction map between amprenavir and PXR. Amprenavir was computationally docked in the ligand binding pocket of the hPXR X-ray crystal structure (3hvl.pdb). The amprenavir-PXR complex was stabilized by three hydrogen bonds with residues Ser247, Gln285 and His407, respectively. The noncovalent contacts with the aromatic side chains Phe288, Trp299, and Tyr306 lining in the subpocket 1 contributed significantly to amprenavir binding. Phe288 was well positioned to engage in π-stacking interactions with the phenyl ring of amprenavir. The interaction diagram reveals that amprenavir occupied the major portion of the large, flexible PXR ligand binding pocket. (C) HepG2 cells were co-transfected with a full-length PXR WT plasmid or mutant PXR plasmids as indicated, together with CYP3A4-luciferase reporter and CMX-β-galactosides plasmid. After transfection, cells were treated with control medium or medium containing amprenavir at indicated concentrations for 24 h. Results are presented as mean ± SEM.
Figure 2.4 Amprenavir induces PXR target gene expression in human cells.

(A and B) Human HepaRG hepatoma cells (A) and LS180 intestinal cells (B) were treated with control medium or medium containing 10 μM amprenavir for 24 h. Total RNA was isolated and gene expression levels were analyzed by QPCR with primers for human CYP3A4, UGT1A1 or MDR1 (n=3, **P<0.01, ***P<0.001; assessed by one-way ANOVA). Results are presented as mean ± SEM.
Figure 2.5 Amprenavir elicits hyperlipidemia in WT, but not in PXR−/− mice.

(A and B) 8-week-old WT (A) and PXR−/− (B) mice were treated with vehicle (corn oil) or 10 mg/kg of amprenavir by oral gavage for 1 week. Plasma total cholesterol levels were measured by a standard method (n= 4-7; *P<0.05; assessed by student’s t-test). (C and D) Plasma cholesterol distribution of WT (C) and PXR−/− mice (D) was analyzed by FPLC. Results are presented as mean ± SEM.
Figure 2.6 Amprenavir elevates atherogenic LDL cholesterol levels in WT mice.

(A and B) Eight-week-old WT (A) and PXR⁻/⁻ (B) mice were treated with vehicle or 10 mg/kg of amprenavir by oral gavage for 1 week. Lipoprotein fractions (VLDL, LDL, and HDL) were isolated and the cholesterol levels of each fraction were measured by a standard method ($n=4-7$; *$P<0.05$; assessed by student’s $t$-test). Results are presented as mean ± SEM.
Figure 2.7 Activation of PXR by amprenavir stimulates the expression of intestinal genes involved in lipid homeostasis.
Eight-week-old WT and PXR-/- mice were treated with vehicle (corn oil) or 10 mg/kg of amprenavir by oral gavage for 1 week. Total RNA was isolated from the small intestine (duodenum) and liver. (A and B) The mRNA levels of known PXR target genes, CYP3A11, GSTA1, and MDR1a, in the intestine (A) and liver (B) were measured by QPCR. (C) Expression levels of intestinal genes involved in lipid homeostasis were measured by QPCR. (n=4-5; *P<0.05, **P<0.01; assessed by student’s t-test). Results are presented as mean ± SEM.

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Chapter 3: Intestinal PXR Links Xenobiotic Exposure and Hypercholesterolemia

3.1 Introduction

Influences of the chemical environment on human health have recently become the subject of intense interest. Mounting evidence shows that endocrine disrupting chemicals (EDCs) can interfere with complex endocrine signaling mechanisms and result in adverse consequences in humans and wildlife [76, 293]. Recent findings have implicated exposure to EDCs in the etiology of cardiovascular disease (CVD) and metabolic disorders [76-78, 293-295]. For instance, higher bisphenol A (BPA) exposure has been consistently associated with CVD in multiple large-scale human population studies [77-79]. Exposure to certain polychlorinated biphenyls (PCBs) induces hypercholesterolemia and promotes atherosclerosis in animals [84, 296]. Circulating PCB levels have been associated with atherosclerotic plaques in elderly individuals [297]. High circulating levels of phthalates are also associated with carotid atherosclerosis [81]. However, the underlying mechanisms responsible for these associations remain largely unknown, which continues to hamper rational assessment of the health risks of EDC exposure.

Many EDCs such as phthalates, PCBs, and BPA and its analogs have been implicated in the activation of the pregnane X receptor (PXR) (also known as steroid and xenobiotic receptor, or SXR) [91, 136, 179, 298]. PXR is a nuclear receptor activated by numerous endogenous hormones, dietary steroids, pharmaceutical agents, and xenobiotic chemicals [89, 91, 95]. PXR functions as a xenobiotic sensor that induces expression of genes required for xenobiotic metabolism in the liver and intestine, including cytochromes P450s (CYP), conjugating enzymes (e.g., glutathione transferase), and ABC family transporters (e.g., multidrug resistance 1 [MDR1]) [90, 91]. In the past decade,
the role of PXR as a xenobiotic sensor has been well established [91]. However, the role of PXR in mediating the pathophysiological effects of EDCs in humans and animals remains elusive.

The identification of PXR as a xenobiotic sensor provided an important tool for the study of new mechanisms through which xenobiotic exposure impacts diseases. Recent evidence indicates that PXR may also play an important role in the regulation of lipid homeostasis [114, 177, 260, 265, 299, 300]. For instance, it is well-known that many clinically relevant PXR ligands (e.g. rifampicin, ritonavir) can elevate plasma lipid levels in patients and increase their CVD risk [52, 261, 263, 264]. A meta-analysis of 7 genome-wide association studies indicated that common genetic variants in PXR can affect plasma lipid levels in humans and 19 PXR SNPs were identified to significantly affect plasma low-density lipoprotein (LDL) cholesterol levels [118]. We recently demonstrated the pro-atherogenic effects of PXR in animals. Chronic activation of PXR elicited by feeding mice the mouse PXR ligand pregnane 16α-carbonitrile (PCN) led to increased levels of plasma total cholesterol and atherogenic lipoproteins LDL and very-low-density lipoprotein (VLDL) in wild-type (WT) mice, but not in PXR-deficient (PXR−/−) mice [177]. Activation of PXR also increased plasma total cholesterol and VLDL levels in apolipoprotein E*3-Leiden mice, which exhibit a human like lipoprotein distribution on a cholesterol-rich diet [260]. In Chapter 2, we identified amprenavir, a widely used anti-retroviral drug, as a potent PXR-selective agonist [299]. Exposure to amprenavir significantly increased plasma total cholesterol and LDL cholesterol levels in WT mice, but not in PXR−/− mice [299].

Despite emerging evidence consistent with the hypothesis that modulation of PXR activity alters lipid homeostasis, the mechanisms underlying PXR ligand-elicited hyperlipidemia remain largely unknown. PXR is expressed at high levels in the liver and intestine, two organs that play a central role in whole-body lipid homeostasis. PXR has been reported to regulate several key hepatic lipogenic genes that promotes dyslipidemia and hepatic steatosis, including
CD36, SCD-1, lipin-1, Insig-1, and S14 [114, 182, 204, 301]; however, the role of intestinal PXR in the regulation of lipid homeostasis remains elusive. Here we report that intestinal PXR plays an important role in linking EDC exposure and hypercholesterolemia. We identified several widely used phthalate substitute plasticizers widely used in food packaging materials, medical devices, cosmetics, and pharmaceutical drugs as agonists of PXR. Tributyl citrate (TBC), one of a large group of Food and Drug Administration (FDA)-approved pharmaceutical plasticizers, is a potent and selective PXR agonist but does not activate other nuclear receptors. Interestingly, TBC is an intestine-specific PXR ligand but does not affect hepatic PXR activity. Short-term TBC exposure increased plasma total cholesterol and atherogenic LDL cholesterol levels in WT mice, but not in PXR−/− mice. We found that TBC-mediated PXR activation stimulated the expression of the intestinal cholesterol transporter Niemann-Pick C1-Like 1 (NPC1L1) and significantly increased lipid uptake by human and murine intestinal cells. We also identified a PXR-binding site in the NPC1L1 promoter, indicating NPC1L1 is a bona fide PXR target gene. These findings provide critical mechanistic insight for understanding the impact of EDC-mediated PXR activation on lipid homeostasis and demonstrate a potential role of PX R in mediating adverse effects of EDCs on CVD risk in humans.

3.2 Materials and Methods

3.2.1 Reagents and Plasmids
TBC, acetyl tributyl citrate (ATBC), di (2-ethylhexyl) phthalate (DEHP), acetyltriethyl citrate (ATEC), diisononyl phthalate (DiNP), triethyl citrate (TEC), di-n-butyl phthalate (DnBP), di-isobutyl phthalate (DiBP), diethyl phthalate (DEP), pregnenolone 16α-carbonitrile (PCN) and rifampicin (RIF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the chemicals were dissolved in dimethyl sulfoxide (DMSO). Human (h) and mouse (m) PXR expression vectors; GAL4 DNA-binding domain (DBD)-linked nuclear receptor ligand-binding domain (LBD) vectors (GAL4-hPXR, GAL4-mPXR, GAL4-rat...
PXR, GAL4-retinoid acid receptor [RARα], GAL4-retinoid X receptor [RXR], GAL4-farnesoid X receptor [FXR], GAL4-liver X receptor [LXR], GAL4-peroxisome proliferator-activated receptor [PPARα, PPARγ] and GAL4-vitamin D receptor [VDR]); and CMX-β-gal expression vectors were described before [179, 222, 266]. VP16-PXR, GAL4-nuclear receptor corepressor (NCoR), GAL4-silencing mediator of retinoid and thyroid hormone (SMRT), GAL4-steroid receptor coactivator (SRC1), GAL4-PPAR binding protein (PBP), PXR-dependent CYP3A4 promoter reporter (CYP3A4XREM-Luciferase), CYP3A2 promoter reporter [(CYP3A2)₃-luciferase], and GAL4 reporter (MH100-Luciferase) were described previously [179, 266, 267]. For the human NPC1L1/DR-4 reporter, 4 copies of the DR-4 element were synthesized and inserted into a pGL3 promoter vector (Promega) by annealing complementary oligonucleotides 5'-GCAGATCACTTGAGGTCAGG-3' containing cohesive ends of the restriction enzyme sites, Kpnl and MluI.

3.2.2 Cell Culture and Transfections
The human intestine epithelial cell line LS180 and hepatic cell line HepG2 were obtained from the American Type Culture Collection. The human hepatoma HepaRG cells were purchased from Life Technologies. Transfection assays were performed as described previously [179, 299]. The cells were transfected with various expression plasmids as well as the corresponding luciferase reporter plasmids, together with CMX-β-galactosidase control plasmids using FuGENE 6 (Roche Diagnostics). The cells were then incubated with the corresponding ligands as indicated in the figure legends for 24 hours, and β-galactosidase and luciferase assays were performed as described [179, 301]. Fold activation was calculated relative to solvent controls. Each data point represents the average of triplicate experiments ± SEM and was replicated in 3-5 independent experiments. For the mammalian 2-hybrid assays, LS180 cells were transfected with GAL4 reporter, VP16-hPXR, and GAL-SRC1, GAL-PBP, GAL-NCoR, and GAL-SMRT [179, 301]. The cells were then treated with compounds at the indicated concentrations.
3.2.3 Animals
C57BL/6 WT mice were purchased from The Jackson Laboratory. PXR-deficient mice (PXR−/−) on C57BL/6 background were employed as described previously [177]. All the animals were housed in the Division of Laboratory Animal Resources, University of Kentucky, as approved by the Institutional Animal Care and Use Committee in the specific pathogen-free environment with a light-dark cycle. Eight-week-old male WT or PXR−/− mice were fed a semisynthetic low-fat AIN76 diet containing 0.02% cholesterol (Research Diets) [177, 178, 270, 299] and treated by oral gavage with vehicle (corn oil) or 10 mg/kg body weight (BW) TBC daily for 7 days. In addition, WT mice were also treated with vehicle control or 10 mg/kg/BW TBC daily by ip injection. On the day of euthanization, mice were fasted for 6 hours after the dark phase (feeding cycle) [177, 178]. Mice were then anesthetized by intraperitoneal injection with Ketamine (Fort Dodge Animal Health). Mice were exsanguinated by left ventricular puncture, and blood was collected into EDTA-containing syringes. Plasma was prepared by spinning at 16,000 g for 10 min. The circulation was flushed with PBS; intestinal and liver tissues were collected and stored in RNA later solution (Life Technologies). Mouse primary hepatocytes and enterocytes were isolated as previously described [302, 303]. Primary cells were cultured in multiwell plates and treated with the indicated compounds before being harvested for quantitative real-time PCR (qPCR) and Western blot analysis or cholesterol uptake assays.

3.2.4 Plasma Analysis
Plasma total cholesterol and triglyceride concentrations were determined enzymatically by colorimetric methods as described previously (Roche) [299, 304]. Lipoproteins fractions were isolated by spinning 60 μl of plasma in a TL-100 ultracentrifuge (Beckman Coulter) at its own density (1.006 g/ml) at 70,000 RPM for 3 hours to harvest the supernatant, and then after adjustment of the infranatant with solid KBr to a density of 1.063 g/ml spinning it for 70,000 RPM for 18 hours to harvest the supernatant [304]. The cholesterol content of each
supernatant and the final infranatant were measured and taken to be very-low-density lipoprotein (VLDL) (<1.006 g/ml), low-density lipoprotein (LDL) (1.006 ≤ d ≤ 1.063 g/ml), and high-density lipoprotein (HDL) (d >1.063 g/ml) cholesterol, respectively. Cholesterol concentrations in all three fractions were then determined enzymatically by a colorimetric method (Roche). Total plasma cholesterol levels were determined enzymatically in the original plasma sample.

3.2.5 Cholesterol Uptake Assay
Cholesterol uptake assay was performed as previously described [305]. In brief, micelles were prepared by mixing 9.7 mM of Taurocholate (Sigma-Aldrich), 6.47 mM of egg yolk L-α-Phosphatidylcholine (Sigma-Aldrich), and 1.5 mM of Cholesterol (Sigma-Aldrich), together with 1 µCi of [1,2-3H(N)]-Cholesterol (PerkinElmer)/µmol of Cholesterol and evaporated under a mild stream of argon. The lipid film was hydrated in serum-free MEM containing 0.5% fatty acid-free BSA (Sigma-Aldrich) and incubated at 37 °C in a rotating incubator. Solutions were filtered through a 0.45 µm surfactant-free cellulose acetate filter (Corning) and used to incubate the LS180 cells or primary enterocytes. After extensive PBS washing, cellular [3H]-cholesterol was counted by a β-counter.

3.2.6 Electrophoretic Mobility Shift Assay (EMSA)
EMSA was performed as described previously [255, 303]. In brief, NPC1L1/DR-4 and CYP3A4/ER-6 probes were created by annealing the oligonucleotides 5’-GGGCAGATCACTTGAGGTCAGGAG-3’ (NPC1L1/DR-4), 5’-GGGCAGAACACTTGAGAACAGGAG-3’ (NPC1L1/DR-4m1), 5’-GGGCAGATCTCTTGAGATCTGAG-3’(NPC1L1/DR-4m2), or 5’-ATATGAACTCAAAGGAGGTCAGTG-3’ (CYP3A4/ER6) to the complementary strand. Double-stranded oligonucleotides were end labeled using T4 polynucleotide kinase (New England Biosciences) and γ-[32P]-ATP (PerkinElmer). Then 5 µl of in vitro-translated PXR or RXR protein was incubated with 2 µg poly d(I-C) (Promega), 2 µl bandshift buffer (50 mM MgCl2 and 340 mM KCl), and 6 µl delta buffer (0.1 mM EDTA, 40 mM KCl, 25 mM
HEPES (pH 7.6), 8% Ficoll 400, and 1 mM dithiothreitol) on ice for 10 minutes. 

$^{32}$P-labeled double-stranded oligonucleotide probe (100,000 cpm) was then 

added, and the reaction was incubated for another 30 minutes on ice. For the 
supershift assays, proteins were incubated with 2 μg of goat anti-PXR (sc-7739; 
Santa Cruz Biotechnology) or rabbit anti-PXR (sc-25381; Santa Cruz 
Biotechnology) antibodies for 1 hour before the addition of $^{32}$P-labeled probe. 
The binding complexes were subjected to electrophoresis in a 6% 
nondenaturing polyacrylamide gel containing 0.5× Tris-borate-
EDTA (TBE). The gels were dried and visualized by exposure to X-ray film.

3.2.7 Chromatin Immunoprecipitation 
ChIP analysis was performed by using an antibody against PXR (sc-
25381; Santa Cruz Biotechnology) and SimpleChIP Enzymatic Chromatin IP Kit 
(Cell Signaling). The precipitated genomic DNA relative to inputs was analyzed 
by semi-quantitative PCR using specific primers and DNA polymerase (Takara). 
The sequences of primer sets used for PCR are listed in Table 3.1.

3.2.8 RNA Isolation and QPCR Analysis 
Total RNA was isolated from mouse tissues and intestinal LS180, 
HepaRG, and primary cells using TRIzol Reagent (Life Technologies) per the 
manufacturer-supplied protocol. QPCR was performed using gene-specific 
primers and the SYBR green PCR kit (Life Technologies) as described 
previously [179, 299]. The primer sets used in this study are listed in Table 3.1.

3.2.9 Western Blot Analysis 
Western blot analysis was performed as previously described [255]. 
Proteins were isolated from cells or mouse tissues by homogenization in 
radioimmunoprecipitation assay buffer with complete mini protease inhibitor 
cocktail (Roche). Protein concentrations were determined by the Pierce BCA 
protein assay kit (Thermo scientific). Anti-PXR antibodies were purchased from 
Santa Cruz Biotechnology (sc-7739); anti-NPC1L1 antibodies were purchased 
from Novus Biologicals (NB400-128); and anti-β-actin antibodies were 
purchased from Sigma-Aldrich (A2066).
3.2.10 Statistical Analysis
Data are expressed as means ± SEM unless otherwise noted. Statistical analysis was performed using a two-sample, two-tailed Student t-test unless otherwise noted, with P < 0.05 regarded as significant. One-way analysis of variance was used when multiple comparisons were made, followed by Dunnett’s t test for multiple comparisons to a control. Two-way analysis of variance was used when multiple comparisons were made, followed by a Bonferroni’s post-hoc test. Analysis was performed using SigmaPlot 13.0.

3.3 Results

3.3.1 FDA-Approved Phthalate Substitute Plasticizers can Activate PXR
Based on previous findings that several plastic-associated chemicals including BPA and phthalates can activate PXR [179, 306], we examined several widely used phthalates and phthalate substitute plasticizers for PXR activation in transient transfection assays. Consistent with previous reports [307, 308], DEHP, DiNP, and ATBC can activate PXR and induce PXR-mediated CYP3A4-luciferase reporter activities (Fig. 3.1, A). TBC, an FDA-approved phthalate substitute plasticizer used in food contact substances and as a pharmaceutical excipient, was a more potent PXR agonist than any of the other tested plasticizers and induced reporter gene activity in a dose-dependent manner (Fig. 3.1, A). We tested the ability of TBC to activate a panel of other nuclear receptors, including mouse PXR, human RARα, RXR, FXR, LXRα, PPARα, PPARγ, VDR, constitutive adrostane receptor, and estrogen receptor α (Fig. 3.1, B). TBC can activate both human and mouse PXR but was unable to activate any of other nuclear receptors. Thus, TBC is a PXR-selective agonist.

Nuclear receptor co-regulators play critical roles in nuclear receptor signaling. We then used a mammalian 2-hybrid assay to evaluate the effects of TBC on PXR co-regulator interaction. TBC promoted the specific interactions between PXR and the coactivators SRC-1 and PBP (Fig. 3.1, C). Consistent
with our previous report [179, 266], unliganded PXR interacted with corepressors, NCoR and SMRT (Fig. 3.1, D), and TBC disrupted this interaction as did the potent human PXR ligand RIF (Fig. 3.1, D). Thus, binding of TBC to PXR inhibits PXR-corepressor interaction and promotes PXR-coactivator recruitment, thereby inducing PXR transcriptional activation in a concentration-dependent manner.

### 3.3.2 TBC is an Intestine-Specific Ligand

We next used human hepatoma HepaRG cells [274] and intestinal LS180 cells [266, 299] to test the effects of TBC exposure on PXR activity and target gene expression. The known human PXR ligand RIF induced the expression of bona fide PXR target genes involved in phase I (CYP3A4), phase II (UGT1A1), and phase III (MDR1) metabolism in both cell lines. Interestingly, TBC stimulated PXR target gene expression in intestinal LS180 cells but not in HepaRG cells (Fig. 3.2, A). We then isolated mouse primary hepatocytes and enterocytes to confirm these findings. As expected, the mouse PXR ligand PCN induced PXR target gene expression in both hepatocytes and enterocytes (Fig. 3.2, B). However, TBC was only able to induce PXR target gene expression in enterocytes but not in hepatocytes (Fig. 3.2, B). We also performed transfection assays in LS180 and HepG2 cell lines. Consistently, TBC did not activate PXR in HepG2 cells but was able to induce PXR reporter activity in LS180 cells after being treated for as few as 3 hours (Fig. 3.3, A and B).

To further investigate the effects of TBC on PXR activity in vivo, WT mice were treated with vehicle (corn oil), 10 mg/kg BW TBC, or PCN daily by oral gavage for 1 week. Consistent with our previous report [303], PCN activated PXR and induced target gene expression in both liver and intestine (Fig. 3.2, C). However, TBC only stimulated expression of known PXR target genes in intestine but not in liver (Fig. 3.2, C). To examine whether lower TBC doses can activate intestinal PXR in vivo, we also treated mice with 2.5 or 5 mg/kg/d TBC by oral gavage for 1 week. Neither 2.5 nor 5 mg/kg/d TBC activated PXR or induced PXR target gene expression in the intestine (Fig. 3.4). Next, to
determine whether the tissue-specific effect of TBC on intestinal PXR is related to poor absorption into the circulation, WT mice were also treated with 10 mg/kg/d TBC by ip deliver. Interestingly, TBC was still unable to induce PXR target gene expression in liver even by ip delivery (Fig. 3.5). Therefore, it is unlikely that the inability of TBC to activate hepatic PXR is due to poor absorption by the intestine. Taken together, these results suggest that exposure to TBC at a relevant dose via oral delivery activates intestinal PXR but does not affect hepatic PXR signaling.

3.3.3 TBC Exposure Elevates Plasma Lipid Levels in WT Mice, but not in PXR−/− Mice

We previously reported that activation of PXR by feeding PCN at a high dose (200 mg/kg in diet) for 2 weeks significantly increased plasma total cholesterol levels and atherogenic LDL and VLDL levels in WT mice [177]. Consistent with our previous report, WT mice treated with a relatively low dose of PCN (10 mg/kg/BW/d via oral deliver) for 1 week also had significantly increased plasma total, LDL, and VLDL cholesterol levels (Fig. 3.6, A). Although TBC only activates intestinal PXR, TBC treatment significantly increased total cholesterol levels compared with those in control WT mice (Fig. 3.6, A). TBC treatment did not affect HDL or VLDL cholesterol levels but significantly increased LDL cholesterol levels in WT mice (Fig. 3.6, A). The relatively stronger effects of PCN on plasma lipid levels could be due to its effects on hepatic PXR signaling. In contrast, TBC did not affect any of the lipoprotein levels in PXR−/− mice (Fig. 3.6, B), indicating that TBC-elicited hypercholesterolemia effects are mediated by PXR signaling. Taken together, these results suggest that activation of intestinal PXR by TBC is sufficient to increase plasma cholesterol levels.

3.3.4 TBC Stimulates the Expression of Intestinal Cholesterol Transporter NPC1L1 and Drives Cholesterol Uptake by Intestinal Cells

Small intestine lipid absorption is the key step for lipid accumulation in the body. Interestingly, we found that TBC stimulated expression of the
transporters responsible for intestinal lipid absorption including CD36 and NPC1L1 in the intestines of WT mice, but not in intestines of PXR<sup>−/−</sup> mice (Fig. 3.7, A). The known PXR target gene, CD36 [114], encodes a fatty acid transporter that plays an important role in intestinal fatty acid absorption and chylomicron production [282, 309]. NPC1L1 is an essential transporter mediating intestinal cholesterol absorption [6, 20, 27]. Western blot analysis also confirmed that TBC induced intestinal NPC1L1 protein levels in WT mice but not in PXR<sup>−/−</sup> mice (Fig. 3.7, B). Consistent with a previous report [310], PXR activation did not affect expression levels of the cholesterol efflux transporters ABCG5 and ABCG8. In addition, the known PXR ligand PCN was also able to induce NPC1L1 expression in intestine (Fig. 3.8), indicating that NPC1L1 is a downstream target of PXR.

To further investigate the effects of TBC on PXR activity and NPC1L1 expression, we isolated primary enterocytes from WT and PXR<sup>−/−</sup> mice. Consistent with in vivo results, TBC treatment increased NPC1L1 mRNA and protein levels in WT enterocytes (Fig. 3.7, C and D). TBC-mediated NPC1L1 induction was abolished in PXR-deficient enterocytes. Because NPC1L1 plays a key role in cholesterol uptake by intestinal cells, we then performed cholesterol uptake assays using primary enterocytes. As expected, TBC treatment increased [H<sup>3</sup>]-cholesterol uptake by primary enterocytes of WT mice but did not affect cholesterol uptake by PXR-deficient enterocytes (Fig. 3.7, E). Taken together, these results indicate a previously unrecognized role of PXR in the regulation of intestinal cell cholesterol uptake.

### 3.3.5 Activation of PXR by TBC Transcriptionally Regulates NPC1L1 Expression and Increases Cholesterol Uptake by Human Intestinal Cells

To determine the impact of TBC-mediated PXR activation on NPC1L1 regulation and cholesterol uptake in human intestinal cells, we used small interfering RNA (siRNA) to successfully reduce PXR expression in human LS180 cells (Fig. 3.9, A). As expected, TBC treatment induced NPC1L1 mRNA levels in control LS180 cells and siRNA-mediated PXR knockdown decreased
TBC-induced NPC1L1 induction (Fig. 3.9, B). In addition, TBC was also able to significantly increase [H\textsuperscript{3}]-cholesterol uptake by LS180 cells (Fig. 3.9, C). LS180 cells transfected with siRNA against PXR can still take up [H\textsuperscript{3}]-cholesterol, which may be due to incomplete inhibition of endogenous PXR expression by siRNA. However, TBC-mediated cholesterol uptake was significantly reduced by siRNA-mediated PXR knockdown. These results confirm that TBC-mediated PXR activation can increase NPC1L1 expression and cholesterol uptake in human intestinal cells.

We next analyzed the promoter of the human NPC1L1 gene and identified a DR-4-type (direct repeat spaced by 4 nucleotides) of nuclear receptor response element (AGATCACTTGAGGTCA), similar to DR-4 element found in other PXR target genes [95]. EMSA confirmed that PXR and RXR heterodimer was able to bind to this DR-4 element as well as the positive control, the ER-6 (everted repeat spaced by 6 nucleotides) element in the CYP3A4 promoter (Fig. 3.10, A). The binding of NPC1L1/DR-4 or CYP3A4/ER-6 by PXR-RXR is specific as excess cold probe decreased PXR-RXR binding to those elements (Fig. 3.10, A). In addition, 2 different anti-PXR antibodies disrupted the protein-DNA complex (Fig. 3.10, B), suggesting that PXR is a component of the protein complex that binds to the NPC1L1/DR4 element. Further, mutations of the DR-4 elements were also able to abolish the binding of PXR-RXR dimers to both mutant DR-4 sites (Fig. 3.10, C).

Next, ChIP analysis demonstrated that TBC can promote the recruitment of PXR onto the NPC1L1 promoter region containing the DR-4 element (Fig. 3.10, D). ChIP on the CYP3A4 promoter region containing the ER-6 element was included as a positive control. Last, 4 copies of the NPC1L1/DR-4 or mutated DR-4 elements were synthesized and inserted into a luciferase reporter vector. Transfection assays were performed to determine whether the DR-4 motif is necessary and sufficient for mediating PXR transactivation. Indeed, both TBC and RIF can activate PXR and increase the NPC1L1/DR-4 reporter gene activity in a dose-dependent manner (Fig. 3.10, E). In contrast, TBC and
RIF had no effect on activity of 2 mutated DR-4 reporters (Fig. 3.10, F and G). Collectively, activation of PXR by TBC transcriptionally regulates NPC1L1 expression and increases cholesterol uptake by human intestinal cells.

3.4 Discussion

Because of their variety and low costs, plastics are fundamental in modern life and plastic production exceeded 300 million tons in 2010 [293]. Plastic-associated chemicals are produced in high volume for use in the production of plastics, including the base chemical BPA and numerous plasticizers. The adverse effects of BPA and several phthalate plasticizers (e.g., DEHP) on human health have attracted considerable attention and engendered controversy in the past few decades, partly due to their endocrine-disrupting properties. We and others have identified BPA, BPA analogs (e.g., bisphenol B and bisphenol AF), and the widely used phthalate plasticizer DEHP as potent PXR agonists [179, 298, 306]. In addition to these well-known EDCs, there are many phthalate substitute plasticizers that have not been tested for endocrine disruption. For example, citrate esters, including TBC, ATBC, and TEC, represent a large group of plasticizers that have been approved by the FDA to be used in food packaging materials, vinyl toys, medical devices, cosmetics, and pharmaceutical drugs [311-314]. ATBC has recently been shown to activate PXR and induce PXR target gene expression [308]. However, it is unclear whether ATBC or other phthalate substitute plasticizers have any adverse effects on the development of complex diseases. In the current study, we demonstrate that TBC is a more potent PXR agonist than ATBC in our assays. Similar to ATBC, TBC activated intestinal PXR and induced PXR target gene expression but did not affect hepatic PXR activity. Nevertheless, TBC-mediated intestinal PXR activation was sufficient to increase plasma cholesterol levels, especially atherogenic LDL levels in WT mice. We then identified a key intestinal cholesterol transporter, NPC1L1, as a direct transcriptional target of
PXR. Activation of PXR stimulated NPC1L1 expression and promoted cholesterol uptake by intestinal cells, which may contribute to TBC-induced hyperlipidemia. These findings demonstrate a previously unrecognized role of intestinal PXR in the regulation of lipid homeostasis.

Whereas the role of PXR in xenobiotic metabolism has been well established, recent studies have revealed a role of PXR in dyslipidemia and atherosclerosis. We previously reported that chronic activation of PXR elicited by feeding mice the potent mouse PXR agonist PCN led to increased levels of plasma total cholesterol and VLDL and LDL in WT mice but not in PXR−/− mice [177]. PCN-mediated PXR activation affected several genes involved in hepatic lipid homeostasis. Because TBC does not activate PXR in the liver, it is unlikely that hepatic PXR signaling contributes to TBC-elicited hypercholesterolemia.

Cholesterol uptake from the intestinal lumen by the enterocytes is the rate-limiting step in cholesterol absorption [309]. NPC1L1, a multitransmembrane protein containing a conserved N-terminal NPC1 domain and a putative sterol-sensing domain, has been established as an essential transporter in mediating intestinal cholesterol uptake [20, 27, 309]. NPC1L1 expression is enriched in the small intestine and is in the brush border membrane of enterocytes [20]. NPC1L1 takes up free cholesterol into cells through vesicular endocytosis and is required for intestinal cholesterol absorption [20, 27, 309]. Indeed, NPC1L1-deficient mice had substantially reduced intestinal cholesterol absorption and plasma cholesterol (particularly LDL) levels and were completely resistant to diet-induced hypercholesterolemia [20, 27]. NPC1L1 is also the molecular target of the clinically used drug ezetimibe, a potent cholesterol absorption inhibitor widely used to treat hypercholesterolemia [20]. Very recently, inactivating mutations in NPC1L1 has been associated with reduced plasma LDL cholesterol levels and as reduced risk of CVD in a large-scale human study [315]. Despite the established function of NPC1L1 in intestinal cholesterol absorption, the transcriptional regulation of NPC1L1 has not been fully understood. Our results suggest that PXR is an important regulator of NPC1L1
transcription. PXR can directly bind to a DR-4 motif in human NPC1L1 promoter and stimulate NPC1L1 expression upon ligand activation. Thus, PXR-mediated NPC1L1 up-regulation may contribute to the TBC and other ligand-induced hypercholesterolemia.

Whereas NPC1L1 plays an essential role in intestinal cholesterol absorption, another PXR-regulated transporter, CD36, mediates enterocyte uptake of fatty acids, which are then converted to triglycerides for transport into chylomicrons [282, 309]. We and others previously reported that activation of PXR induces CD36 expression and increases lipid accumulation in the liver [114], intestine [265], and macrophage [177]. In addition, activation of intestinal PXR can also induce the expression levels of several enzymes involved in intestinal lipid transportation and chylomicron secretion including diacylglycerol acyltransferase 1 and 2 [265]. Therefore, the functions of PXR in intestinal lipid homeostasis are complex, and further studies are needed to define the precise mechanisms through which intestinal PXR modulates lipid homeostasis in animal models as well as in humans.

It is intriguing that TBC can only activate intestinal PXR but not hepatic PXR. Several tissue-specific PXR ligands have been identified [265, 267, 316]. For example, rifaximin, a nonsynthetic antibiotic, has been shown to be a potent intestine-specific PXR ligand [316]. The tissue-specific effect of rifaximin on intestinal PXR was probably related to its poor absorption as rifaximin accumulated in the intestine after oral treatment. However, the reason that TBC cannot activate hepatic PXR is unlikely due to poor absorption by the intestine as TBC was unable to activate hepatic PXR even after ip delivery. Further, TBC treatment did not affect PXR activity in HepaRG and HepG2 cells in vitro. It is possible that hepatic cells can rapidly catalyze TBC. Further studies, including development of appropriate HPLC-tandem mass spectrometry assays and examination of TBC metabolites in vivo and in vitro, will be required to elucidate the detailed mechanisms underlying the tissue-specific effects of TBC and similar chemicals. In addition, we previously demonstrated that tocotrienols,
members of the vitamin E family, show tissue-specific induction of PXR target genes, particularly CYP3A4 [267]. Tocotrienols can up-regulate expression of CYP3A4 in human primary hepatocytes but not in LS180 cells. Nuclear receptor co-regulators play critical roles in nuclear receptor activation and are also involved in the mechanisms underlying the divergent activities of selective estrogen receptor modulators [317, 318]. We found that NCoR is expressed at different levels in intestinal LS180 cells and primary hepatocytes and that tocotrienols can only partially disrupt the interaction of unliganded PXR and NCoR in LS180 cells [267]. Therefore, it is plausible the coregulator difference between liver and intestine may contribute to the tissue-specific effects of TBC.

A fundamental question about all EDC studies is whether low-dose exposure to EDCs can influence human endocrine functions and cause adverse effects. Currently, there is little information about human exposure to TBC. The dose of 10 mg/kg/d TBC used in the present study to treat animals was based on human exposure to other plasticizers such as DEHP, DnBP, and ATBC. A retrospective human biomonitoring study of German adults aged 20 to 29 years showed median daily intakes for DnBP and DEHP of 7 and 4 mg/kg BW/ day, respectively. Fourteen percent of subjects showed DnBP intakes above the tolerable daily intake value of 10 mg/kg BW/d set by the European Food Safety Authority [319, 320]. Patients taking drugs containing another citrate ester plasticizer, ATBC, may be exposed to as much as 20 mg ATBC daily [308]. The 10 mg/kg BW/d TBC dose used in our study was also significantly lower than concentrations experimentally used for DEHP (1000 mg/kg BW/d) and DnBP (2000 mg/kg BW/d) to treat mice in some studies [293, 321]. We have also examined whether lower TBC concentrations can activate PXR in vivo by testing 2 lower doses, 2.5 and 5 mg/kg/d, but neither dose activated PXR. Therefore, the 10 mg/kg/d dose used in this study is close to the lower limit of the TBC concentration that activates PXR in vivo. Considering the wide use TBC in consumer products (e.g., plastic wrap, toys, and drugs), we believe that the dose, which was sufficient to activate PXR activity and cause adverse effects in
vivo, was reasonable. It would be interesting to measure the internal TBC concentrations in human samples and correlate internal TBC concentrations to plasma lipid levels in the future. Such information would provide important insights about the relationship of EDC exposure and disease outcomes in humans.

In addition to TBC, we and others have identified many other environmentally significant chemicals (e.g. BPA, bisphenol B, and DEHP) as PXR agonists. Further, we recently demonstrated that certain EDCs can synergistically activate human PXR [179]. The synergism between different EDCs supports the need to include mixtures for future in vitro and in vivo studies, which may have important implications for environmental chemical risk assessment. In addition to CVD, activation of PXR has been shown to induce tumor aggressiveness in humans and mice [322]. Further studies are needed to investigate whether TBC-mediated intestinal PXR activation can induce tumorigenesis in animal models.

In summary, we have demonstrated that the phthalate substitute plasticizer TBC is potent agonist of PXR. Of particular interest is the finding that TBC activated intestinal PXR but did not affect hepatic PXR activity. Nevertheless, activation of intestinal PXR by TBC was sufficient to increase plasmas cholesterol levels in mice. We then identified the intestinal cholesterol transporter NPC1L1 as a direct transcriptional target of PXR and found that activation of PXR increased cholesterol uptake by intestinal cells. These findings demonstrate a previous unrecognized role of PXR in the regulation of intestine lipid homeostasis. Findings from this study will hopefully stimulate further investigations of phthalate substitute plasticizers, in particular, the mechanisms by which TBC and other chemicals activate PXR, the tissue-specific TBC activation of PXR, and PXR regulation of intestinal lipid homeostasis. Activation of PXR should be taken into consideration for future risk assessment of phthalate substitute plasticizers and related environmental chemicals.
### Table 3.1 Primer sequences for QPCR and ChIP

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Figure 3. Activation of PXR by phthalates and phthalate substitute plasticizers.
Figure 3.1 Activation of PXR by phthalates and phthalate substitute plasticizers.

(A) LS180 cells were cotransfected with full-length human PXR and a CYP3A4-luciferase reporter. Cells were treated with DMSO vehicle, TBC, DEHP, ATBC, ATEC, DiNP, TEC, DnBP, DiBP, and DEP at the indicated concentrations for 24 hours. (B) LS180 cells were cotransfected with a GAL4 reporter and a series of GAL4 DBD-nuclear receptor ligand-binding domain constructs. Cells were treated with DMSO vehicle or 10 µM TBC for 24 hours. CAR, constitutive androstane receptor; ERα, estrogen receptor α. (C and D) LS180 cells were cotransfected with a GAL4 reporter, VP16-hPXR, and expression vector for GAL4 DBD or GAL4 DBD linked to the receptor interaction domains of PXR coactivators (GAL-SRC1 or GAL-PBP) (C) or PXR corepressors (GAL-SMRT or GAL-NCoR) (D). Cells were treated with DMSO vehicle, TBC, or RIF at the indicated concentrations for 24 hours. Data are shown as fold induction of normalized luciferase activity compared with that for DMSO treatment and represent the means from triplicate experiments. Results are presented as mean ± SEM.
Figure 3. TBC activates intestinal PXR but does not affect hepatic PXR activity.
Figure 3.2 TBC activates intestinal PXR but does not affect hepatic PXR activity.
(A) Human HepaRG hepatoma cells and LS180 intestinal cells were treated with control medium or medium containing 10 μM TBC or RIF. Total RNA was isolated, and gene expression levels of human PXR target genes were analyzed by qPCR (n=3; **P<0.01, ***P<0.001; assessed by one-way ANOVA). (B) Primary hepatocytes and enterocytes were isolated from WT mice and were incubated with control medium or medium containing 10 μM TBC or PCN. Total RNA was isolated, and gene expression levels of mouse PXR target genes were analyzed by qPCR (n=3; *P<0.05, **P<0.01, ***P <0.001; assessed by one-way ANOVA). (C) Eight-week-old WT mice were treated with vehicle or 10 mg/kg BW TBC or PCN daily by oral gavage for 1 week. Total RNA was isolated from liver and small intestine. Expression levels of PXR target genes, CYP3A11, GSTA1, and MDR1a, were measured by qPCR (n = 4-5; *P<0.05, **P<0.01, and ***P<0.001 assessed by one-way ANOVA). Results are presented as mean ± SEM.
Figure 3.3 TBC increases PXR reporter activity in human intestinal cells but not in hepatic cells.

(A and B) Human hepatic HepG2 cells (A) or intestinal LS180 cells (B) were cotransfected with full-length hPXR and a CYP3A4-luc reporter. Cells were treated with TBC or RIF at the indicated concentrations for 1, 3, or 6 h. Data are shown as fold induction of normalized luciferase activity compared with DMSO treatment and represent the mean of triplicate experiments. Results are presented as mean ± SEM.
Figure 3.4 TBC does not activate intestinal PXR at 2.5 or 5 mg/kg/day doses.
Eight-week-old WT mice were treated with vehicle control, 2.5, or 5 mg/kg BW of TBC daily by oral gavage for 1 week. Expression levels of PXR target genes, CYP3A11 (A), GSTA1 (B), and MDR1a (C) in the proximal intestine was measured by QPCR (n=5). Results are presented as mean ± SEM.
Figure 3.5 Intraperitoneally delivered TBC does not alter hepatic PXR signaling.

Eight-week-old WT mice were treated with vehicle control or 10 mg/kg BW of TBC daily via intraperitoneal (IP) deliver for 1 week. Expression levels of PXR target genes, CYP3A11, GSTA1, and MDR1a in liver were measured by QPCR (n=5). Results are presented as mean ± SEM.
Figure 3.6 Exposure to TBC induces hyperlipidemia in WT but not in PXR<sup>-/-</sup> mice.

(A) Eight-week-old male WT mice were treated with vehicle or 10 mg/kg BW TBC or PCN daily by oral gavage for 1 week. Plasma total cholesterol levels and lipoprotein levels (LDL, VLDL, and HDL) were measured (n = 6-10; *P<0.05 and ***P<0.001; assessed by one-way ANOVA). (B) Eight-week-old male PXR<sup>-/-</sup> mice were treated with vehicle or 10 mg/kg BW TBC daily by oral gavage for 1 week. Plasma total cholesterol levels and lipoprotein levels (LDL, VLDL, and HDL) were measured (n=4-5). Results are presented as mean ± SEM.
Figure 3.7 Activation of PXR by TBC stimulates the expression of the intestinal cholesterol transporter NPC1L1 in mice and increases cholesterol uptake by murine intestinal cells.

A

B

C

D

E
Figure 3.7 Activation of PXR by TBC stimulates the expression of the intestinal cholesterol transporter NPC1L1 in mice and increases cholesterol uptake by murine intestinal cells.

(A) WT and PXR<sup>−/−</sup> mice were treated with vehicle or 10 mg/kg BW TBC daily for 1 week. Total RNA was isolated from small intestine, and the expression levels of indicated genes were measured by qPCR (n=5-6; *P<0.05; assessed by student’s <i>t</i>-test). (B) Western blot analysis of intestinal NPC1L1 protein levels in control or TBC-treated WT and PXR<sup>−/−</sup> mice. The top band indicates glycosylated NPC1L1. (C and D) Primary enterocytes isolated from WT and PXR<sup>−/−</sup> mice were treated with vehicle control or 10 µM TBC for 3 hours. NPC1L1 mRNA levels were measured by qPCR (n=3; ***P<0.001; assessed by student’s <i>t</i>-test), and protein levels were analyzed by Western blot (D). (E) Primary enterocytes isolated from WT and PXR<sup>−/−</sup> mice were treated with vehicle control or 10 µM TBC for 2 hours, followed by incubation with [3H]-cholesterol and TBC for 1 hour. The cellular cholesterol uptake was then measured (n=3; *P<0.05, **P<0.01; assessed by two-way ANOVA). Results are presented as mean ± SEM.
Figure 3.8 Both TBC and PCN can stimulate intestinal NPC1L1 expression in vivo.

Eight-week-old WT mice were treated with vehicle control, 10 mg/kg BW of TBC, or PCN daily by oral gavage for 1 week. Expression levels of intestinal PXR were measured by QPCR (n=5, *P<0.05, ***P<0.001; assessed by one-way ANOVA). Results are presented as mean ± SEM.
Figure 3.9 TBC promotes cholesterol uptake by human intestinal cells.
(A) Western blot analysis of PXR levels in human intestinal LS180 cells transfected with control siRNA or siRNA against PXR (siPXR).
(B) Control or siPXR LS180 cells were treated with 10 µM TBC for 3 hours, and NPC1L1 expression was analyzed by qPCR (n=3; ***P<0.001; assessed by student’s t-test). (C) Control or siPXR LS180 cells were treated with 10 µM TBC for 2 hours followed by incubation with [³H]-cholesterol and TBC for an additional 1 hour. The cellular cholesterol uptake was then measured (n=3; *P<0.05, **P<0.01; assessed by two-way ANOVA). Results are presented as mean ± SEM.
Figure 3.10 NPC1L1 is a direct transcriptional target of PXR.
Figure 3.10 NPC1L1 is a direct transcriptional target of PXR.

(A) In vitro translated human PXR and RXR, as indicated, were incubated with $^{32}$P-labeled NPC1L1/DR-4 or CYP3A4/ER-6 probes and analyzed by EMSA. Ten- or 25-fold excess of unlabeled NPC1L1/DR-4 or CYP3A4/ER-6 probes were used for the competition experiments. (B) PXR/RXR proteins were incubated with 2 different anti-PXR antibodies, goat anti-PXR (1) or rabbit anti-PXR antibodies (2) for 1 hour before the addition of the $^{32}$P-labeled NPC1L1/DR-4 probe. (C) PXR/RXR proteins were incubated with $^{32}$P-labeled WT NPC1L1/DR-4 or mutated DR-4 (DR-4m1 and DR-4m2) probes and were analyzed by EMSA. (D) LS180 cells were treated with 10 µM TBC for 3 hours, and ChIP analysis was performed to determine the recruitment of PXR onto the NPC1L1 or CYP3A4 promoter. (E–G) LS180 cells were cotransfected with full-length hPXR and RXR expression plasmids along with a synthetic reporter containing 4 copies of NPC1L1/DR-4 element (E) or mutated DR-4 elements, DR-4m1 (F) and DR-4m2 (G). Cells were then treated with DMSO vehicle (control), TBC, or RIF at the indicated concentrations for 24 hours. Data are shown as fold induction of normalized luciferase activity compared with that for DMSO treatment and represent the means for triplicate experiments. Results are presented as mean ± SEM.
Chapter 4: Targeting IKKβ in Adipocyte Lineage Cells for Treatment of Obesity and Metabolic Dysfunctions

4.1 Introduction

Obesity is a rapidly growing epidemic representing a serious threat to the health of populations in an increasing number of countries and the number of overweight and obese individuals are expected to increase to over half of the world’s population by 2030 [323]. There is an urgent need to understand the mechanisms underlying obesity and obesity-related metabolic diseases. Obesity is associated with both increased adipocyte size (hypertrophy) and adipocyte number (hyperplasia). Adipocyte number is a major determinant of fat mass in adults [252, 324] and approximately 10% of the body’s adipocytes are re-generated annually at all adult ages [324]. Obese individuals also have a significantly greater number of adipocytes added per year than lean individuals [252, 324]; suggesting that regulation of new adipocyte production is a potential therapeutic target to treat obesity. However, the mechanisms underlying nutritionally induced hyperplasia remain largely unknown.

It is generally accepted that obesity is associated with a state of chronic low-grade inflammation that is a major contributor to type 2 diabetes and atherosclerosis [325, 326]. Many inflammatory pathways that contribute to the pathogenesis of insulin resistance and atherosclerosis are regulated by the transcriptional factor NF-κB, a master regulator of the innate and adaptive immune responses [238]. IκB kinase (IKK) β is the predominant catalytic subunit of the IKK complex and is required for activation of NF-κB by inflammatory mediators in the canonical or classical activation pathway [238, 239, 255, 271, 327]. It has been well established that overnutrition can lead to IKKβ activation in vitro and in vivo [327-329], and recent studies have implicated IKKβ as a key molecular link between obesity, inflammation and metabolic disorders [325, 330, 331]. For example, diet-induced insulin resistance has been associated with the activation of IKKβ/NF-κB in multiple tissues including
liver, adipose tissue, and brain [241, 244, 247-249, 327, 332]. Deletion of IKKβ in the liver improved diet-induced insulin resistance, and deficiency of IKKβ in myeloid cells rendered global insulin sensitivity upon high-fat (HF) feeding [244]. By contrast, constitutive activation of IKKβ in the liver caused systemic insulin resistance [247]. Activation of IKKβ in the hypothalamus has also been linked to obesity and metabolic disease [249, 333].

We have recently demonstrated that IKKβ functions in smooth muscle cells (SMCs) to regulate vascular inflammatory responses and atherosclerosis development [255]. Of particular interest is that many adipocyte precursor cells express SMC markers and ablation of IKKβ blocked adipocyte differentiation in vitro and in vivo, suggesting that IKKβ functions in adipocyte precursor cells to regulate adipose tissue development [255]. In the present study, we explored a novel and more efficient pharmacological approach to inhibit IKKβ in vivo by using antisense oligonucleotides (ASOs) and found that ASO-mediated IKKβ knockdown ameliorated diet-induced obesity and metabolic disorders in mice. Interestingly, IKKβ ASO also inhibited high-fat diet (HFD)-elicited adipocyte differentiation and reduced adipose tissue growth. As the functions of IKKβ in regulating adipogenesis and adipose tissue development has not been fully understood, we then selectively deleted IKKβ in the white adipose lineage in mice to further elucidate the role of adipose progenitor cell IKKβ signaling in obesity and metabolic function. Deficiency of IKKβ decreased adipogenesis and systemic inflammation elicited by HF feeding, leading to resistance to diet-induced obesity and insulin resistance. Lastly, inhibition of IKKβ in human adipose stem cells also blocked adipogenesis in these cells. Our results establish IKKβ as an important regulator of adipogenesis and adipose tissue development. Overnutrition-mediated IKKβ activation may serve as an initial signal that triggers adipose progenitor cell differentiation in response to consumption of a HFD. Inhibition of IKKβ with antisense therapy may present as a novel therapeutic approach to combat obesity and metabolic dysfunctions.
4.2 Materials and Methods

4.2.1 Animals and Diet
For the IKKβ ASO studies, 8-week-old male C57BL/6 mice (Jackson Laboratory) were fed a normal chow diet (ND) or a Western-type HFD (21.2% fat, 0.2% cholesterol; Harlan Teklad) and received biweekly intraperitoneal injections of either a nontargeting control ASO (5'-CCTTCCCTGAAGGTTCCTCC-3') or an IKKβ targeted ASO (5'-GCAGACTCTCATCCTCCGTC-3') for 8 weeks at a dose of 25 mg/kg body weight/week. The 20-mer phosphorothioate ASOs were designed to contain 2'-O-methoxyethyl groups at positions 1 to 5 and 15 to 20, and were generated and purified by Ionis Pharmaceuticals (Carlsbad, CA). For cell lineage analysis, PDGFRβ-Cre mice [334] were crossed with Rosa26lacZ reporter mice [335] to generate PDGFRβ-Cre/Rosa26lacZ mice. To delete IKKβ in adipocyte lineage cells, mice containing loxP-flanked IKKβ alleles (IKKβ F/F ) [255, 336] was crossed with PDGFRβ-Cre transgenic mice [334] to generate PDGFRβ-Cre/IKKβ F/F mice (termed as IKKβ ΔPDGFRβ). For obesity studies, 4-week-old male IKKβ F/F and IKKβ ΔPDGFRβ littermates were fed a normal chow diet (ND) or a HFD for 16 weeks until euthanization at 20 weeks of age. For the BrdU studies, 4-week-old male IKKβ F/F and IKKβ ΔPDGFRβ littermates were intraperitoneal injected with 50 mg/kg body weight/day BrdU (TCI America) and mice were placed on a HFD for 7 days prior to euthanization. All animals were housed in a specific pathogen-free environment with a light-dark cycle, under a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee.

4.2.2 Metabolic Analysis
Body weight was measured weekly and body composition was measured by NMR spectroscopy (Echo MRI). Intraperitoneal glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed, as previously described [255]. Plasma insulin was measured using a Rat/Mouse Insulin ELISA kit (Millipore). Plasma TNFα levels were measured by a mouse TNFα ELISA kit (BioLegend). Plasma cytokine levels were measured by a mouse cytokine multiplex assay kit.
and a BioPlex 200 system (Bio-Rad Laboratories). For insulin stimulation studies, 20-week-old male IKKβ\textsuperscript{F/F} and IKKβ\textsuperscript{ΔPDGFRβ} littermates or ASO-treated C57BL/6 mice were injected with insulin (0.35 U/kg body weight) into the inferior vena cava [337]. After 5 minutes, mice were euthanized and tissues were collected for protein isolation and Western blot analysis.

4.2.3 Glucose Uptake in Adipose Tissue Explants
Glucose uptake in adipose tissue explants was achieved following a previously described protocol [337]. Briefly, HFD-fed IKKβ\textsuperscript{F/F} and IKKβ\textsuperscript{ΔPDGFRβ} littermates were fasted at the beginning of the light cycle for 7 h and epiWAT was collected. The tissue explants were minced into small pieces and incubated for 2 h in 1% BSA-Krebs-Ringer Bicarbonate (KRB) buffer. The tissues were washed and then incubated with saline or 17 nm insulin for 40 min at 37°C. After the insulin incubation, 1 μCi [\textsuperscript{3}H]-2-deoxyglucose (Perkin Elmer) was added to the buffer and incubated for another 20 min at 37°C and then the tissues were washed with cold 1% BSA-KRB and the weight of the tissues were measured and recorded. The explants were then added to 1 ml 1N NaOH and incubated for 1 h at 65°C. Half of the cell lysate was then used to measure the amount of radioactivity in a scintillation counter and a portion was used to measure the protein concentration.

4.2.4 MRI Analysis and Data Segmentation
HFD-fed IKKβ\textsuperscript{F/F} and IKKβ\textsuperscript{ΔPDGFRβ} littermates were imaged on a 7T ClinScan MRI (Bruker, Ettlingen, Germany) using a 2 point Dixon technique and a 72 mm diameter circularly polarized transmit receive coil at the Magnetic Resonance Imaging and Spectroscopy Center at the University of Kentucky. Anesthesia was induced and maintained using 1-2 % isoflurane in oxygen at a rate of 1.0 L/min. Respiration and temperature were monitored. Temperature was maintained between 36-37 °C using a water bath and tubing. Image data sets including a fat only and water only image were obtained using a dual echo Flash sequence with TR 12ms, TE 2ms and 3.5ms; flip 15°, FOV 80mm x 57.5mm x 31.7, and resolution 0.18mm x 0.18 mm x 0.18 mm. The fat and water images were generated from the in and out of phase images using Siemens Syngo software. FSL software was used for image analysis [338]. The
fat image was resampled to 1.8mm x 0.18mm x 0.18mm using FSL. The visceral cavity was segmented out manually. The total fat volume was calculated by taking a histogram from the fat only image of the visceral cavity and including voxels that were greater than 80% of the fat peak. 80% was chosen to include adipose tissue and exclude voxels from the liver [339].

4.2.5 Histological Analysis
For hematoxylin and eosin staining of liver, tissues were fixed in 4% neutral buffered formalin and embedded in paraffin. 5 μm tissue sections were stained with hematoxylin and eosin following standard protocols. Oil-Red O staining of hepatic neutral lipids was performed as previously described [255]. In brief, livers were embedded in OCT and sectioned at 10 μm. Liver sections were then dried, fixed in 4% PFA, incubated for 5 min in 60% isopropanol then incubation in 0.3% Oil-Red O (Sigma) for 20 min. The livers were counterstained with hematoxylin and mounted with glycerol-gelatin (Sigma).

4.2.6 Immunohistochemistry
Immunohistochemistry was performed on 5 μm adipose tissue sections embedded in paraffin. For F4/80 staining, rehydrated sections were subjected to antigen retrieval by heating in antigen unmasking solution (Vector Laboratories). Slides were cooled and treated with 5% BSA, 3% hydrogen peroxide, and avidin/biotin blocking kit (Vector Laboratories). Sections were incubated with anti-F4/80 (Serotec) or rat IgG (negative control; Jackson Immunoresearch) antibodies at 4°C overnight. Sections were then incubated with biotinylated anti-rat IgG (Vector) for 1 h, VECTASTAIN ABC reagent (Vector Laboratories) for 30 min at room temperature and visualized by the avidin-biotin-complex method using the ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories). Sections were then mounted with glycerol-gelatin. For BrdU staining, sections were incubated with anti-BrdU (Life Sciences; B35130) antibodies for 30 min using a M.O.M. Immunodetection kit (Vector Laboratories) per manufacturer’s instructions. Sections were then counterstained with DAPI. Percent BrdU-positive cells were calculated by dividing BrdU-positive cells by DAPI-positive cells. Two view fields were randomly selected from each mouse for calculation of BrdU-positive cells.
4.2.7  3T3L1 Cell Differentiation and Transfection Assays
3T3-L1 cells were obtained from American Type Culture Collection. For adipocyte differentiation assays, confluent 3T3-L1 cells were exposed to differentiation media (high-glucose DMEM supplemented with 10% FBS and differentiation cocktail including 1 ug/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine[255]. Forty eight hours post-induction, the cells were maintained in high-glucose DMEM containing insulin (1ug/ml) and 10% FBS until they were ready for analysis [255]. The Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) method was used to delete IKKβ in 3T3-L1 cells [340]. Briefly, annealed oligos (5'-CACCGGCGGCGCGGTTGGGGCCATACT-3') were cloned using BsmBI digestion into the lentiCRISPRv2 plasmid (generously provided by Dr. Feng Zhang, Addgene plasmid #52961). The lentivirus was prepared in 293T cells and the viral supernatant was then used to infect 3T3-L1 cells. Cells were selected with puromycin and single clones were expanded and screened by western blot. Further, the Transgenomic Surveyor Mutation Detection Kit (Fisher Scientific) was used to verify genomic mutations within the IKKβ locus. For transfection assays, 3T3-L1 cells were transfected with the TOP-flash reporter [341] and β-gal control plasmid [342] by electroporation using Amaxa Cell Line Nucleofector kit V (Lonza). Two days post-transfection, cells were lysed and luciferase and β-gal values were measured as previously described [299, 342]. We would like to thank Dr. Brett Spear (University of Kentucky) for the TOP-flash reporter.

4.2.8  Adipose SV Cell Isolation and Differentiation
Adipose SV cells and mature adipocytes were isolated as previously described [255]. The SV cells were used for RNA and protein isolation or cultured in 12-well plates for differentiation. For differentiation assays, SV cells were induced by high-glucose DMEM containing dexamethasone (1 μM), isobutylmethylxanthine (0.5 mM), insulin (10 μg/ml), rosiglitazone (1 μM), and 10% FBS until they were ready for analysis [255].

4.2.9  RNA Isolation and QPCR Analysis
Total RNA was isolated from mouse tissues or cells using TRIzol Reagent
QPCR was performed using gene-specific primers and the SYBR Green PCR kit (Life Technologies) as previously described [179, 299]. The sequences of primer sets used in this study are listed in Table 4.1.

### 4.2.10 Western Blot Analysis

Western blot was performed as previously described [255, 271]. Briefly, protein was isolated from cells or tissues with homogenization in RIPA buffer containing complete mini protease inhibitor cocktail (Roche). Protein concentrations were determined by the Pierce BCA protein assay kit (Thermo Fisher Scientific). Anti-IKKβ, anti-IKKα, anti-Smurf2, and anti-TFIIB were purchased from Cell Signaling Technology; anti-β-catenin and anti-β-actin were purchased from Sigma-Aldrich. For immunoprecipitation experiments [255], control or IKKβ knockdown cells, or adipose SV cells, were incubated with 100 nM of PS-341 for 4 h. The whole-cell lysates were isolated, incubated with anti-β-catenin antibody overnight at 4°C, and then incubated with Protein A Agarose beads (Roche) for another 5 h. The samples were washed and analyzed by Western blot using anti-ubiquitin monoclonal antibodies [255].

### 4.2.11 Human Subjects and ADHASC Isolation

Human adipocytes were derived from the differentiation of adult derived human adipocyte stem cells (ADHASC) as described previously [343, 344]. The adipose tissue was from a collagenase digestion of the lipoaspirate of patients undergoing liposuction of subcutaneous fat. These patients were generally young, healthy women undergoing cosmetic procedures, and this method of collection was approved by the University of Kentucky Institutional Review Board. Differentiation was induced using differentiation media as previously described [343, 344]. At least 80 to 90% of the cells developed lipid droplets in 7-10 days.

### 4.2.12 Quantification of Mitochondrial DNA

Genomic DNA was extracted from subcutaneous WAT by the DNAeasy Blood and Tissue kit (Qiagen). QPCR was performed on COX1 mitochondrial DNA and normalized to a nuclear 28S sequence [345, 346]. The sequences of primer sets used in this study are listed in Supporting Information Table 4.1.
4.2.13 Statistical Analysis
All data are presented as the mean ± SEM. Individual pairwise comparisons were analyzed by two-sample, two-tailed Student’s t-test unless otherwise noted, with $P<0.05$ was regarded as significant. Two-way analysis of variance (ANOVA) was used when multiple comparisons were made followed by a Bonferroni multiple comparisons test. $N$ numbers and the statistical tests are listed in figure legends. All statistics were done using SigmaPlot 13.0.

4.3 Results

4.3.1 ASO-Mediated IKKβ Knockdown Protects Mice from Diet-Induced Obesity
We recently demonstrated that IKKβ inhibitors can inhibit adipocyte differentiation in vitro and ameliorate diet-induced adiposity in mice [255]. To explore novel and more efficient pharmacological approaches for IKKβ inhibition, we utilized second-generation ASO targeting technology [347] and investigated the impact of IKKβ ASO treatment on diet-induced obesity and metabolic disorders. Subcutaneous delivery of ASO has been shown to reach a variety of tissues including liver and adipose tissue [348, 349]. Indeed, we found that IKKβ ASO can efficiently decrease IKKβ gene expression in multiple tissues including liver, skeletal muscle, and white adipose tissue (WAT) at a relatively low dose, 25 mg/kg BW/week (Fig. 4.1, A). Interestingly, IKKβ mRNA levels were not significantly altered in brown adipose tissue (BAT) by IKKβ ASO treatment at this dose. Western blot analysis also confirmed the specific and efficient IKKβ knockdown in tissues including liver and WAT as IKKα protein levels were not affected by IKKβ ASO treatment (Fig. 4.1, B).

We next determined whether IKKβ ASO treatment can ameliorate diet-induced obesity. Groups of 8-week-old wild-type male mice were fed a ND or a HFD and were treated with control ASO or IKKβ ASO for 8 weeks (Fig. 4.1, C). HF-feeding increased body weight in mice treated with both control and IKKβ ASOs. However, IKKβ ASO treatment significantly decreased HFD-induced body weight gain and adiposity. While lean mass was slightly but significantly increased in IKKβ ASO-treated mice, fat mass was decreased by 45% in HFD-
fed mice treated with IKKβ ASOs (Fig. 4.1, D). IKKβ ASO was able to decrease both subcutaneous (sub) WAT and visceral WAT including epididymal (epi) and retroperitoneal (retro) fat pads in HFD-fed mice as compared with littermate controls (Fig. 4.1, E).

4.3.2 IKKβ ASOs Improves Insulin Sensitivity and Reverses Hepatic Steatosis in Obese Mice

Obesity is frequently associated with metabolic disorders such as insulin resistance and hepatic steatosis. We next investigated whether IKKβ ASO treatment can protect HFD-fed mice from these disorders. IKKβ ASO-treated mice had decreased fasting plasma glucose and insulin concentrations as compared with control mice (Fig. 4.1, F), suggesting improved diabetic phenotype. Upon glucose and insulin tolerance test, IKKβ ASO-treated mice had improved glucose tolerance and showed an increased hypoglycemic response to the injected insulin (Fig. 4.1, G). To further assess the impact of IKKβ ASO treatment on insulin signaling, HFD-fed mice were injected with insulin prior to euthanization and phosphorylation of Akt was analyzed in multiple tissues. IKKβ ASO was able to enhance phosphorylation of Akt in response to insulin in liver, skeletal muscle and WAT (Fig. 4.1, H). While hepatic IKKβ signaling has been demonstrated to contribute to obesity-associated insulin resistance [244, 247], the role of adipose IKKβ signaling in the regulation of insulin sensitivity has not been well-defined. We then performed adipose glucose uptake assays. Interestingly, adipose tissue explants from IKKβ ASO-treated mice had elevated glucose uptake in the absence of insulin and insulin stimulation further enhanced glucose uptake by adipose tissues (Fig. 4.1, I). Therefore, the enhanced Akt phosphorylation and increased glucose uptake in adipose tissue likely contribute to the improved insulin sensitivity in IKKβ ASO-treated mice. Further, HF feeding also caused lipid accumulation and hepatic steatosis in control mice. However, IKKβ ASO-treated mice were protected from these detrimental effects (Fig. 4.1, J and K). Consistently, hepatic triglyceride and cholesterol contents were significantly reduced in IKKβ ASO-treated mice (Fig. 4.1, L). Collectively, these results suggest that pharmacological inhibition of IKKβ by ASO ameliorates obesity-
associated metabolic disorders in mice.

4.3.3 **IKKβ Regulates Murine Adipocyte Differentiation**

We next investigated whether IKKβ ASO treatment can also affect IKKβ expression in adipocyte precursor cells and affect adipocyte differentiation. Indeed, we found that IKKβ expression was decreased in both adipose SV cells and mature adipocytes in IKKβ ASO-treated mice (Fig. 4.2, A). Consistent with our previous study [255], ASO-mediated IKKβ knockdown diminished the ability of adipose SV cells to differentiate into adipocytes (Fig. 4.2, B). As expected, the expression levels of adipogenic genes including PPARγ, Zfp423, and C/EBPs were significantly decreased in epiWAT from IKKβ ASO-treated mice (Fig. 4.2, C). Further, IKKβ ASO treatment also decreased the expression of a known NF-κB target, Smad ubiquitination regulatory factor 2 (Smurf 2) (Fig. 4.2, D). Smurf2 is an ubiquitin E3 ligase that regulates proteasome-mediated degradation of several proteins including β-catenin [255, 350, 351]. Consistently, ASO-mediated IKKβ knockdown increased nuclear β-catenin protein levels in epiWAT (Fig. 4.2, E). Since Wnt/β-catenin signaling has been well defined to inhibit adipogenesis in vitro and in vivo [352, 353], the increased Wnt signaling likely contributes to the decreased adipogenesis in IKKβ ASO-treated mice.

To further define the role of IKKβ in adipogenesis, we used the CRISPR-Cas9 system to delete the IKKβ gene in murine 3T3-L1 preadipocytes (Fig. 4.2, F). We found that deletion of IKKβ almost completely blocked 3T3-L1 cell differentiation (Fig. 4.2, G). Gene expression analysis showed that mRNA levels of adipogenic genes and adipocyte markers including PPARγ and adiponectin were significantly decreased by IKKβ-deficiency (Fig. 4.2, H). Consistently, deletion of IKKβ decreased the expression of Smurf2 (Fig. 4.2, I) and increased nuclear β-catenin accumulation (Fig. 4.2, J), leading to increased β-catenin activity in these cells (Fig. 4.2, K). We further confirmed that β-catenin ubiquitination was inhibited by IKKβ deficiency in 3T3-L1 cells (Fig. 4.2, L), suggesting the impact of reduced Smurf2 expression. Taken together, these results confirm the important role of IKKβ in adipogenesis and indicate that
pharmacological inhibition of IKKβ by ASO can decrease adipogenesis and diet-induced adiposity.

4.3.4 Targeted Deletion of IKKβ in the White Adipose Lineage

Since ASO treatment affects many tissues and cell types, it is not clear how significantly the ASO-mediated IKKβ knockdown in adipose progenitor contributes to the beneficial effects on obesity and metabolic disorders in mice. Compared with other tissues or cell types, the role of IKKβ signaling in adipose progenitor cells has not been well-defined. To investigate the function of IKKβ signaling in adipose progenitors, we sought to generate a mouse model that selectively lacks IKKβ in white adipose lineage. Recent studies have identified a subset of perivascular cells as adipose progenitors which express multiple mural stem cell markers, including platelet-derived growth factor receptor (PDGFR) β, α smooth muscle actin (SMA), and NG2 [354]. For example, PDGFRβ, a human mesenchymal stem cell (MSC) marker [355], and another isoform of PDGFR, PDGFRα have been confirmed to be adipose progenitor markers by independent groups [356-359]. Consistently, we also found high expression levels of PDGFRβ in adipose SV cells as compared with mature adipocytes (Fig. 4.3, A). We then crossed PDGFRβ promoter-drive Cre mice [334] with Rosa26lacZ reporter mice that express β-galactosidase (lacZ) in target tissues upon Cre-mediated excision of a “Stop” sequence to generate PDGFRβ-Cre/Rosa26lacZ mice (Fig. 4.3, B). While none of the adipose SV cells from control Rosa26lacZ mice were lacZ+ cells, many of the adipose SV cells from PDGFRβ-Cre/ Rosa26lacZ mice were lacZ+ cells (Fig. 4.3, C). Staining of the white adipose depots and tissue sections also indicated that PDGFRβ-Cre is activated in adipose progenitors that give rise to white adipocytes as PDGFRβ generated strong lacZ expressed in adipocytes, in addition to the vasculature (Fig. 4.3, D and E). Taken together, our results are consistent with previous reports [354, 357] and confirm that PDGFRβ is a marker for adipose lineage cells.

We then deleted IKKβ in adipocyte lineage cells by intercrossing PDGFRβ-Cre mice with mice containing loxP-flanked IKKβ alleles (IKKβF/F) [255, 336].

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The resulting PDGFRβ-Cre/IKKβF/F (termed IKKβΔPDGFRβ) mice were viable and appeared indistinguishable from their control IKKβF/F littermates. Western blot analysis confirmed the deletion of IKKβ in WAT including epiWAT and subWAT but not in BAT, liver, and skeletal muscle (Fig. 4.4, A). As expected, PDGFRβ-Cre-mediated IKKβ deletion also caused reduced IKKβ expression in adipose SV cells of IKKβΔPDGFRβ mice (Fig. 4.4, B). Further, IKKβ deficiency reduced NF-κB-mediated proinflammatory gene expression in response to LPS in adipose SV cells of IKKβΔPDGFRβ mice (Fig. 4.4, C-G), suggesting impaired NF-κB activation in these cells.

4.3.5 Deficiency of IKKβ in Adipocyte Lineage Cells Renders Mice Resistant to Diet-Induced Obesity

To determine the role of adipose progenitor IKKβ signaling in obesity, IKKβF/F and IKKβΔPDGFRβ littermates were fed a normal chow diet (ND) or a HFD for 16 weeks. There were no significant differences in body weight for ND-fed mice. Body weight was significantly increased following HFD-feeding for both IKKβF/F and IKKβΔPDGFRβ mice; however, IKKβ deficiency significantly decreased diet-induced bodyweight gain in HFD-fed mice (Fig. 4.5, A). While lean mass was not affected by IKKβ deficiency, HFD-fed IKKβΔPDGFRβ mice had significantly decreased fat mass as compared with control littermates (Fig. 4.5, B).

HFD-fed IKKβΔPDGFRβ mice also had decreased visceral adipose pads including epididymal and retroperitoneal fat pads. However, deficiency of IKKβ did not affect the size or weight of subWAT and BAT (Fig. 4.5-4.6). As subWAT often undergoes “browning” process which enhances oxidative metabolism and protects mice from metabolic dysfunctions [254, 345, 360, 361], we then examined whether deficiency of IKKβ affects “browning” of subWAT in these mice. Interestingly, the expression levels of beige or brown adipocyte markers as well as mitochondrial function-related genes such as UCP-1 and PGC-1α [345, 360, 361] were not affected in subWAT of IKKβΔPDGFRβ mice (Fig. 4.7, A). Consistently, IKKβΔPDGFRβ mice also had comparable mitochondrial content in subWAT as that of control littermates (Fig. 4.7, B). MRI analyses confirmed that IKKβΔPDGFRβ mice had a significantly decreased volume of visceral adipose pads.
tissue but a similar subcutaneous adipose tissue volume as compared with IKKβ<sup>F/F</sup> mice under HF feeding conditions (Fig. 4.5-4.6). We next analyzed the expression levels of IKKβ and adipose progenitor markers in SV cells isolated from subWAT and epiWAT of control mice. Interestingly, IKKβ expression levels were significantly higher in visceral SV cells than subcutaneous SV cells. Further, the expression levels of adipose progenitor markers such as PDGFRβ were much higher in visceral SV cells as compared with subcutaneous SV cells, indicating more abundant adipose progenitor cell population in visceral WAT (Fig. 4.8). Collectively, these results demonstrate that PDGFRβ-Cre-driven IKKβ deficiency in the white adipose lineage decreases diet-induced obesity and visceral adiposity.

### 4.3.6 IKKβ-Deficient Mice are Protected From Obesity-Associated Metabolic Disorders

We next investigated whether deficiency of IKKβ also protected mice from obesity-associated insulin resistance. IKKβ<sup>ΔPDGFRβ</sup> mice had comparable glucose and insulin levels and similar glucose tolerance and insulin tolerance as that of IKKβ<sup>F/F</sup> when fed a ND (Fig. 4.9-4.10). As expected, HF feeding increased plasma glucose and insulin levels in both IKKβ<sup>F/F</sup> and IKKβ<sup>ΔPDGFRβ</sup> mice (Fig. 4.9, A). Although glucose concentrations were not affected by IKKβ deficiency, HFD-fed IKKβ<sup>ΔPDGFRβ</sup> mice had significantly decreased insulin concentrations as compared with HFD-fed control littermates (Fig. 4.9, A). Consistently, IKKβ<sup>ΔPDGFRβ</sup> mice also had improved glucose tolerance and insulin tolerance as compared with IKKβ<sup>F/F</sup> mice under HF feeding conditions (Fig. 4.9, B). PDGFRβ-Cre-mediated IKKβ deletion also enhanced phosphorylation of Akt in response to insulin in WAT but not in liver and skeletal muscle (Fig. 4.9, C). Further, adipose tissue explants from IKKβ<sup>ΔPDGFRβ</sup> mice had significantly increased insulin-stimulated glucose uptake compared with that of IKKβ<sup>F/F</sup> mice (Fig. 4.9, D).

Obesity-associated macrophage infiltration contributes to the development of systemic insulin resistance [362]. Although IKKβ expression levels were not affected in macrophages of IKKβ<sup>ΔPDGFRβ</sup> mice (Fig. 4.9, E), macrophage infiltration was substantially decreased in WAT of IKKβ<sup>ΔPDGFRβ</sup> mice fed a HFD.
suggesting that deficiency of IKKβ in adipose lineage blocks the increased inflammatory infiltrates in WAT under obese conditions. Consistently, the mRNA levels of macrophage markers and several key pro-inflammatory cytokines including TNFα, MCP-1, and IL-1β were significantly decreased in WAT of IKKβΔPDGFRβ mice (Fig. 4.9, G). Next, we measured plasma cytokine levels to determine whether IKKβ-deficient mice also had decreased systemic inflammation. Indeed, deficiency of IKKβ significantly decreased HFD-induced plasma pro-inflammatory cytokines including TNFα, MCP-1 and IL-6 in IKKβΔPDGFRβ mice (Fig. 4.9, H). Taken together, deficiency of IKKβ improved insulin signaling in adipose tissue and protected mice from obesity-associated metabolic disorders.

Deficiency of IKKβ Inhibits Adipogenesis in Mice

We next investigated whether deficiency of IKKβ can decrease HF feeding-elicited adipogenesis *in vivo*. For this experiment, IKKβ F/F and IKKβΔPDGFRβ mice were treated with BrdU during the first week of HF feeding. We then used BrdU as a marker to track newly differentiated adipocytes in these mice after 7d of HFD. Immunostaining of adipose tissue sections with BrdU antibodies revealed that IKKβΔPDGFRβ mice had significantly decreased BrdU positive adipocytes in WAT (Fig. 4.11, A and B), indicating decreased adipogenesis. We also assessed the adipogenic potential of adipose SV cells isolated from visceral adipose tissue of IKKβ F/F and IKKβΔPDGFRβ mice and confirmed that IKKβ deficiency impaired the adipogenic potential of SV cells from IKKβΔPDGFRβ mice (Fig. 4.11, C). Further, the expression levels of adipocyte markers and key adipogenic genes including PPARγ, C/EBPα, and Zfp423, were significantly reduced by IKKβ deficiency (Fig. 4.11, D). Consistent with results from IKKβ ASO treatment and 3T3-L1 experiments (Fig. 2), IKKβΔPDGFRβ mice had decreased Smurf2 levels and increased nuclear β-catenin levels in epiWAT (Fig. 4.11, E and F) as well as in primary adipose SV cells (Fig. 4.11, G and H). Further, deficiency of IKKβ substantially decreased β-catenin ubiquitination in adipose SV cells (Fig. 4.11, I), which likely contributes to the accumulation of nuclear β-catenin in IKKβΔPDGFRβ mice.
4.3.8 Inhibition of IKKβ Decreases Adipogenesis in Human Adipose Stem Cells

In addition to murine 3T3-L1 preadipocytes and primary SV cells, we also determined whether IKKβ regulates the differentiation of human adipose stem cells. Human adipose stem cells, also known as adult-derived human adipose stem cells (ADHASCs) [343, 344, 363], were isolated from healthy subjects. Consistent with previous reports [343, 344, 363], human adipose stem cells were able to efficiently differentiate into mature adipocytes in vitro and at least 80 to 90% of the cells developed lipid droplets in 7-10 days (Fig. 4.12, A). Pharmacological inhibition of IKKβ by BMS-345541 strongly inhibited human adipose stem cell differentiation and repressed human adipogenic gene expression (Fig. 4.12, B). BMS-345541 treatment also led to reduced Smurf2 expression and decreased β-catenin ubiquitination, leading to increased nuclear β-catenin levels (Fig. 4.12, C-E), which likely contribute to the decreased adipogenesis in human adipose stem cells. Collectively, these results demonstrated IKKβ as an important regulator of both human and murine adipocyte differentiation.

4.4 Discussion

As a central coordinator of inflammatory responses, IKKβ signaling in multiple tissues including liver, pancreas, and brain have been associated with obesity and obesity-related metabolic dysfunctions [241, 244, 247, 249, 333, 364]. High doses of IKKβ inhibitors such as salicylates have been used to treat inflammatory conditions including diabetes in humans for more than a century [365, 366]. Moreover, inhibition of IKKβ activity by salicylates also protected mice against insulin resistance triggered by HFD or obesity [241, 248]. Interestingly, long-term anti-inflammatory therapy has also been associated with weight loss in human studies [367]. We recently demonstrated that salicylates and the potent IKKβ inhibitor, BMS-345541, can also inhibit adipocyte differentiation in a dose-dependent manner [255]. In the current study, we demonstrated that IKKβ ASO can efficiently decrease IKKβ expression in
various tissues including liver and WAT, and ameliorate diet-induced obesity and metabolic disorders in mice. To our knowledge, our study is the first to use ASOs targeting IKKβ in vivo and investigate its metabolic impact. Consistent with previous reports demonstrating that hepatic IKKβ signaling contribute to insulin resistance [244, 247], IKKβ ASO-treated mice had improved insulin signaling in the liver. Nevertheless, knockdown of IKKβ in WAT also resulted in enhanced Akt phosphorylation and increased glucose uptake. Therefore, the improved diabetic phenotype in IKKβ ASO-treated mice was likely due to the repressed IKKβ signaling in multiple tissues including liver and WAT. In addition to improved insulin signaling, IKKβ ASO treatment also repressed IKKβ expression in SV cells, leading to decreased adipogenesis in WAT. Collectively, our studies demonstrate IKKβ as a potential target for future anti-obesity drugs and provide evidence for the use of appropriate IKKβ ASOs as a potential therapeutic strategy to treat obesity and metabolic disease (Fig.4.12, A-C).

Despite substantial progress in defining transcription factors such as PPARγ and C/EBPs in the regulation of committed preadipocyte differentiation, the initial signals that trigger adipose progenitor cell commitment to adipocyte lineage in response to overnutrition remained unknown. In addition to its well-established role as a central mediator of inflammation and immune responses, IKKβ/NF-κB signaling plays key roles in the regulation of hematopoietic cell development and stem cell differentiation [350, 368-370]. It has also been reported that NF-κB activity increases during adipocyte differentiation [371] and IKKβ/NF-κB activation is associated with increased adipogenesis and insulin resistance in maternal obesity [372]. Further, pro-inflammatory signals have been demonstrated to be important for adipogenesis in vivo and many IKKβ/NF-κB activators including LPS, IL-1β, and MCP-1 can stimulate adipogenesis and promote adipocyte differentiation [373-375]. While the role of IKKβ signaling in regulating HFD-elicited tissue inflammation and insulin resistance is well recognized [327, 330, 331], it remained elusive if activation of IKKβ also mediates adipogenesis and adipose tissue growth in response to overnutrition. Since ASOs affects many cell types, we then selectively deleted IKKβ in white
adipose lineage in mice and confirmed that deficiency of IKKβ in adipocyte lineage cells decreased adipogenesis and systemic inflammation elicited by HF feeding, leading to resistance to diet-induced obesity and insulin resistance.

Intriguingly, IKKβΔPDGFRβ mice displayed no significant phenotype under standard laboratory conditions but were resistant to obesity when challenged with a HFD, indicating that HFD-induced IKKβ activation in adipose progenitors is essential for HFD-induced adipose tissue growth. Consistent with our previous report [255], deficiency of IKKβ significantly decreased Smurf2 expression and substantially inhibited β-catenin ubiquitination in adipose SV cells, leading to accumulation of nuclear β-catenin and increased β-catenin activity. Wnt/β-catenin signaling plays an important role in the regulation of MSC lineage and has been well defined to inhibit adipogenesis in vitro and in vivo [352, 353, 376]. Consistently, Cre-mediated IKKβ deletion or ASO-mediated IKKβ knockdown impaired the adipogenic potential of adipose SV cells in mice. We also demonstrated, for the first time, that inhibition of IKKβ activity can also block human adipose stem cells differentiation, suggesting that IKKβ plays an important role in regulation of both murine and human adipocyte differentiation.

It is worth noting that IKKβΔPDGFRβ mice had decreased visceral adipose tissue but comparable subcutaneous adipose tissue as control mice when fed a HFD. There is a major ontogenetic difference between visceral and subcutaneous fat as they have different developmental origins [377]. Wang et al. [254] demonstrated that different fat depots have extensive differences in adipogenic potential. Visceral adipose tissue has a high capacity for adipogenesis in vivo but subcutaneous adipogenesis is limited [254]. A recent study also confirmed that HF feeding rapidly and specifically activates adipogenesis in visceral but not subcutaneous depots in mice [378]. Further, subcutaneous fat depot can also undergo extensive "browning" process after cold exposure and the appearance brown-like cells or beige cells are mainly found in subcutaneous fat but not in visceral fat [254]. It has been recently demonstrated that the PDGFRβ-positive adipocyte lineage cells contribute to
beige adipogenesis after prolonged cold exposure [253]. While deficiency of IKKβ did not affect the beige or brown adipocyte marker expression or mitochondrial content in subWAT of IKKβ<sup>ΔPDGFRβ</sup> mice under HF-feed conditions, it would be of interest to investigate whether IKKβ<sup>ΔPDGFRβ</sup> mice have affected beige adipocyte formation or mitochondrial function after chronic cold exposure in the future. Consistent with previous studies demonstrating that human visceral adipose tissue expresses high levels of IKKβ as compared with subcutaneous adipose tissue [379], we also found that IKKβ expression levels were significantly higher in murine visceral SV cells than in murine subcutaneous SV cells. Moreover, the expression levels of adipose progenitor markers including PDGFRβ were much higher in visceral SV cells than in subcutaneous SV cells. It is plausible that visceral adipose tissue has more abundant adipose progenitor population and activation of IKKβ signaling is required for these cells differentiating into adipocytes in response to HF feeding. Deletion of IKKβ in adipocyte lineage cells therefore has more impact on visceral adipose tissue growth than that of subcutaneous adipose tissue when challenged with a HFD. Further studies will be required to determine the detailed mechanisms that account for the different effects of IKKβ deficiency on visceral vs. subcutaneous fat. While both subcutaneous and visceral fat are increased in obese mice and human, accumulation of visceral but not subcutaneous adipose tissue contributes to the increased risk of obesity-associated metabolic dysfunctions [380]. Our data suggest that targeting IKKβ in adipocyte lineage cells may represent a novel therapeutic approach to reduce visceral adipose tissue mass in obesity.

In summary, we have revealed a pivotal role of IKKβ in the regulation of adipocyte differentiation and adipose tissue growth in obesity. ASO-mediated IKKβ knockdown protected mice from diet-induced obesity and metabolic dysfunctions. Deficiency of IKKβ in adipocyte lineage cells also inhibited HF feeding-elicited adipocyte differentiation and adipose tissue growth, leading to resistance to obesity and insulin resistance. Our findings suggest that overnutrition-induced IKKβ activation in adipose progenitors is an important
trigger for adipocyte differentiation and inhibition of IKKβ with antisense therapy may represent as a novel therapeutic approach to combat obesity and related metabolic dysfunctions.
Table 4.1 Primer sequences for QPCR.

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Figure 4.1 Pharmacological inhibition of IKKβ with ASOs protects mice from diet-induced obesity, improves insulin sensitivity, and reverses hepatic steatosis in obese mice.
Figure 4.1 Pharmacological inhibition of IKKβ with ASOs protects mice from diet-induced obesity, improves insulin sensitivity, and reverses hepatic steatosis in obese mice.

(A) IKKβ mRNA expression in liver, kidney, spleen, skeletal muscle (Sk.M.), brown adipose tissue (BAT), subcutaneous white adipose tissue (subWAT), retroperitoneal WAT (retroWAT), and epididymal WAT (epiWAT) from mice treated with control ASO or IKKβ ASO for 8 weeks (n=6-10; *P<0.05, ***P<0.001, assessed by Student’s t-test). (B) Western blot analysis of IKKβ and IKKα expression in liver and epiWAT from mice treated with control or IKKβ ASO for 4 weeks. (C and D) Growth curves (C), and fat mass and lean mass (D) of ND and HFD-fed mice treated with control ASO or IKKβ ASO (n=10 for ND and 30 for HFD; *P<0.05, **P<0.01, and ***P<0.001, assessed by two-way ANOVA). (E) Representative images of adipose depots (top) and weight of adipose depots (bottom) from mice treated with control or IKKβ ASO for 8 weeks (n=20; ***P<0.001, assessed by Student’s t-test). (F) Fasting plasma glucose and insulin levels of HFD-fed mice treated with control or IKKβ ASO (n=29-30; ***P<0.001, assessed by Student’s t-test). (G) Intraperitoneal glucose tolerance test (IPGTT), intraperitoneal insulin tolerance tests (IPITT), and area of curve (AUC) of IPGTT and IPITT of HFD-fed mice treated with control or IKKβ ASO (n=8-10; **P<0.01, ***P<0.001, assessed by Student’s t-test). (H) Western blot analysis of phosphorylated Akt and total Akt levels in liver, epiWAT, and skeletal muscle of control or IKKβ ASO-treated mice injected with saline or 0.35U/kg body weight. (I) Glucose uptake was measured in primary adipose tissues from mice treated with control or IKKβ ASO (n=9; **P<0.01, ***P<0.001, assessed by two-way ANOVA). (J and K): Representative appearance (J) and hematoxylin and eosin (top) and Oil-red-O (bottom) stained sections (K) of livers from mice treated with control or IKKβ ASO (scale bar=100μm). (L) Hepatic cholesterol and triglyceride levels of mice treated with control or IKKβ ASO (n=10; ***P<0.001, assessed by Student’s t-test). Results are presented as mean ± SEM.
Figure 4.2 IKKβ regulates murine adipocyte differentiation.
**Figure 4.2 IKKβ regulates murine adipocyte differentiation.**

(A) IKKβ mRNA expression in adipose SVF and mature adipocytes isolated from epiWAT of mice treated with control or IKKβ ASO (n=6; **P<0.01, assessed by Student’s t-test).  (B) Oil-red-O staining of adipose SV cells isolated from epiWAT of mice treated with control and IKKβ ASO induced by differentiation media (scale bar=100 μm, bottom panels).  (C) Expression of adipogenic genes in epiWAT of control and IKKβ ASO treated mice was measure by QPCR (n=6; **P<0.01, ***P<0.001, assessed by Student’s t-test).  (D) Western blot analysis of Smurf2 protein levels in epiWAT.  (E) Western blot analysis of nuclear β-catenin levels in epiWAT.  (F) Western blot analysis of IKKβ and IKKα protein levels in control 3T3-L1 cells or CRISPR-mediated IKKβ-deficient 3T3-L1 cells.  (G) Oil-red-O staining of control and IKKβ-deficient 3T3-L1 cells induced by differentiation media (scale bar=100μm, bottom panels).  (H) Expression of adipogenic genes and adipocyte markers in control or IKKβ-deficient 3T3-L1 cells (n=6; **P<0.01, ***P<0.001, assessed by Student’s t-test).  (I) Western blot analysis of Smurf2 protein levels in control or IKKβ-deficient 3T3-L1 cells.  (J) Western blot analysis of nuclear β-catenin protein levels in control or IKKβ-deficient 3T3-L1 cells.  (K) β-catenin reporter (TOP-flash reporter) activity in control or IKKβ-deficient 3T3-L1 cells (n=6; ***P<0.001, assessed by Student’s t-test).  (L) Control or IKKβ-deficient 3T3-L1 cells were treated with vehicle control or 100 nM PS-341, as indicated, for 4 h. β-catenin was immunoprecipitated with anti-β-catenin antibodies and then probed with anti-ubiquitin monoclonal antibodies. The whole cell lysates were probed with anti-β-catenin antibodies as an internal control. Results are presented as mean ± SEM.
Figure 4.3 PDGFRβ labels adipose progenitor cells *in vivo*.  
(A) The expression levels of endogenous PDGFRβ in adipose SV cells and adipocytes isolated from epiWAT of mice were measured by QPCR (n=4; ***P<0.001, assessed by Student’s *t*-test).  (B) Schematic of the PDGFRβCre*lacZ*<sub>F/F</sub> mouse model.  (C) Primary adipose SV cells isolated from lacZ<sub>F/F</sub> and PDGFRβCre*lacZ*<sub>F/F</sub> mice were stained with X-Gal (blue) and nuclear fast red (red) (scale bar=100 μm).  (D) EpiWAT of lacZ<sub>F/F</sub> and PDGFRβCre*lacZ*<sub>F/F</sub> mice were stained for β-galactosidase expression (blue) (top). White arrows indicate the vasculature in stained epiWAT of PDGFRβCre*lacZ*<sub>F/F</sub> mice (bottom).  (E) Cross-sections of epiWAT from lacZ<sub>F/F</sub> and PDGFRβCre*lacZ*<sub>F/F</sub> mice were stained for β-galactosidase expression (scale bar=100 μm). Results are presented as mean ± SEM.
Figure 4.4 Generation of mice lacking IKKβ in the white adipose lineage.

(A) Western blot analysis of IKKβ and IKKα protein levels in epiWAT, subWAT, liver, BAT, and skeletal muscle (Sk.M.) of IKKβ<sup>F/F</sup> and IKKβ<sup>ΔPDGFRβ</sup> mice. (B) Western blot analysis of IKKβ and IKKα protein levels in adipose SV cells isolated from IKKβ<sup>F/F</sup> and IKKβ<sup>ΔPDGFRβ</sup> mice. (C-G) Adipose SV cells isolated from IKKβ<sup>F/F</sup> and IKKβ<sup>ΔPDGFRβ</sup> mice were treated with LPS for 3 h. The expression levels for proinflammatory cytokines including TNFα (C), MCP-1 (D), IL-1α (E), IL-1β (F), and IL-6 (G) were examined by QPCR (n=5; **P<0.01, ***P<0.001, assessed by two-way ANOVA). Results are presented as mean ± SEM.
Figure 4.5 Deficiency of IKKβ in adipocyte lineage cells renders mice resistant to diet-induced obesity.

(A) Growth curves of ND or HFD-fed IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice (n=9-18, **P<0.01, ***P<0.001 when comparing HFD-fed IKKβ^{F/F} mice to HFD-fed IKKβ^{ΔPDGFRβ} mice, assessed by Student’s t-test).

(B) Body weight, fat mass, percentage of fat, and lean mass of 10-week-old IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice fed a ND or HFD for 16 weeks (n=7-19; **P<0.01, ***P<0.001, assessed by two-way ANOVA).

(C) Representative photographs of epiWAT, retroWAT, subWAT, and BAT from ND or HFD-fed IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice.

(D and E) Representative coronal section MRI images (D) and visceral and subcutaneous adipose tissue volume (E) of HFD-fed IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} (n=3; **P<0.01, ***P<0.001, assessed by Student’s t-test). Results are presented as mean ± SEM.
Figure 4.6 Loss of adipocyte progenitor IKKβ decreases visceral adiposity in mice fed a HFD. (A) Representative images of IKKβ
\textsuperscript{F/F} and IKKβ\textsuperscript{ΔPDGFRβ} mice fed a ND and HFD. (B) Tissue weight of liver, epiWAT, subWAT, retroWAT and BAT of ND or HFD-fed IKKβ
\textsuperscript{F/F} and IKKβ\textsuperscript{ΔPDGFRβ} mice (top panels), and percentage of body weight of indicated tissues (bottom panels) (n=6-12, **P<0.01, ***P<0.001, assessed by Student’s t-test). Results are presented as mean ± SEM.
Figure 4.7 Deficiency of IKKβ in adipocyte lineage cells does not affect beige and brown adipocyte markers or mitochondrial DNA content in subWAT.

(A) The expression levels of beige or brown adipocyte markers and mitochondrial function-related genes in subWAT of HFD-fed IKKβ\(^{F/F}\) and IKKβ\(^{ΔPDGFRβ}\) mice were analyzed by QPCR (n=6). (B) Quantification of mitochondrial DNA in subWAT of HFD-fed IKKβ\(^{F/F}\) and IKKβ\(^{ΔPDGFRβ}\) mice (n=5). Results are presented as mean ± SEM.
Figure 4.8 Adipose progenitor cell markers are highly enriched in visceral fat.

The expression levels of IKKβ and adipose progenitor cell markers in adipose SV cells isolated from subWAT or epiWAT of WT mice were analyzed by QPCR (n=4; **P<0.01, ***P<0.001, assessed by Student’s t-test). Results are presented as mean ± SEM.
Figure 4.9 IKKβ-deficient mice are protected from obesity-associated metabolic disorders.

(A) Fasting plasma glucose and insulin levels in ND or HFD-fed IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice (n=5-11; **P<0.01, ***P<0.001, assessed by two-way ANOVA). (B) IPGTT, IPITT, and area under the curve (AUC) of IPGTT and IPITT of HFD-fed IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice (n=6-11; *P<0.05, **P<0.01, ***P<0.001, assessed by Student’s t-test). (C) Western blot analysis of phosphorylated Akt and total Akt levels in epiWAT, liver, and skeletal muscle of IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice injected with saline or 0.35U/kg body weight. (D) Glucose uptake was measured in primary adipose tissues from HFD-fed IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice (n=9; ***P<0.001, assessed by two-way ANOVA). (E) Western blot analysis of IKKβ protein levels in peritoneal macrophages of IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice. (F) Representative immunohistochemistry for the macrophage marker, F4/80, in epiWAT from HFD-fed IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice (scale bar=100μm). (G) The expression levels of pro-inflammatory genes and macrophage markers in epiWAT of HFD-fed IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice were measured by QPCR (n=5; *P<0.05, **P<0.01, ***P<0.001, assessed by Student’s t-test). (H) Plasma cytokine levels of ND or HFD-fed IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice (n=5-7; **P<0.01, ***P<0.001, assessed by two-way ANOVA). Results are presented as mean ± SEM.
Figure 4.10 Deficiency of IKKβ in adipocyte lineage cells has no impact of adipose tissue inflammation or glucose and insulin tolerance in mice fed a normal chow.

(A) Representative immunohistochemistry for the macrophage marker, F4/80 in epiWAT from ND-fed IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice (scale bar=100μm). (B) IPGTT, IPITT and area under the curve (AUC) of IPGTT and IPITT of ND-fed IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice (n=5-11). Results are presented as mean ± SEM.
Figure 4.11 Deficiency of IKKβ inhibits adipogenesis in mice.
(A and B) Representative images (A) and quantification (B) of immunostaining for BrdU in epiWAT from BrdU-treated IKKβ\textsuperscript{F/F} and IKKβ\textsuperscript{ΔPDGFRβ} mice fed a HFD for 7 days (n=4-5; **P<0.01, significance assessed by Student’s t-test). The nuclei were stained with DAPI (blue) and the BrdU-positive cells are indicated by arrows. (C) Oil-red-O staining of adipose SV cells isolated from epiWAT of IKKβ\textsuperscript{F/F} and IKKβ\textsuperscript{ΔPDGFRβ} mice induced by differentiation media. (Scale bar=100μm, bottom panels). (D) The expression levels of adipogenic genes and adipocyte markers in adipose SV cells of IKKβ\textsuperscript{F/F} and IKKβ\textsuperscript{ΔPDGFRβ} mice were measured by QPCR (n=5-8; *P<0.05, **P<0.01, ***P<0.001, assessed by Student’s t-test). (E and F) Western blot analysis of Smurf2 protein levels (E) and nuclear β-catenin protein levels (F) in epiWAT of IKKβ\textsuperscript{F/F} and IKKβ\textsuperscript{ΔPDGFRβ} mice. (G and H) Western blot analysis of Smurf2 protein levels (G) and nuclear β-catenin protein levels (H) of adipose SV cells isolated from IKKβ\textsuperscript{F/F} and IKKβ\textsuperscript{ΔPDGFRβ} mice. (I) Adipose SV cells isolated from IKKβ\textsuperscript{F/F} and IKKβ\textsuperscript{ΔPDGFRβ} mice were treated with vehicle control or 100 nM PS-341, as indicated, for 4 h. β-catenin was immunoprecipitated with anti-β-catenin antibodies and then probed with anti-ubiquitin monoclonal antibodies. The whole cell lysates were probed with anti-β-catenin antibodies as an internal control. Results are presented as mean ± SEM.
Figure 4.12 Inhibition of IKK\(\beta\) decreases adipogenesis in human adipose stem cells.
Figure 4.12 Inhibition of IKKβ decreases adipogenesis in human adipose stem cells.

(A) Oil-red-O staining of adult-derived human adipose stem cells induced by differentiation media or media containing 5 μM IKKβ inhibitor BMS-345541 (scale bar=100μm, bottom panels). (B) The expression levels of adipogenic genes and adipocyte markers of human adipose stem cells treated with vehicle control or 5μM BMS-345541 were measured by QPCR (n=4; *P<0.05, **P<0.01, ***P<0.001, assessed by Student’s t-test). (C and D) Western blot analysis of Smurf2 protein levels (C) and nuclear β-catenin protein levels (D) in control or BMS-345541-treated human adipose stem cells. (E) Control or BMS-345541-treated human adipose stem cells were treated with vehicle control or 100 nM PS-341, as indicated, for 4 h. β-catenin was immunoprecipitated with anti-β-catenin antibodies and then probed with anti-ubiquitin monoclonal antibodies. The whole cell lysates were probed with anti-β-catenin antibodies as an internal control. **p<0.01, ***p<0.001. Results are presented as mean ± SEM.
Figure 4.13 Working model of the role of IKKβ signaling in adipocyte lineage cells in obesity.

(A) Under normal feeding conditions, the IKKβ/NF-κB signaling is maintained at basal activity. (B) Under HFD feeding conditions, overnutrition (e.g. excess FFA) activates IKKβ, leading to increased adipogenesis and inflammation. (C) In our current study, pharmacological inhibition or genetic deletion of IKKβ in adipocyte lineage cells impairs adipogenesis and protects mice from HFD-induced obesity and metabolic dysfunctions.
Chapter 5: Future Directions

5.1 Investigate the Consequences Associated with the SNPs Located Within the 3’ UTR of PXR

The SNPs found within PXR that correlate with alterations in cholesterol metabolism are intriguing. Interestingly, many of these SNPs are located within the 3’ UTR of PXR, indicative of extensive post-transcriptional regulation. Whether or not these SNPs are the causal variant in altering cholesterol balance remains to be determined; however, the idea that SNPs elicit changes in host gene expression by altering binding sites of transcription factors and/or microRNAs is well accepted. For instance, Musunuru and colleagues recently demonstrated that a SNP linked to myocardial infarction and high LDL-cholesterol levels disrupts a binding site for the transcription factor CCAAT/enhancer binding protein (C/EBP) in the sortilin 1 (Sort1) gene [381, 382]. Subsequently, the risk allele generates reduced expression of Sort1, ultimately increasing VLDL and LDL cholesterol levels in the blood and increased risk for CAD [381, 382]. It is possible that the SNPs within PXR generate gain-of-function and/or loss-of-function miRNA binding sites that subsequently elicit changes in RNA stability. For example, miR-148a has been recently implicated as a significant modulator of plasma cholesterol by regulating many genes involved in cholesterol homeostasis, including LDLR and ABCA1 [383]. Micro-RNA 148a has also been demonstrated to regulate PXR [117], but whether these SNPs are altering miR148a-binding, or other microRNA binding, remains to be ascertained.

5.2 Determine the Involvement of PXR in Currently Recommended ARV Drug-Associated Dyslipidemia

We recently reported that amprenavir and ritonavir, HIV PIs, are potent PXR agonists and that acute amprenavir exposure induced pro-atherogenic LDL cholesterol levels in WT but not in PXR-deficient mice [299]. Both PIs have
been associated with dyslipidemia and increased risk for CVD in HIV infected individuals [65, 67]. Currently, amprenavir is not widely prescribed, partially due to its deleterious effects on lipid levels, and ritonavir is most commonly used as a booster drug at relatively low doses (e.g. 100 mg/day). In addition to these well-studied PIs, several currently recommended first-line antiretroviral (ARV) drugs including efavirenz, darunavir, and lopinavir also have dyslipidemic effects in HIV patients [61, 67]. Recently, we tested currently recommended first-line HIV drugs from three commonly used drug classes (NNRTIs, NRTIs, and PIs) and found that several widely-prescribed ARV drugs including efavirenz and rilpivirine (NNRTIs), etravirine and darunavir (PIs) can also activate human PXR (Fig A.1). Many of these drugs have been associated with dyslipidemia in HIV patients [67]. For example, efavirenz has profound effects on elevating total and LDL-cholesterol levels in patients [61, 67]. By contrast, the widely used NRTIs, emtricitabine (FTC) and tenofovir, which do not activate PXR, have low risk of dyslipidemia [384-386]. These results suggest the involvement of PXR in ARV drug-associated dyslipidemia.

To further investigate the role of these widely used ARV drugs on PXR activity, we utilized a mammalian two-hybrid system to determine if these drugs can permit association of two well-known coactivators, SRC-1 and PBP. Binding of these ARV drugs to human PXR allowed co-activator/PXR interaction in a dose-dependent manner (Fig. A.2). Similarly, these drugs dose-dependently promoted co-repressor/PXR disassociation (Fig. A.2). To determine whether these ARV drugs act specifically through PXR, we tested their ability to activate a panel of other nuclear receptors, including RXR, FXR, LXRα, PPARα/γ, VDR, and ERα/β. Efavirenz, darunavir, and rilpivirine were unable to activate any of these other nuclear receptors (Fig. A.3), indicating the importance of investigating the effects of these ARV drugs on PXR activity in vivo. These results indicate that these ARV drugs specifically activate PXR and promote coactivator association leading to transcriptional activation.

We are currently investigating the role of these ARV drugs on PXR activity and plasma lipid levels in vivo. We plan to choose ARV drugs from three
commonly used classes: emtricitabine (NRTI), efavirenz (NNRTI), and darunavir (PI). Based on previous pharmacokinetic studies, steady state plasma levels of these compounds reach approximately 10-20 $\mu$M, which is sufficient to activate PXR in cell culture systems [387]. Since emtricitabine does not affect PXR activity, we do not anticipate that it will alter plasma lipid levels \textit{in vivo}. Based on the observation that both efavirenz and darunavir are agonists for both mouse and human PXR, we do not feel it is necessary to include another cohort of mice expressing the huPXR transgene on a mouse PXR-deficient background. Since we previously demonstrated that PXR transcriptionally regulates NPC1L1 [342], we also may consider incorporating ezetimibe into the diet to determine if cholesterol absorption is a major determinant in PXR-elicited hypercholesterolemia. It will be interesting to see if these widely-used drugs can promote hypercholesterolemia \textit{in vivo} in a PXR-dependent manner. All drugs should be assessed for their ability to activate PXR considering the adverse side effects of PXR activation in sterol and energy metabolism.

5.3 \textbf{Investigate the PXR/HNF4$\alpha$ Axis in Regulating MTP}

Mechanisms governing transcriptional regulation of MTP have been investigated by a few groups; however, identification of novel regulators is warranted. A comparative alignment of promoter sequences from different species revealed an evolutionarily conserved 204 bp element upstream from the MTP transcriptional start site that contains several cis elements [7, 44, 388, 389]. For example, three HNF-1 family members synergistically enhance the HNF-4$\alpha$ mediated transactivation of the proximal MTP promoter [388]. Moreover, MTP was identified as a possible PXR-target gene in Chromatin Immunoprecipitation-sequencing (ChIP-seq) assays [390]. Furthermore, PXR has been demonstrated to bind to HNF4$\alpha$ and ligand treatment enhanced this interaction [187]. HNF4$\alpha$ also mediates PXR transactivation of CYP3A4 [391]. ChIP-seq of HNF4$\alpha$ and several nuclear receptors revealed PXR binds within close proximity to all HNF4$\alpha$ binding sites within the genome [392]; whether PXR is directly binding to cis elements in MTP or binding is mediated through
HNF4α remains to be determined.

To gain insight into whether PXR directly regulates MTP expression, we first isolated primary enterocytes, treated with a wide array of PXR agonists and measured expression of genes involved in lipid metabolism. As expected, PXR activation stimulated NPC1L1 mRNA expression with all ligands tested (Fig. A.4). Furthermore, PXR activation also stimulated MTP expression with all ligands tested in a PXR-dependent manner (Fig A.4). These results suggest that PXR is absolutely essential for the xenobiotic-elicited MTP response. In the immediate future, we plan to use ChIP and gel-shift assays to determine if PXR is directly binding to the MTP promoter or if activation is mediated through HNF4α or other factors. These experiments are pivotal in understanding why PXR activation promotes hyperlipidemia.

If PXR does indeed transcriptionally regulate MTP, we plan to examine the role of PXR activation on chylomicron secretion. Since MTP is a major regulator of secretion of apoB-containing lipoproteins, we hypothesize that PXR activation may elicit increases in chylomicron secretion. Once the intestinal-specific PXR-knockout mice are available, we will gavage mice with [H³]-triolein and inject a LPL inhibitor to examine the amount of tritium in the plasma over time, as previously described [276, 393]. Moreover, with our colleagues at the University of Cincinnati, we plan to measure the intestinal uptake, absorption and lymphatic transport of dietary lipid in the conscious mouse [394]. Taken together, the overall contribution of intestinal PXR on whole body metabolism will be investigated in the near future.

5.4 Determine the Role of IKKβ in Hepatic Gluconeogenesis

The regulation of hepatic glucose production (HGP) is a critical step in maintaining blood glucose levels, and pathological changes in gluconeogenesis in the liver are a central characteristic in type-2 diabetes. Arkan and colleagues first demonstrated that mice with hepatocyte-specific deletion of IKKβ had improved glucose tolerance in aged and HFD-fed mice [244]. Utilizing a euglycemic-hyperinsulinemic clamp, the same group demonstrated that these
hepatocyte IKKβ deficient mice had approximately 50% suppression of HGP during insulin clamp, compared to only a 15% reduction in floxed mice fed a HFD [244]. Furthermore, an independent group demonstrated that mice with hepatocyte IKKβ-overexpression were resistant to the suppression of HGP during the insulin clamp [247]. These data demonstrate that the effect of IKKβ in HGP may result from enhanced insulin sensitivity in mice fed a HFD.

In Chapter 4 of this dissertation, we observed a significant decrease in fasting glucose levels and improved glucose tolerance in mice fed a HFD with IKKβ ASO treatment (Fig. 4.1, F and G). Further, mice treated with the IKKβ ASOs had significantly enhanced Akt signaling in the liver, adipose tissue, and skeletal muscle with and without exogenous insulin (Fig. 4.1, H). To examine whether the benefit of reducing IKKβ expression with ASOs on adiposity was HFD-elicited, we treated another cohort of mice fed chow for 8 weeks. Chow-fed mice had no changes in body weight or fat mass, but still had small, albeit significant, increases in lean mass (Fig. 4.1, C and D). Interestingly, however, fasting glucose was decreased and these mice were considerably more glucose tolerant (Fig. A.5, A and B). It would be of interest to examine plasma insulin levels in these mice. Since IKKβ is a negative regulator of insulin signaling by direct phosphorylation of IRS-1 [250], these mice most likely have enhanced hepatic insulin signaling and more suppressed HGP, which would explain the decrease in plasma glucose levels. The IKKβΔPDGFRβ mice did not exhibit changes in plasma blood glucose levels, but that is most likely due to intact IKKβ signaling in the liver in these mice (Fig. 4.4, A). Further, the improvement of glucose tolerance in both models may also be due to enhanced glucose uptake in other tissues, such as skeletal muscle. IKKβΔPDGFRβ mice fed a HFD had increased expression of genes involved in skeletal muscle mitochondrial biogenesis and oxidative metabolism (data not shown). Moreover, IKKβΔPDGFRβ mice exhibited increases in mitochondrial content in skeletal muscle (data not shown). Taken together, these results demonstrate that the improved metabolic parameters associated with IKKβ deletion in adipose progenitor cells is likely attributed to enhanced oxidative metabolism in skeletal muscle.
5.5 Does IKKβ Act as a “Switch” Modulating Stem Cell Commitment?

The IKKβ/NFκB signaling pathway has been demonstrated to play a role in stem cell commitment. Mesenchymal stem cells (MSCs) are multipotent stem cells that differentiate into mesoderm-type cells, including osteoblasts and adipocytes. There has been a lot of work trying to identify the modulators that link MSC commitment between bone and adipose tissue, such as β-catenin. For example, disruption of a negative regulator of Wnt, axin-2, in mice leads to enhanced osteoblast differentiation and matrix mineralization [395]. Mutations in the Wnt antagonist SFRP1 results in high bone mass in mice, suggesting the involvement of canonical Wnt signaling on osteoblast differentiation and bone formation [396]. It was later identified that β-catenin transcriptionally regulates Runx2, a transcription factor that is absolutely essential for osteoblast differentiation [397]. Aside from bone formation, Wnt signaling is a potent suppressor of adipogenesis and fat formation [353]. Thus, signaling pathways converging on Wnt signaling may alter bone and fat formation.

Interestingly, the IKKβ/NFκB signaling pathway exerts opposite effects on mesenchymal stem cell differentiation into bone and adipose tissue. Ligands for IKKβ/NF-κB have been shown to promote adipocyte differentiation and suppress osteoblast formation [253, 254, 350, 374, 378]. Moreover, NFκB inhibits MSC differentiation into bone by promoting β-catenin degradation via the E3 ligases Smurf1 and Smurf2 [350]. The major questions is, are all these effects mediated primarily through NF-κB or does IKKβ mediate NF-κB-independent changes that play a role in the commitment of MSCs? Interestingly, IKKβ has been demonstrated to directly phosphorylate β-catenin [398]. It was initially thought that phosphorylation of β-catenin by IKKβ may stimulate its degradation by the proteasome by enhancing the ubiquitination by β-TrCP; however, no evidence was presented to support this mechanism. Thus, further work identifying the specific amino acids that are phosphorylated by IKKβ and the physiological consequence of the initial phosphorylation is warranted. Taken together, these data demonstrate that IKKβ may sense its
environment (e.g. FFA) and propagate a signaling cascade that initiates stem cell commitment into bone or fat.

5.6 Investigate the Role of PXR and IKKβ in Inflammation

It is evident that reciprocal crosstalk exists between the PXR and NF-κB signaling pathways. What remains to be determined is the precise mechanism. As mentioned above, PXR can suppress NF-κB signaling by a variety of mechanisms, including the physical interaction between PXR and the NF-κB subunit, p65. Furthermore, activation of NF-κB signaling may also suppress PXR signaling by binding to RXR, which may interfere with RXR binding to PXR and ultimately suppress PXR transactivation. Recent work by Ito and colleagues revealed a mechanism linking LXRs to inflammation through its target gene ABCA1 [220]. ABCA1 was demonstrated to alter the lipid membrane composition and disrupt binding of adaptor proteins to the TLRs [220]. Since PXR has also been implicated to regulate ABCA1 [168, 399], it is worth investigating if activation of PXR works in a similar manner by disrupting TLR signaling.

Since IKKβ clearly has NF-κB independent substrates that play a role in many different processes, it is plausible that upon stimulation, IKKβ directly phosphorylates many different nuclear receptors leading to their degradation/suppression in activity. Moreover, IKKβ has the potential to also phosphorylate and regulate the activity of several cofactors needed for proper nuclear receptor signaling. It would be interesting to do phosphoproteomics in cells with and without IKKβ stimulation. This would provide an unbiased approach to identify several signaling pathways that crosstalk and explore novel areas of research. Taken together, there is a lot of room for discovery in this area of research that could lead to druggable targets for the cure of certain inflammatory conditions.

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Appendices

Figure 5.1 Currently recommended HIV drugs activate PXR.
Figure A.1 Currently recommended HIV drugs activate PXR.
(A-D) LS180 cells were transfected with full-length hPXR or full-length mPXR together with a hPXR reporter (CYP3A4-luc) or a mPXR reporter ((CYP3A2)3-luc) and a CMX-β-galactosidase control plasmid. Cells were then treated with HIV protease inhibitors (A, C), non-nucleoside and nucleoside reverse transcriptase inhibitors (B, D), RIF (a hPXR ligand; A, B), or PCN (a mPXR ligand; C, D) at the indicated concentrations for 24 h. Data are shown as fold induction of normalized luciferase activity compared with that for DMSO treatment and represent the means from triplicate experiments. Results are presented as mean ± SEM.
Figure A.2 Currently used HIV drugs promote PXR coactivator association and corepressor disassociation.

(A and B) LS180 cells were cotransfected with a GAL4 reporter, VP16-hPXR, and expression vector for GAL4 DBD or GAL4 DBD linked to the receptor interaction domains of PXR coactivators (GAL-SRC1 or GAL-PBP) (A) or PXR corepressors (GAL-SMRT or GAL-NCoR) (B). Cells were treated with DMSO control, efavirenz, darunavir, rilpivirine, or RIF at the indicated concentrations for 24 hours. Data are shown as fold induction of normalized luciferase activity compared with that for DMSO treatment and represent the means from triplicate experiments. Results are presented as mean ± SEM.
Figure A.3 Widely used HIV drugs are PXR-selective agonists.
LS180 cells were co-transfected with a GAL4 reporter and a series of GAL4 constructs in which the GAL4 DBD is linked to the indicated nuclear receptor LBD. Cells were treated with DMSO control, 10μM efavirenz, 10μM darunavir, or 10μM rilpivirine for 24 h. Data are shown as fold induction of normalized luciferase activity compared with DMSO treatment and represent the mean of triplicate experiments. Results are presented as mean ± SEM.
Figure A.4 PXR activation increases mRNA expression of NPC1L1 and MTP in primary enterocytes isolated from WT but not PXR-deficient mice.

Primary enterocytes were isolated from WT and PXR-deficient mice and were incubated with control medium or medium containing 10 µM amprenavir, TBC, or PCN. Total RNA was isolated and expression of genes involved in lipid metabolism were analyzed by qPCR (n=3; **P<0.01 and ***P <0.001; assessed by one-way ANOVA). Results are presented as mean ± SEM.
Figure A.5 Glucose metabolism is improved in IKKβ ASO mice fed chow.
(A and B) Fasting plasma glucose levels (A) and intraperitoneal glucose tolerance test (B) in chow-fed mice treated with control ASO or IKKβ ASO (n=10; *P<0.05, **P<0.01, ***P<0.001, assessed by Student's t-test compared to control). Results are presented as mean ± SEM.
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