THE ROLE OF HEPATIC NUCLEAR RECEPTORS IN SYSTEMIC LIPID HOMEOSTASIS

by

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ABSTRACT

The nuclear receptor superfamily consists of ligand-activated transcription factors that regulate many genes important in physiological functions such as cellular differentiation as well as glucose and lipid metabolism. Nuclear receptors such as the farnesoid X receptor (FXR) and the liver X receptor function to maintain lipid homeostasis through regulation of genes encoding enzymes, receptors and transporters essential to lipid metabolism. A balance in the intracellular and systemic levels of lipids is crucial for the prevention of diseases such as cholestasis and atherosclerosis. The objectives of this thesis were to examine the role of FXR, a bile acid-activated nuclear receptor, in the regulation of lipid homeostasis and the development of atherosclerosis and to identify and characterize potential novel FXR targets. Through examination, via microarray, northern blot and quantitative PCR, of altered gene expression in various tissues of FXR-deficient mice (FXR-/-) versus wildtype mice, we discovered potential novel targets of this nuclear receptor. Regulatory mechanisms mediating expression of one of the genes altered in FXR-/- mice, apolipoprotein A-IV (apoA-IV), a protein involved in lipid transport and metabolism, were examined using a variety of mouse models and *in vitro* promoter assays. In addition, through the crossing of FXR-/- mice with a mouse model of atherosclerosis, the apoE-deficient mouse (apoE-/-), we were able to study the effect of deletion of this nuclear receptor on the development of atherosclerotic disease. Our results demonstrate that loss of FXR function leads to a disruption in lipid homeostasis through altered gene expression leading to decreased survival and a worsening of atherosclerosis in the apoE-/- background. In addition, our results describe a regulatory mechanism for apoA-IV involving the nuclear receptor hepatic nuclear factor-4α and a coactivator (peroxisome proliferator-activated receptor gamma coactivator-1α) that, in conjunction with glucocorticoids, induce apoA-IV in response to nutritional and metabolic stresses.

LIST OF ABBREVIATIONS AND SYMBOLS USED

α	alpha
β	beta
γ	gamma
δ	delta
α-HDL	alpha high-density lipoprotein
β-oxidation	beta-oxidation
ABC	Adenosine triphosphate-binding cassette transporter
Acetyl coA	acetyl coenzyme A
ADRP	adipocyte differentiation-related protein,
ADRP-/-	adipocyte differentiation-related protein-deficient
Adx	adrenalectomized
ATP	adenosine triphosphate
AF	activation function
AGP	advanced glycation product
ANOVA	analysis of variance
Apo	apolipoprotein
ApoE-/-	apolipoprotein E-deficient
AST	Aspartate amino transferase
BSEP	bile salt export pump
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CD36	cluster of differentiation 36

cDNA	complementary DNA
C/EBP	CAAT/enhancer binding protein
CETP	cholesteryl ester transfer protein
CO_2	carbon dioxide
Cos-7	African Green Monkey kidney fibroblast
COV	coefficient of variance
CREB	cyclic adenosine monophosphate response element binding protein
Cy	cyanine
CYP	cytochrome P450
DMSO	dimethylsulfoxide
DN	dominant negative
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbance assay
ETC	electron transport chain
FADH2	flavin adenine dinucleotide
FAS	fatty acid synthase
FBS	fetal bovine serum
FH	familial hypercholesterolemia
FOI	frequency of incorporation
FPLC	fast protein liquid chromatography
FXR	farnesoid X receptor
$FXR^{-/-}$	farnesoid X receptor-deficient
FXR-/-	farnesoid X receptor and apolipoprotein E-deficient

GR	glucocorticoid receptor
H+E	hematoxylin and eosin
HDL	high-density lipoprotein
HepG2	human hepatocellular carcinoma
HL	hepatic lipase
HF/HC	high fat, high cholesterol
HMG CoA	hydroxy-3-methylglutaryl coenzyme A
HNF-4α	hepatic nuclear factor-4alpha
HRE	hormone response element
IDL	intermediate density lipoproteins
Kb	kilobase
LCAT	lecithin: cholesterol acyl transferase
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LDLR-/-	low-density lipoprotein receptor-deficient
LPL	lipoprotein lipase
LRP	low-density lipoprotein receptor-related protein
LXR	liver X receptor
LXR-/-	liver X receptor-deficient
MAC1	murine macrophage antigen 1
MOPS	3-[N-Morpholino]propanesulfonic acid
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide

NE	norepinephrine
NRF	nuclear respiratory factor
ox-LDL	oxidized low-density lipoprotein
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1α	Peroxisome proliferators-activated receptor gamma coactivator-1alpha
PKA	protein kinase A
PLTP	phospholipid transfer protein
PPAR	peroxisome proliferator-activated receptor
PPARα-/-	peroxisome proliferators-activated receptor alpha-deficient
preß-HDL	pre-beta high density lipoprotein
QPCR	Quantitative real-time polymerase chain reaction
RARESP	retinoic acid responder protein
RNA	ribonucleic acid
RP-II	ribonucleic acid polymerase II
RT-PCR	reverse transcription-polymerase chain reaction
RXR	retinoid X receptor
SD	standard deviation
shRNA	small hairpin ribonucleic acid
SR-A	scavenger receptor class A
SREBP-1c	sterol receptor element binding protein-1c
SR-B1	scavenger receptor-class B1
STZ	streptozocin
T2DM	Type II *diabetes mellitus*

TNF-α	tumour necrosis factor-alpha
UCP	uncoupling protein
VLDL	very low-density lipoprotein
VLDLR	very low-density lipoprotein receptor
WAT	white adipose tissue
WT	wildtype

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CHAPTER 1

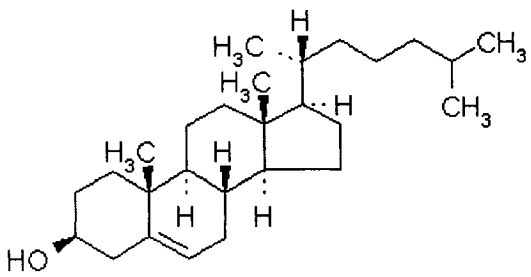
INTRODUCTION

1.1 INTRODUCTION TO LIPIDS

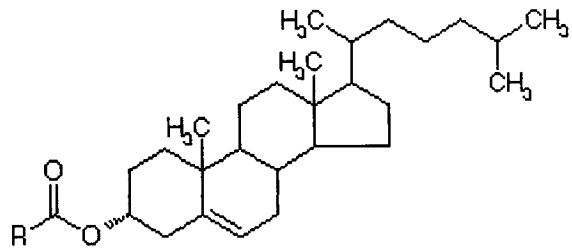
Lipids are a group of molecules that are hydrophobic in nature and are thus insoluble in water. This chemical group includes fats and oils (made of triglycerides), phospholipids, steroids (such as cholesterol) as well as other compounds. Lipids are essential for normal human physiology, but can have undesirable effects at high concentrations. Therefore, levels must be maintained within a narrow range of systemic and intracellular concentrations. Due to the insoluble properties of lipids, transport and storage in various tissues requires specially evolved mechanisms. Accumulation of these insoluble molecules can lead to abnormal cellular function, cell damage and ultimately pathologic gallstone formation and diseases such as atherosclerosis. Two types of lipids of importance in the body that will be discussed at length are cholesterol and triglycerides.

Cholesterol is a sterol molecule consisting of 4 fused cyclic rings with a single alcohol group attached at the carbon-3 position. Cholesterol is crucial to maintaining cell membrane stability and fluidity as well as serving as a necessary precursor for the synthesis of adrenal and sex hormones, bile acids and neurosteroids. In situations where insufficient quantities of cholesterol are obtained from dietary absorption, cholesterol can be synthesized by most tissues through a series of enzymatic steps using acetyl CoA as the precursor [1, 2]. Cholesterol is present in the body as two major forms; free (unesterified) cholesterol (Fig. 1*a*) and cholesteryl esters (Fig. 1*b*) where cholesterol has been esterified to long chain fatty acids. Esterification of cholesterol creates a much less polar molecule and it is in this form that the molecule is stored and transported. In order for dietary cholesterol to be absorbed (in its free state) through the intestine it must first be solubilized through the actions of bile acids. Bile acids are amphipathic molecules

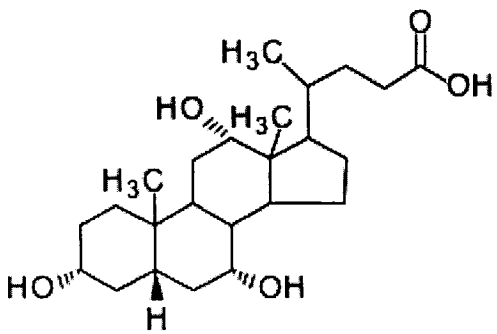
a) Free Cholesterol



b) Esterified Cholesterol



c) Bile Acid



d) Triglyceride

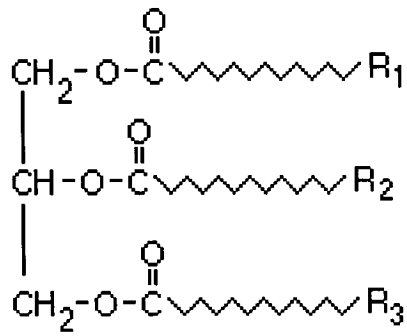


Figure 1.1 Structures of lipids and bile acids.
Cholesterol A) is a sterol molecule and is thus composed of a steroid and an alcohol. The alcohol moiety of cholesterol can become esterified B) for storage within the core of lipoproteins and in cells. Bile acids, such as cholic acid C) are amphipathic, steroid derivatives of cholesterol and contain one or more alcohol groups in addition to a carboxylic acid. Triglycerides D) are the storage form of fatty acids and are composed of a glycerol backbone with three attached fatty acids.

that are synthesized in the liver from cholesterol (see structure in Fig. 1*c*) and subsequently stored in the gall bladder for secretion into the small intestine during a meal. As cholesterol is the precursor for bile acid biosynthesis, this metabolic conversion is a quantitatively important route to eliminate excess cholesterol from the body. Bile acids act as detergents to emulsify lipids into circular masses called micelles, enabling the absorption of these compounds into the enterocytes for distribution in the body. Once emulsified into micelles, cholesterol molecules in the small intestine can now interact with the apical membranes of enterocytes. The exact mechanism of cholesterol absorption from the intestine has long been in contention. Recent research has indicated that rather than passively diffusing across the membrane, a specific transporter called Niemann-pick C1 like 1 is crucial for actively transporting cholesterol from the lumen of the intestine into enterocytes [3]. Inside the enterocytes, dietary cholesterol is packaged into lipoprotein particles called chylomicrons (discussed in the next section) for transport in the blood and delivery to tissues of the body. After a meal (referred to as the postprandial state) endogenous cholesterol stored in the liver is packaged and secreted into VLDL (also discussed in the following section).

Triglycerides are neutral lipids consisting of 3 long chain fatty acids covalently linked to a 3-carbon glycerol backbone (Fig. 1*d*). Triglycerides are the storage form of fatty acids and are catabolised to release their fatty acid components by enzymes called lipases. Fatty acids can also be synthesized from acetyl CoA in a series of metabolic reactions and are utilized in the synthesis of many molecules such as phospholipids, cholesteryl esters and steroid hormones. Fatty acids are also a major source of energy for the body and are stored as triglycerides primarily in the adipose depots of the body for

use in times of nutrient deprivation or high energy demand. In the liver, skeletal and cardiac muscle tissues, fatty acids can enter a catabolic process in mitochondria and peroxisomes called beta (ß)-oxidation that produces adenosine triphosphate as an end product to be used as fuel for many different metabolic processes. Fatty acids can also be converted to glucose in many tissues of the body (excepting adipose) thus providing fuel for glucose-dependent tissues such as the brain that lacks glycerol kinase, a key enzyme for the storage of glucose as glycogen [4].

The intrinsic insolubility of lipids means that these compounds must be specifically packaged and chemically modified for efficient transport through the blood as well as across cellular barriers. The body's ability to transport lipids into and between various tissues is dependent on the formation of particles called lipoproteins.

1.2 LIPOPROTEIN STRUCTURE AND FUNCTION

Lipoproteins are the major form in which lipids are transported between various tissues of the body. As the name suggests, lipoproteins are spherical structures consisting of lipid and protein (Fig. 1.2). The core of lipoproteins consists of varying amounts of cholesteryl esters and triglycerides, encased by a monolayer of phospholipids. The phospholipid monolayer is interspersed with molecules of unesterified cholesterol as well as proteins called apolipoproteins. It is the specific types of apolipoprotein present on the surface of the lipoprotein particle that determine its metabolic fate through specific interactions with receptors and/or enzymes present in the blood or on cells lining blood vessels or tissues. Although a few apolipoproteins are characteristic of certain lipoproteins, a great deal of exchange of these proteins occurs in the plasma compartment and thus lipoproteins can

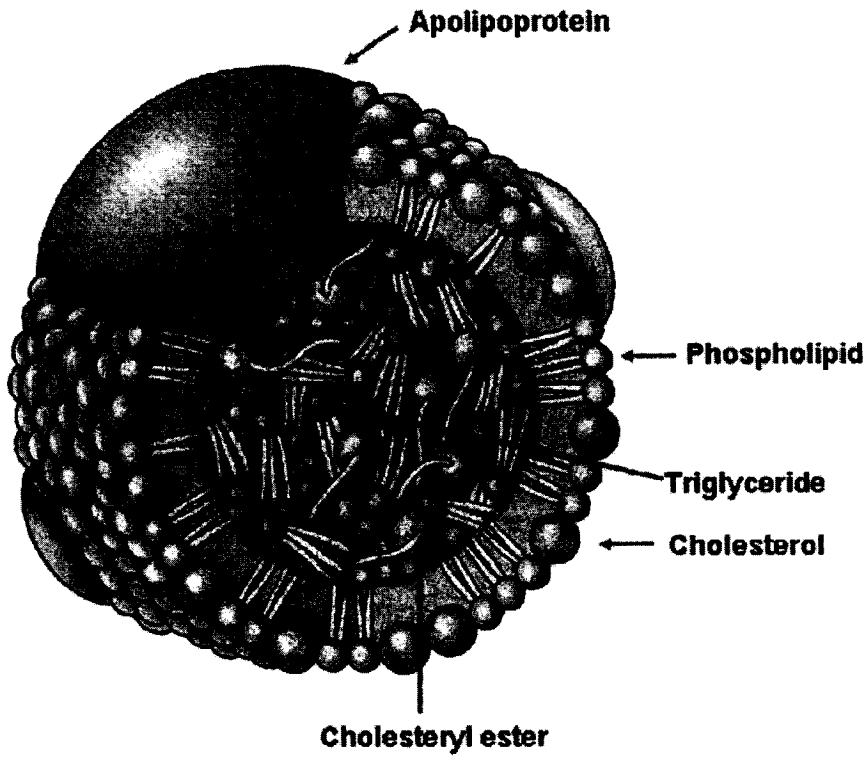


Figure 1.2 General structure of a lipoprotein
Lipoproteins are made up of a core of cholesterol esters and triglycerides surrounded by a phospholipid monolayer. Interspersed within the phospholipid monolayer are molecules of cholesterol and specific proteins called apolipoproteins that are important in determining the metabolic fate of these particles. Figure taken from [5].

"acquire" different apolipoproteins at various stages of their metabolism.

There are 4 major classes of lipoproteins; chylomicrons; very low-density lipoproteins (VLDL); low-density lipoproteins (LDL) and high-density lipoproteins (HDL). These classes of lipoproteins differ with respect to their density, lipid composition, size, apolipoprotein content and physiological roles (Table 1.1). The various lipoprotein classes can be separated based upon buoyant density through the process of ultracentrifugation or based upon size by fast protein liquid chromatography (FPLC) [6, 7]. The largest lipoproteins are the chylomicron and VLDL classes, which both have triglyceride-rich cores and function to deliver fatty acids to peripheral tissues. However, whereas chylomicrons are synthesized and secreted from the small intestine and carry exogenously derived triglycerides from the diet, VLDL particles are made in the liver and carry endogenously synthesized triglycerides. The third class of lipoprotein, called LDL, contains a cholesterol-rich core and is derived through metabolic conversion of VLDL particles. LDL particles contain large amounts of cholesterol rather than triglyceride within their core and function to deliver this cholesterol to various tissues of the body. The final major class of lipoproteins consists of the HDL. The HDL particles are synthesized by the liver as well as the intestine, are protein-rich compared to other lipoproteins and function to remove excess cholesterol from extrahepatic tissues for delivery back to the liver. A more detailed description of each lipoprotein class is given in the following sections.

1.3 CHYLOMICRONS AND DIETARY LIPID ABSORPTION

Intestinal synthesis of chylomicrons is necessary for the absorption of dietary lipids as

Table 1.1 Source, lipid composition and apolipoprotein constituents of lipoproteins. Table taken from [8].

Lipid/ Lipoprotein	Source	Composition						Apolipoprotein
			Total	Percentage of Total Lipid				
		Protein %	Lipid %	TG	Chol	Phosp	Free Chol	
Chylomicron	Intestine	1-2	98-99	88	8	3	1	Major: A-IV, B-48, B-100, H Minor: A-I, A-II, C-I, C-II, C-III, E
VLDL	Major: liver Minor: intestine	7-10	90-93	56	20	15	8	Major: B-100, C-III, E, G Minor: A-I, A-II, B-48, C-II, D
IDL	Major: VLDL Minor: chylomicron	11	89	29	26	34	9	Major: B-100 Minor: B-48
LDL	Major: VLDL Minor: chylomicron	21	79	13	28	48	10	Major: B-100 Minor: C-I, C-II, (a)
HDL_2	Major: HDL_3	33	67	16	43	31	10	Major: A-1, A-II, D, E, F Minor: A-IV, C-I, C-II, C-III
HDL_3	Major: liver and intestine Minor: VLDL and chylomicronremnants	57	43	13	46	29	6	Major: A-1, A-II, D, E, F Minor: A-IV, C-I, C-II, C-III
Chol	Liver and diet		100			70-75	25-30	
TG	Diet and liver		100	100				

VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Chol, cholesterol; TG, triglyceride; Phosp, phospholipid.

well as lipid soluble vitamins (D, E and K) [9]. Fat and cholesterol ingested in the diet enters the intestinal lumen and is emulsified by the actions of bile acids. Triglycerides are partially hydrolysed to monoglycerides and free fatty acids by lipases secreted in the stomach and intestine [10]. The monoglycerides, fatty acids and cholesterol, together with phospholipids secreted in the bile, diffuse across intestinal villi into the enterocytes making up the intestinal wall [11]. The cholesterol, fatty acids and monoglycerides are re-esterified within the enterocytes into cholesteryl esters and triglycerides, respectively, and fuse with the apolipoprotein (apo) B-48 and phospholipids to form chylomicrons which are secreted into lymphatic vessels, see Fig. 1.3 [12, 13]. Chylomicrons travel in the lymph to capillary beds where triglycerides present in these particles are hydrolysed to free fatty acids and glycerol by an enzyme called lipoprotein lipase (LPL), present on the surface of endothelial cells lining blood vessels [14, 15]. The liberated fatty acids can either diffuse through the cell membrane or undergo specific receptor-mediated uptake by receptors such as cluster of differentiation 36 (CD36). The hydrolysis of triglycerides by LPL requires apoC-II as a cofactor, therefore apoC-II is present on all lipoproteins which contain triglycerides [16]. Once devoid of triglycerides, chylomicrons are cholesteryl ester-enriched and are referred to as chylomicron remnants. These remnants are taken up by the liver via a receptor-mediated mechanism through recognition of apoE and apoB48 present on their surface. Receptors mediating the hepatic uptake of chylomicron remnants are the LDL receptor (LDLR) and the LDL receptor-related protein (LRP) [17, 18]. In this fashion, cholesterol obtained in the diet is transported to the liver for metabolism or storage. The dietary fatty acids delivered by chylomicrons can be stored primarily in

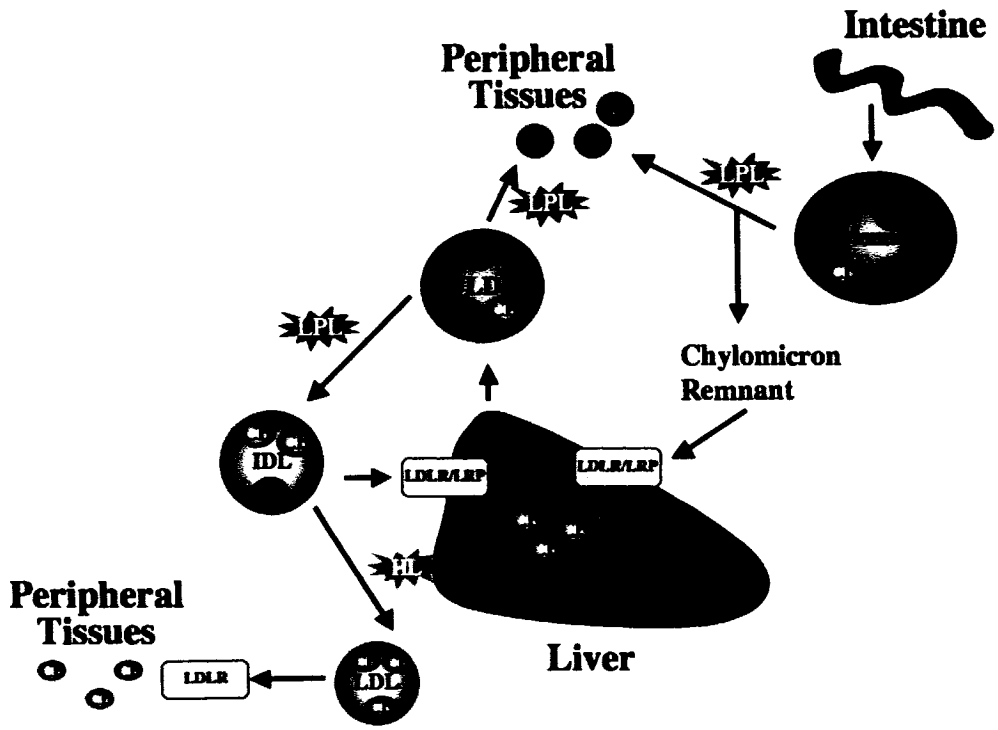


Figure 1.3 Exogenous and endogenous delivery of lipids
Dietary lipids that enter the intestinal lumen are packaged into chylomicrons. These triglyceride-rich lipoproteins encounter LPL present on the endothelial lining of blood vessels. LPL hydrolyzes the chylomicron triglycerides to fatty acids and glycerol that are taken up by peripheral tissues. Chylomicron remnants are then taken up into the liver through receptor-mediated clearance by LRP and LDLR. The liver also synthesizes triglyceride-rich VLDL particles that, like chylomicrons, encounter LPL and therefore function to deliver fatty acids to peripheral tissues. Through diminishment of triglyceride content, VLDL particles become IDL particles. IDL particles can either be taken up into the liver via LDLR or LRP or can be converted to LDL. Conversion of IDL to LDL requires further hydrolysis of the triglyceride content by HL in addition to alterations in apolipoprotein content. LDL functions to deliver cholesterol to peripheral tissues through whole-particle uptake, mediated by LDLRs expressed on cell surfaces.

adipose, oxidized for energy in muscle or liver or utilized to synthesize VLDL particles in the liver.

1.4 VLDL, IDL AND LDL

Chylomicrons deliver dietary lipids from the intestine into the circulation and eventually, to the liver. VLDL, on the other hand, is synthesized and secreted by the liver to transport endogenous triglycerides to other tissues. VLDL particles are packaged and secreted by the liver in a process requiring apoB-100 that is present on their surface (Fig. 1.3). VLDL particles contain mostly triglycerides as well as some cholesterol and like chylomicrons interact with LPL lining the endothelium of capillaries. With the aid of the cofactor apoC-II on the surface of VLDL, LPL hydrolyses the triglyceride core, releasing free fatty acids and glycerol that are taken up by the surrounding tissue. After depletion of most of the triglycerides, the remaining particles are known as intermediate density lipoproteins (IDL) or VLDL remnants. IDL particles can be removed by the liver in a fashion similar to that for chylomicron remnants; through interactions of hepatic receptors with apoE and apoB-100. Alternatively, the remaining triglycerides present in IDL particles can be further hydrolysed by hepatic lipase (HL) an enzyme closely related to LPL [16]. Through enrichment of its cholesterol-rich core and exchange of apolipoproteins on its surface, IDL is converted to LDL [5, 19].

LDL particles are the primary means by which cholesterol is delivered to extrahepatic tissues [20]. LDL particles are recognized by cells through interactions between the apoB-100 present on the lipoprotein and the LDLR present on the target tissue [21]. The actual transfer of cholesterol from LDL occurs by a receptor-mediated event that culminates in endocytosis of the entire LDL particle into the recipient cell [22].

The importance of the LDLR is highlighted by the example of familial hypercholesterolemia (FH) a genetic disorder involving an inactivating mutation in the LDLR. Because the liver can no longer remove LDL particles from the blood through LDLR-mediated uptake, individuals with FH accumulate large amounts of LDL cholesterol in the blood. As increased LDL cholesterol is a causative risk factor in the pathology of atherosclerosis, discussed later in this chapter, individuals with FH exhibit a dramatically elevated risk for cardiovascular disease [23]. Under normal circumstances, concentrations of intracellular cholesterol are tightly regulated to retain homeostatic levels. One of the ways in which intracellular cholesterol is controlled is through the regulation of LDLR protein and messenger ribonucleic acid (mRNA) expression [24]. Through this feedback mechanism, cells can control intracellular cholesterol stores by decreasing LDLR expression and thereby, uptake of LDL cholesterol [25].

1.5 HDL AND REVERSE CHOLESTEROL TRANSPORT

Unlike LDL, which functions to deliver cholesterol from the liver to peripheral tissues, HDL functions to remove excess cholesterol from peripheral tissues and transports it back to the liver. This removal of excess tissue cholesterol for disposal in the liver is called reverse cholesterol transport or RCT, see Fig. 1.4. The first step of RCT involves synthesis and secretion of apoA-I by the liver and intestine. ApoA-I can be secreted from these organs either alone or associated with small amounts of phospholipids and cholesterol in particles called nascent HDL or preß-HDL [26]. Most of the apoA-I proteins initially obtain their phospholipids and cholesterol via interactions with a transporter called the adenosine triphosphate-binding cassette transporter (ABC) A1

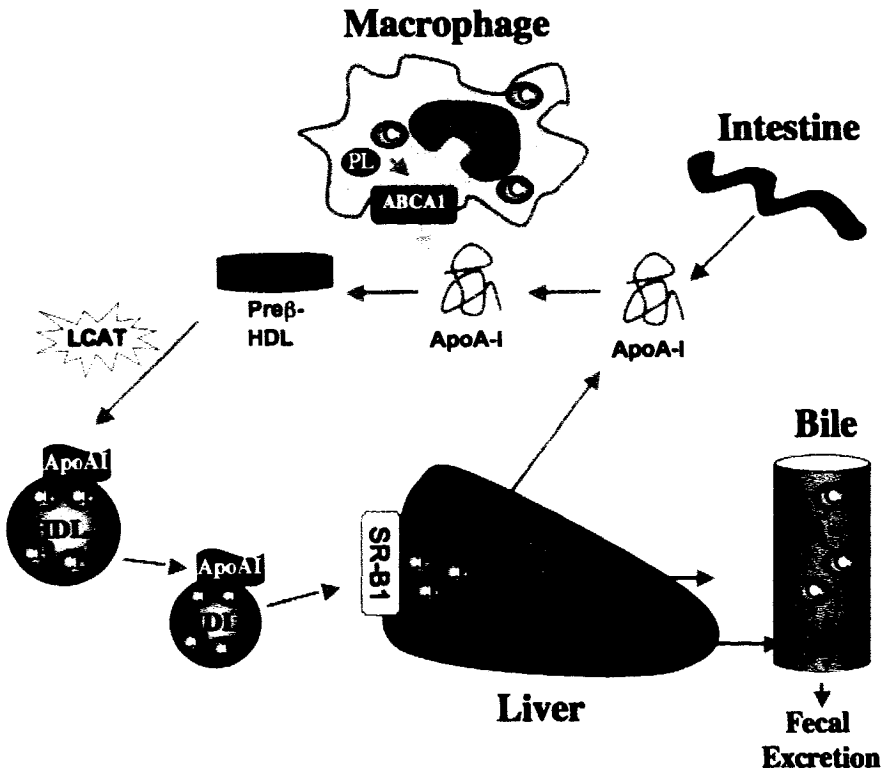


Figure 1.4 Reverse cholesterol transport
HDL functions to remove and transport excess cholesterol from peripheral cells to the liver. Initial HDL particles, usually consisting of just apoA-I are synthesized from liver and intestine. ApoA-I obtains phospholipids (PL) and some cholesterol (C) from interactions with the efflux transporter ABCA1 present on the surface of peripheral cells such as macrophagesto form particles called preβ-HDL. The cholesterol present on the surface of these preβ-HDL particles is esterified through the actions of the enzyme LCAT. Esterified cholesterol (CE) is stored in the core of HDL particles (called HDL_2). Further esterification of cholesterol in HDL_2 particles creates more compact particles called HDL_3. HDL particles interact with a specific HDL receptor called SR-B1 present on the surface of liver cells. SR-B1 functions to transport cholesterol from HDL to hepatocytes where it can be directly secreted into the bile or first converted to bile acids for removal from the body via fecal excretion.

which effluxes lipid (mostly cholesterol) from cells such as macrophages to lipid-poor, nascent HDL [27, 28]. This ABCA1-dependent efflux of lipids from cells occurs through recognition and binding of apoA-I in addition to many other apolipoproteins (ApoE, ApoA-V etc.) present on the surface of HDL [29, 30]. Tangier disease is caused by a defect in the ABCA1 transporter leading to a complete lack of plasma HDL cholesterol in these patients as well as the accumulation of cholesterol in cells such as macrophages [31, 32]. The cholesterol present on the surface of these initial HDL particles is esterified by an enzyme in the plasma known as lecithin: cholesterol acyl transferase (LCAT) which is activated by interactions with apoA-I [33-35]. This esterification allows the cholesterol on the surface of HDL to move into the core, forming a more round and compact particle called alpha HDL (α-HDL), or HDL_2, that can continue to accept cholesterol from peripheral tissues. Further esterification and exchange of lipids from HDL_2 forms smaller particles called HDL_3. After remodeling by LCAT, HDL particles can interact with a receptor called the scavenger receptor-class B1 (SR-B1), present on liver cells, which removes the cholesteryl ester molecules from HDL [36]. Unlike the endocytic process mediated by the LDLR that results in the engulfment of whole LDL particles, the interaction of HDL with SR-B1 involves selective transfer of the cholesteryl esters from the HDL particles. In this manner the remaining lipid-poor HDL particle continues to remove further molecules of excess cholesterol from peripheral tissues [37, 38].

It should be noted that both lipids and apolipoproteins are dynamic and can move between classes of lipoprotein particles. For example, HDL cholesteryl esters can be transferred to triglyceride-rich lipoproteins and LDL and *vice versa* by an enzyme

known as cholesteryl ester transfer protein (CETP) [39-41]. In addition, phospholipids can transfer from chylomicrons and VLDL remnant particles to HDL by an enzyme called phospholipid transfer protein (PLTP) [42, 43]. The specific role of this continuous transfer of lipids between lipoprotein particle classes and in diseases such as atherosclerosis is controversial and requires further study [44]. An important aspect of lipoprotein metabolism crucial to human health is the ratio between blood levels of HDL and LDL. The importance of this ratio in the development of atherosclerotic disease will be discussed later in the introduction.

1.6 THE ROLE OF THE LIVER IN LIPID HOMEOSTASIS

The liver is a large, multi-lobed organ with an attached gall bladder and rich supply of blood supplied via the hepatic artery and portal vein. Hepatocytes make up approximately 60% of the cell types in the liver with the rest being comprised of sinusoid and bile duct epithelial cells, hepatic stellate cells and macrophage-like Kupffer cells [45]. Functionally, the liver is comprised of thousands of substructures called lobules or acini. Blood enters these structures via branches of the hepatic artery and portal vein and filters into sinusoids (Fig. 1.5) that bathe surrounding hepatocytes which act to filter out molecules such as toxicants and foreign bodies [46]. Thus, the liver is essential for detoxification of molecules through biotransformation via cytochromes P450 (CYP) and immunological defense from foreign invaders. Blood collects from the sinusoids into the hepatic venule in the center of the lobule and eventually collects in the vena cava leading back to the heart and lungs. The liver provides many essential functions for the maintenance of systemic lipid homeostasis and is responsible for much of the synthesis of

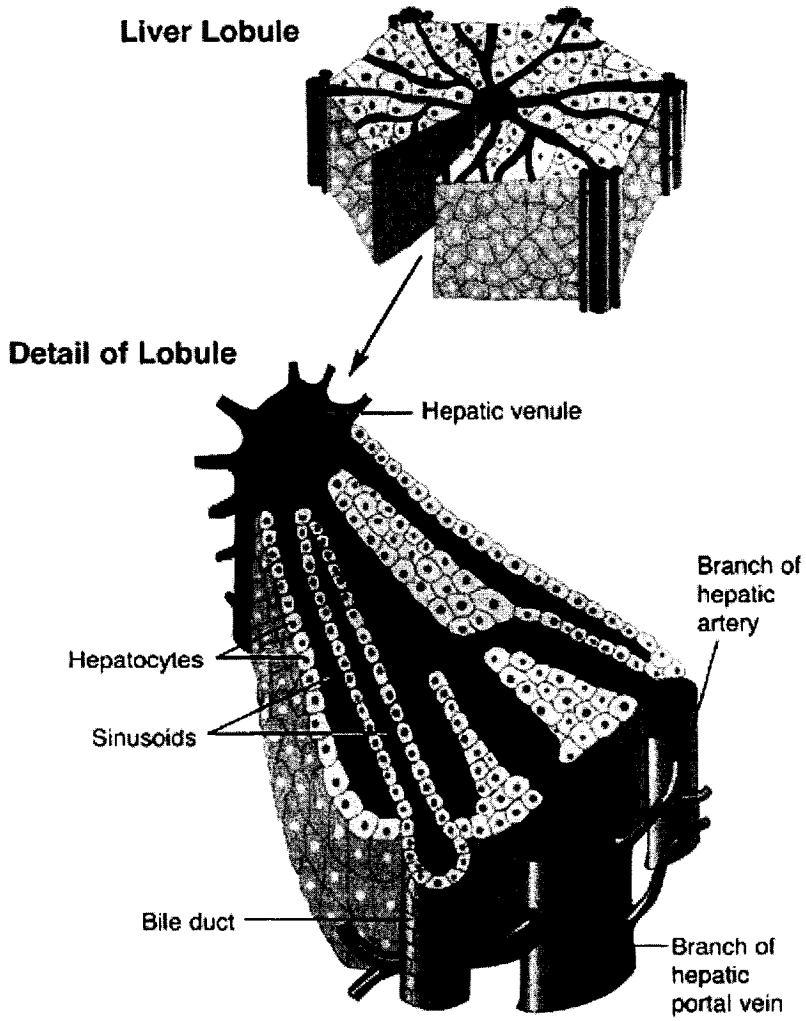


Figure 1.5 Detail of hepatic lobular structure
The liver is composed of thousands of functional substructures called lobules. Blood flows into the sinusoids of lobules via branches of the hepatic artery and portal vein. Hepatocytes lining the sinusoids function to filter toxicants and foreign bodies from the blood. Filtered blood then drains from the sinusoids into a central hepatic venule where it eventually collects in the vena cava to be oxygenated in the lungs. Figure taken from[47].

these molecules as well as their associated lipoproteins and apolipoproteins [48, 49]. The liver contributes approximately 80% of the body's *de novo* cholesterol biosynthesis and almost all production of bile acids [50, 51]. Bile acids are necessary for the absorption of dietary cholesterol and synthesis of these compounds from cholesterol is a major pathway for the elimination of excess levels of this molecule. Other contributions of the liver to lipid pools is through synthesis of VLDL and HDL particles as well as through uptake of HDL, LDL and remnants of chylomicrons and VLDL. In addition, the liver is the main site for synthesis, storage, transportation and degradation of many apolipoproteins important in lipoprotein synthesis and function [52].

Triglyceride-rich VLDL particles are synthesized, secreted and catabolised in the liver [53]. The liver can either synthesize fatty acids or utilize fatty acids acquired from the diet and incorporate them into VLDL particles for secretion and transport to peripheral tissues [54-56]. Systemic clearance of VLDL remnants, or IDL as well as dietary cholesterol-containing chylomicrons is also carried out primarily by the liver [57, 58]. The endothelial structure lining the sinusoids of the liver functions to filter nutrient-rich blood from the digestive tract, gathering chylomicrons as well as VLDL to small areas where they come into contact with lipases (LPL and HL) as well as specific receptors (such as LRP) that take up the remnants into the liver for degradation [59-61]. Since cholesterol-rich LDL is derived from VLDL through metabolic conversion, the liver, through secretion of VLDL, is responsible for much of the circulating levels of both triglycerides as well as cholesterol.

The removal of excess systemic cholesterol by HDL is achieved in part through the synthesis of these particles as well as the removal of their cholesterol by selective

uptake. Circulating levels of HDL are largely dependent on liver function as it is the major site for their synthesis. The liver is also a major site for expression of ABCA1 (necessary for initiation of cholesteryl ester uptake in to these particles) and the HDL receptor (SR-B1) [62]. An indication of the hepatic role in the generation of a functional HDL particle is provided by the phenotype of liver-specific ABCA1-deficient mice whose total HDL cholesterol is decreased by 80% [63]. Since the liver is a major site for the synthesis, remodeling and uptake of HDL, this organ is crucial in determining the efficiency of RCT occurring in the body and the ratio of HDL: LDL particles in the blood.

Excess cholesterol that has accumulated in the liver through uptake of HDL cholesterol must be excreted either directly into the bile or after conversion to bile acids. Cholesterol obtained by the liver through uptake of HDL is favored over other cholesterol for secretion into the bile [65]. Approximately 30-60% of cholesterol secreted into the bile is eventually reabsorbed and stored once again in the gall bladder [64, 65]. Approximately 1/3 of the daily accumulation of cholesterol (100-300 mg/day) in the liver is converted to bile acids with 750-1250 mg/day left unconverted [66, 67]. Bile acids enter the bile in a conjugated form (to taurine or glycine) and are readily reabsorbed (approximately 98%) through the intestinal villi through receptor-mediated transport. A small fraction of bile acids are hydroxylated by gut flora into secondary bile acids which are not efficiently reabsorbed [68]. Although most bile acids secreted into the intestinal tract during a meal are reabsorbed, specific regulatory mechanisms exist that function to maintain homeostatic levels of cholesterol and bile acids. Therefore, in situations of high cholesterol, the conversion of this molecule to bile acids and efflux of bile acids and

cholesterol from the liver can be specifically increased while simultaneously, reabsorption through the intestine can be decreased. Therefore, hepatic secretion of cholesterol into the bile as well as conversion of cholesterol to bile acids represents an efficient mechanism for removal of excess systemic cholesterol. Disruptions in hepatic functions such as altered bile acid transporter expression and nuclear receptor signalling can lead to high cholesterol content in the bile or decreased bile flow causing diseases such as gallstones and cholestasis, respectively [69-71].

The liver is an essential organ for the maintenance of lipid homeostasis. The synthesis of new particles of HDL and VLDL as well as the uptake and secretion of excess cholesterol by the liver is essential for lipid homeostasis in the body. Therefore, the liver is also important in the determination of the blood HDL:LDL ratio. As will be discussed in the following section, the HDL:LDL ratio can be an important determinant in the risk for development of atherosclerotic disease.

1.7 ATHEROSCLEROSIS

The word "atherosclerosis" is derived from the Greek words "athero" and "sclerosis", literally meaning paste and hardness, respectively. This description is fitting as atherosclerosis describes a condition in which fat-filled plaques form in the walls of arteries that can eventually develop into more advanced calcified or hardened lesions. At the most advanced stages of the disease, these plaques or lesions can become larger and significantly occlude the lumen of the artery causing to decreased blood flow through that vessel. Complete blockage of blood flow (ischemia) to the perfused tissue occurs through an acute thrombotic event that can involve essential vessels such as the coronary

arteries, leading to a myocardial infarction (heart attack).

1.7.1 Initiation of Atherosclerosis

Cardiovascular disease characterized by atherosclerosis remains the leading cause of death in Western societies [72, 73]. The inititating event in the pathology of atherosclerosis is still in contention but is thought to involve an insult to the endothelial lining of the vessel caused by such factors as tobacco smoke, diabetes or high blood pressure that can disrupt the normal structure and physiology of the endothelium [74, 75]. This link between endothelial damage and the disease is called the "response to injury" hypothesis [76]. Alterations to the structural integrity of the endothelium expose subendothelial components to factors in the blood (such as lipoproteins) that can then enter through the resulting gaps. The smallest lipoproteins (HDL and LDL) as well as chylomicron and VLDL remnants can easily filter past the damaged endothelium and into the subendothelial space (called the intima). However, circulating LDL particles, unlike HDL, are thought to be more susceptible to trapping within the subendothelial space through ionic interactions between the apoB-100 present on their surface and exposed proteoglycans present in the vessel wall [77]. This trapping of LDL particles is thought to initiate a series of inflammatory responses as a key event in the development of atherosclerotic disease [78, 79]. This theory is referred to as the "response to retention" hypothesis.

1.7.2 Atherosclerosis as a Chronic Inflammatory Condition

LDL particles trapped in the arterial wall can be chemically modified, usually via oxidation, but also through other processes such as acetylation [80-82]. Modification of LDL by oxidation can occur directly through exposure to cellular oxidative waste and/or

endogenous enzymes such as lipooxygenases present in the arterial wall or produced by the macrophages themselves [83-85]. Unlike native LDL, oxidized LDL (ox-LDL) is recognized as a foreign particle by vessel wall macrophages and initiates inflammatory responses in the arterial wall through the activation of these cells [86-88]. Ox-LDL can stimulate expression of specific macrophage cell surface receptors called scavenger receptors. These include CD36 and scavenger receptor class A (SR-A) proteins that mediate uptake of the modified LDL particles [89, 90]. The initiation of inflammation within the vessel wall after damage and subsequent retention of LDL particles leads to the increased expression of certain adhesion molecules as well as chemoattractants by the endothelial cells. Chemoattractants and adhesion molecules act as localizing signals to immune cells and thus initiate a localized infiltration (Fig. 1.6). The increase in cytokines and adhesion molecules by damaged endothelial cells thus attracts white blood cells, such as monocytes (immature macrophages) to the damaged area [82, 91-93]. Monocytes bind to adhesion molecules expressed at the damaged site and transmigrate to the intima of the vessel wall. Once the monocytes enter the vessel wall, they differentiate into macrophages and begin engulfing ox-LDL particles, a step that is crucial for the development of atherosclerosis [94, 95]. Unlike LDLR expression in other tissues of the body, scavenger receptors on macrophages lack regulatory feedback mechanisms and thus accumulate large amounts of lipid via uptake of ox-LDL [96]. These lipid-laden macrophages are referred to as foam cells due to their appearance resulting from the accumulation of large intracellular lipid droplets (Fig. 1.6). The atherogenic potential of LDL cholesterol, coupled with the removal of excess cholesterol from macrophages by

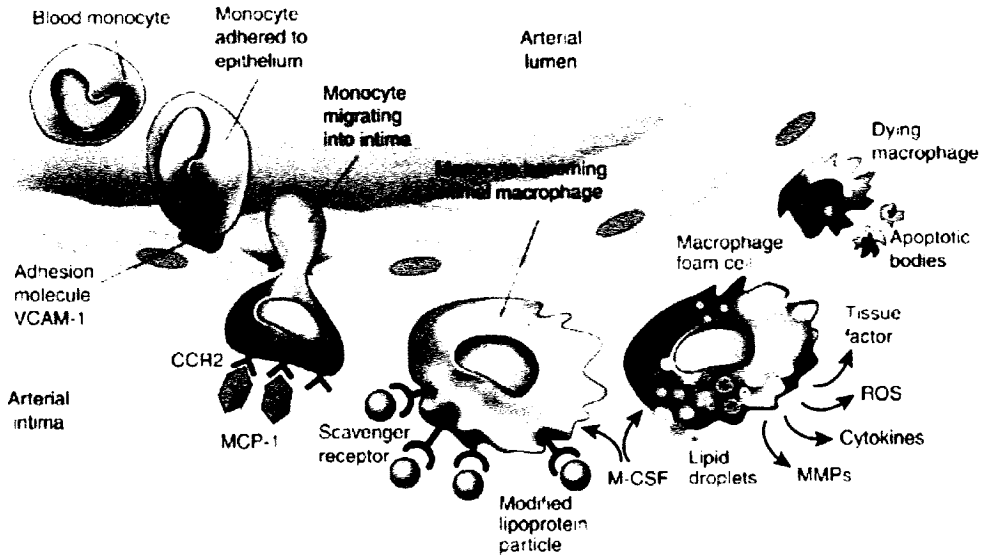


Figure 1.6 Development of foam cells in the arterial wall
The development of macrophages into foam cells within the arterial wall is known to be an essential step in the development of atherosclerosis. Damaged endothelial cells respond through upregulation of expression of cell surface adhesion molecules such as VCAM-1. Blood monocytes attach to these adhesion molecules and migrate through the endothelium into the intima of the vessel wall with the aide of specific chemoattractants such as macrophage chemoattractant protein 1 (MCP-1) released from resident cells. Activated monocytes that have entered the intima differentiate into macrophages. When LDL particles become trapped within the intima, they can become chemically modified and are then recognized as foreign particles by macrophages. The intimal macrophages become known as foam cells once they engulf the modified LDL particles. Foam cells add to the chronic inflammatory state within the intima through their release of inflammatory molecules such as cytokines, reactive oxygen species (ROS). Lack of feedback mechanisms causes foam cells to continue to take up LDL particles, leading to their eventual death within the intima. Figure taken from [97].

interactions with HDL, are consistent with the recognition that a decreased HDL/LDL ratio is a risk factor for atherosclerotic disease [98].

Atherosclerotic plaque progression evolves from early stages consisting of lesions with a small accumulation of foam and smooth muscle cells to later, more advanced plaque stages consisting of an unstable fibrous cap and a necrotic core containing crystallized cholesterol and calcified material (Fig. 1.7). The steps following modification and uptake of ox-LDL by macrophages are complex and often occur simultaneously. Foam cells release cytokines and growth factors that attract additional immune cells including T-cells, macrophages and smooth muscle cells from the underlying medial layer [99, 100]. This cascade of immune cell attraction, infiltration and activation sustains a chronic inflammatory state in the vessel wall leading to necrosis and fibrosis of the plaque. Chronic inflammation at the lesion site leads to the development of additional foam cells that eventually die by necrosis, spilling their accumulated lipid back into the surrounding area causing crystallization of cholesterol in the plaque core. Fibrosis occurs when smooth muscle cells attracted to the plaque site proliferate and deposit fibrotic molecules such as collagen and proteoglycans in an attempt to "heal" the lesion, leading to the formation of a fibrous cap. These fibrotic lesions can be broken down through secretion of proteolytic enzymes called matrix metalloproteinases and collagenases that are produced by macrophages as well as other cell types in the vicinity of the plaque [101-103]. Through the actions of these enzymes, the protective fibrous cap that maintains the integrity of the plaque contents becomes deteriorated and thin, causing the plaque to become unstable, and susceptible to rupture [104, 105]. Other factors that cause plaque instability are calcification and neovascularization. Calcification of the plaque

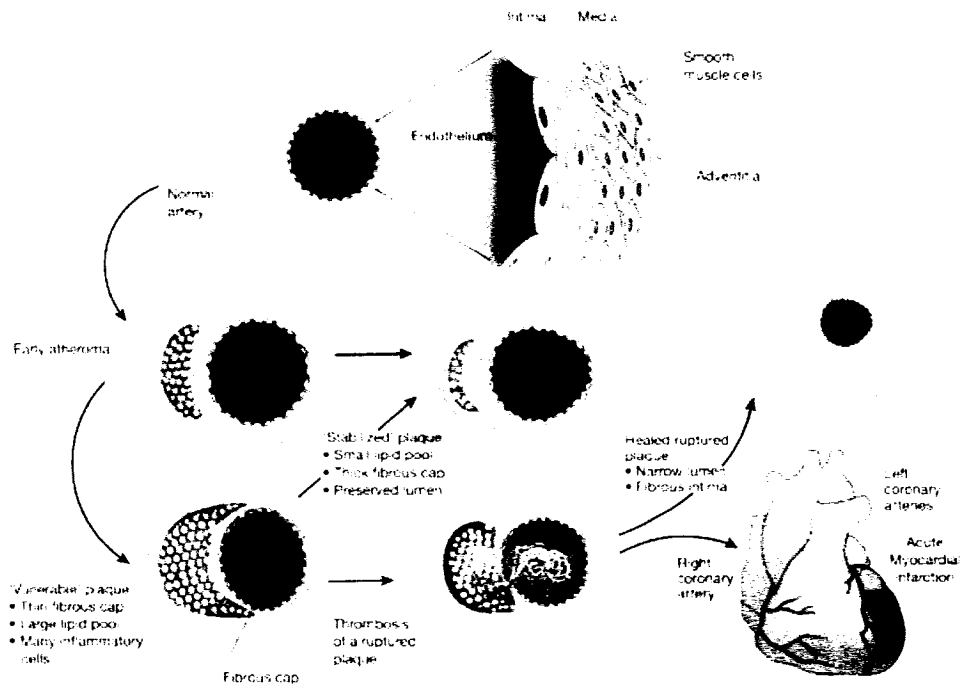


Figure 1.7 Atherosclerotic plaque progression.
A normal artery consists of well defined layers: the outer adventitial layer; the medial layer consisting of mostly smooth muscle cells and the inner intimal layer with endothelium lining the vessel lumen. Early development of an atherosclerotic plaque (atheroma) is characterized by the accumulation of foam cells within the intima. Continued inflammation in the vessel wall leads to the migration of smooth muscle cells from the media that form a fibrous cap which functions to keep the plaque stable. Thinning of this fibrous cap through the actions of matrix metalloproteinases released by foam cells leads to thinning of this protective cap. Thinning of the fibrous cap leads to instability of the plaque and can eventually cause plaque rupture. The rupture of an atherosclerotic plaque triggers the clotting cascade leading to formation of a thrombus. Formation of a thrombus can completely occlude the vessel leading to ischemia of the perfused tissue. If the affected vessel normally supplies the heart, complete occlusion can cause myocardial infarction. Figure taken from [97]

occurs through the deposition of collagen as well as various other proteins such as osteopontin by macrophages and smooth muscle cells [106-108]. Much like a tumour, lesion cores become necrotic (mostly via the death of foam cells) and can induced the growth of new blood vessels to supply the region [109]. Rupture of an unstable plaque exposes the contents of the plaque to factors present in the blood and leads to activation of the body's clotting cascade and the formation of a thrombus (clot) on the surface of the lesion causing occlusion of the vessel lumen [110-112]. Unfortunately, it is not usually until this advanced stage that occlusion of the arteries causes clinical presentations such as angina (chest pain) and life-threatening conditions such as myocardial infarction and stroke [113].

1.7.3 Risk Factors for Atherosclerosis

Atherosclerosis is a complex, multi-factorial disease with a variety of established modifiable (blood lipids, smoking, hypertension, obesity, etc.) and non-modifiable (age, sex, genetics) risk factors. Due to the crucial role of endothelial integrity in the development of atherosclerosis, many risk factors for this disease constitute conditions that can cause damage and dysfunction of the endothelium. For example, hypertension, through the application of shear forces on the endothelium is a known risk factor for plaque development [114].

As outlined in the preceding description of the importance of LDL and HDL in the development and prevention of atherosclerosis, respectively, hypercholesterolemia is an established risk factor especially when characterized by high LDL and low HDL [84] [115, 116]. The importance of cholesterol in the development of atherosclerosis is

highlighted in the case of FH, a well-described inherited disorder involving functional loss of LDL receptors causing hypercholesterolemia and increased susceptibility to atherosclerosis in patients with this genetic abnormality [22]. Despite the well-known association between cholesterol and atherosclerosis, much debate still exists with regard to its importance as a predictor of the disease in humans. For instance, many patients with atherosclerosis do not present with high cholesterol and often the severity of the disease does not correlate to blood cholesterol levels [117].

Diabetes refers to a group of diseases where blood glucose levels are increased due to decreased insulin production or insensitivity to the hormone. The diabetic state can lead to initiation of vessel wall inflammation through the increased formation of advanced glycation products (AGP). These AGPs are formed in the arterial wall through increases in amino-sugar reactions that cross-link backbones of proteins such as collagen and elastin, present in the arterial wall [118, 119]. Age and chemical irritation (by such chemicals as those in tobacco smoke) can also cause changes to the endothelium and thereby increase the risk for atherosclerosis, [98, 120, 121].

More recent factors thought to contribute to the susceptibility to atherosclerosis include high blood levels of a genetic variant of LDL, called lipoprotein(a), as well as triglycerides, homocystein, fibrinogen and chronic inflammatory diseases such as rheumatoid arthritis [122-125].

1.7.4 Therapeutic Strategies for the Treatment and Prevention of Atherosclerosis

Despite the myriad risk factors for the disease, most of the current therapeutic strategies for the treatment and prevention of atherosclerosis act to decrease levels of LDL

cholesterol levels and increase levels of HDL cholesterol. These lipid-lowering therapies include changes to the diet (lower fat content) as well as exercise in addition to pharmacological agents used to target cholesterol synthesis and absorption. To date the most effective agent for the treatment of hyperlipidemia is the statin class of drugs. Statins act to inhibit hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, the enzyme that catalyzes the rate-limiting step in hepatic cholesterol synthesis and thereby inhibit *de novo* synthesis of cholesterol. As hepatic cholesterol synthesis is inhibited, cholesterol stores within hepatocytes are diminished. In response to the diminished intracellular cholesterol, hepatocytes increase LDLR expression and new cholesterol is obtained through increased uptake of LDL particles. Increased hepatic uptake of LDL cholesterol reduces the serum levels of this lipoprotein. These drugs are known to markedly reduce the risk for cardiovascular events and actually reduce the size of existing atherosclerotic plaques [126-128]. In addition to the effective LDL-lowering capacity of statins, it is thought that the atheroprotective effect of these compounds may involve multiple anti-inflammatory effects of these drugs on macrophages, endothelial cells and T-cells [129]. Statins are often given in combination with another class of drugs that target other pathways of lipid metabolism. For example, bile acid-binding resins are a very effective in combination therapy with statins, reducing LDL cholesterol levels by 50% [130]. Bile acid-binding resins, or bile sequestrants, are taken orally and bind to bile acids within the intestinal lumen, preventing their reabsorption [131]. The resulting drop in hepatic bile acids relieves repression of CYP7A1, increasing the conversion of cholesterol to bile acids [132]. Unfortunately, this decrease in hepatic bile acid concentrations also induces HMG CoA reductase, thus increasing *de novo* synthesis of

cholesterol. Therefore, combined treatment of high cholesterol with both a statin and a bile acid resin is an effective way to block both the synthesis of cholesterol and the reabsorption of bile acids, causing a rebound increase in hepatic LDLR expression thus, increasing the uptake of LDL cholesterol from the blood. Recent research has indicated that new and emerging therapies for the treatment of dyslipidemias and/or atherosclerosis may include the targeting of various nuclear receptors (discussed in the following section).

1.8 NUCLEAR RECEPTORS AND LIPID HOMEOSTASIS

The nuclear receptor superfamily consists of ligand-activated transcriptional regulators which function in conjunction with various coactivators and corepressors to activate or repress expression of specific target genes (Fig. 1.8). Presently, 48 nuclear receptors have been discovered in the human genome, many of which are known to have important functions in biological processes including reproduction, development and metabolism [133]. The basic structure of a nuclear receptor consists of deoxyribonucleic acid (DNA) and ligand binding domains as well as activation function domains (AF-1 and AF-2) at one or both ends of the protein that are responsible for binding specific cofactors (coactivators or corepressors). AF-1, usually situated in the amino terminus of the protein, is responsible for ligand-independent activation of nuclear receptor-mediated transcription while AF-2, present in the ligand-binding domain of the carboxy terminus, is responsible for binding specific cofactors in a ligand-dependent manner [135, 136]. Nuclear receptors can bind DNA as monomers, homodimers and/or heterodimers with another nuclear receptor called the retinoid X receptor or RXR [137, 138]. These nuclear

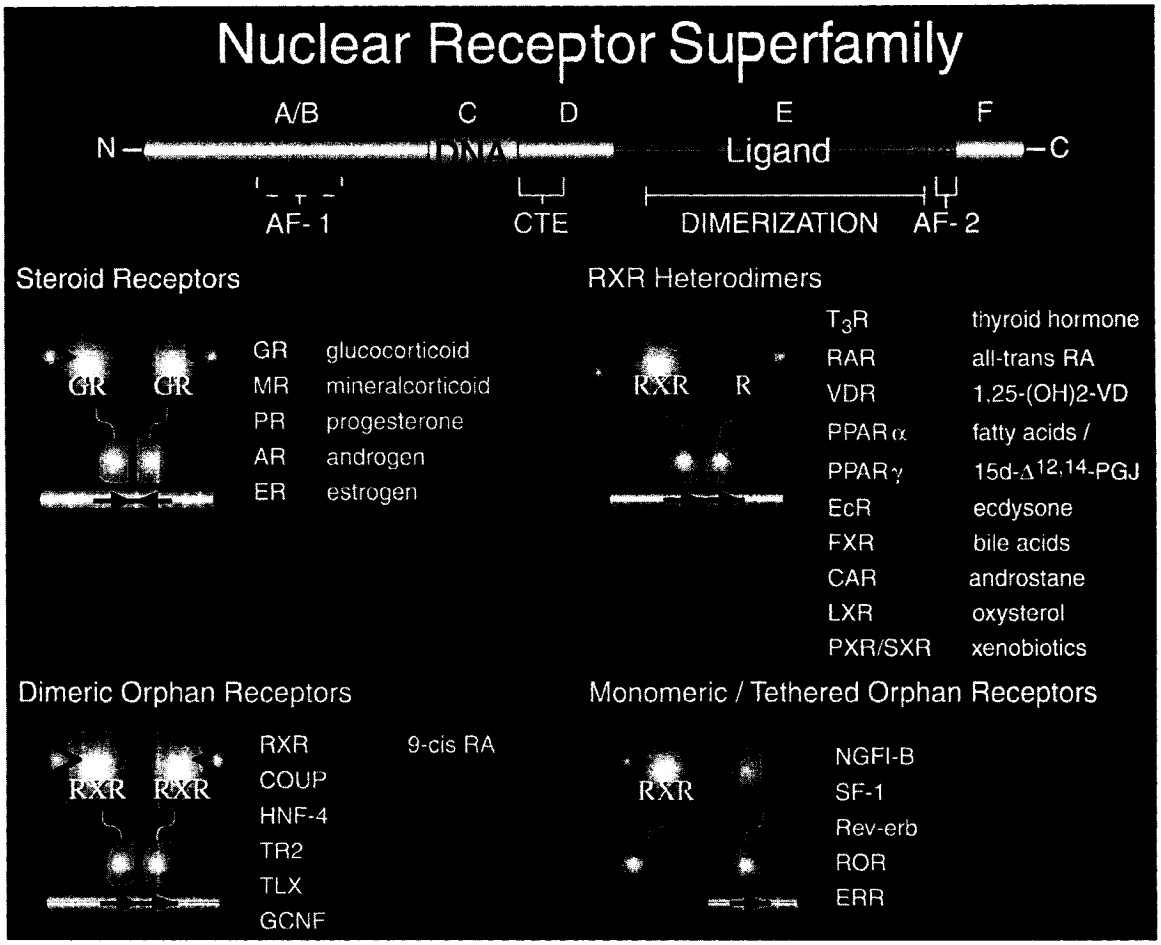


Figure 1.8 The nuclear receptor superfamily.
The nuclear receptor superfamily consists of ligand-activated transcription factors that bind DNA as homodimers (steroid and dimeric orphan receptors), heterodimers with RXR, as monomers or tethered to other nuclear receptors. The basic structure of nuclear receptors consists of ligand-independent and ligand-dependent transactivation domains; AF-1 and AF-2, respectively as well as a DNA binding and ligand-binding domains. Nuclear receptors recognize specific sequences in the promoter regions of target genes. These specific response elements are often made up of two consensus sequences in inverted, everted or direct repeat configurations. Figure taken from [134].

receptors bind DNA at specific sequence sites called response elements that are composed of one or two consensus half sites that are either in a direct, inverted or everted configuration in the promoter regions of target genes.

Most nuclear receptors are present in the nucleus bound to their respective response elements on the DNA but remain inactive until bound by ligand. Once a specific ligand binds to the nuclear receptor a conformational change occurs, allowing the recruitment of coactivators or corepressors that act to bring about transcriptional activation or repression of the target gene, respectively [139]. Coactivators and corepressors known to interact with nuclear receptors include peroxisome proliferators-activated receptor gamma coactivator-1 alpha (PGC-1α) and nuclear corepressor (NCoR), respectively. In order for nuclear receptors to bring about changes in transcriptional levels through alterations in gene expression they must first alter the accessibility of the regulatory region (promoter) to transcriptional machinery [140]. Genomic DNA is organized into coils wrapped around protein structures called nucleosomes that consist of histone proteins. Therefore, in order for gene transcription to occur, DNA must be relaxed in order to allow access to transcriptional machinery. Local alterations of the ultrastructure of DNA at promoter sites are mediated by a group of proteins called cofactors that includes coactivators, corepressors and chromatin remodelers [141]. Coactivators and corepressors do not bind DNA directly but are instead recruited to specific protein motifs in AF-1 and AF-2 regions of nuclear receptors. Coactivators can recruit components of transcriptional machinery such as the RNA polymerase complex as well as chromatin remodelers. Chromatin can be remodeled by coactivators and corepressors directly through intrinsic histone acetyl transferase or

deacetylase activity, respectively or through recruitment of proteins with these enzymatic activities [142]. Nuclear corepressor, or NCoR, is a major corepressor that bind to nuclear receptors such as retinoid X receptor (RXR) and recruits a complex called histone deactetylase complex 3[143, 144]. A major coactivator of nuclear receptor transcription is PGC-1α that functions to recruit histone acetyl transferase complexes such as steroid receptor coactivator-1[145]. Further functions of PGC-1α will be discussed at length in Chapter 4.

1.8.1 Farnesoid X Receptor, Liver X Receptor, Pregnane X Receptor and Vitamin D Receptor

Two nuclear receptors with major roles in cholesterol and triglyceride homestasis are the farnesoid X receptor (FXR) and the liver X receptor (LXR), both of which form obligate heterodimers with RXR [146]. FXR and LXR often function in opposition to maintain lipid homeostasis and thus have been referred to as the "Yin and Yang" of lipid metabolism, see Fig. 1.9 [147]. The opposing roles of FXR and LXR are reflected in their respective endogenous ligands; bile acids are FXR ligands and oxysterols (oxidized forms of cholesterol) are LXR ligands [148-150]. FXR functions mainly in the liver to maintain bile acid homeostasis through prevention of bile acid accumulation. LXR functions to regulate hepatic cholesterol conversion to bile acids and to protect extrahepatic cells such as macrophages from accumulation of large amount of cholesterol.

Once bound by bile acids, FXR functions to repress the transcription of the gene for CYP7A1, the enzyme responsible for catalyzing the rate limiting step in the conversion of cholesterol to bile acids [151]. FXR also acts to activate transcription of the

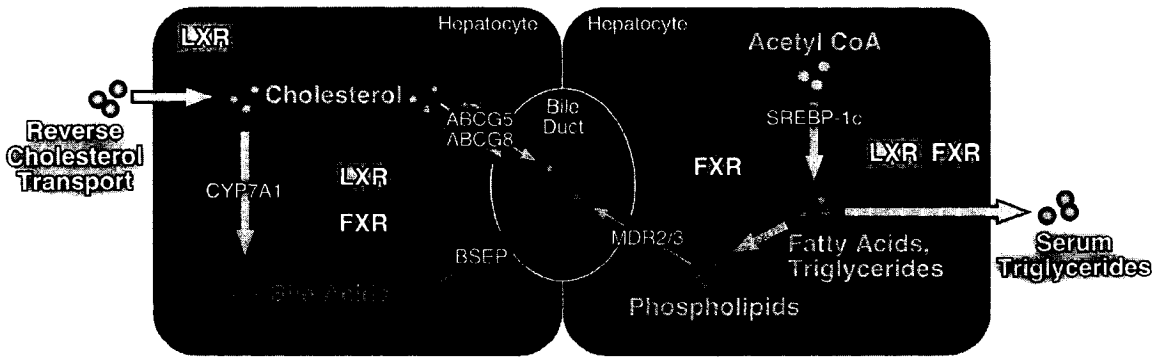


Figure 1.9 Opposing roles of FXR and LXR in lipid homeostasis.
FXR functions in the liver to protect this organ from increasing concentrations of bile acids by decreasing their conversion from cholesterol. FXR represses transcription of the gene coding for CYP7A1, the enzyme responsible for catalyzing the rate limiting step in the conversion of cholesterol to bile acids. FXR also induces the expression of genes coding for bile acid and phospholipid efflux transporters; BSEP and MDR2/3, respectively. FXR also functions to decrease hepatic and blood triglyceride levels through repression of SREBP-1c and induction of apoC-II, respectively. LXR functions in opposition to FXR in the liver by protecting cells from the accumulation of intracellular cholesterol. LXR increases the conversion of cholesterol into bile acids as well as its transport into the bile through increased expression of CYP7A1 and ABCG5/8, respectively. In addition, LXR increases lipogenesis through increased transcription of the gene coding SREBP-1c. Figure taken from[147]

genes coding for the bile salt export pump (BSEP) which normally acts to transport bile acids from the liver into the bile duct [152]. In addition, FXR induces transcription of the gene for the multidrug resistance protein 2 (MDR2), called MDR2 in mice and MDR3 in humans that transports phospholipids into the bile, a function that is important in the solubilization of cholesterol [153]. Therefore, as demonstrated by these examples of target gene regulation, FXR functions to decrease the synthesis of bile acids from cholesterol while increasing the efflux of bile acids and cholesterol from hepatocytes into the bile. The conversion of cholesterol into bile acids represents an important route of elimination of excess cholesterol. Therefore, as a mediator of bile acid homeostasis, FXR is also an important mediator of systemic cholesterol levels. A more detailed discussion of the role of FXR in lipid homeostasis is provided in chapter 2.

The LXR has two isoforms: alpha, with most prevalent expression in liver, and beta, which is more ubiquitous in expression [154, 155]. Oxysterols, ligands for LXR, are intermediate derivatives of cholesterol formed during its conversion to bile acids as well as steroid hormones [156]. LXR is therefore an intracellular cholesterol sensor and functions to increase the catabolism and efflux of this molecule from cells [157, 158]. The gene coding for CYP7A1 is a target of LXR and is activated by this nuclear receptor, thereby increasing the conversion of cholesterol to bile acids [159]. LXR also activates the expression of genes coding for important proteins involved in the efflux of cholesterol from cells (such as the membrane transporters ABCA1, ABCG5, ABCG8 and ABCG1) [160-162]. Therefore, when cells accumulate oxysterols through the metabolism of cholesterol, LXR becomes activated, leading to increased CYP7A1 expression and thereby, increased conversion of cholesterol into bile acids. LXR activation also leads to

increased cholesterol efflux via activation of cholesterol transporters such as ABCA1. In addition to having effects on cholesterol levels, activation of LXR also causes increases in lipogenesis (triglyceride synthesis). The lipogenic properties of LXR are mediated through the activation of genes involved in the synthesis of fatty acids such as the sterol receptor element binding protein-1c (SREBP-1c) and the enzyme fatty acid synthase (FAS) [163-165]. Free cholesterol is a toxic molecule and one mechanism that has evolved in cells to manage increases in this molecule is via esterification, allowing safe storage of cholesterol for later transport or metabolism. The substrates for cholesterol esterification are fatty acids and so the activation of lipogenesis by LXR functions as a protective mechanism to help neutralize toxic intracellular free cholesterol.

Two other nuclear receptors with more minor roles in lipid homeostasis are the vitamin D receptor and the pregnane X receptor. Both of these receptors are activated by bile acids and function to upregulate genes involved in the detoxification of these compounds for more efficient elimination from the body [166-169].

1.8.2 Peroxisome Proliferator-Activated Receptors

There are three members of the peroxisome proliferator-activated receptor (PPAR) family: alpha (α) (highly expressed in the liver) gamma (γ) (highly expressed in adipose and macrophages) and delta (δ) (ubiquitous expression) [170]. These nuclear receptors are also obligate heterodimers with RXR [146]. Endogenous ligands for the PPAR family include saturated and unsaturated long-chain fatty acids and eicosanoid derivatives [171]. These nuclear receptors regulate of many physiological processes such as adipocyte differentiation (PPARγ), fatty acid ß-oxidation (PPARα) and inflammation (PPARδ).

PPARα activates transcription of genes important in the mitochondrial (acyl-coA synthetase) and peroxisomal ß-oxidation (L-bifunctional protein) pathways in the liver and skeletal muscle, thereby increasing the catabolism of fatty acids and the production of adenosine triphosphate, ATP [172, 173]. Thus, as discussed further in Chapter 4, PPARα activation is important in the physiological responses to fasting. Fibrates, a class of lipid-lowering drugs, are known agonists of PPARα [174]. Activation of PPARα increases expression of LPL on the endothelium of capillaries leading to accelerated hydrolysis of blood triglycerides and the increased uptake of fatty acids into tissue [175]. PPARα also increases expression of receptors on liver and intestinal cells responsible for uptake of fatty acids into these organs (such as CD36 and fatty acid transport protein) [176, 177]. Therefore, activation of PPARα by fibrates increases hydrolysis, uptake and degradation of fatty acids, thereby decreasing circulating levels of triglyceride.

Insulin-sensitizing drugs called thiazolidinediones are agonists of PPARγ [178]. The insulin-sensitizing effects of PPARγ are thought to occur primarily through fatty acid uptake and storage in adipose tissue [179]. PPARγ functions to upregulate expression of targets genes coding for receptors involved in the uptake and storage of fatty acids (such as CD36 and phosphoenol pyruvate kinase) into adipose tissue and is also essential to the differentiation of adipocytes [175, 176, 180, 181]. Therefore, through the increased uptake and storage of triglyceride into adipose tissue, PPARγ acts to prevent accumulation of fat in other organs such as liver and muscle, a step that is known to be crucial in the development of insulin resistance [182].

PPARδ is the lesser-known PPAR and its exact function is only now being elucidated. Recently PPARβ was found to have potential importance in lipid homeostasis

and atherosclerosis through its function in macrophages as a sensor for VLDL triglycerides as well as an activator of inflammatory responses in these cells [183, 184]. PPARδ has also been found to be an activator of fatty acid oxidation and utilization in adipose and skeletal muscle and can prevent obesity when activated by specific ligand [185].

1.9 SUMMARY

Lipid homeostasis is essential for normal functioning of the body. The insoluble properties of lipids such as cholesterol and triglycerides have led to the evolution of complex mechanisms for the dietary absorption, transport and storage of these compounds. The toxic nature of free fatty acids and cholesterol can cause cellular dysfunction as well as disease and thus the body must tightly regulate levels of these compounds. Regulation of lipid homeostasis can occur in many tissues through the altered transcription of target genes, such as transporters and enzymes, by nuclear receptors such as FXR, LXR and PPARα. Much of the regulatory mechanisms that function in the maintenance of lipid homeostasis are located in the liver. Dyslipidemia can contribute to diseases such as gallstones, cholestasis and atherosclerosis. Atherosclerosis is a chronic inflammatory disease with complex etiology. Current therapies for the treatment of dyslipidemias and atherosclerosis are focused on increasing HDL/LDL ratios but new approaches may include targeting the activity of nuclear receptors.

CHAPTER 2

LOSS OF FUNCTIONAL FXR INCREASES ATHEROSCLEROTIC LESIONS IN APOE-DEFICIENT MICE

Sections not done by PhD candidate:

Fig. 2.1 was experimental analyses performed by Dr. Gilles Lambert (INSERM, France) who is a research collaborator with our lab. All other experimental data presented in this chapter was produced by the candidate. Assistance with animal blood collection and tissue harvesting was provided by Tanya McCarthy.

Portions of this chapter appeared in the following publication:

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2.1 ABSTRACT

FXR is a bile acid-activated transcription factor that regulates the expression of genes critical for bile acid and lipid homeostasis. The present study was undertaken to investigate the pathological consequences of loss of FXR function on the risk and severity of atherosclerosis. For this purpose, *FXR*$^{-/-}$ mice were crossed with apoE-deficient (*ApoE*$^{-/-}$) mice to generate *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice. Challenging these mice with a high fat, high cholesterol (HF/HC) diet resulted in reduced weight gain and decreased survival compared to *Wildtype (WT)*, *FXR*$^{-/-}$ and *ApoE*$^{-/-}$ mice. *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice also had the highest total plasma lipids and the most atherogenic lipoprotein profile. Livers from *FXR*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice exhibited marked lipid accumulation, focal necrosis (accompanied by increased levels of plasma aspartate amino transferase, AST) and increased inflammatory gene expression. Measurement of *en face* lesion area of HF/HC challenged mice revealed that while *FXR*$^{-/-}$ mice did not develop atherosclerosis, *FXR*$^{-/-}$ *ApoE*$^{-/-}$ mice had approximately double the lesion area compared to *ApoE*$^{-/-}$ mice. In conclusion, loss of FXR function is associated with decreased survival, increased severity of defects in lipid metabolism and more extensive aortic plaque formation in a mouse model of atherosclerotic disease.

Keywords: Farnesoid X Receptor, atherosclerosis, nuclear receptor, Apolipoprotein E, liver

2.2 INTRODUCTION

2.2.1 FXR and LXR as Therapeutic Targets for Hyperlipidemia

The current regime of pharmacological therapies for the treatment of cardiovascular disease remains incomplete, despite the fact that this disease remains the leading cause of death in Western societies [186, 187]. Recent research indicates that future therapies for the treatment and prevention of dyslipidemia and its associated diseases such as atherosclerosis may involve direct targeting of nuclear receptor function. Ideally, targeting nuclear receptor activity will affect numerous downstream pharmacological targets simultaneously. Thus, nuclear receptor targeting represents an attractive strategy by which many physiological processes and risk factors may be modulated. Two nuclear receptors thought to have opposing roles in cholesterol homeostasis are LXR and FXR. FXR functions to protect the liver from accumulation of bile acids by increasing the secretion and repressing the conversion of these molecules from cholesterol. Conversely, LXR functions as a cholesterol sensor and acts to increase its conversion to bile acids in the liver and increases efflux of cholesterol from extrahepatic tissues. The following sections will discuss the potential of LXR and FXR as targets for the treatment and prevention of atherosclerosis.

2.2.2 LXR Deficiency and the Development of Atherosclerosis

LXR is known to protect cells such as macrophages from accumulating large amounts of lipid through positive regulation of genes encoding cholesterol efflux transporters such as ABCA1 [161]. Evidence for the crucial role of this nuclear receptor in lipid homeostasis is highlighted in studies of LXR knockout mice. Mice deficient for the α-isoform of LXR

(LXRα-/-) are characterized by impaired cholesterol and bile acid metabolism and accumulation of hepatic cholesterol when fed a cholesterol-containing diet [157]. In addition, mice deficient for both isoforms of LXR, α and β, have increased LDL and decreased HDL and accumulate foam cells in the arterial wall [188]. Bone marrow transplantation of cells from LXR-/- mice led to increased atherosclerosis in the apoE-/- and LDLR-/- mouse models of atherosclerosis illustrating the importance of this nuclear receptor in macrophage function [189].

2.2.3 LXR Agonism in the Treatment of Dyslipidemia and Atherosclerosis

Given the recent experimental demonstration of the importance of LXR in lipid homeostasis and its role in the prevention of atherosclerotic disease, this nuclear receptor has gained considerable interest as a potential pharmacological target. Early studies using specific agonism of LXR through the use of the synthetic compound T0901317 increased HDL levels in mice and inhibited the development of atherosclerosis in LDLR-/- mice [163, 190]. Unfortunately, as described previously, activation of LXR also leads to increased triglyceride production by activating lipogenesis. Therefore, treatment of mice with T0901317 also causes increased liver fatty acid synthesis, hepatic triglyceride accumulation and increased synthesis of VLDL [191, 192]. The unwanted lipogenic effects of LXR agonism by T0901317 drove the development of a selective modulator of LXR called GW3965 that raises HDL cholesterol in the same manner as T0901317 but fails to induce triglyceride accumulation in the liver [193, 194]. Treatment of mouse models of atherosclerosis with this compound protected against development of the disease [195]. The absence of lipogenic activation by treatment with GW3965 is thought

to be mediated by differential coactivator recruitment however the exact mechanisms have not been elucidated [194]. Thus, LXR represents a promising example of a nuclear receptor targeting for the treatment of dyslipidemia and atherosclerosis.

2.2.4 An Expanded Role for FXR in Lipid Homeostasis

FXR is a nuclear receptor that is activated by binding to bile acids and functions primarily as a bile acid sensor in the liver to induce and repress genes involved in bile acid synthesis and export, respectively. Beyond this function, accumulating evidence strongly supports an expanded role for FXR in the regulation of systemic lipid homeostasis. For example, FXR activates the expression of ApoC-II, the cofactor required for LPL function, leading to decreased blood levels of triglycerides [196]. FXR is also thought to repress expression of the apoC-III gene, an apolipoprotein that inhibits LPL activity [197, 198]. Overexpression of apoC-III has been shown to cause hypertriglyceridemia [199]. Thus, lack of repression of apoC-III expression in FXR-/- mice may also contribute to the hypertriglyceridemia observed in these mice.

FXR functions to protect the liver from increasing concentrations of bile acids through activation of genes encoding efflux transporters as well as suppression of genes encoding enzymes that convert cholesterol to bile acids. Through negative regulation of the conversion of cholesterol to bile acids, a major route for elimination of excess cholesterol, FXR is important in the determination of hepatic and blood levels of cholesterol. FXR has also been shown to repress expression of the gene encoding apoA-I, an essential component of HDL particles, and to induce expression of the VLDL receptor, VLDLR [200, 201]. Although the exact function of VLDLR in the liver is

unknown, overexpression of this receptor has been shown to be beneficial in the prevention of atherosclerosis in a mouse model of the disease [202]. In addition FXR has been found to repress HL, an enzyme important in remodeling and receptor-mediated uptake of all classes of lipoproteins [203, 204]. The correlation of HL activity and atherosclerosis is still unclear and there has been much conflicting data in recent years with evidence to support both anti- and pro-atherosclerotic effects of this enzyme [204]. For example, in mice high HL expression prevents atherosclerosis but deficiency of this enzyme in mice has also been shown to delay atherosclerosis [205, 206]. FXR also activates expression of PLTP which functions in the transfer of phospholipids from VLDL to HDL particles, thereby increasing the formation of HDL cholesterol [207, 208].

With the continued discovery of novel FXR targets with differing or even unknown contributions to atherosclerosis, the exact role of this nuclear receptor in lipid homeostasis and development of atherosclerotic disease has actually become less clear. Further information on the physiological role of FXR in lipid homeostasis was gleaned through the recent development of FXR-deficient mice.

2.2.5 FXR-Deficiency and Lipid Homeostasis

Until recently, no selective ligand for FXR was available for the study of the physiological role of this nuclear receptor in lipid homeostasis. However, phenotypic alterations due to FXR deficiency were studied through development of FXR-/- mice [209]. FXR-/- mice had increased blood levels of cholesterol, triglycerides and bile acids as well as increased VLDL and LDL. These mice also have hepatic accumulation of cholesterol and triglycerides despite having increased secretion of both cholesterol and

bile acids. Phenotypic alterations in FXR-/- mice included decreased hepatic expression of SR-B1, the HDL receptor, as well as decreased HL and LCAT with no changes in hepatic expression of either apoA-I or PLTP [210]. Therefore, despite molecular studies demonstrating repression of HL and apoA-I by FXR, physiological studies using the FXR-/- model have demonstrated the opposite trend. In addition, FXR-/- mice had decreased hepatic expression of ABCG5 and ABCG8, two transporters important in secretion of cholesterol into the bile for elimination [211]. Lack of repression of CYP7A1 by FXR in FXR-/- mice would theoretically lead to increased conversion of cholesterol into bile acids. However, the increased blood levels of cholesterol as well as triglycerides, coupled with increases in LDL and VLDL in the FXR-/- mice indicate a protective role for this nuclear receptor in atherosclerotic disease.

2.2.6 FXR as a Therapeutic Target for the Treatment of Dyslipidemia

Since the development of the FXR-/- mouse, two ligands for FXR have been described; guggulsterone and GW4064 [212-214]. Guggulsterone is a cholesterol- and triglyceride-lowering compound extracted from resin of the mukul myrrh tree (*commiphora mukul*) [215, 216]. This resin has been used for centuries in traditional medicine for the treatment of obesity and dyslipidemias [217]. Recently, guggulsterone was shown to be an antagonist of FXR that lowered hepatic cholesterol accumulation, in mice fed a high fat diet. These results were in opposition to the phenotypic observations made in the FXR-/- mice [214]. Subsequent to this study it was discovered that guggulsterone acted more as a modulator of FXR activity as some targets of FXR were unaffected (such as CYP7A1) or were actually induced (BSEP) in rats treated with this compound [218]. Treatment of rats

in this study led to decreased blood levels of triglycerides and increased HDL. Further insight into the molecular effects of guggulsterone was gained in a recent study which demonstrated that guggulsterone is a promiscuous steroid receptor ligand that binds with much higher affinity to nuclear receptors such as the mineralocorticoid receptor, glucocorticoid receptor (GR) and androgen receptor [219]. Therefore, the hypolipidemic effects of guggulsterone may be mediated through targeting of numerous steroid receptors.

Recently a compound (GW4064) was synthesized that selectively agonizes FXR [212]. When administered to rats via oral dosing for 7 days, GW4064 significantly decreased serum triglycerides and increased HDL cholesterol, results consistent with the previously described phenotype of FXR-/- mice [220]. In addition, treatment with this FXR agonist has been shown to protect from cholestasis as well as to ameliorate diabetes-associated hyperlipidemia in rodent models of these diseases [221, 222]. Primary human hepatocytes and human hepatoma cells exhibit increased uptake and degradation of LDL when treated with GW4064 indicating a protective role for FXR in human liver [223]. Studies using this selective agonist of FXR, in addition to the observed phenotype of FXR-/- mice, support a protective role for FXR in dyslipidemia and atherosclerotic disease.

2.2.7 Mouse Models of Atherosclerosis

In the past decade mice have been used more and more frequently for study of the molecular and pathological mechanisms of atherosclerotic disease. The mouse as a species is highly resistant to atherosclerosis, at least in part due to the fact that virtually

all of their blood cholesterol is transported as HDL. By comparison, 60-65% of blood cholesterol in humans in transported as LDL [224]. In mice there are very few examples of single gene deletions that lead to a greatly increased susceptibility to atherosclerosis. The apoE and LDLR are two genes that when disrupted in mice (separately) lead to profoundly increased susceptibility to development of atherosclerosis and thus represent the most commonly used models of this disease; the apoE-/- mouse and the LDLR-/- mouse [225-227].

ApoE is a protein with numerous functions in lipid homeostasis and is present on all lipoproteins except LDL. Among the most important functions of apoE is that it is essential for receptor-mediated clearance, via LRP and LDLR, of highly atherogenic remnants of chylomicrons and VLDL by the liver [228-230]. Importantly, apoE is expressed in macrophages and is essential to efflux of excess cholesterol to HDL particles in these cells, thereby helping to prevent their development into foam cells [231, 232]. ApoE has also been found to stimulate hepatic production of triglyerides and VLDL particles [233]. The essential function of apoE in lipid homeostasis is clearly demonstrated in the apoE-/-mouse which have cholesterol levels 5-fold higher than control littermates in addition to a 45% decrease in HDL and 68% increase in triglyceride levels. These mice spontaneously develop atherosclerotic plaques and plaque progression and pathology in these mice is very similar to the human disease [234, 235]. There also exists a human form of apoE deficiency called type III hyperlipoproteinemia where apoE is defective in binding to the LDLR and persons with this genetic anomaly are predisposed to early development of atherosclerotic disease [236].

The LDLR functions to clear LDL and IDL from the blood [22]. LDLR-/- mice

have less severe defects in lipid metabolism than apoE-/- mice; cholesterol levels are 2-fold higher than controls with no changes in HDL cholesterol or triglycerides [227]. As a consequence of this less severe phenotype, these mice do not develop atherosclerosis unless fed a diet containing high fat and cholesterol [237]. The human equivalent of the LDLR-/- mice is FH, a condition involving a mutation of the LDLR characterized by high total and LDL cholesterol levels and increased susceptibility to atherosclerosis [238, 239].

2.2.8 Summary and Objectives

The present study was undertaken to examine and clarify the pathological consequences of loss of FXR function on lipid homeostasis and atherosclerotic progression. The results of this study indicate that targeted disruption of the FXR gene leads to more severe atherosclerosis in an established murine model of this disease and provides the first direct evidence linking this receptor to the risk and severity of cardiovascular disease.

2.3 MATERIALS AND METHODS

2.3.1 Animals, Diets and Treatments

ApoE$^{-/-}$ mice were purchased from Jackson laboratories, Bar Harbor, ME. *FXR*$^{-/-}$ mice (from our breeding colony, back-crossed 10 generations to congenic C57BL/6J) were crossed with *ApoE*$^{-/-}$ mice (Jackson Laboratories, Bar Harbor, MA) to obtain the *FXR*$^{-/-}$ *ApoE*$^{-/-}$ mice. The ApoE-/- and FXR-/- genotypes were confirmed using PCR as per a protocol provided by Jackson laboratories as well as a previously established protocol, respectively [209]. The background of all mice used in this study was C57BL/6J. 7-8

week-old male mice were placed on either chow (7% fat) (#F4516, based on AIN-93G diet) or a high fat, high cholesterol (HF/HC) diet containing 16% fat, 1.25% cholesterol (#F4515) (Bioserv, Frenchtown, New Jersey) for 12 weeks. For the GW4064 study, apoE-/- mice were fed the HF/HC diet for 6 weeks before GW4064 (a generous gift from Dr. Tim Willson, GlaxoSmithKline) was administered by oral gavage for 30 additional days. GW4064 was solubilized in 1:4 TWEEN: PEG300 and administered in a dosage of 65 mg/kg once per day. During treatment with GW4064 the mice were maintained on the HF/HC diet. Animals were weighed once per week and food consumption was monitored throughout the study. All animals were housed at room temperature on a 12-hour light/dark cycle and provided food and water *ad libitum*. All procedures were conducted at the Carleton Animal Care Facility in accordance with Canadian Council on Animal Care guidelines.

2.3.2 Plasma Analyses

Blood was collected using heparinized needles after a 6-hour fast and centrifuged at 6700 x g for 5 minutes. Plasma was stored at –20°C. Triglyceride and cholesterol levels were measured at 510 and 500 nm, respectively, using *in vitro* diagnostic reagents and calibrators (Thermo DMA, Arlington, TX) as per the manufacturer's instructions. AST values were measured after dilution of the plasma 1/5 with distilled water and using an Infinity AST Reagent kit (Sigma Diagnostics, St. Louis, MO). Values were determined kinetically by the change in absorbance at 340 nm and at a temperature of 37°C. All absorbance measurements were performed using a 96-well plate reader (PowerWaveX, Bio-Tek Instruments, Inc., Winooski, VT). Blood glucose was measured using a

personal glucose monitoring device (TheraSense FreeStyle, AR-Med Ltd., England).

2.3.3 FPLC and Western Blot Analyses

FPLC separation of plasma lipoproteins from pooled plasma samples (200 μL; n = 3-5) and analysis of lipid content of the subsequent fractions was performed as described previously [240]. Immunoblot analyses of apo's B100, B48, A-I and A-II in the VLDL (V), LDL (L) and HDL (H) fractions (eluting at 14, 20 and 29 mL, respectively) was performed as previously described [209].

2.3.4 Liver Lipid Measurement

Lipids were extracted from liver tissue based on published methods [241]. Solvents were evaporated from the extracted lipids under nitrogen and dissolved in Triton X-100 (Sigma-Aldrich, St. Louis, MO), warmed to 37°C and mixed with water (1:4). The samples were then analyzed for triglyceride and cholesterol content using the same reagents (1 μL sample:100 μL reagent) listed above for plasma lipid analysis.

2.3.5 Histology

After 12 weeks on the diets, the largest lobe of the liver and the top half of the heart were obtained from the mice and embedded in O.C.T. (Sakura Finetek U.S.A., Inc., Torrance, CA) combined with sucrose (20%) and stored at –80°C. 10 μm cryosections were obtained lengthwise through the liver lobe and 5 μm cryosections were obtained in cross-section through the aorta at the origin in the heart. All cryosections were fixed for 1 min in formaldehyde solution, stained for 10 min with Oil Red O (stains

lipids red) and counterstained for 1 min with Hematoxylin (stains nuclei blue) for qualitative observation of lipid accumulation. 5 μm-thick cross-sections were obtained from paraffin embedded liver and stained with hematoxylin and eosin (H+E). *En face* lipid accumulation was determined by removing the aortas from the mice from the ileal bifurcation to the origin at the heart. The heart and the aorta were fixed for a minimum of 2 days in 5% neutral buffered formalin (EM Industries Inc., Gibbstown, NJ) and 0.5x phosphate buffered saline. The aortas were then cut longitudinally, splayed and pinned in a dish which was flooded with Sudan IV stain (stains lipids red) for 8 min, destained in 80% ethanol for 5 min and photographed. Quantitation of plaques from the ileal bifurcation to the origin (not including any branching vessels) was performed using freehand selection of plaques using Image J software (NIH, Bethesda, MD). The additive area of all the plaques in a given aorta was calculated as a percent of the total surface area of the aorta.

2.3.6 Hepatic Gene Expression

Total hepatic ribonucleic acid (RNA) was isolated using Trizol reagent as per the supplier's (Invitrogen, Carlsbad, CA) instructions. Quantitative real-time polymerase chain reaction (QPCR) analysis was performed as previously described [168] with the following modifications. Total RNA (2 μg) was reverse transcribed using Stratascript Reverse Transcriptase (Stratagene, La Jolla, CA) with random hexamers $pd(N)_6$ according to the supplier's instructions. The synthesized complementary DNA (cDNA) was then amplified by QPCR using a Stratagene MX3000p thermocycler in a total volume of 25 μl with Brilliant SYBR Green QPCR Master Mix. Primer sequences are as

follows: murine tumour necrosis factor-α (TNF-α): 5'-CCC TCA CAC TCA GAT CAT CTT CT-3' (forward) and 5'-GCT ACG ACG TGG GCT ACA G-3' (reverse) (accession NM_013693); murine macrophage antigen 1 (MAC1): 5'-GTG GTG CAG CTC ATC AAG AA-3' (forward) and 5'-GCC ATG ACC TTT ACC TGG AA-3' (reverse) (accession M31039) and murine RNA polymerase II (RP-II): 5'-CTG GAC CTA CCG GCA TGT TC-3' (forward) and 5'-GTC ATC CCG CTC CCA ACA C-3' (reverse) (accession U37500). Thermal cycling conditions were identical for each primer pair and were as follows: a single cycle of 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 18 s, and elongation at 72°C for 30 s. Melting curves were generated from 60°C to 94°C at the end of the QPCR protocol to ensure the amplification of a single product. The QPCR products were then separated on a 2.5% agarose gel and visualized by ethidium bromide staining to ensure the formation of a single product at the appropriate size was generated. Relative C_T values were obtained by the $\Delta\Delta C_T$ method [242] using a threshold of 10 standard deviations above background for C_T.

2.3.7 Statistics

All comparisons were performed within diet groups using one-way analysis of variance (ANOVA) with Tukey-Kramer post-analysis, unless otherwise stated. GraphPad Instat (Instat3 V3.0a, GraphPad Software, Inc.) was used for all statistical analyses. Values are expressed as mean ± standard deviation (SD). Differences between groups are considered statistically significant at $P \leq 0.05$.

2.4 RESULTS

In order to study the effect that deletion of FXR has on atherosclerotic disease, *FXR*$^{-/-}$ mice were crossed with *ApoE*$^{-/-}$ mice to generate *FXR*$^{-/-}$*ApoE*$^{-/-}$ animals. Prior to crossing with the *ApoE*$^{-/-}$ mice (pure C57BL/6J background), the existing *FXR*$^{-/-}$ mouse model [209] was backcrossed 10 generations to a congenic C57BL/6J background. We then fed these mice standard rodent chow or the HF/HC containing elevated levels of fat (16% w/w) and cholesterol (1.25% w/w) for a total of 12 weeks. Cholic acid is commonly included in atherogenic diets to increase the dietary absorption of lipids and to increase the inflammatory response. As we have previously shown that dietary cholic acid is extremely toxic to *FXR-/-* mice [209], we omitted this component from our atherogenic diet. As indicators of the general health of mice throughout the 12-week feeding study, starting weights (day 1), cumulative weight gain, food consumption and survival rates were monitored. Starting weights were similar in all experimental groups with the exception of *FXR*$^{-/-}$ *ApoE*$^{-/-}$ mice in the chow fed group that were of lower starting weight than *ApoE*$^{-/-}$ mice on the same diet (Table 2.1). *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice on a HF/HC diet failed to gain weight to a similar magnitude as *WT*, *ApoE*$^{-/-}$ and *FXR*$^{-/-}$ mice on the same diet. *FXR*$^{-/-}$*ApoE*$^{-/-}$ on the HF/HC diet gained approximately 2.8-fold less weight than *FXR*$^{-/-}$ and *ApoE*$^{-/-}$ mice and 3.7-fold less than *WT* mice on the same diet (Table 2.1). The reduced weight gain exhibited by *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice was not due to reduced caloric intake as the food consumption of this group did not differ from that of *WT* or *FXR*$^{-/-}$ (Table 2.1). *ApoE*$^{-/-}$ mice fed a HF/HC diet had the highest food consumption, consuming approximately 1.3-fold more than the other groups on the same diet. Survival rates of the four genotypes of mice on both chow and the HF/HC diet were

Table 2.1 Reduced survival and increased plasma lipids in *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice

Values given as mean±SD. N=5-8. Statistical analyses were performed by one-way ANOVA within each diet group.
[a] P<0.05 vs wildtype, [b] P<0.05 vs *FXR*$^{-/-}$, [c] P<0.05 vs *ApoE*$^{-/-}$

	Initial Body Weight (g)	Cumulative Weight Gain (g)	Food Consumption (g/wk)	Survival (%)	Plasma Cholesterol (mg/dL)	Plasma Triglycerides (mg/dL)	Plasma AST (U/L)	Blood Glucose (mmol/L)
Chow								
Wildtype	22.0 ± 1.0	5.4 ± 1.4	19.6 ± 0.7	100	69 ± 23	30 ± 10	35 ± 14	10.2 ± 1.5
FXR$^{-/-}$	21.2 ± 1.9	5.4 ± 1.3	20.0 ± 1.9	100	139 ± 30[a]	57 ± 6[a]	86 ± 43	11.8 ± 2.3
ApoE$^{-/-}$	24.3 ± 1.5	5.4 ± 2.0	22.9 ± 1.3	100	369 ± 80[a,b]	63 ± 16[a]	.40 ± 23	11.7 ± 2.6
FXR$^{-/-}$*ApoE*$^{-/-}$	20.1 ± 2.9[c]	5.9 ± 1.8	20.4 ± 1.2	100	406 ± 124[a,b]	149 ± 14[a,b,c]	146 ± 49[a,c]	9.9 ± 1.2
HF/HC								
Wildtype	22.9 ± 1.1	8.6 ± 2.7	21.3 ± 2.7	100	96 ± 22	35 ± 9	51 ± 19	10.1 ± 1.7
FXR$^{-/-}$	24.2 ± 1.2	6.2 ± 2.0	18.8 ± 1.3	100	175 ± 28[a]	108 ± 40[a]	219 ± 49[a]	10.8 ± 1.2
ApoE$^{-/-}$	23.7 ± 1.6	6.6 ± 1.6	27.0 ± 0.9[a,b]	100	675 ± 178[a,b]	62 ± 24	92 ± 28[b]	10.7 ± 2.1
FXR$^{-/-}$*ApoE*$^{-/-}$	21.9 ± 1.3	2.3 ± 0.9[a,b,c]	20.4 ± 1.4[c]	66.7	1017 ± 146[a,b,c]	129 ± 41[a,c]	349 ± 151[a,c]	13.8 ± 1.4

100% with the exception of the *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice on HF/HC diet, which exhibited 66.7% survival (Table 2.1). After 12 weeks on regular chow, blood cholesterol levels of *ApoE*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice were significantly greater than *WT* or *FXR*$^{-/-}$ mice fed the same diet, but did not differ significantly from one other. *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice challenged by a HF/HC diet had significantly greater cholesterol levels than all other genotypes on the same diet with blood concentrations approximately 11-fold, 6-fold and 2-fold greater than *WT*, *FXR*$^{-/-}$ and *ApoE*$^{-/-}$, respectively (Table 2.1). After 12 weeks on chow, triglyceride levels in both *FXR*$^{-/-}$ and *ApoE*$^{-/-}$ mice were approximately 2-fold over *WT* mice on the same diet. *FXR*$^{-/-}$*ApoE*$^{-/-}$ triglyceride levels remained significantly higher than the other genotypes on the same diet with blood concentrations of approximately 5-fold, 2-fold and 2-fold greater than *WT*, *FXR*$^{-/-}$ and *ApoE*$^{-/-}$, respectively (Table 2.1). After 12 weeks on a HF/HC diet, blood levels of triglycerides in both *FXR*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice were approximately 3-fold and 2-fold greater than *WT* and *ApoE*$^{-/-}$, respectively. Plasma AST levels for chow fed *FXR*$^{-/-}$ *ApoE*$^{-/-}$ mice were approximately 4-fold greater compared to *WT* and *ApoE*$^{-/-}$ mice fed the same diet (Table 2.1). *FXR*$^{-/-}$ *ApoE*$^{-/-}$mice also had significantly greater plasma AST levels compared to chow fed *FXR*$^{-/-}$ mice. HF/HC feeding led to a general increase of plasma AST levels in all of the genotypes studied. Similar to the chow fed animals, the greatest plasma AST levels measured were those of *FXR*$^{-/-}$ and *FXR*$^{-/-}$ *ApoE*$^{-/-}$ mice. Blood glucose levels did not differ significantly between groups at week 12 of the feeding study (Table 2.1).

Further characterization of alterations in blood lipids due to deletion of FXR in the *ApoE*$^{-/-}$ mouse involved analysis of the lipoprotein profiles of these mice by FPLC (Fig. 2.1). Fractions obtained at elution volumes 14, 20 and 29 mL (corresponding to the

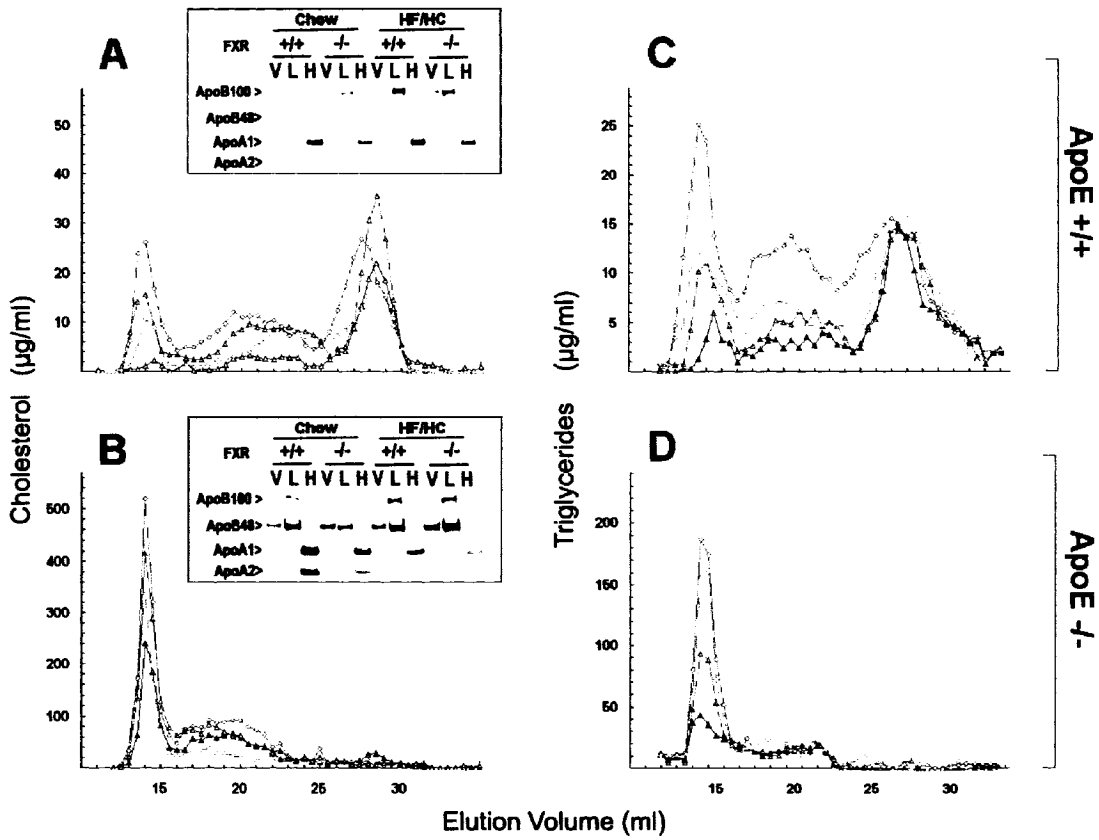

Figure 2.1 FPLC and western blot analysis of plasma lipoprotein and apolipoprotein content.

FPLC cholesterol (A) and triglyceride (C) profiles of wild type mice fed chow (blue) or HF/HC (red) and of FXR (-/-) mice fed chow (yellow) or HF/HC (green). FPLC cholesterol (B) and triglyceride (D) profiles of apoE (-/-) mice fed chow (blue) or HF/HC (red) and of apoE (-/-) FXR (-/-) mice fed chow (yellow) or HF/HC (green). Insets: Apolipoproteins western blot analyses were performed on fractions obtained from FPLC; V=VLDL, L=LDL and H=HDL. Serum (200µl) was pooled from 3-5 animals for each experimental group.

VLDL, LDL and HDL2/3 peaks, respectively) were further characterized by immunoblotting for apolipoprotein content (see Fig. 2.1 *a* and *b* insets). On either chow or the HF/HC diet, *FXR*$^{-/-}$ mice had increased VLDL and LDL lipids as well as reduced HDL2/3 cholesterol, apoA-I and apoA-II, compared with controls. The HDL peak was shifted toward smaller elution volumes (26-28 mL) indicating the presence of large triglyceride-rich HDL1 particles only in the HF/HC fed *FXR*$^{-/-}$ mice (Fig. 2.1*a* and *c*). On the *ApoE*$^{-/-}$ background, FXR deficiency increased the levels of VLDL apoB48 and decreased that of HDL apoA-I and A-II. This was exacerbated by HF/HC feeding with dramatically increased apoB48 associated with VLDL and LDL as well as decreased apoA-I and A-II associated with HDL (Fig. 2.1*b* inset) in *FXR*$^{-/-}$*ApoE*$^{-/-}$mice.

Examination of the lipoprotein distribution patterns for cholesterol in the plasma of chow fed *FXR*$^{-/-}$*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice revealed that *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice had the highest amount of cholesterol in the VLDL fractions. HF/HC feeding of *FXR*$^{-/-}$*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice further increased plasma cholesterol in the form of VLDL in both genotypes. The levels of VLDL and LDL cholesterol were highest in the *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice compared to the other genotypes (Fig 2.1*b*). In addition, *FXR*$^{-/-}$*ApoE*$^{-/-}$ had sharply increased VLDL triglycerides levels compared with *ApoE*$^{-/-}$ animals, upon both diets. Surprisingly, plasma triglycerides, eluting mostly in the VLDL range, were maximal in *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice on both chow and the HF/HC diet (Fig. 2.1*d*).

Given the importance of the liver in lipid homeostasis and RCT, liver histology (H+E examination for necrosis) in HF/HC fed groups in addition to levels of triglyceride and cholesterol (in both chow and HF/HC fed mice) were examined. H+E staining of liver from HF/HC fed mice revealed that both *FXR*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice had areas

of focal necrosis indicated by localized accumulation of cells (most likely neutrophils and/or activated lymphocytes; arrows, Fig. 2.2*a*)[243]. Similar indications of necrosis were not evident in the livers from HF/HC fed *WT* and *ApoE*$^{-/-}$ mice. A qualitative approach to examine hepatic accumulation of lipid was utilized by staining 10 μm sections of liver with Oil Red O (stains lipids a red color Fig 2.2*b*). Livers from *FXR*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice have increased lipid accumulation on a HF/HC diet compared to *WT* and *ApoE*$^{-/-}$ mice on the same diet (seen as large red-colored vacuoles present in liver sections).

Lipids were organically extracted from liver tissue and measured using commercial colorimetric kits for both cholesterol and triglycerides. HF/HC feeding of *FXR*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice led to 4-fold increases in hepatic cholesterol over chow fed mice of the same genotype compared with only 2-fold and 1.6-fold increases in *WT* and *ApoE*$^{-/-}$, respectively (Fig. 2.3*a*). After 12 weeks on a HF/HC diet *FXR*$^{-/-}$ and *FXR*$^{-/-}$ *ApoE*$^{-/-}$ mice had increased hepatic concentrations of cholesterol with increases of approximately 2-fold over both HF/HC fed *WT* and *ApoE*$^{-/-}$, (Fig 2.3*a*). HF/HC feeding increased hepatic triglycerides in *FXR*$^{-/-}$ (5-fold) and *FXR*$^{-/-}$*ApoE*$^{-/-}$ (3-fold) and *WT* mice (2-fold) as compared to respective chow fed controls (Fig. 2.3*b*). Similarly, both *FXR*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice fed the HF/HC diet had increased levels of hepatic triglycerides compared with *WT* and *ApoE*$^{-/-}$ group on the same diet. Hepatic triglyceride concentrations in the *FXR*$^{-/-}$ mice were 2-fold and 3-fold greater than *WT* and *ApoE*$^{-/-}$ on the same diet, respectively (Fig. 2.3*b*). In concordance with the increased hepatic lipid accumulation, the liver weights (as a percentage of body weight) were increased for both HF/HC fed *FXR*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice (5.4± 0.6 and 5.5± 0.6,

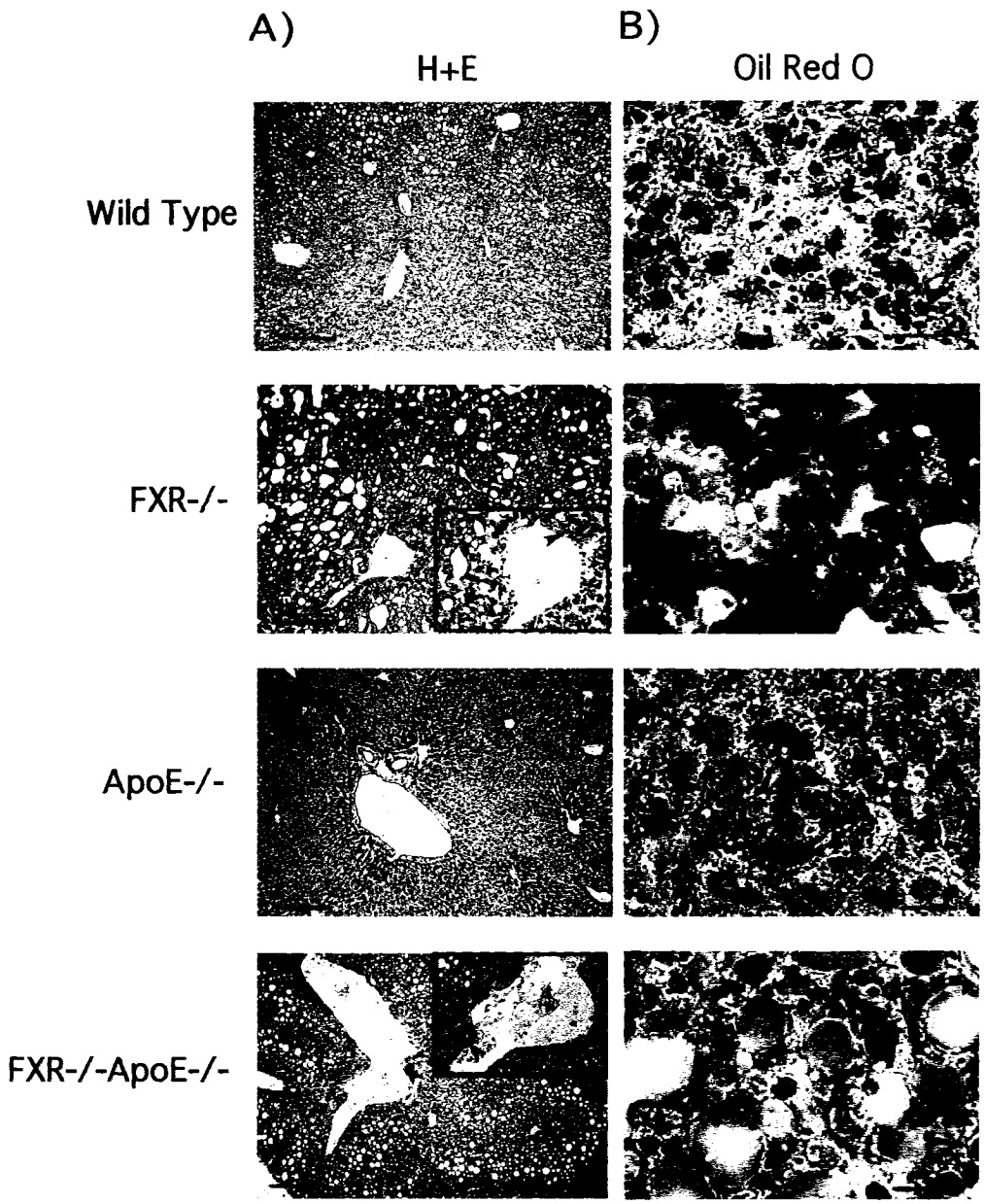

Figure 2.2 Histological analyses of hepatic lipid accumulation and necrosis.
A) H+E stained sections (5 μm) of liver from HF/HC-fed mice (100x). Insets are 40x. Arrowheads indicate accumulation of immune cells. B) Oil-red O-stained sections (5 μm) of liver from HF/HC mice (1000x). Scale bar=50 μm.

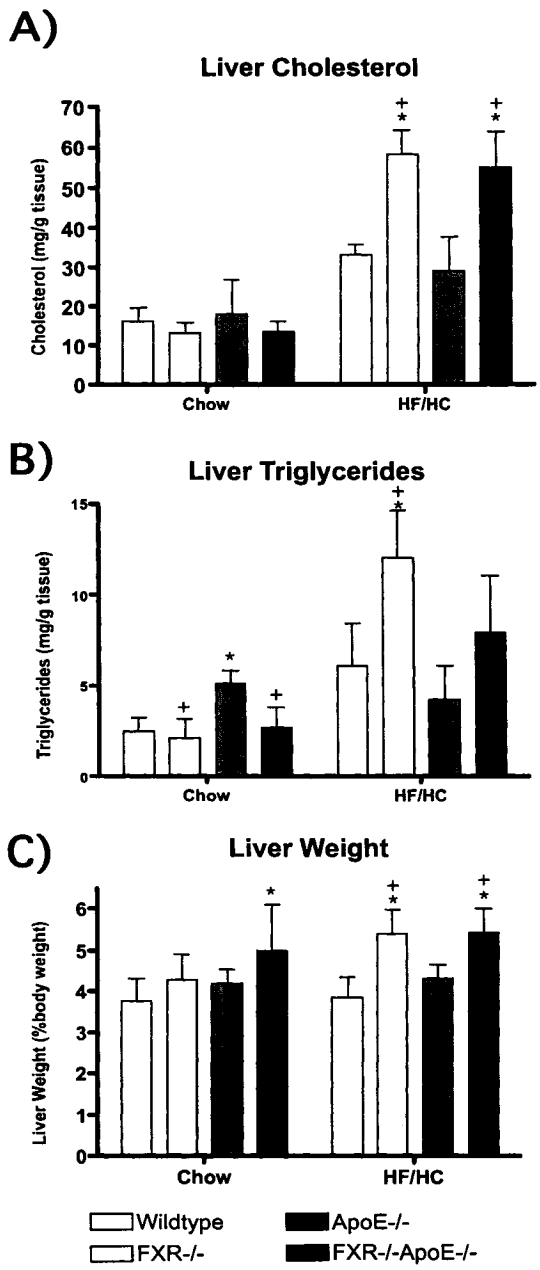

Figure 2.3 Quantitative analyses of hepatic lipid accumulation
Total cholesterol A) triglyceride B) and percent body weight C) of livers from chow and HF/HC-fed mice. *$p<0.05$ versus wildtype and +$p<0.05$ versus ApoE-/- on the same diet. N=3 for hepatic lipids,

respectively) compared to *WT* (3.9± 0.5) and *ApoE*$^{-/-}$ (4.3± 0.4) (Fig. 2.3*c*) mice fed the same diet.

In addition to histological and lipid analyses of livers from these mice, expression of the genes for TNFα and MAC1 (markers of inflammation) was examined using QPCR. Chow fed *FXR*$^{-/-}$, *ApoE*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice had approximately 3-4-fold higher levels of TNFα expression compared to *WT* mice fed the same diet (Fig. 2.4*a*). HF/HC feeding dramaticallyincreased TNFα mRNA levels in both *FXR*$^{-/-}$ and *ApoE*$^{-/-}$ mice to approximately 10-fold over chow fed *WT* mice. *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice exhibited a synergistic increase in TNFα gene expression to approximately 40-fold over *WT* values (Fig. 2.4*a*). Similar to the TNFα expression patterns, both *FXR*$^{-/-}$ and *ApoE*$^{-/-}$ mice fed chow had similar mRNA expression (approximately 5-fold over *WT*) of hepatic MAC1 (Fig. 2.4*b*). Chow fed *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice had approximately 12-fold higher expression than *WT* mice on the same diet. HF/HC feeding led to increases in MAC1 mRNA levels (approximately 15-17-fold over chow fed *WT*) in both *FXR*$^{-/-}$ and *ApoE*$^{-/-}$ mice. Similar to TNFα expression, MAC1 transcript levels showed a synergistic increase in the HF/HC fed *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice (approximately 68-fold higher than *WT*) (Fig. 2.4*b*).

Determination of atherosclerosis was performed using cross-sectional as well as *en face* representations of aortas (stained for lipid-filled plaques) from all animals on both chow and the HF/HC diet (Fig. 2.5 and 2.6). Examination of both *en face* and cross-sectional aortas from the chow fed mice revealed that neither *WT* nor *FXR*$^{-/-}$ animals had any detectable plaques on this diet (Fig. 2.5 and 2.6). Both *FXR*$^{-/-}$*ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice fed chow had small amounts of detectable plaques in both cross-sectional and *en face* representations (Fig. 2.5 and 2.6). The extent of atherosclerosis in aortas from these

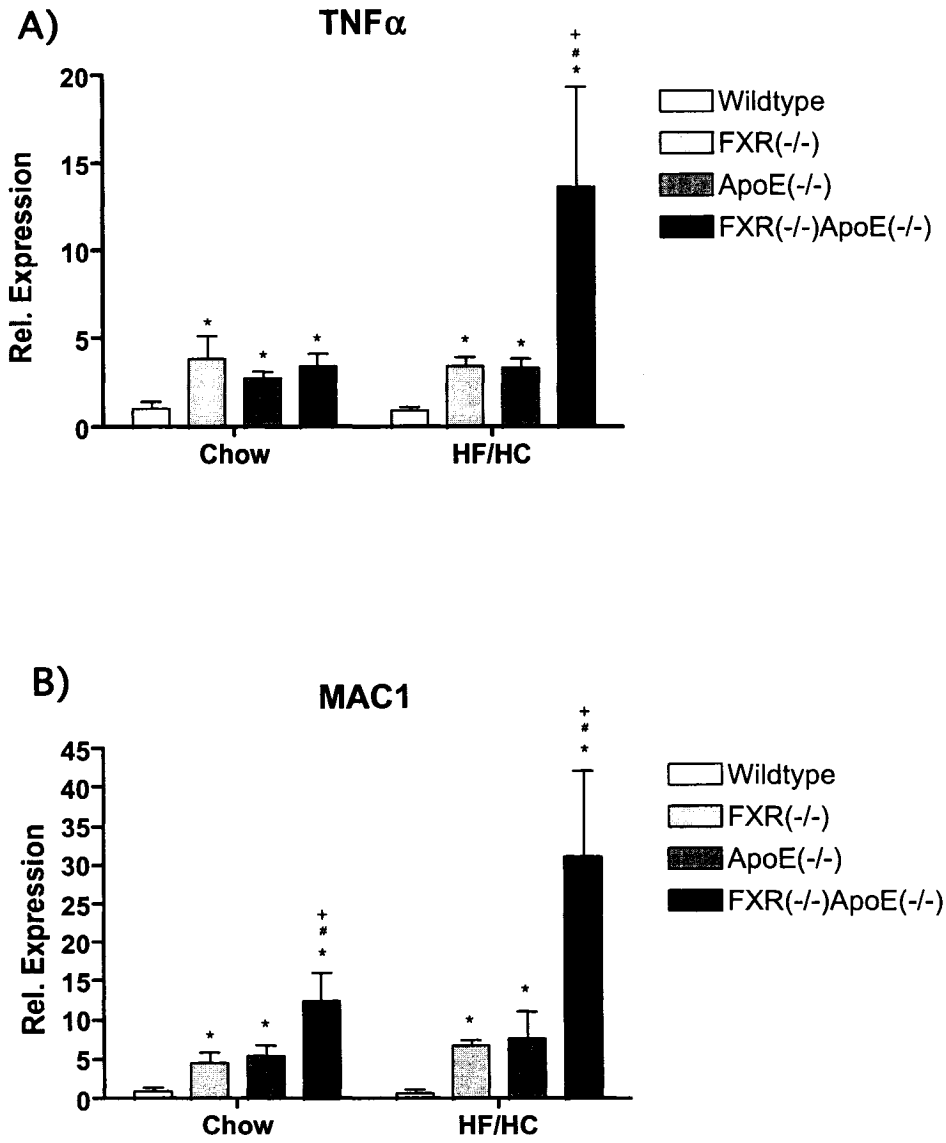

Figure 2.4 Quantitative analysis of hepatic inflammatory gene expression.
Real-time quantitative PCR of A) TNFα and B) MAC1 mRNA levels. All values were normalized to RP-II mRNA levels and are expressed as the fold difference relative to chow fed wildtype mice.*$p<0.05$ versus wildtype, #$p<0.05$ versus FXR-/- and +$p<0.05$ versus ApoE-/- on the same diet. N=4-5

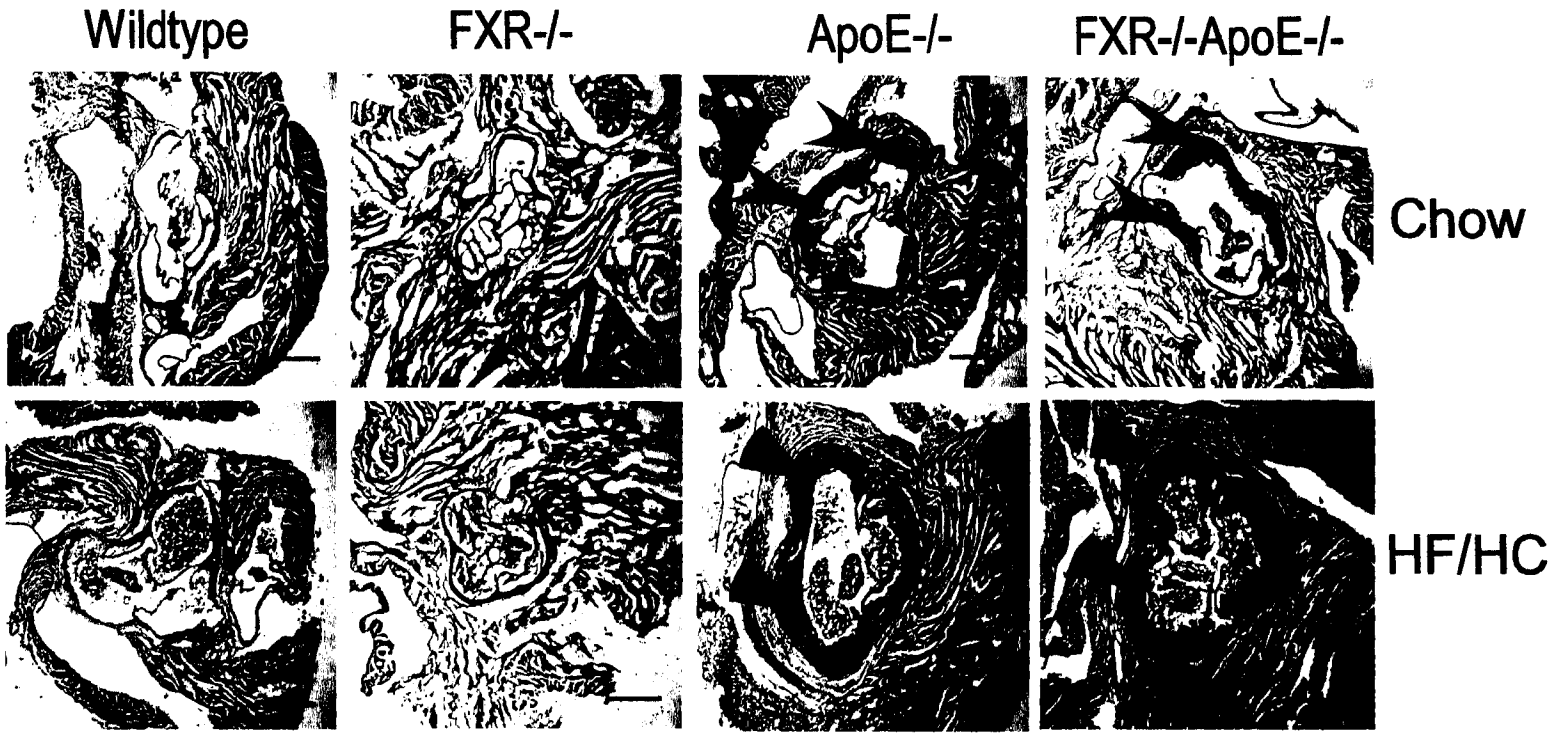

Figure 2.5 Cross-sectional analysis of aortic atherosclerosis in chow and HF/HC fed mice.
5 μm, Oil Red O-stained cross-sections through aortic roots, 700 μm past appearance of the aortic valve, 1000x. Scale bar=50 μm
Arrowheads and wedges indicate atherosclerotic plaques in chow and HF/HC fed animals, respectively.

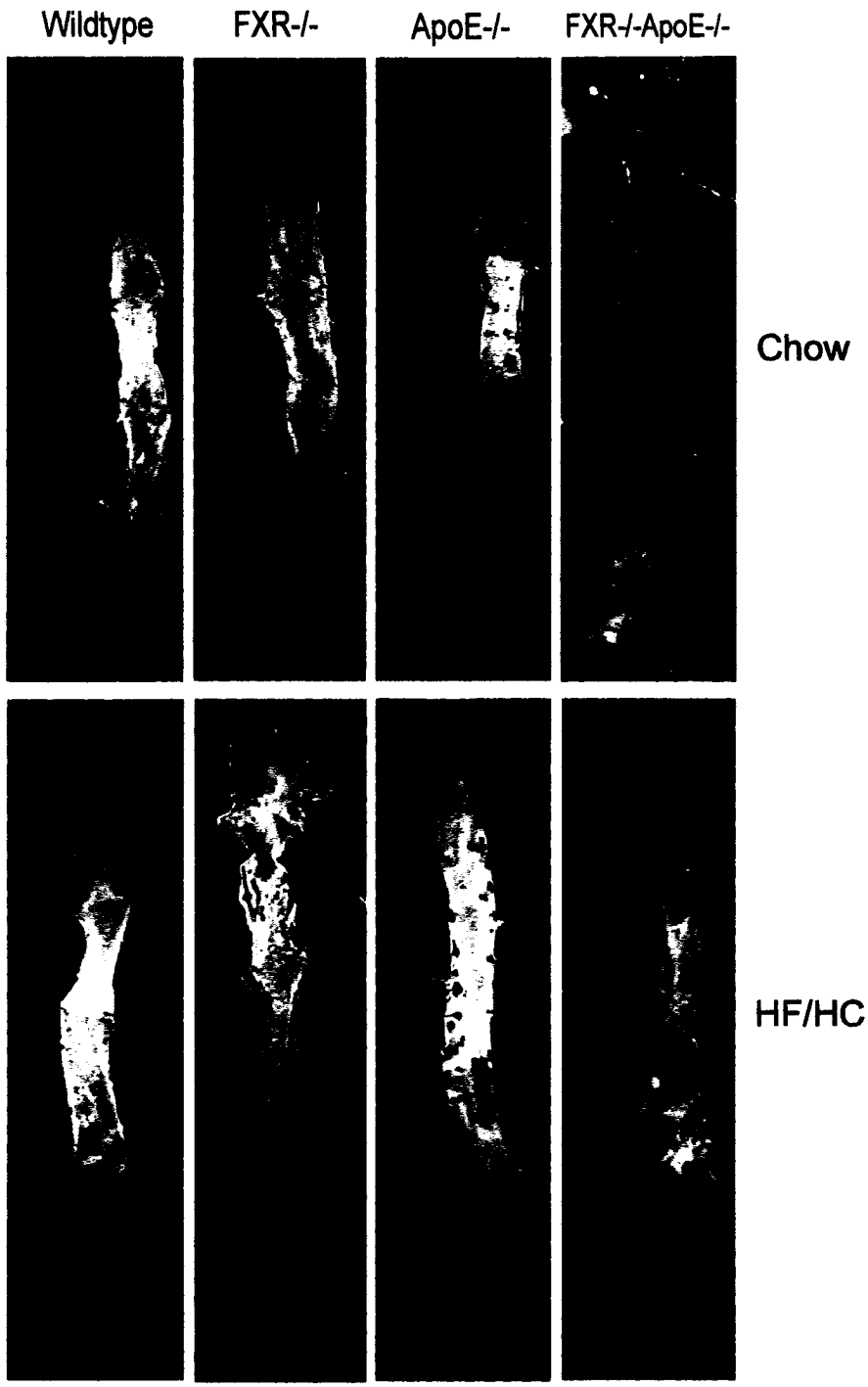

Figure 2.6 *En face* analysis of aortic atherosclerosis in chow and HF/HC fed mice. *En face* staining of Sudan IV-stained aortas from chow and HF/HC-fed mice.

chow fed *ApoE*$^{-/-}$*FXR*$^{-/-}$ and *ApoE*$^{-/-}$ mice, as determined by quantitation of *en face* plaques, was not significantly different (Fig. 2.7*a*). Challenging both *WT* and *FXR*$^{-/-}$ mice with a HF/HC diet did not result in detectable atherosclerotic plaques as evidenced by cross-sectional or *en face* aortas from these mice (Fig. 2.5 and 2.6). In contrast, HF/HC feeding of *ApoE*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice resulted in large increases in the extent of atherosclerosis in aortas of these mice (Fig. 2.5 and 2.6). Quantitation of atherosclerosis in *en face* aortas from these mice revealed that the *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice had approximately double the amount of plaques compared to the *ApoE*$^{-/-}$ mice (37.2%± 11.4 relative to 18.2%±3.2) (Fig. 2.7*b*).

In order to further study the effect of FXR on atherosclerotic disease, *ApoE*$^{-/-}$ mice were fed a HF/HC diet for a total of 10 weeks. After 6 weeks of this diet, the *ApoE-/-* mice were administered GW4064 via oral gavage treatment in addition to the HF/HC diet for 30 days. Blood cholesterol levels of GW4064 treated *ApoE-/-* mice were significantly decreased from those of vehicle treated *ApoE-/-* mice at 2, 3 and 4-weeks of treatment (Fig. 2.8*a*). Atherosclerosis was assessed in the vehicle and GW4064-treated *ApoE-/-* mice via Sudan-IV staining of en face aortas (Fig. 2.8*b*). The extent of atherosclerosis in aortas from the GW4064- and vehicle-treated *ApoE*$^{-/-}$ mice, as determined by quantitation of *en face* plaques, was not significantly different (Fig. 2.8*c*).

2.5 DISCUSSION

The present study investigated the pathological consequences of deficient FXR function for the development of atherosclerotic disease. Challenging mice that lacked both functional FXR and ApoE genes with a HF/HC diet led to a failure to gain weight to a

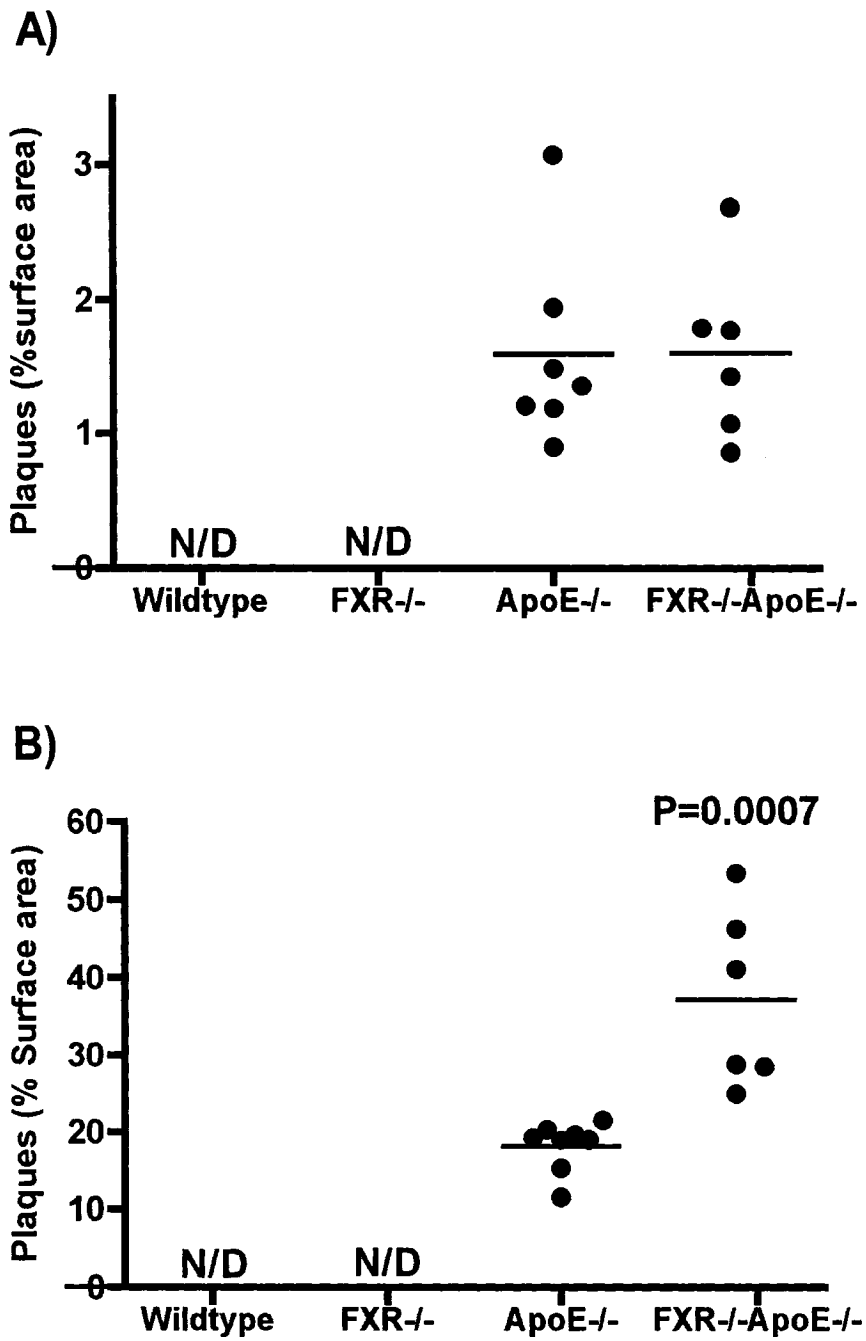

Figure 2.7 Quantitation of aortic atherosclerosis in chow and HF/HC fed mice. A) Quantitation of atherosclerotic plaques in chow fed mice (% total en face aorta surface area). B) Quantitation of atherosclerotic plaques in HF/HC fed mice (% total en face aorta surface area). N=5-8. P=0.0007 compared to ApoE(-/-) mice on a HF/HC diet (Mann-Whitney U test).

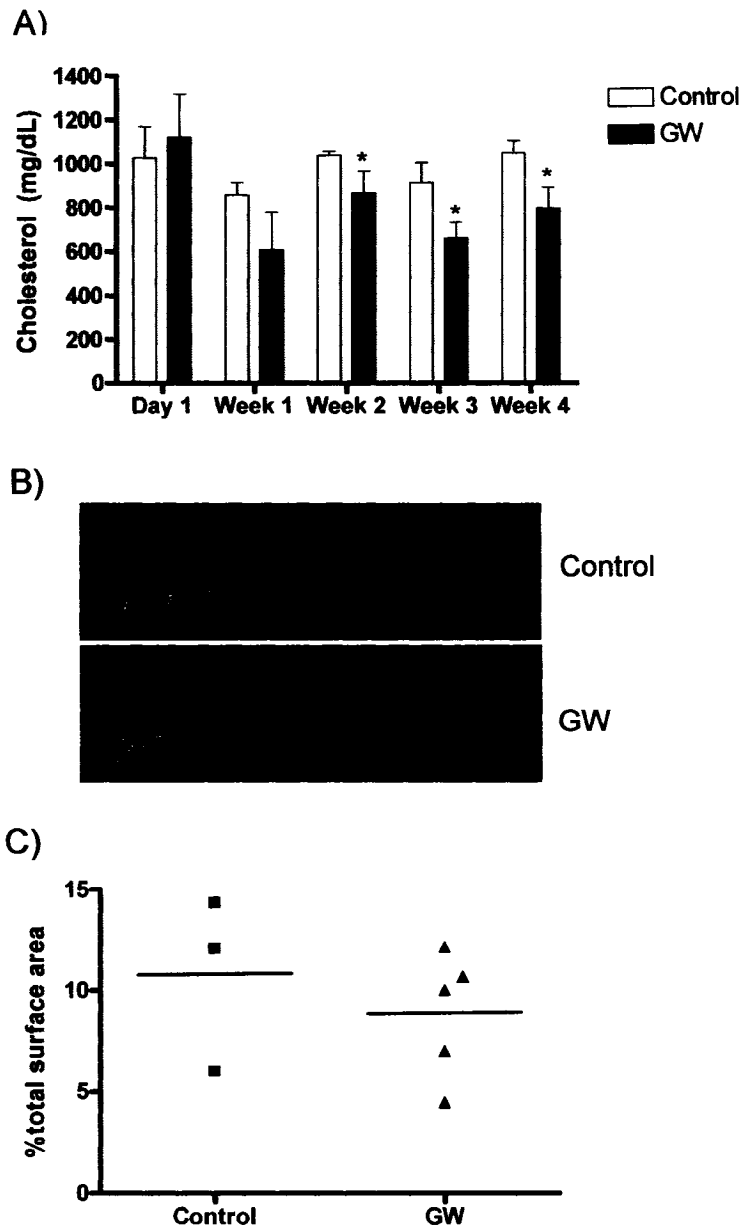

Figure 2.8 Analyses of blood cholesterol and atherosclerosis in GW4064 treated apoE-/- mice.

A) Cholesterol levels at day 1 and weeks 1-4 of chow and HF/HC-fed apoE-/- mice treated for 30 days with GW4064. Representative photos B) and quantitation C) of *en face*, Sudan-IV-stained aortas from HF/HC-fed apoE-/- mice treated for 30 days with GW4064. *P<0.05 vs control (vehicle treated) mice (unpaired t test). N=3-5.

similar extent and decreased survival compared to *WT*, *FXR*$^{-/-}$ or *ApoE*$^{-/-}$ mice fed the same diet. Loss of functional FXR also led to further increases of total blood cholesterol and triglyceride levels, as well as increased VLDL and LDL in the *ApoE*$^{-/-}$ model. HF/HC fed *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice experienced the greatest detrimental effects on hepatic status as evidenced by massive lipid accumulation, focal necrosis and markedly increased inflammatory gene expression and plasma AST levels. Ultimately, all of these phenotypic characteristics were associated with the most severe degree of atherosclerotic lesion formation and the lowest survivability in the HF/HC fed *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice compared to the other genotypes studied. Treatment of HF/HC fed *ApoE*-/- mice with GW4064 resulted in decreased cholesterol in these mice but no change in the extent of atherosclerotic disease.

Our previous work demonstrated decreased hepatic expression of the scavenger receptor B1 in *FXR*$^{-/-}$ mice. This was associated with decreased hepatic uptake of HDL cholesterol as well as increased synthesis of highly atherogenic apoB-containing lipoproteins [210]. Additionally, *FXR*$^{-/-}$ mice exhibit hyperabsorption of cholesterol [210] and triglycerides (unpublished results) from the intestine. All of these factors are believed to contribute to the proatherogenic plasma total lipid and lipoprotein profile exhibited by *FXR*$^{-/-}$ mice. ApoE is required for hepatic LDL-receptor-mediated clearance of remnants of VLDL in the liver [244] and consistent with previous studies [245, 246], we observed an almost complete absence of HDL in the plasma of *ApoE*$^{-/-}$ mice. Thus, the extremely atherogenic plasma lipid and lipoprotein profile exhibited by *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice, particularly when challenged with a HF/HC diet, reflects the striking genetic interaction between the loss of function of the *FXR* and *apoE* alleles.

Histological analysis of livers from HF/HC fed mice revealed that both *FXR*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice revealed areas of focal necrosis that were absent from the livers of *ApoE*$^{-/-}$ and *WT* mice fed the same diet. Consistent with this observation, HF/HC fed *FXR*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice also exhibited the highest levels of plasma AST which may have resulted from the release of this enzyme from necrotic hepatocytes. Hepatic lipid accumulation is a hallmark feature of the pathogenesis of necrotic liver diseases such as non-alcoholic steatohepatitis [247]. Thus, given the massive hepatic accumulation of lipid in both the *FXR*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice it is not surprising that chronic HF/HC feeding caused damage to the livers of these animals. Furthermore, *FXR*$^{-/-}$ mice present with a cholestatic phenotype characterized by elevated plasma bile acids [209]. This may have also contributed to the hepatic necrosis seen in *FXR*$^{-/-}$ and *FXR*$^{-/-}$*ApoE*$^{-/-}$ when fed a HF/HC diet. Indicators of an altered inflammatory state in the liver included changes in expression of the immune receptor MAC1 and the inflammatory cytokine TNFα. MAC1, also known as CD11b/CD18 and complement receptor 3, is predominantly expressed on macrophages, monocytes, neutrophils and natural killer cells and is involved in many functions including activation and migration of these cells [248]. TNFα is a pro-inflammatory cytokine released by tissue macrophages and T-cells at a site of injury and essentially acts to activate and attract other immune cells [249]. TNFα has also been shown to induce the upregulation of MAC1 in neutrophils [250]. Therefore, the dramatic increase of MAC1 and TNFα mRNA levels in the livers of HF/HC fed *FXR*$^{-/-}$ *ApoE*$^{-/-}$ most likely represented the combined effects of inflammation-induced expression of these genes in hepatocytes and infiltrating cells of the immune system such as macrophages and neutrophils. In addition, increased TNFα levels in the *FXR*$^{-/-}$*ApoE*$^{-/-}$

mice may have added to the induction of MAC1 expression in these immune cells. Combined with the failure to gain as much body weight and reduced survival compared to the other genotypes, these data suggest that hepatic toxicity and severe dysfunction occurred in the HF/HC challenged *FXR*$^{-/-}$*ApoE*$^{-/-}$ mice.

Although loss of FXR function alone was not sufficient to cause atherosclerosis when mice were challenged with a HF/HC diet, combination of FXR-deficiency with that of ApoE resulted in a dramatic worsening of the disease. Unlike humans, mice transport the majority of plasma lipids in HDL particles and this is thought to contribute to the inherent resistance of this species to atherosclerotic disease. Compared to *WT*, *FXR*$^{-/-}$ mice exhibit a proatherogenic plasma lipoprotein profile characterized by the presence of increased amounts of highly atherogenic VLDL and LDL particles in the plasma. Therefore, it was somewhat surprising that no atherosclerosis was detected in the *FXR*$^{-/-}$ mice fed the HF/HC diet. ApoE is synthesized in large quantities by the liver, is a constituent of all lipoproteins except LDL and functions as a ligand for receptors that clear chylomicrons and VLDL remnants from the blood [245]. ApoE is also synthesized by monocytes and macrophages, and is thought to promote cholesterol efflux and modulate inflammatory responses in atherosclerotic vessels [246]. Thus, the increased susceptibility of *ApoE*$^{-/-}$ mice to spontaneous- and diet-induced atherosclerotic lesions results from a loss of critical functions at least two sites, the liver and macrophage. In contrast, while FXR has well defined regulatory roles in hepatic function, expression of this receptor in macrophages is undetectable (unpublished results and [251]). Thus, despite the severe proatherogenic plasma lipid profile of *FXR*$^{-/-}$ mice, it is likely that macrophages in the vessel walls of these mice remain resistant to lipid accumulation and

plaque formation. Only when the loss of functional FXR was combined with the loss of ApoE was a genetic interaction resulting in more severe dyslipidemia, impaired hepatic function and atherosclerosis revealed.

Recently, treatment with the selective FXR agonist GW4064 was shown to prevent the development of gallstones in a mouse model of the disease [252]. Importantly, this study demonstrated that modulation of FXR could be used to treat a specific disease process and more generally illustrated the therapeutic potential of targeting this receptor. Although treatment of HF/HC fed apoE-/- mice with GW4064 for 30 days did not prevent the development of atherosclerosis, this treatment did improve the high blood cholesterol levels present in these mice. Presently, however, the benefits of manipulating FXR function as a novel therapeutic approach for atherosclerosis are not clear. Presumably, activation of FXR *in vivo* would lead to decreased conversion of cholesterol to bile acids, an effect detrimental to the treatment of coronary artery disease and hyperlipidemia. However, data generated in the present study as well as previous work with $FXR^{-/-}$ mice has demonstrated that the role of FXR in lipid homeostasis is more complicated than previously thought. For instance, $FXR^{-/-}$ mice exhibit increased intestinal absorption of cholesterol and triglycerides, decreased hepatic uptake of HDL and increased synthesis of apoB-containing lipoproteins [210]. All of these characteristics associated with the loss of FXR function argue that antagonism of this receptor would lead to an undesirable disruption of systemic lipid homeostasis that ultimately may be detrimental to the treatment of atherosclerosis. In addition, it is not known whether modulation of hepatic FXR function can affect the inflammatory state at the level of the vessel wall in addition to that seen in the liver in this study. Studies of the lipid-lowering

mechanism of an herbal product, guggulsterone, revealed antagonism of FXR and a lowering of hepatic cholesterol accumulation [213, 214] as well as hypolipidemic effects and increased hepatic LDL uptake [253]. These findings lead to the question of why antagonism of FXR by guggulsterone is lipid-lowering while targeted disruption of the FXR gene leads to hyperlipidemia and increased atherosclerosis when combined with loss of apoE function. One answer to this controversy may be provided by recent work demonstrating that guggulsterone antagonizes a number of nuclear receptors (including glucocorticoid, mineralocorticoid and androgen receptors) with up to 100-fold greater potency than that exhibited for FXR [219]. Furthermore, guggulsterone is also a potent activator of a number of other nuclear receptors including the estrogen, progesterone and pregnane X receptor [254]. Therefore, the *in vivo* effects of guggulsterone are likely to be mediated by several mechanisms other than antagonism of FXR.

In conclusion, the present study demonstrates that loss of FXR function causes increased atherosclerosis in the ApoE-deficient mouse model of this disease. Other outcomes precipitated by the loss of FXR in this model included decreased weight gain and survival, increased hepatic and plasma lipids, increased hepatic inflammation and a more severe plasma lipoprotein profile. In addition, treatment of the apoE-/- model of atherosclerosis with a specific FXR agonist led to decreased blood cholesterol levels in these mice despite a lack of effect on the extent of atherosclerosis. This study is the first to demonstrate a pathogenic role for FXR in atherosclerosis using an *in vivo* model of the disease. In addition, this study is the first to demonstrate the effects of chronic administration of a specific FXR agonist in a mouse model of atherosclerosis. In contrast to other nuclear receptors with roles in macrophage lipid homeostasis (e.g LXRs,

PPARs), the worsening of atherosclerosis caused by targeted deletion of FXR appears to be a consequence of the loss of function of this receptor in liver and gut only. Our data demonstrate the pathological consequences of a lack of FXR function and the potential for genetic interactions of deleterious mutations of the gene for this nuclear receptor with other gene mutations known to increase the risk for cardiovascular disease.

CHAPTER 3

DISRUPTION OF ENERGY METABOLISM IN THE FXR-/- MOUSE

3.1 ABSTRACT

FXR is a nuclear receptor that functions to maintain bile acid homeostasis through regulation of genes involved in the conversion of cholesterol to bile acids as well as those involved in bile acid transport. Work presented in this thesis demonstrated an expanded role for FXR in systemic lipid homeostasis through observations of the hyperlipidemic FXR-/- mouse. In addition, this work has also established the importance of FXR in the development of atherosclerosis. Although many observations have been made regarding the phenotype of FXR-/- mice, a thorough analysis of altered gene expression of these mice has yet to be performed. Thus, through the use of cDNA microarray and northern blot analyses of of FXR-/- versus WT mice, we have discovered alterations in several hepatic genes including the adipose differentiation-related protein, apoA-IV and retinoic acid responder protein. Through the use of *in vitro* promoter assays it was determined that FXR does not directly regulate transcription of these genes. However, FXR-/- mice may have metabolic abnormalities as exhibited by decreased weight gain when fed both chow and HF/HC diets despite having normal food consumption. To further characterize potential metabolic abnormalities in these mice, alterations in gene expression were examined by QPCR in both white and brown adipose. Our results demonstrate that loss of FXR function in mice leads to a disruption in metabolic homeostasis as evidenced through decreased gene expression of leptin in white adipose tissue (WAT) as well as increased expression of PGC-1α and uncoupling protein 1 (UCP-1) in brown adipose. These disruptions in FXR-/- metabolism may create an altered hormonal environment responsible for changes in hepatic gene expression observed in these mice.

3.2 INTRODUCTION

As outlined previously, the liver is essential for systemic metabolic homeostasis. Brown and white adipose tissues are also important in this respect. In order to discuss altered gene expression in white and brown adipose as a result of FXR deficiency, a brief introduction into the normal function of these tissues in necessary.

3.2.1 Cellular Respiration

Cellular respiration involves the oxidation of nutrients such as glucose to water and carbon dioxide, and in the process of releasing energy that is stored in molecules of ATP. The first steps in cellular respiration occur in the cytosol and involve glycolysis that catabolizes glucose to pyruvate. In the mitochondrial matrix, pyruvate molecules are oxidized to form acetyl coA. Acetyl coA then enters the Krebs, or citric acid cycle which, through a series of enzymatic steps produces the molecules nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2). These two molecules then enter the electron transport chain (ETC), a series of protein complexes present on the inner mitochondrial membrane. The function of the ETC is to shuttle electrons from NADH and FADH2 to various protein complexes and finally to a molecule of oxygen, forming water. The energy released as electrons is transferred down the ETC and used by protein complexes to pump protons against the gradient into the intermembrane space. The importance of this proton pumping lies in the fact that the only route for these protons to move down their gradient and reenter the matrix is by passing through the ATP synthase complex. Thus the energy produced by the transfer of electrons is harnessed, or coupled, to ATP synthesis.

3.2.2 Brown Adipose and Adaptive Thermogenesis

Mammals have evolved to function at optimal environmental temperatures. When outside temperatures drop below these levels, various acute physiological and behavioral measures such as vasoconstriction, piloerection and curling-up of the body are initiated in order to keep the body at optimal temperature [255]. A long-term measure that has evolved in mammals to help maintain body temperature during persistent cold conditions is the induction of adaptive thermogenesis. In mammals the major site of adaptive thermogenesis is brown adipose tissue and induction of this process occurs in both humans and rodents [256, 257]. A crucial step in initiation of adaptive thermogenesis is the cold-induced release of norepinephrine (NE) or adrenaline from sympathetic nerve fibers innervating brown adipocytes (Fig. 3.1). NE acts on β_3-adrenergic receptors on the surface of brown adipocytes, initiating a signaling cascade that activates adenylyl cyclase, increases the intracellular concentration of cyclic adenosine monophosphate (cAMP) and activates protein kinase A (PKA) [258, 259]. The major goal of NE signaling in brown adipose is to increase lipolysis of stored triglyceride, releasing free fatty acids used as fuel for thermogenesis [260, 261]. Therefore, one of the targets of NE signaling in brown adipocytes is hormone sensitive lipase when becomes phosphorylated by PKA breaks down triglyceride stores into free fatty acids, Fig. 3.1 [262]. Fatty acids that are released from stores move into mitochondria where they are oxidized through the β–oxidative process to produce acetyl CoA. Acetyl coA enters the citric acid cycle to produce substrates for the ETC and ATP synthesis. In normal, coupled electron transport, ATP molecules are formed from the movement of protons down their gradient through the ATP synthase complex. In brown adipose tissue there are high concentrations of a

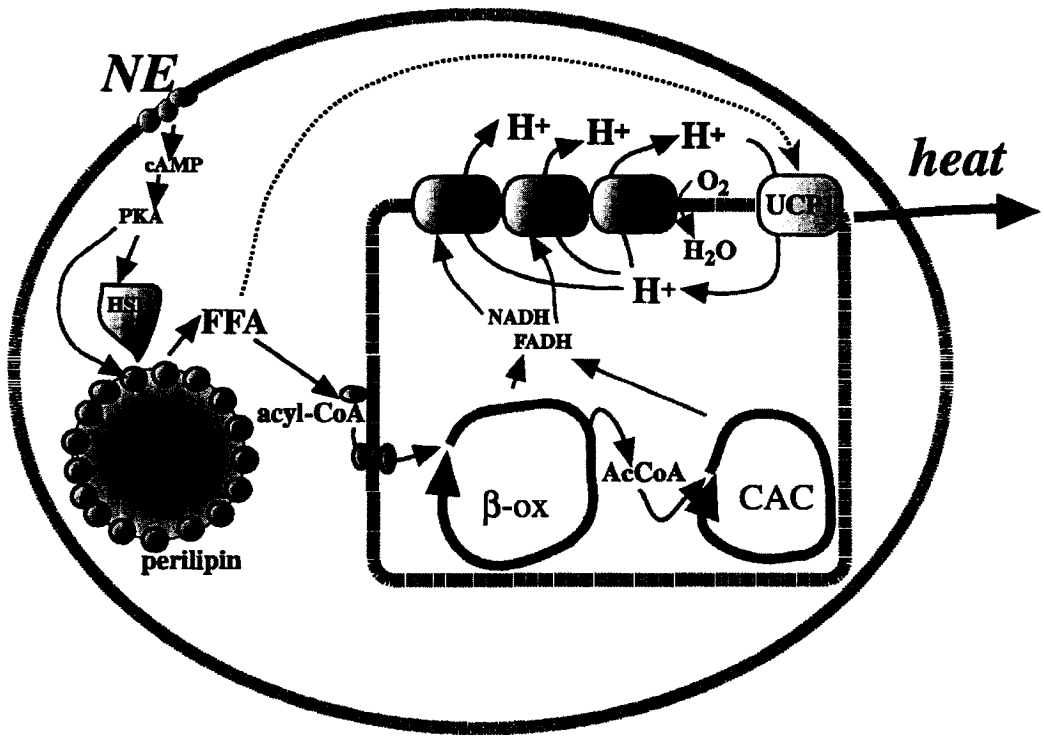

Figure 3.1 Adaptive thermogenesis in brown adipose
Exposure to cold temperatures stimulates the release of norepenephrine (NE) from sympathetic neurons adjacent to brown adipocytes. NE binds to β_3-adrenergic receptors on the surface of brown adipocytes and stimulates a signaling cascade, activating PKA. PKA phosphorylates many different targets including hormone-sensitive lipase (HSL) that then acts to break down triglyceride stores into free fatty acids (FFA). Acyl CoA synthases in the cytosol esterify fatty acids to acyl-CoA which then enters the mitochondrion where it is then oxidized to acetyl CoA through β-oxidation (β-ox). Acetyl CoA then enters the citric acid cycle (CAC). Both β-ox and the CAC produce molecules of FADH and NADH, substrates for the electron transport chain (ETC). The ETC shuttles electrons from substrates through its various protein complexes, pumping protons (as H+) into the intermembrane space. These protons flow down their gradient back into the mitochondrial matrix through an ATP synthase complex, thus coupling the transfer of electrons to the synthesis of ATP. In adaptive thermogenesis, this process becomes uncoupled through the actions of an uncoupling protein (UCP-1) that acts as a proton pump, causing leakage in the system so that the energy released from the transfer of electrons is released as heat instead of being trapped in molecules of ATP. Figure taken from [257].

protein called UCP-1 [263, 264]. Although the exact mechanism is unclear, it is thought that UCP-1, with fatty acids serving as cofactors, acts as a proton carrier independent of ATP synthase to move protons down their concentration gradient, thereby accelerating cellular respiration without having to form ATP molecules. Thus UCP-1 acts to "uncouple" the ETC from ATP synthesis. Instead of forming ATP, the energy released from the transfer of electrons down the ETC is released as heat. Although it was originally thought that adaptive thermogenesis could only occur in brown adipose, it has been discovered that this process can also occur in other tissues of the body such as skeletal muscle. The uncoupling proteins (UCP-2 and -3) may mediate this adaptive thermogenesis in these tissues [265, 266].

Another target of adrenergic signaling in brown adipose is the transcriptional coactivator PGC-1α [267]. Increases in cAMP due to β_3-adrenergic receptor activation induce expression of PGC-1α that acts in turn to coactivate the transcriptional activity of various nuclear receptors such as PPARα, PPARγ and thyroid hormone receptor on the UCP-1 promoter [257, 268, 269]. In addition, PGC-1α acts to coactivate nuclear respiratory factor-1 and -2 (NRF-1 and NRF-2) actions on promoters of target genes involved in mitochondrial biogenesis [270, 271].

Adaptive thermogenesis is regulated by many different hormones and nutritional states. Decreased sympathetic stimulation that occurs in fasting mediates decreases in the body's metabolic rate as well as increase the efficiency of essential metabolic processes. Thus, adaptive thermogenesis is decreased during this state [272]. By contrast, obesity is associated with decreased metabolic efficiency and increased adaptive thermogenesis [273]. Leptin, a hormone that is increased in obesity, serves to increase sympathetic

activity. Thus, brown fat thermogenesis is increased in obesity [274, 275]. Other hormones important in determining the level of adaptive thermogenesis in brown adipose are insulin, glucocorticoids, glucagon and the thyroid hormone [276-278].

3.2.3 White Adipose

In recent years it has become apparent that WAT has additional roles in the body outside that of a storage depot for triglycerides. WAT produces and secretes many different proteins including hormones such as leptin and resistin, cytokines such as TNF-α and proteins important for lipoprotein metabolism such as apoE and CETP [279, 280]. The functions of leptin and adiponectin will be discussed in this section.

Leptin, the protein deficient in ob/ob mice, is an important molecule with many functions in the body [281]. Levels of leptin expression and secretion increase with the level of obesity in the body and fall after weight loss in both rodents and humans [282, 283]. The receptor for leptin is expressed in many tissues including the hypothalamus and WAT [284]. Important physiological effects of leptin include suppression of appetite and increased energy expenditure. The anorexigenic effects of leptin may be mediated indirectly through interactions with the neuroendocrine system and resultant suppression of transcription of neuropeptide Y, a protein with appetite-inducing properties [285]. Leptin also directly affects tissue metabolism by decreasing triglyceride and fatty acid synthesis and increasing fatty acid oxidation and thermogenesis, thus improving glucose homeostasis and insulin sensitivity [286-289].

Expression of leptin by adipose tissue is directly related to fat content of the body. However, levels of leptin expressed by WAT can be modulated by many different

factors. Stimulation of leptin expression can occur through increases in insulin, glucocorticoids and cytokines while repression of leptin expression by WAT can occur via exposure to cold, fasting and increased sympathetic tone [290-294].

Another protein released by WAT, adiponectin, has been found to have important roles in metabolism. Unlike leptin, expression levels of adiponectin in WAT drop in response to obesity [295]. Adiponectin binds to its receptors in hepatic and skeletal muscle tissues and signals an increase in glucose utilization and catabolism of fatty acids, thereby increasing insulin sensitivity of these tissues [296-299].

3.2.4 Summary and Objectives

Given that a thorough comparison of altered hepatic gene expression in FXR-/- and WT mice has yet to be performed, we endeavored to discover potential novel FXR targets through examination of altered gene expression in these mice. Characterization of genes with altered expression in FXR-/- mice is essential for further insight into FXR function in lipid homeostasis and atherosclerotic disease. We demonstrate, through cDNA microarray and northern blot analysis of chow and HF/HC-fed mice, increased hepatic expression of apoA-IV and decreased expression of adipose differentiation-related protein and retinoic acid responder protein in FXR-/- mice. Through the use of *in vitro* promoter analyses we demonstrate a lack of direct regulation by FXR in the transcription of these genes. Further examination of altered phenotype of FXR-/- mice revealed a resistance to weight accumulation on both chow and HF/HC diets. This apparent lack of weight gain in FXR-/- mice was accompanied by increased expression of genes important

in adaptive thermogenesis in brown adipose tissue as well as a decreased expression of the leptin gene in WAT.

3.3 MATERIALS AND METHODS

3.3.1 Animals and Diets

The background of all mice used in this study was C57BL/6J. *FXR*$^{-/-}$ mice (from our breeding colony, back-crossed 10 generations to congenic C57BL/6J) and C57Bl/6J mice (controls) were used in all studies. 7-8 week-old male mice were placed on either chow (7% fat) (#F4516, based on AIN-93G diet) or a HF/HC diet containing 16% fat, 1.25% cholesterol (#F4515) (Bioserv, Frenchtown, New Jersey) for 16 weeks. Weights of individual mice were measured weekly using a standard scale. Weekly weights of mice were calculated as a percent of their respective weights measured at day 1 of the feeding study. Mice were housed at room temperature under a 12 h light/dark cycle and provided food and water *ad libitum*. All procedures were conducted at the Carleton Animal Care Facility in accordance with Canadian Council on Animal Care guidelines.

3.3.2 RNA Isolation and QPCR

Total hepatic and brown adipose RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) as per the supplier's instructions. Total WAT RNA was isolated using the RNeasy Mini Kit (Qiagen, Mississauga, ON). Total RNA (2 μg) from white and brown adipose was reverse transcribed using Stratascript Reverse Transcriptase (Stratagene, La Jolla, CA) with random hexamers pd$(N)_6$ according to the supplier's instructions. The synthesized cDNA was then amplified by QPCR using a Stratagene MX3000p

thermocycler in a total volume of 25 μl with Brilliant SYBR Green QPCR Master Mix. QPCR Murine primer sequences are as follows: PGC-1α: 5'-GAA GTG GTG TAG CGA CCA ATC-3' (Forward) and 5'-AAT GAG GGC AAT CCG TCT TCA-3' (reverse); ADRP: 5'-CTT ATG TCA GTA CAA AGA TCA-3' (forward) and 5'-GTA CTG GTC TAC CAG CAG CTC-3' (reverse); leptin: 5'-GAC ACC CCT GTG TCG GTT C-3' (forward) and 5'-CTG CGT GTG TGA AAT GTC ATT G-3' (reverse); adiponectin: 5'-AGC CGC TTA TAT GTA TCG CTC A-3' (forward) and 5'-TGC CGT CAT AAT GAT TCT GTT GG-3' (reverse); UCP1: 5'-CAC TCA GGA TTG GCC TCT ACG-3' (forward) and 5'-GGG GTT TGA TCC CAT GCA GA-3' (reverse) and GAPDH: 5'-GAA GGT CGG TGT GAA CGG ATT TGG C-3' (forward) and 5'-TTG ATG TTA GTG GGG TCT CGC TCC TG-3' (reverse). Thermal cycling conditions were performed as previously described [300]. Relative C_T values were obtained by the $\Delta\Delta C_T$ method [242] using a threshold of 10 standard deviations above background for C_T.

3.3.3 Northern Blot

For northern blot, 10 μg of hepatic RNA from FXR-/- and WT mice (fed HF/HC or chow diets for 16 weeks) was separated on a 1% agarose gel containing 0.22 M formaldehyde and 20 mM (3-[N-Morpholino]propanesulfonic acid) or MOPS in buffer containing 0.01 M MOPS and 0.2 M formaldehyde and transferred overnight to a Immobilon charged nylon membrane (Millipore, Bedford, MA) using downward capillary action and 20X SSC as the transfer buffer. The membrane was UV crosslinked and dried at 65° for 1 h. DNA probes were prepared in a total volume of 42 μl using Prime-it RmT Random Primer Labeling kit, 75 ng of probe, 5 μl of $^{32}\alpha$-P-dCTP and 3 μl of Klenow enzyme as per manufacturer's instructions (Stratagene). $^{32}\alpha$-P-CTP-incorporated probes were

filtered through a microspin G-50 column (Amersham Biosciences, Piscataway, NJ), diluted in water and heated to 95° for 10 minutes. Probe incorporation of $^{32}\alpha$-P-CTP was assessed through addition of 2 μl of probe to scintillation fluid and measurement of counts per minute (cpm) using a LS 6500 scintillation counter (Beckman Coulter, Missisauga, ON). Prepared probes were added to 10 ml of PerfectHyb (Sigma-Aldrich, St. Louis, MO) hybridization buffer to a total of 2 x 10^6 cpm. RNA-containing membranes were added to glass tubes containing the prepared probes in hybridization buffer and incubated, while rotating, at 65° overnight. Blots were washed using standard protocols and placed in phosphorimaging screen cassettes overnight and visualized using a Molecular Dynamics Storm 840 Phosphorimager system (Sunnyvale, CA). The cDNA probes for murine ADRP, ApoC-II, ApoA-IV, RARESP and β-actin were prepared by restriction enzyme digestion of cDNA-expressing PCRII plasmids previously cloned in our lab using a TOPO cloning kit (Invitrogen) and cDNA from reverse transcribed mouse liver RNA.

3.3.4 Microarray Analyses

Total hepatic RNA was isolated using the Trizol method (as described previously). RNA from FXR-/- and WT male mice was pooled (3-5 animals) to obtain a total quantity of 10 μg. Messenger RNA was reverse transcribed from total RNA using poly-thymidine oligonucleotides and amino allyl-dUTP using the Stratagene Fairplay II cDNA generation kit as per manufacturer's instructions (Stratagene). The amino-modified cDNA from each genotype was labeled individually with one of 2 different fluors, either cyanine (Cy) 3 or 5, which conjugate to the modified amino groups and label the cDNA green or red,

respectively. The number of Cy-labeled nucleotides incorporated per 1000 bp, the frequency of incorporation (FOI), was determined for each labeling reaction using a 96-well plate spectrophotometer (PowerWaveX, Bio-Tek Instruments, Inc., Winooski, VT). The FOI of each labeling reaction was determined by measuring the ratio of 550/260 nm or 650/260 nm for the Cy3 and Cy5 reactions, respectively. The minimum acceptable FOI used for the cDNA microarray hybridization was 15. The volume of each labeling reaction containing 20 pmoles of labeled cDNA was then used for hybridization. DIG Easy Hybridization solution (Roche Diagnostics, Laval, QC) was incubated for 2 min at 65° with yeast tRNA, calf thymus DNA and poly $d(A)_{40-60}$ before incubation for 2 min at 65° with Cy-labeled cDNA using a previously described protocol (University Health Network Microarray Centre, Toronto, ON). Microarray chips were incubated with Cy-labeled cDNA at 42° overnight. Two 15.4 K mouse microarray chips (Microarray Centre, Ontario Cancer Institute) were hybridized in separate chambers to obtain reciprocally-labeled microarrays. After overnight incubation, microarray chips were washed 3X at 50°C for 15 min each in 1X SSC, 0.1% SDS. Next the microarray chips were briefly rinsed (2X in 1X SSC and then 2X in 0.1X SSC, both at room temperature) prior to analysis. Microarray chips were scanned using a Genepix scanner (Molecular Devices Corporation, Sunnyvale, CA). Data management and analysis was performed using GeneTraffic software (Stratagene). Genes of interest were those with at least 2 spots detected on each chip (both reciprocally-labeled chips) with mean log2 ratios that were $\geq 0.6 \leq$ (greater or equal to a 1.5-fold induction or less than or equal to -1.5-fold repression). In addition, the coefficient of variance (COV) was defined to be less than or

equal to 0.5 (50%). COV is determined by dividing the standard deviation by the average fold expression from both chips.

3.3.5 Promoter Constructs

The pGL3-mADRP construct containing 2.1 kilobase (Kb)
of the mouse ADRP promoter was generously donated by Ronald Evans (Howard Hughes Medical Institute, La Jolla, CA) [183]. The pGL3-RARESPROM construct containing 2.0 Kb of the mouse RARESP promoter region was previously cloned in our lab using primers that amplified the genomic sequence from -1989 to +39. The pGL3-heC3A4 construct containing the human apoA-IV promoter fused to the upstream apoC-III enhancer was created using an original construct (eC3A4-CAT)[301] [301], generously donated by Agnès Ribeiro (INSERM, Université Pierre et Marie Curie, Paris, France) as the template. Further information on the cloning of this promoter construct is described in the materials and methods section of chapter 4.

3.3.6 Nuclear Receptor Constructs

Expression constructs for murine PGC-1α, PPARβ, FXR and PPARγ were created by reverse transcription-polymerase chain reaction (RT-PCR) amplification of C57BL/6J mouse liver total RNA followed by insertion into the BamHI sites of pSG5. The following primers were used in amplification of PGC-1α from mouse DNA: 5'-AAA AGG ATC CAG CTG GAT GGC TTG GGA CAT GTG-3' (forward) and 5'-AAA AGG ATC CGG AAC ACG TTA CCT GCG CAA GCT T-3' (reverse). Underlined sequences

correspond to restriction enzyme recognition sites. All other nuclear receptor constructs were generous gifts from Frank Gonzalez (NIH, Bethesda, MD).

3.3.7 Cell Culture and Transfections

Human hepatocellular carcinoma, HepG2, (ATCC) were maintained at 37°C in 5% carbon dioxide (CO_2) in media containing phenol red-free Dulbecco's modified Eagle's medium (Hyclone, Logan, UT), 1 mM sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich) and 10% heat-inactivated charcoal dextran-stripped fetal bovine serum, FBS (Gemini Bioproducts, Woodland, CA). For transfections, HepG2 were plated at a density of 120,000 cells/mL (0.5 mL per well) in 24-well plates and transfected 24 h later. For the promoter regulation studies, cells were transfected with: 150 ng of luciferase reporter construct (ADRP, apoA-IV or RARESP); 50 ng of nuclear receptor expression construct (PPARγ, PPARβ or FXR); 125 ng of pCMV-βgal; 350 ng PGC-1α or PSG5 to a total of 700 ng. For the serum treatment experiment, cells were transfected with 150 ng of promoter construct as well as 400 ng of PBSK. A ratio of 4 μL/μg DNA of TransIT-LT1 (Mirus, Madison, WI) was used for all transfections. Cells were treated 24 h post-transfection with dimethylsulfoxide (DMSO) as vehicle; 50 μM Rosiglitazone; 1 μM GW4064 or 50 μM Bezafibrate. DMSO reached a final concentration of 0.25% in all wells. Cells were harvested 24 h post-treatment using Reporter Lysis Buffer (Promega, Madison, WI) per manufacturer's instructions. Cell lysates were assayed for firefly luciferase and β-galactosidase activity as described in the methods section of chapter 4.

3.3.8 Statistics

All comparisons were performed using unpaired t tests. GraphPad Instat (Instat3 V3.0a, GraphPad Software, Inc.) was used for all statistical analyses. Values are expressed as mean ± SD. Differences between groups were considered statistically significant at $P \leq 0.05$.

3.4 RESULTS

In order to identify potential novel regulatory targets of FXR, differential gene expression was performed with liver mRNA from FXR-/- and WT mice fed a chow or HF/HC diet using cDNA microarrays. Microarray analysis of chow-fed FXR-/- and WT mice revealed several altered genes in FXR-/- mice that may have importance in lipid metabolism. FXR-/- mice had 1.5-fold increased hepatic mRNA expression of apoA-I compared to WT mice fed the same diet (Table 3.1). These results are consistent with literature findings that bile acid-activated FXR suppresses apoA-I transcription [200]. Also in concurrence with previously reported data indicating FXR induces apoC-II transcription [196], hepatic apoC-II expression was decreased in FXR-/- mice by approximately 3-fold compared to WT mice on the same chow diet (Table 3.1). In addition, hepatic expression of VLDLR was decreased by approximately 1.8-fold in FXR-/- mice (Table 3.1). This altered expression of VLDLR in FXR-/- mice is consistent with the finding that reported FXR-mediated induction of VLDLR expression [201]. Another gene with altered expression in FXR-/- mice as indicated through microarray analysis was apoA-IV that was increased in these mice by approximately 2-fold (Table 3.1). In addition to these genes, thought to have roles in lipid metabolism, a gene called

Table 3.1 Fold expression of hepatic genes in FXR-/- versus WT mice fed chow and HF/HC diets.

	Diet	
Gene	**Chow**	**HF/HC**
Acetyl CoA acyltransferase 1A	-2.44	NC
Adaptor-related complex 3D	NC	-8.26
ADRP	NC	-3.51
Alanine aminotransferase 2	2.21	NC
ApoA-I	1.50	NC
ApoC-II	-3.06	-2.95
ApoA-IV	2.09	NC
CYP17A1	2.85	NC
FAS	NC	-2.80
Hydroxysteroid dehydrogenase-1, delta<5>-3-beta	NC	2.94
Inhibitor of DNA binding-2	-2.29	NC
Malic enzyme-1	NC	-3.48
Metallothionein-1	16.61	NC
RARESP	-4.45	NC
VLDLR	-1.80	NC

NC = no change as determined using criteria described in Materials and Methods

the retinoic acid responder protein (RARESP) was decreased by 4.5-fold in FXR-/- mice (Table 3.1). Although little is yet known in the literature regarding the physiological function of this protein, it is thought to have immune functions through induction of macrophage activation and neutrophil maturation [302, 303]. Our lab is currently investigating the physiological functions of RARESP (now called chemerin) as a mediator of adipocyte differentiation and function. In order to further characterize changes in gene expression that result from deficiency of FXR, FXR-/- and WT mice were challenged for 16 weeks with a HF/HC diet and hepatic RNA was subsequently used in additional microarray analyses. Similar to microarray results generated using chow-fed RNA, apoC-II mRNA expression was decreased (by approximately 3-fold) in FXR-/- mice compared to WT mice fed a HF/HC diet (Table 3.1). In addition, two other genes with function in lipid storage and metabolism: adipophilin (also called adipocyte differentiation-related protein, or ADRP) and FAS had decreased expression in FXR-/- mice by approximately 3.5- and 2.8-fold, respectively (Table 3.1). ADRP is highly expressed in mature adipocytes and is believed to function in the uptake and storage of fatty acids [304, 305]. The exact function of ADRP in liver is not clear but recent studies of ADRP-/- mice have demonstrated normal adipogenesis and protection from fatty liver development [306]. In addition, this protein has been found to inhibit VLDL secretion from liver cells [307].

To confirm the alterations in hepatic gene expression observed between FXR-/- and WT mice fed both chow and HF/HC diets using microarray analysis, mRNA from both genotypes from both feeding groups was used in northern blot analysis. Although microarray analysis did not reveal altered expression of the ADRP gene in FXR-/- mice

fed a chow diet, northern blot analysis indicated that ADRP expression was decreased in these mice after 16 weeks on both chow and HF/HC diets compared to WT mice (Fig. 3.2). Upon HF/HC feeding, ADRP expression increased in WT mice but remained near chow-fed levels in FXR-/- mice (Fig. 3.2). Consistent with the microarray results, apoC-II expression levels were decreased in FXR-/- compared to WT mice on both chow and HF/HC diets (Fig. 3.2). ApoA-IV mRNA expression was increased in FXR-/- mice compared to WT mice fed chow diet, results which were consistent with those obtained using microarray analysis (Fig. 3.2). HF/HC feeding appeared to increase hepatic expression of apoA-IV in WT but not FXR-/- mice so that mRNA expression levels of this gene were similar between the two genotypes fed HF/HC diet (Fig. 3.2). Northern blot expression levels of RARESP mRNA were decreased in chow fed FXR-/- mice compared to WT mice, a result consistent with that found using microarray analysis (Fig. 3.2). HF/HC feeding increased hepatic mRNA expression of RARESP in both the FXR-/- and WT groups (Fig. 3.2). Although not indicated in the microarray analysis, the decreased hepatic mRNA expression of RARESP in FXR-/- mice seen in chow fed mice persisted after 16 weeks on the HF/HC diet (Fig. 3.2).

To further characterize novel candidate FXR target genes identified by microarray and northern blot analyses, promoter constructs of three putative FXR targets: ADRP, apoA-IV and RARESP, were used in transient transfections of HepG2 cells. PPARβ as well as both PPARα and PPARγ nuclear receptors have been shown to induce activity of the ADRP promoter [183, 308-310]. Examination of ADRP promoter activity demonstrated an induction of approximately 2-fold when treated with ligands for PPARγ and PPARβ; rosiglitazone and bezafibrate, respectively (Fig. 3.3*a*). Addition of the

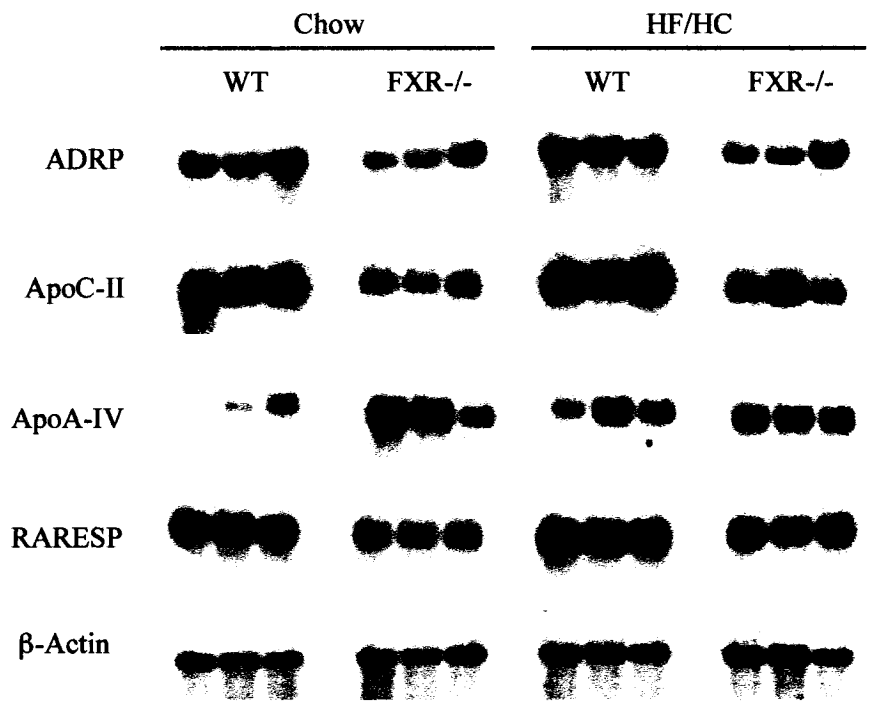

Figure 3.2 Northern blot analyses of hepatic gene expression.
Northern blot analyses of hepatic ADRP, ApoC-II, ApoA-IV, RARESP and β-actin expression in chow and HF/HC fed FXR-/- and WT mice.

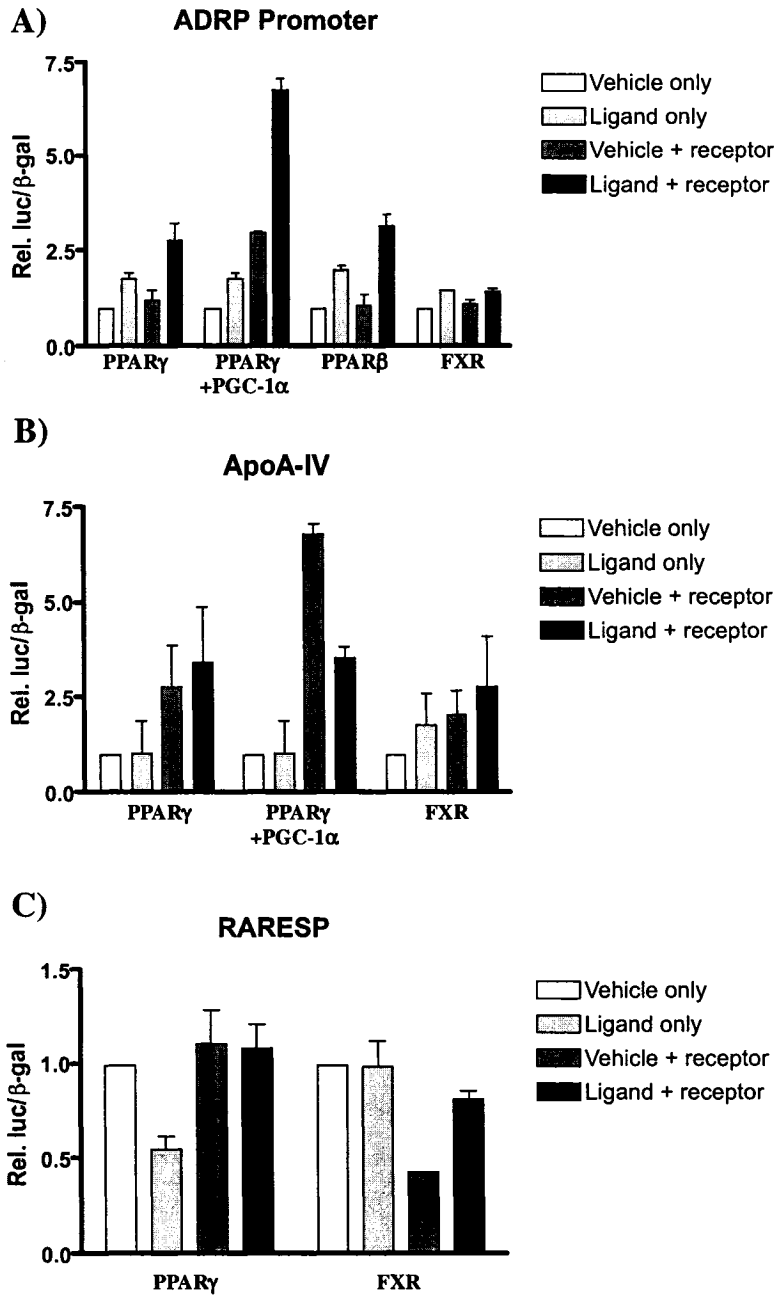

Figure 3.3 Promoter analyses of putative FXR targets
HepG2 cells were co-transfected with constructs containing the A) ADRP B) apoA-IV or C) RARESP promoter regions. Cells were treated 24 h post-transfection and harvested after an additional 24 h. Ligands for PPARγ, PPARβ and FXR are rosiglitazone, bexafibrate and GW4064, respectively. Luciferase values were normalized to individual β-galactosidase activities for the same well. Promoter luciferase activities were reported as fold activation over activity with vehicle-treated PSG5 control vector.
Values are mean ± SD of three replicates obtained from a single experiment.

synthetic FXR ligand GW4064 slightly induced the ADRP promoter by 1.5-fold (Fig. 3.3*a*). Cotransfection of a PPARγ-expressing construct with a construct expressing its coactivator PGC-1α, but not PPARβ- or FXR-expressing constructs, induced ADRP promoter activity by approximately 3-fold (Fig. 3.3*a*). Addition of specific ligands to HepG2 cells cotransfected with the ADRP promoter and nuclear receptor-expressing constructs demonstrated induction in promoter activity with activated PPARγ, PPARγ in addition to PGC-1α and PPARβ by approximately 3-, 7- and 3-fold, respectively (Fig. 3.3*a*). Addition of GW4064 to cells transfected with ADRP promoter- and FXR-expressing constructs did not induce activity of this promoter (Fig. 3.3*a*).

Treatment of HepG2 cells transfected with the apoA-IV promoter region with rosiglitazone or GW4064 did not induce activity over that with vehicle alone (Fig. 3.3*b*). Cotransfection of the apoA-IV promoter with PPARγ- and PGC-1α-expressing constructs induced promoter activity by 2.5- and 6.5-fold, respectively while cotranfection of the FXR-expressing construct did not alter promoter activity (Fig. 3.3*b*). Addition of rosiglitazone to cells cotransfected with apoA-IV promoter- and PPARγ-expressing constructs did not further increase its activation over vehicle treated controls (Fig. 3.3*b*). Addition of rosiglitazone to cells cotransfected with the apoA-IV promoter-, PPARγ- and PGC-1α-expressing constructs repressed activation of the promoter from that of vehicle treated controls, from 6.5-fold down to 3.5-fold, indicating that coactivation of this promoter may not be mediated through PPARγ (Fig. 3.3*b*). Treatment of cells cotransfected with the apoA-IV promoter- and FXR-expressing constructs with GW4064 slightly induced promoter activity from 2-fold (vehicle treated controls) to 2.5-fold (Fig. 3.3*b*).

Examination of RARESP promoter regulation revealed that treatment with rosiglitazone repressed promoter activity by 50% and treatment with GW4064 had no effect (Fig. 3.3*c*). Treatment of HepG2 cells cotransfected with the RARESP promoter- and PPARγ-expressing constructs with rosiglitazone had no effect on RARESP promoter activity (Fig. 3.3*c*). Cotransfection of cells with the RARESP promoter and FXR-expressing constructs with GW4064 reduced promoter activity by 50% and treatment with GW4064 brought promoter activity levels back to baseline (Fig. 3.3*c*).

The apparent lack of specific regulation by FXR on the ADRP, apoA-IV and RARESP promoters indicated that the phenotypic environment of FXR-/- mice may result in altered hepatic gene expression. Thus, changes in blood levels of hormones, such as glucocorticoids or insulin, or increases in blood lipids due to deficiency of FXR could alter hepatic expression of these genes. In order to test this hypothesis, HepG2 cells were transfected with promoter constructs for the ADRP, apoA-IV and RARESP promoters and treated with pooled plasma from either WT or FXR-/- mice. ADRP promoter activity was unaffected by treatment with 5% plasma but was decreased in a dose-dependent manner by treatment with 10 and 25% WT plasma to 70 and 60% of control, respectively (Fig. 3.4*a*). Treatment of the ADRP promoter with plasma from FXR-/- mice also reduced promoter activity to 85% with 5 and 10% and reduced its activity further to 60% of control with treatment of 25% plasma (Fig. 3.4*b*).

The apoA-IV promoter was transfected into HepG2 cells and treated with plasma from FXR-/- and WT mice. Addition of 5, 10 and 25% plasma from WT mice decreased promoter activity to 90, 80 and 50% of control, respectively (Fig. 3.4*b*). Treatment of the apoA-IV promoter with 5, 10 and 25% plasma from FXR-/- mice also reduced activity,

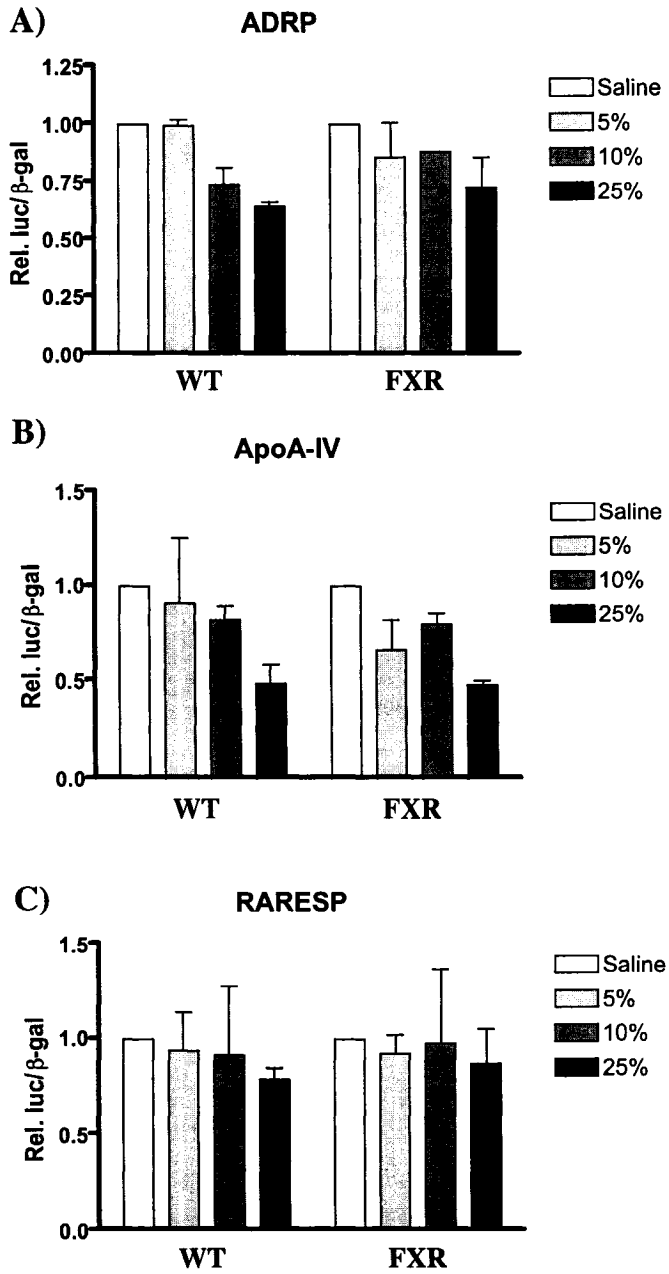

Figure 3.4 Treatment of putative FXR target gene promoters with WT and FXR-/- plasma.

HepG2 cells were co-transfected with constructs containing the A) ADRP B) apoA-IV or C) RARESP promoter regions. Cells were treated 24 h post-transfection with fresh plasma (5, 10 and 25% of total media) from FXR-/- or WT mice (3 animals pooled per genotype). Cells were harvested 24 h post-treatment. Luciferase values were normalized to individual β-galactosidase activities for the same well. Promoter luciferase activities were reported as fold activation over saline-treated. Values are mean ± SD of three replicates obtained from a single experiment.

to 60, 80 and 50% of control, respectively (Fig. 3.4*b*).

Treatment of RARESP promoter-transfected HepG2 cells with 5 and 10% plasma from WT mice decreased activity to 90% while treatment with 25% plasma decreased activity to 75% of control (Fig. 3.4*c*). Addition of 5 and 25% plasma from FXR-/- mice reduced activity of the RARESP promoter by 0.9- and 0.8-fold, respectively while treatment with 10% plasma had no effect (Fig. 3.4*c*).

Through the use of promoter regulation studies using constructs expressing nuclear receptors as well as treatment with plasma from FXR-/- mice, ADRP, apoA-IV and RARESP promoters did not appear to be regulated in a direct manner by FXR. Nor did it appear that exposure to any alterations in blood elements such as hormones in FXR-/- mice were responsible for altered expression of these promoters, at least with acute (24 h) exposure. Although acute exposure of promoter expressing constructs to plasma from FXR-/- mice did not appear to alter activity in a specific manner, chronic exposure to altered blood lipids or hormones in plasma from FXR-/- mice may affect hepatic expression of ADRP, apoA-IV and RARESP. In order to further characterize phenotypic differences in FXR-/- mice compared to WT, weight gain of these mice was monitored over 16 weeks of feeding with chow and HF/HC diets. Examination of weight gain (measured as a percentage of day 1 weight) over 16 weeks on a chow diet, FXR-/- mice gained significantly less weight than WT mice at all time points, from weeks 1 through 16, with 15% weight gain compared to 30% in WT mice (Fig. 3.5*a*). Treatment of FXR-/- and WT mice with a HF/HC diet for 16 weeks caused increased weight gain in both genotypes of mice (Fig. 3.5*b*). Although the marked differences in weight gain between FXR-/- and WT mice fed a chow diet were lessened in HF/HC-fed mice, there

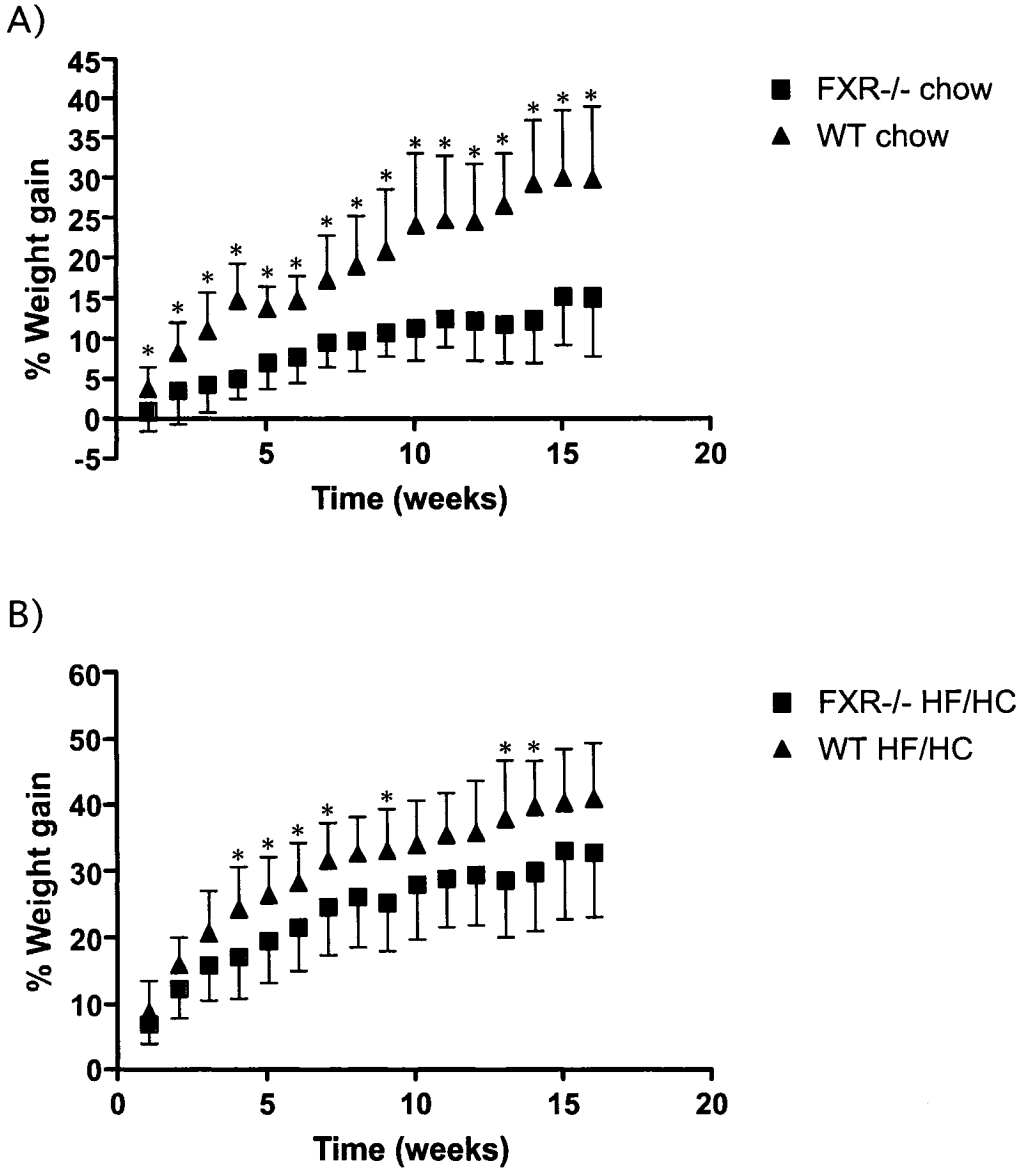

Figure 3.5 Weight gain of FXR-/- and WT mice on chow and HF/HC diets.
Weight gain of FXR-/- and WT mice over 16 weeks on A) chow and B) HF/HC diets. Weight gain is reported as a percent of day 1 body weight. *P<0.05 vs FXR-/- on same diet (unpaired t-test). N=9-10 animals per group.

remained significant decreases in FXR-/- weight gain, by approximately 7%, at weeks 4-7, 9 and 14-14 (Fig. 3.5*b*). Decreased weight gain in FXR-/- mice may indicate either a decreased food consumption or an increased metabolism in these mice. Both HF/HC and chow fed FXR-/- and WT mice consumed an average of 20 g of food per week. An important aspect of murine metabolism is that of adaptive thermogenesis that occurs in brown adipose tissue. One of the main regulators of adaptive thermogenesis is PGC-1α [267]. In addition, an important protein in brown adipocyte adaptive thermogenesis is UCP-1 [264]. Examination of brown adipose gene expression in FXR-/- and WT mice by QPCR demonstrated an increased mRNA expression of both PGC-1α and UCP-1 by approximately 2.5-fold (Fig. 3.6*a*). Expression of ADRP was decreased in brown adipose of FXR-/- mice to approximately 25% of WT expression levels (Fig. 3.6*a*).

In addition to thermogenesis in brown adipose tissue, white adipose is also important in metabolism both through its function as a storage depot of triglycerides as well as through its release of hormones important in appetite and metabolism. Two of the hormones expressed in adipose tissue are adiponectin and leptin. Examination of leptin mRNA expression in FXR-/-mice revealed a decrease to approximately 25% of WT levels while expression of another hormone, adiponectin, was unchanged (Fig. 3.6*b*).

3.5 DISCUSSION

The present study demonstrates altered liver, brown adipose and WAT expression of several genes with importance in lipid and energy metabolism in FXR-/- mice. ADRP, apoA-IV and RARESP are genes with altered expression in FXR-/- mouse livers and

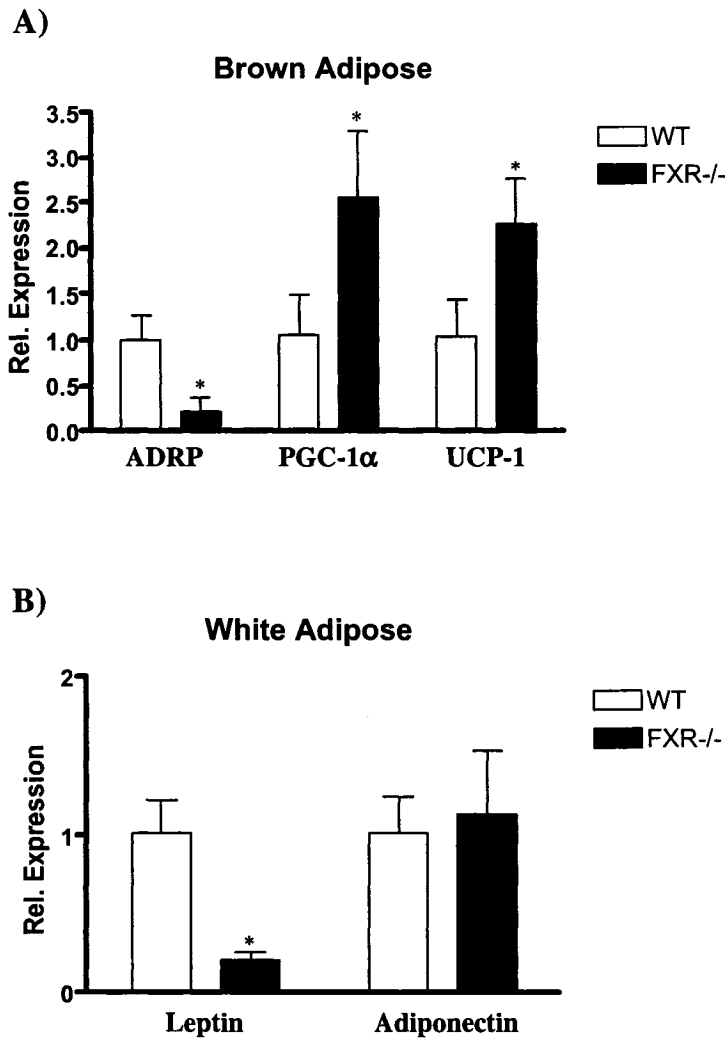

Figure 3.6 Differential gene expression in adipose tissue of FXR-/- and WT mice. Real-time quantitative PCR analysis of A) brown adipose mRNA expression of ADRP, PGC-1α and UCP-1 and B) brown adipose mRNA expression of leptin and adiponectin in FXR-/- and WT mice. All values were normalized to GAPDH expression and are expressed as the fold difference relative to WT controls. *P<0.05 vs WT (unpaired t-test). All values are mean ± SD. N=5-8 for brown adipose and N=4-6 for white adipose.

whose regulation by FXR has not been previously described. Despite decreased hepatic expression of RARESP and ADRP and increased expression of apoA-IV in FXR-/- mice, *in vitro* promoter analyses revealed no direct regulation by FXR on the transcriptional level of these genes. In addition, treatment with plasma from FXR-/- mice had no effect on promoter activity of these genes. Further examination of the FXR-/- mouse phenotype demonstrated a marked resistance to weight gain on a chow diet and, to a lesser extent, on a HF/HC diet. Investigation into the causative mechanisms behind this weight gain resistance revealed a disruption in energy metabolism in both WAT and brown adipose characterized by decreased leptin and increased expression of adaptive thermogenic genes, respectively.

Alterations in the hepatic expression of many genes have been previously found for FXR-/- mice [210]. Direct regulation of some of these genes by FXR has been demonstrated, including that for apoA-I, apoC-II, FAS and VLDLR [196, 200, 201, 311]. Despite the overt hyperlipidemic phenotype of FXR-/- mice, there have been no reports of the use of microarray analyses to examine altered hepatic mRNA expression. Therefore, this techniques was employed in order to discover potential novel targets of FXR-/- in these mice. Microarray and northern blot analyses of liver from chow and HF/HC fed FXR-/- versus WT mice revealed altered transcriptional levels of known targets of this nuclear receptor as well as a few genes whose altered transcription had not been previously described in these mice (ADRP, RARESP and apoA-IV). Despite the altered expression of these genes in the FXR-/- mouse however, promoter analyses did not reveal a direct regulation by FXR that could explain the altered transcription of these genes in the FXR-deficient mice.

ADRP is a protein with established functions in WAT that contribute to lipid storage [312]. Little was known regarding the function of ADRP in other tissues until recently. The recent advent of the ADRP-/- mouse has demonstrated that these mice are protected from developing fatty livers [306]. Similarily, knockdown of ADRP in liver cells has recently been shown to decrease cytosolic lipid droplets and increase the flow of fatty acids into β-oxidation [307]. Increases of ADRP in liver cells increased triglyceride accumulation and prevented the incorporation of fatty acids into triglycerides and VLDL particles [307]. Accumulation of liver lipids in FXR-/- mice has been demonstrated by both qualitative analysis using oil red O staining as well as quantitative analysis of lipids extracted from these tissues (see Fig. 2.2 and 2.3 from Chapter 2). Therefore, the decrease in ADRP gene expression in FXR-/- livers suggests negative regulation of this protein in response to hepatic lipid accumulation. This decrease in ADRP may function as a hepatoprotective mechanism by shunting toxic free fatty acids into β-oxidation and VLDL secretion. Further evidence in support of this hypothesis is provided by the increased hepatic VLDL secretion in FXR-/- mice [210]. ADRP expression has previously been shown to require insulin and is increased by treatment with this hormone in skeletal muscle [313, 314]. Thus, the decrease in hepatic ADRP expression in FXR-/- mice could also be due to an increased insulin resistance in these mice. Indeed, recent studies have demonstrated glucose and insulin resistance in FXR-/- mice and have highlighted a role for this nuclear receptor in glucose homeostasis [222, 315].

ApoA-IV is an apolipoprotein expressed in liver and intestine that associates with HDL, VLDL and chylomicron particles [316]. ApoA-IV expression in the intestine is stimulated by the ingestion of fat and associates with newly synthesized chylomicrons

[317]. Despite this association with intestinal fat absorption, apoA-IV-/- mice have normal lipid absorption, suggesting other roles for this protein in lipid metabolism [318]. ApoA-IV has been suggested to function in lipoprotein modification, such as by enabling apoC-II to act as an efficient cofactor for LPL-mediated hydrolysis of triglycerides within HDL and VLDL particles [319]. ApoA-IV has also been demonstrated to stimulate LCAT remodeling of HDL cholesterol [320]. Although factors that modulate expression of hepatic apoA-IV are not yet well characterized, hepatic apoA-IV expression does not always correlate well with apoB-secretion from liver and may be increased by hepatic triglyceride accumulation [321, 322]. In addition, hepatic expression of apoA-IV may be modulated in glucose homeostasis, as insulin has been shown to inhibit liver expression of this apolipoprotein [323]. ApoA-IV-/- mice have decreased HDL levels associated with an increased catabolism of these particles [318]. Together these data indicate that hepatic secretion of apoA-IV is involved in HDL metabolism and that expression of this protein may be increased in insulin resistance. FXR-/- mice have increased hepatic secretion of apoB-containing lipoproteins and increased plasma HDL levels associated with a reduced clearance of these particles associated with decreased SR-B1 expression [210]. Therefore, the increased hepatic expression of apoA-IV observed in FXR-/- mice may occur through mechanisms related to insulin resistance and/or increased triglyceride accumulation occurring in these cells. The increased apoA-IV content of HDL particles secreted from FXR-/- liver may be another mechanism by which the clearance of these particles is inhibited.

RARESP is a recently discovered molecule that is expressed and secreted from many tissues, especially liver and lung, and functions as a potent chemoattractant

molecule [324, 325]. The receptor for RARESP is expressed in white blood cells such as macrophages and T-cells and is modulated by pro-inflammatory cytokines [303, 326]. Liver from FXR-/- mice, as previously shown by our lab, has increased cellular infiltrate and increased expression of TNFα and MAC-1, indicative of an increased inflammatory state in this tissue (see Fig. 2.2 and 2.4, Chapter 2). Although the function of RARESP in liver has not been previously described, the decreased expression of this molecule in FXR-/- hepatocytes may be the product of an anti-inflammatory response in an attempt to protect from hepatotoxicity in these mice.

Feeding of FXR-/- mice with chow or HF/HC demonstrated a resistance to weight gain in these mice. Since these mice ingested similar amounts of food as their WT counterparts, the decrease in weight accumulation in these mice indicated a metabolic disturbance. FXR-/- mice also had decreased WAT expression of leptin. Recent studies have demonstrated a key role for FXR in induction of differentiation in adipocytes in WAT and pre-adipocytes from FXR-/- mice have impaired differentiation [327, 328]. Therefore, it is likely that the observed decreased weight accumulation in these mice results from a failure of existing pre-adipocytes to differentiate and store fat. However, due to the fact that FXR-/- mice fed a HF/HC diet demonstrated less difference in weight gain when compared to WT mice, it is likely that FXR is not absolutely required for adipose development.

As described previously, a metabolic function of leptin is the induction of thermogenesis in brown adipose. Paradoxically, despite the decreased leptin expression in FXR-/- mice, brown adipose thermogenic genes were upregulated. A recent study may have the answer to this apparent contradiction; bile acids have been found to bind to a G-

protein coupled receptor on brown adipoytes to induce adaptive thermogenesis [329]. Therefore, the increases in plasma bile acid concentration in FXR-/- mouse may act at the level of the brown adipocyte to induce thermogenic mechanisms, further contributing to prevention of weight accumulation in these mice.

In conclusion, the present study demonstrates that disruption of the FXR gene in mice has profound effects on bile acid, lipid and energy homeostasis mediated through both direct and indirect mechanisms. Although many genes are directly regulated by FXR through its regulation of transcription, multiple environmental factors present in FXR-/mice may also affect gene expression in various tissues, contributing to the hyper-lipidemia and decreased weight gain observed in these mice.

CHAPTER 4

ApoA-IV IS REGULATED BY NUTRITIONAL AND METABOLIC STRESS: INVOLVEMENT OF GLUCOCORTICOIDS, HNF-4α AND PGC-1α

Sections not done by PhD candidate:

Figs. 4.5*c*, 4.6*b* and 4.8*b* were experimental analyses performed by Dr. Gilles Lambert (INSERM, France) who is a research collaborator with our lab. All other experimental data presented in this chapter was produced by the candidate. Liver tissue from liver-specific HNF-4α-/- mice, used in Fig. 4.12*b*, was provided by Dr. Yusuke Inoue and Dr. Frank Gonzalez.

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4.1 ABSTRACT

ApoA-IV is a 46-kDa glycoprotein that associates with HDL and triglyceride-rich lipoproteins. Blood levels of apoA-IV generally correlate with triglyceride levels and are increased in diabetic patients. The present study investigated the mechanisms regulating *in vivo* expression of apoA-IV in the liver and intestine of mice in response to changes in nutritional status. Fasting markedly increased liver and ileal apoA-IV mRNA and plasma protein concentrations. This induction was associated with increased serum glucocorticoid levels and was abolished by adrenalectomy. Treatment with dexamethasone increased apoA-IV expression in adrenalectomized mice. Marked increases of apoA-IV expression were also observed in two murine models of diabetes. Reporter gene analysis of the murine and human apoA-IV/CIII promoters revealed a conserved cooperative activation by the hepatic nuclear factor-4α (HNF-4α) and PGC-1α, but no evidence of a direct regulatory role for GR. Consistent with these *in vitro* data, induction of apoA-IV in response to fasting was accompanied by increases in HNF-4α and PGC-1α expression and was abolished in liver-specific HNF-4α-/- mice. Together these results indicate that induction of apoA-IV expression in fasting and diabetes likely involves PGC-1α-mediated coactivation of HNF-4α in addition to glucocorticoid-dependent actions.

SUPPLEMENTARY KEY WORDS: Glucocorticoid Receptor, Fasting, Diabetes, Adrenalectomy

4.2 INTRODUCTION

4.2.1 Insulin and the Fed State

In the body, blood glucose levels are normally kept within a narrow range as determined by the ratio of the hormones insulin and glucagon. In the fed state, insulin is the major regulator of carbohydrate and lipid metabolism (Fig. 4.1). When food is ingested, blood glucose levels rise, signaling the β–cells of the pancreas to secrete insulin. Insulin binds to its specific receptor on the surface of cells and directs the uptake of glucose (through induction of glucose transporter expression) and storage of this nutrient for future metabolism. Insulin has a number of tissue-specific effects. For instance, insulin action in the liver promotes the uptake and storage of glucose as glycogen through upregulation of genes coding for glucose transporters and enzymes that catalyze various steps in glycogenesis, respectively. In the liver, insulin also induces glycolysis and synthesis of fatty acids from the glycolysis product, pyruvate, mediated through insulin induction of the SREBP-1c gene [330, 331]. Importantly, insulin also functions in the liver to inhibit enzymes involved in glucose synthesis (gluconeogenesis) and breakdown of glycogen, glycogenolysis [331, 332]. In skeletal muscle and adipose, insulin promotes uptake of glucose. In adipose tissue, this increase in glucose uptake is accompanied by the synthesis of fatty acids from pyruvate and subsequent storage of these molecules as triglycerides [333]. The regulation of insulin-mediated pathways includes many different levels of control such as allosteric, posttranslational and transcriptional [331].

4.2.2 Glucagon and the Fasted State

Once the nutrients from a meal are no longer in circulation, the body enters the post-

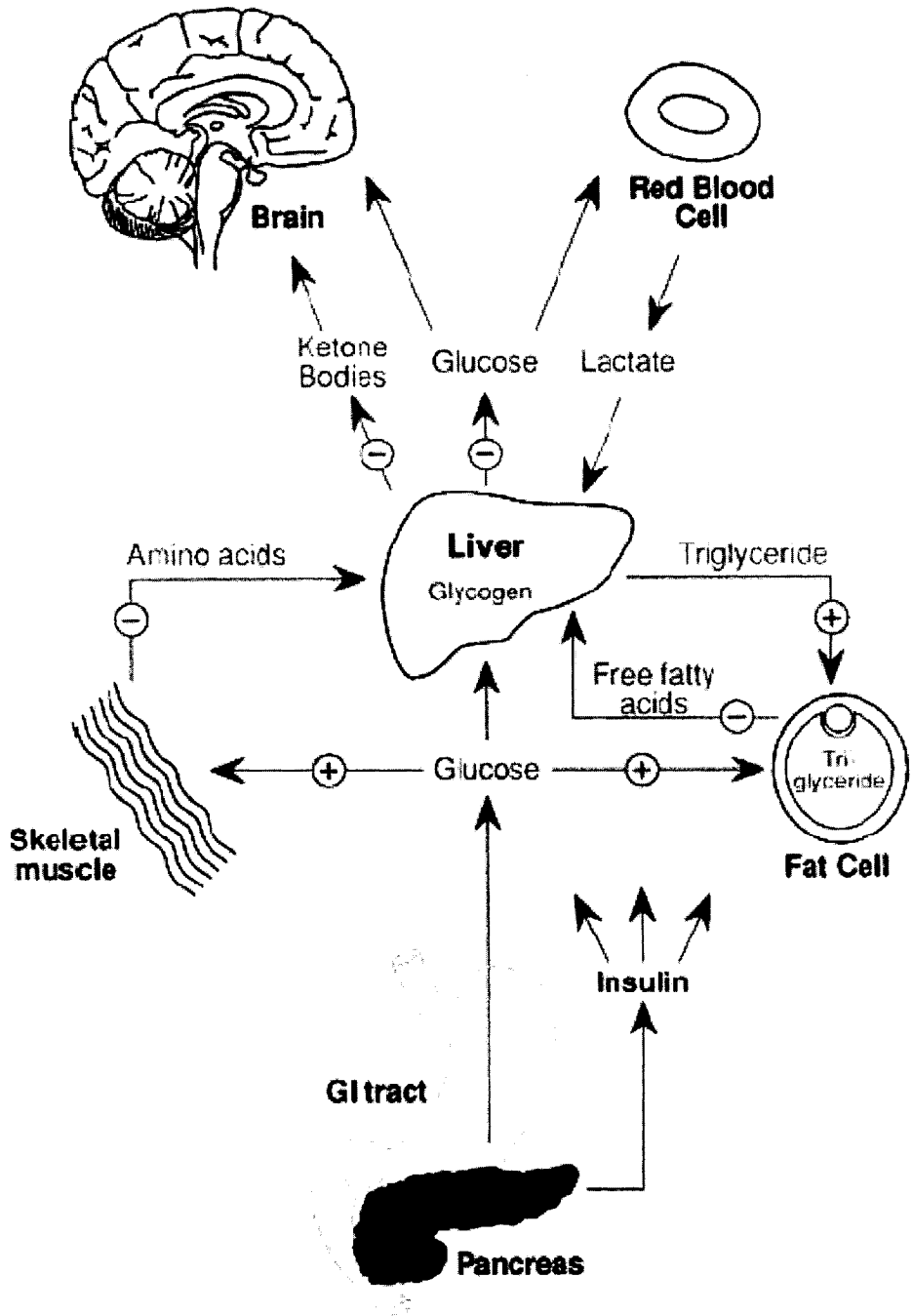

Figure 4.1 Physiological functions of insulin.
When food is ingested, rising levels of glucose signal the β cells of the pancreas to secrete insulin. Three major tissues of the body that are sensitive to insulin are skeletal muscle, adipose and liver. The actions of insulin are to lower blood glucose levels and promote storage of energy from nutrients through the increased uptake of glucose by skeletal muscle and adipose and storage as glycogen in addition to increased uptake of fatty acids by adipose and storage as triglyceride. Insulin also acts to repress breakdown of protein into amino acids and triglycerides into fatty acids in skeletal muscle and adipose, respectively, in addition to repression of ketogenesis in liver. Figure taken from [334].

absorptive, or fasted, state. In this catabolic state the body begins to break down stored nutrients for energy or for the synthesis of other essential molecules and acts to maintain stable blood glucose levels. During fasting sufficient blood glucose levels are maintained in large part by the liver, in addition to some contribution by the kidney and small intestine during prolonged fasts [331, 335]. When blood glucose levels decline, this signals α cells of the pancreas to secrete the hormone glucagon. Glucagon signals the liver to break down its stores of glycogen (by stimulation of glycogenolysis) and synthesize new glucose through gluconeogenesis in order to maintain blood glucose levels within normal range (Fig. 4.2). Glucagon, through binding to its receptor, activates a signaling pathway that increases intracellular cAMP levels. In turn, cAMP inhibits insulin activity and thus, SREBP-1c-mediated lipogenesis and glycolysis are also inhibited (Fig. 4.2). Increases in cAMP also activate PKA that phosphorylates many downstream targets including transcription factors such as cAMP response element binding protein (CREB). CREB activates the transcription of genes important in gluconeogenesis such as phosphoenolpyruvate carboxykinase (PEPCK), the enzyme that catalyzes the rate-limiting step in gluconeogenesis, and the cofactor PGC-1α [336, 337]. PGC-1α has been shown to induce the transcription of the PEPCK gene through coactivation of HNF-4α as well as GR transcriptional activity on the promoter region of this gene (Fig. 4.2) [338]. HNF-4α, in addition to two other transcription factors, CAAT/enhancer binding protein (C/EBP) α and β, bind to the promoter region and activate trancription of the gene for glucose-6-phosphatase, the enzyme that catalyzes the last step in gluconeogenesis [339]. Further physiological roles of both HNF-4α and PGC-1α will be discussed later in this introduction. White adipose lipolysis, mediated by

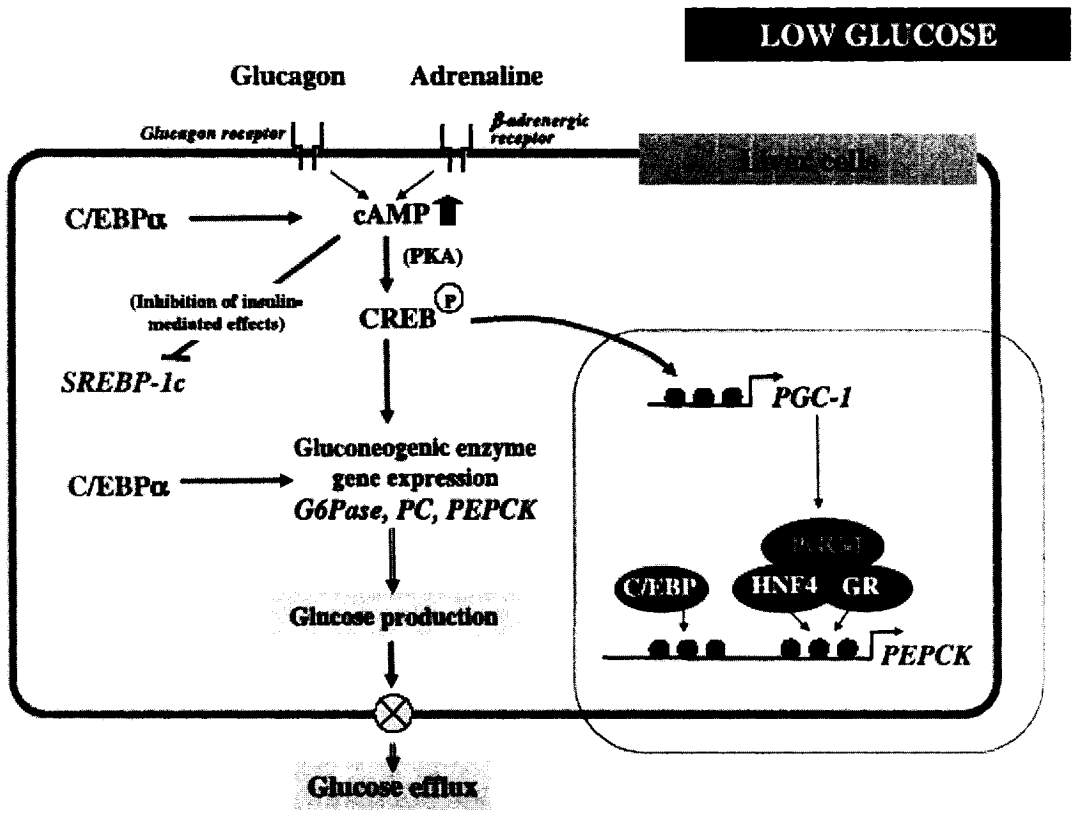

Figure 4.2 Hepatic gluconeogenic responses to fasting.
Decreases in blood glucose levels signal pancreatic α cells and adrenal medullary cells to secrete the hormones glucagon and adrenaline, respectively. In the liver, glucagon binds to its receptor, initiating a cell-signaling cascade that leads to increases in intracellular levels of cAMP. The enzyme PKA becomes activated by the cAMP and phosphorylates many target proteins including CREB. Phosphorylated CREB, in addition to C/EBPα, participates in the activation of gene expression of many genes encoding enzymes important in gluconeogenesis including glucose 6-phosphatase and PEPCK. One gene with importance in the gluconeogenic responses of the liver is the coactivator PGC-1α. Phosphorylated CREB acts in the induction of the PGC-1α gene. PGC-1α acts to coactivate HNF-4α and GR at the promoter of the PEPCK gene leading to increased transcription. Figure taken from [331].

HSL, is stimulated by rises in cAMP that occur as a result of glucagon and adrenergic signaling [340]. WAT lipolysis is thus inhibited by insulin signaling which activates phosphodiesterases, enzymes that degrade cAMP [341]. Lipolysis in WAT releases free fatty acids which are taken up by the liver and converted into ketones, used as fuel for the brain and peripheral tissues [333].

4.2.3 Diabetes and Atherosclerotic Disease

Diabetes (*diabetes mellitus*) is characterized by the accumulation of blood glucose due to deficiencies in either insulin production or tissue sensitivity to hormone signaling. Type I diabetes is an autoimmune disorder in which the insulin-producing β-cells of the pancreas are destroyed by the body's own immune system. Patients with this disorder have deficient insulin levels which can be supplemented with recombinant insulin. Type II diabetes (T2DM) is the most common form of the disease and is characterized by the development of a resistance to the effects of insulin [342]. Known risk factors for the development of T2DM are obesity and a sedentary lifestyle as insulin resistance is known to occur in fat and unexercised muscle. Although the molecular mechanisms that cause T2DM are still in debate, it is known that decreased responsiveness to insulin causes a reflexive increase in secretion of the hormone from β-cells leading to eventual failure of these cells [334].

Patients with T2DM are at a 3-4-fold higher risk of mortality by cardiovascular disease [343, 344]. As described previously, a chronic diabetic state can cause damage to blood vessels due to the increased production of AGPs in arterial wall components. In addition, T2DM is associated with low HDL and high triglyceride levels [345-347].

Much like in the fasted state, insulin resistance results in a catabolic state characterized by increases in adipose lipolysis, hepatic uptake of free fatty acids and synthesis of triglycerides. Lowered HDL levels in diabetes are thought to be caused by metabolic disturbances associated with increased triglycerides [348]. Enhanced hepatic secretion of VLDL particles is thought to occur in T2DM, thus leading to increased triglyceride levels [349, 350]. Hypertriglyceridemia is an independent risk factor for the development of atherosclerosis [351]. An increase in plasma triglycerides leads to accelerated exchange of this lipid from VLDL to HDL particles in exchange for cholesterol, via CETP-mediated transfer [352, 353]. The enrichment of the triglyceride content of HDL creates a smaller, more dense particle that is metabolized more readily by the body, thus an accelerated clearance of HDL occurs in patients with T2DM [354, 355] [356]. Prolonged residence of VLDL also increases the amount of CETP-mediated lipid exchange between these particles and LDL, leading to the development of smaller, denser LDL which are even more likely than regular-sized LDL to enter vessel walls thus further increasing the risk for cardiovascular disease in diabetic patients [357].

4.2.4 Mouse Models of Diabetes

Mice are often used to study the causative and pathological mechanisms of diabetic disease. A common mechanism used to mimic the pathology that occurs in type I diabetes is through chemical destruction of pancreatic β-cells by streptozocin (STZ). STZ (2-deoxy-2(3-methyl-3-nitrosoureido)-D-glucopyranose) is a broad-spectrum antibiotic with diabetogenic properties [358, 359]. Although the exact mechanisms of STZ toxicity to β-cells are not clear, it is known that the drug is taken up into β-cells via the glucose

transporter 2 and acts to alkylate DNA causing its fragmentation as well as to increase reactive oxygen species and nitric oxide synthase causing permanent loss of β- cells [360, 361].

T2DM can be studied in mice through a number of different models. One model commonly used is the leptin-deficient ob/ob mouse, discovered in 1950 [281, 362]. As previously discussed in chapter 3, leptin is a hormone released from WAT that acts as a satiety signal in the brain, limiting food intake and increasing energy expenditure. Therefore, deficiency in leptin causes ob/ob mice to develop extreme obesity to the point where they can no longer groom themselves. Concurrent with obesity, ob/ob mice develop insulin resistance, high blood glucose and glucocorticoid levels, thus providing an excellent model for T2DM research.

4.2.5 Glucocorticoids and Glucose Homeostasis

Glucocorticoids are steroid hormones that are released from the adrenal cortex and function to activate the GR, a member of the nuclear receptor superfamily [363]. Cortisol and corticosterone are the main glucocorticoids in humans and mice, respectively and function through the ubiquitously expressed GR to regulate many different physiological processes such as growth, glucose and lipid metabolism as well as inflammatory responses [364-366]. Therefore, exposure to excessive glucocorticoids, as seen in Cushing's syndrome, can lead to insulin resistance, glucose tolerance, decreased growth rate, bone loss and obesity [367, 368]. Rises in glucocorticoids can also occur in cases of insulin or leptin deficiency as well as fasting [369].

A drop in plasma glucose directly stimulates the adrenal cortex to release

glucocorticoids that act in a permissive manner with glucagon to induce gluconeogenesis and maintain blood glucose levels during fasting [370-372]. One of these gluconeogenic targets of GR is PEPCK [373]. GR inhibits glucose uptake and glucose catabolism in adipose and muscle, thereby increasing accessibility of the liver to the gluconeogenic substrates: fatty acids and amino acids [369, 374]. Two prevalent pathologies associated with diabetes are unchecked gluconeogenesis (primarily in the liver) and hyperlipidemia. Therapeutic inhibition of adipose and liver GR expression improves both of these conditions, suggesting a causative role for GR in the pathology of diabetes [375, 376]. Further evidence of the importance of GR activity in the pathology of diabetes is evidenced by studies demonstrating that increased activity of this receptor in either skeletal muscle or the liver can cause the development of insulin resistance [377, 378].

4.2.6 HNF-4α, PGC-1α and Glucose Homeostasis

Two additional transcription factors known to be important for regulating gluconeogenic responses (in both diabetes and fasting) are HNF-4α and the transcriptional coactivator PGC-1α [338]. HNF-4α is an orphan nuclear receptor meaning that its physiological ligand has not yet been identified. HNF-4α is highly expressed in the liver, intestine and pancreas and is important in the maintenance of metabolic homeostasis through regulation of many genes, including those encoding apolipoproteins and enzymes important in lipid, amino acid and glucose metabolism [379, 380]. A mutation of the HNF-4α gene in humans is known to cause maturity onset diabetes of the young [381]. Although the standard gene knockout of HNF-4α is embryonically lethal in mice, liver-specific disruption of this gene using the Cre-recombinase/loxp system has been

performed. These mice have phenotypic abnormalities that include hepatic lipid accumulation, elevated serum bile acids, decreased blood cholesterol and decreased hepatic secretion of VLDL [382]. As discussed previously, HNF-4α and PGC-1α interact at the PEPCK gene promoter. The induction of the PEPCK gene by GR requires the binding of HNF-4α to promoter sites within the GR regulatory region [383].

PGC-1α is a coactivator of transcription with important functions in glucose homeostasis. PGC-1α coactivation is known to be important in the induction of mitochondrial biogenesis, respiration and thermogenesis in response to cold, fasting and prolonged exercise [384, 385]. Thus, PGC-1α is highly expressed in tissues with abundant mitochondria and high energy requirements such as liver, heart, brown fat and brain [267]. In the liver, PGC-1α is induced synergistically by cAMP and glucocorticoids (which rise in diabetes and fasting) and acts to coactivate the transcription of several genes important to gluconeogenesis including PEPCK and glucose-6-phosphatase [338]. PGC-1α coactivates both GR and HNF-4α on the PEPCK gene promoter. In fact, HNF-4α is required for PGC-1α coactivation of several gluconeogenic genes but not those involved in ketogenic and β-oxidation [386]. Therefore, PGC-1α is a stress-induced coactivator that responds to low blood glucose levels by increasing nuclear receptor-mediated the activation of gluconeogenic responses in the liver.

4.2.7 ApoA-IV

ApoA-IV is a 46 kDa glycoprotein found primarily in triglyceride-rich chylomicrons and HDL [316]. Synthesis of apoA-IV from the small intestine is proposed to occur in response to ingestion of fat [317]. In humans, the majority of circulating plasma apoA-

IV is found associated with HDL, some of which is thought to transfer from chylomicrons and VLDL [387, 388]. In rodents, apoA-IV is present in plasma in HDL particles with 59% of the apoA-IV being synthesized in the intestine and the remainder by the liver [389, 390]. While the physiological functions of apoA-IV remain to be fully elucidated, a number of functions have been proposed. These include acting as a satiety signal; aiding in apoC-II transfer to triglyceride-rich lipoproteins; increasing LPL activity and stimulating LCAT activity [319, 320, 391, 392]. Studies of an apoA-IV knockout mouse demonstrated no abnormalities in lipid absorption or feeding behavior, suggesting that although apoA-IV is synthesized in the intestine, the major function may lie elsewhere [318]. Importantly, apoA-IV knockout mice exhibited a marked decrease in HDL cholesterol due to an increased catabolism of these particles. Consistent with this, overexpression of liver apoA-IV in mouse models of atherosclerosis protected from atherosclerotic disease [393]. In further support of a protective role for apoA-IV in atherosclerosis, studies have demonstrated an inverse relationship of apoA-IV levels and risk of coronary heart disease in human subjects [394, 395].

Few studies have attempted to elucidate the regulation of apoA-IV at the promoter level. One such study has demonstrated that basal apoA-IV levels in the intestine depend on the orphan nuclear receptor estrogen-related receptor alpha in combination with PGC-1α [396]. In addition, the orphan nuclear receptors HNF-4α and -4gamma (HNF-4γ) have been shown to contribute to apoA-IV expression in the intestinal villi [397]. Another study has demonstrated induction of ApoA-IV in the liver (but not the intestine) of mice treated with an LXR agonist [398]. Additional insight into a possible regulatory mechanism of apoA-IV comes from recent work that has indicated modulation of apoA-

IV levels by glycemic status. For instance, insulin-dependent diabetic patients had higher apoA-IV levels (independent of triglyceride or HDL levels) that were closely related to control of glycemia [399]. In addition, mRNA levels of apoA-IV increased in the livers of rats treated with insulin [400].

4.2.8 Summary and Objectives

Given that variations in plasma apoA-IV levels occur in response to nutritional and hormonal status, we endeavored to investigate the mechanisms by which apoA-IV synthesis in the liver and intestine is regulated in response to fasting as well as diabetic states. We demonstrate that apoA-IV protein and mRNA levels increase in mouse liver and ileum during fasting and diabetes and that these increases occur in a glucocorticoid-dependent fashion. In vitro analyses revealed that human and mouse apoA-IV promoter activity is markedly induced by the interactions of PGC-1α and HNF-4α. Evidence for this interaction were supported by in vivo studies demonstrating parallel induction of PGC-1α, HNF-4α and apoA-IV mRNA in fasting and an absence of basal apoA-IV expression and induction in HNF-4α liver-specific knockout mice during fasting.

4.3 MATERIALS AND METHODS

4.3.1 Mice and In Vivo Protocols

All fasting studies began in the late afternoon and continued overnight for a total of 24 h. For the fasting time-course, mice were deprived of food at the beginning of the dark cycle and sacrificed 6-, 12- and 24 h later. In addition, one group was fasted for 24 h after which free access to food was given for an additional 24 h. PPARα-/- and liver-specific

HNF4α-/- mice were obtained from our breeding colony and have been previously described [382, 401]. Dexamethasone (Sigma-Aldrich, St. Louis, MO) treatment consisted of 4 daily intra-peritoneal (I.P.) injections of 50 mg/kg dexamethasone (concentrated solution dissolved in 65% Cremophor EL (Sigma-Aldrich) and 35% ethanol and subsequently diluted 1:5 in 5% glucose solution). Streptozocin treatment consisted of 2 consecutive daily I.P. injections of 250 mg/kg streptozocin (Sigma-Aldrich) in sodium citrate buffer (pH 4.5) after which the diabetic mice (blood glucose levels of ≥15 mmol/L) were housed for 4 weeks. Adrenalectomized (Adx) and sham-operated mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed for 1 week (all mice were given 0.9% normal saline to drink) prior to commencement of either a 24 h fast or dexamethasone treatment (as per above protocol). Ob/Ob mice were also obtained from Jackson laboratories. All mice were of C57BL/6J background and were approximately 7-8 weeks of age with the exception of the ob/ob mice and littermate controls (12 weeks of age) and were housed at room temperature under a 12 h light/dark cycle and provided food and water ad libitum. All procedures were conducted at the Carleton Animal Care Facility in accordance with Canadian Council on Animal Care guidelines.

4.3.2 Hepatic and Ileal Gene Expression

Total hepatic and ileal RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) as per the supplier's instructions. Total RNA (2 μg) was reverse transcribed using Stratascript Reverse Transcriptase (Stratagene, La Jolla, CA) with random hexamers $pd(N)_6$ according to the supplier's instructions. The synthesized cDNA was then

amplified by quantitative PCR using a Stratagene MX3000p thermocycler in a total volume of 25 μl with Brilliant SYBR Green QPCR Master Mix. Murine primer sequences are listed in Table 4.1. Thermal cycling conditions were performed as previously described [300]. Relative C_T values were obtained by the $\Delta\Delta C_T$ method [242] using a threshold of 10 standard deviations above background for C_T.

4.3.3 Plasma Corticosteroid Analysis

Blood was collected from Adx and sham-operated mice (fed or fasted for 24 h) via cardiac puncture using heparanized needles and centrifuged at 6700 x g for 5 minutes. The resulting plasma samples were treated with a steroid displacement reagent, diluted 50-fold and assayed for corticosteroid levels in a corticosteroid enzyme-linked immunosorbance assay (ELISA) as per the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN).

4.3.4 Western Blot Analysis

Immunoblot analyses of apos B100, B48, A-I and A-II were performed as previously described [209]. For immunoblot analysis of plasma apoA-IV protein, 50 μg of plasma protein was separated on a 10% acrylamide gel, transferred onto a 0.45 μm nitrocellulose membrane (Whatman Inc., Florham Park, NJ) and blocked for 1 hour with 5% milk in Tris-Buffered Saline Tween-20 (TBS-T). The membrane was then probed for 2 hours at room temperature with polyclonal anti-mouse ApoA-IV-IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a dilution of 1/1000 in 1% milk in TBS-T. The membrane was then incubated with HRP-conjugated rabbit antigoat IgG (Sigma-Aldrich) at a dilution of 1/10000 (in 1% milk in TBS-T) for 1 h at room temperature. Antibody binding was

Table 4.1: Real-time quantitative PCR primer sequences

Primer Name	Primers (5'-3')	Accession Number
RP-II	CTG GAC CTA CCG GCA TGT TC GTC ATC CCG CTC CCA ACA C	M12130
ApoA-IV	CAG TGA GGA GCC CAG GAT GTT TCT ACA GCC TCC TTG GCA TT	BC050149
ApoA-I	GGC ACG TAT GGC AGC AAC AT GAC TAA CGG TTG AAC CCA GAG	NM_009692
Cyp3a11	TGG TCA AAC GCC TCT CCT TG TGA ATG TGG GGG ACA GCA AAG	NM_007818
PGC-1α	GAA GTG GTG TAG CGA CCA ATC AAT GAG GGC AAT CCG TCT TCA	NM_008904
ApoC-III	TAC AGG GCT ACA TGG AAC AAG C CAG GGA TCT GAA GTG ATT GTC C	NM_023114
Cyp4a14	GTC TCT CGG GGA GCA ATA TAC G ACC AAT CCA GGG AGC AAA GAA	BC089609
HNF-4α:	CAC GCG GAG GTC AAG CTA C CCC AGA GAT GGG AGA GGT GAT	NM_008261

detected using the ECL plus chemiluminescence kit (Amersham Biosciences, Piscataway, NJ) and imaged using a Storm 840 phosphorimager (Molecular Dynamics).

4.3.5 ApoA-IV Promoter Constructs

The pGL3-heC3A4 construct containing the human apoA-IV promoter fused to the upstream apoC-III enhancer was created using an original construct (eC3A4-CAT)[301], generously donated by Agnès Ribeiro (INSERM, Université Pierre et Marie Curie, Paris, France) as the template. The primers used for the generation of this construct are listed in Table 4.2. Amplification of the promoter region was performed using Elongase PCR (Invitrogen). The PCR product was digested with KpnI/MluI and ligated into the pGL3-Basic vector (Promega, Madison, WI) previously digested with the same enzymes. For generation of the mouse apoA-IV promoter construct, mouse genomic DNA was isolated from a male C57BL/6J ear tag using phenol:chloroform:isoamyl alcohol organic extraction (25:24:1), precipitated using 1/10 volume of sodium acetate and 2.5 volume of ethanol, washed in ethanol and used as a template for Elongase (Invitrogen) PCR amplification. The mouse ApoC-III/ApoA-IV intergenic region (approximately -5808 to +25), which contains both the apoA-IV proximal promoter as well as the apoC-III enhancer [396], was cloned into pGL3-Basic vector (pGL3-meC3A4). Primers used for amplification of the intergenic region are listed in Table 4.2. The PCR product was digested with MluI/BglII and ligated into the PGL3-Basic vector. All constructs were verified by restriction mapping and sequencing.

Table 4.2: Oligomer sequences used for promoter and expression constructs
Underlined sequences correspond to restriction enzyme sites used for cloning
Bolded sequence corresponds to stop site

Construct Name	Primers (5'-3')
pGL3-heC3A4	AAAA GGT ACC AAT GCA TGT TGA CGG GAC CGT CG AAAA ACG CGT AGT TGA CTG AAG CTC AGA GCC AGC CAG A
pGL3-meC3A4	AAAA ACG CGT CAT TAC CTG GAG TAG CTA GCT GC AAAA AGA TCT GTG TAC CTG TGT CCC TGT GCA G
DN-rHNF-4α	AAAA GGT ACC ATG GAC ATG GCT GAC TAC AGT GCT (N terminal) AAAA AAG CTT **TCA** CAG GTT GTC AAT CTT GGC CAT (C terminal)
pENTR-sh-hHNF4α	CAC CGC ACT CGA AGG TCA AGC TAT GCG AAC ATA GCT TGA CCT TC (+) AAAA GCA CTC GAA GGT CAA GCT ATG TTC GCA TAG CTT GAC CTT C (-)
PSG5-mPGC-1α	AAAAGGATCCAGCTGGATGGCTTGGGACATGTG AAAAGGATCCGGAACACGTTACCTGCGCAAGCTT
PSG5-mGR	AAAAGGATCCGCCAATGGACTCCAAAGAATCCTTA AAAAAGATCTGCAGTCATTTCTGATGAAACAGAAGC

4.3.6 Dominant-Negative rHNF4α-, shRNA- and Nuclear Receptor-Expressing Constructs

The construct expressing a dominant negative (DN) form of rat HNF-4α was generated using primers based on those designed by Ladias *et al.* [402]. Sequences of the primers used to generate the DN rat HNF-4α (DN-rHNF-4α) are listed in Table 4.2. The PCR product was digested with HindIII/KpnI and ligated into pShuttle-CMV (Stratagene) previously digested with the same enzymes. The construct expressing small hairpin (sh)RNA for human HNF-4α was generated using oligomers designed using Block-iT RNAi Designer Web Software (Invitrogen). Double stranded oligomers were annealed and ligated into the pENTR/U6 vector using the Block-iT U6 RNAi Entry Vector Kit according to the manufacturer's instructions (Invitrogen). Sequences of the oligonucleotides used for generation of the pENTR-sh-hHNF4α are listed in Table 4.2. Expression constructs for murine GR and PGC-1α were created by RT-PCR amplification of C57BL/6J mouse liver total RNA using the primers shown in supplemental Table 2 followed by insertion into the BamHI/BglII or BamHI sites of pSG5, respectively. The identity of the constructs was verified by restriction digest and sequencing. The expression construct for rat HNF4α has been described previously [403].

4.3.7 Cell Culture and Transfections

HepG2 and African Green Monkey kidney fibroblast (Cos-7) cells (ATCC) were maintained in complete media containing phenol red-free Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) supplemented with 1 mM sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich) and 10% heat-inactivated charcoal

dextran-stripped FBS (Gemini Bioproducts, Woodland, CA). Cells were maintained at 37°C in 5% CO_2. For transfections, HepG2 and Cos-7 cells were plated at a density of 150,000 and 120,000 cells/mL (0.5 mL per well) in 24-well plates and transfected 24 h later.

For transfections of dominant-negative HNF-4α or HNF-4α shRNA-expressing constructs, cells were transfected with: 120 ng of luciferase reporter construct; 100 ng of pCMV-Bgal or PRL-TK; 280 ng of PGC-1α or PSG5. In addition, for shRNA transfections, 75, 150 and 275 ng of pENTR-hHNF-4α or pENTR-lacZ (expresses shRNA for lacZ) was added and 75, 150 and 275 ng of pShuttle-DN-rHNF-4α or pshuttle-CMV was added for dominant negative transfections. For all other transfections, cells were transfected with: 150 ng of luciferase reporter construct; 50 ng of nuclear receptor expression construct; 125 ng of pCMV-βgal; 350 ng PGC-1α or PSG5 to a total of 700 ng. All cells were transfected using 4 μL/μg DNA of TransIT-LT1 (Mirus, Madison, WI). Cells were treated 24 h post-transfection with DMSO as vehicle, or 50 μM dexamethasone. DMSO reached a final concentration of 0.25% in all wells. Cells were harvested 24 h post-treatment using Reporter Lysis Buffer (Promega, Madison, WI) per manufacturer's instructions. For those transfection experiments that did not require treatment, cells were harvested 36 h post-transfection. Cell lysates were assayed for firefly luciferase using the Luciferase Assay System or renilla luciferase (for shRNA transfections) using the Dual Luciferase Reporter System (Promega). Activity of luciferase(s) was measured using a Luminoskan Ascent (Thermo Labsystems, Franklin, MA). Luciferase activity (in relative light units) was corrected for lysate renilla luciferase

activity or β-galactosidase activity that was measured calorimetrically using a Power-WaveX microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT).

4.3.8 Statistics

All comparisons were performed across groups using one-way ANOVA with Tukey-Kramer post-analysis, unless otherwise stated. GraphPad Instat (Instat3 V3.0a, GraphPad Software, Inc.) was used for all statistical analyses. Values are expressed as mean ± SD. Differences between groups were considered statistically

significant at $P \leq 0.05$.

4.4 RESULTS

In order to examine apoA-IV expression during changes in nutritional status, WT (C57Bl/6J) mice were fasted for 6, 12 and 24 h and one group was given 24 h access to food after a 24 h fast (re-fed). After each fasting time point, liver and ileal apoA-IV mRNA expression was measured by QPCR. QPCR analysis revealed a time-dependent induction of liver apoA-IV mRNA levels to approximately 4.5-, 20- and 15-fold in the fasted mice compared to fed controls (Fig. 4.3*a*). Re-feeding of 24 h fasted mice for an additional 24 h did not reverse this increase in apoA-IV mRNA levels. The genes encoding apoA-I and apoC-III are present in a cluster that also includes the gene for apoA-IV. These three genes have a number of common regulatory sequences present within the intergenic region located between the apoA-IV and apoC-III coding regions [404]. Therefore, to determine the specificity of induction of apoA-IV mRNA in fasted

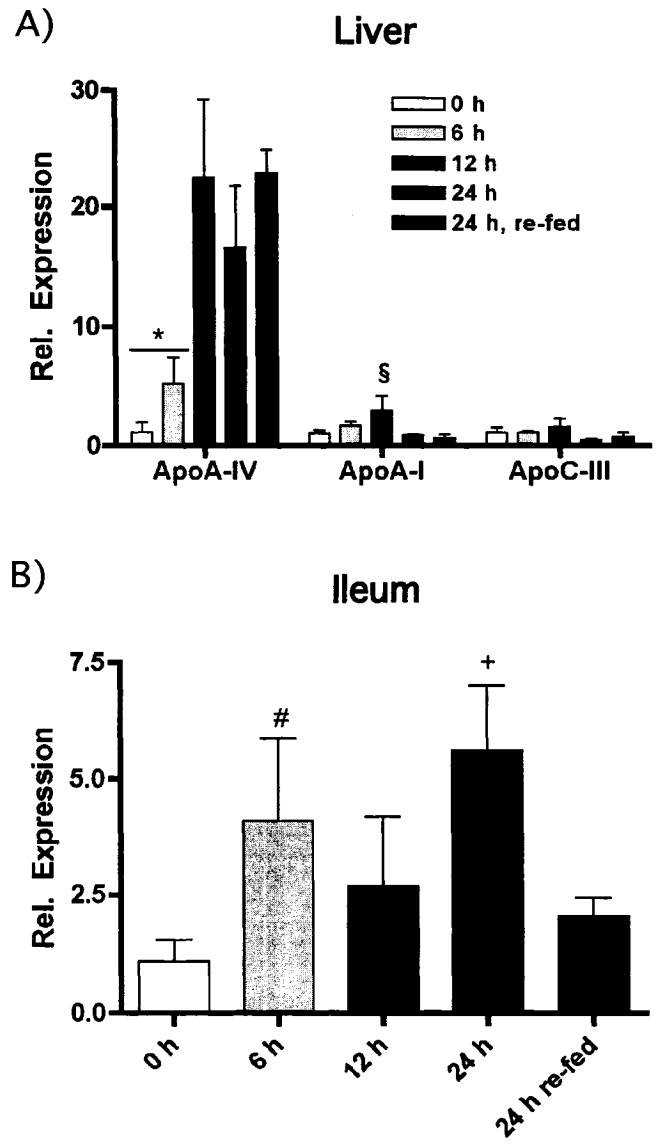

Figure 4.3 Hepatic and ileal apolipoprotein mRNA levels are increased in fasting. Real-time quantitative PCR analysis of apoA-IV, apoA-I and apoC-III mRNA expression in liver A) and apoA-IV expression in the ileum B) after 0, 6, 12 and 24 h fasts and 24 h fast followed by a 24 h re-feed. All values were normalized to RP-II mRNA levels and are expressed as the fold difference relative to 0 h fasted controls. *P<0.05 vs 12 and 24 h fasted and 24 h re-fed. §P<0.05 vs 0 and 24 h fasted and 24 h re-fed. #P<0.05 vs 0 h fasted. +P<0.05 vs 0 h fasted and 24 h re-fed. All values are mean ± SD, n=3-4.

liver, it was necessary to also examine apoA-I and apoC-III expression in the livers of these mice. Examination of apoA-I mRNA expression revealed a small, transient increase by 12 h of fasting (3-fold over controls) that was abolished at the 24 h fasting time point (Fig. 4.3*a*). ApoC-III mRNA expression in fasted liver was not altered at any of the time points examined (Fig. 4.3*a*). Ileal expression of apoA-IV mRNA was also examined in fasted mice and was induced to approximately 4- and 6-fold over fed controls at the 6 and 24 h time points and was decreased to control levels after 24 h of re-feeding (Fig. 4.3*b*).

PPARα is a nuclear hormone receptor that mediates many hepatic responses to fasting by regulating the expression of genes with key roles in energy homeostasis (33). Therefore, in order to determine if PPARα was involved in the induction of apoA-IV in fasting, PPARα-/- mice were fasted for 24 h and examined for changes in expression of both apoA-IV mRNA (liver) and protein (plasma). Consistent with the results of the fasting time course (Fig. 4.3*a*), liver apoA-IV mRNA levels were induced to approximately 19-fold over fed controls by a 24 h fast (Fig. 4.4*a*). Basal apoA-IV mRNA expression was elevated (approximately 3.5-fold over WT) in PPARα-/- mouse livers (Fig. 4.4*a*). Examination of apoA-IV mRNA levels in fasted PPARα-/- mice revealed a similar magnitude of induction to that seen in the control mice; approximately 15-fold over WT controls (Fig. 4.4*a*). When apoA-IV expression in fasted PPARα-/- was compared to the high basal expression in PPARα-/- fed mice, the induction level was reduced to approximately 4-fold (Fig. 4.4*a*). QPCR analysis of Cyp4a14 mRNA expression, an established PPARα target gene [405], demonstrated a complete lack of expression in both fed and fasted PPARα-/- mice (Fig. 4.4*b*). In contrast, fasting induced

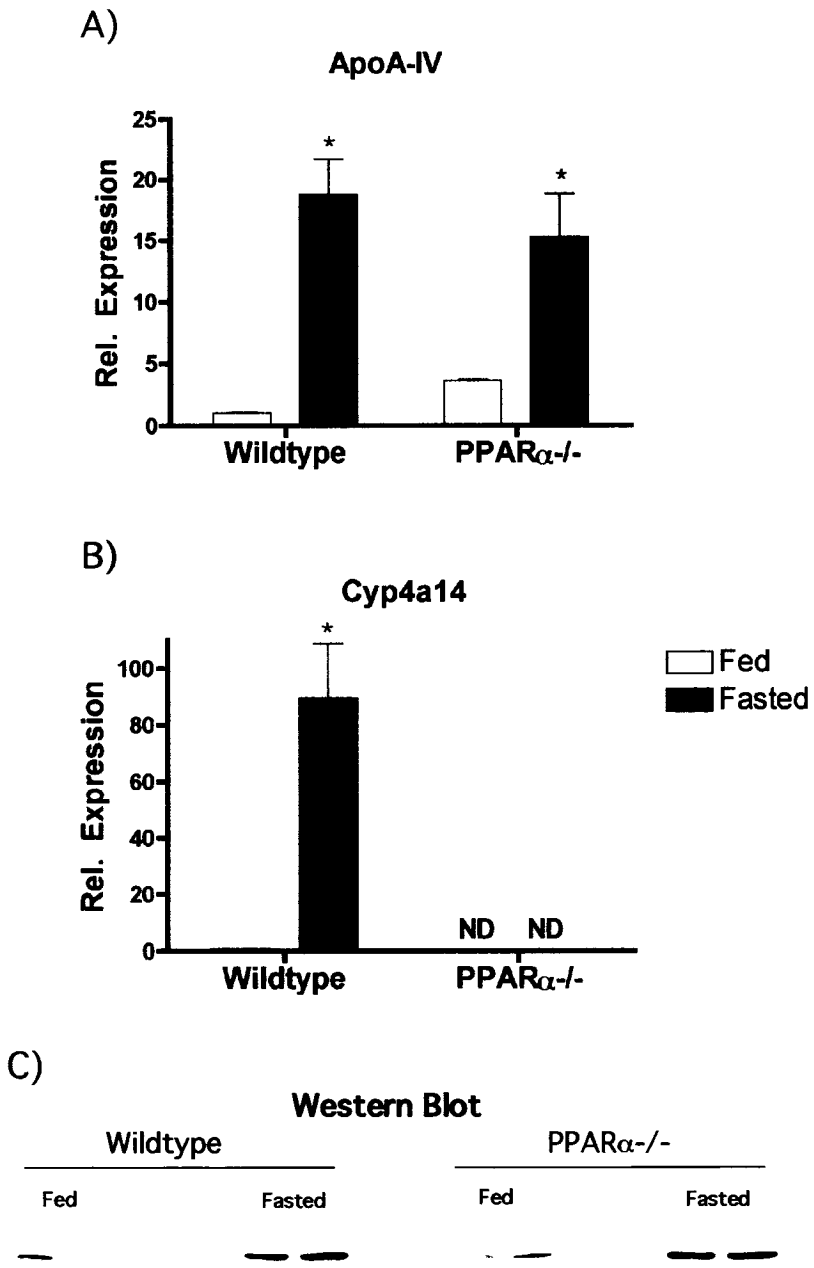

Figure 4.4 Fasting apoA-IV induction is not abolished in PPARα-/- mice. Real-time quantitative PCR analysis of hepatic mRNA expression of A) apoA-IV and B) Cyp4a14 in 24 h fasted wildtype and PPARα-/- mice. All values were normalized to RP-II mRNA levels and are expressed as the fold difference relative to wildtype fed controls. C) Western blot analysis of ApoA-IV plasma protein levels in wildtype and PPARα-/- mice fasted for 24 h. *P<0.05 vs respective wildtype values. All values are mean ± SD, n=3-4.

liver Cyp4a14 mRNA in WT mice by approximately 90-fold over fed controls (Fig 4.4*b*). Protein expression in plasma of fasted and fed PPARα-/- and WT mice was determined by western blot. Consistent with the increases in apoA-IV mRNA, protein expression of apoA-IV was increased in the plasma of both WT and PPARα-/- mice fasted for 24 h (Fig. 4.4*c*).

Food deprivation is known to elevate plasma levels of corticosteroids through activation of the hypothalamic-pituitary-adrenal axis [369]. Therefore, in order to further characterize the regulatory mechanisms responsible for the induction of apoA-IV expression in fasting, Adx mice were fasted for 24 h. ELISA was used to determine plasma corticosteroid concentrations in sham-operated and Adx mice. Plasma corticosteroid levels in sham-operated mice were increased by a 24 h fast (approximately 2.5-fold) over levels seen in fed controls (Fig. 4.5*a*). Adrenalectomy of mice resulted in a decrease of corticosteroid levels (by approximately 2.5-fold) when compared to the sham-operated control and this level was not significantly increased by a 24 h fast (Fig. 4.5*a*). Liver mRNA expression of apoA-IV in fasted, Adx mice was examined by QPCR. Consistent with previous results, fasting sham-operated mice for 24 h resulted in induction of liver apoA-IV mRNA to approximately 19-fold over fed, sham-operated controls (Fig. 4.5*b*). Adrenalectomized mice that had been fasted for 24 h lacked significant induction of liver apoA-IV mRNA expression (Fig. 4.5*b*). Ileal mRNA expression of apoA-IV was also induced approximately 24-fold and this induction was abolished by adrenalectomy (Fig. 4.5*b*). Plasma concentrations of apolipoproteins in Adx and sham-operated mice were examined by western blot. ApoB-100 and apoB-48 levels

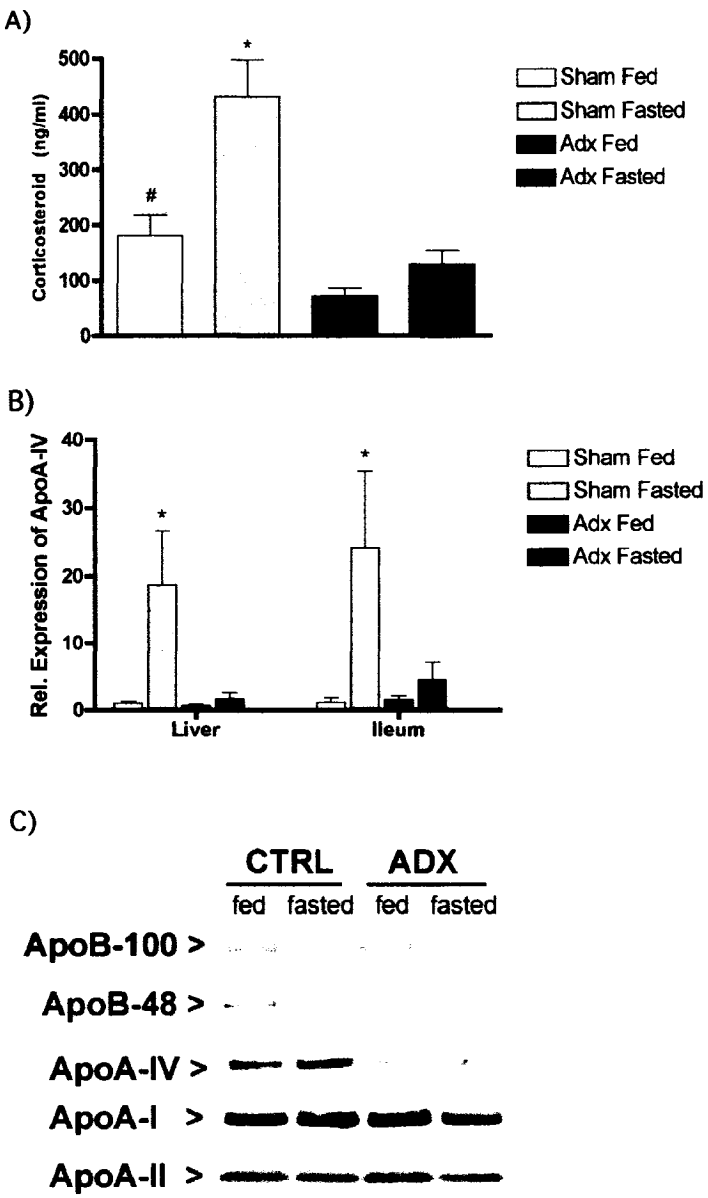

Figure 4.5 ApoA-IV induction is abolished in adrenalectomized mice.
A) ELISA detection of plasma corticosteroid levels in adrenalectomized and sham-operated mice (fed or fasted for 24 h). Real-time quantitative PCR analysis of B) hepatic and ileal apoA-IV mRNA expression. All values were normalized to RP-II mRNA expression and are expressed as the fold difference relative to sham-operated mice (fed). C) Western blot analysis of plasma lipoproteins from the adrenalectomized and sham-operated mice (fed or fasted for 24 h). *P<0.05 vs all other groups. #P<0.05 vs adrenal-ectomized fed. All values are mean ± SD, n=5.

were decreased while apoA-IV protein levels were increased in fasted, sham-operated mice over that detected in controls (Fig. 4.5*c*). Adx mice had decreased plasma apoA-IV protein levels relative to the control that were not increased by a 24 h fast (Fig. 4.5*c*). Western blot analysis also revealed that protein levels of apoA-I and apoA-II were not altered in response to either fasting or adrenalectomy (Fig. 4.5*c*). Further investigation of the role of corticosteroids in the *in vivo* induction of the apoA-IV gene was examined by treatment of WT (C57BL/6J) mice with dexamethasone. QPCR analysis of liver apoA-IV mRNA demonstrated induction (approximately 7-fold) over vehicle treated controls (Fig. 4.6*a*). Similarly, the mRNA levels of Cyp3a11, an established dexamethasone-responsive gene [406], were increased by approximately 5-fold (Fig. 4.6*a*). Hepatic expression of HNF-4α and PGC-1α was also increased in dexamethasone-treated mice by 1.6- and 4.6-fold over vehicle treated controls, respectively (Fig. 4.6*a*). Western blot analysis revealed decreases in both apoB-100 and apoB-48 plasma proteins in dexamethasone-treated compared to vehicle- treated controls (Fig. 4.6*b*). Consistent with mRNA levels, plasma protein expression of apoA-IV was increased with dexamethasone treatment (Fig. 4.6*b*). Plasma protein levels of apoA-I and apoA-II were also increased in dexamethasone treated mice (Fig. 4.6*b*). FPLC separation and subsequent western blotting of plasma revealed that the increase in apoA-IV protein occurred solely in the HDL fraction (data not shown). To determine if exogenous glucocorticoid treatment could induce apoA-IV in adrenalectomized mice, mice were treated with dexamethasone. This treatment resulted in a 20-fold induction of apoA-IV mRNA levels (Fig 4.6*c*).

Glucocorticoids are a causative factor in many of the pathologies associated with diabetes, including hyperlipidemia and hyperglycemia [368, 375, 407]. The diabetic liver,

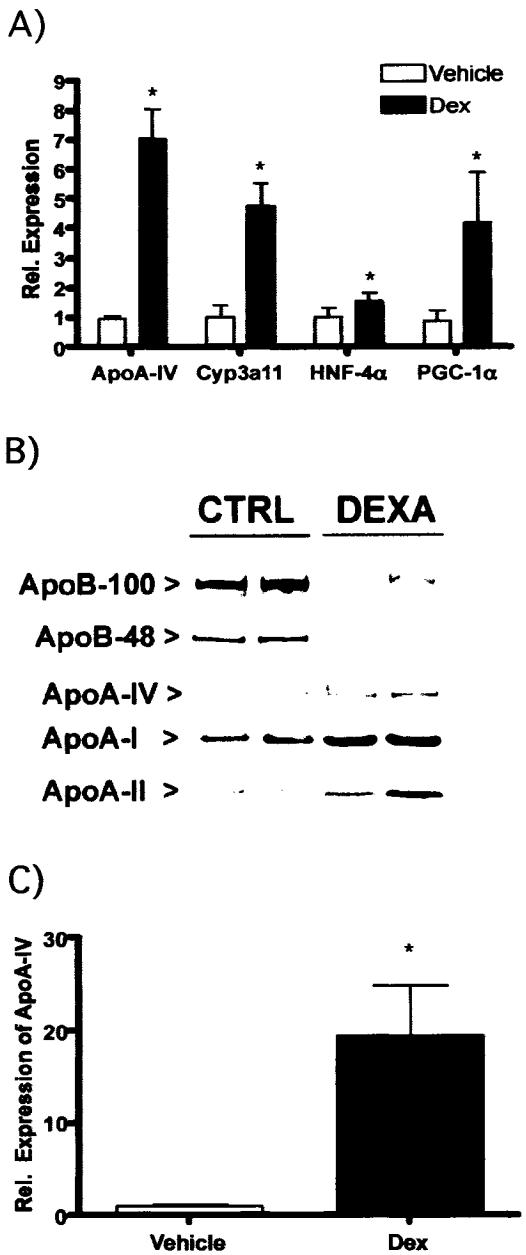

Figure 4.6 Exogenous glucocorticoids increase apoA-IV expression.
A) Real-time quantitative PCR analysis of hepatic apoA-IV, Cyp3a11, HNF-4α and PGC-1α mRNA expression in dexamethasone-treated mice. All values were normalized to RP-II mRNA levels and are expressed as the fold difference relative to vehicle-treated mice. B) Western blot analysis of plasma lipoproteins from dexamethasone-treated mice. C) Real-time quantitative PCR analysis of hepatic apoA-IV mRNA expression in dexamethasone treated, adrenalectomized mice. *P<0.05 vs respective vehicle-treated controls (unpaired t-tests). All values are mean ± SD, n=4-5.

being unable to properly sense circulating glucose levels, responds similarly to the fasted liver; by increasing gluconeogenesis. Given the importance of glucocorticoids in hepatic responses to fasting and diabetes and the demonstrated importance of corticosteroids in the *in vivo* induction of apoA-IV in fasting, we endeavored to examine expression of apoA-IV in two animal models of diabetes. *In vivo* expression of apoA-IV in diabetes was first investigated by induction of type-I diabetes in C57BL/6J mice by streptozocin injection. High blood glucose (≥15mmol/L) levels were confirmed in STZ-injected mice compared to vehicle-treated controls. In addition, triglyceride and cholesterol levels were increased in STZ-injected mice by 2- and 4-fold, respectively (Fig. 4.7*a*). At 4-weeks post-injection, liver mRNA expression of apoA-IV was increased approximately 3.5-fold over vehicle treated controls (Fig. 4.7*b*). Liver expression of the apoA-I and apoC-III genes were also altered in STZ treated mice; a 2-fold decrease and a 4-fold increase in apoA-I and apoC-III mRNA levels were measured, respectively (Fig. 4.7*b*). Plasma protein levels of apoA-IV in STZ treated mice were measured by western blot and found to be increased in the diabetic mice compared with vehicle treated mice (Fig. 4.7*c*). The inductive effect of the diabetic state on apoA-IV expression was further examined using ob/ob mice, a model of T2DM (40). QPCR analysis of liver mRNA expression in ob/ob mice demonstrated a large induction of apoA-IV expression (approximately 125-fold) over that measured in control littermates (Fig. 4.8*a*). Analysis of mRNA expression of the apoA-I and apoC-III genes revealed no changes in levels of either transcript in the ob/ob mice (Fig. 4.8*a*). Consistent with the inductive effect of type I diabetes on plasma protein levels of apoA-IV, the ob/ob model of T2DM also demonstrated an increase in the protein compared to WT littermate controls (Fig. 4.8*b*).

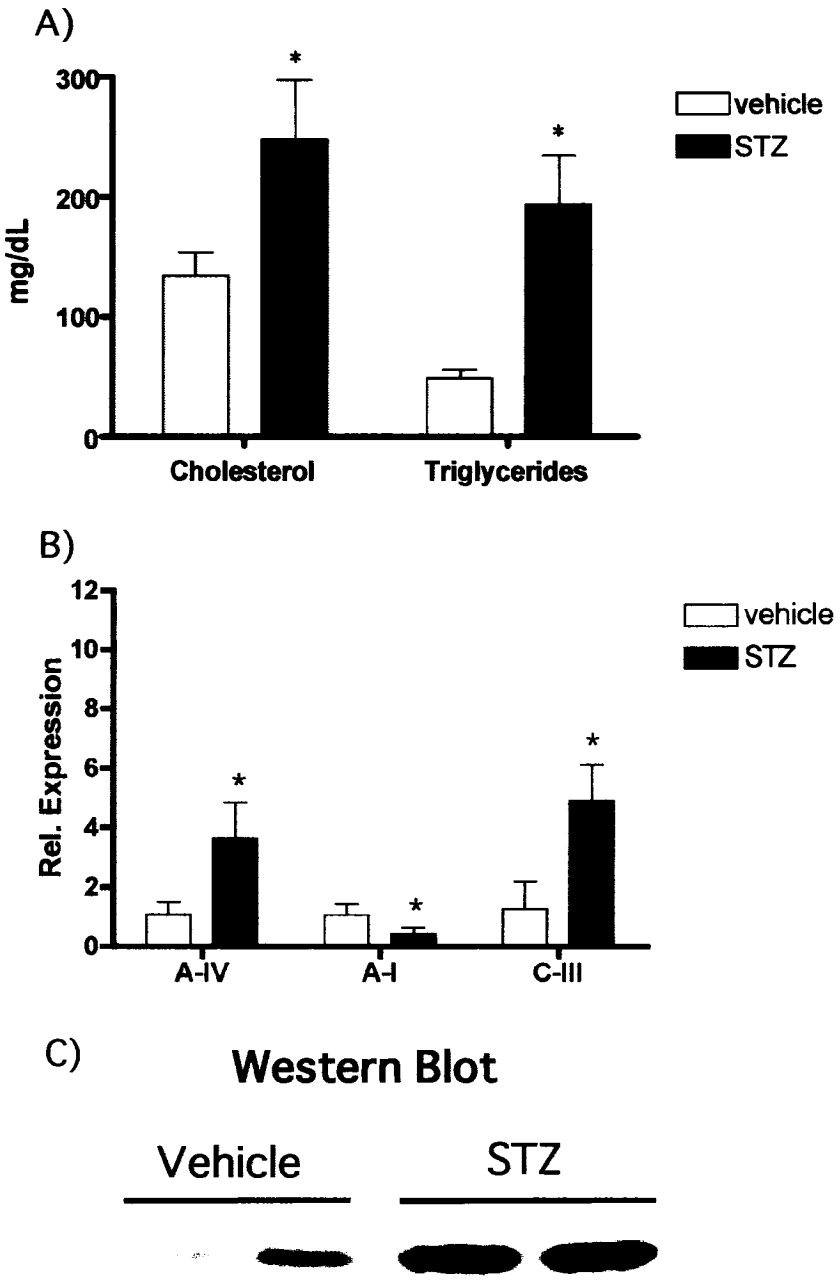

Figure 4.7 ApoA-IV mRNA and serum protein levels are increased in type I diabetes. A) Plasma cholesterol and triglyeride levels of 4 week post-streptozocin injected mice. B) Real-time quantitative PCR analysis of hepatic mRNA expression of apoA-IV, apoA-I and apoC-III in mice 4 weeks post-streptozocin injection. C) Western blot analysis of apoA-IV plasma protein levels in mice 4 weeks post-streptozocin injection. All values were normalized to RP-II expression and are expressed as the fold difference relative to vehicle-treated controls. *P<0.05 vehicle trated controls All values are mean ± SD. N=4-5.

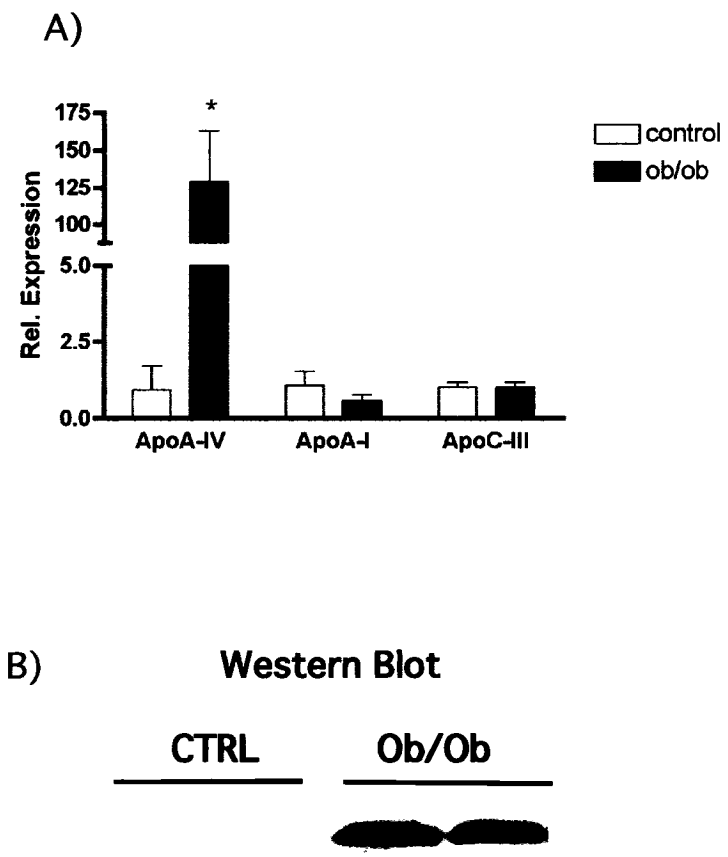

Figure 4.8 ApoA-IV mRNA and serum protein levels are increased in type II diabetes.

A) Real-time quantitative PCR analysis of hepatic mRNA expression of apoA-IV, apoA-I and apoC-III in 12-week old Ob/Ob mice. D) Western blot analysis of apoA-IV plasma protein levels in 12 week-old Ob/Ob mice. All values were normalized to RP-II expression and are expressed as the fold difference relative to littermate controls. *P<0.05 vs littermate controls.. All values are mean ± SD. N=4-5.

Given the requirement for adrenal corticosteroids for the induction of apoA-IV during fasting and the ability of dexamethasone, a synthetic corticosteroid, to increase expression of this gene *in vivo*, the role of the GR in regulating apoA-IV promoter activity was investigated. HNF-4α has been shown to regulate to apoA-IV expression *in vivo* [301] and PGC-1α is known to contribute to a number of the changes in gene expression that occur during fasting. As such, experiments were designed to test the effect of transfection of a mouse promoter reporter construct containing both the apoC-III enhancer region and the apoA-IV promoter region (designated mEC3A4) into HepG2 cells in combination with control (pSG5) vector, GR- or HNF-4α-expressing constructs in the presence or absence of a PGC-1α–expressing construct. Both the human and mouse apoC-III/A-IV intergenic regions contain three conserved hormone response elements (HREs) that bind HNF-4α; one HRE in each of the proximal and distal regions of the apoA-IV promoter and one HRE in the apoC-III enhancer region [301, 397, 408, 409]. A schematic representing both the human and mouse apoA-IV promoter constructs used in the transient transfection experiments is provided in Fig. 4.9*a*. Cotransfection of the PGC-1α construct with pSG5 resulted in increased activation of mEC3A4 (approximately 15-fold) and was not further activated by addition of dexamethasone (Fig. 4.9*b*). Transfection of the GR construct alone did not activate mEC3A4 however cotransfection with the PGC-1α construct increased activity (approximately 15-fold) and was not further activated by treatment with dexamethasone (Fig 4.9*b*). Transfection of the HNF-4α construct alone caused induction of mEC3A4 (5-fold) and this induction was synergistically increased by the cotransfection of the PGC-1α construct, inducing activity by approximately 65-fold (Fig. 4.9*b*). Treatment with dexamethasone did not affect the

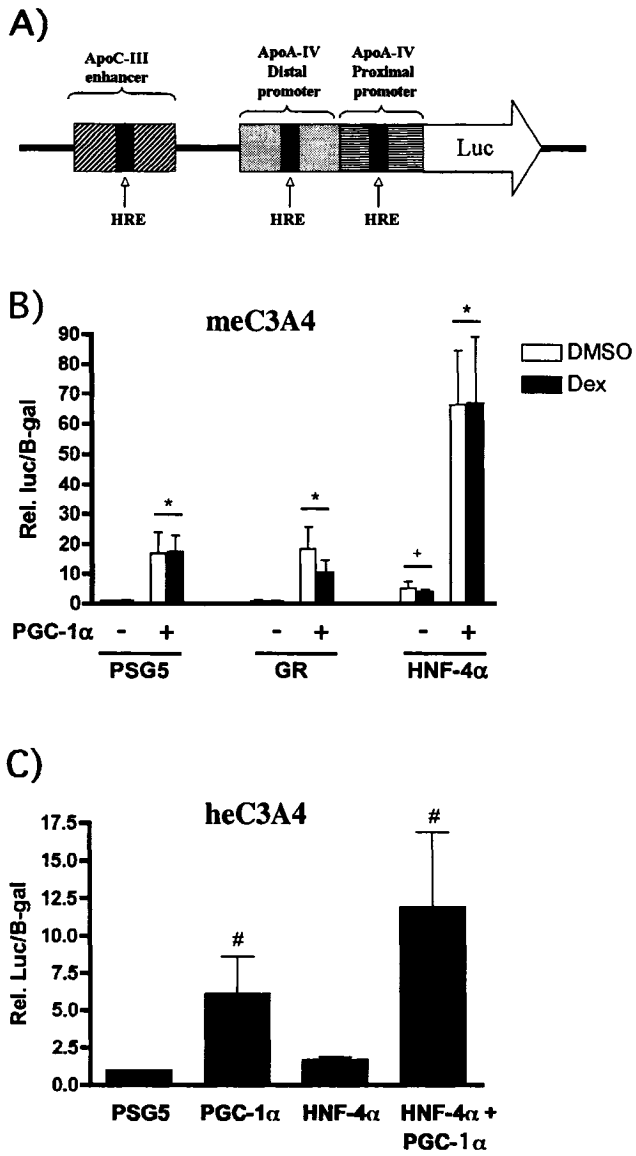

Figure 4.9 HNF-4α and PGC-1α activate murine and human apoA-IV promoter activity.

A schematic representing the mouse and human apoA-IV promoter constructs containing 3 HREs known to bind HNF-4α (A). Cotransfection of HepG2 cells with (B) the mouse or (C) human apoA-IV/C-III intergenic region in addition to vector (PSG5) or constructs expressing GR or HNF-4α. Cells were treated 24 h post-transfection with either 50 μM dexamethasone or DMSO and harvested 24 h later. Luciferase values were normalized to individual β-galactosidase activities. Promoter luciferase activities were reported as fold activation over activity with PSG5. *P<0.05 vs expression construct alone (PSG5, GR or HNF-4α) with same treatment. +P<0.05 vs transfection with PSG5 or GR alone (unpaired t tests). #P<0.05 vs all other groups. All values are mean ± SD, n=3.

activity of mEC3A4 either in the presence of the HNF-4α construct alone or in combination with the PGC-1α construct (Fig. 4.9*b*). To determine if the regulatory mechanisms demonstrated with the murine apoA-IV promoter were conserved, the human apoA-IV construct was also examined. This construct contained the apoC-III enhancer fused to the apoA-IV promoter and was designated hEC3A4. Consistent with the pattern of regulation seen in the mouse promoter, transfection of the PGC-1α construct alone caused activation of hEC3A4 by approximately 7-fold (Fig. 4.9*c*). Transfection of the HNF-4α construct alone did not activate hEC3A4 while the combination with the PGC-1α construct synergistically induced activity by approximately 11-fold (Fig. 4.9*c*). The observation that PGC-1α could activate apoA-IV promoter activity in the absence of cotransfected receptor indicated an interaction with an endogenous nuclear receptor. Given that PGC-1α, when cotransfected with HNF-4α, led to a synergistic activation of the apoA-IV promoter, we hypothesized this nuclear receptor was involved. To determine whether the basal activation of the apoA-IV promoter region by cotransfection of PGC-1α alone was due to endogenous HNF-4α in HepG2 cells, similar transfections were performed in Cos-7 cells, which do not express high levels of HNF-4α. Transfection of either the PGC-1α or HNF-4α constructs alone did not cause activation of the mEC3A4 construct in these cells (Fig. 4.10*a*). Consistent with data obtained in the HepG2 cells, cotransfection of both HNF-4α and PGC-1α-expressing constructs in Cos-7 cells caused a synergistic induction of mEC3A4 by approximately 170-fold (Fig. 4.10*a*). A similar pattern of regulation of the human apoA-IV promoter was seen in transfections of Cos-7 cells where neither the PGC-1α nor the HNF-4α construct alone could induce the hEC3A4 construct but cotransfection of the

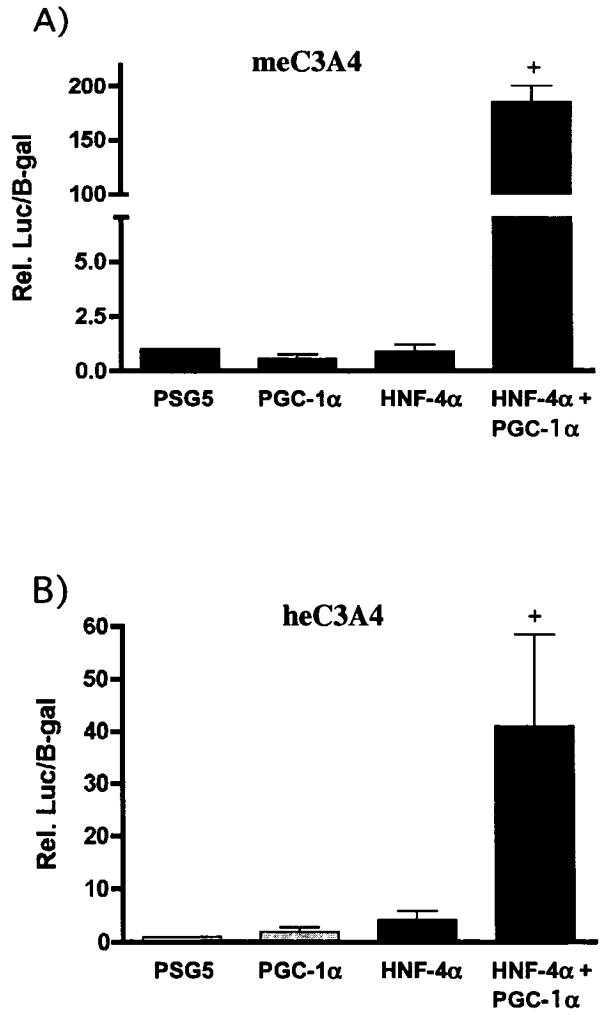

Figure 4.10 Lack of induction of mouse and human apoA-IV promoters by PGC-1α in non-hepatic cells.

Cos-7 cells were co-transfected with constructs containing the A) mouse or B) human apoA-IV/C-III regulatory regions in addition to either PGC-1α or HNF-4α expressing constructs, alone or in combination. Cells were harvested 24 h post-transfection. Luciferase values were normalized to individual β-galactosidase activities for the same well. Promoter luciferase activities were reported as fold activation over activity with pSG5. +$P<0.05$ vs all other groups. Values are mean ± SD, n=3.

two led to a synergistic induction of approximately 40-fold (Fig. 4.10*b*). Therefore, the activating effect of PGC-1α alone on hEC3A4 in HepG2 cells was likely due to the presence of endogenous HNF-4α. To confirm the contribution of endogenous HNF-4α to mEC3A4 activation in HepG2 cells, two independent approaches were used to disrupt this effect. The first used a vector expressing a DN form of HNF-4α. Transfection of 75, 150 and 275 ng of this construct caused a dose-dependent decrease in coactivation of mEC3A4 by PGC-1α (Fig. 4.11*a*). The second approach knocked-down endogenous HNF-4α expression using a human HNF-4α-shRNA expression vector. Transfection with the lowest amount of this construct (75 ng) abolished the coactivation of mEC3A4 by PGC-1α (Fig. 4.11*a*). Similar to results seen with the mEC3A4 construct, the DN rHNF-4α-expressing construct caused a dose-dependent decrease in coactivation of the hEC3A4 construct by PGC-1α (Fig. 4.11*b*). Similarly, cotransfection of the lowest amount of human HNF-4α shRNA-expressing construct (75 ng) abolished the coactivation of hEC3A4 by PGC-1α (Fig. 4.11*b*).

Further characterization of the role of HNF-4α and PGC-1α in activation of apoA-IV was assessed *in vivo* by QPCR analysis of livers from 0, 6, 12, 24 h fasted and 24 h fasted, re-fed mice. Hepatic expression of PGC-1α was induced in 6, 12, and 24 h fasted mice (approximately 3-, 6- and 3.5-fold, respectively) and decreased to control values after 24 h access to food (Fig. 4.12*a*). Similarly, hepatic mRNA expression of HNF-4α was induced by a 6 and 12 h fast however, decreased to control values by a 24 h fast and remained at this level after 24 h access to food (Fig. 4.12*a*). Further insight into the *in vivo* roles of HNF-4α and PGC-1α was determined through a 24 h fast of HNF-4α

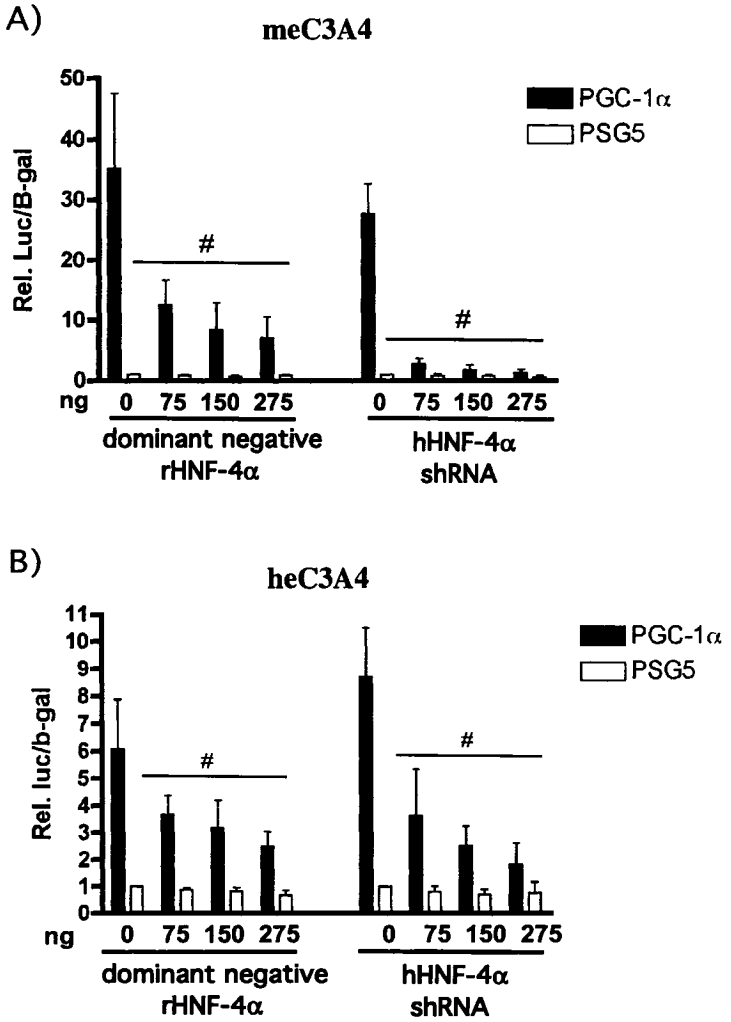

Figure 4.11 HNF-4α is required for PGC-1α-coactivation of the mouse and human apoA-IV promoters.

HepG2 cells were co-transfected with a construct containing the A) mouse or B) human apoA-IV/C-III regulatory regions with or without the addition of a PGC-1α-expressing construct and 75, 150 and 275 ng of constructs expressing either a dominant-negative rat HNF-4α or a human HNF-4α shRNA (hHNF4α). Cells were harvested 36 h post-transfection. Firefly luciferase values were normalized to renilla luciferase activity for the same well. Promoter luciferase activities were reported as fold activation over activity with control vector (pshuttle or the pENTR-lacZ construct that expresses shRNA for lacZ). #P<0.05 vs PGC-1α plus 275ng control vector (either pENT-lacZ or TK-RL). Values are mean ± SD, n=3.

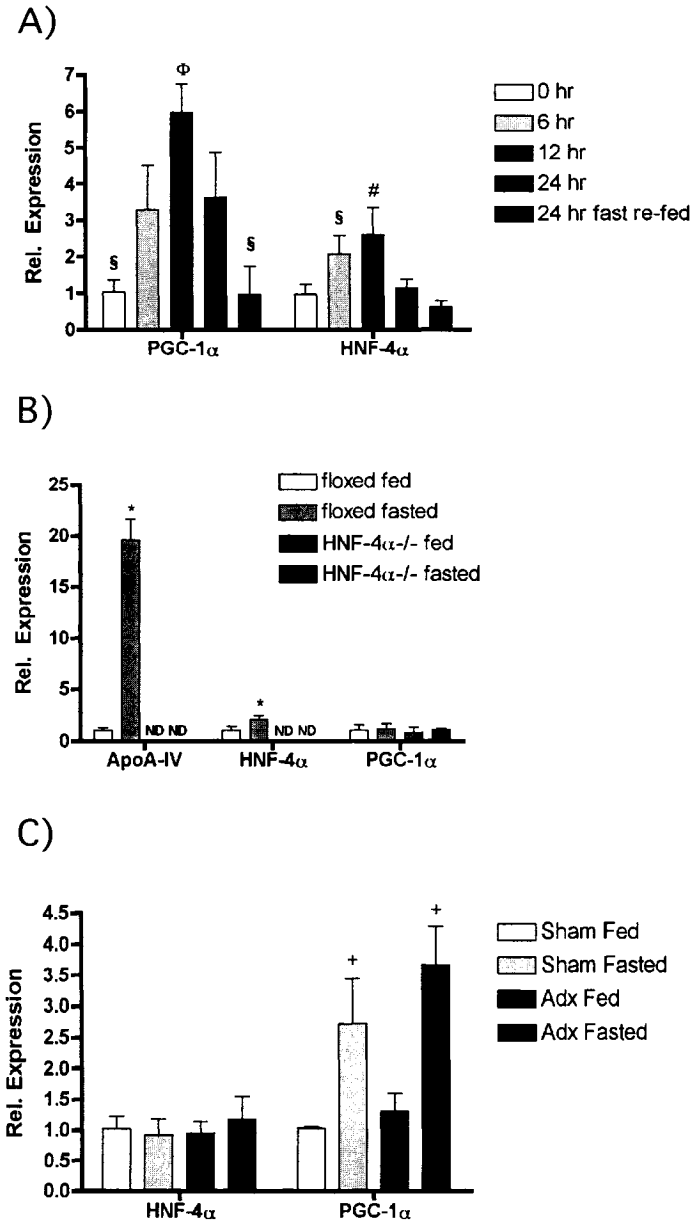

Figure 4.12 Fasting induces HNF-4α and PGC-1α expression *in vivo*.
Real-time quantitative PCR analysis of hepatic mRNA expression of A) PGC-1α and HNF-4α after 0, 6, 12 and 24 h fasts and 24 h fast followed by a 24 h re-feed B) ApoA-IV, HNF-4α and PGC-1α in fed or 24 h fasted HNF-4α-/- mice and floxed controls and C) HNF-4α and PGC-1α in adrenalectomized and sham-operated mice (fed or fasted for 24 h). All values were normalized to RP-II mRNA levels and are expressed as the fold difference relative to controls (0 h fasted, floxed fed or sham fed). §P<0.05 vs 24 h. ΦP<0.05 vs 0, 6 and 24 h fasted re-fed. #P<0.05 vs 0, 24 and 24 h fasted re-fed. *P<0.05 vs floxed fed. +P<0.05 vs all other groups. ND= non-detectable. Values are mean ± SD, n=3.

liver-specific knockout mice. ApoA-IV mRNA expression in floxed mouse controls was induced approximately 20-fold by a 24 h fast (Fig. 4.12*b*). Expression of apoA-IV mRNA was not detectable in HNF-4α liver-specific knockouts and was not induced by a 24 h fast (Fig 4.12*b*). HNF-4α mRNA expression was increased by a 24 h fast in floxed controls (approximately 2-fold) and was not detectable in knockout livers (Fig. 4.12*b*). Hepatic mRNA expression of PGC-1α in these mice was unaltered by the lack of HNF-4α expression in the knockout mice, compared to floxed controls, and was not induced by a 24 h fast in either genotype (Fig. 4.12*b*). To examine the role of corticosteroids in PGC-1α and HNF-4α expression *in vivo*, mRNA expression of these two genes was examined by QPCR analysis of 24 h fasted, adrenalectomized mice. Examination of liver mRNA expression of HNF-4α revealed no change in HNF-4α due to fasting in either the sham-operated or Adx mice (Fig. 4.12*c*). PGC-1α liver mRNA expression was increased due to fasting in sham-operated mice (approximately 2.5-fold) and induction of this gene was even greater in fasted, Adx mice (approximately 3.5-fold) (Fig. 4.12*c*).

4.5 DISCUSSION

The present study demonstrates profound changes of hepatic and ileal apoA-IV expression during states of nutritional and metabolic stress. Challenging mice with a 24 h fast led to increases in liver and ileal apoA-IV mRNA and plasma apoA-IV protein levels. These increases did not occur in adrenalectomized mice, indicating a requirement for endogenous corticosteroids. Treatment with dexamethasone as well as induction of diabetes in mice also induced apoA-IV gene and protein expression. *In vitro* analyses revealed that the mouse and human apoA-IV promoters were induced by PGC-1α and

HNF-4α, but not GR. Inhibition of endogenous HNF-4α function by expression of a dominant negative HNF-4α or shRNA for HNF-4α abolished the coactivation of the promoter by PGC-1α. Consistent with a PGC-1α/HNF-4α mechanism of induction, *in vivo* findings indicate that induction of these genes occurs in parallel with that of the apoA-IV gene during fasting.

Many physiological changes occur in the liver during fasting, including increased oxidation of fatty acids. This catabolic process involves many enzymatic steps, some of which are regulated at the transcriptional level by PPARα [410]. PPARα is also known to regulate expression of the genes encoding apoA-I and apoC-III [411, 412] and PPARα-/- mice exhibit hepatic accumulation of lipids when challenged with clofibrate or Wy-14,643 [401]. Examination of apoA-IV expression PPARα-/- mice revealed that hepatic mRNA and plasma protein levels were increased by fasting to a level identical to that for WT mice. However, due to the high basal level of apoA-IV mRNA, the relative increase by fasting in the PPARα-/- mice was lower than that seen for the WT mice. Given that PPARα-/- mice did not exhibit superinduction of apoA-IV expression with fasting, it is likely that the mechanism(s) contributing to the increased basal expression of this apolipoprotein are also related to perturbations of energy homeostasis in these mice. A recent study found that female PPARα-/- mice had increased secretion of hepatic VLDL [413]. Given that apoA-IV is a component of this lipoprotein, it is possible that the increased basal expression of mRNA and protein for apoA-IV detected in the current study is functionally related to this increased VLDL synthesis and secretion. Further studies comparing apoA-IV expression in fasted and fed female as well as male PPARα-/- mice would help further characterize this phenotype.

Another physiological change that occurs during fasting is an increase in glucocorticoid levels. Glucocorticoids antagonize the physiological effects of insulin in large part by inhibiting tissue uptake of glucose and by increasing hepatic gluconeogenesis [368]. Glucocorticoids have also been known to cause increases in apoA-I expression levels through an unknown indirect mechanism [414, 415]. In the present study, fasting caused increases in plasma corticosteroid levels. Adrenalectomy abolished both the fasting-induced increases in plasma corticosteroids and apoA-IV expression. Furthermore, treatment of Adx mice with an exogenous glucocorticoid (dexamethasone) induced expression of the apoA-IV gene in these mice. This demonstrated that the transcriptional components required for glucocorticoid-induced expression of apoA-IV were present and functional in Adx mice. Taken together, these data strongly support that the *in vivo* induction of apoA-IV is mediated by the release of an adrenal corticosteroid, most likely a glucocorticoid hormone. While the fasting-induced expression of apoA-IV was relatively selective for this apolipoprotein, dexamethasone treatment was associated with increased expression of other apolipoproteins such as apoA-I, A-II and E. Thus, it is likely that the dose of dexamethasone used in this study was also associated with non-specific effects independent of those involved in the fasting associated induction of apoA-IV.

Glucocorticoids are increased in plasma by insulin deficiency [369] and are thought to contribute to many of the pathological changes that occur in diabetes such as hyperlipidemia and hyperglycemia [375]. Indeed, GR inactivation in liver or adipose leads to amelioration of hyperglycemia as well as hyperlipidemia in animal models of diabetes [375, 416]. Induction of type-I diabetes in mice by STZ treatment led to

increases in liver apoA-IV mRNA and plasma protein that persisted even at 4 weeks post-injection. These STZ-treated mice also had increased plasma triglycerides and cholesterol. Consistent with current literature (5,6), apoA-IV was detected solely in the HDL fraction of FPLC separated plasma from mice. Therefore, increases in apoA-IV in the type I diabetic mice are likely to be associated with HDL cholesterol and not triglycerides. Ob/ob mice are leptin deficient and become obese and insulin resistant at an early age. Thus, these mice are often used as a model of T2DM [417]. Ob/ob mice have glucocorticoid levels 14-fold higher than controls and adrenalectomy of these mice leads to a reversal of obesity [418, 419]. In the present study, liver apoA-IV mRNA and plasma protein levels were greatly increased in 12-week old ob/ob mice. Together, these results indicate that the induction of apoA-IV expression that occurs in fasting or diabetes entails a common glucocorticoid-dependent mechanism. Consistent with these *in vivo* findings in mice, apoA-IV protein levels are increased in diabetic patients [420].

Despite the glucocorticoid dependent induction of apoA-IV observed *in vivo*, functional analyses of the murine and human apoA-IV promoters failed to provide evidence for direct activation by the GR. The orphan nuclear receptor HNF-4α, in addition to PGC-1α, is known to be important in mediating gluconeogenic responses to fasting in the liver (35). The human apoA-IV promoter has two HRESs for HNF-4α in addition to one other HRE in the adjacent apoC-III enhancer [397]. Studies with transgenic mice that express human apoA-IV under the control of the native promoter demonstrated that the distal HRE is required for basal expression in the intestine [301]. Consistent with this, HNF-4α liver-specific knockout mice had no detectable basal expression of hepatic apoA-IV and no induction of this gene was seen in the present

study when these mice were fasted. Expression of PGC-1α alone was able to activate expression of the murine and human apoA-IV/C-III promoter constructs in HepG2 cells that expresses endogenous HNF4α, but not in Cos-7 cells that lack expression of this nuclear receptor. Cotransfection of expression constructs for HNF-4α and PGC-1α synergistically increased activation of these promoter constructs. Cotransfection of DN HNF-4α- or HNF-4α shRNA-expressing constructs abrogated induction of the apoA-IV promoter by PGC-1α alone in HepG2 cells. This provided convincing evidence that HNF-4α and PGC-1α were mutually required for the activation of this promoter *in vitro*. This interaction of HNF-4α and PGC-1α at the mouse promoter is consistent with the recent findings of Rhee *et al* [421]. Our empirical demonstration that this interaction also activates expression of the human apoA-IV highlights the evolutionary conservation and likely, physiological importance of this response across species.

Consistent with the promoter analyses, induction of hepatic HNF-4α and PGC-1α expression was observed *in vivo* as early as 6 h following the start of fasting. Maximal levels of HNF4α and PGC1α mRNA occurred 12 h after the start of fasting and preceded the maximum levels of apoA-IV mRNA seen at 24 h. These results, in addition to the abrogation of response in liver-specific HNF-4α knockout mice, are consistent with an essential role for both HNF-4α and PGC1-α in fasting-induced hepatic apoA-IV mRNA expression *in vivo*. The coincident induction of PGC-1α, HNF-4α and apoA-IV is also consistent with our *in vitro* findings that HNF-4α and PGC-1α interact to activate expression of the apoA-IV promoter. In agreement with these findings, ecotopic overexpression of PGC-1α or RNAi-mediated repression of endogenous PGC-1α

expression was recently reported to increase or decrease, respectively, hepatic apoA-IV mRNA levels [421].

Intriguingly, examination of Adx mice revealed that while apoA-IV induction was absent, fasting-induced increases of PGC-1α mRNA levels were similar to that seen for sham-operated animals. Thus, while our data strongly support a role for PGC-1α in the induction of apoA-IV by fasting, increased expression of this transcription factor alone is clearly not sufficient for this response. It is possible that glucocorticoids contribute to apoA-IV induction through an independent signaling pathway or alternatively, by modifying the transcriptional activating function of HNF-4α and PGC-1α. For example, a negative regulatory motif that represses transcriptional activity has been identified in the PGC-1α protein [422]. Phosphorylation of this regulatory domain by p38 mitogen-activated protein kinase relieves this functional repression and permits transcriptional co-activation by PGC-1α. It is possible that adrenal glucocorticoid release acts upon an analogous regulatory mechanism that serves to activate the transcriptional activity of PGC-1α and/or HNF-4α *in vivo*. Alternatively, rises in glucocorticoid levels lead to altered insulin levels and activity, changes in lipid metabolism and induction of gluconeogenesis in the liver. It is possible that apoA-IV gene activity is induced by an environmental stimulus (such as glucose or ketogenic products) that is required for the actions of HNF-4α and PGC-1α on the promoter. Studies on the complex regulation of the apoA-I gene have demonstrated that the induction by prolonged fasting is correlated with rises in ketone bodies [423]. In addition, the apoA-I gene has also been found to be responsive to glucocorticoid levels through an indirect mechanism thought to involve an increase in the amount and/or activity of another nuclear receptor, HNF-3β [415].

Therefore, the *in vivo* role for glucocorticoids in the induction of the apoA-IV gene in fasting and diabetes is likely mediated through an indirect mechanism which may include altered hormonal signaling, environmental stimuli or the activation of another as yet unknown transcription factor.

In conclusion, the present study demonstrates that hepatic and ileal apoA-IV gene expression are dramatically induced by fasting in a glucocorticoid-dependent manner. ApoA-IV is also induced in two established mouse models of diabetes. Despite the requirement for glucocorticoids, analysis of the mouse and human apoA-IV promoters indicates a direct regulatory role for HNF-4α and PGC-1α but not GR. While HNF-4α and PGC-1α are necessary for the induction apoA-IV during fasting and diabetes, ourdata suggest a more complex and highly conserved *in vivo* mechanism for the regulation of apoA-IV during times of nutritional and/or metabolic stress.

CHAPTER 5

DISCUSSION

5.1 Summary of Experimental Findings

The work presented in this thesis investigated the role of FXR in lipid homeostasis and atherosclerotic disease with the objectives of identifying and characterizing novel gene targets of this nuclear receptor. Results presented in this thesis demonstrate the novel finding that deficiency of FXR worsens the atherogenic profile and increases the severity of atherosclerosis in the apoE-/- mouse model of the disease. The search for novel gene targets of FXR indicated a number of hepatic genes with functions in lipid homeostasis with altered expression in FXR-/- mice. Although none of the genes studied were directly regulated by FXR at the gene level, interesting observations were made regarding the phenotype of the FXR-/- mice. These mice had a resistance to weight gain that was accompanied by altered gene expression in both brown and white adipose tissue. These changes in adipose gene expression indicated that these mice had increased adaptive thermogenesis and decreased lipid stores in white and brown adipose, respectively. The regulation of ApoA-IV, one of the genes with increased hepatic expression in FXR-/- mice, was also investigated. Results of this study indicated that this gene is highly induced during nutritional stresses, such as fasting and diabetes, and likely involves interactions of HNF-4α and PGC-1α in addition to glucocorticoid signalling. The study of nuclear receptor roles in regulation of physiological processes has become a very popular and fast-paced field of molecular research with the goal of discovering new therapeutic targets for the treatment of disease. The results presented in this thesis demonstrate the complex nature of nuclear receptor biology and contribute novel experimental findings to this field.

5.2 FXR: *In Vivo* Functions and Future Studies

The first study presented in this thesis (Chapter 2) investigated the effect of FXR deficiency in the ApoE-/- model of the disease. Given the hyperlipidemic effect of FXR deficiency, the worsened atherogenic profile and severity of atherosclerotic disease found in the HF/HC fed FXR-/-ApoE-/- mice was not surprising. Unexpectedly, however, loss of FXR function in this model also led to decreased weight gain and survival of these mice when challenged with a HF/HC diet. In addition, examination of WAT gene expression in FXR-/- versus WT mice (Chapter 3) demonstrated a decreased expression of leptin. However, despite the role of this hormone as a satiety factor, neither the FXR-/-ApoE-/- mice nor the FXR-/- mice had increased food intake. These results were consistent with a recent study demonstrating decreased adipose mass and decreased leptin secretion in FXR-/- mice with no change in food intake [328]. Recent evidence has demonstrated an inductive role of FXR in adipose differentiation and adipogenesis that may explain the observed resistance of FXR-/-ApoE-/- mice to weight gain and the decreased leptin expression in white adipose stores from FXR-/- mice [327, 328].

It is not clear why the HF/HC fed FXR-/-ApoE-/- mice had decreased survival in this study. One possibility is that the deaths that occurred in these mice were caused by myocardial infarction. There are few studies on spontaneous myocardial infarction in mice. However, a recent study describes how mice deficient for both ApoE and SR-B1 develop myocardial infarction and die prematurely [424]. It is interesting to note that FXR-/- mice have decreased hepatic expression of SR-B1 [210]. Therefore, it is possible that the combination of apoE-deficiency and the decreased expression of SR-B1 caused by FXR-deficiency may have contributed to the decreased survival rate of these mice.

Conversely, the increased hepatic inflammation, lipid accumulation and necrosis in FXR-/-ApoE-/- mice may have caused hepatic failure and death.

A recent study demonstrated that FXR deficiency ameliorates the severity of the atherosclerosis in LDLR-/- mice [425]. These results demonstrated increased blood levels of cholesterol and triglyceride due to FXR deficiency in this mouse model, results consistent with those from our study. However, HF/HC (21% cholesterol and 1.25% fat) feeding of male FXR-deficient LDLR-/- mice increased only blood triglycerides. In contrast, female mice in that study had increased blood cholesterol and triglycerides and a worsened atherogenic profile, results consistent with our findings. It is unclear why the results of this study using male mice contrasts with that from our lab. However, it is likely that differences between the apoE-/- and LDLR-/-mouse models used are responsible for this discrepancy. While LDLR-/- mice are deficient in hepatic lipid uptake of LDL particles, apoE-/- mice are deficient in both clearance of LDL as well as efflux of lipid from macrophages. In the FXR-/-ApoE-/- mice, vessel wall macrophages are defective in the efflux of cholesterol and thus develop into foam cells and initiate an inflammatory cascade leading to development of atherosclerotic plaques. In the LDLR-/-FXR-/- mice, macrophage lipid efflux is intact. The proposed mechanism by which FXR deficiency is atheroprotective in the male LDLR-/-FXR-/- mice is via decreased CD36 expression in macrophages, as well as decreased circulating LDL. Therefore, despite increases in blood cholesterol, the macrophage will recognize and take up less ox-LDL (via CD36) and therefore protect against the development of atherosclerosis. However, this does not explain why these mice had decreased LDL levels nor why female mice did not exhibit lowered cholesterol or protection from atherosclerotic disease.

Despite the opposing results of this study, recent publications have demonstrated the lipid-lowering effects of the FXR agonist GW4064, lending support to our hypothesis that FXR function is atheroprotective [220-222]. In addition, our investigation into the effects of long-term treatment of apoE-/- mice with GW4064 demonstrated a decrease in blood cholestesterol in these mice with no apparent effect on the severity of atherosclerosis. Again, these results indicate a lipid-lowering effect of FXR agonism. It is clear, however, that further study into the effects of FXR on atherosclerosis is needed in order to clarify the role of this nuclear receptor in the pathology of this disease. These future studies should include a thorough comparison of the effects of FXR deficiency on blood lipids, lipoprotein profiles and severity of atherosclerosis in both the LDLR-/- and apoE-/- models of the disease, including a comparison of female and male groups in each genotype. Ideally, further studies of FXR function should also include an investigation of the effects of GW4064 on blood lipids, lipoproteins and atherosclerotic disease, including the effects of various doses, lengths of treatment and times of intervention.

Comparison of WT and FXR-/- hepatic gene expression revealed repression of the genes for RARESP and ADRP as well as induction of the apoA-IV gene (see Chapter 3). However, FXR was not found to directly regulate promoter activity of any of these genes *in vitro*. Despite this fact, the discovery of altered expression of the RARESP gene in FXR-/- mice prompted further enquiries into its physiological role. Indeed, current studies investigating the role of RARESP in adipogenesis and inflammation are ongoing in our lab. In addition, discovery of the increased expression of hepatic apoA-IV in FXR-/- mice led to further studies into the mechanisms of regulation of this gene (Chapter 4). Thus, examination of altered gene expression in FXR-/- liver, while unsuccessful in

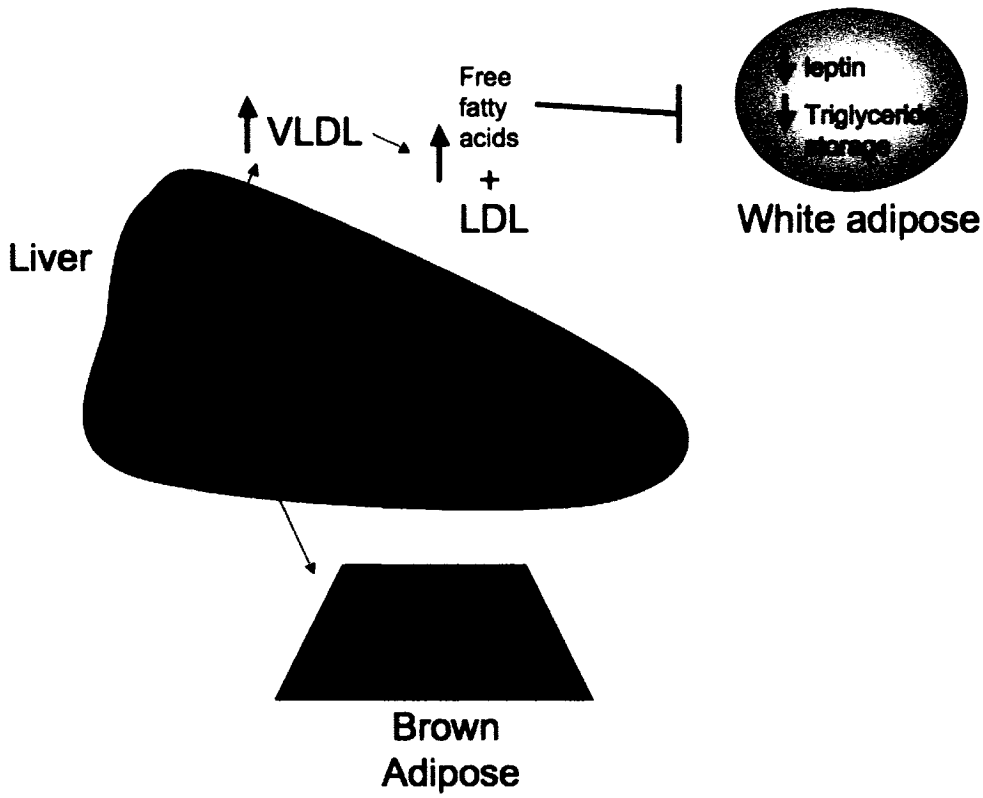

Figure 5.1 Altered hepatic, brown adipose and white adipose lipid metabolism in FXR-/- mice.

FXR-/- mice accumulate hepatic triglycerides, cholesterol and bile acids. Accumulation of these molecules causes increased inflammation and toxicity of hepatocytes. Increases in bile acid levels in the blood signal an increase in adaptive thermogenesis in brown adipocytes. Increases in hepatic triglycerides induce VLDL synthesis and secretion from liver. Lipolysis of circulating VLDL releases free fatty acids and VLDL is converted to LDL. FXR-/- mice have deficient adipogenesis and store less free fatty acids as triglycerides. Because of insufficient adipose storage, free fatty acid levels rise in the blood of these animals.

revealing novel therapeutic targets of this nuclear receptor, led to further study of these genes and revealed their potential importance in the pathologies associated with obesity and diabetes. The phenotypic changes that occur in liver and adipose lipid metabolism of FXR-/- mice are summarized in Fig. 5.1.

Greater understanding of the physiological roles of FXR is occuring at an exponential rate; the majority of the literature regarding FXR has been published only within the last 6 years. These novel roles for FXR include the aforementioned adipogenic and insulin signaling roles, in addition to modulation of endothelial cell function, protection from gall bladder disease and intestinal bacterial defense [252, 426, 427]. Needless to say, interest in the potential therapeutic implications of FXR modulation for the treatment of human diseases on the rise. Although a mutation in the gene for FXR has not yet been associated with a known human disease, decreases in hepatic FXR gene expression have been found to correlate with cholestatic diseases [428, 429]. Thus, future studies will likely include research into FXR agonism for the treatment of human cholestasis [430-432].

5.3 ApoA-IV: Regulation and Function in Fasting

Gene expression of apoA-IV was increased in FXR-/- mice. At the time of initial experimentation, current literature indicated that the apoA-IV gene was expressed and regulated in the small intestine in response to lipid ingestion. Little was known, however, regarding the function or regulation of this protein in liver. Concurrent with experimental work regarding apoA-IV regulation presented in this thesis, a study was published that demonstrated *in vivo* induction of hepatic, but not ileal, expression of this gene by LXR

agonism [398]. The increased hepatic apoA-IV expression in this study correlated with apoA-IV protein secretion and was associated with HDL particles. However, FXR-/- mice display decreased expression of ABCG5, ABCG8 and FAS, all genes known to be induced through increased LXR activity, see chapter 3 and [162, 210, 311]. In addition, the increased apoA-IV expression present in FXR-/- mice occurs in ileum as well as liver [210]. Therefore, it was unlikely that the increased expression of apoA-IV in FXR-/- mice was due to increased activity of LXR on the promoter [164]. It was thus more likely that apoA-IV induction in FXR-/- mice was mediated through changes in the lipid environment such as the increased accumulation of hepatic triglyceride and cholesterol that occurs in these mice, as discussed in Chapter 4. Another possibility may relate to the decreased WAT expression and secretion of leptin that occurs in FXR-/- mice. Leptin and apoAIV expression are thought to be inversely correlated [433, 434]. However, it remains to be seen how the repressive effects of leptin on apoA-IV expression are mediated.

Having observed the increased hepatic apoA-IV expression and associated metabolic disturbances present in FXR-/- mice led to the hypothesis that apopA-IV could be modulated through alterations in nutritional state, such as that of fasting. In support of this hypothesis, studies presented in Chapter 4 demonstrated a marked time-dependent increase in hepatic and ileal expression of apoA-IV, despite little effect on the expression of other apolipoprotein genes. In addition, this fasting induction of apoA-IV was independent of PPARα expression. Basal hepatic expression of apoA-IV was higher in PPARα-/- mice than WT. PPARα-/- mice have altered lipid metabolism and demonstrate increased plasma cholesterol and HDL [412]. Thus, the increased apoA-IV expression in livers of these mice is likely associated with HDL synthesis.

Induction of hepatic expression of the apoA-IV gene also occurred in response to treatment with dexamethasone. When adrenal glands were removed from mice, apoA-IV induction in fasting did not occur. Given the results of these two *in vivo* studies, it appeared that glucocorticoid release from adrenal glands during fasting was responsible for the induction of hepatic apoA-IV expression. Despite the *in vivo* importance of glucocorticoid signaling in apoA-IV induction during fasting, in vivo promoter analyses indicated that GR did not activate the apoA-IV promoter. However, coexpression of both PGC-1α and HNF-4α, two genes important for gluconeogenic responses, induced apoA-IV promoter activity in a glucocorticoid-independent manner. Since these transfections were carried out using a hepatically-derived cell line, it was presumed that endogenous PGC-1α, GR and HNF-4α expression was intact. One profound difference that exists between the *in vivo* and *in vitro* situations studied is that the *in vitro* situation lacks hormonal and neuronal signaling. In fasted adx mice, hepatic PGC-1α expression was induced. Both dexamethasone and cAMP can induce hepatic PGC-1α gene expression individually, but can also act synergistically [338]. Therefore, although glucocorticoid release was absent in the fasted adx mice, increased intracellular cAMP (due to glucagon signaling) was able to induce expression of PGC-1α. Although glucagon signaling is intact in adx mice and thus induced hepatic PGC-1α expression during fasting, the lack of induction of apoA-IV by fasting in these mice indicates some requirement for glucocorticoid signaling in order to enable HNF-4α and/or PGC-1α function. In addition, as discussed in Chapter 4, glucocorticoid signaling may increase factors such as glucose or ketone bodies in the blood that may mediate apoA-IV expression indirectly. It is also possible that an adrenergic signal is required for apoA-IV induction in fasting. Thus,

future studies into the fasting induction of hepatic apoA-IV expression should include a comparison of the gene expression in fasted mice lacking the adrenal medulla with mice lacking the adrenal cortex. In this fashion, any neuronal signaling through norepinephrine or epinephrine release by the adrenal medulla can be separated from the signaling of glucocorticoids released from the adrenal cortex.

The physiological role of apoA-IV during fasting remains unknown. Fasting, in addition to the induction of gluconeogenesis, results in the increased uptake and catabolism of fatty acids in the liver while uptake of fatty acids into adipose is inhibited. As demonstrated in Chapter 4, the induction of apoA-IV gene expression during fasting is likely mediated by HNF-4α and PGC-1α interactions on the promoter. These two transcription factors have known importance in gluconeogenic responses to fasting [338]. In our studies, apoA-IV was induced in the mouse liver in response to fasting, glucocorticoid treatment and diabetes, all conditions in which hepatic gluconeogenesis is induced. However, these conditions are also associated with hepatic accumulation of lipids. Adenoviral injection of PGC-1α into mice causes increases in VLDL triglyceride as well as increased hepatic apoA-IV and apoC-II gene expression [408]. This increase in VLDL triglyceride is consistent with what occurs during fasting; the liver, in response to the increased intake of free fatty acids, induces their oxidation as well as their incorporation into new triglyceride molecules [435]. However, our studies have found that plasma apoA-IV is detectable only in the HDL fraction of plasma from fasted mice. In addition, apoA-IV-/- mice have decreased HDL but overexpression of this gene in apoE-/- mice has been shown to protect against atherosclerosis in a manner that does not involve HDL [318, 393]. It is also thought that the atheroprotective effect of apoA-IV

could be mediated through its potent antioxidative effect in lesion sites [436]. Thus, despite over 30 years of research, the exact functions of apoA-IV and how this apolipoprotein affects lipoprotein metabolism are still in debate and require further study.

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