The Pennsylvania State University

The Graduate School

Department of Biochemistry, Microbiology and Molecular Biology

NOVEL REGULATION OF LIPID HOMEOSTASIS

BY THE AH RECEPTOR

A Dissertation in

Biochemistry, Microbiology and Molecular Biology

by

Rachel Tanos

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August, 2012

The dissertation of Rachel Tanos was reviewed and approved* by the following:

Gary H. Perdew
John T. and Paige S. Smith Professor in Agricultural Sciences
Director of the Center for Molecular Toxicology and Carcinogenesis
Dissertation Advisor
Chair of Committee

Jeffrey M. Peters
Distinguished Professor of Molecular Toxicology and Carcinogenesis

Richard J. Frisque
Professor of Molecular Virology

Ross C. Hardison
T. Ming Chu Professor in Biochemistry and Molecular Biology

Joshua D. Lambert
Assistant Professor of Food Science

Scott B. Selleck
Professor of Biochemistry and Molecular Biology
Head of the Department of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School

ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor. Activation of AHR has been associated with toxicity through its binding to dioxin response element (DRE) sequences in its target genes (e.g. *CYP1A1)*. Evolutionary conservation of the receptor and the phenotype of mice lacking the AHR suggest an endogenous role for the receptor beyond responding to xenobiotic exposure. Based on previous work in our lab showing the ability of the receptor to alter gene expression independent of DRE sequences, we set out to explore a possible involvement of the receptor in key cellular pathways. We performed a microarray analysis on liver isolated from ligand-treated transgenic mice expressing a wild-type *Ahr* or a DRE-binding mutant *Ahr* (A78D). The results revealed that AHR ligand treatment suppressed cholesterol synthesis but did not require DRE binding. In order to confirm this finding in humans, primary human hepatocytes were administered an AHR ligand and subsequent analysis of mRNA levels revealed a significant trend of repression in fatty acid and cholesterol synthesis genes. Our lab has also established the ability of the ligand SGA360 to activate AHR without inducing DRE-mediated activity. Since toxicity associated with hyperactivation of the AHR is a DRE-mediated event and our microarray data revealed that DRE-binding is not essential for the regulation of our genes of interest, we tested the effect of SGA360 in primary human hepatocytes. Selective activation of the receptor showed a significant attenuation in the expression of our target genes and the ability of the receptor to inhibit the compensatory mechanism of statins on cholesterol synthesis gene expression. In addition, SGA360 exhibited a higher degree of repression of fatty

acid and cholesterol biosynthesis gene expression than the AHR agonist BNF. Mirroring

our gene expression results, a significant repression of fatty acid and cholesterol secretion

was observed in human cells. In an effort to elucidate the mechanism of this regulation by

AHR, we investigated the activity of AHR on the sterol element binding proteins

(SREBPs), the key regulators of both pathways. Our results indicated the targeted

proteosomal degradation of the cleaved active form of those transcription factors when

AHR is activated causing the attenuation of their transcriptional signaling. The discovery

of AHR as a regulator of fatty acid and cholesterol biosynthesis pathways independently

of its DRE-binding and the ability to selectively activate the receptor for this purpose

suggests that AHR may be a previously unrecognized therapeutic target.

TABLE OF CONTENTS

Chapter 2 AH RECEPTOR REGULATES THE CHOLESTEROL BIOSYNTHETIC PATHWAY IN A DIOXIN RESPONSE ELEMENT-INDEPENDENT MANNER

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ACC, Acaca, acetyl-coA carboxylase
ADD1, adipocyte determination and differentiation dependent factor 1
AHR, Aryl hydrocarbon Receptor
$Ahr^{fx/fx}$, genotype of mice possessing AHR^d allele flanked by two loxP sites
Ahr^b, high affinity mAHR allele
Ahr^d, low affinity mAHR allele
Alb, albumin promoter
α-MEM, minimal essential medium with alpha modification
ANOVA, analysis of variance
ARNT, AHR nuclear translocator
bHLH, basic helix-loop-helix
BNF, β-naphthoflavone
Cre, cre DNA-recombinase
$Cyp1a1$, Cytochrome P450 1A1
$Cyp1b1$, Cytochrome P450 1B1
DiMNF, 3',4'-dimethoxy-α-naphthoflavone
DRE, Dioxin Responsive Element
DTT, Dithiothreitol
ER, Estrogen Receptor or endoplasmic reticulum
FAS, fasn, fatty acid synthase
FBS, Fetal Bovine Serum
FDFT1, farnesyl-diphosphate farnesyl transferase
hAHR, human AHR
HIF, Hypoxia Inducible Factor
HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase
HSP90, 90 kDa heat shock protein
Insig1, insulin induced gene 1
LDLR, low density lipoprotein receptor
LSS, lanosterol synthase
mAHR, mouse AHR
mAHR-A78D, transgenic DRE-binding mutant mice
MENG, 25 mM MOPS/2mM EDTA/0.02% NaN3/10% Glycerol
MUFA, monounsaturated fatty acid
NLS, nuclear localization signal
PBS, phosphate buffered saline
PCR, polymerase chain reaction,
RT-PCR, reverse transcription-polymerase chain reaction
S1P/S2P, site 1 protease or site 2 protease
TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin
SAhRM, selective AHR modulator
SCAP, SREBP cleavage activating protein
SCD1, stearoyl-CoA desaturase 1

SQLE, squalene epoxidase
SFA, saturated fatty acid
SRE, sterol response element
SREBP, sterol response element binding protein
Ttr, transthyretin promoter
VLDL, very low density lipoprotein
WAT, white adipose tissue
WT, wild type C57/BL6
XAP2, hepatitis B virus X-associated protein 2

ACKNOWLEDGEMENTS

Chapter 1

INTRODUCTION

The aryl hydrocarbon receptor (AHR) is a ligand inducible transcription factor that regulates transcription of a diverse array of genes, including Phase I and Phase II enzymes in xenobiotic metabolism. The AHR is activated by a structurally diverse group of ligands, some of which are toxic environmental contaminants, endogenously generated compounds or dietary compounds. Sustained AHR activation by some of these ligands is thought to be responsible for mediating a toxicological response through inappropriate activation of its target genes. Many of the xenobiotics that bind to the AHR also induce expression of enzymes that metabolize the AHR ligand and induce the generation of toxic intermediates capable of causing cellular damage. In contrast, the AHR is also implicated in the regulation of key physiological pathways in the immune and reproductive systems, the liver, and in cardiac and vascular development, among others [1-3].

1.1 bHLH-PAS PROTEINS

The AHR belongs to the basic helix-loop-helix - Per ARNT Sim (bHLH/PAS) family of transcription factors [4]. Members of this family are distinguished from other bHLH protein subfamilies by the PAS domain that houses the dimerization and/or repression domains. The PAS domain is a conserved region of 250-300 amino acids containing two highly conserved regions of approximately 50 amino acids, referred to as PAS-A and PAS-B repeats. Functionally, the PAS domain aids in protein interaction (with other PAS and non-PAS proteins) whereas the bHLH region functions in heterodimer formation between binding partners and the basic region is directly involved in DNA binding. Members of this family are thought to play a role in sensing

environmental and developmental cues, such as light, oxygen, and embryogenesis signaling. The Per protein was the first PAS protein to be discovered and, unlike ARNT and Sim, it does not possess a bHLH motif. It has been shown to play a role in regulating circadian rhythm through CLOCK/BMAL1 heterodimer formation [4], while SIM (single minded) proteins have been implicated in cell lineage midline development [5]. ARNT (Ah Receptor Nuclear Translocator) binds to AHR to form a heterodimer capable of activating the transcription of target genes [6, 7]. ARNT is also capable of heterodimerizing with other bHLH-PAS proteins, such as HIF1α (Hypoxia inducible factor 1α), during hypoxic conditions and is thus sometimes referred to as HIF1β [4]. Interestingly, a number of coactivators with a bHLH-PAS domain have been identified and the role for this domain in coactivators remains to be defined. Several coactivators with this domain (e.g SRC-1 and NcoA-2) play an important role in AHR mediated transcription [8].

1.2 AHR LIGANDS

1.2.1 Exogenous AHR Ligands

Ligands for the AHR include many planar, hydrophobic polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs), which are widespread environmental contaminants. PAHs are known to be involved in adverse health effects and have been extensively studied as potent carcinogens that are bioactivated. They are formed as a result of incomplete burning of fuel (coal, oil, and gas) and are also present in cigarette smoke and smog [9].

HAHs include the polychlorinated-dibenzo-p-dioxins (PCDDs), biphenyls (PCBs) and dibenzofurans (PCDFs). The prototypical AHR agonist 2,3,7,8-tetrachlorodibenzo-ρ-dioxin also known as TCDD or dioxin, the most potent AHR ligand, capable of generating maximal CYP1A1 gene induction at very low concentrations (e.g. 10 nM). This extreme potency is partially attributed to the resistance of TCDD to metabolism, resulting in a long half-life in the living organism. It is generated during combustion processes such as incineration of waste, fuel burning (coal, wood and oil), and as a side product of chemical manufacturing, such as pesticides and herbicides. Exposure to TCDD and other HAHs results in adverse health effects both in animal models and humans, including chloracne (skin disorder), reproductive and birth defects, cancer and suppression of the immune system [10].

An array of chemicals are AHR ligands and activate AHR transcriptional activity, including certain drugs and industrial chemicals. For instance, benzimidazoles such as omeprazole, used in the treatment of heartburn and peptide ulcer [11], can mediate transcription of AHR target genes in humans [12-14] without binding AHR within the ligand binding pocket. However, omeprazole fails to activate the murine AHR, which therefore provides proof that the human and mouse AHR have different affinities and thus might have different biochemical roles. The AHR can bind to a variety of structurally dissimilar compounds with varying affinity and sensitivity in different species.

1.2.2 Endobiotic AHR Ligands

Although the AHR has been identified as an exogenous chemical sensor, studies from *ahr*-null animals have demonstrated an endogenous role for the receptor. Mice lacking AHR exhibit multiple physiological phenotypes, comprising a reduction in peripheral lymphocytes, decreased liver weight, vascular abnormalities in the heart and liver, diminished fertility and overall slower growth, that indicate a constitutive role for the receptor in the absence of exogenous ligands [3].

A number of endobiotic compounds have been shown to activate the AHR, including indole 3-carbinol (I3C) and indole acetic acid, arachidonic acid metabolites, leukatriene A4 metabolites and tryptophan metabolites. I3C is found in cruciferous vegetables and, through acid condensation in the stomach, gives rise to the potent AHR ligand indolo-[3,2*b*]-carbazole (ICZ). However, ICZ has been found to be rapidly metabolized and is thus unlikely to be involved in high level systemic AHR regulation [15]. Hemin, biliverdin and bilirubin are also capable of activating the receptor but at very low affinities. The levels at which these compounds exist under normal physiological conditions are not effective in eliciting AHR activation, but some diseases such as Crigler-Najjar syndrome (hyperbilirubinemia) lead to increased levels of bilirubin and thus AHR activation [16, 17].

The arachidonic acid derivatives, 12(R)-hydroxy-5(Z),8(Z),10(E),14(Z)-eicosatetraenoic acid (12(R)-HETE) and lipoxin A4, both endogenously produced compounds, were found to be potent AHR agonists capable of displacing TCDD from AHR and inducing DNA binding and CYP1A1 transcriptional activity [18, 19]. Nuclear accumulation of AHR has been demonstrated upon treatment with tryptophan

metabolites, indigo and indirubin, first identified in human urine. At high concentrations, these compounds are capable of displacing TCDD from the AHR and activating a very similar subset of genes [20]. Interestingly, indirubin proved to be a very potent selective inducer of the human AHR, compared to the mouse protein [21]. Another endogenous modulator is 7-ketocholesterol (7-KC), a key oxysterol present in human serum. In contrast to the agonistic activity of the aforementioned endogenous ligands, 7-KC was shown to be an antagonist. Its concentration in human blood plasma and tissues was found to be sufficient to displace TCDD from the AHR [22]. Given that the AHR is capable of binding to a structurally diverse group of compounds, it is possible that there exists a myriad of endogenous ligands for the receptor. Whether any of these endogenous AHR modulators are physiologically important remains to be determined.

1.2.3 Selective AHR Modulators

Sustained activation of AHR by xenobiotic agonists is linked to toxicity through a predominantly DRE-mediated mechanism. Our lab has established the ability of a number of selective ligands to activate the receptor which then represses cytokine-mediated induction of the acute phase response gene serum amyloid A3 (*Saa3*), yet fails to induce gene expression through binding to its cognate response element. We have succeeded in identifying a number of selective AHR modulators (SAhRMs) first through the structural modification of the WAY-169916 compound, a selective ligand to both the ER and the AHR, resulting in the characterization of SGA360 [23]. In addition, 3′,4′-dimethoxy-α-naphthoflavone (DiMNF) was identified as an effective SAhRM through

compound screening [24]. These compounds failed to induce CYP1A1 levels in HepG2 cells but they were still capable of attenuating TCDD-mediated induction of luciferase activity in a dose-dependent manner. Competition binding assays using a photoaffinity ligand demonstrated the ability of those ligands to bind the ligand-binding pocket of AHR. Repression of cytokine mediated *Saa1* and *Saa3* induction by SAhRM treatment was shown to occur in an AHR-dependent manner without inducing dimerization with ARNT. This correlates with an inability to invoke AHR/ARNT dimer formation and subsequent binding to its cognate element, as evidenced by gel shift assays and a failure to activate DRE-regulated genes. The loss of DRE-complex formation might be explained by a conformational change in a selective ligand bound AHR that facilitates protein-protein interactions, yet does not mediate heterodimerization with ARNT. However, this change in the receptor's affinity does not limit its ability to repress non-DRE mediated genes such as *Saa3,* supporting the notion that the SAhRMs could be used to therapeutically utilize the AHR in the treatment of inflammatory disorders [23-25]. Chemical structures of selected AHR ligands are provided in Figure 1.1.

Figure 1.1
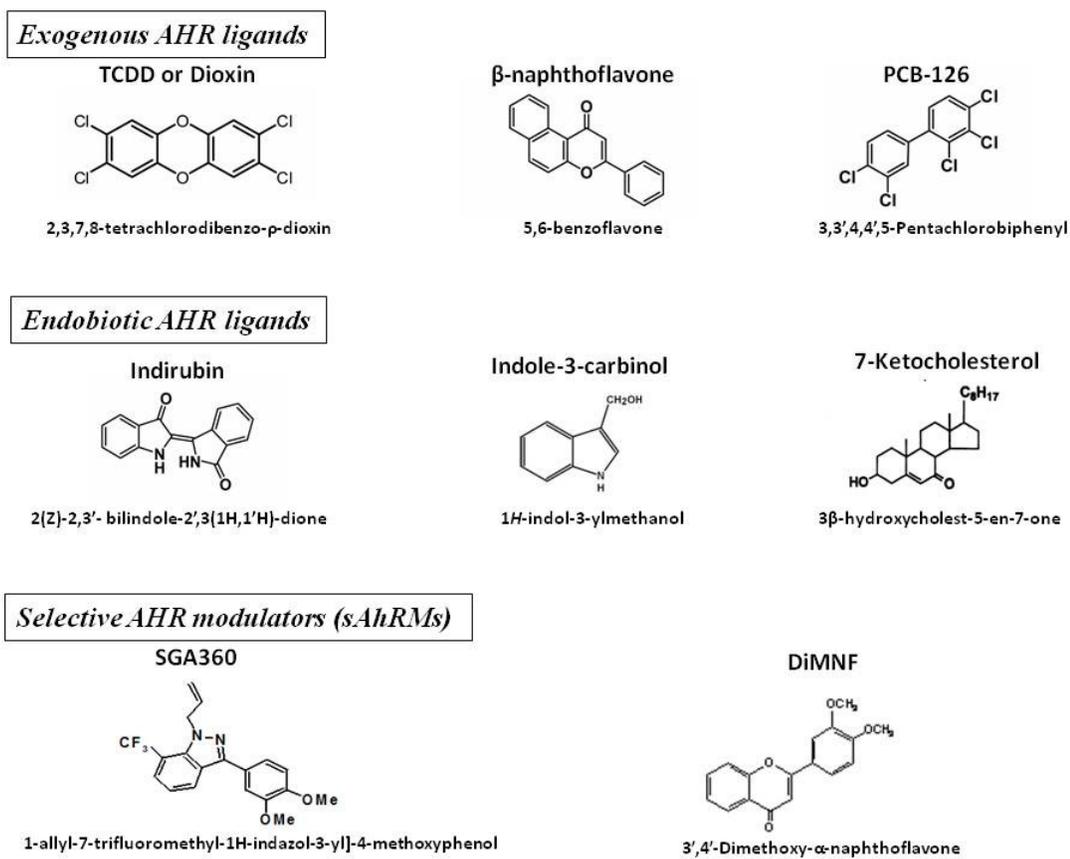
Figure 1.1 **Structure of exogenous, endogenous and selective ligands of the AHR.**

1.3 AHR: PATHWAY AND INTERACTING PROTEINS

1.3.1 Unliganded AHR Complex

The AHR resides in the cytosol within a core complex of ~280 kDA. This stable protein complex is composed of the AHR, two molecules of heat shock protein 90 (Hsp90), p23 and the hepatitis B-virus X-associated protein 2 (XAP2). The proper folding of the receptor into a ligand-binding conformation was shown to be dependent on the presence of the Hsp90 protein dimer [26, 27] This phenomenon has already been observed with other steroid receptors such as the glucocorticoid and progesterone receptors [28]. p23 is associated with the complex through its interaction with Hsp90 [29] with no evidence of any essential role in AHR function [30, 31]. Another member of the AHR cytoplasmic complex is the immunophilin homolog XAP2. This protein interacts with Hsp90 and AHR through its tetracopeptide repeat domains (TPR), inhibiting receptor nuclear translocation in the absence of exogenous ligand binding. This is performed through disrupting the ability of the importin B protein to recognize the nuclear translocation signal of AHR [32]. Although XAP2 represses the transcription of the Hepatitis B virus X protein [33], it has been shown to either enhance or repress AHR transcriptional activity [32, 34, 35]. XAP2 also influences AHR cytosolic compartmentalization, interaction with p23, protein stability and degradation, possibly through prevention of ubiquitination of the receptor [36-39]. When not bound to a ligand, the AHR complex undergoes rapid nucleo-cytoplasmic shuttling [28, 40]. While the significance of this shuttling has not yet been elucidated, nuclear import of the AHR is

known to be facilitated in part through its nuclear localization sequence in association with the nuclear pore proteins β-importin [32]. In addition, its export to the cytoplasm is mediated by the exporter protein CRM-1[41]. Although XAP2 appears to play a critical role in determining the cellular localization of the receptor [32], the cause and significance of the shuttling of unliganded AHR is not yet known and suggests a role for the receptor in other signaling processes in the absence of an exogenous ligand.

1.3.2 Ligand Dependent Nuclear Translocation and Heterodimer Formation

Ligand binding triggers AHR/hsp90 complex translocation from the cytoplasm to the nucleus where AHR dissociates from hsp90/XAP2 and heterodimerizes with its nuclear partner ARNT [36, 42] (See Figure 1.2). Using mutational analysis, it has been shown that AHR/ARNT interaction occurs through the bHLH domain [42] with the PAS domain serving as their secondary interaction motif. The resulting AHR/ARNT heterodimer is capable of binding a consensus sequence (TNGCGTTG) named dioxin or xenobiotic response elements (DRE or XRE) in the promoter of their target genes. Although both proteins are indispensible for binding those sequences, ARNT is known to recognize the GTTG half-site while AHR recognizes the TNGC half-site [43, 44]. The recruitment of coregulators, chromatin modifying proteins and other transcriptional machinery components [8] activates the transcription of a number of genes involved in Phase I and Phase II metabolism. It is important to note here that the transactivation domain of AHR plays a dominant role in the context of this heterodimer compared to

ARNT since mutant ARNT lacking the C-terminal transactivation domain is still capable

of driving transcription of DRE-responsive genes [45].

Figure 1.2
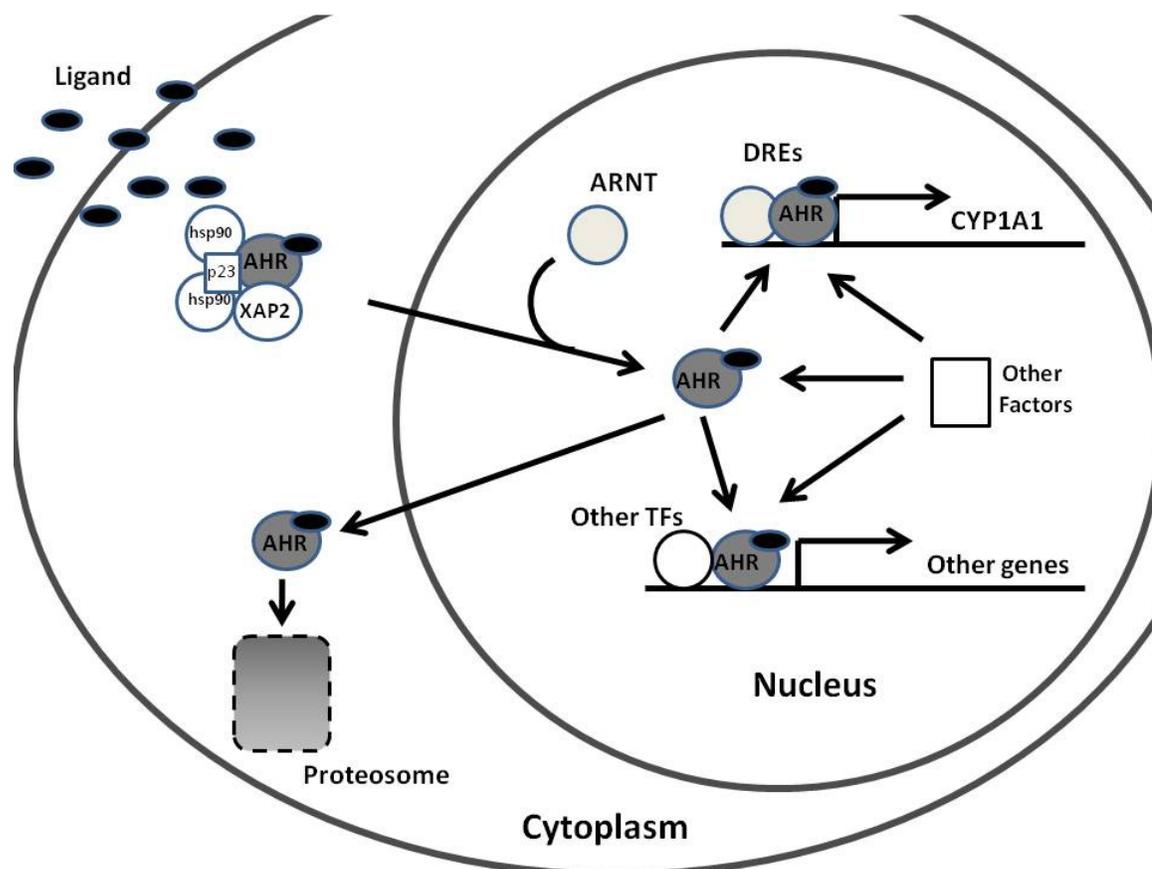
Figure 1.2 **Ligand activation of the AHR.**
Ligand activation of the AHR induces its nuclear translocation and heterodimerization with ARNT. The ARNT/AHR heterodimers then binds to dioxin responsive elements (DREs) leading to AHR-target gene activation. AHR has also been shown to affect gene expression independently of DRE sequences and through he interaction with other transcription factors (TFs). The liganded receptor is eventually targeted to proteosomal degradation.

1.3.3 AHR Interaction with Coregulators

AHR mediated transcriptional activation requires several coregulators influencing chromatin remodeling, transcription, initiation, elongation, mRNA splicing, and translation, thus playing an important role in the modulation of AHR activity in response to a given ligand or in a gene or tissue specific context. These factors can either enhance or repress genetic targets of AHR and include the steroid receptor coactivator-1 (SRC-1), nuclear receptor coactivator-2 (NcoA-2), p300/CBP/cointegrator-associated protein (p/CIP) [8, 46], CREB binding protein (CBP) [45] and the nuclear factor Sp1 [47] among others.

Several studies have pointed to the ability of AHR to modulate the expression of a number of genes without requiring binding to its cognate response element, instead the AHR interacts with other transcription factors [48, 49]. Crosstalk between AHR and other receptors and transcription factors would also explain at least in part the numerous endogenous functions of the AHR following its activation by exogenous stimuli.

1.3.4 AHR Interaction with Other Receptors and Transcription Factors

Interaction between the AHR and the estrogen and androgen receptors (ER and AR) has already been established. In fact, dioxins have well-described anti-estrogenic effects, including the inhibition of estrogen-induced uterine enlargement, MCF-7 cell growth and target gene induction [50, 51]. Furthermore, impaired ovarian follicle formation has been observed in mice lacking AHR expression [52]. In a similar fashion, dioxin exhibits both androgenic and anti-androgenic effects on prostate development

[53]. It has also been demonstrated that AHR is capable of blocking ER-mediated transcription at certain promoters, such as Cathepsin D, through direct competition for specific DNA binding sites [54]. Likewise, more recent evidence suggests the ability of ligand-activated ERα to transrepress AHR-mediated transcription by being recruited to the AHR transcriptional complex at the promoter of its target genes [49].

The RelA subunit of NF-κB, a player in the inflammatory and growth signaling, has also been shown to interact with AHR [48]. Evidence from MCF-10F human breast cancer cells indicates that this interaction leads to the activation of c-myc through the binding of a NF-κB element in its promoter in the absence of any AHR exogenous ligand [55]. However, liganded AHR and activated NF-κB were shown to antagonize each other through reciprocal inhibition of binding to their respective cognate DNA element [48, 56]. Other proteins reported to intersect with AHR include the constitutive androstane receptor (CAR) [48], the glucocorticoid receptor (GR) [57, 58], and the peroxisome proliferators activated receptors (PPAR) [59].

The most recent addition to the list of AHR interacting proteins is the transcription factor SREBP1c. SREBP1 was demonstrated to physically interact with AHR and to interfere with its positive regulation of murine Th17 cell differentiation. Based on the SREBP1c and AHR overlapping binding sites on the IL17 promoter, the reported data also suggested a displacement of the AHR from its DNA binding by SREBP1 following LXRα activation [60].

1.3.5 AHR and Ubiquitination

Among the adverse effects exerted by AHR following dioxin exposure is the modulation of sex steroid hormone signaling. A number of studies aimed at elucidating the mechanism of AHR mediated alterations in the functions of other transcription factors have been reported. As previously mentioned, the ligand-activated AHR was found to directly associate with ERα and AR modulating their function both positively and negatively. Later reports demonstrated the ability of AHR to promote the proteolysis of those transcription factors through the assembly of a ubiquitin ligase complex cullin 4B (CUL4-B[Ahr]) defining a new role for the receptor as a ligand dependent E3 ubiquitin ligase. In this regard, AHR acts as a substrate-recognition subunit within the CUL4-B[Ahr] complex to recruit ERα and AR and target them for proteosomal degradation [61]. This is supported by studies showing that treatment with an AHR agonist (3MC and βNF), as well as expression of constitutively active AHR, lead to a pronounced decrease in endogenous ERα, ERβ and AR protein levels without alteration in their mRNA levels [62]. This degradation is abrogated in the presence of the proteasome inhibitor MG-132, indicating a ubiquitin-proteasome system through which AHR represses the ER and AR pathways. Further evidence comes from in vivo studies demonstrating degradation of ERα and AR in the prostate and the uterus in mice treated with an AHR ligand, while the same treatment fails to affect their protein levels in AHR-deficient mice [52, 63].

1.4. PHYSIOLOGICAL ROLE OF AHR

The expression of the AHR spanning a wide range of tissues and the discovery of endogenous ligands for the receptor imply its role in normal physiological homeostasis. Numerous studies focusing on its toxicity in mice and humans, as well as targeted deletion of the AHR in mice, has helped unravel a role for the receptor in several key cellular functions.

1.4.1 AHR and Reproduction

Cross-talk between the AHR and steroid receptor pathways guides the influence of the AHR expression and activation over the reproductive system in rodents and humans. This is supported by exposure studies in humans showing that TCDD significantly affects offspring sex ratios [64], while the 2,4,5-trichlorophenoxyacetic acid ligand is linked to sexual dysfunction and reduced libido [65]. In rodent studies, *ahr*-null female mice suffer from uterine vascular hypertrophy and reduced fertility due to disrupted ova release and follicle maturation [1, 2]. In addition, dioxin was shown to disrupt luteal cell secretion of progesterone, sexual differentiation and neuroendocrine functions in female animal models [66, 67]. In male rodents, TCDD exposure significantly reduces sperm quality and production as well as testosterone concentration and synthesis in Leydig cells [68, 69]. Although the AHR has a less pivotal role in the reproductive system of male mice when compared to females, the receptor still plays an important role in proper male reproductive organ development and function.

1.4.2 AHR and Organ Development

Ahr-null mice indicate a role for the receptor in the development of a number of organ systems including but not limited to the liver, kidney, skin, and heart. AHR deficiency has been associated with a marked dermal and cardiac fibrosis, gastric hyperplasia, and hyperkeritinosis [2]. Activation of AHR with dioxin produces similar cardiac and renal developmental defects both in utero and exposed adult mice [70]. In contrast, transgenic mice expressing a constitutively active AHR exhibit abnormal lesions and increased weight in several organs such as the liver and the kidney [71].

1.4.3 AHR and the Immune System

Numerous studies indicate a role for the receptor in mediating adverse immunological responses following excessive activation. Exposure to AHR ligands has been correlated with thymic atrophy and immune system impairment in mice and increased incidence of lymphoma and leukemia in humans [72-74]. Dioxin was found to affect the adaptive immune response; mice developmentally exposed to TCDD display reduced numbers of T cells and IFN-γ-producing cells in the lymph nodes [75]. At the same time, dioxin treatment was found to enhance resistance to certain infections like *Streptococcus pneumoniae* and listerosis, without triggering the expected enhanced inflammatory response or adaptive immune response disruption [76, 77].

Knock-out studies also indicate an endogenous role for the receptor. Reduced numbers of peripheral lymphocytes [73] and disrupted B-lymphocyte differentiation are evident in AHR-deficient mice [78]. Ligand activation of the AHR results in the repression of the acute-phase gene *Saa3* in mouse hepatocytes [79]. This involvement of

the receiver in immune system development and regulation further supports the

hypothesis of an endogenous role for the receptor in addition to its role in xenobiotic

metabolism.

1.5 AHR TARGET GENES

Activation of AHR leads to the transcriptional induction of several known genes,

some of which are Phase I and Phase II drug metabolizing enzymes. In addition, AHR

regulates other non-metabolizing target genes that play a critical role in development and

tumor promotion.

1.5.1 Phase I Enzymes

The phase I enzymes regulated by the AHR include members of the cytochrome

P450 1 family of enzymes: CYP1A1, CYP1B1 and CYP1A2. They metabolize a wide

variety of substrates while activating some of them to carcinogenic intermediates.

Levels of expression of CYP1A1 and 1A2 proteins in liver, lung and kidney have

been found to be highly dependent on AHR and they can metabolize many AHR ligands

as well [73]. CYP1A1 constitutive expression is generally very low and its induction is

primarily dependent on activated AHR. It is expressed in extrahepatic tissues and is

known to metabolize a large variety of ligands [80]. Examples of such compounds

include benzo-[a]-pyrene (B[a]P), which is found in cigarette smoke and can be

metabolized by CYP1A1 to form the mutagen B[a]P-7,8-diol-9,10-epoxide as well as

7,12-dimethylbenzanthracene (DMBA), and benzo[b]fluoanthrene [80, 81].

CYP1B1 has been shown to metabolize many of the same substrates as CYP1A1 and has been demonstrated to be mostly expressed in extrahepatic tissues. However unlike 1A1, its constitutive expression and regulation is not primarily dependent on AHR. For example, both cyclic AMP [82] and the estrogen receptor [83] have been shown to regulate CYP1B1 expression and may be more important in terms of mediating toxicity [81, 84]. Examples of CYP1B1 substrates include the arachidonic acid [85] and 17 β-estradiol, the latter substrate can be metabolized to the carcinogenic metabolite 4-hydroxyestradiol [86].

CYP1A2 is primarily expressed in the liver and thus is the primary CYP1 member that plays a significant role in the metabolism of therapeutic drugs. CYP1A2 is known to metabolize toxins like aflatoxin B1, drugs such as acetaminophen (anti-inflammatory) and zoxaolamine (muscle relaxant) and caffeine [87, 88]. Expression of CYP1A2 is highly elevated by exposure to cigarette smoke causing poor efficacy of certain drugs in smokers [88].

The most recently discovered CYP member regulated by the AHR is CYP2S1. It is expressed in the lungs, intestine and skin and is capable of metabolizing relatively small xenobiotics like naphthalene and retinoic acid [89-92]. Further work is needed to clearly define the substrates for CYP2S1 and its physiologic importance.

1.5.2 Phase II Enzymes

Phase II enzymes are known to conjugate bulky functional groups onto Phase I metabolites, rendering them more hydrophilic and ready to be cleared from the body through the bile. The AHR specifically induces the expression of a number of Phase II

genes. Uridine diphosphate glucuronosyl transferase (UGT1A1) has been established as an AHR inducible gene through TCDD, PCB and 3-methylcholanthrene rat liver treatment [93, 94]. This enzyme plays a key role in the metabolism of bilirubin, a potential AHR ligand. Mutational studies have shown lack of functional UGT1A1 results in hyperbilirubinemia (Crigler-Najjar syndrome or Gilbert syndrome) [93]. NADP H-quinone reductase possesses several DRE sequences in its promoter that are important to its induction by AHR ligands. This enzyme plays a role in the breakdown of toxic quinones and in keeping an intracellular redox balance through maintaining cellular oxidants in an active state [95]. Aldehyde dehydrogenase enzymes are involved in the metabolism of aldehydes produced by the body through retinoic acid synthesis as well as amino acid, lipid and carbohydrate metabolism. Expression of these enzymes has also been demonstrated to be induced by the AHR ligands 3-MC and β-naphthoflavone (BNF) [72].

1.5.3 Other Genes Regulated by the AHR

The wide screening of new target genes for AHR using high throughput gene technologies, namely microarrays, has helped uncover new physiological roles for the receptor with evidence of a number of genes regulated by AHR and independent of xenobiotic metabolism. Recently, AHR has been shown to indirectly regulate CAR [96], indirectly induce the expression of the insulin growth factor binding protein 1 (IGFBP 1) [97] and to positively affect retinoic acid receptor activity [98]. TCDD induces the expression of transforming growth factor (TGF)-α and the plasminogen activator inhibitor-1 [99]. The AHR plays a direct role in the expression of COX2 and Il6 [100].

Our lab has established the ability of the receptor to repress the activation of the acute-phase genes *SAA3* without requiring DRE binding [79]. All these factors indicate an important endogenous role for the receptor beyond toxicity and the diversity of the genes and pathways influenced by AHR.

1.6 AHR DOMAIN AND FUNCTIONS

The AHR structure is composed of several domains organized roughly into two halves, the amino (N)-terminal and the carboxy (C)-terminal. The N-terminal portion contains the bHLH-PAS domain that mediates binding to HSP90, XAP2, ARNT and AHR ligand [101, 102]. This half also houses the nuclear localization domain (NLS) and the nuclear export signal (NES) [103] that facilitates nucleo-cytoplasmic shuttling and translocation of the receptor following ligand binding. The C-terminal portion of the receptor comprises the transactivation domain (TAD) divided into acidic, glutamine-rich and proline-serine-threonine rich (PST) subdomains [104]. This domain controls recruitment of co-factors (co-activators or co-repressors) to the transcription complex on the promoters of target genes.

1.6.1 Inter and Intra-Species Differences in AHR Gene Sequences and Response to TCDD

Evolutionary conservation of the AHR is marked by significant divergence in the receptor's size (from 95 kDa to 125 kDa) and amino acid sequence across species [105-108]. There are substantial species differences in their degree of TCDD responsiveness.

For instance while the guinea pig shows a high sensitivity to TCDD (LD_{50} 1µg/kg) [109, 110], the hamster is 100-fold more resistant [111]. Within the rat species, Han-Wistar rats are 1000-fold more resistant to TCDD than Long-Evans rats, making them resistant to TCDD toxicity and lethality [112]. The mechanism for these observations has not yet been established. Variation in sensitivity is also seen in mouse strains; DBA/2 mice are about 10-fold more resistant than their C57BL/6J counterparts [113]. With the same set of genes induced by the treatment, the Ahr[d] form of the receptor still requires a dose 10-fold higher than that for Ahr[b] [114]. The mechanism for this effect is due to a point mutation in the ligand binding domain. In general terms, there is a significant homology in the first half of the receptor (N terminal) across species but the second half (C terminal), containing the transaction domain is not as well conserved. Compared to the AHR in other species, hamsters have an extended transactivation domain with more glutamine residues [107]. Whether this causes an increased sensitivity to TCDD has yet to be established. The carboxy terminal domain of the receptor in the Han-Wistar rats also shows a loss of either 38 or 43 amino acids [115]. The guinea pig and human versions of the receptor show high similarity [108]. The receptor in the DBA2 mouse harbors a point mutation in the ligand binding domain (amino acid valine at 375) rendering the receptor not very efficient in ligand binding [116]. The human ligand binding domain of the receptor is structurally similar to the DBA2 with a mutation at the amino acid valine 381. Consequently, the hAHR has a 10-fold lower ligand binding affinity than the Ahr[b] allele, which most likely confers HAH-toxicity resistance in humans [117].

1.6.2 Human AHR Polymorphism

A number of polymorphisms in the human AHR have been reported, most of them involve the transactivation domain of the receptor (P517S, R554K, and V570I). However, the effect of the R554K variant is not well delineated: two studies concluded the variant had no effect on AHR mediated transcriptional activity [118, 119], while a third study claimed an increased CYP1A1 activity in the R554K polymorph [120]. The variant V570I has only been observed in conjunction with R554K and the P517S polymorph [121]. None of these polymorphisms has shown a variation in the level of CYP1A1 expression, except for the double and triple variant receptor which fails to activate expression of CYP1A1 in transient cell transfection experiments [121]. Other polymorphisms that show no effect on AHR-induced activity include a M786V substitution in the coding region, a single nucleotide substitution in the 5′-untranslated region [118], and a silent substitution in codon 44 [119]. Intriguingly, a variation in the activity of CYP1A1 has been reported in the human population with no correlation to AHR polymorphism [122]. Knowing that the main methods for testing the phenotype of a given AHR allele are through assessing CYP1A1 activity in peripheral blood lymphocytes or mutant expression constructs in vitro and detection of urinary blood metabolites for CYP1A2 activity [122], it is possible that another battery of genes under the regulation of AHR might be affected by the different variants of the receptor. This variation in receptor activity could affect the metabolism of drugs by enzymes regulated by the AHR but this has yet to be determined. It is important to note that no AHR polymorphism affecting the receptor's ligand binding, dimerization with ARNT, nuclear

localization and export, protein stability and coactivator recruitment has been found, suggesting high conservation of the AHR, possibly due to its constitutive role in normal human homeostasis.

1.7 AHR AND LIPID METABOLISM

Microarray studies performed in mice have revealed that daily exposure to low levels of TCDD had a profound impact on the expression of genes involved in circadian rhythm, cholesterol biosynthesis, fatty acid synthesis, and glucose metabolism in the liver [123]. A similar study performed in rats revealed that high levels of TCDD exposure were required to alter genes involved in cholesterol metabolism and bile acid synthesis and transport [124]. This observation is also supported by a study indicating a disruption in lipid metabolism in male guinea pigs through changes in expression of cholesterol synthesis genes following TCDD treatment [125]. These results are consistent with TCDD-induced anorexia and wasting syndrome in rats, characterized by weight loss, muscle atrophy and a loss of appetite [126]. Results from human exposure studies revealed a significant disruption in lipid metabolism and high cholesterol and triglyceride levels in blood of workers exposed to TCDD [127]. AHR involvement in hepatotoxicity, hepatic steatosis and wasting syndrome has already been documented in rodents, further linking the receptor to hepatic lipid metabolism [128]. Taken together, these results strongly suggest the involvement of AHR in the regulation of cholesterol and fatty acid homeostasis in rodents and humans.

The essential roles for fatty acids and cholesterol and the human diseases caused by disorders in their metabolism prompted the study of their mode of regulation in an effort to control their levels in vivo [129]. In the body, fatty acids and cholesterol are either derived from the diet or from *de novo* synthesis occurring mainly in the liver through the lipogenic and the mevalonate pathways respectively. Each of these pathways comprises several enzymes, such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) in lipogenesis, and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), farnesyl-diphosphate farnesyltransferase (FDFT1), squalene epoxidase (SQLE) and lanosterol synthase (LSS) in cholesterol synthesis. All these enzymes have been shown to be under the regulation of the transcription factors sterol element binding proteins 1 and 2 (SREBP1c and SREBP2) [130].

1.8 THE STEROL REGULATORY ELEMENT BINDING PROTEINS (SREBPs)

1.8.1 SREBP Gene Structure and Function

SREBP proteins belong to the basic helix-loop-helix zipper (bHLHZ) family of transcription factors. There are two major mammalian SREBP genes: *SREBF1* gives rise to 2 distinct proteins SREBP1a and -1c through alternative splicing, and *SREBF2* produces a single SREBP2 domain. SREBP1 and SREBP2 proteins share 47% homology. The SREBP1c rat homolog was initially named adipocyte determination and differentiation dependent factor 1 (ADD1) for its involvement in adipogenesis [131]. Due

to its longer NH2-terminal transactivation domain, SREBP1a is a more potent transcriptional activator compared to SREBP1c. However, the SREBP1c isoform is more predominantly expressed in mouse and human tissues, with especially high levels in the liver, white adipose tissues (WAT), skeletal muscle, adrenal gland and brain. In contrast, SREBP1a is highly expressed in cultured cell lines and tissues undergoing high cell proliferation such as the spleen and the intestine [132]. Hepatic overexpression of SREBP1c in isolated hepatocytes increases the expression of lipogenic genes whereas hepatic overexpression of SREBP2 in mice causes a preferential increase in the expression of cholesterol synthesis genes and, to lesser a extent, genes involved in fatty acid synthesis [133-135] (See Figure 1.3).

When cholesterol levels in the cells are sufficient, the ~120kDa SREBP precursor protein is anchored in the endoplasmic reticulum (ER) and nuclear membrane by virtue of its 2 membrane-spanning helices, with its both amino-terminal transcription factor domain and its carboxy-terminal regulatory domain facing the cytoplasm. The inactive form of the protein is bound to the cholesterol-sensing SREBP cleavage activating protein (SCAP), which in turn binds reversibly to an ER-resident membrane protein insulin induced gene 1 (Insig-1). Under low cholesterol levels, Insig-1 detaches from the SREBP:SCAP complex allowing the latter to move to the golgi apparatus through COP II vesicles. In the golgi, sequential cleavage of the SREBP protein by 2 distinct proteases site-1 protease (S1P) and site-2 protease (S2P), releases the cytoplasmic N-terminal domain of the protein. This then translocates to the nucleus to bind specific sterol regulatory elements (SRE), thus upregulating the synthesis of enzymes involved in sterol biosynthesis and uptake in order to restore cholesterol levels [129, 136-139]. In contrast,

SREBP1c is under the regulation of high glucose and insulin levels [134, 140] through an

unknown mechanism and primarily activates expression of genes involved in fatty acid,

triglyceride and phospholipid pathway.

Figure 1.3
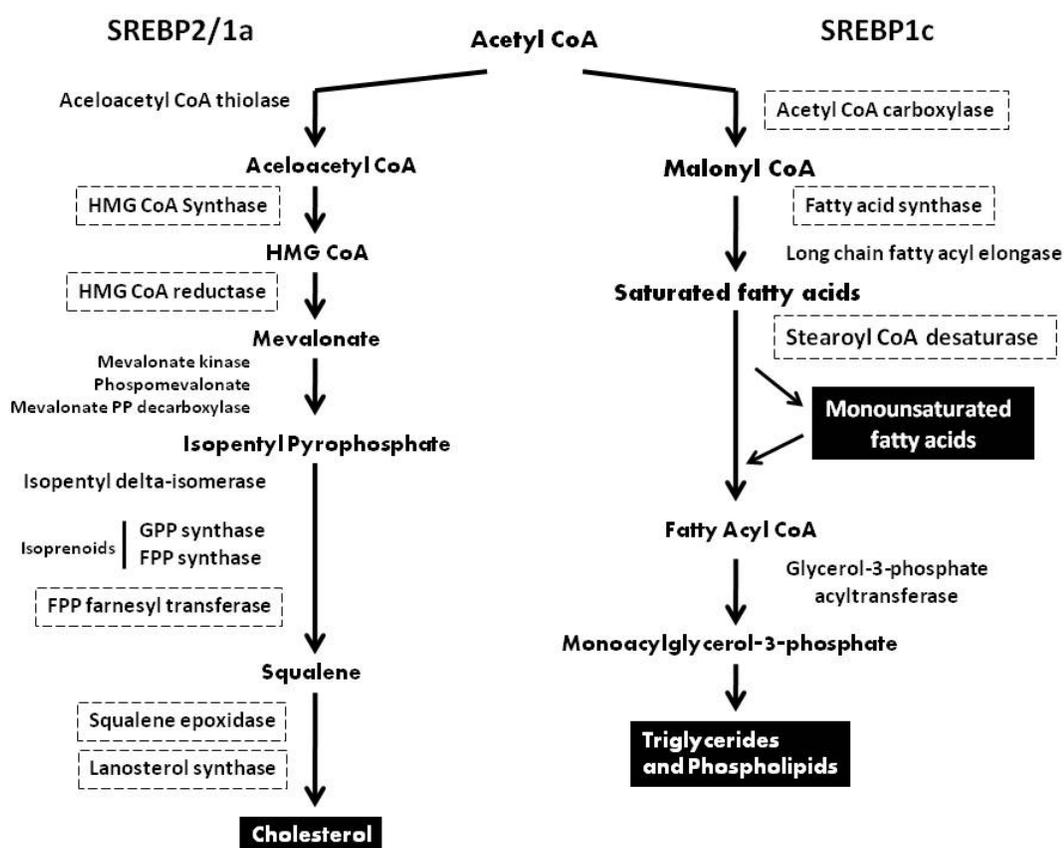
Figure 1.3 **SREBP regulation of the cholesterol and fatty acid synthesis pathways.**
> SREBP1c controls the regulation of fatty acid, triglycerides and phospholipids synthesizing enzymes. SREBP2 regulated the expression of cholesterol biosynthetic gene expression. Highlighted enzymes are evaluated as target of AHR regulation in our study.

1.8.2   Regulation of SREBP Transcription Factors

Regulation of SREBP transcriptional activity on their target genes comprises three major levels: transcription, proteolytic cleavage, and ubiquitination and degradation of nuclear SREBPs.

1- Transcriptional Control of SREBPs

The first insights into the transcriptional regulation of SREBP1c came from studies using fasting/refeeding regimes in rodents. Changes in nutritional status were shown to regulate the expression of SREBP1c in the liver, skeletal muscle and WAT; fasting depressed SREBP1c expression whereas refeeding using a high carbohydrate diet increased its levels markedly, with only minor effects on the expression of the other SREBP isoforms [141-144]. More recent experiments demonstrated the induction of SREBP1c transcription by insulin, leading to a parallel increase in expression of both the ER-membrane bound precursor and its nuclear form [145]. These effects of insulin on SREBP1c are counter-regulated by glucagon via cAMP [134]. The nuclear receptor LXRα can also induce the transcription of SREBP1c and subsequently the lipogenic genes in the liver. In fact, LXRα-deficient animals exhibit reduced basal expression of SREBP1c, FAS, ACC, and SCD1 [146, 147]. In addition, LXRα agonists selectively increase the transcription levels of those genes, resulting in a concomitant increase in the rate of fatty acid synthesis [147-149].

Sterol depletion has already been established as the primary cause for induction of the transcription of SREBP1a and SREBP2. Transcription of SREBP2 was also shown to be mediated by a feed-forward mechanism in which nuclear SREBP2 binds SRE sequences in the promoter region of the *SREBF2* gene and induces its own transcription

[150]. This mechanism is also observed in the transcriptional regulation of SREBP1c [151].

2- SREBPs Activation by Proteolytic Cleavage

SREBP processing from the ER to the Golgi Apparatus, where it is subsequently cleaved by S1P and S2P, can be controlled by cellular sterol content. This sterol-dependent trafficking between the ER and the Golgi requires an intact sterol-sensing domain in the SCAP protein. Cholesterol binds directly to this domain and modulates the SCAP conformation to control its protein-protein interaction with the Insig protein responsible for the ER retention of SREBP-SCAP [152, 153]. Nonetheless, this sterol sensing SREBP processing does not apply to all three SREBP isoforms, with other factors such as insulin involved in SREBP1c cleavage. Sterols were demonstrated to have no effect on the SREBP1c isoform in vivo. Instead, changes in nutritional status and insulin levels primarily regulate SREBP1c [141, 142].

3- Ubiquitination and Degradation of SREBPs

In the nucleus nSREBPs are modified by ubiquitination and rapid degradation by the 26S proteasome [154], as evidenced by the use of proteasome inhibitors. These effects were shown to require both a functional transactivation and DNA binding domain in SREBPs and to occur through a transcription dependent pathway, which involves the proteosome, thereby terminating their transcriptional signal [155]. Recently, nuclear

SREBP1c was found to be phosphorylated at two sites and negatively regulated by GSK-β [156]. This phosphorylation served as a docking site for fbw7, the substrate recognition component of a specific SCF ubiquitin ligase [157]. Thus, DNA binding provides a link between phosphorylation, ubiquitination and rapid degradation of active SREBP. In this way, sustained activation of lipid and cholesterol synthesis target genes of SREBP would require a continuous supply of processed SREBP molecules, providing tight regulation over sterol and fatty acid synthesis cellular levels.

1.8.3 Statins (HMG-CoA Reductase Inhibitors)

Since there is normally strict control over the rate of cholesterol synthesis, diseases caused by high cholesterol in the blood, namely familial hypercholesterolemia and atherosclerosis, are treated with a low cholesterol diet coupled with drugs inhibiting cholesterol synthesis pathway. Statins, the most important class of lipid-lowering agents, are competitive inhibitors for the rate-limiting enzyme, HMGCR. Generally, they have been shown to be very effective in lowering cellular cholesterol, thus stimulating the upregulation of the low density lipoprotein receptors (LDLR) in the liver, allowing rapid removal of cholesterol from the circulation [158]. Additionally, they have a wide-spectrum of non-lipid mediated favorable pleiotropic impacts on inflammation, cancer, hypertension, and cardiac hypertrophy [159, 160] among others. Nonetheless, in certain cases prolonged statin therapy has been associated with hepatotoxicity, rhabdomyolysis (pathological skeletal muscle breakdown) leading to acute renal failure due to tubular necrosis [159, 161-163], and compromised cardiac function [164].

Several studies attribute some of those effects to the blocking of the isoprenoid branch that stems from the mevalonate pathway beyond the step catalyzed by the inhibited enzyme. This branch of the pathway produces the non-sterol isoprenoid metabolites involved in numerous functions in the body. These metabolites include heme A and the ubiquinone coenzyme Q10 [162], both of which are essential in mitochondrial electron transport [161], small GTP-binding proteins such as Rho and Ras, involved in post-translational lipid modification by prenylation [160], and dolichol, which is required for glycoprotein synthesis [161]. Furthermore, being the rate-limiting enzyme of the pathway, HMGCR is under feedback control by sterol synthesis end-products: sterols, cholesterol and bile acids. In fact, the compensatory mechanisms exerted on the enzyme results in an alteration in its rate of synthesis and degradation [163, 165] and subsequently in all the levels of the enzymes involved, through an increase in the active SREBP2 (nSREBP) levels. High liver enzymes have been postulated to account for the hepatotoxicity seen in certain patients. This increase in SREBP expression associated with statin treatment has another major adverse effect: it increases the levels of the HMGCR expression, which opposes the competitive inhibition of the statins on the pathway. Theoretically, inhibition of HMGCR with drugs that are non-competitive could improve the level of cholesterol synthesis inhibition and, as a consequence, the serum LDL lowering effect of statins.

The side effects of statins as well as the substantial numbers of people who either do not tolerate them or whose LDL levels are not lowered adequately, suggests that new drugs should be designed to address this issue. Recent studies have aimed to target enzymes involved in the committed sterol branch of the pathway. Such inhibition would

therefore spare isoprenoid synthesis. It was also desirable to inhibit the pathway before

the formation of the first 4-ring precursor structure of cholesterol, lanosterol. This was

based on the assumption that accumulation of such a hydrophobic compound would be

associated with toxicity [163]. Classes of drugs have been developed to target and inhibit

the following enzymes: FDFT1, SQLE, LSS. [161] and they were shown to have fewer

secondary effects [166]. However, to the best of our knowledge, to this date no inhibitor

has entered clinical trials. Reasons range from toxicity issues due to accumulation of one

or more metabolites to ineffectiveness of the drug *in vivo* [163, 164, 167, 168].

1.8.4 Fatty Acid Synthesis Inhibitors

ACC is the rate limiting enzyme for the synthesis of long-chain fatty acids

through the carboxylation of acetyl-CoA into malonyl CoA, a fatty acid acyl-chain

elongation unit [169]. Malonyl-CoA plays a crucial role in the control of β-oxidation, is

elevated in response to insulin and glucose in muscle tissues, and decreases during

physical activity. It is thus believed to act as a metabolic switch and a player in the

pathogenesis of insulin resistance in muscle tissue of obese patients [170-173]. FAS is a

critical enzyme highly expressed in the liver and adipose tissue [174] and responsible for

the terminal catalytic step involving the synthesis of saturated fatty acids (SFAs). FAS

inhibitors have been proven to be effective anti-obesity agents in rodents causing a

significant reduction in food intake along with a profound weight loss [175, 176]. The

major product of FAS is palmitate, a 16-carbon long-chain fatty acid, through malonyl-

Coa and acetyl-CoA condensation [175]. Palmitate (C16:0) can undergo elongation by

FAS III forming stearate (C18:0), this product is a key substrate for the rate-limiting

enzyme SCD1. SCD1 catalyzes the conversion of SFAs into the mono unsaturated fatty acids (MUFAs) such as oleate (C18:1), through the introduction of the first double bond in the *cis*-Δ9 position. This makes the SCD1 a critical mediator of fatty acid synthesis and a key regulatory factor of body adiposity [177]. MUFAs are considered to be major components of various lipids, including triglycerides, cholesterol esters, phospholipids and wax esters [178]. Mice with targeted disruption of SCD1 exhibit a decrease in the content of their hepatic triglycerides and cholesterol esters [179, 180] coupled with very low levels of triglycerides in the VLDL and LDLR fraction compared to their wild-type counterparts. In addition, they show increased energy expenditure and less fat accumulation in their adipose tissues [181, 182], as well as enhanced insulin sensitivity in liver [183], adipose tissue [184] and skeletal muscle [182]. While leptin-deficient ob/ob mice are obese and accumulate high amounts of lipid in the liver, SCD1 deficiency completely corrects the hypometabolic phenotype and hepatic steatosis in these mice [185]. Conversely, elevated SCD1 levels in humans positively correlate with higher plasma triglycerides, increased BMI, and high insulin levels [186, 187].

1.9 OVERVIEW AND SIGNIFICANCE

Nuclear receptors such as the estrogen receptor and the glucocorticoid receptor have been shown to function through alternate mechanisms in the absence of DNA-binding. Our lab has already established that DRE-binding was not necessary for ligand activated AHR to directly attenuate acute-phase gene expression, namely *Saa3,* in the liver [79]. Moreover, evolutionary conservation of the receptor and the *ahr*-null mice

phenotype suggest an endogenous role for this receptor. A growing list of AHR target genes that play a role in cell proliferation, cell cycle control, epithelial-mesenchymal transition and inflammation (e.g., slug, epiregulin) have been identified [188, 189] clearly pointing to a physiological role for the AHR beyond regulating xenobiotic metabolism.

1.9.1 Implication of AHR in cholesterol and fatty acid synthesis

Our focus on the involvement of AHR in regulating fatty acid and cholesterol biosynthesis genes emerges from previous microarray studies performed in mice and rats, and clinical studies in humans exposed to TCDD showing alteration of lipid metabolism mentioned earlier in this chapter. Furthermore, the involvement of AHR in lipid metabolism might explain the wasting syndrome seen in *ahr*-null mice characterized by loss of fat weight and lowered cholesterol biosynthesis. *Schiller et al.* [190] have shown that rats treated with TCDD exhibited a 10% decrease in body weight after one week with a marked increase in triglyceride and cholesterol in the blood, consistent with the observed human phenotype. This observation is also supported by a study that revealed a disruption in lipid metabolism in male guinea pigs through changes in expression of cholesterol synthesis genes following TCDD treatment [31]. A study done in rats using TCDD revealed that the extent of the inhibition of the fatty acids and cholesterol synthesis pathways was dose-dependent [191]. Although those studies were important in establishing a link between the receptor and the cholesterol and fatty acid synthesis pathways following TCDD exposure, no assessment of any change in the expression levels of fatty acid and cholesterol synthesis genes in humans has been performed and no attempt at elucidating the mechanism through which the AHR is contributing to this

regulation has been reported. In the same context, our preliminary microarray studies from transgenic DRE-binding mutant mAHR-A78D mice exposed to the AHR ligand BNF imply a non-DRE mode of action for this receptor in the regulation of lipogenesis and thus establish the need to expand upon these studies.

1.9.2 Selective Activation of AHR in the Regulation of Cholesterol and Fatty Acid Synthesis

The side effects of statins and their ineffectiveness in some patients prompts the need for new drugs that would target other cholesterol biosynthesis enzymes present in the committed sterol pathway, namely FDFT1, SQLE and LSS. Additionally, inhibitors of the fatty acid synthesis enzymes ACC, FAS and SCD1 have shown promising results including lower plasma triglyceride levels, enhanced insulin sensitivity and reduced fat accumulation among others. Additionally, the benefits of SREBP1c and 2 cleavage inhibition on fatty liver and hyperlipidemia have already been reported [192, 193].

Sustained activation of AHR by xenobiotic agonists, like TCDD, is known to lead to toxicity. Since the toxicity resulting from AHR hyperactivation is mediated through a DRE-mediated response, the discovery that the AHR can coordinately modulate the expression of cholesterol and fatty acid biosynthetic genes in a DRE-independent manner is very promising. As previously stated our laboratory has identified several selective ligands namely SGA 360 and DiMNF capable of efficiently repressing acute-phase gene expression (e.g. *SAA1*) through AHR and yet fail to mediate significant DRE-driven transactivation effects. In this context, the transactivation domain of AHR would still be capable of recruitment co-factors that might play a role in the modulation of transcription.

Treatment with a selective ligand might also tilt the overall AHR pool towards repressing cholesterol and fatty acid biosynthesis away from potentially toxic DRE-driven genes. Determining the ability of a selective ligand to activate the receptor's function to repress the lipid biosynthesis pathways without inducing xenobiotic metabolism would be essential if the receptor were to be therapeutically utilized.

Chapter 2

AH RECEPTOR REGULATES THE CHOLESTEROL BIOSYNTHETIC PATHWAY IN A DIOXIN RESPONSE ELEMENT-INDEPENDENT MANNER

2.1 ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor. Activation of AHR mediates the expression of target genes (e.g. *CYP1A1),* by binding to dioxin response element (DRE) sequences in their promoter region. To understand the multiple mechanisms of AHR-mediated gene regulation, a microarray analysis on liver isolated from ligand-treated transgenic mice expressing a wild-type *Ahr* or a DRE-binding mutant *Ahr* (A78D) on an *ahr*-null background was performed. Results revealed that AHR DRE-binding is not required for suppression of genes involved in cholesterol synthesis. Quantitative RT- PCR performed on both mouse liver and primary human hepatocyte RNA demonstrated a coordinate repression of genes involved in cholesterol biosynthesis, namely *HMGCR*, *FDFT1*, *SQLE* and *LSS* following receptor activation. An additional transgenic mouse line was established expressing a liver-specific *Ahr-A78D* on a $Cre^{Alb}/Ahr^{Fx/Fx}$ background. These mice displayed a similar repression of cholesterol biosynthetic genes compared to $Ahr^{Fx/Fx}$ mice, further indicating that the observed modulation is AHR-specific and occurs in a DRE-independent manner. Elevated hepatic transcriptional levels of the genes of interest were noted in congenic C57BL/6J-Ah^d allele mice, when compared to the WT C57BL/6J mice, which carry the Ah^b allele. Down-regulation of ARNT levels using siRNA in a human cell line revealed no effect on the expression of cholesterol biosynthetic genes. Finally, cholesterol secretion was shown to be significantly decreased in human cells following AHR activation. These data firmly establish an endogenous role for AHR as a regulator of the cholesterol biosynthesis

pathway independent of its DRE-binding ability and suggest that AHR may be a

previously unrecognized therapeutic target.

2.2 INTRODUCTION

The aryl hydrocarbon receptor is a ligand activated transcription factor belonging to the basic helix-loop-helix – Per ARNT Sim family of transcription factors. Ligands for the AHR include the planar, hydrophobic halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons, many of which are environmental contaminants. Activation of AHR by xenobiotic agonists such as TCDD, a prototypic potent ligand, is known to have toxic consequences, illustrating its role as an exogenous chemical sensor. Atypical ligands include bilirubin and indirubin [194, 195]. The presence of potent endogenous ligands for the human AHR exhibiting agonistic activities such as kynurenic acid [196] and 3-indoxyl sulfate [197] have been identified.

Upon ligand binding, the AHR heterodimerizes with the AHR nuclear translocator protein (ARNT), another bHLH-PAS family member [3]. The AHR/ARNT heterodimer represents a fully competent transcription factor capable of binding a consensus sequence known as dioxin or xenobiotic response element (DRE or XRE). This specific high affinity interaction stimulates transcription of xenobiotic target genes, including cytochrome P450s *CYP1A1* and *CYP1B1*, which are involved in the metabolism of xenobiotics. Interestingly, the regulation of xenobiotic metabolism in tissues (e.g. intestinal tract) by the AHR is important in the clearance of endogenous and exogenous compounds [198]. *Ahr*-null mice exhibit a defined set of physiological phenotypes comprising a reduction in peripheral lymphocytes, vascular abnormalities in the heart and liver, diminished fertility, and overall slower growth, all of which indicate a constitutive role for the receptor [3]. A growing list of AHR target genes has been identified that clearly point to a physiological role for the AHR beyond regulating

xenobiotic metabolism. AHR target genes that play a role in cell proliferation, cell cycle control, epithelial-mesenchymal transition and inflammation (e.g., slug, epiregulin) have been identified [188, 189].

Our laboratory has already established that DRE-binding was not necessary for ligand activated AHR to directly attenuate acute-phase gene expression, namely *Saa3* [79]. This result has led us to test the hypothesis that the AHR can regulate gene expression in the absence of DRE binding in the liver. Using a transgenic mouse model that expresses the DRE-binding mutant AHR-A78D and microarray analysis, we examined the genes that are altered by activation of this receptor. Upon injection with the AHR agonist BNF, the major class of genes markedly repressed was directly involved in cholesterol metabolism. We found a similar change in primary human hepatocytes following receptor activation, demonstrating receptor involvement in regulating cholesterol synthesis both in vivo in mice, and in human cells. Absence of the AHR in mice and human cells correlated with an increased level of expression of those enzymes, further proving an endogenous role of the receptor in cholesterol homeostasis in the absence of any exogenous ligand. Finally, we demonstrated that repression of cholesterol synthesis gene expression was mirrored by a repression in the rate of cholesterol secretion in primary human hepatocytes.

2.3 MATERIALS AND METHODS

Cell Culture

Hep3B cells, a human hepatoma-derived cell line, were maintained in α-minimal essential medium (Sigma, St. Louis, MO), supplemented with 8% fetal bovine serum (FBS) (HyClone Labs, Logan, UT), 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma) in a humidified incubator at 37°C, with an atmospheric composition of 95% air and 5% CO_2.

Primary Human Hepatocytes

Primary human hepatocytes were obtained from the University of Pittsburgh, through the Liver Tissue Cell Distribution System, NIH Contract #N01-DK-7-0004 /HHSN267200700004C. Cells were kindly provided by Curt Omiecinski and Stephen Strom. Hepatocytes were received plated in collagen-coated T25 flasks. Upon arrival, the media were changed to William's Media E supplemented with 1% penicillin/streptomycin, 10 mM HEPES, 20 μM glutamine, 25 nM dexamethasone, 10 nM insulin, 30 mM linoleic acid, 1 mg/ml BSA, 5 ng/ml selenious acid, 5 μg/ml transferrin. Within 4–16 h, a 10 mg/ml stock solution of Matrigel (BD Biosciences, San Jose, CA), diluted to a final concentration of 225 μg/ml, was added dropwise to the culture media and evenly distributed by gentle swirling. The media were subsequently changed every 2 days until cells were harvested. Cells were exposed to BNF (10μM) or carrier solution for 5 h.

RNA Isolation and Reverse Transcription

RNA samples were isolated from cell cultures and mouse livers using TRI Reagent according to the manufacturer's specifications (Sigma Aldrich). cDNA was generated using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA).

Quantitative PCR

Sequences of primers (Table 2.1) were designed to detect mRNA levels. PerfeCTa™ SYBR® Green SuperMix for iQ (Quanta Biosciences, Gaithersburg, MD) was used and analysis was conducted using MyIQ software (Bio-Rad Laboratories, Hercules, CA).

Gene Silencing

Specific protein levels were decreased in Hep3B cells using the Dharmacon small interfering RNA (siRNA) (control oligonucleotide D001810-0X, AHR oligonucleotide J004990-07, ARNT oligonucleotide D007207-01). Hep3B cells were used for their transfection efficiency compared to Huh-7 cells used in other experiments. Electroporation/nucleofection was performed using the Amaxa nucleofection system essentially as described in the manufacturer protocols. Briefly, the cells were washed and suspended at a concentration of 2.0×10^6/100 µl of nucleofection solution. Control or targeted siRNA was added to the sample for a final concentration of 1.5 µmol/liter. The samples were electroporated using the manufacturer's T16 high efficiency program and

plated into six-well dishes in complete medium. 72 h post-electroporation, RNA and

protein samples were isolated.

Table 2.1 **Primers used for quantitative cholesterol synthesis genes RT- PCR.**

Mouse			
	Cyp1a1	F	5' AGAGGTTGGCGACTTTGACCCTTA 3'
		R	5' ACCTCCCGAAACTGATTGCTGAGA 3'
	Hmgcr	F	5' ATGCCTTGTGATTGGAGTTGGCAC 3'
		R	5' AACTCTGGCAAAATGGCTGAGCTG 3'
	Fdft1	F	5' TGCCTCAGAGTTTGAAGACCCCAT 3'
		R	5' TCCTGAGGCCAAAACTTCCTTCCT 3'
	Sqle	F	5' TTGTTGCGGATGGACTCTTCTCCA 3'
		R	5' GTTGACCAGAACAAGCTCCGCAAA 3'
	Lss	F	5' ATCCAAGCACTGTTAGAGGCAGGT 3'
		R	5' TCCAGTGTGCTGAAGGAGAAACCA 3'
	Rpl13	F	5' TTCGGCTGAAGCCTACCAGAAAGT 3'
		R	5' GCATCTTGGCCTTTTCCTTCCGTT 3'

Human	CYP1A1	F	5' TCTTCCTTCGTCCCCTTCAC 3'
		R	5' TGGTTGATCTGCCACTGGTT 3'
	HMGCR	F	5' GTTTGCCCTCAGTTCCAACTCACA 3'
		R	5' GACATGCAGCCAAAGCAGCACATA 3'
	FDFT1	F	5' GCAGCAGCCTGAAAACTTGCTACA 3'
		R	5' AGTTGTGTAACAGCGGGACCTTCT 3'
	SQLE	F	5' ACTTCTCGTCCTGGGACACTGTTT 3'
		R	5' GGTGAAAGTGGCAATGCCCAGAAA 3'
	LSS	F	5' CATCAACATGCTTGTGCGCTGGTA 3'
		R	5' ATTTTCATGCCGTCAAGGCCCATC 3'
	RPL13	F	5' CCTGGAGAAGAGGAAAGAGA 3'
		R	5' GAGGACCTCTGTGTATTTGTCAA 3'
	CYP51	F	5' GAGTGATGCTGCTGCACTGCTTTT 3'
		R	5' ACATCGTATGCAACTCCCTTCCCA 3'
	HMGCS1	F	5' AAAGTACCAAGACTCCCTGCCACA 3'
		R	5' ATCCCATTCCTCCAACTGTTCCCA 3'
	IDI1	F	5' AATCCAGCCGAGCTTGAGGAAAGT 3'
		R	5' ATTCCAAGCTCAGCTTTCAGCCGT 3'
	HSD17B7	F	5' AAGGCAAGGAACCCTACAGCTCTT 3'
		R	5' AATATTGCCGGCATCAACAGCGTC 3'

Mice

The cDNA for the mouse A78D-*Ahr* or the wild-type *Ahr* were inserted into the

pTTR1 EXV3 vector (obtained from Dr. Terry Van Dyke, University of North Carolina),

which mediates hepatocyte specific expression through the *Ttr* promoter. A78D-*Ahr*^{Ttr}

and *Ahr*^{Ttr} fragments were then microinjected into C57BL/6J fertilized eggs at the Penn

State University Transgenic Mouse Facility. Transgenic mice A78D-*Ahr*^{Ttr} *and Ahr*^{Ttr}

were mated with *Ahr*$^{(-/-)}$ and the albumin promoter-driven, *Cre* recombinase-expressing

Cre^{Alb}*Ahr*$^{Fx/Fx}$ mice (a kind gift from Christopher Bradfield, University of Wisconsin) to

produce transgenic A78D-*Ahr*^{Ttr}-*Ahr*$^{(-/-)}$, *Ahr*^{Ttr} *Ahr*$^{(-/-)}$, A78D-*Ahr*^{Ttr} *Cre*^{Alb}*Ahr*$^{Fx/Fx}$ and

Ahr^{Ttr} *Cre*^{Alb}*Ahr*$^{Fx/Fx}$. Congenic *Ah*^{d} and wild-type mice (C57BL/6J) were purchased from

The Jackson Laboratory (Bar Harbor, ME). All mice were genotyped using relevant primers: Ahr^{Ttr} (5'AAA GTC CTG GAT GCT GTC CGA G 3' and 5' CAG ACA TGA TAA GAT ACA TTG ATG 3'), Cre^{Alb} (5' CCA GCA ACA TTT GGG CCA GCT AAA 3' and 5' GCC GCA TAA CCA GTG AAA CAG CAT 3'), $Ahr^{(-/-)}$ (5' CAG TGG GAA TAA GGC AAG AGT GA and AGG GAG ATG AAG TAT GTG TAT GTA 3'), Ahr^{Fx} (5' GTC ACT CAG CAT TAC ACT TTC TA 3' and 5' AGT GGG AAT AAG GCA AGA GTG A 3'). Mice were housed on corncob bedding in a temperature- and light-controlled facility and given access to food (chow diet) and water ad libitum. Mice were maintained in a pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of the Pennsylvania State University. Adult (10-12 weeks) female mice of different genotypes were injected intraperitoneally with BNF at 50 mg/kg dissolved in corn oil or with corn oil alone for 5 h. Mice were sacrificed via CO_2 inhalation.

DNA Microarray: Isolation of RNA and Data Analysis

Livers were isolated from mice (3 per group) injected with BNF (50 mg/kg) or corn oil for 5 h. DNA microarray analysis of those samples was performed as described (fla, murray gary tox sci 2010). GeneChip Operating Software (Affymetrix) was utilized to preprocess CAB/CEL files generated from scanned DNA microarrays. Data quality was initially assessed by checking the array image, B2 oligo performance, average background to noise ratios, poly-A controls, hybridization controls, and the 3# to 5# probe set ratios for control genes. DNA microarray data were normalized using Probe Logarithmic Intensity Error (PLIER-MM) approximation algorithm (Affymetrix Expression Console Software 1.1).

Protein and RNA Preparation

Mouse liver samples were collected and frozen immediately in liquid nitrogen before storage at -80°C, RNA was isolated using TRI Reagent (Sigma). Livers were homogenized in MENG buffer (25 mM MOPS, 2 mM EDTA, 0.02% NaN_3, and 10% glycerol, pH 7.5) with protease inhibitors (Sigma) using a Dounce homogenizer. Hep3B extracts were prepared in MENG buffer, 1% NP-40 and proteinase inhibitors. Cell homogenates were then centrifuged at 14,000 x g for 10 minutes and proteins were analyzed.

Immunoblotting

Mouse liver and cell extracts were resolved on 8% SDS-tricine polyacrylamide gels. Proteins were transferred to PVDF membrane and detected using the mouse AHR monoclonal antibody RPT1 (Thermo Scientific). All other primary antibodies were from Santa Cruz Biotechnology (1/1000 dilution) and were visualized using a secondary biotinylated antibody and $[I^{125}]$streptavidin followed by autoradiography.

Lipid Extraction and GC-MS Analysis

Primary human hepatocytes were treated with BNF (10µM) every 12 h for 48 h and the media was changed every 24 h. Lipids were extracted from the media using the Bligh and Dyer method [199]. Cholestanol (Sigma) was used as an internal standard. Subsequently, two aliquots of the sample were evaporated to dryness under a stream of nitrogen. A 99:1 mixture of BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) and

TMCS (trimethylchlorosilane) was added, vortexed for 60 seconds, and the sample heated for 60 min at 60-65°C. Samples were analyzed by GC-MS using an Agilent 6890 gas chromatograph coupled to a Waters GCT classic mass spectrometer. Samples were separated on a DB5 (5% phenyl-methylpolysiloxane) capillary column (20 m length, 180 µm diameter 0.15 µm film thickness). Samples were injected onto the column using a split/splitless injector in splitless mode, the initial temperature of 50°C was held for 2 min, and then heated at 15°C/min to a final temperature of 300°C which was held for 3 min. Helium was used as the carrier gas at a constant flow of 0.5ml/min. Mass spectra were acquired in electron ionization mode over the mass range 45-500 Da, scan time was 0.2 seconds and five scans were summed with a 0.05 seconds interscan delay. Values represent average of 3 wells run in duplicate.

LDH Assay

Possible toxicity caused by ligand treatment on primary human hepatocytes was assessed using *In Vitro* Toxicology Assay Kit, Lactic Dehydrogenase (Sigma).

Statistical Analysis

Data were analyzed using the Mann-Whitney U-test in GraphPad Prism (v.5.01) software to determine statistical significance between treatments. P-values < 0.05 were considered statistically significant (*$P<0.05$; **$P<0.01$;***$P<0.001$).

2.4 RESULTS

Characterization of a Transgenic Mouse Lines Expressing a DRE-Binding Mutant or WT AHR.

Our laboratory has previously established that A78D modification in the mouse AHR is sufficient to render the receptor unable to bind DRE sequences without compromising its other functions [200]. In the current study, we wanted to further address the ability of the AHR to affect hepatic gene expression *in vivo* independent of its DRE binding activity. For this purpose, we cloned the wild-type (WT) *Ahr* and the A78D-*Ahr* vector under the regulation of the hepatocyte-specific transtherytin (TTR) promoter. We established the *Ahr*^{Ttr} and A78D-*Ahr*^{Ttr} expression vectors in mice, which were then backcrossed onto an *ahr*-null background. The resulting mice were *ahr*-null with either the WT or the DRE-binding mutant form of the receptor expressed exclusively in the hepatocytes. Figure 2.1A confirms that the A78D modification completely abolishes BNF-dependent induction of DRE-driven *Cyp1a1* activity. To ensure that the expression of the transgene was intact, liver proteins were subjected to western blot analysis and the results revealed a similar level of expression. Finally, a photo-affinity ligand experiment demonstrated that the ligand-binding ability of the receptor was not affected by the mutation (Figure 2.1B).

Figure 2.1
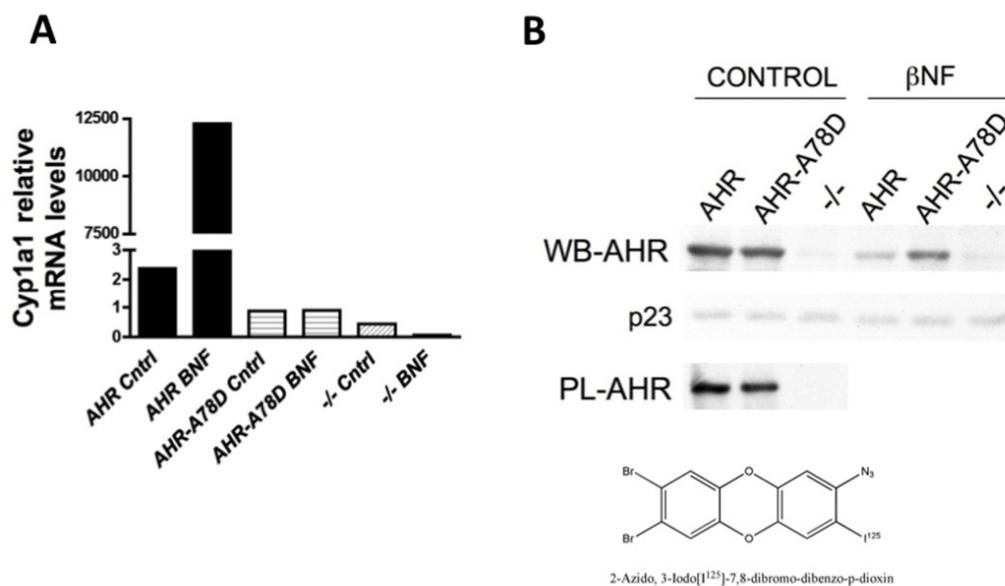
Figure 2.1 **Characterization of *Ahr* transgenic mouse lines.**
   A. BNF-dependent activation of *Cyp1a1*, a DRE-driven gene, is completely
   abolished in A78D-*AhrTtr Ahr(-/-)* (AHR-A78D) transgenic mice to levels similar
   to *Ahr* (-/-). **B.** Liver protein expression of transgenic mice confirms a similar
   expression of the A78D-*AhrTtrAhr(-/-)* and the *AhrTtrAhr(-/-)* (AHR) proteins in
   control and BNF-treated mice. Ligand binding shows the ability of the mutant
   receptor to bind a photoaffinity ligand (PL-AHR). The structure of the
   photoaffinity ligand is shown.

DNA Microarray Analysis.

Transcript profiling was performed on liver RNA isolated from mice of each genotype: *ahr*-null and our transgenic mouse lines $Ahr^{Ttr}Ahr^{(-/-)}$ and A78D-Ahr^{Ttr} -$Ahr^{(-/-)}$. Subsequent data analysis pointed to a suppression of a large subset of genes involved in the cholesterol biosynthesis pathway when AHR was activated regardless of its ability to bind the consensus DRE sequence (Table 2.2 and Figure 2.2). Conversely, no change in the transcript levels of those genes was noted when *ahr*-null mice were similarly treated, further indicating that the observed change in gene expression in the Ahr^{Ttr} and A78D-Ahr^{Ttr} transgenic mice was AHR-mediated.

Table 2.2 **Liver gene expression of transgenic A78D-*Ahr*^{Ttr} *Ahr*$^{(-/-)}$ or *Ahr*^{Ttr} *Ahr*$^{(-/-)}$ treated with BNF^a.**

	Gene Title	Gene Symbol	Ratio WT +/- BNF	Ratio A78D +/- BNF	Ratio Ahr (-/-) +/- BNF
***	Cytochrome P450, family 51	Cyp51	-1.4	-1.4	-0.1
	Down syndrome critical region gene 1-like 1	Dscr1l1	-1.4	-0.6	0.2
***	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	Hmgcs1	-1.3	-1.4	0.2
***	Lanosterol synthase	Lss	-1.3	-0.8	-0
***	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	Hmgcr	-1.2	-0.6	0.4
***	Farnesyl diphosphate farnesyl transferase 1	Fdft1	-1.2	-1.2	0
***	Isopentenyl-diphosphate delta isomerase	Idi1	-1.1	-1.4	-0.1
	Coenzyme Q10 homolog B (S. cerevisiae)	Coq10b	-1.1	-0.4	0
	NAD(P) dependent steroid dehydrogenase-like	Nsdhl	-1.1	-0.6	0.1
***	Sterol-C4-methyl oxidase-like	Sc4mol	-1	-1.2	-0.2
***	Acetyl-Coenzyme A acetyltransferase 2	Acat2	-1	-0.4	0.2
	Proprotein convertase subtilisin/kexin type 9	Pcsk9	-1	-0.9	0.2
***	Hydroxysteriod (17-beta) dehydrogenase 7	Hsd17b7	-1	-0.7	0.3
	Retinol dehydrogenase 11	Rdh11	-0.9	-0.8	0.2
***	Phosphomevalonate kinase	Pmvk	-0.8	-0.8	0.4
	Retinol dehydrogenase	Rdh11	-0.8	-0.6	0.1
	Macrophage activation 2 like	Mpa2l	-0.8	-0.5	-0.2
***	7-dehydrocholesterol reductase	Dhcr7	-0.6	-0.6	-0.3
***	Squalene epoxidase	Sqle	-0.6	-1.2	0
***	Mevalonate (diphospho) decarboxylase	Mvd	-0.6	-0.4	0
	UDP-glucose ceramide glucosyltransferase	Ugcg	-0.6	-0.4	-0.1
	Latrophilin 2	Lphn2	-0.6	-0.5	-0.2
	Carboxylesterase 5	Ces5	-0.6	-0.4	0.1
	Camello-like 2	Cml2	-0.5	-0.4	-0.1
	Cytochrome b5 type B	Cyb5b	-0.5	-0.6	0
	Cytochrome b5 type B	Cyb5b	-0.4	-0.6	-0
	Antiproteinase, antritrypsin, member 7	Serpina7	-0.4	-0.6	-0.1
	Hydroxyacyl glutathione hydrolase	Hagh	-0.4	-0.4	-0.1
	Carboxylesterase 3	Ces3	-0.4	-0.9	-0.2
	Immunity-related GTPase family, M	Irgm	-0.4	-0.4	0.1
	Dimethylglycine dehydrogenase precursor	Dmgdh	-0.4	-0.4	-0.1

^{a} Results indicate ratio of BNF- compared to vehicle-treated mice. Genes that are directly part of the cholesterol biosynthesis pathway are indicated (***).

Figure 2.2

Acetoacetyl CoA

*** *HMG CoA synthase*

HMG CoA

*** *HMG-CoA reductase*

Mevalonate

Mevalonate kinase
*** *Phosphomevalonate kinase*
*** *Mevalonate PP decarboxylase*
GPP synthase
*** *IPP isomerase*
*** *FPP synthase*
Squalene synthase

Squalene

*** *Squalene epoxidase*
*** *Lanosterol synthase*
*** *CYP51*
Lathosterol oxidase
*** *7-dehydrocholesterol reductase*
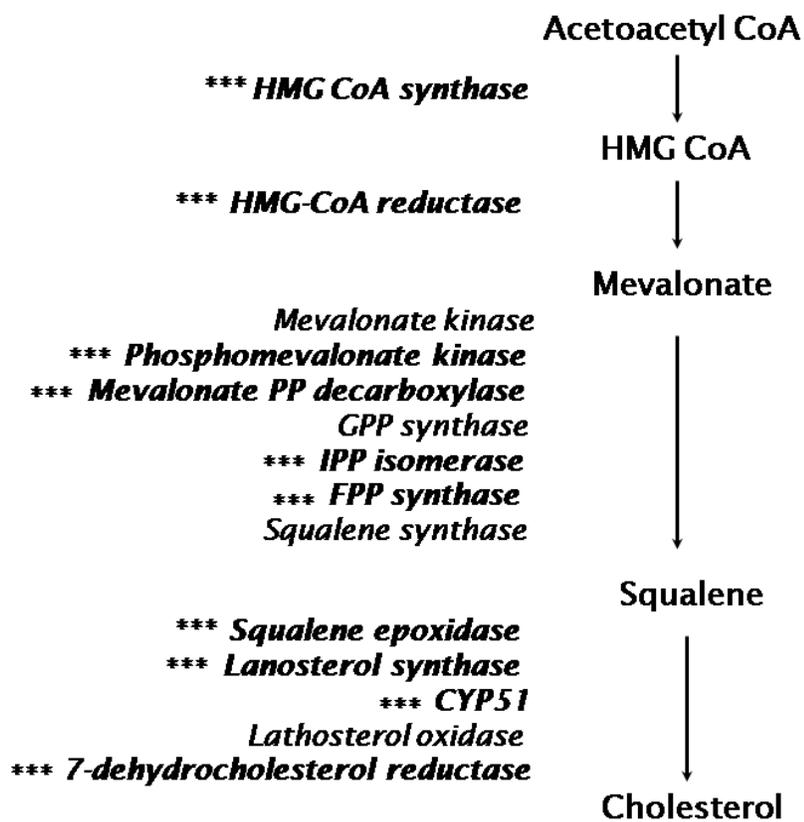
Cholesterol

Figure 2.2 **Cholesterol biosynthetic genes expression downregulated by AHR.**
Cholesterol biosynthetic genes downregulated by AHR activation in a
DRE-binding independent mechanism (based on our microarray data) are
indicated (***).

Regulation of Cholesterol Synthesis Gene Expression by AHR in Vivo.

In order to validate our microarray data, we injected WT mice with BNF. *Cyp1a1* levels were utilized as a positive control for receptor activation (Figure 2.3A and B). Hepatic RNA levels of selected cholesterol synthesis genes, including the gene encoding the pivotal rate-limiting enzyme of the cholesterol synthesis pathway *HMGCR*, were revealed to be significantly repressed when BNF was administered. Interestingly, SREBF2 expression showed no significant change. From this point on, we decided to focus on the genes encoding the most studied and critical enzymes in the mevalonate pathway: *hmgcr*, *fdft1, sqle*, and *lss*. These enzymes have been the subject of extensive studies to find a new therapeutic target in order to down-regulate the activity of the cholesterol synthesis pathway [166].

Figure 2.3
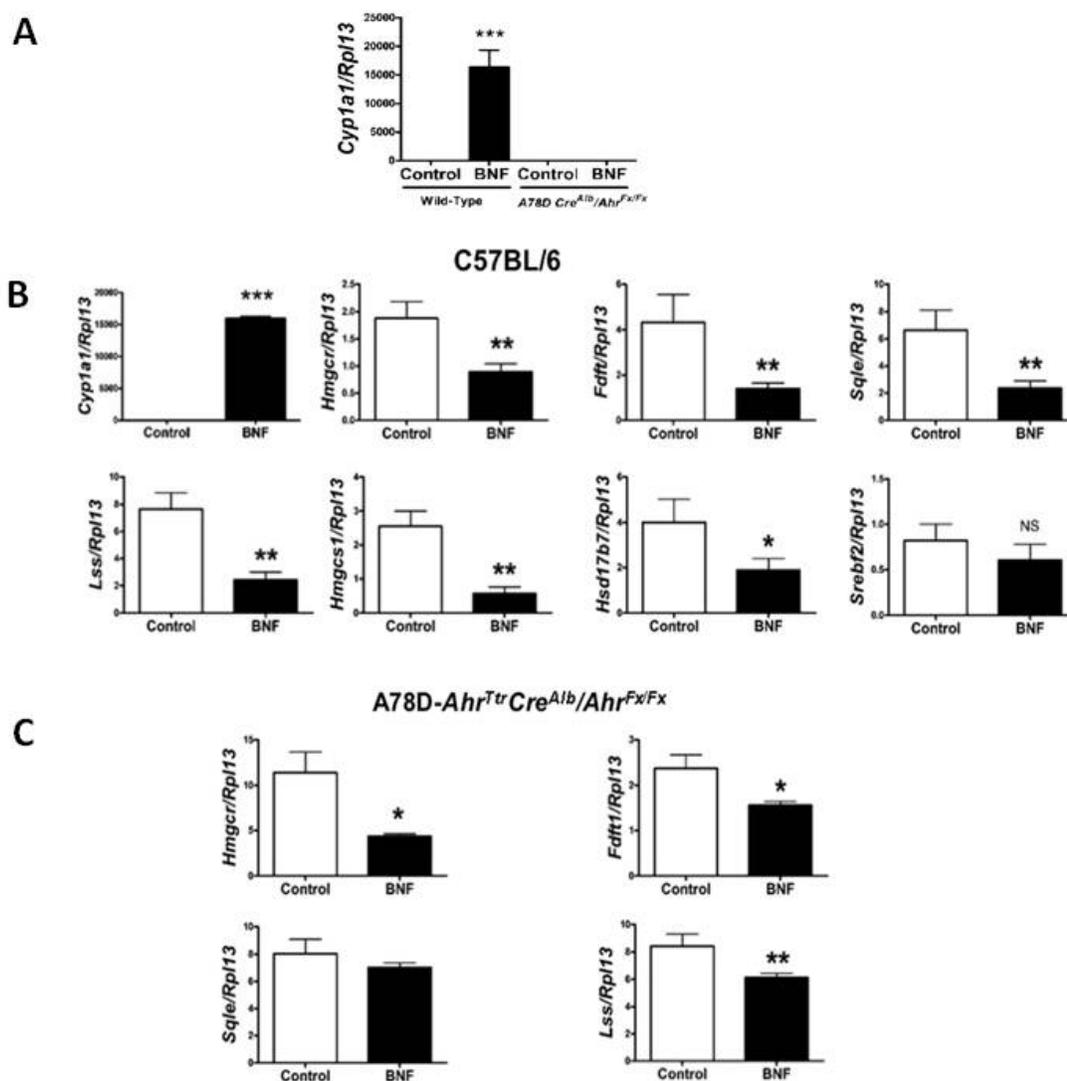
Figure 2.3 **Repression of cholesterol synthesis gene expression following wild-type and DRE binding mutant AHR activation in mice.**

    A. Hepatic *Cyp1a1* levels confirm the inability of the mutant receptor in A78D-Ahr *Cre*^{Alb} *Ahr*$^{Fx/Fx}$ transgenic mice to upregulate its expression compared to C57BL/6 (WT) mice following AHR activation. **B.** Normalized hepatic RNA expression of cholesterol metabolism genes in wild-type mice (6 female mice per group) injected with BNF versus control mice are shown. **C.** Normalized hepatic mRNA levels from A78D-*Ahr*^{Ttr} *Cre*^{Alb}*Ahr*$^{Fx/Fx}$ mice injected with BNF compared to vehicle-treated mice (6 female mice per group). (Figure 2.3B and 2.3C are different experiments).

Regulation of Cholesterol Synthesis Gene Expression by AHR in a DRE-

Independent Manner.

Due to the compromised physiological condition of *ahr*-null mice, we decided to establish the A78D-*Ahr^Ttr^* transgene on a *Cre^Alb^* background. The resulting mice expressed the *Ahr^Fx/Fx^* receptor in all tissues except in hepatocytes, where the A78D-*Ahr^Ttr^* transgene was present and failed to induce *Cyp1a1* (Figure 2.3A). Figure 2.3C, a similar repression in the expression of hepatic cholesterol synthesis genes occurs in the A78D-*Ahr^Ttr^* *Cre^Alb^* *Ahr^Fx/Fx^* mice when the receptor was activated. The mutation did not affect the basal level of expression of our genes of interest (Figure 2.4). The levels of these transcripts in the liver exhibited no difference upon BNF treatment of *Cre^Alb^* *Ahr^Fx/Fx^* mice, further confirming that the BNF effect in WT and transgenic DRE-binding mutant mice was mediated through AHR (Figure 2.5).

Figure 2.4
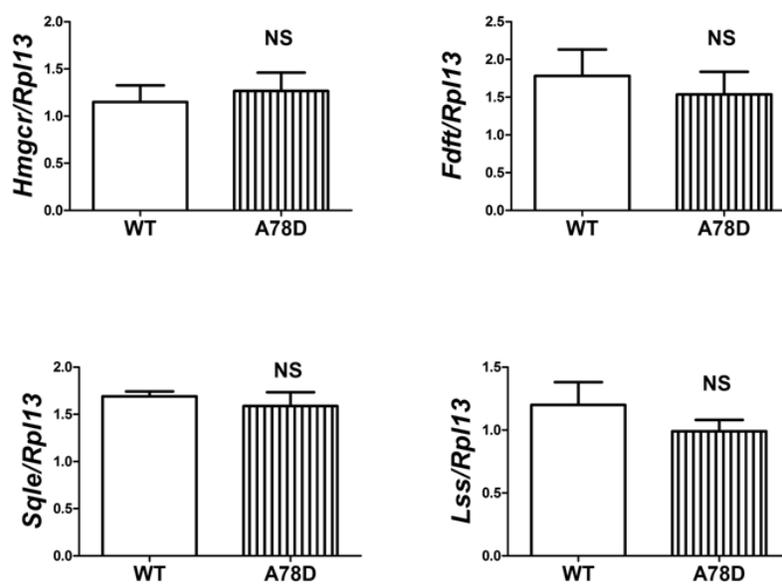
Figure 2.4 **DRE-binding mutation in AHR does not affect the expression of cholesterol synthesis genes.**
Hepatic RNA levels of C57BL/6 (WT) and A78D-*Ahr Cre^{Alb}/Ahr^{Fx/Fx}* non-treated female mice show no difference in basal expression.

Figure 2.5
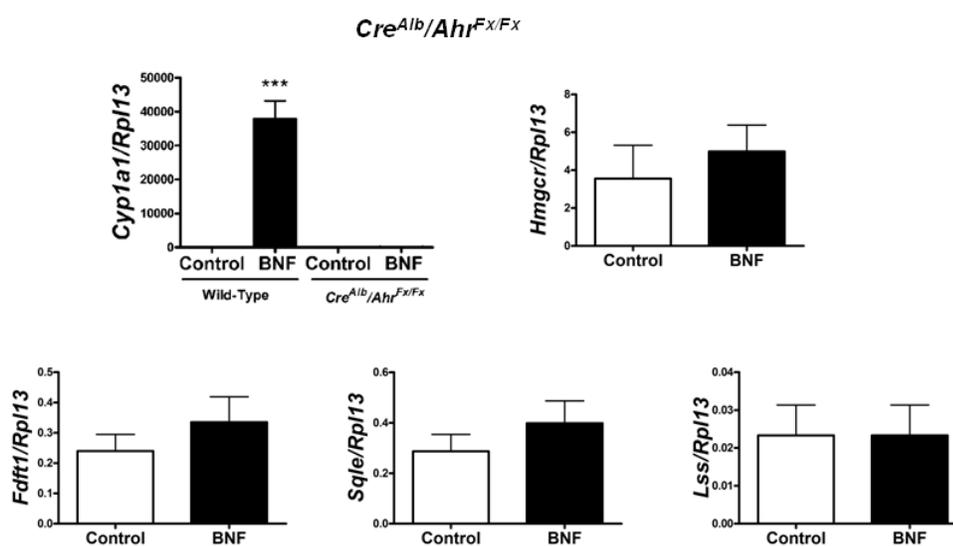
Figure 2.5 **Levels of cholesterol metabolism gene expression in $Cre^{Alb}Ahr^{Fx/Fx}$ following ligand treatment.**

No change was detected in the hepatic mRNA expression levels of our target genes in $Cre^{Alb}Ahr^{Fx/Fx}$ mice upon BNF treatment (n=6 per group). *Cyp1a1* levels are used as a control to confirm activation of the receptor in wild-type mice but not in $Cre^{Alb}Ahr^{Fx/Fx}$ mice.

Constitutive Role for AHR in the Regulation of Cholesterol Synthesis Gene Expression.

We tested whether there is a difference in constitutive expression of genes in the cholesterol biosynthetic pathway between Ah^d allele (low ligand affinity) on a C57BL6/J background and Ah^b allele (high affinity) in C57BL6/J mice. These two allelic forms of the AHR differ in their ability to mediate induction of AHR activity upon ligand treatment. Higher transcriptional levels of cholesterol synthesis genes were noted in Ah^d congenic mice compared to C57BL/6 mice, suggesting a role for endogenous AHR ligands in modulating the expression of cholesterol synthesis genes (Figure 2.6A). In contrast to the extensive repressive activity observed in C57BL/6 mice, no significant differences were noted in the BNF-treated in Ah^d congenic mice compared to control (Figure 2.7). This supports the notion that receptor activation by a ligand mediates suppression of cholesterol synthesis gene expression. Next, whether the presence of the AHR constitutively attenuates the expression of cholesterol synthesis genes was examined. To directly test this hypothesis, we assessed the constitutive hepatic levels of *hmgcr*, *fdft1*, *sqle* and *lss* between $Cre^{Alb}Ahr^{Fx/Fx}$ and C57BL/6 mice. Results revealed that an absence of the receptor correlated with a significant elevated level of gene expression and corresponding protein levels of these enzymes (Figure 2.6B and Figure 2.8).

Figure 2.6
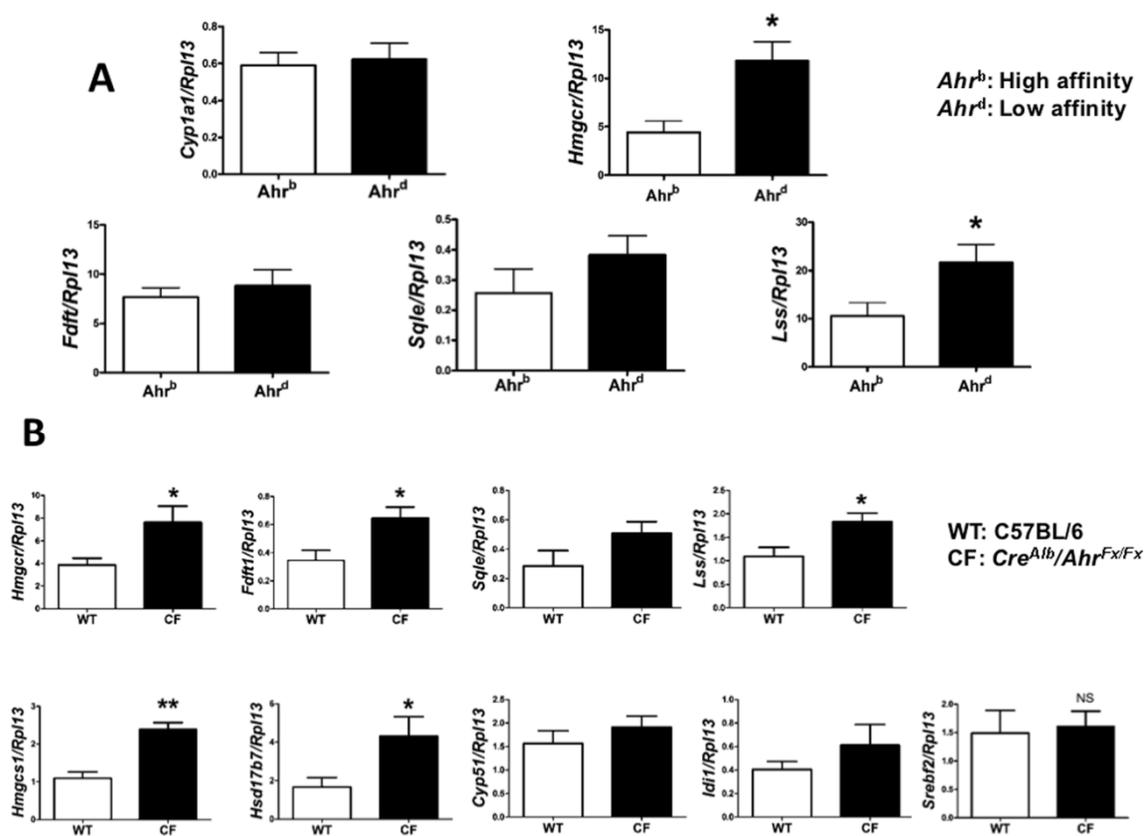
Figure 2.6 **AHR unresponsive allele and AHR absence correlated with a higher expression of cholesterol synthesis genes.**

> **A.** Normalized hepatic mRNA of cholesterol biosynthetic genes in *Ahb* and *Ahd* congenic mice (6 female mice per group) are shown in the absence of any exogenous ligand. **B.** Normalized mRNA hepatic expressions of selected cholesterol biosynthetic genes in $Cre^{Alb}Ahr^{Fx/Fx}$ mice (CF) are compared to C57BL/6J (WT) mice (6 female mice per group) in the absence of exogenous ligand.

Figure 2.7
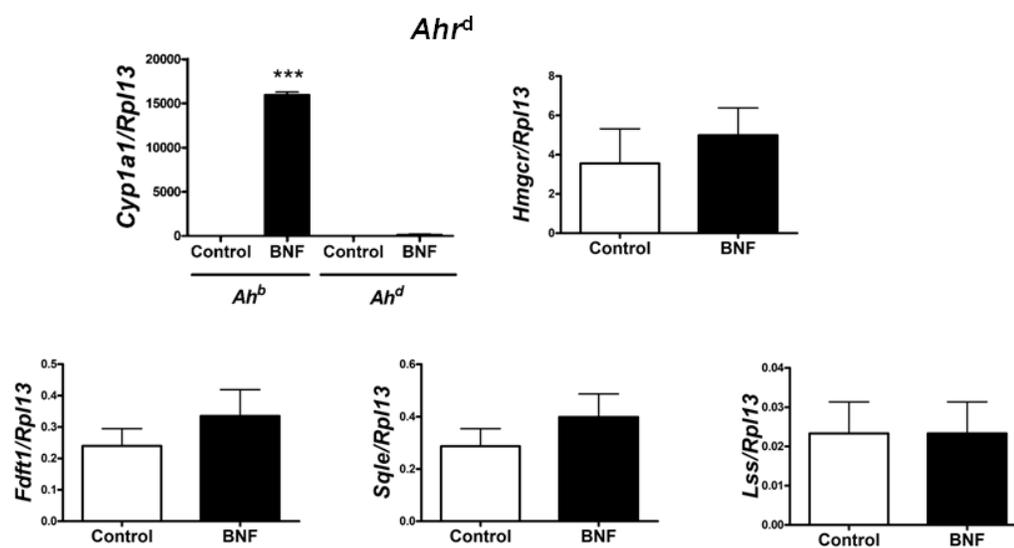
Figure 2.7 **Levels of cholesterol metabolism gene expression in AHR-unresponsive mice following ligand treatment.**

No change was detected in the hepatic mRNA expression levels of our target genes in Ah^d congenic mice upon BNF treatment. *Cyp1a1* levels are used as a control to confirm activation of the receptor in wild-type mice but not in Ah^d congenic mice.

Figure 2.8
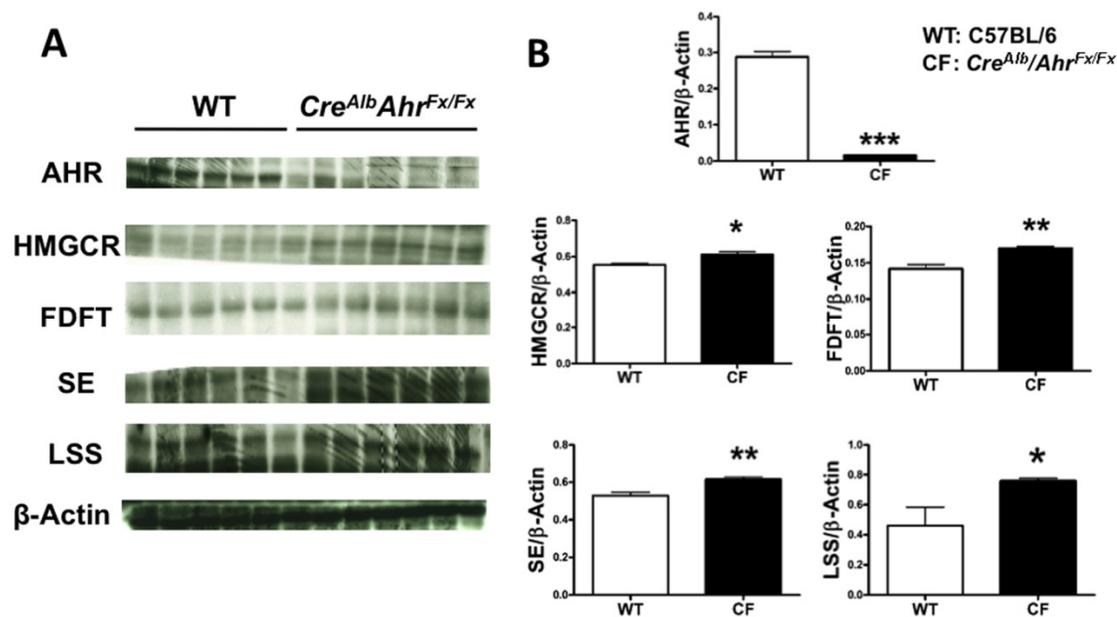
Figure 2.8 **AHR absence in mice correlated with a higher protein expression of cholesterol synthesis genes.**
    A. Hepatic protein expressions of cholesterol biosynthetic genes in liver-specific $Cre^{Alb}Ahr^{Fx/Fx}$ mice (CF) are compared to C57BL/6J (WT) mice (6 female mice per group) in the absence of exogenous ligand. **B.** Quantification of protein expression normalized to β-actin.

Role for AHR in the Regulation of the Genes in the Cholesterol Synthetic Pathway in Primary Human Cells.

Primary human hepatocytes were administered BNF and subsequent analysis of mRNA levels revealed a significant decrease in the four core *de novo* cholesterol biosynthesis genes (Figure 2.9). We also examined other enzymes in the pathway, *CYP51, HMGCS1, HSD17B7*, and *IDI1*; they showed a similar trend of repression further suggesting a general regulation of the cholesterol biosynthetic pathway by AHR. However, SREBP2 expression levels were not altered by BNF treatment. In order to further explore the mechanism of this regulation, AHR siRNA was used to decrease expression of the AHR in human Hep3B cells. Similar to our in vivo results in mice, enhanced expression of the mRNA and protein levels of the genes of interest correlated with lower AHR levels (Figure 2.10). Consistent with our mouse data, this establishes a role for AHR in the regulation of cholesterol homeostasis in humans.

ARNT has been linked to cholesterol homeostasis through its regulation of the ATP-binding cassette transporter A1 (ABCA1), a major reverse cholesterol transporter [201]. Agonist treatment leads to the formation of AHR-ARNT heterodimer formation, the ablation of ARNT expression can assess whether this heterodimer plays a role in gene repression studied here. Unlike AHR, partial absence of ARNT in Hep3B cells had no effect on the mRNA levels of cholesterol synthesis genes, suggesting that the AHR mediates gene repression in the absence of heterodimer formation (Figure 2.11). This result is consistent with the ability of the DRE-binding mutant AHR to repress the genes described here.

Figure 2.9
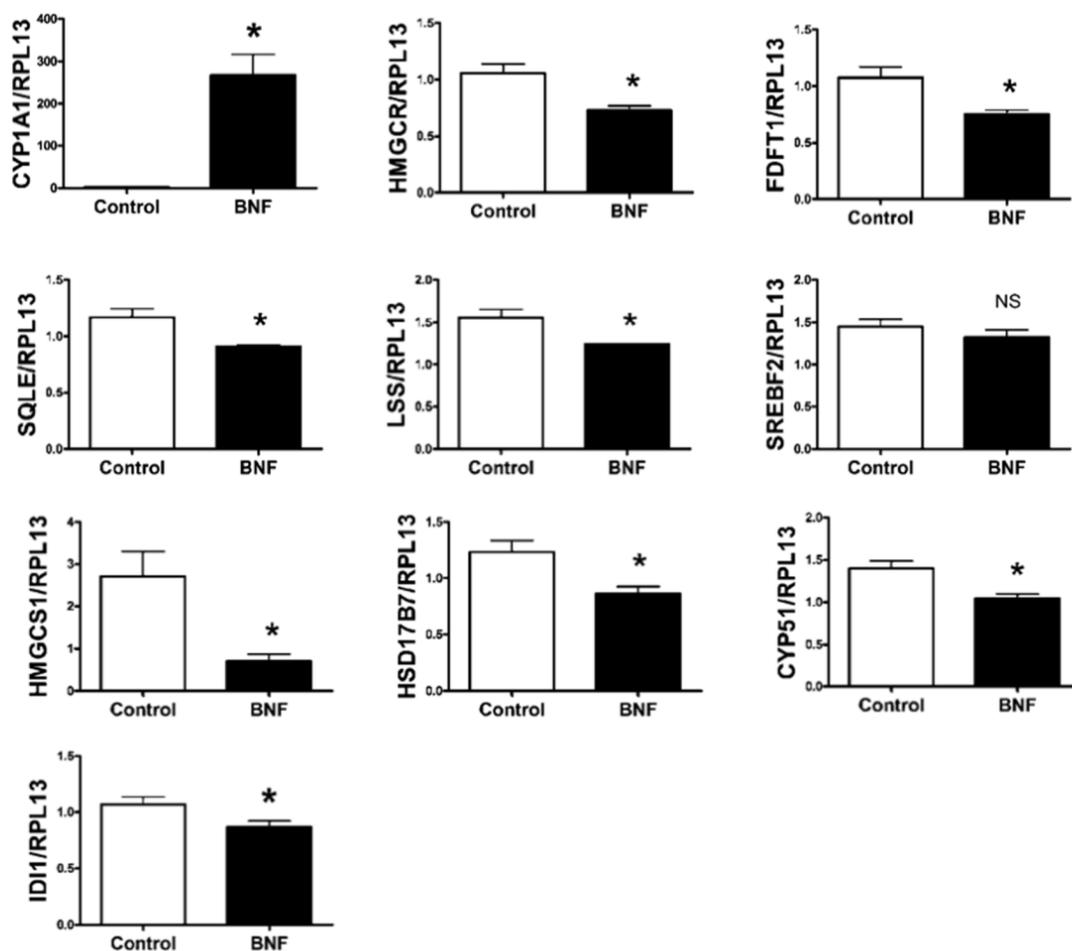
Figure 2.9 **Repression of cholesterol synthesis gene expression following AHR activation in primary human hepatocytes.**
Normalized mRNA expression of selected cholesterol biosynthesis genes of primary human hepatocytes (3 wells per group) is shown.
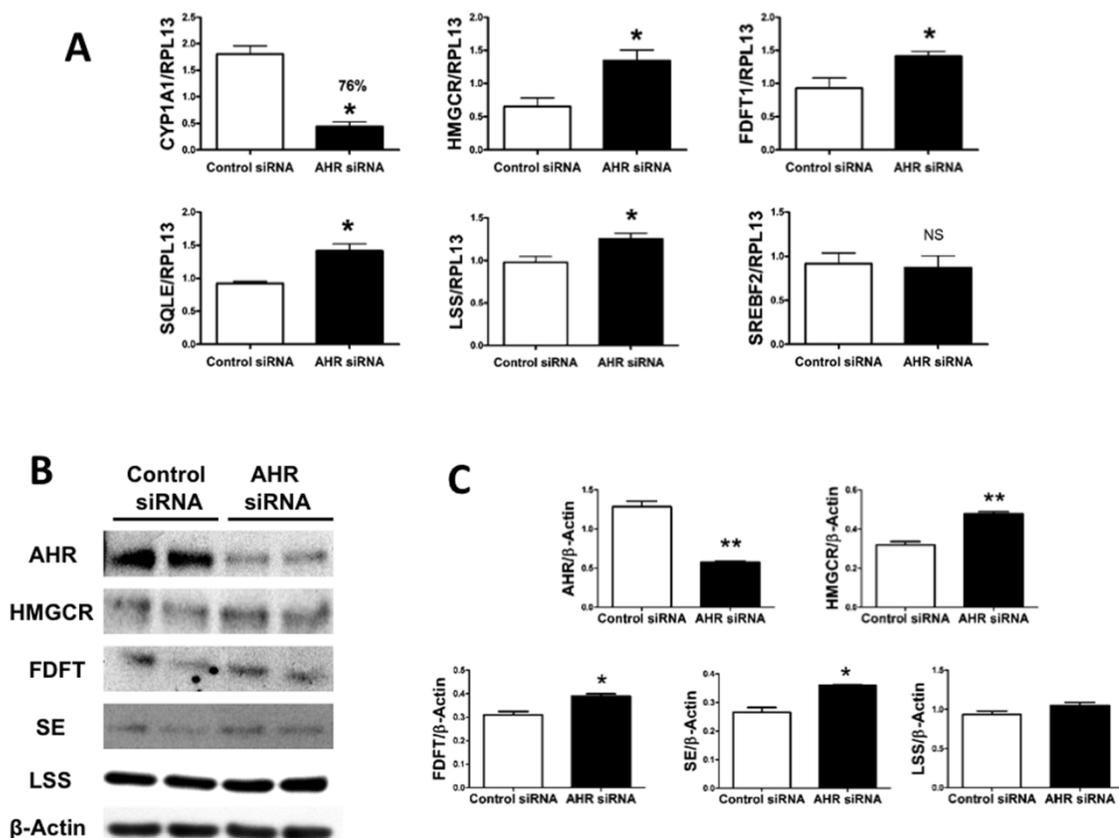
Figure 2.10 **Lower levels of AHR in human cells correlated with higher cholesterol synthesis gene expression.**

> **A.** Normalized RNA hepatic expression of selected cholesterol biosynthesis genes in Hep3B cells where AHR has been down-regulated (AHR siRNA) are compared to control cells in the absence of exogenous ligand. CYP1A1 levels are used to confirm down-regulation of the receptor. **B.** Hepatic protein expression of selected cholesterol biosynthetic genes in AHR down-regulated Hep3B cells (AHR siRNA) are compared to control cells in the absence of exogenous ligand. **C.** Quantification of protein expression is normalized to β-actin.

Figure 2.11

A

B
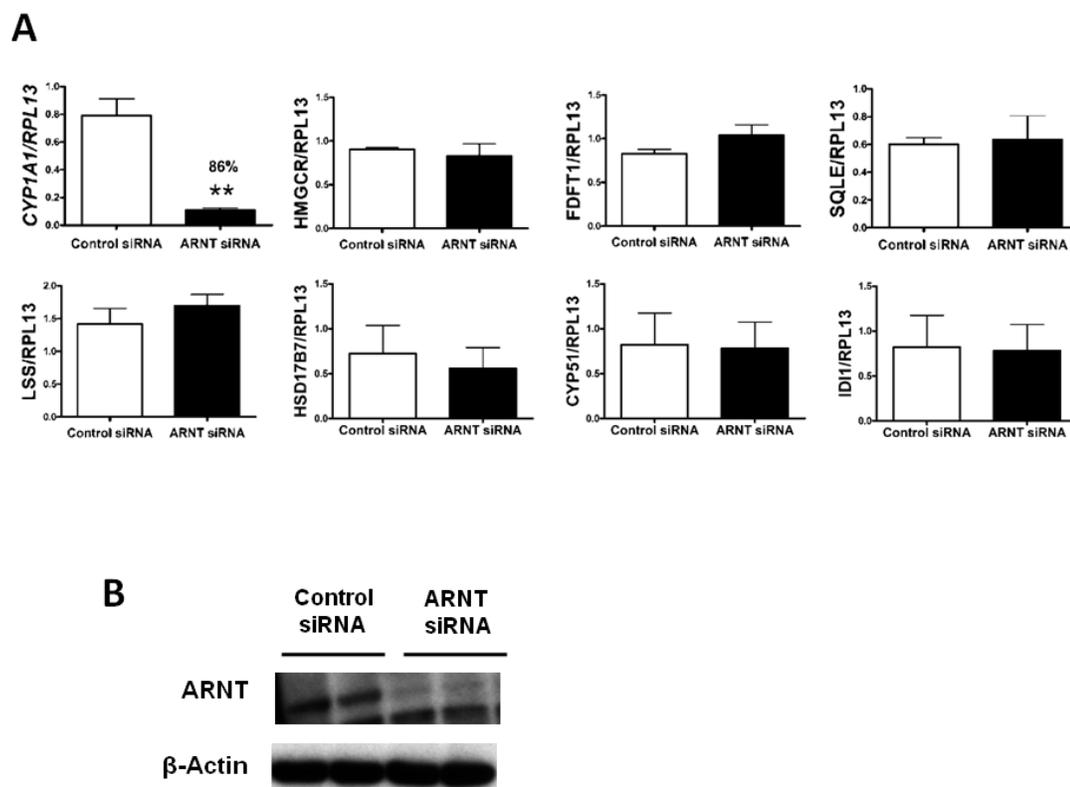
Figure 2.11 **ARNT ablation in human cells fails to alter expression of cholesterol synthesis genes.**
    A. Normalized mRNA expression levels of selected cholesterol biosynthetic genes in ARNT-downregulated Hep3B cells (3 wells per group) are compared to control cells (control siRNA) in the absence of exogenous ligand. CYP1A1 levels are used to confirm dowregulation of ARNT. **B.** Western blot confirms downregulation of ARNT protein.

Cholesterol Synthesis Is Repressed in Primary Human Hepatocytes Following AHR Activation.

Based on our findings, one might speculate that the decrease in the expression of all cholesterol synthesis genes examined in mice and humans would result in a lowered cholesterol synthesis and subsequent secretion. For this purpose, we treated primary human hepatocytes with BNF and used the GC-MS technique to quantify cholesterol in the media. As expected, treated cells showed a significant repression in the levels of secreted cholesterol indicating an overall regulation of the pathway by AHR (Figure 2.12).

Figure 2.12
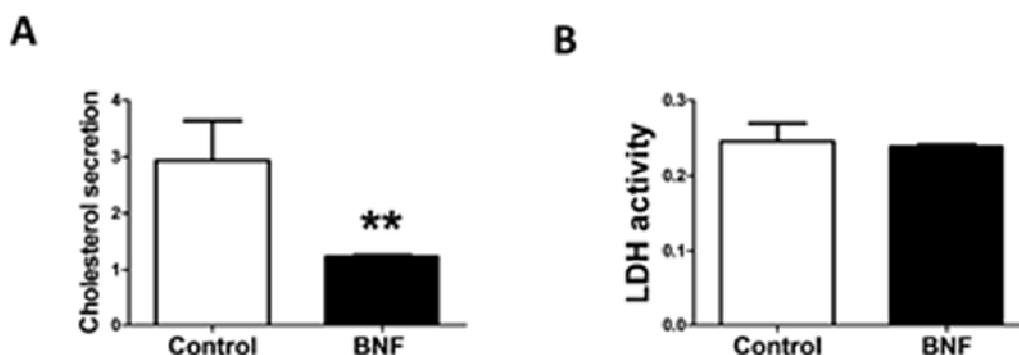
Figure 2.12 **Repression of cholesterol synthesis in primary human hepatocytes following AHR activation.**
A. Synthesized cholesterol levels from media of BNF-treated cells compared to control cells are analyzed by GC-MS and normalized to an internal standard. **B.** Cytotoxicity assay demonstrates cell viability is not affected by BNF treatment.

Table 2.3 **Effects of the genetic manipulations of the AHR gene in mice.**
Ah^d allele is a low ligand affinity version of the receptor. Ah^b allele is the high affinity dominant allele.

	Allele	DRE activity	Non-DRE activity	Effect on cholesterol synthesis
C57BL/6 (WT)	Ah^b	+	+	+
AHR- A78D	Ah^b	-	+	+
Ah^d congenic	Ah^d	+	+	-
$Cre^{Alb} Ahr^{Flox/Flox}$	-	-	-	-

2.5 DISCUSSION

In the current study, we have established a transgenic mouse model that demonstrates the ability of AHR to modulate expression of a number of genes that encode for enzymes involved in the cholesterol synthetic pathway independent of DRE-binding leading to a repression in cholesterol secretion rate. There has been extensive interest in assessing the effect of TCDD exposure on serum lipid levels and more recently on the expression of lipid metabolism genes in rodents using microarrays [123, 124], but little progress has been made with regard to AHR modulation of those genes in vivo in the absence of exogenous ligand. Evolutionary conservation of the receptor coupled with the *ahr*-null mice phenotype suggests a role for the receptor beyond mediating xenobiotic metabolism. Differential constitutive expression of cholesterol biosynthesis genes between $Cre^{Alb}Ahr^{Fx/Fx}$ mice and AHR-silenced human cell lines and their AHR expressing counterparts, as well as between Ah^d and Ah^b allele congenic mice suggests a function for the AHR in cholesterol homeostasis. Although, whether there is an endogeneous ligand(s) that mediates this effect is not known. Considering that cholesterol and oxysterols are involved in feedback regulation of cholesterol homeostasis, it will be important to test whether there are sterols that can act as AHR ligands. Indeed, 7-ketocholesterol is a key oxysterol present in serum that has been shown to be an AHR antagonist [22]. Clearly, there is a need to examine the ability of other endogenous metabolites to act as AHR ligands and regulate cholesterol biosynthesis.

Circadian clocks synchronize the rhythmic activation of selective pathways in energy metabolism in mammalian tissues. Major metabolic pathways, including glucose and lipid metabolism as well as mitochondrial fuel oxidation, exhibit diurnal rhythms.

Crosstalk between the AHR signaling pathway and the circadian rhythm is believed to occur [202]. Concomitantly, AHR expression has been shown to take place in a circadian-dependent fashion displaying dual peaks. Superimposing the circadian expression of the AHR and the rate-limiting enzyme HMGCR reveals inverse peaks of expression [202, 203]. This observation is in accordance with our results showing a higher expression of cholesterol biosynthetic enzymes with the absence of AHR both in vivo in mice, and in human cells. The integration of the circadian clock and energy metabolism and its ability to respond to a variety of exogenous stimuli, including chemical and metabolic signals, makes AHR a very likely candidate for genetic regulation of this lipid metabolic pathway. Our hypothesis for an adaptive endogenous role for the AHR is also supported by the fact that CYP1A1 and CYP1B1 are known to modulate cellular levels of a variety of lipid signaling molecules [85] and their high physiological levels observed in sections of human coronary arteries were shown to be an adaptive response to chronic arterial levels of shear stress [204]. Furthermore, shear modified LDL can lead to AHR activation in liver derived cell lines by an unknown mechanism; this observation would be consistent with a feedback regulation that attenuates cholesterol biosynthesis [205].

Our microarray and transgenic mouse studies show that the DRE-binding mutant AHR is still capable of modulating the expression of cholesterol synthesis genes upon ligand activation. Based on these observations coupled with the fact that SREBP2 levels remain unchanged both in mice and humans, one may speculate that AHR may be attenuating hepatic transcription of cholesterol biosynthetic genes through interaction with the transcription factor SREBP2 and/or through interference with co-factor

recruitment. This hypothesis is supported by the ability of the AHR and SREBP2 to physically interact with other transcription factors and the physiological interaction between AHR and SREBP1 in T cells [60, 206]. It is also worth noting that AHR has been shown to regulate the expression of CAR and FXR, nuclear receptors involved in the regulation of lipid synthesis [96, 124]. Thus, it would be interesting to explore the possible involvement of these two receptors, along with the lipid-activated nuclear receptor PXR[207], in AHR-mediated regulation of cholesterol biosynthesis.

Since the toxicity resulting from AHR activation is mediated through DRE-binding, the discovery that the AHR can coordinately attenuate the expression of cholesterol biosynthetic genes and subsequently cholesterol biosynthesis in a DRE independent manner is very an important observation. We have recently described that the AHR can be activated by selective Ah receptor modulators (SAhRM) to repress cytokine-mediated acute-phase gene expression in the liver, it will be important to test whether these compounds will also attenuate cholesterol biosynthesis [23]. Thus, whether AHR can be used as a therapeutic target to repress the expression of cholesterol synthesis genes in vivo and thereby lower cholesterol synthesis rate inducing LDL receptors will be investigated in chapter 4.

Chapter 3

ROLE OF THE AH RECEPTOR IN HOMEOSTATIC CONTROL OF FATTY ACID SYNTHESIS IN THE LIVER

3.1 ABSTRACT

We have previously demonstrated a role for the aryl hydrocarbon receptor (AHR) in the attenuation of the cholesterol biosynthesis pathway. This regulation did not require that the AHR bind to its cognate response element. Based on these observations and other reports depicting a role for AHR in lipid metabolism, we chose to investigate the involvement of the receptor in the regulation of the fatty acid synthesis pathway in mice and humans. For this purpose, C57BL/6J, liver-specific transgenic DRE-binding mutant AhR (A78D-Ahr^{Ttr} Cre^{Alb} $Ahr^{fx/fx}$) and Cre^{Alb} $Ahr^{fx/fx}$ mice were treated with an AHR ligand and hepatic mRNA expression levels of key fatty acid genes (e.g. *Acaca*, *Fasn, Scd1*) were measured. The basal levels of those genes were also compared between C57BL6/J and hepatic AHR deficient mice, as well as between Ah^b and Ah^d congenic mice. To extend these results to humans, fatty acid gene expression in human cells were compared to AHR-silenced cells. In addition, primary human hepatocytes were treated with an AHR ligand to assess alterations in gene expression and fatty acid synthesis. These studies indicated that the AHR constitutively attenuates the expression of key fatty acid synthesis genes in the absence of binding to its cognate response element. In addition, activation of AHR led to further repression of the expression of these genes and a decrease in overall fatty acid synthesis and secretion in human hepatocytes. Based on our results, we can conclude that increased AHR activity represses fatty acid synthesis, suggesting it may be a future therapeutic target.

3.2 INTRODUCTION

Recent studies have begun to reveal the involvement of the AHR in several basic physiologic activities which has been supported by studies showing that the AHR regulates an array of genes; including, *Ereg, Il6, and Ptgs2*. The phenotype exhibited by mice specifically lacking the AHR includes a reduction in peripheral lymphocytes, vascular abnormalities and diminished fertility, thus linking the receptor to many endogenous biological functions [3]. The endobiotic role of AHR is further supported by the identification of an endogenous agonist for the receptor, such as leukotriene A4 metabolites [194], kynurinic acid [196] and 3-indoxyl sulfate [197]. In addition, activated AHR has been observed to interact with other transcription factors, namely the nuclear factor kappa-light-chain-enhancer (NF-κB), retinoblastoma protein, and the estrogen receptor, thereby modulating their transcriptional regulation of target genes [208, 209].

Based on our recent findings showing the role of AHR in the regulation of the cholesterol biosynthesis pathway, as well as studies pointing to lipid metabolism disruption associated with AHR activation, we set out to examine the relationship between the AHR and the transcriptional regulation of enzymes involved in the fatty acid synthesis pathway. In particular, we focused on the enzymes that are key regulators of fatty acid synthesis, such as ACC, FAS and SCD1. We used the AHR agonist BNF to activate the receptor in C57BL/6J and a transgenic mouse model expressing a hepatic DRE-binding mutant AHR (A78D-AHR). As seen with the cholesterol synthesis genes, activation of AHR was able to attenuate the expression of lipogenic genes *Acaca*, *Fasn* and *Scd1*, through a DRE-independent mechanism. A similar repression was observed in

primary human hepatocytes treated with BNF, accompanied by lower fatty acid production and secretion. Finally, we also demonstrated that AHR plays a constitutive role in the repression of fatty acid synthesis in mice and humans.

3.3 MATERIALS AND METHODS

Cell Culture

Hep3B cells, a human hepatoma-derived cell line, were maintained in α-minimal essential medium (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (HyClone Labs, Logan, UT), 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma) in a humidified incubator at 37°C, with an atmospheric composition of 95% air/5% CO_2.

Primary Human Hepatocytes

Primary human hepatocytes were obtained from the University of Pittsburgh, through the Liver Tissue Cell Distribution System, NIH Contract #N01-DK-7-0004 /HHSN267200700004C. Culture details have been reported in chapter 2. 48 h following Matrigel (BD Biosciences, San Jose, CA) addition, cells were exposed to BNF (10μM) or carrier solvent for 48 h. BNF treatment was replenished every 12 h.

RNA Isolation and Reverse Transcription

RNA samples were isolated from cell cultures and mouse livers using TRI Reagent according to the manufacturer's specifications (Sigma Aldrich). cDNA was generated using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA).

Quantitative PCR

Sequences of primers (Table 1) were designed to detect mRNA levels. PerfeCTa™ SYBR® Green SuperMix for iQ (Quanta Biosciences, Gaithersburg, MD) was used and analysis was conducted using MyIQ software (Bio-Rad Laboratories, Hercules, CA).

Table 3.1 **Primers used for quantitative fatty acid synthesis genes RT- PCR.**

Mouse	Cyp1a1	F	5' AGAGGTTGGCGACTTTGACCCTTA 3'
		R	5' ACCTCCCGAAACTGATTGCTGAGA 3'
	Acaca	F	5' TAACAGAATCGACACTGGCTGGCT 3'
		R	5' ATGCTGTTCCTCAGGCTCACATCT 3'
	Fasn	F	5' GGTGTGGTGGGTTTGGTGAATTGT 3'
		R	5' TCACGAGGTCATGCTTTAGCACCT 3'
	Scd1	F	5' TCCCTCCGGAAATGAACGAGAGAA 3'
		R	5' AGTGCAGCAGGACCATGAGAATGA 3'
	Srebp1	F	5' TATGGAGGGCATGAAACCCGAAGT 3'
		R	5' TTGACCTGGCTATCCTCAAAGGCT 3'
	Rpl13	F	5' TTCGGCTGAAGCCTACCAGAAAGT 3'
		R	5' GCATCTTGGCCTTTTCCTTCCGTT 3'

Human	CYP1A1	F	5' TCTTCCTTCGTCCCCTTCAC 3'
		R	5' TGGTTGATCTGCCACTGGTT 3'
	ACACA	F	5' TTAACAGCTTGTGAGTCTGGCTGT 3'
		R	5' AACACTCGATGGAGTTTCTCGCCT 3'
	FASN	F	5' TGTTCCTCGGAGTGAATCTGGGTT 3'
		R	5' AGGTTTGATGCCTCCTTCTTCGGA 3'
	SCD1	F	5' GCAAACACCCAGCTGTCAAAGAGA 3'
		R	5' TCGCAAGAAAGTGGCAACGAACAC 3'
	SREBP1	F	5' AGATGCGGAGAAGCTGCCTATCAA 3'
		R	5' TTATTCAGCTTTGCCTCAGTGCCC 3'
	RPL13	F	5' CCTGGAGAAGAGGAAAGAGA 3'
		R	5' GAGGACCTCTGTGTATTTGTCAA 3'

Gene Silencing

AHR and ARNT levels were decreased in Hep3B cells using siRNA as previously described. RNA and protein samples were isolated 72 h post-electroporation.

Mice

Transgenic A78D-Ahr^{Ttr} $Cre^{Alb}Ahr^{fx/fx}$ mice were generated as described previously in chapter 2. Transgenic $Cre^{Alb}Ahr^{fx/fx}$ were a kind gift from Christopher Bradfield, University of Wisconsin. Congenic Ah^d and wild-type mice (C57BL/6J) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed on corncob bedding in a temperature- and light-controlled facility and given access to food and water ad libitum. Mice were maintained in a pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of the Pennsylvania State University. Adult (10-12 weeks) female mice of different genotypes were injected intraperitoneally with BNF at 50 mg/kg dissolved in corn oil or with corn oil alone. After 5 h mice were sacrificed and livers harvested.

Protein and RNA Preparation

Mouse liver samples were collected and frozen immediately in liquid nitrogen before storage at -80°C, RNA was isolated using TRI Reagent (Sigma). Livers and Hep3B extracts were prepared as reported previously in chapter 2.

Immunoblotting

Mouse liver and cell extracts were resolved on 8% SDS-tricine polyacrylamide gels. Proteins were transferred to PVDF membrane and detected using the AHR antibody RPT1 (Thermo Scientific), ACC, and SCD1 antibodies (Santa Cruz Biotechnology), or FAS antibody (BD Biosciences). All antibodies were visualized using the appropriate biotinylated secondary antibody and [I^{125}]streptavidin followed by autoradiography.

Lipid Extraction and GC-MS Analysis

Primary human hepatocytes were treated for 48 h with BNF (10μM), which was replenished at 12 h intervals. Lipids were extracted from the media and analyzed by GC-MS as described in the previous chapter. Heptadecanoic acid (Sigma) was used as an internal standard. Values represent an average of 3 wells run in duplicate.

Statistical Analysis

Data were analyzed using t-test and ANOVA in GraphPad Prism (v.5.01) software to determine statistical significance between treatments. P values < 0.05 were considered statistically significant (*P<0.05; **P<0.01;***P<0.001).

3.4 RESULTS

AHR Regulates Fatty Acid Synthesis Gene Expression in C57BL/6J Mice.

As a first approach to target the regulation of lipogenic genes by AHR, we treated C57BL/6J mice with the AHR agonist BNF for 5 h. Hepatic mRNA analysis revealed a significant and collective attenuation of the expression levels of the three rate-limiting enzymes of fatty acid synthesis *Acc*, *Fas* and *Scd1*. *Cyp1a1* mRNA levels were determined as a positive control to illustrate the level of receptor activation (Figure 3.1A). In contrast, BNF failed to alter mRNA levels of the transcription regulator SREBP1c the master regulator of fatty acid synthesis. Given the short time of treatment, these observations might suggest that AHR is directly influencing the regulation of the 3 genes examined at the promoter level.

Regulation of Key Genes Involved In Fatty Acid Synthesis by the AHR Is DRE-Independent.

Our previous work, as well as that of other laboratories, has demonstrated the ability of AHR to function through multiple mechanisms other than DRE-mediated activity [3, 79, 210]; for this reason we opted to test whether this regulation is DRE-specific. We have established a suitable transgenic mouse model to test such a hypothesis. Using a hepatocyte-specific TTR promoter, a transgenic mouse (A78D-*Ahr*^{Ttr} *Cre*^{Alb} *Ahr*$^{fx/fx}$) was designed to express the DRE-binding mutant form of the receptor (A78D-AHR) in the liver, which also has the endogenous AHR gene deleted. In response to BNF treatment, these transgenic mice showed a similar marked attenuation in the

expression levels of the genes of interest (Figure 3.1B), establishing the ability of the receptor to repress *Scd1* and *Fas* expression independent of DRE-mediated activity in hepatocytes. As shown in Fig. 3.1C, these repressive events were not observed in *Cre^{Alb} Ahr$^{fx/fx}$* mice that lack AHR expression in hepatocytes, demonstrating that the observed effect exerted by BNF in C57BL/6 and A78D-*Ahr^{Ttr} Cre^{Alb} Ahr$^{fx/fx}$* mice is thus mediated through the AHR.

Figure 3.1
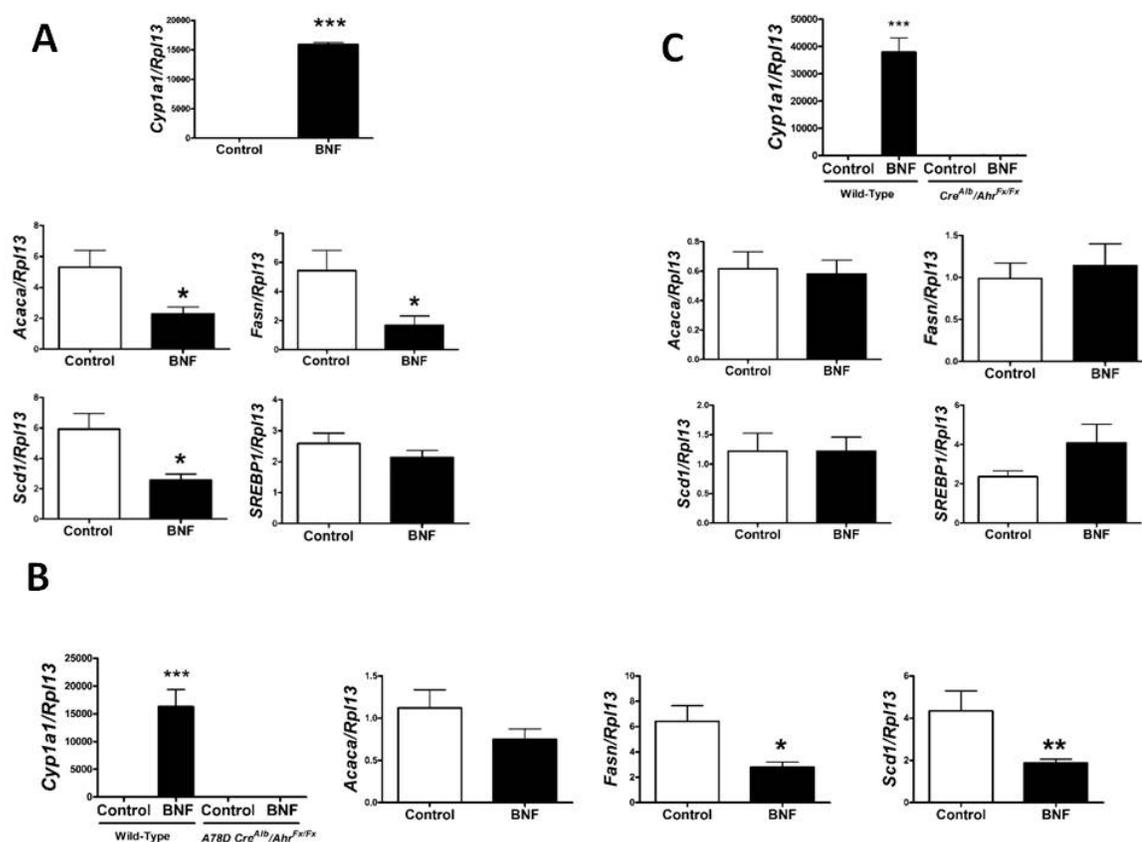
Figure 3.1 **AhR negatively regulates the expression of fatty acid synthesis genes in the mouse in a DRE-independent manner.**

A. Normalized RNA expression of hepatic fatty acid synthesis genes in C57BL/6J mice (6 female mice per group) injected with BNF, compared to control mice. **B.** Normalized hepatic fatty acid gene transcripts from A78D-AhR^{Ttr} Cre^{Alb} $AhR^{Fx/Fx}$ mice (6 female mice per group) injected with BNF versus control mice. **C.** Normalized hepatic mRNA levels of fatty acid synthesis genes in BNF-treated versus vehicle-treated Cre^{Alb} $AhR^{Fx/Fx}$ mice (6 female mice per group). Cyp1a1 mRNA levels illustrate the lack of DRE-mediated activity in mice that lack hepatic expression of the AHR.

AHR Constitutively Represses the Expression of Fatty Acid Synthesis Genes in Mice.

Mice with a targeted global deletion of AHR exhibit a set of phenotypes, including decreased liver weight and compromised immune and reproductive systems, that points to an endogenous role for the receptor independent of its xenobiotic function. In order to probe for a constitutive role of the receptor in fatty acid synthesis, we looked for differences in the basal expression level of hepatic fatty acid genes between C57BL/6J and $Cre^{Alb} Ahr^{fx/fx}$ mice. Remarkably, mRNA expression levels of the *Acaca, Fasn and Scd1* genes were significantly lower when AHR was present, suggesting a constitutive activity of the receptor in the repression of those genes (Figure 3.2A).

Several AHR endogenous ligands have been identified in our laboratory and have been shown to modulate the receptor's activity [194, 196, 197]. To investigate the presence of an endogenous ligand exhibiting agonist activity, we compared the mRNA levels of fatty acid genes in mice expressing the Ah^d allele (low ligand affinity) in a congenic C57BL6/J background and C57BL6/J mice, which express the Ah^b allele (high ligand affinity). Relatively higher mRNA levels were noted in Ah^d congenic mice especially for SCD1 (Figure 3.2B) suggesting a role for an endogenous ligand in the constitutive regulation of those genes by AHR.

Figure 3.2
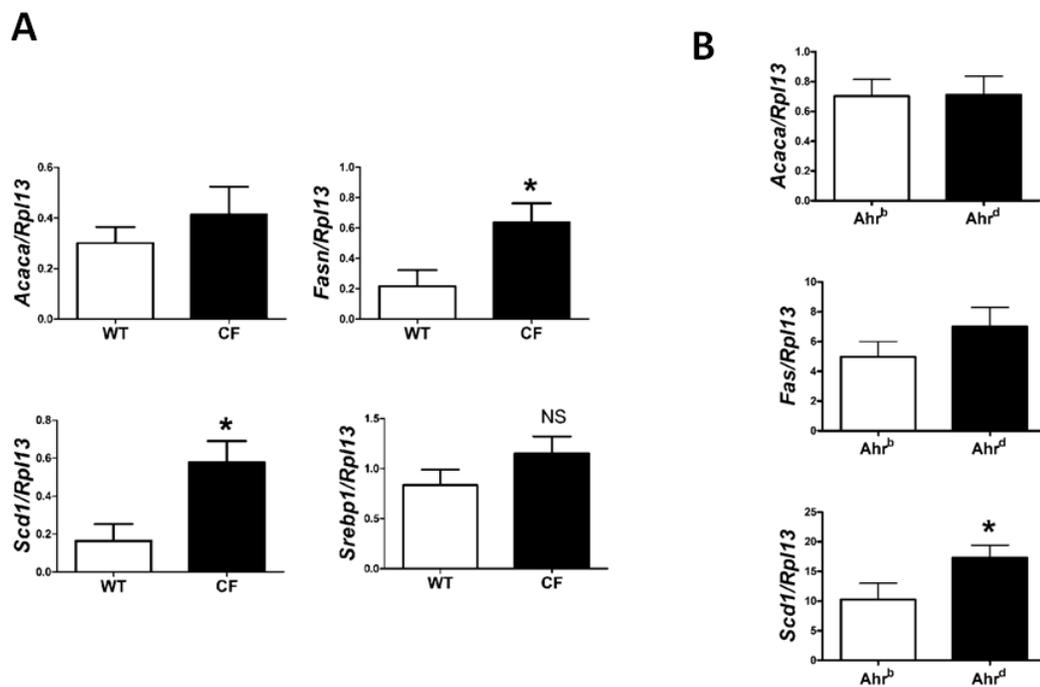
Figure 3.2 **AHR plays a constitutive role in the repression of fatty acid synthesis genes in mice.**

> **A.** Normalized hepatic mRNA levels of fatty acid genes of liver-specific Cre^{Alb} $AhR^{Fx/Fx}$ mice (CF) compared to C57BL/6J (WT) mice (6 female mice per group) in the absence of any exogenous ligand. **B.** Normalized hepatic expression of fatty acid synthesis genes in non-treated Ah^b and Ah^d congenic mice (4 female mice per group).

AHR Constitutively Represses the Expression of Fatty Acid Synthesis Genes in a Human Hepatoma Cell Line.

To examine whether AHR-mediated regulation of the fatty acid synthesis genes is relevant in humans, we largely ablated AHR levels in Hep3B cells, a human hepatoma cell line, using siRNA, and measured levels of fatty acid synthesis gene expression using quantitative real-time PCR and western blot. We observed higher levels of the three fatty acid synthesis gene transcripts (Figure 3.3A) and their respective proteins (Figure 3.3B) upon repression of AHR levels. These results are consistent with the data obtained in mice, indicating the AHR constitutively represses the expression of fatty acid synthesis genes in humans as well.

Figure 3.3
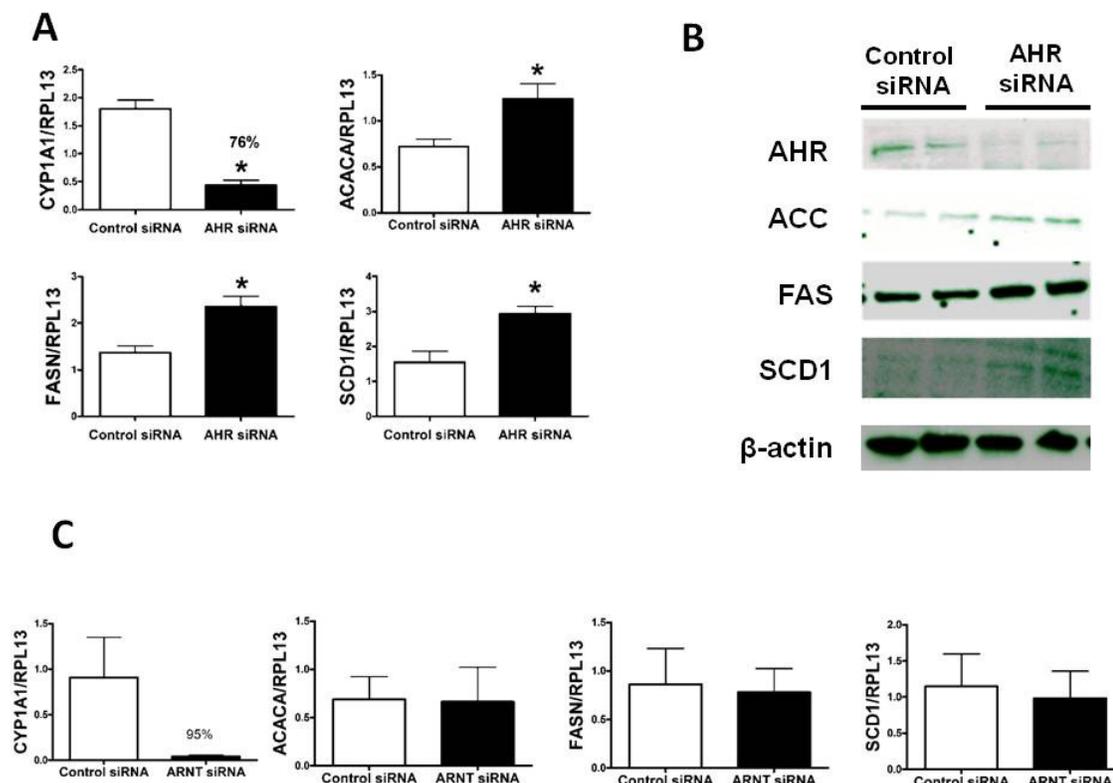
Figure 3.3 **Repression of AHR expression correlates with increased expression of fatty acid synthesis genes in human cells.**
Normalized hepatic RNA expression (**A**) and protein expression (**B**) of fatty acid synthesis genes in control Hep3B cells compared to cells where AHR expression has been repressed (AHR siRNA). **C.** Effect of repression of ARNT expression in Hep3B cells (ARNT siRNA) on the hepatic expression of fatty acid synthesis genes compared to control cells. CYP1A1 levels in (**A**) and (**C**) are used to confirm the functional down regulation of AHR/ARNT transcriptional activity.

AHR Represses Fatty Acid Synthesis Gene Expression Independent of ARNT Expression.

DRE-mediated AHR activity is known to take place through heterodimerization with its partner ARNT that renders the receptor capable of binding its cognate response element in the promoter region of target genes. Figure 3.3C shows that unlike AHR, ablation of ARNT expression in Hep 3B cells has no effect on the expression of the fatty acid synthesis genes. Constitutive CYP1A1 mRNA levels were almost totally repressed by ARNT siRNA treatment, demonstrating that ARNT expression was ablated. This further supports the idea that AHR is capable of mediating regulation of the fatty acid synthesis genes in the absence of heterodimer formation and thus independent of DRE-binding in humans.

AHR is Capable of Suppressing Fatty Acid Synthesis Gene Expression in Primary Human Hepatocytes.

Since AHR has been shown to behave differently in humans then in and mice, we sought to examine whether activation of the receptor by an exogenous ligand could also modulate the expression of fatty acid synthesis genes in humans and if this in turn translates to attenuation of fatty acid synthesis. Similar to our results in rodents, ACACA, FASN and SCD1 mRNA expression levels were markedly reduced following AHR activation in primary human hepatocytes accompanied by unchanged SREBP1c mRNA levels (Figure 3.4A).

AHR Activation Represses Fatty Acid Secretion from Primary Human Hepatocytes.

Analysis of FA composition by GC-MS of lipids extracted from the primary human hepatocyte media revealed that BNF-induced alterations in several fatty acid products such that the secretion of saturated (myristic acid C14:0, palmitic acid C16:0, and stearic acid C18:0) and monounsaturated (palmitoleic acid C16:1, and oleic acid C18:1) fatty acids were significantly reduced (Figure 3.4B). Thus, these results suggest that lower enzyme levels led to reduced fatty acid synthesis and subsequent reduced fatty acid secretion. These results establish a functional consequence to the observed repression of fatty acid synthesis gene expression observed in primary human cells after AHR activation. LDH cytotoxicity assay reveals that BNF treatment did not affect cell viability.

Figure 3.4
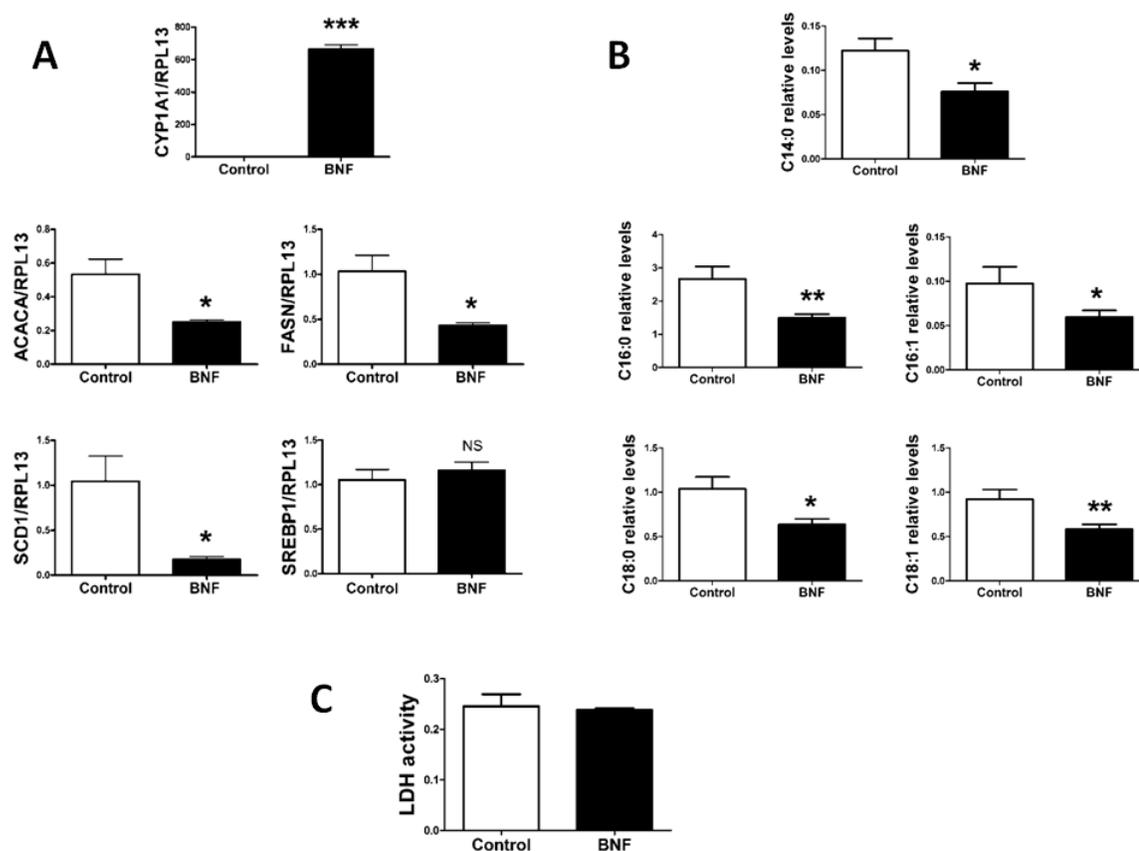
Figure 3.4 **AHR activation represses fatty acid synthesis in primary human hepatocytes.**

> **A.** Normalized RNA expression of fatty acid synthesis genes of primary human hepatocytes following BNF-treatment (3 wells per group) are shown. **B.** Secreted fatty acid levels extracted from the media were analyzed by GC-MS and normalized to an internal standard. **C.** LDH cytotoxicity assay is shown.

3.5 DISCUSSION

Previous studies examining the role of AHR in the control of the lipogenic pathway have focused on the effect of TCDD exposure in rodents [123, 124]. However there have been no investigations into the constitutive role of the AHR in rodents or the effect of AHR activation on de novo lipogenesis in humans. Here we have reported that the AHR-mediated repression of three rate-limiting enzymes of the fatty acid synthesis pathway, ACC, FAS and SCD1, both in mice and humans, which leads to a subsequent decrease in fatty acid production. Furthermore, we demonstrated that AHR involvement in this regulation occurs in a DRE-independent manner. Indeed, AHR involvement in fatty acid metabolism is consistent with both the wasting syndrome, characterized by body fat loss, and the hypoinsulinemia observed in TCDD-treated animals [3], as well as reports describing disruption of fatty acid and triglyceride levels in workers exposed to dioxin [127]. Microarray and serum data from rats injected with TCDD have shown repression of *Fasn* expression and triglyceride levels [124]. Paradoxically, a mouse study showed repression of *Acaca* on microarrays while *Fasn* transcript levels were increased in liver [123]. It is important to note here that previous studies exploring a role for AHR in fat metabolism and transport involved the use of TCDD. Although this compound is known to be a highly potent ligand for AHR, multiple reports argue for an independent role for this compound. In fact, dioxin alone has been shown to bind and disrupt the three-dimensional structure of lipoprotein molecules, rendering them incapable of binding to their cognate receptor [211] and attenuating insulin-induced glucose uptake in differentiated 3T3-L1 adipocytes, all in an AHR-independent manner [212]. Hence, the

development of insulin resistance, hepatic steatosis and elevated triglyceride levels following exposure to TCDD might be occurring at least in part through a mechanism independent of AHR. In addition, the highly toxic properties of TCDD complicate the interpretation of gene expression data.

There is a marked diurnal rhythm in the rate of fatty acid synthesis in the liver of lean mice [213]. Likewise, AHR is known to exhibit rhythmic expression with its highest peak levels coinciding with the lowest fatty acid synthesis rates [202]. Moreover, the rise in fatty acid synthesis at the beginning of the dark cycle occurs after the decrease in AHR levels. This pattern seems even more pronounced in genetically obese (*ob/ob*) mice [213]. Considering that liver AHR levels ebb and flow according to a circadian rhythm that inversely correlates with fatty acid synthesis, our current results would support the idea of a constitutive role for AHR in the repression of fatty acid synthesis both in mice and humans.

SREBP1c is the central regulator of the transcriptional activity of genes involved in fatty acid biosynthesis. Our results indicate a down regulation of those genes in AHR ligand-treated mice and primary human hepatocytes and their increased expression in the absence of AHR, suggesting a possible interaction between AHR and SREBP1c. Indeed a recent report demonstrated a physical interaction between the two factors in murine T cells, with a possible inhibition of SREBP1c activity by the presence of the AHR [60]. In addition, we have previously demonstrated that AHR constitutively regulates the transcriptional targets of SREBP2 [210] in a similar fashion, making it highly possible that the AHR interacts and/or affects the activity of both SREBP family members. However, one should not dismiss a possible involvement of other factors implicated in

the expression of fatty acid synthesis genes, such as PPARα and LXR [214]. Clearly, further studies are needed to precisely define the mechanism of AHR-mediated repression of fatty acid synthesis.

Recent studies have extensively focused on the promise of fatty acid synthesis enzyme inhibitors in the treatment of metabolic syndrome, hepatic steatosis and diabetes. Inhibitors of ACC were effective in reducing plasma triglyceride levels and body fat mass, inhibiting fatty acid biosynthesis, stimulating fatty acid oxidation and, most importantly, improving insulin sensitivity in rodents [215]. On the other hand, experimental results from animals treated with a FAS inhibitor showed significant reduction in food intake and adipose tissue [216]. Finally, SCD1 inhibitors have been tested in mice and revealed protection against obesity, hepatic steatosis and improved insulin sensitivity along with preclinical antidiabetic and antidyslipidemic efficacy [217]. However, despite the fact that the effects of SCD1 inhibitors are quite striking, they do not always result in lower plasma triglyceride levels as seen with ACC inhibition [218]. Our present investigation clearly demonstrates that AHR has a major impact on the lipogenesis pathway by directly regulating the expression of three rate-limiting enzymes.

Our results are the first to establish the role of the AHR in the hepatic regulation of three rate-limiting hepatic fatty acid synthesis genes, as well as the role of the receptor and the effect of its activation on the production of fatty acid synthesis products in primary human hepatocytes. Moreover, we demonstrate the complexity of AHR activation by an agonist and its effect on the expression of a variety of genes through DRE and non-DRE mechanisms. In conclusion, evidence of DRE-independent AHR

regulation of the lipid biosynthesis pathway shows promise for the therapeutic use of the

AHR for the treatment of non-alcoholic fatty liver disease, obesity and cancer.

Chapter 4

SELECTIVE ACTIVATION OF AHR EXERTS REPRESSIVE EFFECTS ON HEPATIC CHOLESTEROL AND FATTY ACID SYNTHESIS

4.1 ABSTRACT

Activation of AHR by an exogenous ligand is known to mediate the expression of target genes (e.g. *CYP1A1),* by binding to dioxin response element (DRE) sequences in their promoter region. We have previously demonstrated the ability of the receptor to attenuate the expression of cholesterol and fatty acid synthesis genes in a DRE-independent manner in mice and humans. Our laboratory has already established the ability of the selective ligand SGA360 to activate the receptor without inducing its DRE-binding. To test the effect of the receptor's selective activation in this context, C57BL/6J mice and primary human hepatocytes were exposed to SGA 360 and showed a dramatic repression in the hepatic expression of cholesterol and fatty acid synthesis genes accompanied by an overall lower cholesterol and fatty acid secretion in human cells. Interestingly, this regulation did not seem to affect the hepatic expression of the fatty acid β-oxidation genes. Additionally, selective activation of the receptor was capable of overcoming the statin-induced upregulation of cholesterol synthesis gene expression and showed a synergistic suppression of cholesterol synthesis. In order to explore the mechanism of action behind this regulation, we studied the regulatory effect of AHR on the transcription factors regulating those pathways the sterol element binding proteins (SREBPs). Our results indicated significant proteosomal mediated degradation of the active form of the SREBP proteins following AHR activation. These observations clearly establish a role for AHR as a key regulator of lipid homeostasis and indicate the potential use of this receptor as a target for the treatment of metabolic diseases and cancer.

4.2 INTRODUCTION

Fatty acids and cholesterol perform numerous essential cellular activities. This has prompted a detailed analysis of the mechanisms of regulation that mediate their levels *in vivo* [129, 219]. A variety of human diseases is caused or results in alterations in fatty acid and cholesterol homeostasis, including cardiovascular diseases and diabetes. In vivo, fatty acids and cholesterol are either derived from the diet or from *de novo* synthesis, which occurs mainly in the liver through the lipogenesis and the mevalonate pathways, respectively [163]. Each biosynthetic pathway is comprised of a series of enzymes shown to be under the regulation of the basic helix-loop-helix zipper (bHLH-ZIP) family of transcription factors the sterol element binding proteins (SREBPs). Those transcription factors reside anchored in the endoplasmic reticulum/golgi apparatus and under low cholesterol levels the transcriptionally active portion of the protein is cleaved and translocates to the nucleus. This is the major event that leads to activation of SREBP2 and subsequent expression of genes involved in cholesterol biosynthesis and uptake [130, 136]. In contrast, SREBP1c is known to be also under the regulation of high glucose and insulin levels [134, 140] in order to activate expression of genes involved in fatty acids, triglycerides and phospholipids pathways.

We have established in the two previous chapters the ability of the Ah receptor to attenuate the expression of fatty acid and cholesterol synthesis genes through a DRE-independent mechanism without affecting the levels of expression of their transcription factors SREBP1c and -2 respectively. Our discovery of a dual regulation of the fatty acid and cholesterol synthesis gene expression through AHR activation in primary human

hepatocytes may have several therapeutic applications. However, sustained activation of AHR by xenobiotic agonist like BNF is known to alter xenobitic metabolism and other DRE dependent changes in gene expression. The discovery that AHR can coordinately modulate the expression of fatty acid and cholesterol biosynthetic genes in a DRE-independent manner is very promising.

Based on these observations, one may speculate that AHR might be contributing to the hepatic transcription of fatty acid and cholesterol biosynthetic genes through interaction with the transcription factor SREBP1c and 2. Such mechanism has precedent from previous studies demonstrating AHR interaction with NF-κB subunits and estrogen receptor (ER)-dependent signal transduction [3]. A physiological interaction between AHR and SREBP1c in T cells has already been documented further supporting a possible negative regulation of the lipogenic genes by AHR through SREBP1c [60]. On the other hand, SREBP2 has also been shown to physically interact with ubiquitous transcription factors Sp1 and NF-Y to synergistically augment the transcription of its target genes [206, 220] making an interaction between AHR and SREBP2 very plausible. Regulation of SREBPs levels is also known to occur through alteration in its transcription levels, its cleaving process in the endoplasmic reticulum and the golgi apparatus, and the degradation of its active cleaved form. AHR has been shown to act as a E3 ubiquitin ligase and modulate the function of several steroid receptors such as the estrogen receptor and the androgen receptor through assembling a ubiquitin ligase complex CUL4B and subsequent ubiquitination and proteosomal degradation [61, 221, 222].

Our lab has previously established the ability of the selective ligand SGA360 to bind AHR and subsequently represses cytokine-mediated acute-phase gene induction (e.g. *Saa3*),

yet this ligand fails to elicit receptor binding to DRE sequences [23]. In this study, we report

the ability of SGA360 to activate the AHR in mice and primary human hepatocytes to

coordinately modulate gene expression of a number of key enzymes involved in fatty acid

and cholesterol synthetic pathways, independent of DRE-mediated transcription. In addition,

we demonstrate that AHR activation leads to the degradation of the cleaved form of

SREBP1c and -2. The proteosomal targeting of those SREBPs by AHR results in

attenuation in the transcription of SREBP target genes. SAhRM activation of the AHR leads

to an overall decrease in fatty acid and cholesterol synthesis and subsequent secretion in

primary human hepatocytes.

4.3 MATERIALS AND METHODS

Materials

SGA360 (1-Allyl-3-(3,4-dimethoxyphenyl)-7-(trifluoromethyl)-1H-indazole) was synthesized as previously described [23]. GNF351 (N-[2-(3H-indol-3-yl)ethyl]-9-isopropyl-2-(5-methyl-3-pyridyl)purin-6-amine) was acquired from the Genomics Institute of the Novartis Research Foundation (San Diego, CA). BNF was purchased from Iodofine, lovastatin from AG Scientific, and MG-132 from Calbiochem.

Cell Culture

Hep3B, Huh-7 and COS-1 cells were maintained in α-minimal essential medium (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (HyClone Labs, Logan, UT), 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma) in a humidified incubator at 37°C, with an atmospheric composition of 95% air/5% CO_2. Upon lovastatin and ligand treatment, media was switched to 5% lipid-reduced fetal bovine serum (HyClone) and was changed every 24 h. Treatment with BNF (10μM), SGA 360 (10μM) was replenished every 12 h, lovastatin (10μM) every 24 h for a 48 h period.

Primary Human Cells

Primary human hepatocytes were obtained from the University of Pittsburgh, through the Liver Tissue Cell Distribution System, NIH Contract #N01-DK-7-0004 /HHSN267200700004C. Cells were kindly provided by Curt Omiecinski and Stephen Strom. Culture details are reported in chapter 2. Primary human adipocytes were purchased from PromoCell and maintained according to the manufacturer's instructions. Cells were

exposed to BNF (10μM), SGA 360 (10µM or 20 µM), or GNF351 (500nM), or carrier

solvent replenished every 12 h for 48 h. Lovastatin (10µM) treatment was replenished and

media was changed every 24 h.

Quantitative PCR

Sequences of primers were designed to detect mRNA levels. Primes are listed in

chapter 2 and 3. PerfeCTa™ SYBR® Green SuperMix for iQ (Quanta Biosciences,

Gaithersburg, MD) was used and analysis was conducted using MyIQ software (Bio-Rad

Laboratories, Hercules, CA).

Gene Silencing and Cell Proliferation

AHR were decreased in Hep3B cells using siRNA as previously described in chapter

2. Cells were counted after 48 h treatment.

Mice

Wild-type (C57BL/6J) were purchased from The Jackson Laboratory (Bar Harbor,

ME). AhR knockout (AhR$^{-/-}$) mice in a C7BL/6J background were a kind gift from Dr.

Bradfield (McArdle Laboratory for Cancer Research, University of Wisconsin–Madison

Medical School). Mice were housed on corncob bedding in a temperature- and light-

controlled facility and given access to food and water ad libitum. Mice were maintained in a

pathogen-free facility and treated with humane care with approval from the Animal Care

and Use Committee of the Pennsylvania State University. Adult (10-12 weeks) male mice

were administered SGA360 at 20 mg/kg by gavage every 12 h for a 24 h period after which they were sacrificed by CO_2 and livers and white adipose tissue were harvested.

RNA and Protein Preparation

Mouse liver samples were collected and frozen immediately in liquid nitrogen before storage at -80°C. RNA samples were isolated from cell cultures and mouse livers using TRI Reagent according to the manufacturer's specifications (Sigma Aldrich). cDNA was generated using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Livers and primary human hepatocytes extracts were prepared as reported in chapter 2. For Huh-7 whole cell extracts, we used MENG (25 mM MOPS, 2 mM EDTA, 0.02% NaN3, and 10% glycerol, pH 7.4) with 300mM NaCl and 1% NP-40 coupled with proteinase inhibitors.

Immunoblotting

Cell extracts were resolved on 8% SDS-tricine polyacrylamide gels. Proteins were transferred to PVDF membrane and detected using the AHR antibody RPT1 (Thermo Scientific), SREBP2 (BD Biosciences) and SREBP1 (AbCam). All the other primary antibodies were from Santa Cruz Biotechnology (1/1000 dilution). Antibodies were visualized using the appropriate biotinylated secondary antibody and $[I^{125}]$streptavidin followed by autoradiography.

Lipid Extraction and GC-MS Analysis

Lipids were extracted from the media and analyzed by GC-MS as described in chapter 2. Cholestanol (Sigma) and Heptadecanoic acid (Sigma) were used as an internal standard. Values represent an average of 3 wells run in duplicate.

Red Oil Staining

Cells were washed with PBS, fixed with 10% formaldehyde in PBS for 30 min then stained with staining solution (5mg/mL Oil Red O powder in 60:40 isopropanol: water). Stain was extracted using 60:40 isopropanol:water for 1 h and quantified at 490nm.

Gal4-Mammalian-One Hybrid Assay

Active hSREBP2 gene sequence was cloned into a pM vector. COS-1 cells (American Type Culture Collection, Manassas, VA) were seeded in 6-well plates 24 h before transfection with 200ng of either pM-control (empty vector) or pM-hSREBP2 along with their pFR-Luc target reporter sequence (200ng) and β-Gal vector (200ng) with/without pcDNA3-hAHR using LipofectAMINE reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The activity of each sample was measured using a TD-20e luminometer (Turner Systems, Sunnyvale, CA) using Luciferase Assay Substrate (Promega, Madison, WI) as suggested by the manufacturer.

Nuclear Extract and Cytosolic Fraction

Cells were trypsinized, washed with PBS and resuspended in 1mL of MENG + inhibitors and homogenized with a stainless steel homogenizer. After centrifugation (1,000g

for 20 min), supernatant represented the cytosolic fraction and the pellet was washed with MENG, resuspended in MENG + 500mM NaCl and centrifuged at 42,000g for 30 min to generate the nuclear extracts.

Electromobility Shift Assay

Huh-7 nuclear extracts (5µl of 1mg/mL lysate) were incubated with 1.5 µl of HEDG buffer (25 mM HEPES, 1 mM EDTA, 10 mM sodium molybdate, and 10% (v/v) glycerol, pH 7.5) along with 5µL 3x oligo buffer, 1µL dI/dC (500ng/µL), 1µL of E. Coli DNA (1mg/mL) for 15 min at room temperature. P-labeled SRE probe [using a T4 kinase kit (BioLabs) and purified with Quick Spin columns (TE) (Roche)] was added to each reaction and incubated for 15 min. A total of 10µl of lysate was resolved using a 6% DNA-retardation gel (Invitrogen), which was then fixed, vacuum-dried, and visualized.

Statistical Analysis

Data were analyzed using t-test and ANOVA in GraphPad Prism (v.5.01) software to determine statistical significance between treatments. P values < 0.05 were considered statistically significant (*P<0.05; **P<0.01;***P<0.001).

4.4 RESULTS

Selective Activation of AHR by SGA 360 Attenuates the Hepatic Expression of Cholesterol and Fatty Acid Synthesis Genes in Mice.

To assess the effect of selective ligand activation of AHR in mice, we treated C57BL/6J mice with SGA 360. Hepatic expression of select cholesterol and fatty acid synthesis genes were significantly repressed upon exposure (Figure 4.1A). In contrast, the levels of the transcription factors SREBP1c and -2 remained unaffected. It is important to note that although the LDL receptor is also transcriptionally regulated by SREBP2, the ligand did not have a significant negative effect on its levels. Next, we assessed the expression of an array of genes involved in fatty acid β-oxidation in response to SGA360 treatment (Figure 4.1B). The expression of the rate-limiting enzyme carnitine palmitoyltransferase 1(*cpt1*) as well as acyl-CoA oxidase (*acox*), medium chain acyl-CoA dehydrogenase (*mcad*), and the fatty acid binding protein (*fabp*) were not altered (Figure 4.2). Treatment of *ahr*-null mice with SGA360 revealed no difference in gene expression of the cholesterol and fatty acid synthesis genes examined in figure 1, establishing that the transcriptional effects observed in wild-type mice are AHR-mediated (Figure 4.3A,B). The ability of SGA 360 to display a similar effect to previously described treatments with BNF on the expression of these genes provides evidence that the AHR-mediated regulation is DRE-independent. Adipose tissue is an important site of trigyceride synthesis and exhibits relatively low AHR levels, thus it was of interest to test whether SGA360 would influence gene expression in this tissue.

Figure 4.1
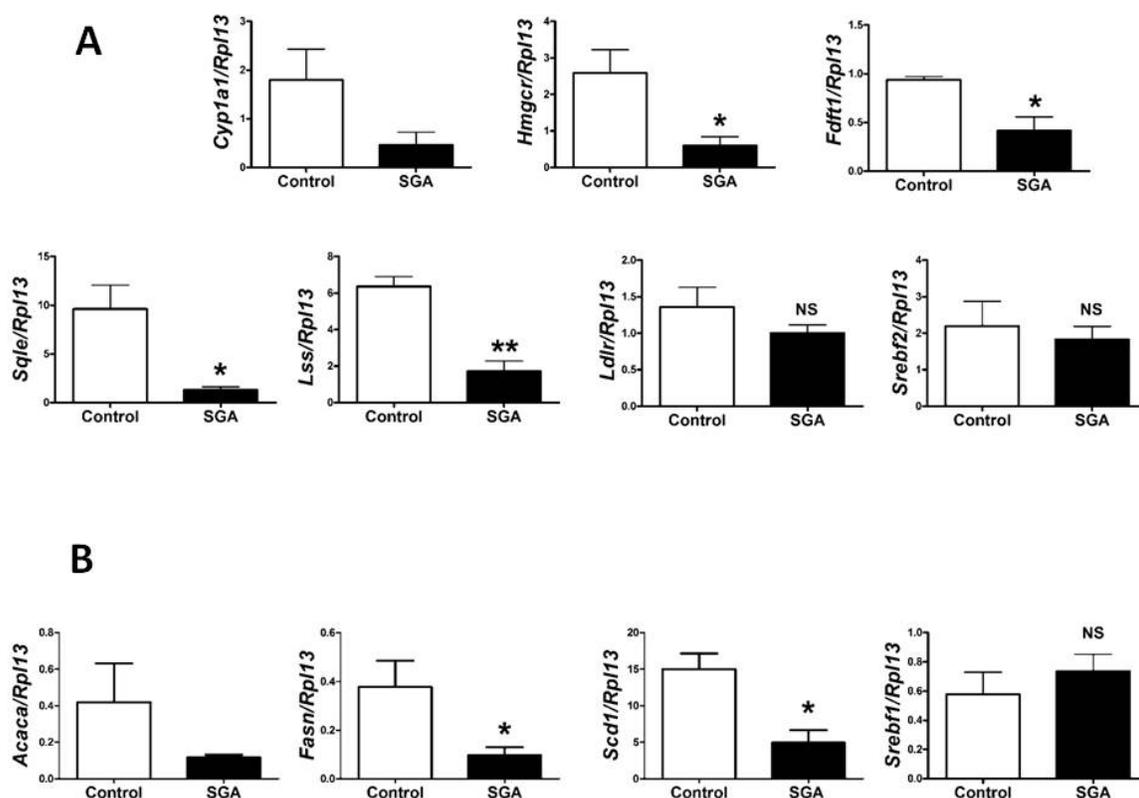
Figure 4.1 **Selective activation of AHR by SGA 360 has repressive effects on the expression of cholesterol and fatty acid synthesis genes in mice.**
Normalized RNA expression of hepatic cholesterol synthesis genes (**A**) and fatty acid synthesis genes (**B**) in C57BL/6J mice (3 male mice per group) administered SGA 360 (SGA) (20 mg/kg) by gavage compared to vehicle treated mice (control) are shown.

Figure 4.2
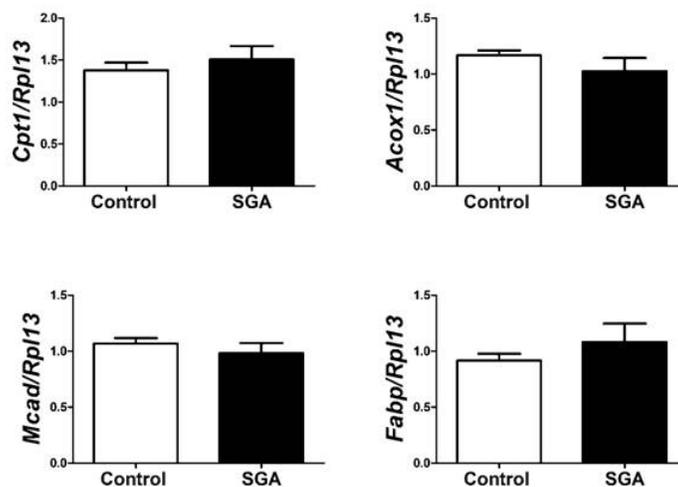
Figure 4.2 **Selective activation of AHR by SGA 360 has no effect on the expression of fatty acid β-oxidation genes in mice.**

Normalized RNA hepatic expression of selected fatty acid β-oxidation genes in C57BL/6J mice (3 male mice per group) treated with SGA 360 (SGA) (20 mg/kg) by gavage are compared to vehicle treated (control) mice.

Figure 4.3
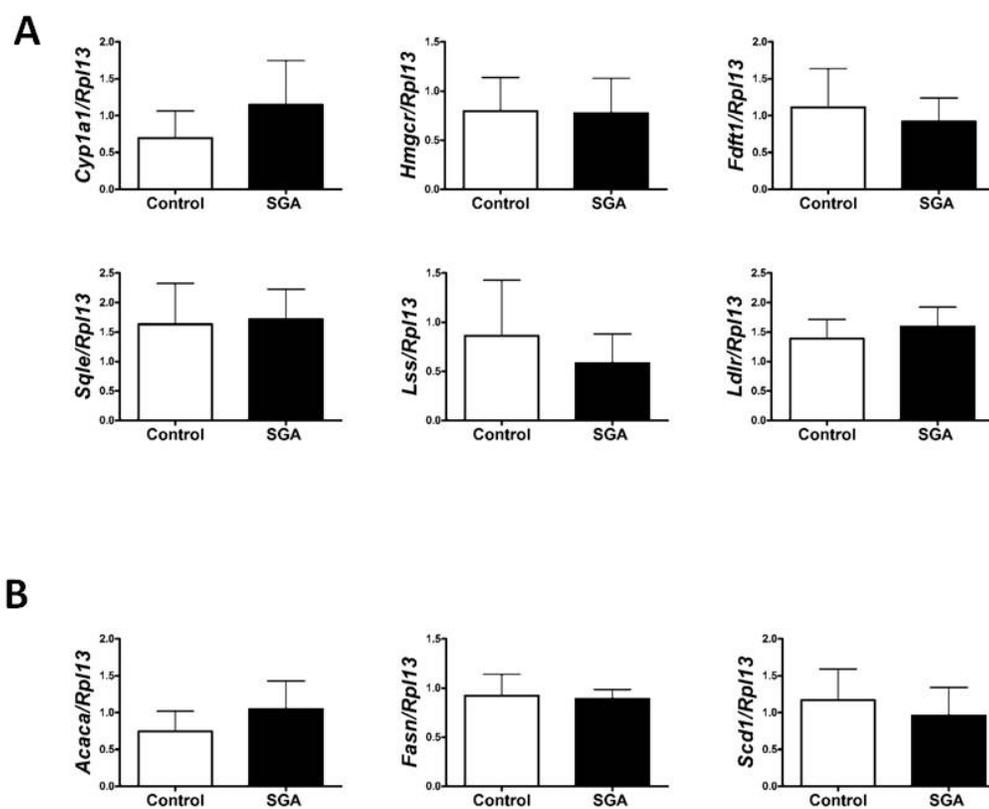
Figure 4.3 **SGA 360 shows no activity on cholesterol and fatty acid synthesis gene expression in *ahr*-null mice.**

> Normalized RNA expression of hepatic cholesterol synthesis genes (**A**) and fatty acid synthesis genes (**B**) in *ahr*-null mice (3 male mice per group) treated with with SGA 360 (SGA) (20 mg/kg) by gavage are compared to vehicle treated (control) mice.

The level of expression of four key genes in the fatty acid synthesis pathway were assessed and none of these genes were repressed by SGA360 (Figure 4.4). However, *Acaca* was significantly induced.

Figure 4.4
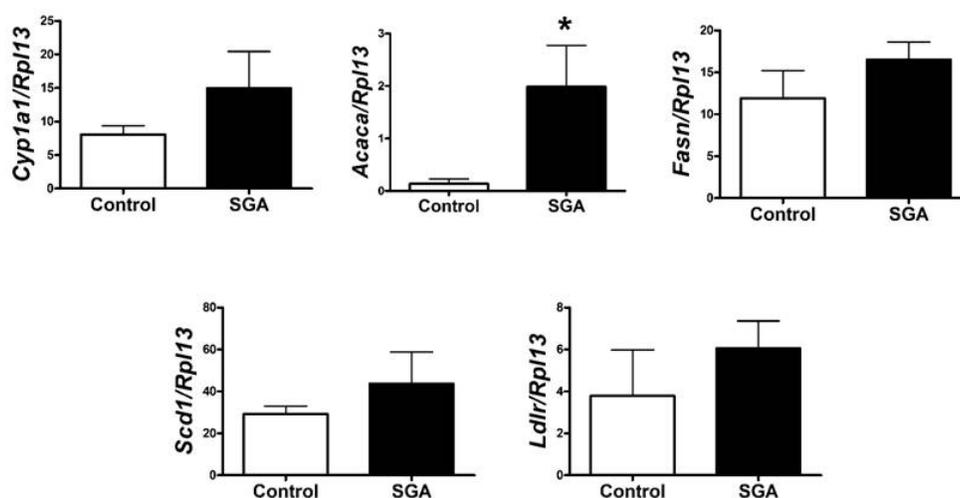
Figure 4.4 **SGA 360 repressive activity on fatty acid synthesis genes is absent in mouse white adipose tissue.**

Normalized RNA expression of fatty acid synthesis genes in white adipose tissue of C57BL/6J mice (3 male mice per group) administered SGA 360 (SGA) (20 mg/kg) by gavage compared to vehicle treated (control) mice is shown.

Activation of AHR by an Agonist or a Selective Ligand Can Overcome the Compensatory Activation of Cholesterol Synthesis Genes Following Statin Treatment in Primary Human Hepatocytes.

Prolonged statin treatment is known to induce an upregulation of cholesterol biosynthetic gene expression. A co-treatment with the AHR agonist BNF (Figure 4.5) or the selective ligand SGA360 (Figure 4.6) were successful in coordinately repressing the expression of all the examined enzymes back to and in some cases lower than the control levels with a more modest effect on LDL receptor expression. Quantitative protein blot analysis of SGA360 treated hepatocytes confirmed the ability of a SAhRM to attenuate statin-induced upregulation of genes in the cholesterol biosynthesis pathway (Figure 4.7). We observed similar effects with the use of another AHR selective ligand, 3',4'-dimethoxy-αNF (DiMNF) (data not shown). Additionally, the expression of the key fatty acid synthesis genes was repressed by SGA360 exposure (Figure 4.8A). Consistent with the mouse adipose tissue, SGA360 had no effect on the expression of fatty acid synthesis genes in primary human adipocytes (Figure 4.8B) and concomitantly the lipid content of adipocytes was not affected (Figure 4.8C).

Figure 4.5
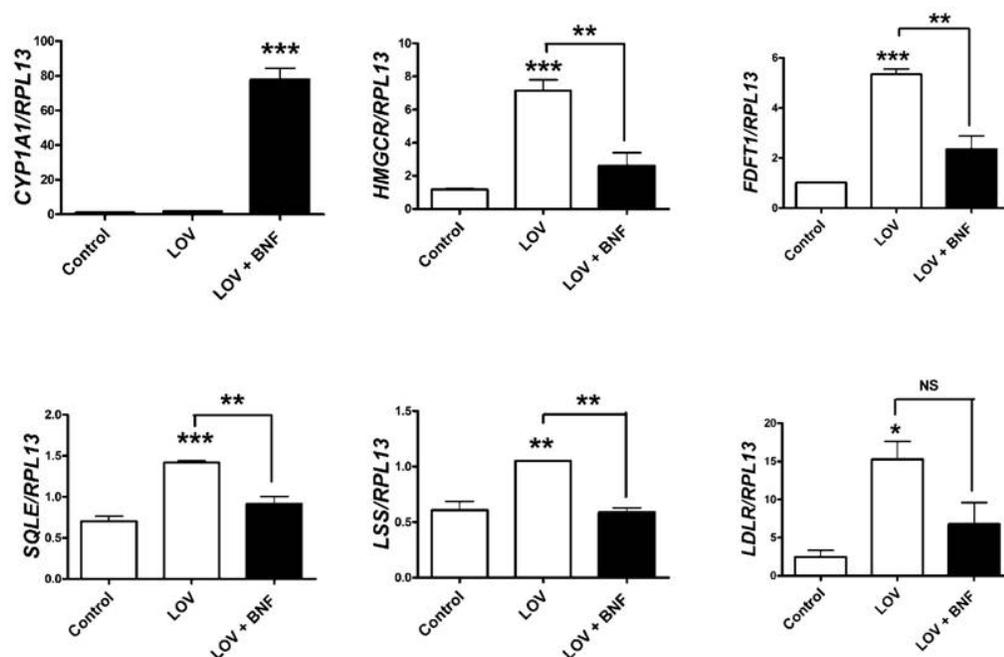
Figure 4.5 **AHR activation by an agonist can overcome statin-induced cholesterol synthesis gene expression in primary human hepatocytes.**
Normalized RNA expression of cholesterol synthesis genes of primary human hepatocytes following lovastatin (LOV) (10 µM) with or without BNF (10 µM) treatment (3 wells per group) for 48 h are shown.

Figure 4.6
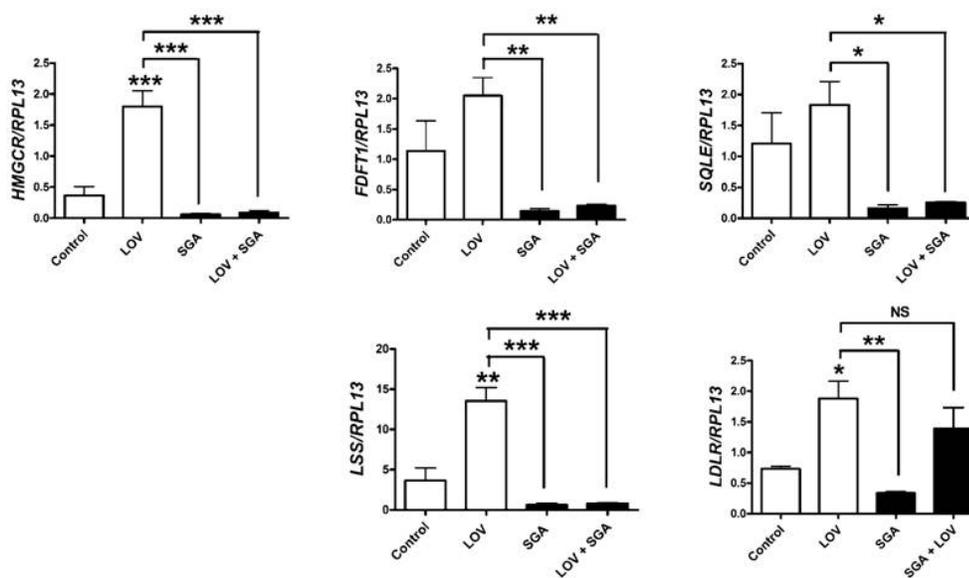
Figure 4.6 **AHR activation by SGA360 represses the compensatory upregulation of cholesterol synthesis gene expression following statin treatment in primary human hepatocytes.**
Normalized RNA expression of cholesterol synthesis genes of primary human hepatocytes following lovastatin (LOV) (10 µM) with or without SGA 360 (SGA) (10 µM) treatment (3 wells per group) for 48 h is shown.

Figure 4.7
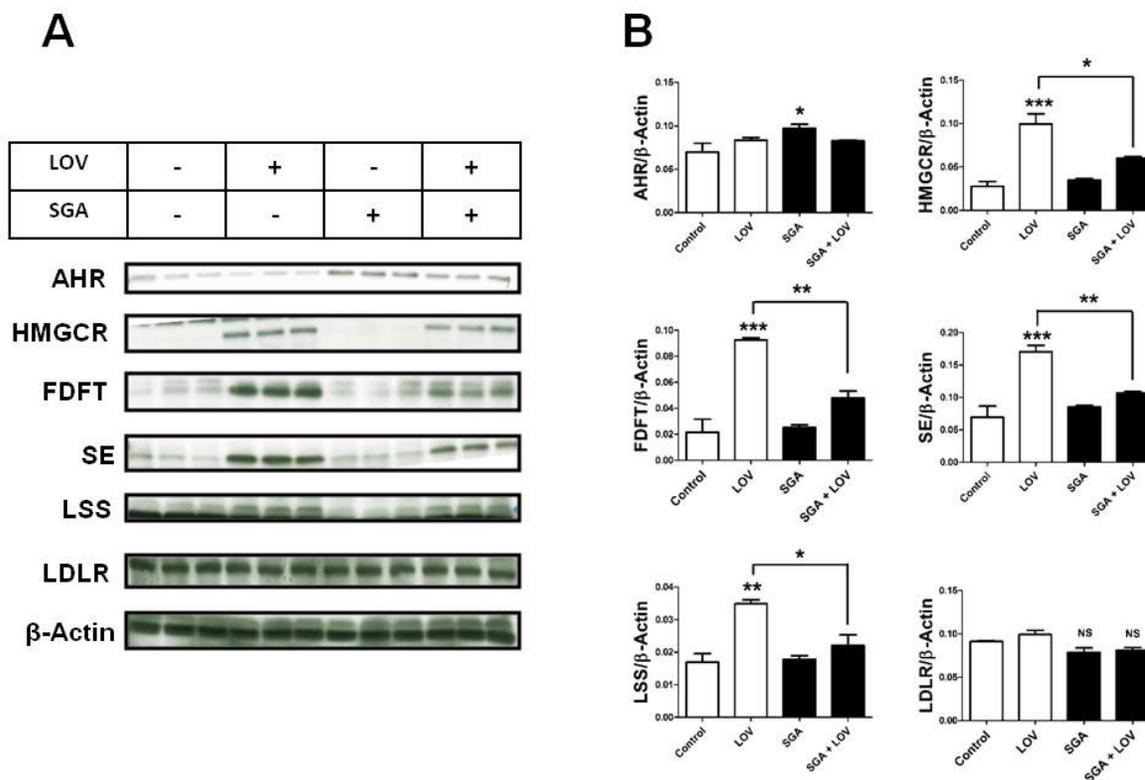
Figure 4.7 **AHR activation by SGA360 coordinately represses the compensatory upregulation of cholesterol synthesis enzyme proteins in primary human hepatocytes following statin treatment.**
    A. The hepatic increase in protein expression of selected cholesterol biosynthetic genes following lovastatin (LOV) (10 µM) treatment (48 h) is attenuated in the presence of SGA360 (SGA) (10 µM) (3 wells per group). **B.** Quantification of protein expression normalized to β-actin is shown.

Figure 4.8
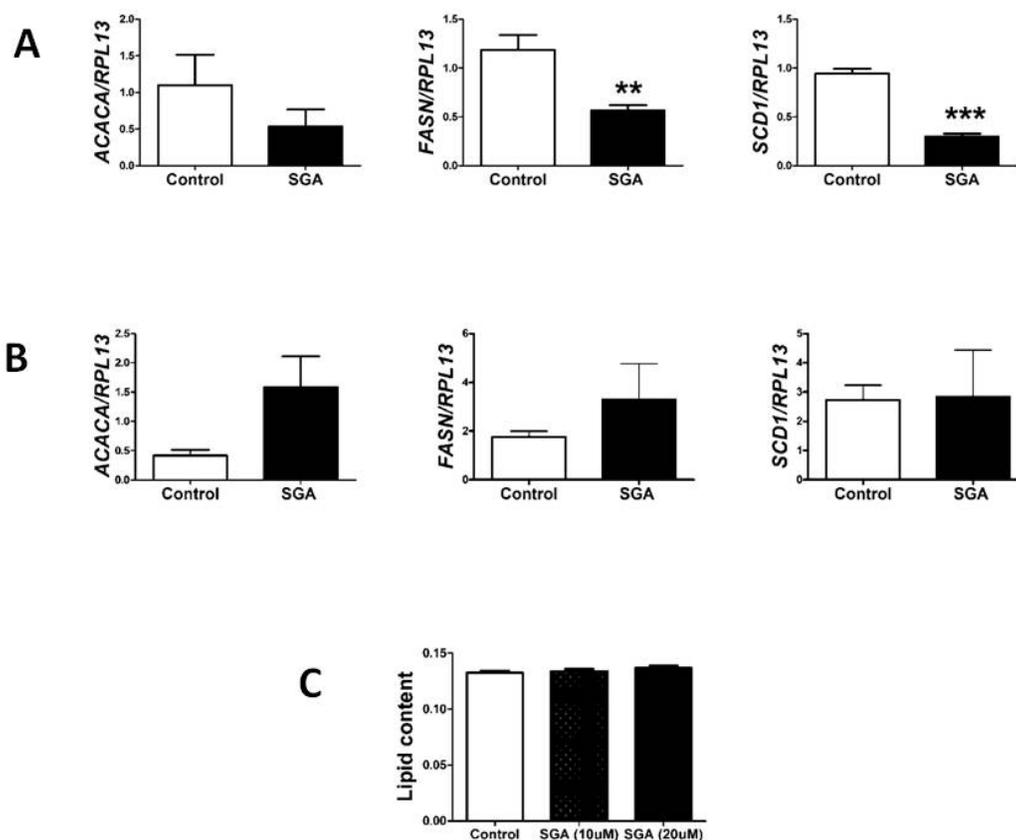
Figure 4.8 **SGA360 suppresses fatty acid synthesis gene expression in primary human hepatocytes with no effect on primary human adipocytes.**
Normalized mRNA expression of selected fatty acid biosynthesis genes of SGA360 (SGA) (10 µM) treated primary human hepatocytes (48 h) (**A**) and primary human adipocytes (4 days) (**B**) compared to control cells (3 wells per group) are shown. **C.** Quantification of lipid accumulation of primary human adipocytes (3 wells per group) by red oil stain following SGA360 treatment (10 µM or 20 µM) for 4 days is shown.

Cholesterol and Fatty Acid Synthesis Are Suppressed in Primary Human Hepatocytes upon SGA360 Treatment.

GC-MS analysis was used to quantify the cholesterol and fatty acids in lipid extracted from treated primary human hepatocytes media. Consistent with the gene expression result, a significant attenuation in the secretion levels of cholesterol (Figure 4.9A) and certain fatty acids (Figure 4.9B) by the hepatocytes was observed illustrating the ability of a SAhRM to regulate the cholesterol and lipogenesis pathways. Additionally, an additive attenuation of cholesterol secretion was observed following co-treatment with lovastatin and an AHR ligand (Figure 4.9A). To ensure that the effects seen with SGA 360 were not due to toxicity an LDH assay was performed and no toxicity was observed (Figure 4.9C).

AHR Selective Activation Inhibits Human Hepatoma Cell Proliferation.

Tumors exhibit high metabolic activity in order to support fast cell growth, proliferation and signaling. The anti-tumorigenic effects of statins and the potential use of fatty acid enzyme inhibitors in cancer treatment are well established. Given that the AHR is shown to be involved in the negative regulation of both pathways, we tested the effect of AHR ablation via siRNA exposure and the ability of SAhRM treatment to decrease cellular proliferation in a human hepatoma cell line (Hep3B). The presence of the AHR and activation by SGA360 is linked to decreased cell proliferation compared to AHR-silenced cells (Figure 4.10A). While statin treatment alone also exhibited a decrease in cell proliferation, this effect was even more pronounced in cells co-treated with SGA360 (Figure 4.10B).

Figure 4.9
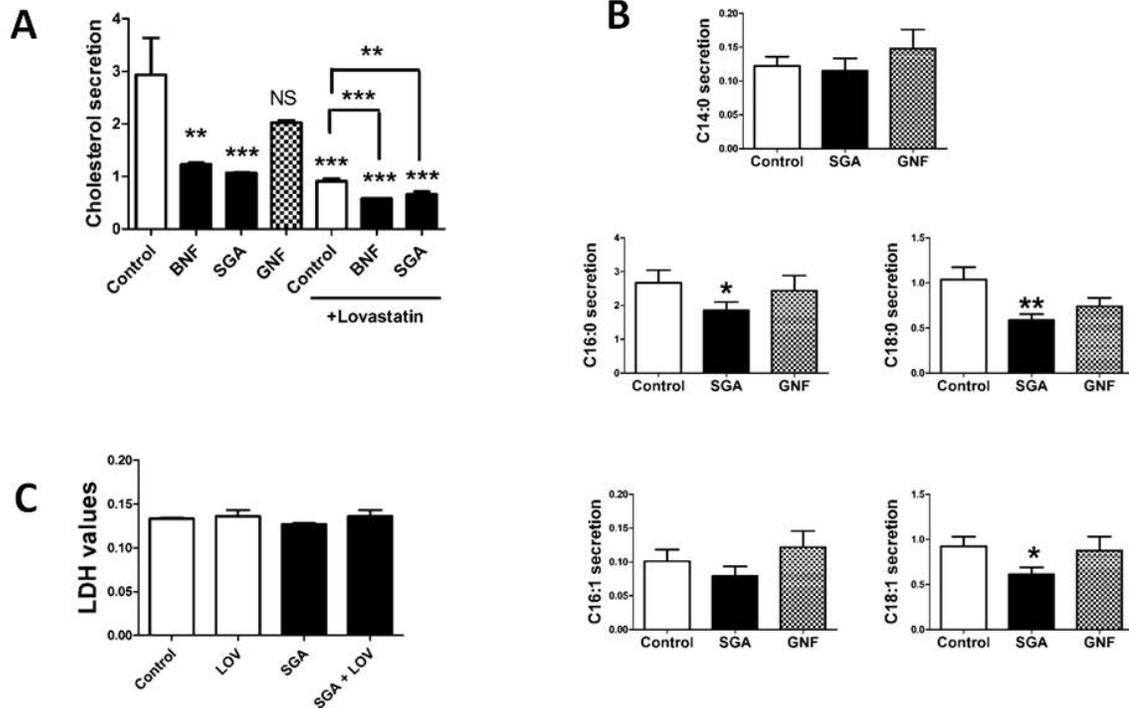
Figure 4.9 **Cholesterol and fatty acid synthesis are repressed in primary human hepatocytes following AHR activation.**
> Levels of synthesized cholesterol (**A**) and fatty acids (**B**) from media of primary human hepatocytes treated with the agonist BNF (10 µM), the selective ligand SGA 360 (SGA) (10 µM), the antagonist GNF351 (GNF) (500 nM) with or without lovastatin (LOV) (10 µM) for 48 h are compared to control cells. **C.** LDH levels demonstrate that cell viability is not affected by lovastatin or SGA360 treatment.

Figure 4.10
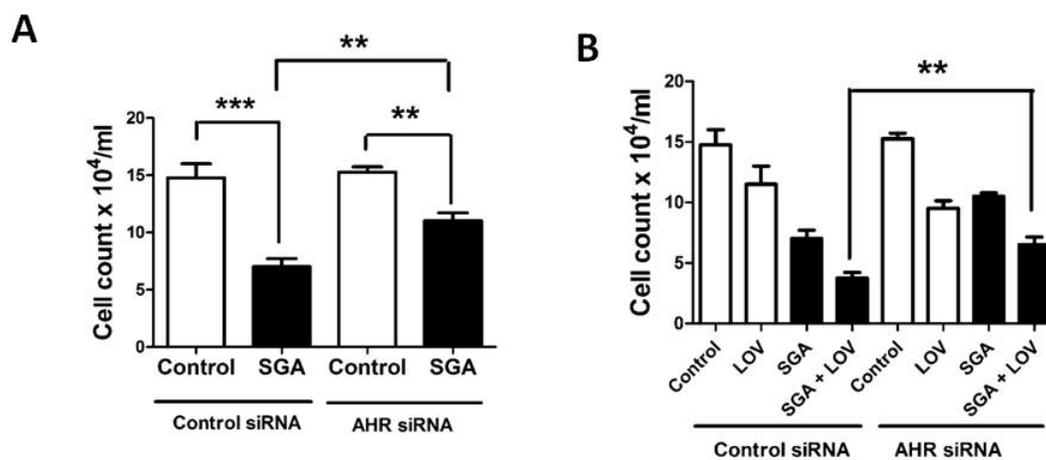
Figure 4.10 **AHR activation decreases cell number of a human hepatoma cell line.**

> **A.** Cell count of Hep3B cells where AHR has been down-regulated (AHR siRNA) is compared to control cells in the presence or absence of SGA treatment (SGA) (10 µM). **B.** Effect of statin treatment (lovastatin LOV 10 µM) coupled with SGA360 (10 µM) on cell proliferation is assessed in AHR-silenced Hep3B cells compared to control cells. Cells were treated for 48 h prior to cell count. Values represent average of 3 wells. **A** and **B** are the same experiment.

AHR Plays a Role in SREBP Activation and Binding on Its Response Element.

We next explored the possible mechanism of SAhRM attenuation of cholesterol and fatty acid synthesis pathways. The most direct mechanism would be to decrease SREBP1/2 mediated transcriptional activity expression through a direct interaction between SREBP2 and AHR. This possibility was tested using a Gal4-mammalian-one hybrid system. Increasing levels of transient AHR expression in COS-1 cells were able to negatively affect SREBP2-mediated transcription activity (Figure 4.11A). In Hep3B cells that express AHR, SGA360 treatment was effective in ablating SREBP2 activation even when coupled with statin treatment (Figure 4.11B). To test the effect of AHR activation on SREBP2 binding to its cognate response element derived from a target gene, a gel shift assay was performed. Results indicated a decrease binding of SREBP2 in Huh-7 nuclear extracts isolated from cells treated with an AHR agonist or a selective ligand (Figure 4.12). The ligand treatments were also able to completely eliminate the increased presence of SREBP2 bound to its cognate response element following statin treatment.
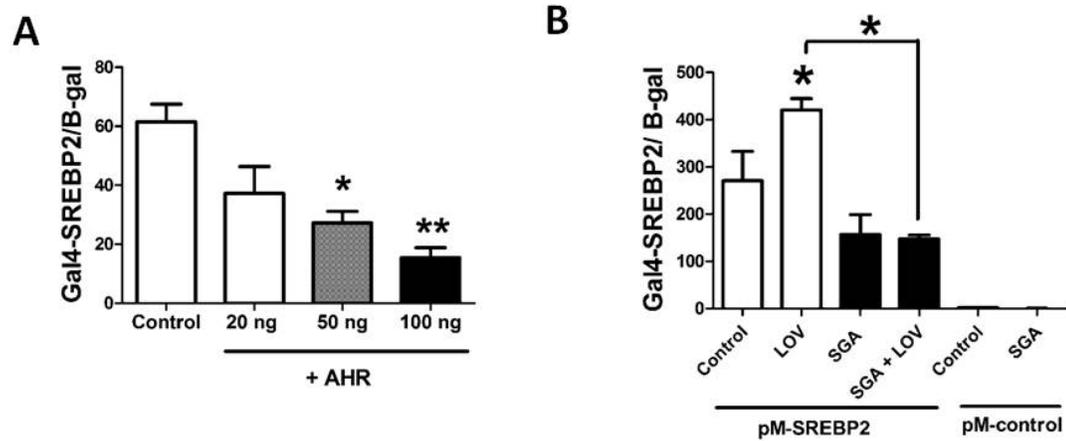
Figure 4.11

Figure 4.11 **AHR presence and selective activation modulates SREBP2 activity.**
A. The effect of increased presence of unliganded AHR on SREBP2 activity
in COS-1 cells is compared to absence of the receptor (3 wells per group, 24
h post-transfection). **B.** Effect of AHR activation by SGA360 (SGA) (10 μM)
on SREBP2 activity in the presence or absence of lovastatin (LOV) (10 μM)
compared to control cells is shown (3 wells per group, 24 h treatment).

Figure 4.12

SREBP2 response element (SRE) Probe
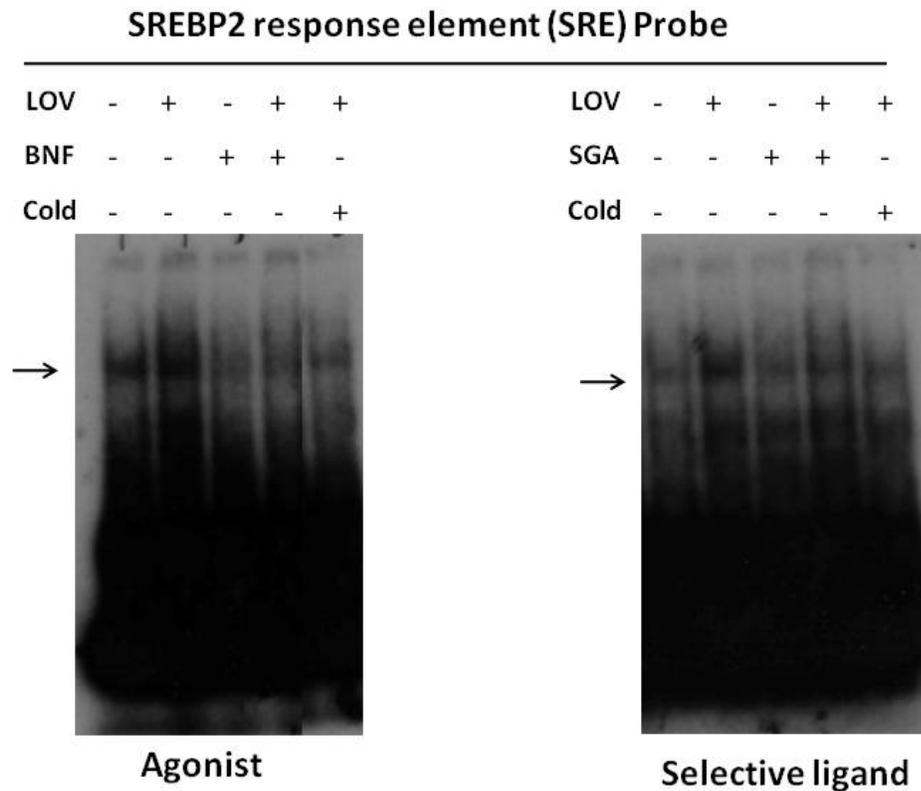
Figure 4.12 **AHR activation disrupts SREBP2 presence and binding to its cognate response element.**

Nuclear extracts of Huh-7 cells treated with lovastatin (LOV) (10 µM) with BNF (10 µM) or SGA360 (SGA) (10 µM) for 48 h were subjected to EMSA using a labeled SRE probe. A cold SRE probe was utilized for competition binding and band specificity.

AHR Targets SREBP for Degradation.

The attenuation of SREBP2 DNA binding activity in nuclear extracts following

AHR ligand treatment led us to evaluate its transcriptionally active form in nuclear and

cytoplasmic fractions. Regulating the active amount of this transcription factor or its

translocation to the nucleus is a potential way to modulate the expression of cholesterol

biosynthesis genes. Huh-7 cells were first treated with statins in order to increase the levels

of their SREBP2 pool (uncleaved ~120kDa and transcriptionally active ~68kDa). Following

AHR agonist or selective ligand treatment, lower levels of the cleaved SREBP2 protein

were found in both the nucleus and the cytoplasm (Figure 4.13A). Whole cell extract

analysis also showed a time-dependent alteration in the protein levels of cleaved SREBP2

(Figure 4.13B) indicating that AHR activation is not compromising nuclear translocation but

rather the total pool of active SREBP2. Since the treatment had no effect on the levels of the

nascent form of the protein, we concluded that the receptor's regulation was not affecting

SREBP processing in the endoplasmic reticulum or the golgi. On the basis of these

observations and previous studies showing that the receptor is capable of acting as an E3

ligase, we speculated that the receptor might be targeting the active SREBP2 protein for

proteosomal degradation. In fact, the proteosome inhibitor MG-132 was able to rescue the

active SREBP2 protein from degradation (Figure 4.14A). To further investigate the ability

of AHR to regulate the degradation of both SREBP members, the active forms of SREBP1c

and -2 were transfected into COS-1. Subsequent SGA360 treatment decreased the level of

SREBP1/2 (Figure 4.14B).

Figure 4.13
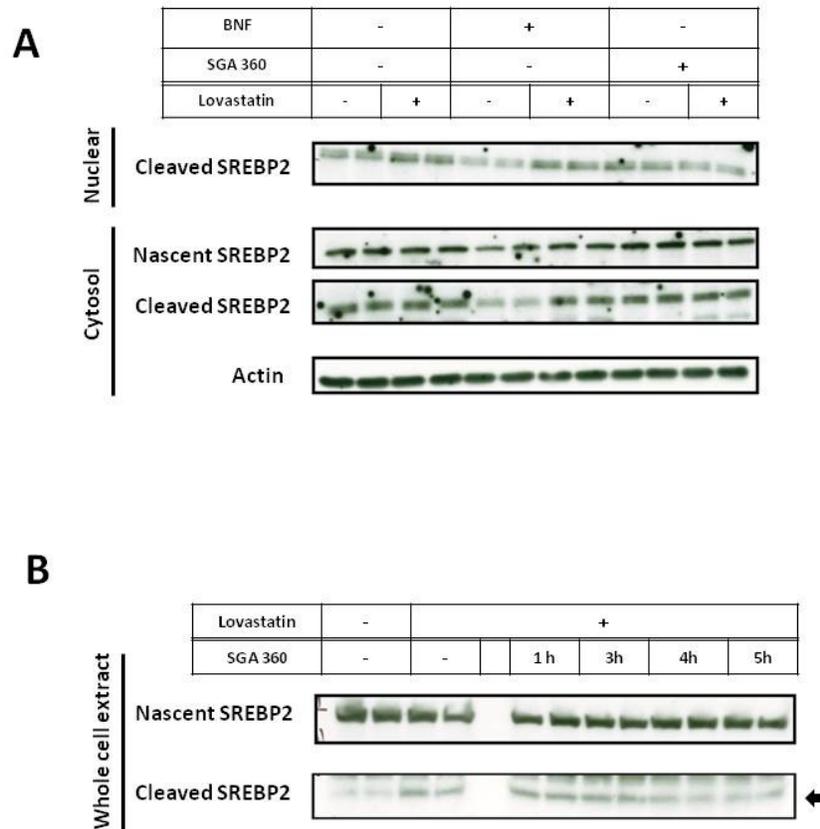
Figure 4.13 **AHR activation mediates the degradation of active SREBP2 proteins.**
> **A.** Nuclear and cytosolic protein fractions of Huh-7 cells treated with lovastatin (10 µM) and BNF (10 µM) or SGA360 (10 µM) for 48 h are shown. Levels of uncleaved SREBP2 protein and its cleaved active form were evaluated (2 wells per treatment). **B.** Whole cell extracts of HUH-7 cells exposed to lovastatin (10 µM) for 48 h coupled with a time-course AHR activation by SGA360 (10 µM) are shown. Levels of the nascent and cleaved forms of SREBP2 proteins were determined (2 wells per treatment).

Figure 4.14
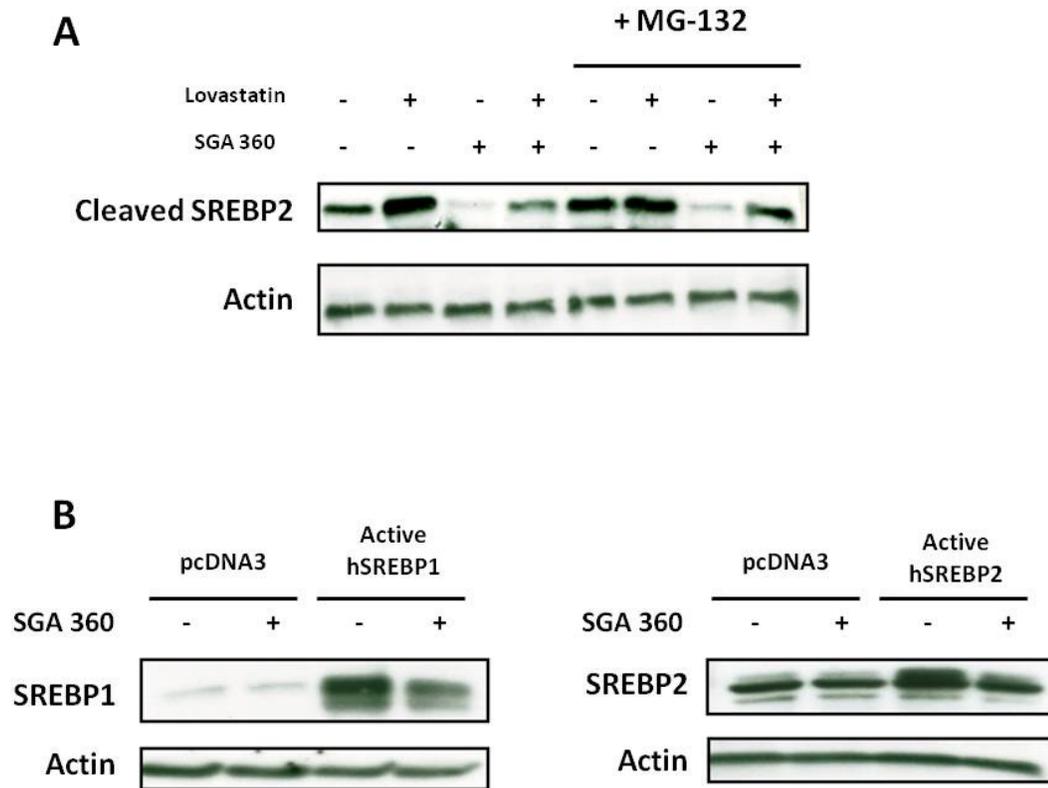
Figure 4.14 **AHR mediates the proteosomal degradation of active SREBP proteins.**
> **A.** Whole cell extracts of Huh-7 cells exposed to lovastatin (10µM) for 48 h followed by a 5 h SGA (10µM) treatment with or without the presence of the proteosome inhibitor MG132 (10µM). Levels of cleaved SREBP2 proteins were determined (each band represents 2 wells). **B.** pcDNA3, pcDNA3-hSREBP1c (active form), pcDNA3-hSREBP2 (active form) and pcDNA3-hAHR were transfected into COS-1 cells. SREBP protein levels were determined following treatment with SGA360 (10µM) for 5 h.

4.5 DISCUSSION

Selective activation of AHR with SGA360 was able to suppress the hepatic expression of cholesterol and fatty acid synthesis genes more significantly than the agonist BNF, both in mice and human in an AHR dependent manner. This suppression in mRNA and protein expression was mirrored by attenuation in fatty acid and cholesterol secretion in human cells. Both SGA360 and BNF are capable of inducing degradation of transcriptionally active SREBP2. However, an agonist induces AHR degradation resulting in a dramatic decrease in AHR protein levels [223, 224]. In contrast, SGA360 does not induce turnover of the AHR, which could result in a more sustained attenuation of SREBP1/2 levels. This concept coupled with the lack of dioxin response element driven activity would suggest that SAhRM would exhibit a limited subset of activities and thus have significant therapeutic potential. Expression of fatty acid synthesis genes was not affected in adipose tissue where AHR levels are undetectable providing evidence that SAhRM will exhibit tissue specific effects. This is also of importance if the receptor were to be utilized therapeutically since unlike metabolic target enzymes, AHR is not ubiquitously expressed and thus provides a more targeted approach to influencing liver metabolism, while minimizing the effects on other tissues. For instance, SCD1 inhibition has been linked to dry eye, squinting and alopecia believed to be due to depletion of essential SCD1-derived lubricating lipids in the eye and skin. This is also of importance for statin treatment exhibiting its most pronounced adverse effects in skeletal muscles, neurons and the kidney [159, 161-163] where AHR levels are low [106, 225]. In contrast, AHR targeting will most likely exhibit some of the beneficial pleiotropic effects of statins in the lung, heart and liver where it is highly expressed [226].

LDLR transcripts were slightly repressed upon co-treatment with statins and an AHR agonist. In contrast, LDLR mRNA and protein levels following SGA360 co-treatment were not affected. Since LDLR expression is also regulated by SREBP2, this indicates a more complex regulation of this gene compared to the other cholesterol synthesis genes tested. Interestingly, the induction of SREBP degradation by AHR would likely be similar to the phenotype of S1P knock-out mice, where SREBP processing is compromised. In these mice, a similar repression on hepatic cholesterol and fatty acid biosynthesis was observed and although they showed lower LDL receptor levels, they demonstrated reduced plasma and triglyceride levels [227]. In contrast, a study in human cells assessing the effect of a S1P inhibitor reported that the LDL receptor levels were not significantly affected [228].

Tumors exhibit a higher rate of glucose metabolism, lactate production and biosynthesis of lipids than normal tissues and show overexpression and hyperactivity of lipogenic enzymes making the critical enzymes of cholesterol and fatty acid synthesis attractive targets for disrupting cancer development. In fact, statin treatment has long been linked to anti-cancer effects including prostate, colon, lung and pancreatic cancer by inducing autophagy and cell death while causing no hindrance to normal cells [226]. Along the same lines, cancer cells targeted with FAS inhibitors show decreased proliferation and increased apoptosis [229] while inhibition of SCD1 activity severally impairs the capacity of lung and prostate cancer cell progression, survival and invasiveness, and greatly reduces tumor formation in mice [230, 231]. Interestingly, AHR has already been shown to function as a tumor suppressor of liver carcinogenesis and progression when AHR-silenced liver hepatocytes acquired a pro-proliferative and anti-apoptotic phenotype following exposure to

a chemical carcinogen [232]. The ability of AHR to target all the examined enzymes in the cholesterol and fatty acid synthesis pathways might explain its tumor suppressor characteristic also reported in this study and might provide a new way for targeting cancer development and treatment.

Our results also indicate degradation of the active SREBP transcription factors regulating both pathways. Inhibition of the cleavage of those proteins should lead to an accumulation of the uncleaved ER membrane-bound form which we did not observe after SAhRM treatment. Thus we speculate that AHR-mediated regulation is solely occurring through proteosomal degradation, as evidenced by the ability of a proteosomal inhibitor to block SAhRM-mediated SREBP2 degradation. Although reports have shown that ubiquitination and proteosomal degradation of SREBP proteins are a key process for the tight regulation of cellular sterol and fatty acid levels, to our knowledge no transcription factor or compound controlling or activating this degradation has ever been reported. Nonetheless, numerous studies have focused on the promise of drugs targeting the processing of SREBP in order to lower their nuclear levels and subsequently the expression of their target genes. For example, the small molecule Betulin inhibits the maturation of SREBP by inducing SCAP:INSIG interaction causing a decrease in the biosynthesis of cholesterol and fatty acids. In vivo, Betulin was shown to improve hyperlipidemia and insulin resistance and to reduce atherosclerotic plaques [192]. Because expression of SREBPs is also regulated through SREs in their promoter, the reduction of processed SREBP is speculated to lead to a two-fold inhibition of their target genes [227].

The ubiquitin-proteosome system is known to play a pivotal role in cellular homeostasis through the degradation of major regulatory proteins in numerous processes

including cell cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis [233]. We have previously demonstrated that AHR plays a constitutive role in the suppression of the cholesterol and fatty acid synthesis pathways in mice and humans suggesting a role for the receptor as a major regulator of lipid homeostasis

SUMMARY AND CONCLUSIONS

The AHR was originally identified as a sensor for environmental contaminants and a regulator of xenobiotic metabolism. However, it has since then been shown to be part of numerous critical cellular pathways. Previous work in our lab and others has indicated the ability of the AHR to function without binding to its cognate response element. Therefore, we wanted to get a clearer understanding of the battery of genes targeted and regulated by AHR independent of DRE binding. Overall, the goal of this thesis was to identify new target genes and functions regulated by AHR by mechanisms distinct from the classical DRE-mediated mode of gene regulation.

In this study we showed that AHR is involved in the negative regulation of the cholesterol and fatty acid synthesis pathways in a DRE-independent manner. Although this regulation was found to be constitutive, activation of the receptor by an agonist or a selective ligand was able to repress the cholesterol and fatty acid synthesis in primary human hepatocytes. Moreover, we found that this regulation involves the targeted degradation of the transcription factors SREBPs governing the expression of these enzymes through a ubiquitin-proteasome system.

In chapter 2, we generated a transgenic mouse model that expresses the DRE-binding mutant mAhr-A78D on an *ahr*-null background and subsequent data analysis pointed to a suppression of a large subset of genes involved in the cholesterol biosynthesis pathway when AHR was activated regardless of its ability to bind DRE sequences in the promoter region of various genes. Primary human hepatocytes administered an AHR agonist

showed a similar regulation with an overall lower cholesterol secretion observed, providing evidence of a global regulatory role for this receptor on the cholesterol synthesis pathway.

In chapter 3, we took a similar approach to test the involvement of the receptor in fatty acid synthesis since the enzymes in both pathways are transcriptionally regulated by the same family of proteins (SREBPs). Results from this study also showed a role for the receptor in the repression of the fatty acid synthesis genes both in mice and humans leading to attenuation of fatty acid synthesis secretion in human cells. This regulation was also occurring independently of DRE-binding and similar to our results in chapter 2, unlike their target genes the transcript levels of the SREBPs proteins remained unchanged following agonist treatment suggesting a more complex regulation by AHR.

Elucidating the mode of action of this regulation and the ability to selectively activate the receptor were the focus of chapter 4. Results indicated a proteosomal targeting of both SREBP1c and -2 proteins upon AHR ligand activation causing the observed repression in the expression of the cholesterol and fatty acid synthesis genes and eventual attenuated cholesterol and fatty acid synthesis. A co-treatment with statins demonstrated the ability of AHR selective ligands to hinder the compensatory up-regulation of the cholesterol synthesis genes seen with statin treatment. Since this regulation appeared to occur in a DRE-independent manner in mice, the effect of the AHR selective ligand on primary human hepatocytes further established this finding in humans and indicated the possibility of modulating AHR therapeutically without inducing toxicity.

It is also important to note here that previous studies exploring a role for AHR in fat metabolism and transport involved the use of TCDD. Although this compound is known to be a highly potent ligand for AHR, multiple reports argue for an independent role for this

compound. In fact, dioxin alone has been shown to bind and disrupt the three-dimensional structure of lipoprotein molecules, rendering them incapable of binding to their cognate receptor [211] and attenuating insulin-induced glucose uptake in differentiated 3T3-L1 adipocytes, all in an AHR-independent manner [212]. Hence, the development of insulin resistance, hepatic steatosis and elevated triglyceride levels following exposure to TCDD might be occurring at least in part through a mechanism independent of AHR. In addition, the highly toxic properties of TCDD complicate the interpretation of gene expression data. Another possible explanation for this result is a high dose of TCDD likely leads to a dramatic reduction in total AHR levels and in cytoplasmic AHR in hepatocytes. This in turn would limit the ability of the AHR that can enter into protein-protein interactions, leading to a result similar to what is seen when the AHR is knockdown with siRNA (Figure 2.8, 2.10, 3.2 an 3,3). In contrast, an AHR selective ligand treatment seems to stabilize the levels of the receptor (Figure 4.7) leading to a more pronounced effect in human hepatocytes compared to agonist treatment (Figure 4.5 and 4.6) without inducing CYP1A1 expression.

Our results are the first to establish the role of the AHR in the hepatic regulation of all the examined cholesterol and fatty acid synthesis genes including the rate-limiting enzymes, and the effect of the receptor's activation on the cholesterol and fatty acid synthesis products in primary human hepatocytes. Such modulation should not interfere with the isoprenoid branch of the cholesterol synthesis pathway believed to account for the adverse effects of statins. However, taking into consideration the effectiveness of statins in lowering blood cholesterol and increasing LDL receptor levels as well as their pleiotropic favorable effects, a combination therapy using statins and an AHR ligand might give a synergistic cholesterol lowering effect [130, 234]. Furthermore, the use of chemical

inhibitors of a given ubiquitous enzyme such as statins or SCD1 inhibitors may have an effect in every tissue and cell type. In contrast, an AHR ligand will exert its activity dependent on the expression pattern of AHR in various tissues. For instance, the AHR exhibits very low levels in mature adipose tissue and there is almost no AHR expression in skeletal muscle. Thus, activation of the AHR will have a more targeted effect on liver cholesterol and fatty acid synthesis. Moreover, the coordinate repression in both pathways by a single receptor suggests that the recurrent compensatory mechanism of biological pathways or the concern of metabolite accumulation/depletion of key cell signaling fatty acid molecules following drug targeting of single enzyme might be less likely to take place. Although to the best of our knowledge no study has attempted to inhibit more than one enzyme at a time, our observations suggest that selective activation of the AHR might be a particularly effective treatment to coordinately repress the two major pathways associated with cardiovascular diseases, the leading cause of human death world-wide (the World Health Organization, Sept 2011).

In the future, it would be interesting to study if SREBP degradation is the only mechanism through which AHR is exerting its repressive effect on fatty acid and cholesterol biosynthesis. And whether SGA360 is capable of ameliorating diet-induced obesity, decreasing lipid contents in serum and tissues and increasing insulin sensitivity in vivo would also be important to explore. For this purpose, a high carbohydrate diet could be used to induce hepatic steatosis and treatment with an AHR selective ligand could be tested for protection from liver injury and induced obesity [235]. Alternatively, selective activation of AHR could also be tested in several mouse lines namely leptin-deficient mice (ob/ob), a

model of type 2 diabetes and obesity [236] and apolipoprotein-null mice (Apoe -/-) [237], a

model that exhibits lipid abnormalities and atherosclerosis even on low-cholesterol-diet.

BIBLIOGRAPHY

1. Robles, R., et al., *The aryl hydrocarbon receptor, a basic helix-loop-helix transcription factor of the PAS gene family, is required for normal ovarian germ cell dynamics in the mouse.* Endocrinology, 2000. **141**(1): p. 450-3.

2. Fernandez-Salguero, P.M., et al., *Lesions of aryl-hydrocarbon receptor-deficient mice.* Vet Pathol, 1997. **34**(6): p. 605-14.

3. Beischlag, T.V., et al., *The aryl hydrocarbon receptor complex and the control of gene expression.* Crit Rev Eukaryot Gene Expr, 2008. **18**(3): p. 207-50.

4. Gu, Y.Z., J.B. Hogenesch, and C.A. Bradfield, *The PAS superfamily: sensors of environmental and developmental signals.* Annu Rev Pharmacol Toxicol, 2000. **40**: p. 519-61.

5. Nambu, J.R., et al., *The Drosophila single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development.* Cell, 1991. **67**(6): p. 1157-67.

6. Hoffman, E.C., et al., *Cloning of a factor required for activity of the Ah (dioxin) receptor.* Science, 1991. **252**(5008): p. 954-8.

7. Reyes, H., S. Reisz-Porszasz, and O. Hankinson, *Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor.* Science, 1992. **256**(5060): p. 1193-5.

8. Beischlag, T.V., et al., *Recruitment of the NCoA/SRC-1/p160 family of transcriptional coactivators by the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator complex.* Mol Cell Biol, 2002. **22**(12): p. 4319-33.

9. Hankinson, O., *The aryl hydrocarbon receptor complex.* Annu Rev Pharmacol Toxicol, 1995. **35**: p. 307-40.

10. Huff, J.E., et al., *Long-term hazards of polychlorinated dibenzodioxins and polychlorinated dibenzofurans.* Environ Health Perspect, 1980. **36**: p. 221-40.

11.   Hellstrom, P.M. and S. Vitols, *The choice of proton pump inhibitor: does it matter?* Basic Clin Pharmacol Toxicol, 2004. **94**(3): p. 106-11.

12.   Backlund, M., et al., *Signal transduction-mediated activation of the aryl hydrocarbon receptor in rat hepatoma H4IIE cells.* J Biol Chem, 1997. **272**(50): p. 31755-63.

13.   Dzeletovic, N., et al., *Regulation of dioxin receptor function by omeprazole.* J Biol Chem, 1997. **272**(19): p. 12705-13.

14.   Backlund, M. and M. Ingelman-Sundberg, *Different structural requirements of the ligand binding domain of the aryl hydrocarbon receptor for high- and low-affinity ligand binding and receptor activation.* Mol Pharmacol, 2004. **65**(2): p. 416-25.

15.   Bjeldanes, L.F., et al., *Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol in vitro and in vivo: comparisons with 2,3,7,8-tetrachlorodibenzo-p-dioxin.* Proc Natl Acad Sci U S A, 1991. **88**(21): p. 9543-7.

16.   Sinal, C.J. and J.R. Bend, *Aryl hydrocarbon receptor-dependent induction of cyp1a1 by bilirubin in mouse hepatoma hepa 1c1c7 cells.* Mol Pharmacol, 1997. **52**(4): p. 590-9.

17.   Phelan, D., et al., *Activation of the Ah receptor signal transduction pathway by bilirubin and biliverdin.* Arch Biochem Biophys, 1998. **357**(1): p. 155-63.

18.   Chiaro, C.R., R.D. Patel, and G.H. Perdew, *12(R)-Hydroxy-5(Z),8(Z),10(E),14(Z)-eicosatetraenoic acid [12(R)-HETE], an arachidonic acid derivative, is an activator of the aryl hydrocarbon receptor.* Mol Pharmacol, 2008. **74**(6): p. 1649-56.

19.   Schaldach, C.M., J. Riby, and L.F. Bjeldanes, *Lipoxin A4: a new class of ligand for the Ah receptor.* Biochemistry, 1999. **38**(23): p. 7594-600.

20.   Adachi, J., et al., *Comparison of gene expression patterns between 2,3,7,8-tetrachlorodibenzo-p-dioxin and a natural arylhydrocarbon receptor ligand, indirubin.* Toxicol Sci, 2004. **80**(1): p. 161-9.

21.   Flaveny, C.A., et al., *Ligand selectivity and gene regulation by the human aryl hydrocarbon receptor in transgenic mice.* Mol Pharmacol, 2009. **75**(6): p. 1412-20.

22.   Savouret, J.F., et al., *7-ketocholesterol is an endogenous modulator for the arylhydrocarbon receptor.* J Biol Chem, 2001. **276**(5): p. 3054-9.

23.   Murray, I.A., et al., *Development of a selective modulator of aryl hydrocarbon (Ah) receptor activity that exhibits anti-inflammatory properties.* Chem Res Toxicol, 2010. **23**(5): p. 955-66.

24.   Murray, I.A., et al., *Suppression of cytokine-mediated complement factor gene expression through selective activation of the Ah receptor with 3',4'-dimethoxy-alpha-naphthoflavone.* Mol Pharmacol, 2011. **79**(3): p. 508-19.

25.   Murray, I.A., et al., *Evidence for ligand-mediated selective modulation of aryl hydrocarbon receptor activity.* Mol Pharmacol, 2010. **77**(2): p. 247-54.

26.   Coumailleau, P., et al., *Definition of a minimal domain of the dioxin receptor that is associated with Hsp90 and maintains wild type ligand binding affinity and specificity.* J Biol Chem, 1995. **270**(42): p. 25291-300.

27.   Pongratz, I., G.G. Mason, and L. Poellinger, *Dual roles of the 90-kDa heat shock protein hsp90 in modulating functional activities of the dioxin receptor. Evidence that the dioxin receptor functionally belongs to a subclass of nuclear receptors which require hsp90 both for ligand binding activity and repression of intrinsic DNA binding activity.* J Biol Chem, 1992. **267**(19): p. 13728-34.

28.   Richter, K. and J. Buchner, *Hsp90: chaperoning signal transduction.* J Cell Physiol, 2001. **188**(3): p. 281-90.

29.   Nair, S.C., et al., *A pathway of multi-chaperone interactions common to diverse regulatory proteins: estrogen receptor, Fes tyrosine kinase, heat shock transcription factor Hsf1, and the aryl hydrocarbon receptor.* Cell Stress Chaperones, 1996. **1**(4): p. 237-50.

30. Cox, M.B. and C.A. Miller, 3rd, *Cooperation of heat shock protein 90 and p23 in aryl hydrocarbon receptor signaling.* Cell Stress Chaperones, 2004. **9**(1): p. 4-20.

31. Flaveny, C., G.H. Perdew, and C.A. Miller, 3rd, *The Aryl-hydrocarbon receptor does not require the p23 co-chaperone for ligand binding and target gene expression in vivo.* Toxicol Lett, 2009. **189**(1): p. 57-62.

32. Petrulis, J.R., et al., *The hsp90 Co-chaperone XAP2 alters importin beta recognition of the bipartite nuclear localization signal of the Ah receptor and represses transcriptional activity.* J Biol Chem, 2003. **278**(4): p. 2677-85.

33. Kuzhandaivelu, N., et al., *XAP2, a novel hepatitis B virus X-associated protein that inhibits X transactivation.* Nucleic Acids Res, 1996. **24**(23): p. 4741-50.

34. Meyer, B.K., et al., *Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity.* Mol Cell Biol, 1998. **18**(2): p. 978-88.

35. LaPres, J.J., et al., *ARA9 modifies agonist signaling through an increase in cytosolic aryl hydrocarbon receptor.* J Biol Chem, 2000. **275**(9): p. 6153-9.

36. Meyer, B.K. and G.H. Perdew, *Characterization of the AhR-hsp90-XAP2 core complex and the role of the immunophilin-related protein XAP2 in AhR stabilization.* Biochemistry, 1999. **38**(28): p. 8907-17.

37. Kazlauskas, A., L. Poellinger, and I. Pongratz, *The immunophilin-like protein XAP2 regulates ubiquitination and subcellular localization of the dioxin receptor.* J Biol Chem, 2000. **275**(52): p. 41317-24.

38. Petrulis, J.R., N.G. Hord, and G.H. Perdew, *Subcellular localization of the aryl hydrocarbon receptor is modulated by the immunophilin homolog hepatitis B virus X-associated protein 2.* J Biol Chem, 2000. **275**(48): p. 37448-53.

39.   Hollingshead, B.D., J.R. Petrulis, and G.H. Perdew, *The aryl hydrocarbon (Ah) receptor transcriptional regulator hepatitis B virus X-associated protein 2 antagonizes p23 binding to Ah receptor-Hsp90 complexes and is dispensable for receptor function.* J Biol Chem, 2004. **279**(44): p. 45652-61.

40.   Pollenz, R.S. and E.R. Barbour, *Analysis of the complex relationship between nuclear export and aryl hydrocarbon receptor-mediated gene regulation.* Mol Cell Biol, 2000. **20**(16): p. 6095-104.

41.   Berg, P. and I. Pongratz, *Two parallel pathways mediate cytoplasmic localization of the dioxin (aryl hydrocarbon) receptor.* J Biol Chem, 2002. **277**(35): p. 32310-9.

42.   Whitelaw, M., et al., *Ligand-dependent recruitment of the Arnt coregulator determines DNA recognition by the dioxin receptor.* Mol Cell Biol, 1993. **13**(4): p. 2504-14.

43.   Swanson, H.I., W.K. Chan, and C.A. Bradfield, *DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins.* J Biol Chem, 1995. **270**(44): p. 26292-302.

44.   Bacsi, S.G., S. Reisz-Porszasz, and O. Hankinson, *Orientation of the heterodimeric aryl hydrocarbon (dioxin) receptor complex on its asymmetric DNA recognition sequence.* Mol Pharmacol, 1995. **47**(3): p. 432-8.

45.   Ko, H.P., et al., *Dioxin-induced CYP1A1 transcription in vivo: the aromatic hydrocarbon receptor mediates transactivation, enhancer-promoter communication, and changes in chromatin structure.* Mol Cell Biol, 1996. **16**(1): p. 430-6.

46.   Kumar, M.B. and G.H. Perdew, *Nuclear receptor coactivator SRC-1 interacts with the Q-rich subdomain of the AhR and modulates its transactivation potential.* Gene Expr, 1999. **8**(5-6): p. 273-86.

47.   Kobayashi, A., K. Sogawa, and Y. Fujii-Kuriyama, *Cooperative interaction between AhR.Arnt and Sp1 for the drug-inducible expression of CYP1A1 gene.* J Biol Chem, 1996. **271**(21): p. 12310-6.

48.   Tian, Y., et al., *Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity.* J Biol Chem, 1999. **274**(1): p. 510-5.

49.   Beischlag, T.V. and G.H. Perdew, *ER alpha-AHR-ARNT protein-protein interactions mediate estradiol-dependent transrepression of dioxin-inducible gene transcription.* J Biol Chem, 2005. **280**(22): p. 21607-11.

50.   Astroff, B., B. Eldridge, and S. Safe, *Inhibition of the 17 beta-estradiol-induced and constitutive expression of the cellular protooncogene c-fos by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the female rat uterus.* Toxicol Lett, 1991. **56**(3): p. 305-15.

51.   Boverhof, D.R., et al., *Inhibition of estrogen-mediated uterine gene expression responses by dioxin.* Mol Pharmacol, 2008. **73**(1): p. 82-93.

52.   Baba, T., et al., *Intrinsic function of the aryl hydrocarbon (dioxin) receptor as a key factor in female reproduction.* Mol Cell Biol, 2005. **25**(22): p. 10040-51.

53.   Lin, T.M., et al., *Effects of aryl hydrocarbon receptor null mutation and in utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure on prostate and seminal vesicle development in C57BL/6 mice.* Toxicol Sci, 2002. **68**(2): p. 479-87.

54.   Gillesby, B.E., et al., *Identification of a motif within the 5' regulatory region of pS2 which is responsible for AP-1 binding and TCDD-mediated suppression.* Biochemistry, 1997. **36**(20): p. 6080-9.

55.   Kim, D.W., et al., *The RelA NF-kappaB subunit and the aryl hydrocarbon receptor (AhR) cooperate to transactivate the c-myc promoter in mammary cells.* Oncogene, 2000. **19**(48): p. 5498-506.

56.   Ke, S., et al., *Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor-alpha and lipopolysaccharide.* J Biol Chem, 2001. **276**(43): p. 39638-44.

57.   Weber, L.W., et al., *Correlation between toxicity and effects on intermediary metabolism in 2,3,7,8-tetrachlorodibenzo-p-dioxin-treated male C57BL/6J and DBA/2J mice.* Toxicol Appl Pharmacol, 1995. **131**(1): p. 155-62.

58.   Sweeney, M.H., et al., *Review and update of the results of the NIOSH medical study of workers exposed to chemicals contaminated with 2,3,7,8-tetrachlorodibenzodioxin.* Teratog Carcinog Mutagen, 1997. **17**(4-5): p. 241-7.

59.   Alexander, D.L., et al., *Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis.* J Cell Sci, 1998. **111 (Pt 22)**: p. 3311-22.

60.   Cui, G., et al., *Liver X receptor (LXR) mediates negative regulation of mouse and human Th17 differentiation.* J Clin Invest, 2011. **121**(2): p. 658-70.

61.   Ohtake, F., Y. Fujii-Kuriyama, and S. Kato, *AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions.* Biochem Pharmacol, 2009. **77**(4): p. 474-84.

62.   Ohtake, F., et al., *Intrinsic AhR function underlies cross-talk of dioxins with sex hormone signalings.* Biochem Biophys Res Commun, 2008. **370**(4): p. 541-6.

63.   Mimura, J. and Y. Fujii-Kuriyama, *Functional role of AhR in the expression of toxic effects by TCDD.* Biochim Biophys Acta, 2003. **1619**(3): p. 263-8.

64.   Mocarelli, P., et al., *Paternal concentrations of dioxin and sex ratio of offspring.* Lancet, 2000. **355**(9218): p. 1858-63.

65.   Moses, M., et al., *Health status of workers with past exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin in the manufacture of 2,4,5-trichlorophenoxyacetic acid: comparison of findings with and without chloracne.* Am J Ind Med, 1984. **5**(3): p. 161-82.

66.   Petersen, S.L., S. Krishnan, and E.D. Hudgens, *The aryl hydrocarbon receptor pathway and sexual differentiation of neuroendocrine functions.* Endocrinology, 2006. **147**(6 Suppl): p. S33-42.

67. Gregoraszczuk, E.L., E. Zabielny, and D. Ochwat, *Aryl hydrocarbon receptor (AhR)-linked inhibition of luteal cell progesterone secretion in 2,3,7,8-tetrachlorodibenzo-p-dioxin treated cells.* J Physiol Pharmacol, 2001. **52**(2): p. 303-11.

68. Gray, L.E., Jr., *Xenoendocrine disrupters: laboratory studies on male reproductive effects.* Toxicol Lett, 1998. **102-103**: p. 331-5.

69. Baba, T., et al., *Disruption of aryl hydrocarbon receptor (AhR) induces regression of the seminal vesicle in aged male mice.* Sex Dev, 2008. **2**(1): p. 1-11.

70. Aragon, A.C., et al., *In utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure: effects on fetal and adult cardiac gene expression and adult cardiac and renal morphology.* Toxicol Sci, 2008. **101**(2): p. 321-30.

71. Brunnberg, S., et al., *The constitutively active Ah receptor (CA-Ahr) mouse as a potential model for dioxin exposure--effects in vital organs.* Toxicology, 2006. **224**(3): p. 191-201.

72. Silkworth, J.B., T. Lipinskas, and C.R. Stoner, *Immunosuppressive potential of several polycyclic aromatic hydrocarbons (PAHs) found at a Superfund site: new model used to evaluate additive interactions between benzo[a]pyrene and TCDD.* Toxicology, 1995. **105**(2-3): p. 375-86.

73. Fernandez-Salguero, P., et al., *Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor.* Science, 1995. **268**(5211): p. 722-6.

74. Kimbrough, R.D. and C.A. Krouskas, *Human exposure to polychlorinated biphenyls and health effects: a critical synopsis.* Toxicol Rev, 2003. **22**(4): p. 217-33.

75. Vorderstrasse, B.A., J.A. Cundiff, and B.P. Lawrence, *Developmental exposure to the potent aryl hydrocarbon receptor agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin Impairs the cell-mediated immune response to infection with influenza a virus, but enhances elements of innate immunity.* J Immunotoxicol, 2004. **1**(2): p. 103-12.

76.   Vorderstrasse, B.A. and B.P. Lawrence, *Protection against lethal challenge with Streptococcus pneumoniae is conferred by aryl hydrocarbon receptor activation but is not associated with an enhanced inflammatory response.* Infect Immun, 2006. **74**(10): p. 5679-86.

77.   Shi, L.Z., et al., *The aryl hydrocarbon receptor is required for optimal resistance to Listeria monocytogenes infection in mice.* J Immunol, 2007. **179**(10): p. 6952-62.

78.   Thurmond, T.S., et al., *The aryl hydrocarbon receptor has a role in the in vivo maturation of murine bone marrow B lymphocytes and their response to 2,3,7,8-tetrachlorodibenzo-p-dioxin.* Toxicol Appl Pharmacol, 2000. **165**(3): p. 227-36.

79.   Patel, R.D., et al., *Ah receptor represses acute-phase response gene expression without binding to its cognate response element.* Lab Invest, 2009. **89**(6): p. 695-707.

80.   Hasler, J.A., *Pharmacogenetics of cytochromes P450.* Mol Aspects Med, 1999. **20**(1-2): p. 12-24, 25-137.

81.   Shimada, T. and Y. Fujii-Kuriyama, *Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1.* Cancer Sci, 2004. **95**(1): p. 1-6.

82.   Zheng, W., et al., *Cell selective cAMP induction of rat CYP1B1 in adrenal and testis cells. Identification of a novel cAMP-responsive far upstream enhancer and a second Ah receptor-dependent mechanism.* Arch Biochem Biophys, 2003. **416**(1): p. 53-67.

83.   Tsuchiya, Y., et al., *Human CYP1B1 is regulated by estradiol via estrogen receptor.* Cancer Res, 2004. **64**(9): p. 3119-25.

84.   Buters, J.T., et al., *Cytochrome P450 CYP1B1 determines susceptibility to 7, 12-dimethylbenz[a]anthracene-induced lymphomas.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 1977-82.

85. Choudhary, D., et al., *Metabolism of retinoids and arachidonic acid by human and mouse cytochrome P450 1b1.* Drug Metab Dispos, 2004. **32**(8): p. 840-7.

86. Chen, H., W.N. Howald, and M.R. Juchau, *Biosynthesis of all-trans-retinoic acid from all-trans-retinol: catalysis of all-trans-retinol oxidation by human P-450 cytochromes.* Drug Metab Dispos, 2000. **28**(3): p. 315-22.

87. Liang, H.C., et al., *Cyp1a2(-/-) null mutant mice develop normally but show deficient drug metabolism.* Proc Natl Acad Sci U S A, 1996. **93**(4): p. 1671-6.

88. Nebert, D.W., R.A. McKinnon, and A. Puga, *Human drug-metabolizing enzyme polymorphisms: effects on risk of toxicity and cancer.* DNA Cell Biol, 1996. **15**(4): p. 273-80.

89. Rivera, S.P., et al., *A novel promoter element containing multiple overlapping xenobiotic and hypoxia response elements mediates induction of cytochrome P4502S1 by both dioxin and hypoxia.* J Biol Chem, 2007. **282**(15): p. 10881-93.

90. Saarikoski, S.T., et al., *Localization of cytochrome P450 CYP2S1 expression in human tissues by in situ hybridization and immunohistochemistry.* J Histochem Cytochem, 2005. **53**(5): p. 549-56.

91. Saarikoski, S.T., et al., *Identification of genetic polymorphisms of CYP2S1 in a Finnish Caucasian population.* Mutat Res, 2004. **554**(1-2): p. 267-77.

92. Ingelman-Sundberg, M., *Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms.* Naunyn Schmiedebergs Arch Pharmacol, 2004. **369**(1): p. 89-104.

93. Van Birgelen, A.P., et al., *Subchronic effects of 2,3,7,8-TCDD or PCBs on thyroid hormone metabolism: use in risk assessment.* Eur J Pharmacol, 1995. **293**(1): p. 77-85.

94. Saarikoski, S.T., et al., *Induction of UDP-glycosyltransferase family 1 genes in rat liver: different patterns of mRNA expression with two inducers, 3-methylcholanthrene and beta-naphthoflavone.* Biochem Pharmacol, 1998. **56**(5): p. 569-75.

95.   Alsharif, N.Z., W.J. Schlueter, and S.J. Stohs, *Stimulation of NADPH-dependent reactive oxygen species formation and DNA damage by 2,3,7,8-tetrachlorodibenzo-p-dioxin in rat peritoneal lavage cells.* Arch Environ Contam Toxicol, 1994. **26**(3): p. 392-7.

96.   Patel, R.D., et al., *Aryl-hydrocarbon receptor activation regulates constitutive androstane receptor levels in murine and human liver.* Hepatology, 2007. **46**(1): p. 209-18.

97.   Murray, I.A. and G.H. Perdew, *Omeprazole stimulates the induction of human insulin-like growth factor binding protein-1 through aryl hydrocarbon receptor activation.* J Pharmacol Exp Ther, 2008. **324**(3): p. 1102-10.

98.   Widerak, M., et al., *The aryl hydrocarbon receptor activates the retinoic acid receptoralpha through SMRT antagonism.* Biochimie, 2006. **88**(3-4): p. 387-97.

99.   Bryant, P.L., et al., *Effects of TCDD on Ah receptor, ARNT, EGF, and TGF-alpha expression in embryonic mouse urinary tract.* Teratology, 1997. **55**(5): p. 326-37.

100.   Totlandsdal, A.I., et al., *Diesel exhaust particles induce CYP1A1 and pro-inflammatory responses via differential pathways in human bronchial epithelial cells.* Part Fibre Toxicol, 2010. **7**: p. 41.

101.   Burbach, K.M., A. Poland, and C.A. Bradfield, *Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor.* Proc Natl Acad Sci U S A, 1992. **89**(17): p. 8185-9.

102.   Fukunaga, B.N., et al., *Identification of functional domains of the aryl hydrocarbon receptor.* J Biol Chem, 1995. **270**(49): p. 29270-8.

103.   Ikuta, T., et al., *Nuclear localization and export signals of the human aryl hydrocarbon receptor.* J Biol Chem, 1998. **273**(5): p. 2895-904.

104.   Ma, Q., L. Dong, and J.P. Whitlock, Jr., *Transcriptional activation by the mouse Ah receptor. Interplay between multiple stimulatory and inhibitory functions.* J Biol Chem, 1995. **270**(21): p. 12697-703.

105.   Poland, A. and E. Glover, *Variation in the molecular mass of the Ah receptor among vertebrate species and strains of rats.* Biochem Biophys Res Commun, 1987. **146**(3): p. 1439-49.

106.   Dolwick, K.M., et al., *Cloning and expression of a human Ah receptor cDNA.* Mol Pharmacol, 1993. **44**(5): p. 911-7.

107.   Korkalainen, M., J. Tuomisto, and R. Pohjanvirta, *Restructured transactivation domain in hamster AH receptor.* Biochem Biophys Res Commun, 2000. **273**(1): p. 272-81.

108.   Korkalainen, M., J. Tuomisto, and R. Pohjanvirta, *The AH receptor of the most dioxin-sensitive species, guinea pig, is highly homologous to the human AH receptor.* Biochem Biophys Res Commun, 2001. **285**(5): p. 1121-9.

109.   Schwetz, B.A., et al., *Toxicology of chlorinated dibenzo-p-dioxins.* Environ Health Perspect, 1973. **5**: p. 87-99.

110.   McConnell, E.E., et al., *The comparative toxicity of chlorinated dibenzo-p-dioxins in mice and guinea pigs.* Toxicol Appl Pharmacol, 1978. **44**(2): p. 335-56.

111.   Henck, J.M., et al., *2,3,7,8-tetrachlorodibenzo-p-dioxin: acute oral toxicity in hamsters.* Toxicol Appl Pharmacol, 1981. **59**(2): p. 405-7.

112.   Pohjanvirta, R., et al., *Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on plasma and tissue beta-endorphin-like immunoreactivity in the most TCDD-susceptible and the most TCDD-resistant rat strain.* Life Sci, 1993. **53**(19): p. 1479-87.

113.   Chapman, D.E. and C.M. Schiller, *Dose-related effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in C57BL/6J and DBA/2J mice.* Toxicol Appl Pharmacol, 1985. **78**(1): p. 147-57.

114.   Poland, A. and E. Glover, *Characterization and strain distribution pattern of the murine Ah receptor specified by the Ahd and Ahb-3 alleles.* Mol Pharmacol, 1990. **38**(3): p. 306-12.

115. Pohjanvirta, R., et al., *Point mutation in intron sequence causes altered carboxyl-terminal structure in the aryl hydrocarbon receptor of the most 2,3,7,8-tetrachlorodibenzo-p-dioxin-resistant rat strain.* Mol Pharmacol, 1998. **54**(1): p. 86-93.

116. Poland, A., D. Palen, and E. Glover, *Analysis of the four alleles of the murine aryl hydrocarbon receptor.* Mol Pharmacol, 1994. **46**(5): p. 915-21.

117. Ema, M., et al., *Dioxin binding activities of polymorphic forms of mouse and human arylhydrocarbon receptors.* J Biol Chem, 1994. **269**(44): p. 27337-43.

118. Cauchi, S., et al., *Polymorphisms of human aryl hydrocarbon receptor (AhR) gene in a French population: relationship with CYP1A1 inducibility and lung cancer.* Carcinogenesis, 2001. **22**(11): p. 1819-24.

119. Kawajiri, K., et al., *Polymorphisms of human Ah receptor gene are not involved in lung cancer.* Pharmacogenetics, 1995. **5**(3): p. 151-8.

120. Smart, J. and A.K. Daly, *Variation in induced CYP1A1 levels: relationship to CYP1A1, Ah receptor and GSTM1 polymorphisms.* Pharmacogenetics, 2000. **10**(1): p. 11-24.

121. Wong, J.M., A.B. Okey, and P.A. Harper, *Human aryl hydrocarbon receptor polymorphisms that result in loss of CYP1A1 induction.* Biochem Biophys Res Commun, 2001. **288**(4): p. 990-6.

122. Harper, P.A., et al., *Polymorphisms in the human AH receptor.* Chem Biol Interact, 2002. **141**(1-2): p. 161-87.

123. Sato, S., et al., *Low-dose dioxins alter gene expression related to cholesterol biosynthesis, lipogenesis, and glucose metabolism through the aryl hydrocarbon receptor-mediated pathway in mouse liver.* Toxicol Appl Pharmacol, 2008. **229**(1): p. 10-9.

124. Fletcher, N., et al., *2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) alters the mRNA expression of critical genes associated with cholesterol metabolism, bile acid biosynthesis,*

and bile transport in rat liver: a microarray study. Toxicol Appl Pharmacol, 2005. **207**(1): p. 1-24.

125. Nishiumi, S., et al., *Involvement of SREBPs in 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced disruption of lipid metabolism in male guinea pig.* Toxicol Appl Pharmacol, 2008. **229**(3): p. 281-9.

126. Tuomisto, J.T., et al., *TCDD-induced anorexia and wasting syndrome in rats: effects of diet-induced obesity and nutrition.* Pharmacol Biochem Behav, 1999. **62**(4): p. 735-42.

127. Pelclova, D., et al., *Lipid metabolism and neuropsychological follow-up study of workers exposed to 2,3,7,8- tetrachlordibenzo- p-dioxin.* Int Arch Occup Environ Health, 2002. **75 Suppl**: p. S60-6.

128. Angrish, M.M., et al., *Aryl hydrocarbon receptor-mediated induction of Stearoyl-CoA desaturase 1 alters hepatic fatty acid composition in TCDD-elicited steatosis.* Toxicol Sci, 2011. **124**(2): p. 299-310.

129. Robichon, C. and I. Dugail, *De novo cholesterol synthesis at the crossroads of adaptive response to extracellular stress through SREBP.* Biochimie, 2007. **89**(2): p. 260-4.

130. Bennett, M.K., et al., *Selective binding of sterol regulatory element-binding protein isoforms and co-regulatory proteins to promoters for lipid metabolic genes in liver.* J Biol Chem, 2008. **283**(23): p. 15628-37.

131. Tontonoz, P., et al., *ADD1: a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation.* Mol Cell Biol, 1993. **13**(8): p. 4753-9.

132. Shimomura, I., et al., *Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells.* J Clin Invest, 1997. **99**(5): p. 838-45.

133.   Horton, J.D., et al., *Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12027-32.

134.   Foretz, M., et al., *Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes.* Proc Natl Acad Sci U S A, 1999. **96**(22): p. 12737-42.

135.   Horton, J.D., et al., *Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2.* J Clin Invest, 1998. **101**(11): p. 2331-9.

136.   Shimano, H., *Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes.* Prog Lipid Res, 2001. **40**(6): p. 439-52.

137.   Brown, M.S. and J.L. Goldstein, *The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor.* Cell, 1997. **89**(3): p. 331-40.

138.   Brown, M.S. and J.L. Goldstein, *A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood.* Proc Natl Acad Sci U S A, 1999. **96**(20): p. 11041-8.

139.   Brown, M.S., et al., *Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans.* Cell, 2000. **100**(4): p. 391-8.

140.   Hegarty, B.D., et al., *Distinct roles of insulin and liver X receptor in the induction and cleavage of sterol regulatory element-binding protein-1c.* Proc Natl Acad Sci U S A, 2005. **102**(3): p. 791-6.

141.   Horton, J.D., et al., *Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice.* Proc Natl Acad Sci U S A, 1998. **95**(11): p. 5987-92.

142.   Kim, J.B., et al., *Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1.* J Clin Invest, 1998. **101**(1): p. 1-9.

143. Bizeau, M.E., et al., *Skeletal muscle sterol regulatory element binding protein-1c decreases with food deprivation and increases with feeding in rats.* J Nutr, 2003. **133**(6): p. 1787-92.

144. Commerford, S.R., et al., *In vivo regulation of SREBP-1c in skeletal muscle: effects of nutritional status, glucose, insulin, and leptin.* Am J Physiol Regul Integr Comp Physiol, 2004. **287**(1): p. R218-27.

145. Azzout-Marniche, D., et al., *Insulin effects on sterol regulatory-element-binding protein-1c (SREBP-1c) transcriptional activity in rat hepatocytes.* Biochem J, 2000. **350 Pt 2**: p. 389-93.

146. Peet, D.J., et al., *Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha.* Cell, 1998. **93**(5): p. 693-704.

147. Repa, J.J., et al., *Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta.* Genes Dev, 2000. **14**(22): p. 2819-30.

148. Schultz, J.R., et al., *Role of LXRs in control of lipogenesis.* Genes Dev, 2000. **14**(22): p. 2831-8.

149. Laffitte, B.A., et al., *Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue.* Proc Natl Acad Sci U S A, 2003. **100**(9): p. 5419-24.

150. Sato, R., et al., *Sterol-dependent transcriptional regulation of sterol regulatory element-binding protein-2.* J Biol Chem, 1996. **271**(43): p. 26461-4.

151. Amemiya-Kudo, M., et al., *Promoter analysis of the mouse sterol regulatory element-binding protein-1c gene.* J Biol Chem, 2000. **275**(40): p. 31078-85.

152. Yang, T., et al., *Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER.* Cell, 2002. **110**(4): p. 489-500.

153.   Yabe, D., M.S. Brown, and J.L. Goldstein, *Insig-2, a second endoplasmic reticulum protein that binds SCAP and blocks export of sterol regulatory element-binding proteins.* Proc Natl Acad Sci U S A, 2002. **99**(20): p. 12753-8.

154.   Hirano, Y., et al., *Direct demonstration of rapid degradation of nuclear sterol regulatory element-binding proteins by the ubiquitin-proteasome pathway.* J Biol Chem, 2001. **276**(39): p. 36431-7.

155.   Sundqvist, A. and J. Ericsson, *Transcription-dependent degradation controls the stability of the SREBP family of transcription factors.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 13833-8.

156.   Kim, K.H., et al., *Regulatory role of glycogen synthase kinase 3 for transcriptional activity of ADD1/SREBP1c.* J Biol Chem, 2004. **279**(50): p. 51999-2006.

157.   Sundqvist, A., et al., *Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCF(Fbw7).* Cell Metab, 2005. **1**(6): p. 379-91.

158.   Stossel, T.P., *The discovery of statins.* Cell, 2008. **134**(6): p. 903-5.

159.   Kiortsis, D.N., et al., *Statin-associated adverse effects beyond muscle and liver toxicity.* Atherosclerosis, 2007. **195**(1): p. 7-16.

160.   Jasinska, M., J. Owczarek, and D. Orszulak-Michalak, *Statins: a new insight into their mechanisms of action and consequent pleiotropic effects.* Pharmacol Rep, 2007. **59**(5): p. 483-99.

161.   Flint, O.P., et al., *Inhibition of cholesterol synthesis by squalene synthase inhibitors does not induce myotoxicity in vitro.* Toxicol Appl Pharmacol, 1997. **145**(1): p. 91-8.

162.   Hargreaves, I.P., et al., *The effect of HMG-CoA reductase inhibitors on coenzyme Q10: possible biochemical/clinical implications.* Drug Saf, 2005. **28**(8): p. 659-76.

163. Charlton-Menys, V. and P.N. Durrington, *Human cholesterol metabolism and therapeutic molecules.* Exp Physiol, 2008. **93**(1): p. 27-42.

164. Chugh, A., A. Ray, and J.B. Gupta, *Squalene epoxidase as hypocholesterolemic drug target revisited.* Prog Lipid Res, 2003. **42**(1): p. 37-50.

165. Bengoechea-Alonso, M.T. and J. Ericsson, *SREBP in signal transduction: cholesterol metabolism and beyond.* Curr Opin Cell Biol, 2007. **19**(2): p. 215-22.

166. Charlton-Menys, V. and P.N. Durrington, *Squalene synthase inhibitors : clinical pharmacology and cholesterol-lowering potential.* Drugs, 2007. **67**(1): p. 11-6.

167. Huff, M.W. and D.E. Telford, *Lord of the rings--the mechanism for oxidosqualene:lanosterol cyclase becomes crystal clear.* Trends Pharmacol Sci, 2005. **26**(7): p. 335-40.

168. Menys, V.C. and P.N. Durrington, *Squalene synthase inhibitors.* Br J Pharmacol, 2003. **139**(5): p. 881-2.

169. Wakil, S.J., J.K. Stoops, and V.C. Joshi, *Fatty acid synthesis and its regulation.* Annu Rev Biochem, 1983. **52**: p. 537-79.

170. Bavenholm, P.N., et al., *Fatty acid oxidation and the regulation of malonyl-CoA in human muscle.* Diabetes, 2000. **49**(7): p. 1078-83.

171. Saha, A.K., T.G. Kurowski, and N.B. Ruderman, *A malonyl-CoA fuel-sensing mechanism in muscle: effects of insulin, glucose, and denervation.* Am J Physiol, 1995. **269**(2 Pt 1): p. E283-9.

172. Ruderman, N.B., et al., *Malonyl CoA as a metabolic switch and a regulator of insulin sensitivity.* Adv Exp Med Biol, 1998. **441**: p. 263-70.

173. Ruderman, N.B., et al., *Malonyl-CoA, fuel sensing, and insulin resistance.* Am J Physiol, 1999. **276**(1 Pt 1): p. E1-E18.

174. Menendez, J.A. and R. Lupu, *Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis.* Nat Rev Cancer, 2007. **7**(10): p. 763-77.

175. Loftus, T.M., et al., *Reduced food intake and body weight in mice treated with fatty acid synthase inhibitors.* Science, 2000. **288**(5475): p. 2379-81.

176. Makimura, H., et al., *Cerulenin mimics effects of leptin on metabolic rate, food intake, and body weight independent of the melanocortin system, but unlike leptin, cerulenin fails to block neuroendocrine effects of fasting.* Diabetes, 2001. **50**(4): p. 733-9.

177. Dobrzyn, P., et al., *Loss of stearoyl-CoA desaturase 1 inhibits fatty acid oxidation and increases glucose utilization in the heart.* Am J Physiol Endocrinol Metab, 2008. **294**(2): p. E357-64.

178. Ntambi, J.M. and M. Miyazaki, *Recent insights into stearoyl-CoA desaturase-1.* Curr Opin Lipidol, 2003. **14**(3): p. 255-61.

179. Miyazaki, M., et al., *Stearoyl-CoA desaturase 1 gene expression is necessary for fructose-mediated induction of lipogenic gene expression by sterol regulatory element-binding protein-1c-dependent and -independent mechanisms.* J Biol Chem, 2004. **279**(24): p. 25164-71.

180. Miyazaki, M., Y.C. Kim, and J.M. Ntambi, *A lipogenic diet in mice with a disruption of the stearoyl-CoA desaturase 1 gene reveals a stringent requirement of endogenous monounsaturated fatty acids for triglyceride synthesis.* J Lipid Res, 2001. **42**(7): p. 1018-24.

181. Ntambi, J.M., et al., *Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity.* Proc Natl Acad Sci U S A, 2002. **99**(17): p. 11482-6.

182. Rahman, S.M., et al., *Stearoyl-CoA desaturase 1 deficiency elevates insulin-signaling components and down-regulates protein-tyrosine phosphatase 1B in muscle.* Proc Natl Acad Sci U S A, 2003. **100**(19): p. 11110-5.

183.   Gutierrez-Juarez, R., et al., *Critical role of stearoyl-CoA desaturase-1 (SCD1) in the onset of diet-induced hepatic insulin resistance.* J Clin Invest, 2006. **116**(6): p. 1686-95.

184.   Rahman, S.M., et al., *Stearoyl-CoA desaturase 1 deficiency increases insulin signaling and glycogen accumulation in brown adipose tissue.* Am J Physiol Endocrinol Metab, 2005. **288**(2): p. E381-7.

185.   Cohen, P., et al., *Role for stearoyl-CoA desaturase-1 in leptin-mediated weight loss.* Science, 2002. **297**(5579): p. 240-3.

186.   Attie, A.D., et al., *Relationship between stearoyl-CoA desaturase activity and plasma triglycerides in human and mouse hypertriglyceridemia.* J Lipid Res, 2002. **43**(11): p. 1899-907.

187.   Hulver, M.W., et al., *Elevated stearoyl-CoA desaturase-1 expression in skeletal muscle contributes to abnormal fatty acid partitioning in obese humans.* Cell Metab, 2005. **2**(4): p. 251-61.

188.   Ikuta, T. and K. Kawajiri, *Zinc finger transcription factor Slug is a novel target gene of aryl hydrocarbon receptor.* Exp Cell Res, 2006. **312**(18): p. 3585-94.

189.   Patel, R.D., et al., *The aryl hydrocarbon receptor directly regulates expression of the potent mitogen epiregulin.* Toxicol Sci, 2006. **89**(1): p. 75-82.

190.   Schiller, C.M., et al., *Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and fasting on body weight and lipid parameters in rats.* Toxicol Appl Pharmacol, 1985. **81**(2): p. 356-61.

191.   Lakshman, M.R., et al., *Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on de novo fatty acid and cholesterol synthesis in the rat.* Lipids, 1988. **23**(9): p. 904-6.

192.   Tang, J.J., et al., *Inhibition of SREBP by a small molecule, betulin, improves hyperlipidemia and insulin resistance and reduces atherosclerotic plaques.* Cell Metab, 2011. **13**(1): p. 44-56.

193. Moon, Y.A., et al., *The Scap/SREBP pathway is essential for developing diabetic fatty liver and carbohydrate-induced hypertriglyceridemia in animals.* Cell Metab, 2012. **15**(2): p. 240-6.

194. Chiaro, C.R., et al., *Leukotriene A4 metabolites are endogenous ligands for the Ah receptor.* Biochemistry, 2008. **47**(32): p. 8445-55.

195. Dabir, P., et al., *Aryl hydrocarbon receptor is activated by glucose and regulates the thrombospondin-1 gene promoter in endothelial cells.* Circ Res, 2008. **102**(12): p. 1558-65.

196. DiNatale, B.C., et al., *Kynurenic acid is a potent endogenous aryl hydrocarbon receptor ligand that synergistically induces interleukin-6 in the presence of inflammatory signaling.* Toxicol Sci, 2010. **115**(1): p. 89-97.

197. Schroeder, J.C., et al., *The uremic toxin 3-indoxyl sulfate is a potent endogenous agonist for the human aryl hydrocarbon receptor.* Biochemistry, 2010. **49**(2): p. 393-400.

198. Ito, S., et al., *Dietary phytochemicals regulate whole-body CYP1A1 expression through an arylhydrocarbon receptor nuclear translocator-dependent system in gut.* J Clin Invest, 2007. **117**(7): p. 1940-50.

199. Bligh, E.G. and W.J. Dyer, *A rapid method of total lipid extraction and purification.* Can J Biochem Physiol, 1959. **37**(8): p. 911-7.

200. Levine, S.L., et al., *A tetratricopeptide repeat half-site in the aryl hydrocarbon receptor is important for DNA binding and trans-activation potential.* Mol Pharmacol, 2000. **58**(6): p. 1517-24.

201. Ugocsai, P., et al., *HIF-1beta determines ABCA1 expression under hypoxia in human macrophages.* Int J Biochem Cell Biol, 2010. **42**(2): p. 241-52.

202. Shimba, S. and Y. Watabe, *Crosstalk between the AHR signaling pathway and circadian rhythm.* Biochem Pharmacol, 2009. **77**(4): p. 560-5.

203. Acimovic, J., et al., *CREM modulates the circadian expression of CYP51, HMGCR and cholesterogenesis in the liver.* Biochem Biophys Res Commun, 2008. **376**(1): p. 206-10.

204. Conway, D.E., et al., *Expression of CYP1A1 and CYP1B1 in human endothelial cells: regulation by fluid shear stress.* Cardiovasc Res, 2009. **81**(4): p. 669-77.

205. McMillan, B.J. and C.A. Bradfield, *The aryl hydrocarbon receptor is activated by modified low-density lipoprotein.* Proc Natl Acad Sci U S A, 2007. **104**(4): p. 1412-7.

206. Bennett, M.K., et al., *Co-stimulation of promoter for low density lipoprotein receptor gene by sterol regulatory element-binding protein and Sp1 is specifically disrupted by the yin yang 1 protein.* J Biol Chem, 1999. **274**(19): p. 13025-32.

207. Handschin, C. and U.A. Meyer, *Regulatory network of lipid-sensing nuclear receptors: roles for CAR, PXR, LXR, and FXR.* Arch Biochem Biophys, 2005. **433**(2): p. 387-96.

208. Vogel, C.F. and F. Matsumura, *A new cross-talk between the aryl hydrocarbon receptor and RelB, a member of the NF-kappaB family.* Biochem Pharmacol, 2009. **77**(4): p. 734-45.

209. Kharat, I. and F. Saatcioglu, *Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin are mediated by direct transcriptional interference with the liganded estrogen receptor. Cross-talk between aryl hydrocarbon- and estrogen-mediated signaling.* J Biol Chem, 1996. **271**(18): p. 10533-7.

210. Tanos, R., et al., *Ah receptor regulates the cholesterol biosynthetic pathway in a dioxin response element-independent manner.* Hepatology, 2012.

211. Arehart, E., et al., *Dioxin alters the human low-density and very low-density lipoprotein structure with evidence for specific quenching of Trp-48 in apolipoprotein C-II.* Biochemistry, 2004. **43**(26): p. 8503-9.

212. Hsu, H.F., et al., *Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on adipogenic differentiation and insulin-induced glucose uptake in 3T3-L1 cells.* J Hazard Mater, 2010. **182**(1-3): p. 649-55.

213.   Hems, D.A., E.A. Rath, and T.R. Verrinder, *Fatty acid synthesis in liver and adipose tissue of normal and genetically obese (ob/ob) mice during the 24-hour cycle.* Biochem J, 1975. **150**(2): p. 167-73.

214.   Jump, D.B., et al., *Fatty acid regulation of hepatic gene transcription.* J Nutr, 2005. **135**(11): p. 2503-6.

215.   Harwood, H.J., Jr., *Acetyl-CoA carboxylase inhibition for the treatment of metabolic syndrome.* Curr Opin Investig Drugs, 2004. **5**(3): p. 283-9.

216.   Zhao, W.H., et al., *Weight-reducing effect of Acer truncatum Bunge may be related to the inhibition of fatty acid synthase.* Nat Prod Res, 2011. **25**(4): p. 422-31.

217.   Oballa, R.M., et al., *Development of a liver-targeted stearoyl-CoA desaturase (SCD) inhibitor (MK-8245) to establish a therapeutic window for the treatment of diabetes and dyslipidemia.* J Med Chem, 2011. **54**(14): p. 5082-96.

218.   Flowers, M.T., et al., *Cholestasis and hypercholesterolemia in SCD1-deficient mice fed a low-fat, high-carbohydrate diet.* J Lipid Res, 2006. **47**(12): p. 2668-80.

219.   Espenshade, P.J. and A.L. Hughes, *Regulation of sterol synthesis in eukaryotes.* Annu Rev Genet, 2007. **41**: p. 401-27.

220.   Dooley, K.A., S. Millinder, and T.F. Osborne, *Sterol regulation of 3-hydroxy-3-methylglutaryl-coenzyme A synthase gene through a direct interaction between sterol regulatory element binding protein and the trimeric CCAAT-binding factor/nuclear factor Y.* J Biol Chem, 1998. **273**(3): p. 1349-56.

221.   Ohtake, F., et al., *Cross-talk of dioxin and estrogen receptor signals through the ubiquitin system.* J Steroid Biochem Mol Biol, 2011. **127**(1-2): p. 102-7.

222.   Ohtake, F., Y. Fujii-Kuriyama, and S. Kato, *[Transcription factor AhR is a ligand-dependcnt E3 ubiquitin ligase].* Tanpakushitsu Kakusan Koso, 2007. **52**(15): p. 1973-9.

223. Swanson, H.I. and G.H. Perdew, *Half-life of aryl hydrocarbon receptor in Hepa 1 cells: evidence for ligand-dependent alterations in cytosolic receptor levels.* Arch Biochem Biophys, 1993. **302**(1): p. 167-74.

224. Song, Z. and R.S. Pollenz, *Functional analysis of murine aryl hydrocarbon (AH) receptors defective in nuclear import: impact on AH receptor degradation and gene regulation.* Mol Pharmacol, 2003. **63**(3): p. 597-606.

225. Gassmann, K., et al., *Species-specific differential AhR expression protects human neural progenitor cells against developmental neurotoxicity of PAHs.* Environ Health Perspect, 2010. **118**(11): p. 1571-7.

226. Mihos, C.G., M.J. Salas, and O. Santana, *The pleiotropic effects of the hydroxy-methyl-glutaryl-CoA reductase inhibitors in cardiovascular disease: a comprehensive review.* Cardiol Rev, 2010. **18**(6): p. 298-304.

227. Yang, J., et al., *Decreased lipid synthesis in livers of mice with disrupted Site-1 protease gene.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13607-12.

228. Hawkins, J.L., et al., *Pharmacologic inhibition of site 1 protease activity inhibits sterol regulatory element-binding protein processing and reduces lipogenic enzyme gene expression and lipid synthesis in cultured cells and experimental animals.* J Pharmacol Exp Ther, 2008. **326**(3): p. 801-8.

229. Kuhajda, F.P., et al., *Fatty acid synthesis: a potential selective target for antineoplastic therapy.* Proc Natl Acad Sci U S A, 1994. **91**(14): p. 6379-83.

230. Hess, D., J.W. Chisholm, and R.A. Igal, *Inhibition of stearoylCoA desaturase activity blocks cell cycle progression and induces programmed cell death in lung cancer cells.* PLoS One, 2010. **5**(6): p. e11394.

231. Fritz, V., et al., *Abrogation of de novo lipogenesis by stearoyl-CoA desaturase 1 inhibition interferes with oncogenic signaling and blocks prostate cancer progression in mice.* Mol Cancer Ther, 2010. **9**(6): p. 1740-54.

232. Fan, Y., et al., *The aryl hydrocarbon receptor functions as a tumor suppressor of liver carcinogenesis.* Cancer Res, 2010. **70**(1): p. 212-20.

233. Hershko, A. and A. Ciechanover, *The ubiquitin system.* Annu Rev Biochem, 1998. **67**: p. 425-79.

234. Grigore, L., G.D. Norata, and A.L. Catapano, *Combination therapy in cholesterol reduction: focus on ezetimibe and statins.* Vasc Health Risk Manag, 2008. **4**(2): p. 267-78.

235. Alwayn, I.P., et al., *Omega-3 fatty acid supplementation prevents hepatic steatosis in a murine model of nonalcoholic fatty liver disease.* Pediatr Res, 2005. **57**(3): p. 445-52.

236. Drel, V.R., et al., *The leptin-deficient (ob/ob) mouse: a new animal model of peripheral neuropathy of type 2 diabetes and obesity.* Diabetes, 2006. **55**(12): p. 3335-43.

237. Buzello, M., et al., *The apolipoprotein e knockout mouse: a model documenting accelerated atherogenesis in uremia.* J Am Soc Nephrol, 2003. **14**(2): p. 311-6.

APPENDIX

Portions of this thesis, including texts and figures were from the following peer reviewed articles:

Tanos, R, Patel RD, Murray IA, Smith PB, Patterson AD, Perdew GH. (2012) Aryl hydrocarbon receptor regulates the cholesterol biosynthetic pathway in a dioxin response element-independent manner. *Hepatology*.

Tanos, R, Murray IA, Smith PB, Patterson AD, Perdew GH. (2012) Role of the Ah receptor in homeostatic control of fatty acid synthesis in the liver. *Toxicol sci*.

VITA

RACHEL TANOS

EDUCATION

2007 - 2012   Doctor of Philosophy in Biochemistry, Microbiology and Molecular
              Biology – The Pennsylvania State University, USA
2005 - 2007   Master of Science in Biochemistry – American University of Beirut,
              Lebanon
2001 - 2004   Bachelor of Science in Biology (*Magna Cum Laude*) - Notre Dame
              University, Lebanon

PUBLICATIONS

Tanos, R., Patel, R.D., Murray, I.A., Smith, P.B., Patterson, A.D., and Perdew, G.H. Ah receptor regulates the cholesterol biosynthetic pathway in a dioxin response element-independent manner. Hepatology. 2012.

Smith, K.J., Murray, I.A., **Tanos, R.**, Tellew J., Boitano, A.E., Bisson, W., Kolleri, S.K., Cooke, M.P., and Perdew, G.H. Identification of a high affinity ligand that exhibits complete Ah receptor antagonism. J. Pharmacol Exp Ther. 2011.

Murray, I.A., Flaveny, C.A., Chiaro, C.R., Sharma, A.K., **Tanos, R.S.**, Schroeder, J.S., Amin S.G., Bisson, W.H., Kolluri, S.K., and Perdew, G.H. Suppression of cytokine-mediated complement factor gene expression through selective activation of the Ah receptor with 3', 4'-dimethoxy-α-naphthoflavone. Mol Pharmacol. 2011.