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The Contribution of Cholesterol-7alpha-hydroxylase, Paraoxonase-1, and Thioredoxin-interacting protein in Lipid Metabolism

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

In

Biology

by

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2009

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Chair

University of California, San Diego

San Diego State University

2009

Dedication

This work is dedicated to my family and friends.

Especially,
Donald Milton Ratliff and Julie Pham Ratliff
Thank you.

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List of Abbreviations

ASK1 – Apoptosis Signal-regulating Kinase 1

ABCA1 – ATP Binding Cassette Protein A1

ABCG1 – ATP Binding Cassette Protein G1

ABCG5 – ATP-Binding Cassette Transporter G5

ABCG8 – ATP-Binding Cassette Transporter G8

ACAT-1 – Acyl-CoA:Cholesterol Acyltransferase 1

ACAT-2 – Acyl-CoA:Cholesterol Acyltransferase 2

ACC – Acetyl-Coenzyme A Carboxylase

AMPK – AMP-activated protein kinase

Angptl3 – Angiopoietin-Like Protein 3

Angptl4 – Angiopoietin-Like Protein 4

AP-1 – Activator protein 1

Apo A-I – Apolipoprotein A-I

Apo B – Apolipoprotein B

Apo C-I – Apolipoprotein C-I

Apo C-II – Apolipoprotein C-II

Apo C-III – Apolipoprotein C-III

Apo E – Apolipoprotein E

APOBEC-1 – Apo B mRNA-Editing Enzyme Catalytic Polypeptide 1

ApoER2 – Apolipoprotein E Receptor 2

BARE – Bile Acid Response Element

CETP – Cholesterol Transfer Protein

ChREBP – Carbohydrate Response Element Binding Protein

COUP-TFII – Chicken Ovalbumin Upstream Promoter-Transcription Factor II

CYP27A1 – Cholesterol 27-hydroxylase

CYP7A1 – Cholesterol 7α-hydroxylase

EL – Endothelial Lipase

FAS – Fatty Acid Synthase

FGF15 – Fibroblast Growth Factor 15

FGF19 – Fibroblast Growth Factor 19

FGFR4 – Fibroblast Growth Factor Receptor 4

FOXO1 – Forkhead Box O1

FXR – Farsenoid X Receptor

GPAT – Glycerol-3-Phosphate Acyltransferase

GPIHBP1 – Glycosylphosphatidylinositol-Anchored HDL-Binding Protein

GR – Glucocorticoid Receptor

HDL – High-Density Lipoprotein

HIF-1α – Hypoxia Inducible Factor 1 alpha

HL – Hepatic Lipase

HMG-CoA – 3-hydroxy-3-methyl-glutaryl-CoA

HNF4 – Hepatocyte Nuclear Factor 4

HSP70 – Heat Shock Protein 70

HSPG – Heparin Sulfate Proteoglycan

IDL – Intermediate-Density Lipoprotein

IL-1 – Interleukin 1

JNK – c-Jun N-Terminal Kinase

LCAT – Lecithin:Cholesterol Acyltransferase

LDL – Low-Density Lipoprotein

LDLR – Low-Density Lipoprotein Receptor

LPL – Lipoprotein Lipase

LRH-1 – Liver Receptor Homolog

LRP – Low-Density Lipoprotein Receptor Related Protein

LXR – Liver X Receptor

MAP – Mitogen Activated Pathway

MTP – Microsomal Triglyceride Transfer Protein

NDST1 – N-acetyl Glucosamine N-deacetylase-N-sulfotransferase 1

NPC1L1 – Niemann-Pick C1-Like1

PDI – Protein Disulfide Isomerase

PGC-1α – PPAR gamma Co-activator 1 alpha

PLTP – Phospholipid Transfer Protein

PON1 – Paraoxonase 1

PPARα – Peroxisome Proliferator-Activated Receptor alpha

PPARδ – Peroxisome Proliferator-Activated Receptor delta

PXR – Pregnane X Receptor

RCT – Reverse Cholesterol Transport

RXR – Retinoid X Receptor

S1P – Site-1 Protease

S2P – Site-2 Protease

SCAP – SREBP Cleavage-Activating Protein

SCD1 – Stearoyl-CoA Desaturase 1

SHP – Small Heterodimer Partner

SR-BI – Scavenger Receptor B-I

SREBP-2 – Sterol Regulatory Element Binding Protein 2

SREBP-1c – Sterol Regulatory Element Binding Protein 1c

TNFα – Tumor Necrosis Factor alpha

Txnip – Thioredoxin Interacting Protein

VDUP1 – Vitamin D_3 Up-Regulated Protein

VEGF – Vascular Endothelial Growth Factor

VLDL – Very Low Density Lipoprotein

VLDLR – Very Low Density Lipoprotein Receptor

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the co-authors listed in this publication directed and supervised the research which forms the basis for this chapter.

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Chapter Four, in part, is currently being prepared for submission for publication of the material. The dissertation author was the primary researcher and author of this material.

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Presentations

Kern Aspen Lipid Conference – Aspen, CO (Aug 2007)

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ABSTRACT OF DISSERTATION

The Contribution of Cholesterol-7alpha-hydroxylase, Paraoxonase-1, and Thioredoxin-interacting protein in Lipid Metabolism

By

Eric Pham Ratliff

Doctor of Philosophy in Biology

University of California, San Diego, 2009

San Diego State University, 2009

Professor Christopher C. Glembotski, Chair

The studies presented herein examine the role of cholesterol-7α-hydroxylase, paraoxonase-1, and thioredoxin-interacting protein in various aspects of lipid metabolism. Cholesterol-7α-hydroxylase (CYP7A1) is responsible for the conversion of cholesterol into bile acids. Hepatic over-expression of CYP7A1 maintained cholesterol homeostasis in low-density lipoprotein receptor (LDLR)-deficient mice that were fed a cholesterol-enriched diet. The presence of the CYP7A1 transgene altered the expression of many genes in the liver and the ileum. In particular, the expansion of the bile acid pool reduced ileal expression of Niemann-Pick C1-like 1 (NPC1L1), in an farsenoid X receptor (FXR)-independent manner, which was associated with diminished cholesterol absorption.

The expression of paraoxonase 1 (PON1) correlates inversely with the formation of atherosclerotic lesions. Through the activation of FXR, bile acids increase the ileal expression of fibroblast growth factor 15 (FGF15) which binds to fibroblast growth factor receptor 4 (FGFR4) leading to diminished hepatic expression of PON1 and CYP7A1. In contrast to hepatic PON1 mRNA expression, a strong correlation exists between plasma PON1 activity levels and plasma HDL cholesterol levels. Indeed, mice that have virtually undetectable levels of plasma HDL cholesterol, such as ATP Binding Cassette Protein A1 (ABCA1)-deficient mice, have only minute plasma PON1 protein levels. However, the administration of reconstituted HDL to ABCA1-deficient mice is sufficient to generate a transient increase in mature HDL particles containing PON1.

Disruption of thioredoxin-interacting protein (Txnip) in mice leads to metabolic abnormalities, especially in the fasted state. Fasting Txnip knockout mice display high plasma triglyceride and ketone levels, as well as decreased plasma glucose levels. The hypertriglyceridemia observed in Txnip knockout mice is due to an impaired clearance of triglycerides, which correlated with decreased LPL mRNA expression and decreased LPL activity in the skeletal muscle and heart. Additional metabolic alterations in fasting Txnip knockout mice include impaired AMP-activated protein kinase (AMPK) activation and diminished oxidative capacity of glucose, 3-hydroxybutyrate, and fatty acids.

Chapter 1

Literature Review

Atherosclerosis – Plasma Lipoproteins

Cardiovascular disease is the leading cause of death and illness in technologically advanced societies (1, 2). Atherosclerosis, the primary cause of cardiovascular disease, is characterized by the accumulation of lipids in the arterial wall (3). Circulating plasma lipoproteins, derived from the liver and the intestine, are the major contributors of these lipids (see below). Specifically, high circulating levels of low-density lipoprotein (LDL) cholesterol leads to prolonged residence in the arterial wall, where LDL is subject to oxidative modifications (1, 4, 5). Monocytes/macrophages uptake oxidized LDL and become lipid-laden foam cells, one of the later stages in the development of atherosclerotic plaques (2).

Circulating plasma lipoproteins exist in two major classes: lipoproteins involved in 'forward' lipid transport and lipoproteins involved in 'reverse' lipid transport (6). Very low-density lipoprotein (VLDL) and chylomicrons, synthesized by the liver and intestine, respectively, transport lipids (mainly in the form of triglycerides) to peripheral tissues for storage/utilization ('forward' lipid transport) (6). The loss of triglycerides from the core of VLDL particles leads to the generation of intermediate density lipoproteins (IDLs) and cholesterol-enriched LDLs (6). Both

plasma cholesterol levels and plasma triglyceride levels have an influence on atherogenesis: cholesterol deposition in the arterial wall is the primary event in atherosclerosis (2) and a meta-analysis revealed that plasma triglyceride levels are an independent risk factor for cardiovascular disease (7).

In contrast, high-density lipoproteins (HDLs), 'reverse' lipid transport lipoproteins, transport cholesterol from peripheral tissues to the liver for removal from the body (6). Thus, plasma HDL levels are considered protective against the formation of atherosclerosis (8). Indeed, several studies indicated that plasma HDL levels are inversely correlated with the development of atherosclerosis (8, 9).

Importance of cholesterol in maintaining cell integrity

The relative free cholesterol/phospholipid ratio is one of the major determinants of membrane viscosity, which is important for membrane function and, thus, must be contained within narrow limits (10). As an illustration, in rat liver, the rough endoplasmic reticulum has a cholesterol/phospholipid ratio of 0.1, while the plasma membrane has a cholesterol/phospholipid ratio of 0.8 (11). The high cholesterol content in the plasma membrane is especially important for creating lipid rafts, microdomains that allow signaling events to occur (12). Therefore, it is important to maintain cellular cholesterol levels through influx, efflux, biosynthesis, and esterification (13).

Acyl-CoA:cholesterol acyltransferase (ACAT)

As previously described, it is important to maintain the free cholesterol/phopholipid ratio of the plasma membrane. Accumulation of too much free cholesterol is toxic to cells (14). In order to maintain free cholesterol levels, cells convert free cholesterol into non-toxic cholesterol esters, through the action of acyl-CoA:cholesterol acyltransferase (ACAT) (14). ACAT resides in the endoplasmic reticulum where it catalyzes the formation of cholesterol esters from cholesterol and long chain fatty acids, thereby allowing the cell to store cholesterol as a cytosolic lipid droplet (15).

There are two isoforms of ACAT: ACAT-1 and ACAT-2. ACAT-1 is expressed in multiple tissues, including the adrenal gland, heart, spleen, kidney, and the lymph nodes, as well as in macrophages (16, 17). In contrast, ACAT-2 is expressed primarily in the liver and the intestine (18); it is important to note that these tissues are responsible for the formation of VLDL and chylomicrons, respectively (see below).

Cholesterol absorption

In order to obtain the cholesterol necessary to maintain the plasma membrane and the membrane of organelles, organisms acquire cholesterol from two sources: exogenous sources and endogenous cholesterol biosynthesis. Organisms obtain exogenous cholesterol through absorption in the gastrointestinal tract.

Early studies have suggested that cholesterol is absorbed into the brush border membrane of the intestine cholesterol via passive diffusion (19). However, recent studies indicate that cholesterol absorption is a protein mediated process (20). Within the past 15 years, ezetimibe was discovered as an efficient cholesterol lowering drug that inhibits intestinal cholesterol absorption (21). Further studies indicated that ezetimibe was a direct binding target of Niemann-Pick C1-like 1 (NPC1L1), which is located on the brush border membranes of jejunum enterocytes and was found to be essential for cholesterol absorption (20, 22). Subsequent studies using ezetimibe suggested that NPC1L1 plays a role in cholesterol traffic from the plasma membrane to the endoplasmic reticulum (23).

Aside from NPC1L1, additional studies have implicated Scavenger Receptor B-1 (SR-BI) and CD-36 to the ezetimibe-sensitive pathway of cholesterol absorption (24); these lipid transporters/receptors were found to be responsible for the initial uptake of cholesterol, which is prior to the action of NPC1L1 (24, 25).

ACAT-2, which is responsible for the esterification of cholesterol in the intestine has also been implicated in intestinal cholesterol absorption (15). Ablation of ACAT-2 protected mice from diet-induced hypercholesterolemia and significantly reduced cholesterol absorption (26). These studies indicated that ACAT-2 knockout mice secreted chylomicrons devoid of cholesterol esters, thereby contributing to lower plasma cholesterol levels in these mice (26).

Alternatively, ATP-binding cassette (ABC) transporters ABCG5 and ABCG8 have been implicated in the removal of cholesterol, as well as non-cholesterol sterols,

from cells (27-29). ABCG5 and ABCG8 function as obligate heterodimers to promote cholesterol and sterol efflux from cells (30). Individuals with mutations in either ABCG5 or ABCG8 exhibit sitosterolemia (a condition in which excess plant-derived sterols are present in the plasma), along with increased plasma cholesterol levels (31, 32). Transgenic expression of human ABCG5 & ABCG8 resulted in decreased fractional cholesterol absorption (27). In contrast, ablation of ABCG8 increased fractional cholesterol absorption (29).

Cholesterol biosynthesis by SREBP-2 and HMG-CoA Reductase

In addition to acquiring cholesterol from exogenous sources, cells are capable of synthesizing cholesterol endogenously. In fact, cells can make a sufficient amount of cholesterol to maintain their plasma membrane (33). Endogenously synthesized cholesterol derives all of its carbon atoms from acetate (34). The rate-limiting step in cholesterol biosynthesis is the conversion of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) to mevalonic acid, a reaction catalyzed by HMG-CoA reductase, which is located in the endoplasmic reticulum and in peroxisomes (35, 36). In turn, the expression of HMG-CoA reductase is regulated by sterol regulatory element binding proteins (SREBPs), in particular SREBP-2 (35).

As the maintenance of cellular cholesterol is very important, an intricate mechanism has developed to control HMG-CoA reductase expression, through SREBP-2. Briefly, the SREBP family of transcription factors (including SREBP-2) is bound to the endoplasmic reticulum-membrane when they are synthesized (37). In

order to enter the nucleus and become activated, SREBPs need to be cleaved by site-1 protease (S1P) and site-2 protease (S2P) in the Golgi apparatus (38, 39). SREBP cleavage-activating protein (SCAP) is necessary for the transport of SREBPs to the Golgi apparatus (39, 40). When cholesterol levels are high, the conformation of SCAP is altered thereby preventing the SCAP/SREBP complex from transporting to the Golgi apparatus (39, 40). Thus, when excess cholesterol levels are present, SREBP-2 is unable to translocate to the nucleus and promote the transcription of HMG-CoA reductase (41).

Triglyceride Biosynthesis

During the fed state, when insulin levels are high, the mammalian liver converts excess glucose (carbohydrate) into triglycerides that can be utilized by/stored in peripheral tissues (42). Triglyceride biosynthesis is promoted by the insulin-responsive transcription factor SREBP-1c, another member of the SREBP family of nuclear receptors (39). Previous studies suggested that liver X receptor (LXR) can enhance SREBP-1c expression and stimulate fatty acid synthesis in a insulin-responsive manner (43). In addition to insulin, recent studies also indicate that glucose itself is an agonist for LXR and can promote the induction of lipogenic genes (44).

SREBP-1c target genes include: glycerol-3-phosphate acyltransferase (GPAT), fatty acid synthase (FAS), acetyl-coenzyme A carboxylase (ACC), and stearoyl-CoA desaturase (SCD1) (39). GPAT catalyzes the first step in triglyceride synthesis (45).

FAS, ACC, and SCD1 are essential for the generation of fatty acids such as palmitate and oleate (39). In addition to SREBP-1c, LXR, in of itself, and carbohydrate response element binding protein (ChREBP) have been implicated in the expression of these lipogenic genes (46, 47).

Secretion of VLDL and chylomicrons

Apolipoprotein B Secretion

Plasma lipoproteins, namely VLDL and chylomicrons, transport cholesterol and triglycerides through the bloodstream where they can be utilized by peripheral tissues (6). These triglyceride-rich lipoproteins originate from two sources: the liver and the small intestine (6). Chylomicrons are synthesized by the intestinal epithelium and transport dietary lipids into circulation; these lipoproteins have a half-life of less than 10 min. and are quickly cleared from the plasma (48). In contrast, VLDL particles are secreted by the liver and transport endogenously synthesized lipids to the periphery (6). Atherogenic LDL is derived from VLDL (49) and can remain in plasma for several days (half-life of 2-3 days) (48).

Apolipoprotein (apo) B is essential for the production of hepatic atherogenic lipoproteins. There are two different forms of apo B found in the plasma: apo B48 and apo B100. Apo B48 is a truncated form of apo B100, wherein 48 refers to the percent sequence of apo B100 (50). Apo B48 mRNA is created from the apo B100 gene in which the cytosine residue at position 6666 is edited to a uracil residue by the cytidine deaminase apo B mRNA-editing enzyme catalytic polypeptide 1 (APOBEC-1),

thereby converting a glutamine codon into a stop codon (51). Nascent lipoproteins made by the liver and intestine contain one apo B100/VLDL particle (52) and one apo B48/chylomicron (53). In humans, apo B100 is produced exclusively in the liver and apo B48 is produced exclusively in the intestine (54). In contrast, in mice apo B100 is produced exclusively in liver while both the liver and intestine can create apo B48 (54).

Apo B has unique structural traits that allow it to be an ideal transport vesicle for lipids in plasma (55). Specifically, three amphipathic α helical domains separated by two amphipathic β sheets (NH_2-α1-β1-α2-β2-α3-COOH), which have hydrophobic and hydrophilic surfaces, are located throughout the apo B100 molecule (56). These properties of apo B100 allow a VLDL particle to have a surface monolayer, consisting of phospholipid and free cholesterol, and a core consisting of triglycerides and cholesterol esters (56, 57). The active synthesis of each of these lipids has been suggested as being essential for the secretion of VLDL. For example, the active synthesis of phosphatidylcholine was found to be necessary for VLDL secretion in rat primary hepatocytes (58). Rats given lovastatin, an HMG-CoA reductase inhibitor, exhibited a 50% reduction in VLDL secretion (59). Inhibition of ACAT-2, which is essential for providing cholesterol esters to newly secreted VLDL particles (60), resulted in decreased apo B secretion in HepG2 cells (61). Treatment of HepG2 cells with a fatty acid synthesis inhibitor also diminished apo B secretion (62).

Early studies using electron microscopy noted the presence spherical particles similar to VLDL particles in the lumen of the endoplasmic reticulum in the liver (63).

Later studies combining electron microscopy with immunology revealed that apo B was present in the rough endoplasmic reticulum unassociated with lipid (64); in contrast, apo B was associated with lipoprotein particles in terminal junctions between the rough and smooth endoplasmic reticulum (64). These initial observations provided vast insight regarding the secretion of apo B-containing lipoproteins. Subsequent studies demonstrated that apo B is co-translationally bound to the endoplasmic reticulum membrane (65).

Early studies indicated that microsomal triglyceride transfer protein (MTP) promotes the transfer of lipids to liposomes suggesting that MTP may play an important role in the assembly of lipoproteins (66). Indeed, subsequent studies demonstrated that MTP, working in complex with protein disulfide isomerase (PDI), facilitated the transfer of neutral lipid to apo B100 as it is being translated in the endoplasmic reticulum (67). Additional studies have demonstrated that MTP is limiting in the production of VLDL secretion (68). The importance of MTP in apo B secretion is exemplified in abetalipoproteinemia patients, a disease in which individuals lack of apo B-containing lipoproteins in plasma (56); in 1992, it was discovered that these individuals had a mutation in gene encoding MTP (69).

Of the lipids that are present in a nascent VLDL particle, triglycerides comprise the bulk of the lipid found in VLDL particle (70). In fact, during the initial lipidation of apo B, MTP transfers triglyceride and cholesterol esters into apo B at a ratio of 1:0.6 (71); in order to become a secretion-ready mature lipoprotein particle,

apo B must subsequently acquire additional bulk lipid via a mechanism that is not well understood (70).

Apolipoprotein B Degradation

Early studies in rat hepatocytes found that many apo Bs are degraded prior to secretion, suggesting that the failure to adequately lipidate the nascent apo B particle leads to intracellular degradation (72). Further studies noted the presence of apo B N-terminal peptides in the rough ER, indicating that the degradation of apo B could occur at the same place as its synthesis (73). In contrast, limited degradation of apo B was found in the Golgi apparatus (74). Combined, these studies suggest that the failure of apo B to translocate to the Golgi apparatus would target apo B towards the degradation pathway (74).

The translocation of apo B is regulated by many processes. The translocation efficiency of apo B was found to be inversely proportional to the length of the apo B polypeptide, suggesting that larger nascent apo B chains have higher exposure to the cytosol where proteosomal degradation can take place (75). Further studies implicated MTP (76) and the availability of lipid (77) in facilitating the translocation of apo B across the ER. As a result of impaired translocation, apo B increases its association with the cytosolic chaperone HSP70 and is degraded via an the ubiquitin-proteosome pathway (78).

Additional pathways have also been proposed to mediate apo B degradation. For example, recent studies have implicated a role for the LDL receptor (LDLR) in modulating apo B secretion (79). In fact, the localization of the LDLR in the ER

promoted the pre-secretory degradation of apo B (80). A re-uptake pathway, possibly mediated by the LDLR (79, 81), as well as a non-proteosomal pathway (82, 83) are other pathways that have been shown to mediate apo B degradation.

Utilization of triglycerides

Along with the hepatic production of triglyceride-rich VLDL and the secretion of chylomicrons from the intestine, plasma triglyceride levels are maintained through the subsequent hydrolysis of these particles (84). The extra-hepatic enzyme lipoprotein lipase (LPL) is responsible for the hydrolysis of a majority of triglycerides found in plasma (85). LPL is highly expressed in the heart, skeletal muscle, and adipose tissue and becomes a functionally active homodimer in the Golgi appartus (86, 87). LPL is subsequently translocated to the vascular endothelium where it hydrolyzes triglycerides found in the core of circulating apolipoprotein C-II (apo C-II) containing lipoproteins (88, 89); apo C-II serves as an activator/cofactor for LPL (90, 91).

It was long thought that LPL binds to heparin sulfate proteoglycans (HSPGs) on the vascular endothelium (92). However, ablation of *N*-acetyl glucosamine *N*-deacetylase-*N*-sulfotransferase 1 (NDST1), which is involved in heparin sulfate biosynthesis, in endothelial cells did not elevate plasma triglyceride levels (93). Instead, recent evidence implicates endothelial expressed glycosylphosphatidylinositol-anchored HDL-binding protein (GPIHBP1) as the bridge between LPL and the vascular endothelium (94, 95). Ablation of the *GPIHBP1* gene in mice impaired lipolytic processing of chylomicrons resulting in plasma triglyceride

levels between 2000 – 4000 mg/dl (in contrast, *GPIHBP1*$^{+/+}$ mice have plasma triglyceride levels below 50 mg/dl) (94, 96); recent studies suggest that GPIHBP1 binds to an enzymatically-active pool of LPL *in vivo* (96). Indeed, it was found that the N-terminal acidic domain in GPIHBP1 is essential for binding the positively-charged heparin-binding domain of LPL (95). Recent studies identified an individual with severe chylomicronemia that had a mutation (Q115P) in GPIHBP1, thus implicating a role for GPIHBP1 in human physiology (97). The (Q115P) mutation in GPIHBP1 was found to be unable to bind to LPL (97).

During the post-prandial period, LPL activity is increased in adipose tissue, thereby promoting the storage of VLDL-derived triglycerides (98). However, during the fasting state, LPL expression is increased in the heart and skeletal muscle, thereby conserving glucose for utilization by the brain (89, 99). In order to appropriately regulate LPL activity, the mammalian body has developed multiple methods to control LPL activity, which includes regulation of LPL mRNA expression and post-translational control (88, 89).

During the post-prandial state, there is an increase in plasma insulin levels. Insulin, along with its role in glucose homeostasis, has been shown to have a direct stimulatory effect on LPL in adipose tissue (88). For example, previous studies have found that insulin elicited a 10-fold increase of LPL release from adipocytes *in vitro* (100). In contrast, during the fasting state, LPL activity in adipose tissue is diminished due to increased intracellular degradation of the LPL protein (101). Specifically, during the fasting state, LPL has a high mannose content that retains it in the ER, thus

rendering it catalytically inactive (89, 102); during the post-prandial period, these mannose side-chains are cleaved allowing for the proper secretion of LPL (102).

In addition to post-translational modifications, LPL activity in the heart and skeletal muscle has been shown to be modulated by changes in LPL mRNA expression. In fact, fasting-induced up-regulation of LPL mRNA expression in the heart and skeletal muscle was shown to correlate well with LPL activity in these tissues (103). LPL gene expression in skeletal muscle can be regulated by the transcription factors persoxisome proliferator-activated receptor delta (PPARδ) (104) and forkhead box O 1 (Foxo1) (105).

In addition to post-translational modifications and changes in mRNA expression, LPL activity can be regulated by the presence of circulating apolipoproteins, specifically apo C-I and apo C-III. Recent studies using human apo C-I transgenic mice revealed that apo C-I can inhibits the lipolytic activity of LPL (106). In regards to apo C-III, ablation of apo C-III in mice leads to very low plasma triglyceride levels (107), while administration of apo C-III decreased LPL activity *in vitro* (108).

Recent studies established that two members of the angiopoietin family, angiopoietin-like protein (Angptl) 3 and Angptl4, also modulate lipolysis by inhibiting LPL activity (109). Angptl3 is expressed primarily in the liver (110) and has been suggested to inhibit LPL through its interaction with HSPG (111, 112). Recent studies, however, suggest that Angptl3 can bind to and inhibit LPL directly (113).

Angptl4 is a secreted protein that is highly expressed in adipose and liver and, to a lesser extent, in skeletal muscle and heart (114). Angptl4 was found to inhibit LPL directly by converting catalytically active LPL dimers into inactive monomers (115, 116). In addition, previous studies demonstrate that intravenous administration of Angptl4 protein in the KK/San hypolipidemic mice increased plasma triglyceride levels nearly 5-fold (117), and that transgenic expression of Angptl4 in mice increased plasma triglycerides 3-fold in the fed state (116). In contrast, antibody inhibition of Angptl4 resulted in a 2-fold reduction in plasma triglycerides (118).

Clearance of VLDL & LDL

As discussed above, the removal of triglycerides from the core of VLDL particles leads to the generation of cholesterol-enriched LDL particles (6). The liver is responsible for the clearance of a majority of these LDL particles through the action of the LDLR (119), a SREBP target gene (39). The LDLR recognizes apo B100 after it has undergone conformational changes (due to removal of core lipid) from a newly secreted VLDL particle (120); apo B48 lacks the epitope that LDLR recognizes and is thus unable to clear apo B48-containing particles (121). Further studies have also indicated that the LDLR can bind to and remove apo E-containing particles (122), which is of importance because a secreted VLDL particle could acquire apolipoproteins, including apo E and apo C (6). As a result, LDLR-deficient mice have a 2.5-fold increase in plasma cholesterol levels (123); cholesterol feeding exacerbates this phenotype in LDLR-deficient leading to plasma cholesterol levels

exceeding 2000 mg/dl (in contrast, wild-type mice exhibited plasma cholesterol levels of < 200 mg/dl) (122, 123).

The modest change in plasma triglyceride levels in LDLR-deficient mice (123) suggests that there are other receptors/pathways responsible for the clearance of triglyceride rich lipoproteins. Recently, the role of liver HSPGs in mediating this clearance has been exemplified in liver-specific NDST1 knockout mice (93). Ablation of hepatic *Ndst1* in mice lead to a two-fold increase in plasma triglyceride levels and an increase in plasma apo B100, apo B48, and apo E levels, suggesting that HSPGs play a significant role in the clearance of these apolipoprotein-containing particles (93); this pathway was found to be independent of the LDLR as ablation of the LDLR exacerbated the phenotype (93). The relative contribution of hepatic HSPG in mediating the clearance of triglyceride-rich lipoprotein particles may lie in its sheer abundance on the plasma membrane; there are 100-fold more HSPGs than LDLRs on the hepatocyte plasma membrane (124).

Additional receptors mediating the clearance of triglyceride-rich lipoproteins include the very low density lipoprotein receptor (VLDLR), the low density lipoprotein receptor (LRP), and the apo E receptor 2 (apoER2) (125). The VLDLR is highly expressed in extra-hepatic tissues, such as the heart, muscle and adipose, and is involved in the peripheral uptake of VLDL triglcyerides (126, 127). Studies using LRP-deficient mice suggest that LRP plays a significant role in the clearance of apo B48-containing particles, but not apo B100-containing particles (128). The apoER2 is expressed in the liver and binds to apo E-containing VLDL particles (129, 130).

Reverse Cholesterol Transport

Reverse cholesterol transport (RCT) is the process by which excess cholesterol found in peripheral tissues is returned to the liver where it can be removed from the body (6). The initial step in RCT is the transport of unesterfied cholesterol to high-density lipoproteins (HDLs) (131). As the accumulation of cholesterol in arteries is a major step in atherogenesis, the importance of maintaining HDL levels to promote RCT is exemplified (132).

HDL levels are determined by biogenesis, remodeling in the plasma compartment, and catabolism (133). The major protein constituent of HDL is apolipoprotein A-1 (apo A-1), which is primarily expressed in the liver and the intestine (134). The primary sequence of apo A-1 predicts eight 22-mer and two 11-mer amphipathic α-helices that interact with lipid (135). Apo A-1 is known to exist in three different forms (136). Following its secretion from the liver or intestine (137), apo A-1 is found in a lipid-free/lipid-poor, pre-β-migrating form, where pre-β refers to its migration pattern on an agarose gel (138). The lipid-free/lipid-poor apo A-1 interacts with ATP binding cassette protein A1 (ABCA1), which facilitates the transfer of phospholipids and cholesterol to apo A-1 generating a second form, a discoidal, pre-β-migrating HDL disc (139, 140). In contrast, the majority of apo A-1 is found in plasma as a component of spherical, α-migrating HDL particle (138); remodeling of the HDL particle mediated by LCAT alters the electrophoretic mobility of the HDL particle from a discoidal pre-β-migrating particle to a spherical α-migrating particle (141). Further HDL remodeling that occurs in plasma is mediated

by the enzymes phospholipid transfer protein (PLTP), cholesterol transfer protein (CETP, which is present in humans but not mice), hepatic lipase (HL), and endothelial lipase (EL) (142, 143). Unlike apo B-containing lipoprotein particles, in which apo B remains with the particle throughout (144), lipid-poor apo A-1 can be released as a result of the remodeling process and can be subsequently lipidated by ABCA1 (6, 143, 145).

The complex HDL maturation processes facilitates the removal of cholesterol from cells (i.e. reverse cholesterol transport (RCT)) (142, 146). HDL particles remove cholesterol from peripheral cells by accepting ABCA1 and ATP binding cassette protein G1 (ABCG1) mediated cholesterol efflux; lipid-poor apo A-1 particles and spherical HDL particles are the preferred substrates for ABCA1 and ABCG1, respectively (147). HDL cholesterol is subsequently removed from the body through biliary cholesterol excretion or bile acid production, as described below.

ABCA1

Although apo A-1 is the major protein constituent in plasma HDL, ABCA1 has been shown to play a pivotal role in modulating plasma HDL levels (148, 149). For example, ablation of apo A-1 in mice results in a 70% reduction of HDL cholesterol levels; the apolipoprotein composition of the resulting HDL particles in apo A-1 knockout mice is altered and becomes enriched with apo E (150). In contrast, ablation of ABCA1 in mice completely abolishes plasma HDL cholesterol levels (148). Individuals with Tangier disease, due to homozygous mutations in ABCA1 (151), are

characterized with having a virtual absence of HDL cholesterol and have a 4-fold higher incidence of cardiovascular disease (152).

ABCA1 is expressed in several tissues including macrophages, liver, and intestine and is up-regulated by the activation of LXR (153, 154). However, plasma HDL cholesterol levels are primarily modulated by the expression of ABCA1 in the liver and the intestine. Liver-specific ablation of ABCA1 reduces plasma HDL-cholesterol levels by 70% in mice (155), while intestine-specific ablation of ABCA1 reduces plasma cholesterol levels by 30% (156).

ABCA1 is found on the plasma membrane and, through its association with lipid-poor apo A-1 or apo E, facilitates the transfer of lipids to HDL (157, 158). Alternatively, apo A-1 bound to ABCA1 can be endocytosed and the apo A-1 particle could be lipidated in late endosomes within the cell (159); nascent HDL could then be secreted via the retro-endocytosis pathway (159). Without ABCA1-facilitated transfer of phospholipids to lipid-poor apo A-1, the lipid-poor apo A-1 is quickly catabolized by the kidney (160, 161). Thus, *ABCA1*-knockout mice have extremely low apo A-1 levels, resulting in greatly diminished HDL levels, although these mice secrete normal levels of apo A-1 (148, 162).

Clearance of HDL

Uptake and removal of HDL cholesterol by the liver represents an integral part of RCT pathway (6). Scavenger receptor B-1 (SR-B1) is highly expressed in the liver and has been shown to play a pivotal role in this process (164). SR-B1 is responsible

for clearing the lipid components of HDL, but not the apolipoprotein components, which is predominately apo A-1 (164, 165). Ablation of SR-B1 impaired selective cholesterol uptake from HDL in mice (165). In contrast, SR-B1 over-expression was associated with reduced plasma HDL levels and increased biliary cholesterol (166).

Removal of cholesterol from the body/Bile acids

The removal of cholesterol from the body can be mediated by two pathways: bile acid sythesis and direct secretion into bile (28, 167). A majority of cholesterol removed from the body requires the generation of bile acids (168, 169). There are two major pathways controlling bile acid synthesis: the classical or "neutral" pathway and the alternative or "acidic" pathway (170). The alternative pathway is primarily catalyzed by the action of cholesterol 27-hydroxylase (CYP27A1), which accounts for ~45% of the bile acid pool in mice (170-172). The classical pathway of bile acid synthesis is catalyzed by cholesterol-7α-hydroxylase (CYP7A1), which is responsible for the majority of bile acid production (173). In humans, up to 90% of bile acid production is mediated through the classical pathway (174).

The generation of bile acids plays an important role in the absorption of dietary fats and cholesterol (167). In addition, bile acids were found to be effective detergents aiding in the absorption of lipid-soluble vitamins (175). Interestingly, the mammalian body has developed several mechanisms by which bile acids feed-back to inhibit additional bile acid synthesis (as described below).

Direct secretion into bile represents a significant pathway by which cholesterol is removed from the body. Biliary cholesterol secretion is mediated by the ABC transporters, ABCG5 and ABCG8 (28). Overexpression of ABCG5 and ABCG8 in mice increased biliary cholesterol levels four-fold (27); in contrast, ablation of ABCG5 and ABCG8 decreased biliary cholesterol levels by ~80% (28).

Cholesterol-7α-hydroxylase

Given the importance of CYP7A1 in maintaining cholesterol homeostasis, it is easy to understand why the expression of CYP7A1 needs to be tightly controlled. Of note, the expression of CYP7A1 displays a circadian cycle, in which it is most highly expressed just prior to feeding (176).

There are a number of trans-acting factors that regulate CYP7A1 expression. Specifically, there are two bile acid response elements (BARE) in the CYP7A1 promoter (170). In mice, LXR forms a heterodimer with retinoid X receptor (RXR) which binds to the BAREI in the CYP7A1 promoter to activate transcription (177). In contrast, functionally active LXR sites in the human CYP7A1 promoter are lacking (178). Alternatively, hepatocyte nuclear factor 4 (HNF4) (179), chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) (180), and liver receptor homolog (LRH-1) (181) have been shown to bind to the BAREII in the CYP7A1 promoter. Further studies implicated HNF4 as essential for basal CYP7A1 expression (182) and that PPAR gamma co-activator 1α (PGC-1 α) interacts with HNF4 to promote the expression of CYP7A1 (183).

Along with transcriptional activation, CYP7A1 expression is controlled via transcriptional repression. Earlier studies noted that administration of cholesterolamine, a bile acid sequestrant that prevents its reabsorption via the intestine, resulted in increased bile acid output (175); this study demonstrated that the bile acid synthesis is controlled via a self-regulating mechanism: increased bile acid levels diminish the production of bile acids (175). Mechanistically, bile acids were found to repress CYP7A1 expression in a farsenoid X receptor (FXR) – dependent manner (184); bile acids are the major natural ligands for FXR (185). Ligand-activated FXR promotes the expression of small heterodimer partner (SHP), a transcriptional co-repressor (186). SHP was found to bind LRH-1, causing it to dissociate from the BAREII sequence in the CYP7A1 promoter (187). However, SHP knockout mice were still sensitive to bile acid-mediated repression of CYP7A1 (188), indicating that there are additional FXR-dependent, SHP-independent mechanisms responsible for bile acid mediated repression of CYP7A1.

Later studies implicated a FXR-FGF15-FGFR4 pathway for bile acid-mediated repression of CYP7A1 (189-191). Early studies by Holt et. al., demonstrated that administration of fibroblast growth factor 19 (FGF19) to HepG2 cells decreased CYP7A1 mRNA expression (192). In contrast, ablation of FGF15, the murine homologue of human FGF19 (193), resulted in increased expression of CYP7A1 (189). Furthermore, ablation of the FGF15 specific receptor, fibroblast growth factor receptor 4 (FGFR4) (194), also exhibited constitutively increased CYP7A1 expression

(195). Subsequent studies implicated Klotho beta as being essential for this pathway (196).

Additional studies have also indicated that bile acids induce the expression of tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1), which represses CYP7A1 expression through the activation of the c-Jun N-terminal kinase (JNK) pathway, independent of FXR (197, 198). Furthermore, pregnane X receptor (PXR) has been shown to interact with HNF4α and disrupt the association of PGC-1α from the CYP7A1 promoter, thereby repressing CYP7A1 expression (199).

As previously described, CYP7A1 plays an essential role in bile acid biosynthesis. CYP7A1 is a microsomal cytochrome P450 enzyme that places a hydroxyl group onto the 7α position of cholesterol, which is the primary and rate-limiting step in bile acid synthesis through the classical pathway (170). The clearance of cholesterol into bile through bile acid synthesis or direct bile secretion also represents the final step in reverse cholesterol transport (131). Previous studies have exemplified the importance of CYP7A1 in removal of cholesterol in the body. Specifically, transgenic expression of CYP7A1 in the liver increased the expression of the LDL receptor (200) and protected mice from diet-induced atherosclerosis (201).

Due to its role in bile acid synthesis, ablation of CYP7A1 has deleterious effects on post-natal development in mice; ~85% of CYP7A1-knockout mice die within 18 days of birth (202). Bile acid deficiency resulted in the failure of CYP7A1-knockout mice to absorb a sufficient amount of vitamins to survive (202); vitamin

supplementation, along with bile acid-feeding, restored vitamin D_3 and E in the serum and fat, respectively, in these mice (203).

Paraoxonase-1

Part of the inverse correlation between plasma HDL cholesterol levels and the susceptibility to coronary artery disease is due to the presence of paraoxonase-1 (PON1) (2, 204, 205). Studies in humans and experimental animals show that plasma levels of PON1 activity vary inversely with the formation of atherosclerotic lesions (206-213). For example, genetic ablation of PON1 in mice markedly increased the formation of atherosclerotic lesions (214), whereas over-expression of a PON1 transgene prevented atherosclerosis (210). Most of the PON1 present in plasma is associated with HDL, which may explain the well-established athero-protective effect of HDL (205, 206, 215). Specifically, the anti-inflammatory properties of HDL are dependent, at least in part, on the presence of PON1 (205); dissociation of PON1 from HDL causes the HDL particle to become atherogenic (205).

PON1 is predominately expressed in the liver (216); however, data regarding the transcriptional activation of the *PON1* gene *in vivo* is limited (217). However, previous studies have shown that SREBP-2/Sp1 (218) and the aryl hydrocarbon receptor (AhR) (219) can increase PON1 promoter activity and mRNA expression *in vitro*. In contrast, the effect of cytokines on the repression of PON1 mRNA expression has been studied extensively. Previous studies have shown that administration of the endotoxin lipopolysaccharide (LPS) reduced PON1 mRNA

expression in mice due, in part, to the induction of the cytokines TNFα and IL-1 (220). Additional studies have also indicated that bile acids may have an inhibitory effect on PON1 gene expression (216).

Previous studies have demonstrated that PON1 is expressed on the plasma membrane and is inefficiently secreted (221, 222). The unusual retention of the amphipathic α-helical N-terminal signal sequence of PON1 may play an important physiological function by allowing PON1 to associate with lipoproteins, the vehicles which carry it in plasma (223). Indeed, deletion of the N-terminal signal sequence prevents the association of PON1 with HDL and inactivates PON1 enzyme activity (224-226).

PON1 exhibits multiple enzyme activities including acting as an organophosphate esterase, a carboxyl esterase (227, 228), and a phospholipase A2 (205, 229). Recent detailed structure-activity analysis using a wide variety of substrates indicates that PON1 is also a lactonase having high activity toward many lactones formed as oxidation products from polyunsaturated fatty acids (i.e. arachidonic and docosahexanoic acids) (224, 230). Since many of these oxidized fatty acids are potent modulators of enzymes involved in lipid metabolism and inflammation (e.g. 5-lipoxygenase and phospholipase A2), it has been proposed that PON1 modifications may affect atherogenesis by altering the bioactivity of oxidized fatty acid lactones (224).

Thioredoxin-interacting protein

A critical role for thioredoxin-interacting protein (Txnip) in regulating triglyceride metabolism was established when it was found that the *Txnip* gene was mutated in the *Hyplip1* locus of HcB-19 mice, which was originally characterized as a mouse model of familial combined hyperlipidemia (231, 232). Previous studies demonstrated that HcB-19 mice displayed an abnormal fasting phenotype, which included a significant increase in plasma triglyceride levels, as compared to control C3H mice (233).

Txnip, a ubiquitously expressed 46 kDa protein (234), was originally identified and characterized as vitamin D_3 up-regulated protein (VDUP1) using HL-60 cells (235); subsequently, Txnip was found to bind to and inhibit thioredoxin-mediated reduction of disulfide bonds, hence where it derived its name (234). In addition to vitamin D_3, further studies have demonstrated that glucose can up-regulate Txnip expression (236). In contrast, Txnip expression is decreased following oxidative stress (237) or treatment with insulin (238).

Structurally, Txnip is a member of the alpha-arrestins protein family (239, 240). However, many studies attribute the physiological actions of Txnip to its ability to bind to thioredoxin-1. The thioredoxin system is an integral, ubiquitously expressed thiol-reducing anti-oxidant system found in organisms ranging from yeast to mammals (241-245). This system includes thioredoxin, thioredoxin reductase and NADPH as its important constituents (242, 246). Thioredoxin reduces oxidized cysteine groups of various proteins, which in turn, can act as a metabolic signal for

various cellular processes (247). Thioredoxin has a catalytic site containing two cysteine residues (Cys32 and Cys35) that are responsible for its unique biological properties and action (248, 249). Once thioredoxin reduces its target protein, thioredoxin itself becomes oxidized (242). Oxidized thioredoxin can become active again after it is reduced by thioredoxin reductase, which, in turn, is reduced by NADPH (242).

The thioredoxin system, which can be regulated by Txnip, has been shown to play a critical role in the expression of several genes. Specifically, thioredoxin has been shown to modulate the activities of various transcription factors including NF-κB, p53, Sp1, glucocorticoid receptor (GR), activator protein 1 (AP-1), hypoxia inducible factor 1α (HIF-1α), and peroxisome proliferator activated-receptor α (PPARα) (250-252). As an example, thioredoxin has been shown to activate HIF-1α, which, in turn, promotes the expression of the major angiogenic factor, vascular endothelial growth factor (VEGF) (253).

Along with the modulation of plasma triglyceride levels, Txnip has been linked to various disease conditions, such as cancer and impaired glucose metabolism. Up-regulation of Txnip in various cancer cell lines led to a reduction in cell growth, suggesting that Txnip is a tumor suppressor (254); Txnip presumably regulates cell proliferation through its regulation of thioredoxin (255). Indeed, Txnip expression has been shown to be down-regulated in various cancers including, but not limited to, breast cancer (256), hepatic tumors (257), gastrointestinal cancer (258), colon cancer (259), B cell lymphoma (260), and T-cell leukemia (261). In this regard, the

relationship between apoptosis signal-regulating kinase-1 (ASK-1) and thioredoxin has been extensively characterized and is the most well-known (250, 262, 263). Using yeast-two hybrid screening, thioredoxin was found to directly interact with the N-terminal portion of ASK-1 and inhibit ASK-1 pro-apoptotic activity (264). Briefly, reduced thioredoxin binds to and inhibits ASK-1; however, under conditions of oxidative stress, thioredoxin becomes oxidized releasing ASK-1, thereby promoting apoptosis (264). ASK-1 is an important activator of the c-Jun N-terminal kinase (JNK) and the p38 MAP kinase pathways (265) and has been shown to play a role in mediating TNF-α induced apoptosis (266).

Given that Txnip expression is modulated by glucose (236), as well as insulin (238), it is not surprising that Txnip plays a role in glucose homeostasis. Ablation of *Txnip* in mice improves glucose tolerance (267) and increases hepatic glucose production (268). In addition, recent studies have indicated that Txnip regulates peripheral glucose uptake in humans; specifically, elevations in *Txnip* expression correlated with inhibition of glucose uptake (269). Although this phenomena was initially attributed to redox circuitry and thioredoxin (270), recent studies demonstrate that the ability of Txnip to inhibit glucose uptake is independent of its ability to bind to thioredoxin (240, 271). These recent findings may shed light on other Txnip-associated metabolic phenotypes that are independent of thioredoxin.

Goals of this study

The goals of this thesis are to examine various aspects of lipid metabolism in relation to CYP7A1, PON1, and Txnip. Specifically, in Chapter 2, the goal of this research was to examine and characterize LDL receptor deficient mice that expressed a CYP7A1 transgene. These studies extend upon previous studies in lab that demonstrated that transgenic expression of CYP7A1 maintained plasma cholesterol levels by up-regulating the LDL receptor (Figure 1.1) (200).

In Chapter 3, the goal of this research was to determine the mechanism by which bile acids diminish the expression of CYP7A1 and PON1. Previous studies have implicated inflammatory cytokines, as well as FXR activation, in this process (Figure 1.2); however, additional studies suggested that other mechanisms exist (188). Further studies were performed to increase plasma HDL levels and plasma PON1 levels.

In Chapter 4, the goal of this research was to examine the role of Txnip in modulating plasma triglyceride levels. Previous studies have implicated Txnip in a variety of metabolic actions, including acting as a tumor suppressor and altering glucose homeostasis (Figure 1.3).

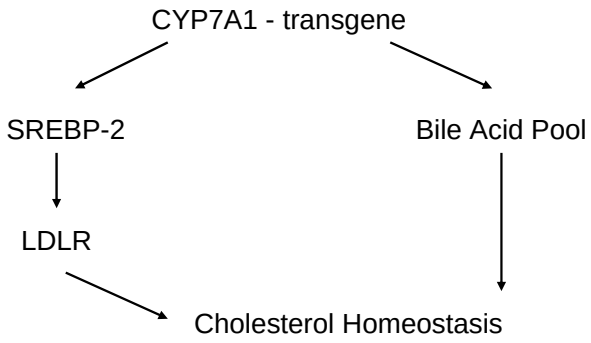


Figure 1.1. Transgenic expression of CYP7A1 improves cholesterol homeostasis. Mice expressing a CYP7A1 transgene have increased hepatic SREBP-2 expression and LDLR expression. Further, the CYP7A1 transgene increased the bile acid pool. Combined, these contributed to improved cholesterol homeostasis in CYP7A1 transgenic mice.

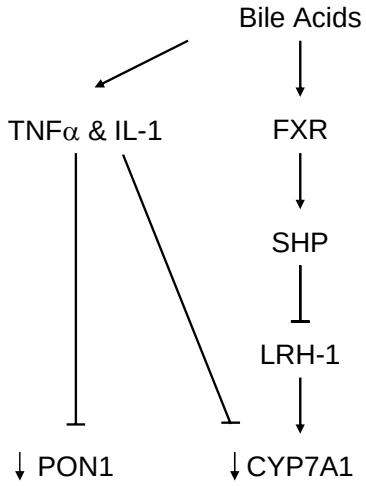


Figure 1.2. Bile acids diminish hepatic expression of CYP7A1 and PON1. Previous studies demonstrated that bile acid feeding increases the expression of inflammatory cytokines, which lead to diminished CYP7A1 and PON1 expression. Additional studies demonstrated that bile acids provide a ligand for FXR, which leads to the up-regulation of the FXR-target gene, SHP. The SHP co-repressor prevents the binding of LRH-1 to the BARE I site on the CYP7A1 promoter thereby decreasing the expression of CYP7A1.

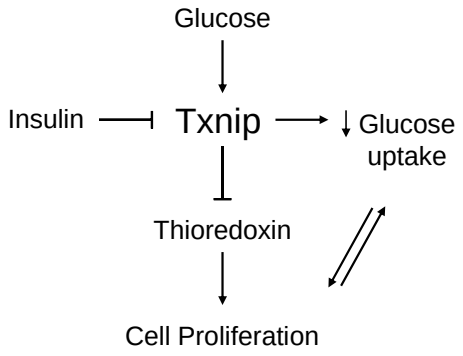


Figure 1.3. The effect of Txnip on thioredoxin and glucose homeostasis. Previous studies demonstrated that Txnip expression increases with glucose but is decreased by insulin. Txnip, in turn, diminishes glucose uptake in tissues. In addition, Txnip has been shown to bind to and inhibit thioredoxin, which promotes cell proliferation. Thus, Txnip is viewed as a tumor suppressor. Further, glucose homeostasis and cell growth are intimately linked.

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Chapter 2

Transgenic expression of CYP7A1 in LDL receptor-deficient mice blocks diet-induced hypercholesterolemia

Abstract

Constitutive expression of a cholesterol-7α-hydroxylase (CYP7A1) transgene in LDL receptor-deficient mice blocked the ability of a cholesterol-enriched diet to increase plasma levels of apolipoprotein B-containing lipoproteins. LDL receptor-deficient mice expressing the CYP7A1 transgene exhibited complete resistance to diet-induced hypercholesterolemia and to the accumulation of cholesterol in the liver. Hepatic mRNA expression of liver X receptor-inducible ABCG5 and ABCG8 was decreased in CYP7A1 transgenic, LDL receptor-deficient mice fed a cholesterol-enriched diet. Thus, increased biliary cholesterol excretion could not account for the maintenance of cholesterol homeostasis. CYP7A1 transgenic, LDL-receptor deficient mice fed the cholesterol-enriched diet exhibited decreased jejunal Niemann-Pick C1-Like 1 (NPC1L1) mRNA expression, an important mediator of intestinal cholesterol absorption. A taurocholate-enriched diet also decreased NPC1L1 mRNA expression in a farsenoid X receptor-independent manner. Reduced expression of NPC1L1 mRNA was associated with decreased cholesterol absorption (~20%, $P < 0.05$) exhibited by CYP7A1 transgenic LDL receptor-deficient mice fed the cholesterol-

enriched diet. The combined data show that enhanced expression of CYP7A1 is an effective means to prevent the accumulation of cholesterol in the liver and of atherogenic apolipoprotein B-containing lipoproteins in plasma.

Introduction

Hepatic expression of cholesterol-7α-hydroxylase (CYP7A1), the enzyme regulating the conversion of cholesterol to bile acids, plays a crucial role in eliminating excess cholesterol from the body (reviewed in (1)). Studies in rats show that biliary excretion of bile acids and cholesterol account for ~85% of the cholesterol eliminated from the body (2). The ability of the cholesterol-to-bile acid pathway to vary in parallel to changes in the endogenous cholesterol pool plays an important role in maintaining whole body cholesterol homeostasis (3, 4). In rodents such as mice and rats, sterol-mediated activation of the nuclear liver X receptor (LXR) increases CYP7A1 gene expression and, thus, provides a mechanism to maintain cholesterol homeostasis (5, 6). In contrast to the rat and mouse CYP7A1 genes, the human CYP7A1 gene does not display LXR-mediated enhancement of transcription (7, 8).

By altering hepatic expression of sterol-regulatory element binding protein (SREBP)-mediated genes, CYP7A1 has a marked influence on many processes controlling lipoprotein production as well as LDL receptor-dependent removal from plasma (9, 10). Hepatic expression of CYP7A1 indirectly controls plasma LDL levels by influencing the expression of LDL receptors (11). Studies showing that the expression of CYP7A1 in non-hepatic CHO cells enhanced the expression of LDL receptors further indicate the critical role for cholesterol catabolism in maintaining cholesterol homeostasis (9). Transgenic expression of CYP7A1 in hepatoma cells (10), CHO cells (12), and in macrophages (13) also activated SREBP-mediated LDL

receptor gene expression. These findings led to the proposal that transgenic expression of CYP7A1 in mice would reduce diet-induced hypercholesterolemia by increasing SREBP-mediated transcription of the LDL receptor and, thus, increasing hepatic LDL uptake. This hypothesis was tested in mice that expressed a CYP7A1 transgene whose liver-specific expression was controlled by the human apo E enhancer element (14). The data showed that constitutive expression of the CYP7A1 transgene in mice resulted in increased SREBP-mediated expression of the LDL receptor and reduced susceptibility to diet-induced hypercholesterolemia (15).

In this study, we examined how expression of the CYP7A1 transgene affects diet-induced hypercholesterolemia in mice lacking functional LDL receptors. Our results show that expression of the CYP7A1 transgene in LDL receptor deficient mice protected the mice from diet-induced hypercholesterolemia via a mechanism linked to increased conversion of cholesterol to bile acids and expansion of the bile acid pool size, which, in turn was associated with decreased intestinal expression of Niemann-Pick C1-Like 1 protein (NPC1L1) and reduced cholesterol absorption.

Materials and Methods

Mice and Diets

All mice used were between 6 and 8 weeks at the beginning of the experiment. C57BL/6 and LDL receptor-deficient mice were originally purchased from Jackson Laboratory, and colonies were maintained at San Diego State University. The generation of CYP7A1 transgenic mice has been previously described (14). CYP7A1-transgenic, LDL receptor deficient mice and non-transgenic littermates exhibited similar weight gain and appeared normal for the duration of the experiments (data not shown). Farsenoid X receptor (FXR) deficient mice were a generous gift from Dr. Frank Gonzalez (National Cancer Institute). Mice were maintained on a 12 h light/12 h dark cycle (light cycle from 6:00 AM to 6:00 PM). Mice were fed a standard chow (Harlan Teklad number 5001) before the beginning of the experiment. During the 2 weeks of experimental feeding, mice were fed a diet consisting of 1.25% cholesterol (Harlan Teklad number TD 96335). In studies examining NPC1L1 expression, mice were fed a diet consisting of chow, 20% olive oil, 2% cholesterol, and 0.5% taurocholate. All procedures were approved and performed in accordance with the Institutional Animal Care and Use Committee at San Diego State University.

Plasma lipids and Hepatic cholesterol levels

After an overnight fasting period, the mice were anesthetized with isofluorane at mid-light and blood was collected via retro-orbital puncture. Blood was centrifuged

for 15 min at 4ºC, and the resulting plasma samples were assayed using the commercially available Infinity Cholesterol Reagent Kit (Thermo Electron Corp.) and Wako Triglycerides Kit (Wako Chemicals USA). HDL levels were determined after treating samples with non-HDL cholesterol precipitant (Boehringer Mannheim). Plasma was injected onto a Superdex 200 column (Amersham Parmacia Biotech) with a mobile-phase buffer consisting of 10 mM Tris, 1 mM EDTA, and 150 mM NaCl. Fractions (0.5 ml) were collected at a flow rate of 0.5 ml/min. Individual fractions were assayed for cholesterol as described above. Hepatic cholesterol levels were determined after the homogenization of the liver samples (~50 mg) and subsequent chloroform-methanol (2:1) extraction. Stigmasterol was added before extraction and used as an internal control. Separation of cholesterol and stigmasterol was performed using a TR-50MS column (Thermo Electron Corp.) on a Trace gas chromatograph (Thermo Electron Corp.).

Western Blot Analysis

Plasma samples were diluted with 1 M Tris (pH 6.7) and water followed by the addition of SDS sample buffer (pH 8.0). Hepatic samples were obtained after homogenization of tissue (~200 mg) in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% sodium lauryl sulfate (SDS), 1% Triton X-100, and 1% deoxycholate. Crude extracts were centrifuged at 13,000 rpm for 20 min. Protein concentrations from the resulting supernatant were determined using the Bio-Rad Protein Assay Kit. Thirty micrograms of protein from each sample was added to SDS

sample buffer (pH 6.7). Samples were separated using a 4-12% Tris-glycine gradient gel (Invitrogen) and then electroblotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). Nonspecific binding sites were blocked using 10% nonfat dry milk/Tris Buffered Saline with Tweeen 20. Immunodetection was performed after incubation with the appropriate primary and secondary antibodies (Kirkengaard & Perry Lab) using ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech). Blots were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech). Antibodies to murine LDL receptor were a generous gift from Dr. Joachim Herz (University of Texas Southwestern Medical Center). Immunodetection of albumin (plasma) and tubulin (liver lysate) was performed as a loading control.

Generation of proprotein convertase subtilisin/kexin type 9 antibody

The murine of proprotein convertase subtilisin/kexin type 9 (PCSK9) amino acid sequence (SwissProt accession number Q80W65) was analyzed using Invitrogen peptideselect design tool (https://peptideselect.invitrogen.com/peptide/). The segment corresponding to amino acids 460-477 was submitted to BioSource (Quality Controlled Biochemicals), where the peptide was synthesized, conjugated to Keyholelimpet hemocyanin, and injected into two rabbits. Antiserum was affinity-purified and recognized two bands corresponding to the precursor and cleaved form of PCSK9, 76 and 62 kDa, respectively.

Liver and Jejunum RNA isolation and cDNA synthesis

After the experimental feeding period, the mice were anesthetized with isofluorane and sacrificed at the mid-light period after an overnight fast. In studies examining NPC1L1 mRNA expression, mice were euthanized at mid-dark under nonfasing conditions. Livers were excised and immediately flash-frozen in liquid nitrogen. Intestines were removed and flushed twice with ice-cold PBS. The first 5 cm from the cecum was considered to be the ileum, and the segment corresponding to 5-15 cm from the cecum was considered to be the jejunum. The segments corresponding to the ileum and the jejunum were excised and flash-frozen in liquid nitrogen. RNA was isolated from frozen tissue using the Versagene RNA Tissue Kit and DNase-treated using the Versagene DNase Kit (Gentra Systems) following the manufacturer's protocol for 80 mg of tissue. RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad), which consists of a mixture of random primers, oligo(dT), and iScript reverse transcriptase.

Quantitative PCR Analysis

Quantitative PCR, using SYBR Green detection chemistry, was performed on an IQ-cycler (Bio-Rad). Primer sequences are shown in Table 2.1. Melt curve analyses of all real-time PCR products were performed and shown to produce a single DNA duplex. Quantitative measurements were determined using the ΔΔ Ct method and expression of GAPDH was used as the internal control.

Cholesterol Absorption

Mice were fed the 1.25% cholesterol diet for two weeks before the cholesterol absorption studies. After a 4 h fast, mice were given an oral gavage of 4 μCi [^{3}H]sitostanol (American Radiochemicals, Inc.) and 4 μCi of [^{14}C]cholesterol (New England Nuclear) dissolved in olive oil. Mice were returned to their cages and were allowed free access to the cholesterol-enriched diet. After 72 h, feces were collected, homogenized, and extracted using chloroform-methanol (2:1). The organic layer was dried down using N_2, 10 ml of Ecolite was added (ICN Radiochemicals), and the ratio of [^{14}C]cholesterol to [^{3}H]sitostanol was measured using a β-scintillation counter (Beckman Instruments).

Statstical Analysis

Statistical analysis between groups of mice were performed using Student's *t*-test (two-tailed, unpaired). All values are reported as means $\pm$ S.D.

Results

Expression of the CYP7A1 transgene blocks diet-induced hypercholesterolemia via a mechanism independent of the LDL receptor

The presence of the CYP7A1 transgene in LDL receptor-deficient mice did not affect plasma total cholesterol and HDL cholesterol levels when mice were fed a standard chow diet (Figure 2.1A). However, when challenged with a cholesterol-enriched diet, LDL receptor-deficient mice expressing the CYP7A1 transgene displayed a marked resistance to diet-induced hypercholesterolemia (Figure 1A). In contrast to the modest ~20% increased in plasma total cholesterol levels observed in LDL receptor-deficient mice expressing the CYP7A1 transgene, nontransgenic LDL receptor-deficient mice displayed an ~6-fold increase in plasma total cholesterol levels in response to the cholesterol-enriched diet (Figure 2.1A). The accumulation of cholesterol in the plasma of nontransgenic LDL receptor-deficient mice was attributed to an increase in VLDL, intermediate density lipoprotein, and LDL (Figure 2.1B) and was associated with increased plasma levels of both apolipoprotein B-100 and apolipoprotein B-48 (Figure 2.1C).

Although the presence of the CYP7A1 transgene in LDL receptor-deficient mice did not affect hepatic cholesterol levels when mice were fed a chow diet, it completely blocked the accumulation of both free and esterified cholesterol caused by the cholesterol-enriched diet (Figure 2.1D). These data indicate that the expression of

the CYP7A1 transgene blocks diet-induced hypercholesterolemia via a mechanism independent of the LDL receptor.

The CYP7A1-transgene blocks diet-induced hypercholesterolemia via a mechanism not dependent upon increased mRNA expression of hepatic lipoprotein receptors and LXRα-regulated ABC transporters

The presence of the CYP7A1 transgene did not increase the hepatic mRNA expression of low-density lipoprotein receptor-related protein (LRP)α, scavenger receptor A, scavenger receptor class B type 1, or the VLDL receptor (Figure 2.2A). The presence of the CYP7A1 transgene actually reduced mRNA expression of scavenger receptor A and VLDL receptor. These data suggest that increased expression of these hepatic lipoprotein receptors is not responsible for the resistance to diet-induced hypercholesterolemia caused by the expression of the CYP7A1 transgene in LDL receptor-deficient mice.

The ABC transporter family members ABCA1 (16), ABCG5/ABCG8 heterodimers (17) and ABCG1 (18) are transcriptionally regulated by LXRα and have been shown to promote cholesterol excretion from cells. The expression of the CYP7A1 transgene in LDL receptor-deficient mice was associated with decreased hepatic expression of LXRα as well as ABCG5, ABCG8, and ABCG1 (Figure 2.2B). Thus, enhanced cholesterol excretion mediated by the expression of these ABC transporters could not account for the resistance to diet-induced hypercholesterolemia afforded by expression of the CYP7A1 transgene in LDL receptor-deficient mice.

CYP7A1-transgene induces expression of SREBP-2 and PCSK9

Expression of the CYP7A1 transgene in LDL receptor-deficient mice fed the cholesterol-enriched diet resulted in increased expression of SREBP-2 mRNA as well as the expression of many SREBP-mediated gene targets controlling hepatic lipogenesis (Figure 2.3A). These findings are similar to those obtained by expressing a CYP7A1 transgene in cultured hepatoma cells (10) and in C57BL/6 mice (14, 15).

Of all the mRNAs quantitated, the SREBP-2 target, PCSK9 (19, 20), exhibited the largest induction (i.e. ~60-fold) in response to the expression of the CYP7A1 transgene in LDL receptor-deficient mice (Figure 2.3A). PCSK9 has been identified as a proteinase K-like subtilase (21). Adenoviral overexpression of PCSK9 in mice caused hypercholesterolemia as a result of reduced LDL uptake attributed to enhanced degradation of hepatic LDL receptors (22, 23). Conversely, ablation of PCSK9 gene expression in mice led to decreased plasma LDL cholesterol attributable to increased expression of hepatic LDL receptors (24). Because PCSK9 appears to be a negative regulator of hepatic LDL receptor protein expression, the ~60-fold increase in PCSK9 mRNA expression in LDL receptor-deficient mice caused by the CYP7A1 transgene (Figure 2.3A) could not account for their resistance to diet-induced hypercholesterolemia.

Factors in addition to PCSK9 determine its ability to degrade hepatic LDL receptors

Because transgenic expression of CYP7A1 increased the expression of PCSK9 mRNA ~16-fold in C57BL/6 mice (Figure 2.3B), we examined how this affected hepatic expression of LDL receptor protein. The 2-fold increased expression of LDL receptor mRNA displayed by livers from CYP7A1 transgenic mice (Figure 2.3B) corresponded to a similar ~2-fold increase in LDL receptor protein (Figure 2.3C). Thus, the CYP7A1 transgene did not decrease the expression of LDL receptor protein relative to expression of its mRNA. Although expression of the CYP7A1 transgene did not affect the relative level of the 76 kDa precursor form of PCSK9, it increased by ~3-fold the relative amount of the mature 62 kDa form (Figure 2.4D). These data suggest that expression of the CYP7A1 transgene increased the expression of PCSK9 mRNA, PCSK9 protein, and its activity, as determined by its ability to process the 76 kDa precursor form to the 62 kDa form (Figure 2.4). The lack of a decrease in LDL receptor protein in wild-type mice expressing the CYP7A1 transgene suggests that factors in addition to the presence of mature 62 kDa PCSK9 determine its ability to degrade hepatic LDL receptors.

The CYP7A1-transgene blocks diet-induced hypercholesterolemia in part by decreasing intestinal expression of NPC1L1 and cholesterol absorption

NPC1L1 is a transmembrane protein expressed in the jejunal segment of the intestine and plays an important role in regulating cholesterol absorption (25-28). In

mice fed the cholesterol-enriched diet, jejunal expression of NPC1L1 mRNA was reduced by >40% ($P < 0.005$) in LDL receptor-deficient mice expressing the CYP7A1 transgene (Figure 2.4A). These data suggest that that reduced cholesterol absorption may contribute to the resistance of LDL receptor-deficient mice expressing the CYP7A1 transgene to diet-induced hypercholesterolemia. Indeed, expression of the CYP7A1 transgene in LDL receptor-deficient mice fed the cholesterol-enriched diet resulted in an ~20% reduction in cholesterol absorption ($P < 0.05$) (Figure 2.4B).

The CYP7A1-transgene reduces intestinal expression of NPC1L1 independent of its activation of FXR

The bile acid pool of mice expressing the CYP7A1 transgene is increased by 2-fold (15). Bile acids bind to and activate the nuclear receptor FXR (29). Increased bile acid pool size in mice expressing the CYP7A1 transgene may explain why mRNA levels of the hepatic FXR-inducible gene bile salt export pump are increased ~2-fold in the livers of CYP7A1 transgenic LDL receptor-deficient mice when fed the cholesterol-enriched diet (Figures 2.5A). Similarly, intestinal mRNA expression of the FXR-inducible gene ileal bile acid binding protein was increased by ~2-fold in LDL receptor-deficient mice that expressed the CYP7A1 transgene (Figures 2.5B).

To examine whether bile acid induction of FXR was responsible for the decreased expression of NPC1L1 mRNA in LDL receptor-deficient mice expressing the CYP7A1 transgene (Figure 2.4A), C57BL/6 mice (expressing the LDL receptor) were fed a diet containing 0.5% taurocholate. The taurocholate-containing diet

decreased the expression of NPC1L1 mRNA to a similar extent as that observed in mice expressing the CYP7A1 transgene (Figure 2.5C). Feeding the taurocholate-containing diet to mice expressing the CYP7A1 transgene did not further reduce NPC1L1 mRNA expression (Figure 2.5C). We have shown that feeding the taurocholate-containing diet to wild-type C57BL/6 mice expressing the CYP7A1 transgene does not increase the bile acid pool size beyond the already 2-fold increase associated with expression of the CYP7A1 transgene (15). These data suggest that increased bile acid pool size is responsible for the decreased expression of NPC1L1 mRNA. Because feeding the taurocholate-containing diet to FXR-deficient mice caused an ~50% reduction in NPC1L1 mRNA (Figure 2.5D), which is similar to the reduction observed in the wild type (Figure 2.5C), bile acids reduce NPC1L1 mRNA expression via a mechanism independent of FXR.

Discussion

The results of these studies demonstrate that expression of a CYP7A1 transgene in LDL receptor-deficient mice completely prevents diet-induced hypercholesterolemia. Further analysis indicates that CYP7A1-mediated conversion of cholesterol to bile acids evokes several complementary mechanisms that facilitate the maintenance of cholesterol homeostasis even in the absence of functional LDL receptors. Studies in CYP7A1 knockout mice demonstrated that a minimal amount of bile acids is necessary for the intestinal absorption of cholesterol and other essential fat soluble nutrients (30). Our findings indicate that expansion of the endogenous bile acid pool via the expression of the CYP7A1 transgene actually reduced cholesterol absorption by decreasing jejunal expression of NPC1L1 mRNA. Enhanced conversion of cholesterol to bile acids in combination with reduced cholesterol absorption act in concert to eliminate cholesterol from the body and maintain cholesterol homeostasis.

Analysis of the hepatic gene expression indicated that the CYP7A1 transgene did not enhance the expression of genes responsible for receptor-mediated lipoprotein removal from plasma (LRP1, SRA, SR-B1, VLDL receptor) (Figure 2.2A) or biliary cholesterol excretion (ABCG5, ABCG8) (Figure 2.2B). The findings that expression of the CYP7A1 transgene in C57BL/6 mice (expressing LDL receptors) reduced biliary cholesterol excretion (15) further support this conclusion. Because ABCG5 and ABCG8 play important roles in mediating biliary cholesterol excretion, the lack of

an induction in their mRNA expression suggests that the CYP7A1 transgene prevented hepatic cholesterol accumulation by converting it to bile acids, not by enhancing biliary cholesterol excretion.

The CYP7A1 transgene is expressed exclusively in the liver; hepatic microsomes obtained from mice expressing this CYP7A1 transgene exhibit a ~6-fold increase in CYP7A1 enzyme activity and a 2-fold increase in bile acid pool size (14). Normally, CYP7A1 is only expressed in a "few" parenchymal cells located in the pericentral zone of the liver acinus (31). Expression of CYP7A1 mRNA changes rapidly (half-life of mRNA < 0.5 min (32)) and varies widely in response to diet, diurnal cycle, and changes in the size of the bile acid pool (reviewed in (1)). Since the CYP7A1 transgene is driven by the constitutive and highly active liver-specific enhancer element from the human apolipoprotein E gene (14, 33), its functional importance is magnified with regard to the mass conversion of hepatic cholesterol into bile acids. Thus, the most parsimonious interpretation of the combined data is that increased conversion of hepatic cholesterol to bile acids is the primary mechanism responsible for blocking diet-induced hypercholesterolemia in LDL receptor-deficient mice expressing the CYP7A1 transgene. Our studies also revealed secondary intestinal responses to the doubling of the endogenous bile acid pool size in CYP7A1 transgenic mice (14). These include decreased jejunal mRNA expression of NPC1L1 mRNA (Figure 2.4A), which can account for the ~20% decrease in cholesterol absorption exhibited by LDL receptor-deficient mice expressing the CYP7A1 transgene (Figure 2.4B) (26). Our findings showing that feeding mice a taurocholate-containing diet

reduced the expression of NPC1L1 mRNA in wild-type mice, but not in mice expressing the CYP7A1 transgene, indicate that bile acids negatively affect the expression of NPC1L1 mRNA. Additional studies showing that FXR-deficient mice also decreased the expression of NPC1L1 mRNA in response to the taurocholate-containing diet (Figure 2.5D) indicate that bile acids repress NPC1L1 expression via a mechanism independent of FXR activation.

Our findings demonstrate the important influence biliary bile acids have on intestinal function and gene expression. The findings showing that bile acids reduce the mRNA expression of NPC1L1, whose gene product regulates cholesterol absorption, are unexpected because it has been generally assumed that bile acids promote cholesterol absorption (30). Bile acids are essential to emulsify cholesterol and allow it to be absorbed in the proximal intestine (34). A plethora of FXR-dependent (29) and independent mechanisms (35-38) are evoked to prevent the accumulation of potentially cytotoxic bile acids. These mechanisms limiting the expansion of the circulating bile acid pool are graphically illustrated in the CYP7A1 transgenic mice whose bile acid pool is doubled (14) but cannot be expanded further by feeding a taurocholate-containing diet (15). Because of saturation of the processes responsible for returning intestinal bile acids to the enterohepatic circulation, bile acids are lost via fecal excretion. Decreased expression of NPC1L1 mRNA (Figure 2.4A) and the corresponding ~20% reduction in cholesterol absorption (Figure 2.4B) also contribute to the elimination of cholesterol via fecal excretion.

Enhanced conversion of cholesterol to bile acids, biliary bile acid excretion, and fecal bile acid loss provide the most direct mechanism to prevent the accumulation of excess cholesterol in liver and plasma. Clearly, CYP7A1 is an effective therapeutic target to prevent diet-induced hypercholesterolemia.

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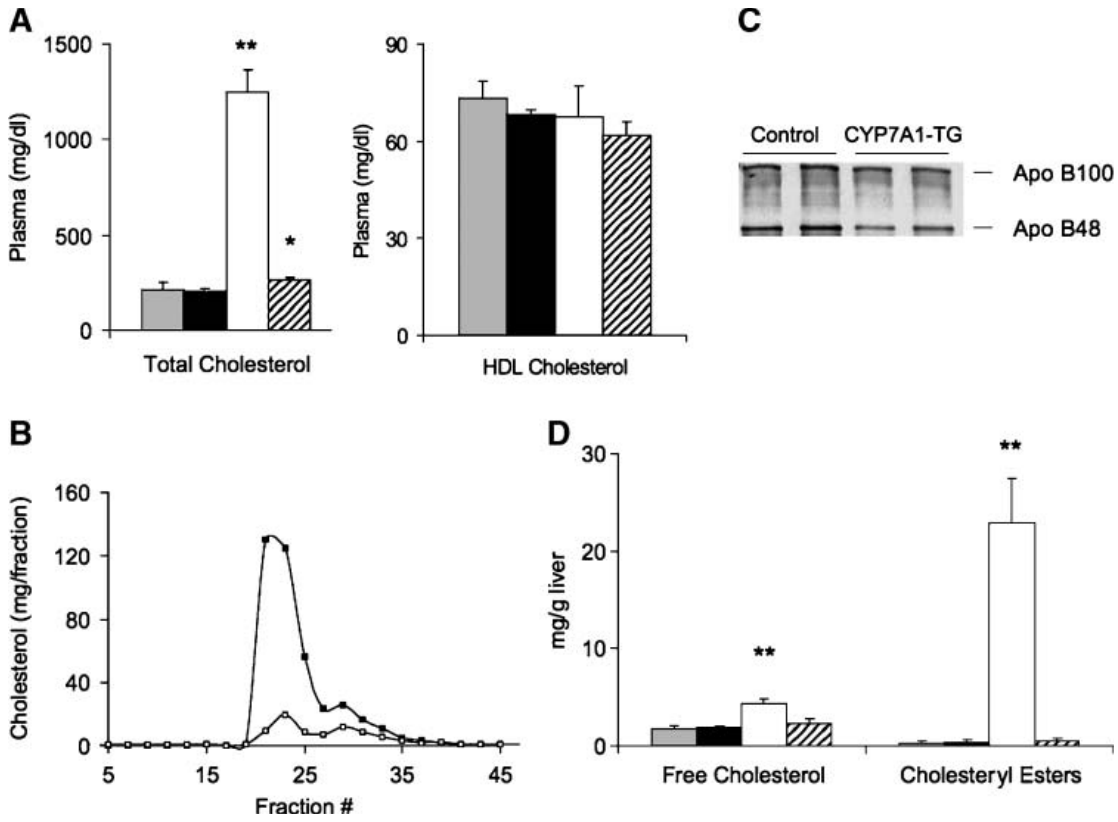


Figure 2.1. Transgenic expression of cholesterol-7α-hydroxylase (CYP7A1) prevents diet-induced hypercholesterolemia in the absence of the LDL receptor. A: LDL receptor-deficient mice were fed either a chow diet (shaded bars) or a 1.25% cholesterol diet (open bars), and CYP7A1 transgenic, LDL receptor-deficient mice were fed either a chow diet (closed bars) or a 1.25% cholesterol diet (hatched bars). After an overnight fast (~18 h), mice were euthanized and livers and plasma were obtained. Plasma total cholesterol and HDL cholesterol levels were measured using commercially available enzyme assays. B: Profiles of lipoproteins fractionated by fast-protein liquid chromatography of pooled plasma samples from LDL receptor-deficient and CYP7A1 transgenic, LDL receptor-deficient mice (n = 5) fed the 1.25% cholesterol diet for 16 weeks. Closed squares denote $LDLR^{-/-}$ mice and open squares denote the CYP7A1-TG, $LDLR^{-/-}$ mice. C: Plasma apolipoprotein B protein levels were determined by Western blot. Apolipoprotein B-100 (Apo B100) and apolipoprotein B-48 (Apo B48) bands are indicated. A representative blot from multiple experiments is shown. TG, transgene. D: Hepatic free cholesterol and cholesteryl ester contents were determined by flame ionization detection after gas chromatography. Mean values ± SD (n = 3–6) are shown. * $P < 0.05$, ** $P < 0.0005$ for differences between chow and 1.25% cholesterol diets within a group.

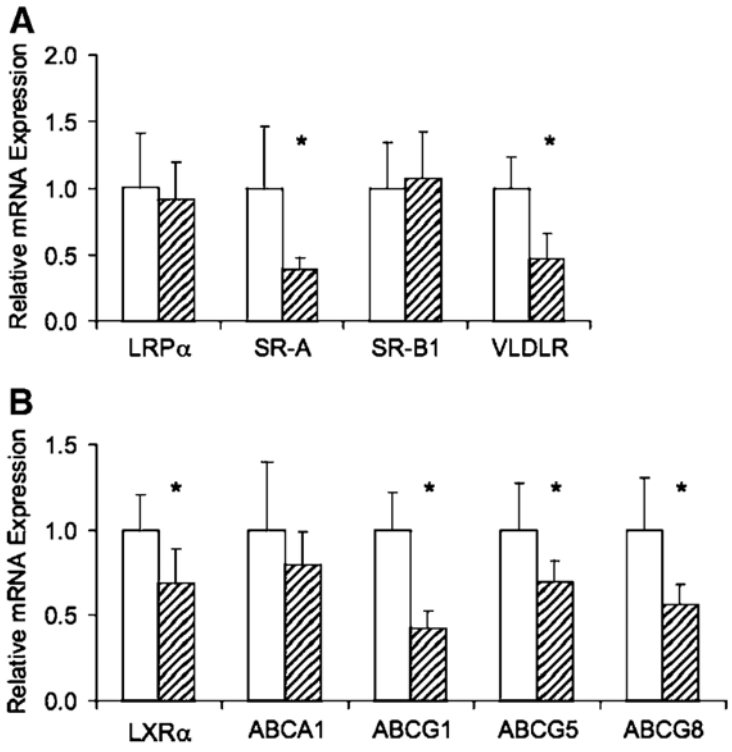


Figure 2.2 The protective effect of the CYP7A1 transgene is independent of increased mRNA expression of hepatic lipoprotein receptors and liver X receptor α (LXRα)-mediated cholesterol excretion.
LDL receptor-deficient mice (open bars) and CYP7A1 transgenic, LDL receptor-deficient mice (hatched bars) were fed a 1.25% cholesterol-containing diet for 2 weeks. Hepatic mRNA expression is shown for the cholesterol receptors LRPα, scavenger receptor A (SR-A), scavenger receptor class B type I (SR-B1), and VLDL receptor (VLDLR) (A) and for LXRα and the LXRα target genes ABCA1, ABCG1, ABCG5, and ABCG8 (B). mRNA expression was normalized with LDL receptor-deficient expression equal to 1. Mean values ± SD (n = 5) are shown. * $P < 0.05$.

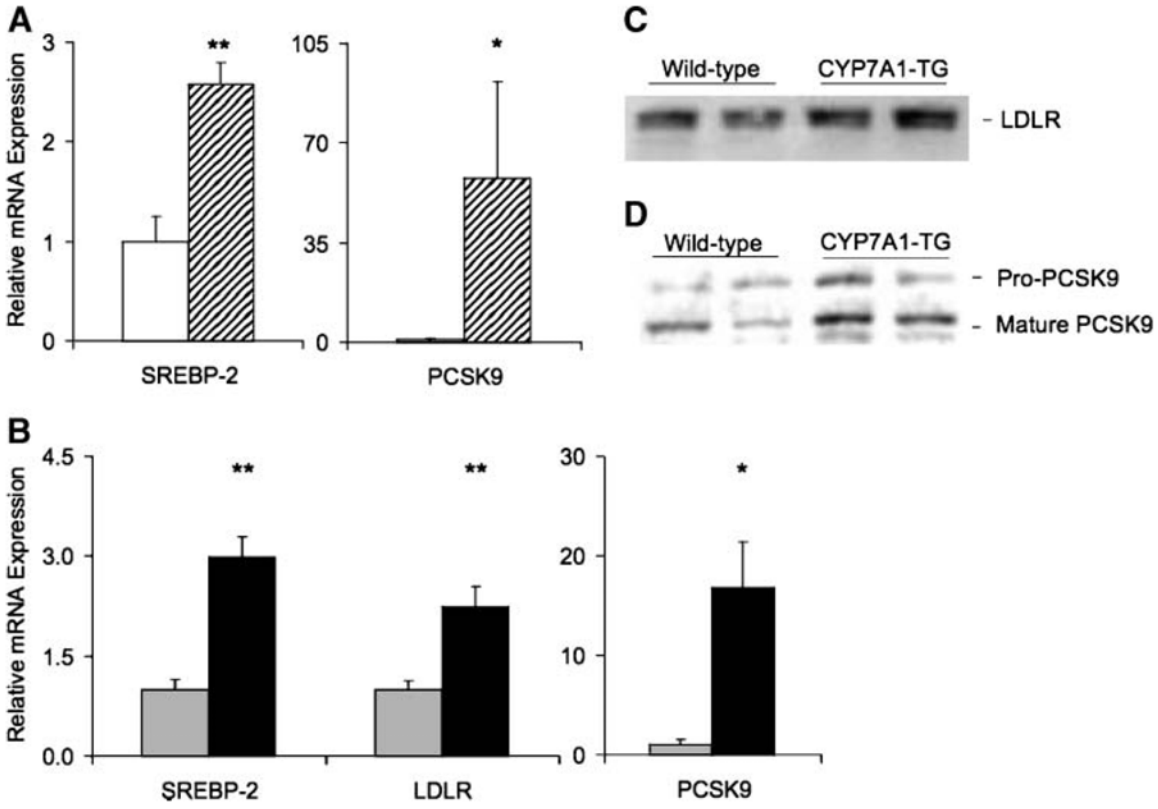


Figure 2.3. Examination of proprotein convertase subtilisin/kexin type 9 (PCSK9) in CYP7A1 transgenic (TG) mice.

A: LDL receptor-deficient mice (open bars) and CYP7A1 transgenic, LDL receptor-deficient mice (hatched bars) were fed a 1.25% cholesterol-containing diet for 2 weeks. B–D: Wild-type mice (shaded bars) and CYP7A1 transgenic mice (closed bars) mice were sustained on a chow diet. After an overnight fast (~18 h), mice were euthanized and livers and plasma were obtained. Hepatic mRNA expression of sterol-regulatory element binding protein-2 (SREBP-2), LDL receptor (LDLR), and PCSK9 was determined by quantitative real-time PCR and normalized with LDL receptor-deficient or wild-type expression equal to 1 (A, B). Hepatic PCSK9 (C) and LDL receptor (D) protein levels were determined by Western blot. Mean values ± SD (n = 4–5) are shown. * $P < 0.01$, ** $P < 0.005$.

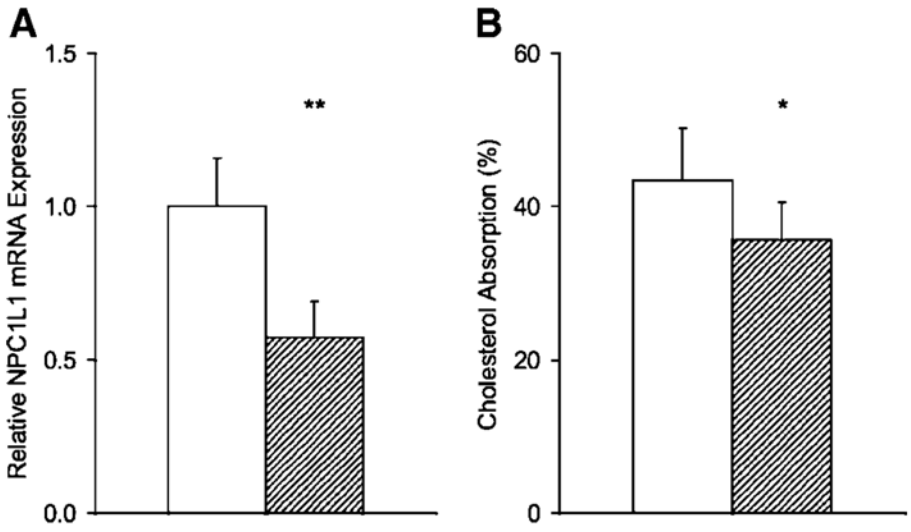


Figure 2.4. Transgenic expression of CYP7A1 reduces jejunal Niemann-Pick C1-Like 1 protein (NPC1L1) mRNA expression and cholesterol absorption.
LDL receptor-deficient mice (open bars) and CYP7A1 transgenic, LDL receptor-deficient mice (hatched bars) were fed a 1.25% cholesterol-containing diet for 2 weeks. A: Jejunal mRNA expression of NPC1L1. B: Percentage cholesterol absorption as determined by the fecal dual isotope method described in Materials and Methods. mRNA expression was normalized with LDL receptor-deficient expression equal to 1. Mean values ± SD (n = 5–6) are shown. * $P < 0.05$, ** $P < 0.005$.

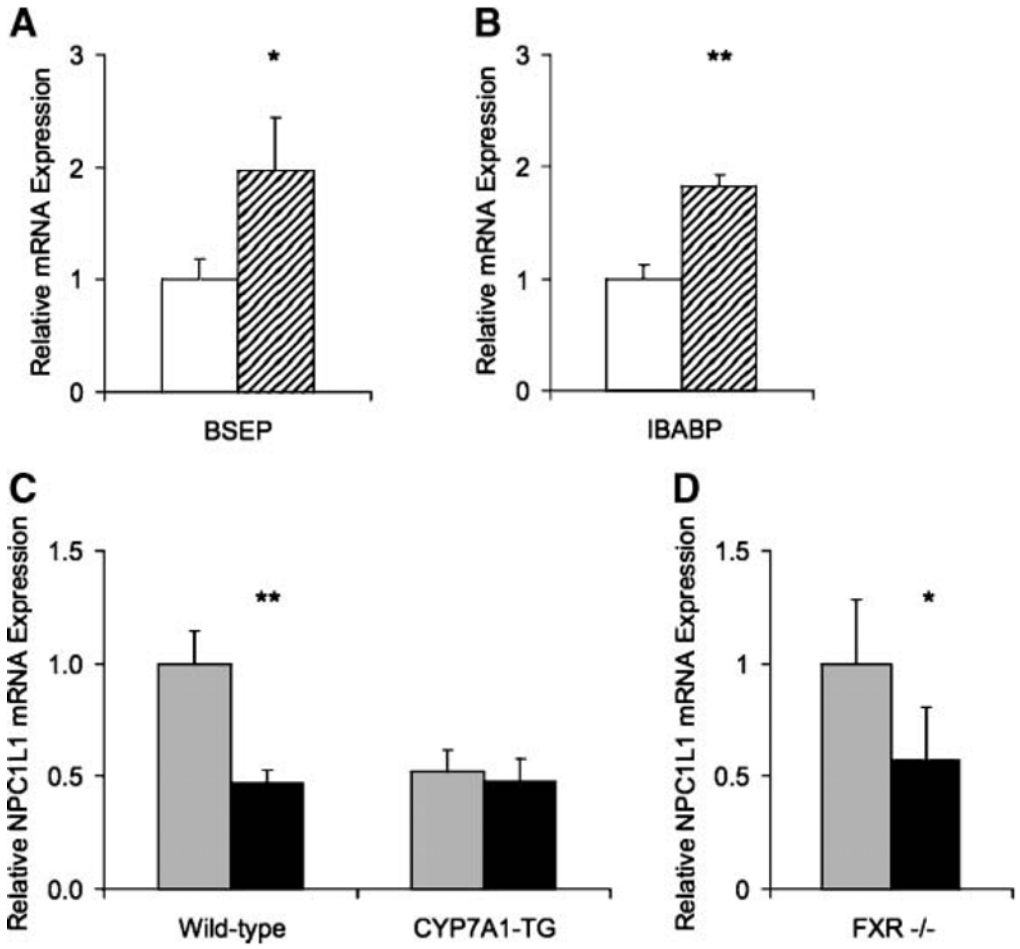


Figure 2.5. Bile acid-mediated reduction of NPC1L1 is independent of farnesoid X receptor (FXR).

A, B: LDL receptor-deficient mice (open bars) and CYP7A1 transgenic, LDL receptor-deficient mice (hatched bars) were fed a 1.25% cholesterol-containing diet for 2 weeks. Hepatic mRNA expression of bile salt export pump (A) and ileal mRNA expression of ileal bile acid binding protein (B) are shown. C, D: Wild-type, CYP7A1 transgenic (TG), and FXR-deficient ($FXR^{-/-}$) mice were sustained on a chow diet (shaded bars) or fed a taurocholate-containing diet (closed bars). Jejunal mRNA expression of NPC1L1 (C) and whole intestine mRNA expression of NPC1L1 (D) are shown. mRNA expression was normalized with LDL receptor-deficient or wild-type and FXR-deficient expression on chow equal to 1. Mean values ± SD (n = 5) are shown. * $P < 0.05$, ** $P < 0.005$.

Gene	Forward 5' - 3'	Reverse 5' - 3'
ABCA1	TCGCACTTTCCGAATAAAGC	GGAGTTGGATAACGGAAGCA
ABCG1	TCGAATTCAAGGACCTTTCC	CCACTGTTGAATTTCCCAGA
ABCG5	TGGATCCAACACCTCTATGCTAAA	GGCAGGTTTTCTCGATGAACTG
ABCG8	TGCCCACCTTCCACATGTC	ATGAAGCCGGCAGTAAGGTAGA
BSEP	CACCCTAGTTGGAGAAGGAGG	ATCTTGGGTTTCCGTATGAGG
IBABP	TTGAGAGTGAGAAGAATTACGATGAGT	TTTCAATCACGTCTCCTGGAA
LDLR	AGGCTGTGGGCTCCATAGG	TGCGGTCCAGGGTCATCT
LRP$^{\alpha}$	AACTTGCAGCCTAAGCAGTT	GCAATCTCTTTCACCGTCACA
LXR$^{\alpha}$	GCTCTGCTCATTGCCATCAG	TGTTGCAGCCTCTCTACTTGGA
NPC1L1	ATCCTCATCCTGGGCTTTGC	GCAAGGTGATCAGGAGGTTGA
PCSK9	CACATTGCAGACACCATTTTG	GCAGATGAGGACATCTGGAAG
SRA	CATGAACGAGAGGATGCTGACT	GGAAGGGATGCTGTCATTGAA
SRBI	GTCATAGCCCCACAGGATCTC	GTGAGCCTGAAGCTGATGATG
SRA	CATGAACGAGAGGATGCTGACT	GGAAGGGATGCTGTCATTGAA
SREBP-2	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA
VLDLR	GGCAACATAACATGTAGTGCAGA	GAGCACAGTCCAGCTCATCAC
GAPDH	GTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTG

Supplemental Figure 2.I. Primers used for quantitative real-time PCR.

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Chapter 3

Bile-acid mediated repression of PON1 mRNA expression and HDL biogenesis

Introduction

Plasma high density lipoproteins (HDL) cholesterol levels correlate inversely with the susceptibility to coronary artery disease (1, 2). Indeed, individuals that lack HDL cholesterol, due to a mutation in the gene encoding ATP binding cassette protein A1 (ABCA1), have over a four-fold increase in cardiovascular disease (3). ABCA1 plays a critical role in maintaining plasma HDL levels (4). ABCA1 is highly expressed in the liver and the intestine and facilitates the transfer of phospholipids to the major protein constituent of HDL, apolipoprotein A-1 (apo A-1). Without ABCA1-facilitated transfer of phospholipids, the lipid-poor apo A-1 remains a pre-β-migrating HDL particle that is quickly catabolized by the kidney (5, 6).

Following the initial ABCA1-mediated lipidation of apo A-1, HDL particles containing apo A-1 are extensively remodeled in the plasma. Through the action of lecithin:cholesterol acyltransferase (LCAT), the newly secreted discoidal, pre-β-migrating HDL acquires cholesterol esters in its core and is converted into spherical, α-migrating HDL particle (7). Spherical HDLs are subsequently remodeled in the plasma through the actions of phospholipid transfer protein (PLTP), cholesterol transfer protein (CETP), hepatic lipase (HL), and endothelial lipase (EL) (8, 9). While

being remodeled in plasma, HDL particles freely exchange apolipoproteins, such as apo A, apo E, and apo C, thereby releasing lipid-poor apo A-I, which can generate new HDL particles (10, 11).

At least part of the correlation between plasma HDL cholesterol levels and atherosclerosis is due to the presence of paraoxonase 1 (PON1) on HDL particles; studies in humans and experimental animals show that plasma levels of PON1 activity vary inversely with the formation of atherosclerotic lesions (12-19). In addition, dissociation of PON1 from HDL causes the HDL particle to become atherogenic (20).

PON1 is predominately expressed in the liver and is found in plasma as an HDL-associated enzyme (21, 22). Unlike most secreted proteins, PON1 is secreted with an intact N-terminal signal sequence (21, 23). The unusual retention of the amphipathic α-helical N-terminal signal sequence of PON1 may play an important physiological function by allowing PON1 to associate with lipoproteins, the vehicles which carry it in plasma (23).

Bile acids have been shown to repress PON1 mRNA expression, which correlated with a decrease in PON1 activity (24). However, the mechanism by which bile acids mediate the repression of the *PON1* gene is unclear. Another atherosclerosis-protective gene, cholesterol-7α-hydroxylase (*CYP7A1*), has also been shown to be down-regulated by bile acids, through a farsenoid X receptor (FXR)–dependent manner (25); bile acids are the major natural ligands for FXR (26). Ligand-activated FXR promotes the expression of small heterodimer partner (SHP), a transcriptional co-repressor, leading to the repression of the *CYP7A1* gene (27).

However, SHP knockout mice were still sensitive to bile acid-mediated repression of CYP7A1 (28), indicating that there are additional FXR-dependent, SHP-independent mechanisms responsible for bile acid mediated repression of CYP7A1.

In the present study, we examined the down-regulation of *PON1* expression by bile acids and have uncovered a novel mechanism by which bile acids repress PON1 mRNA expression through fibroblast growth factor receptor 4 (FGFR4). Further, our results demonstrate that the generation of HDL particles facilitated the hepatic secretion of PON1 in the normally HDL-deficient ABCA1 knockout (*Abca1*$^{-/-}$) mouse model.

Materials and Methods

Mice and Diets

The generation of CYP7A1-transgenic,LDLR knockout mice has been previously described (29, 30). FGFR4 knockout mice (129SV background) were a generous gift from Dr. McKeehan (Texas A&M University) and were generated as described (31, 32). FXR knockout mice were a generous gift from Dr. Frank Gonzalez (National Cancer Institute) (33). ABCA1 knockout mice (4) and their respective control mice were a generous gift from Dr. Omar L. Francone or from a colony originally purchased from Jackson Laboratory and maintained at San Diego State University. Mice were maintained on a 12 hr light – 12 hr dark cycle and were fed a standard chow (Harlan Teklad no. 5001) diet for the duration of the experiments. All procedures were approved and performed in concordance with the Institutional Animal Care and Use Committee at San Diego State University.

Liver RNA isolation, Ileal RNA isolation, cDNA synthesis, and Real-time PCR

After euthanization, livers were removed and flash frozen in liquid N_2. The intestine was removed and flushed with ice-cold PBS. The portion of the intestine, a length of 5 cm from the cecum, was considered the ileum, which was excised and flash frozen in liquid N_2. RNA was isolated from frozen tissue using the Versagene RNA Tissue kit (Gentra Systems). cDNA was made using the iScript cDNA synthesis

kit (BioRad). Real-time PCR experiments were performed as described previously (34).

HepG2 Experiments

HepG2 cells, cultured as described (35) were treated with 160 ng/mL FGF19, a generous gift from Genentech and harvested as indicated.

Plasma collection and FPLC analysis

Plasma samples from apo A-1 transgenic, LDLR knockout mice, apo A-1/LDLR double knockout mice, and respective control LDLR knockout mice were a generous gift from Dr. David T. Valenta and Dr. Linda K. Curtiss (The Scripps Research Institute). Mice were anesthetized at the indicated times blood was collected via retro-orbital puncture. For analysis of α-migrating apo A-1 particles, blood was collected in tubes containing EDTA. Pooled plasma samples from *ABCA1*$^{-/-}$ mice, rHDL treated mice (200 μL) was separated using two Superdex 200 columns connected in tandem (Amersham Pharmacia Biotech) with a mobile phase buffer consisting of 10 mM Tris, 1 mM EDTA, and 150 mM NaCl. Fractions corresponding to α-migrating and pre-β-migrating particles were concentrated using Microcon centrifugation filter devices following the manufacturer's protocol (Millipore). Plasma PON1 activity (EDTA-sensitive arylesterase activity) was measured spectrophotometrically at 270 nm over time, using phenylacetate as the substrate.

Preparation of rHDL and D4F:POPC complexes

Reconstituted HDL (rHDL) was a generous gift from Dr. Henry J. Pownall and Dr. Baiba K. Gillard was made as previously described using the cholate dialysis method (36). Mice were injected with 0.875 mg of rHDL in PBS based upon the Bradford method. D4F:POPC complexes were a generous gift from Dr. G.M. Anantharamaiah. Mice were injected with 0.5 mg D4F:POPC complexes in PBS based upon the Bradford method. POPC (Avanti Polar Lipids, Inc.) was incorporated into liposomes via sonication.

Western Blot Analysis

Plasma samples, diluted with 0.1 M Tris (pH 6.7), and liver samples (30 μg) were added to SDS sample buffer. Due to the low amount of plasma PON1 present in *ABCA1*$^{-/-}$ mice, in studies using rHDL treatment or D4F:POPC treatment, plasma was diluted 1/5 for the western blot analysis. Samples were separated using a 4-20% Tris-Glycine gradient gel (Invitrogen), then electroblotted onto polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech). Immuno-detection was performed following incubation with respective primary antibodies: anti-mouse PON1, a generous gift from Dr. Michael N. Oda, anti-human apo A-1, a generous gift from Dr. Linda K. Curtiss, and anti-mouse apo A-1 (MBL Scientific).

Analysis of α-migrating apo A-1 particles

For α-migrating particle analysis, plasma samples were separated using a 0.75% agarose gel. Plasma samples were diluted with PBS and mixed with 2X native TBE sample buffer. Samples were run at 90V for 2.5 hrs (37). Alternatively, plasma samples were separated using 4-20% Native PAGE, which was run at 90V for 18 hrs (37).

Statstical Analysis

Statistical analysis between the groups of mice were performed using Student's t-test (double tailed, unpaired). All values are reported as mean $\pm$ S.D.

Results

Regulation of CYP7A1 and PON1 expression via FGFR4

Bile acids have been shown to inhibit the expression of both PON1 and CYP7A1 (24). Although the mechanism underlying this phenomenon is unknown in regards to PON1, previous studies demonstrated a FXR-SHP-LRH-1 mediated pathway which confers the repression of CYP7A1 (27). However, there appears to be an additional SHP-independent pathway by which bile acids mediated repression of CYP7A1 (28). Similarly, our studies demonstrated that there was a significant decrease in endogenous CYP7A1 expression without an induction of SHP expression in CYP7A1-transgenic, LDLR knockout mice (Figure 3.1). Simultaneous studies in Dr. Davis's lab demonstrated that bile acid-mediated repression of PON1 occurred in an FXR-dependent manner, which was not dependent on the induction SHP expression (data not shown) (34).

Recent studies in HepG2 cells demonstrated that fibroblast growth factor 19 (FGF19), which is an FXR target gene, suppressed the expression of CYP7A1 (38). FGF19 is unique from other FGFs in that FGF19 binds exclusively to fibroblast growth factor receptor 4 (FGFR4), which leads to the auto-phosphorylation of FGFR4 and an initiation of downstream signaling events (38, 39). To determine if signaling through FGFR4 could contribute to the bile acid-mediated suppression of PON1 and CYP7A1 *in vivo*, FGFR4 knockout mice were fed a bile acid-containing diet. Indeed,

feeding a bile acid-containing diet to FGFR4 knockout mice did not reduce the hepatic expression of either PON1 or CYP7A1 (Figure 3.2).

Unlike human FGF19, which is expressed in the liver and the ileum, the murine homolog FGF15 is exclusively expressed in the ileum (38, 40). Looking upstream of FGFR4, feeding a bile-acid containing diet increased the expression of ileal expression of FGF15 (Figure 3.3A). In contrast, FXR knockout mice were unable to up-regulate FGF15 expression (Figure 3.3A), which compliments previous studies demonstrating that FGF15 is an FXR-regulated gene (40). Finally, to determine if FGF15/FGF19 was capable of suppressing PON1 expression, FGF19 was administered to HepG2 cells. Indeed, FGF19 decreased the expression of PON1 and CYP7A1 (Figure 3.3B). This effect was independent of an induction of the inflammatory cytokine TNFα (34), which has previously been shown to suppress the expression of both PON1 and CYP7A1 .

Plasma PON1 levels correlate well with plasma HDL cholesterol levels

Following the publication of these earlier findings, an association between plasma PON1 and HDL cholesterol levels was noted. Specifically, plasma PON1 activity levels correlated most closely with plasma HDL cholesterol levels, but not hepatic PON1 mRNA expression (Figure 3.4). In another set of studies, plasma PON1 protein levels correlated with plasma HDL cholesterol levels and plasma apo A-1 levels (as described in (37)) (Figure 3.5).

Plasma HDL particles facilitate the secretion of PON1

Subsequent experiments were undertaken to increase plasma PON1 protein levels. It is important to note that the majority of PON1 resides in the membrane fractions of the liver (41). Further, previous studies have demonstrated that peritoneal macrophages expressing PON1 (42), as well as CHO cells expressing PON1 (43, 44), retain PON1 as an integral membrane protein. As a result, both of these cell lines displayed an inefficient secretion of PON1 (42, 44).

Given the unique association of PON1 with HDL particles (22), plasma levels of PON1 were measured in ABCA1 knockout (*Abca1*$^{-/-}$) mice, a mouse model having a marked deficiency in HDL (4). *Abca1*$^{-/-}$ mice exhibited a dramatic reduction in plasma PON1 activity, as well as in PON1 protein levels (Figure 3.6A & B). The changes in plasma PON1 activity and protein were independent of an alteration in hepatic PON1 mRNA expression and protein levels (Figure 3.6C & D). Combined, these data indicate that the decreased plasma PON1 enzyme activities and protein levels exhibited by *Abca1*$^{-/-}$ mice are a result of a post-translational mechanism. Furthermore, the reduction in plasma PON1 levels correlated with diminished plasma apo A-1 levels (Figure 3.6E).

Intravenous injection of rHDL increases plasma PON1 and endogenous apo A-1 levels in ABCA1 knockout mice

To determine if the availability of HDL particles was sufficient to increase plasma PON1 levels, *Abca1*$^{-/-}$ mice were intravenously injected with reconstituted

HDL (rHDL), containing 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) and isolated human apo A-1 (45); POPC provides a lipid core for apo A-1 thereby creating an "HDL-like particle" that is similar to a nascently secreted HDL particle. By using this preparation of rHDL, we were able to discriminate between the exogenously injected apo A-1 (human apo A-1) and the endogenous apo A-1 pool for our further studies.

Interestingly, rHDL treatment increased plasma HDL cholesterol levels and plasma PON1 activity levels 240 min post-injection in *Abca1*$^{-/-}$ mice (Figure 3.7A & B). The increase in PON1 activity corresponded to the gradual increase in PON1 protein following rHDL injection (Figure 3.8A). Maximal plasma levels of PON1 were achieved 240 min post-injection (data not shown). The secretion of PON1 in *Abca1*$^{-/-}$ mice required the intact rHDL particle as lipid-free apo A-1 and POPC liposomes, individually, were unable to facilitate the secretion of PON1, as determined by plasma PON1 activity (Figure 3.7C & D).

Under basal conditions, *Abca1*$^{-/-}$ mice have diminished levels of apo A-1 (Figure 3.6E). Surprisingly, injection of human apo A-1-containing rHDL, increased endogenous mouse apo A-1 levels (Figure 3.8C). These findings appear in contrast to previous studies that show human apo A-1 transgenic mice exhibit diminished endogenous apo A-1 levels (46). Following rHDL injection in *Abca1*$^{-/-}$ mice, the endogenous apo A-1 levels gradually increased, peaking at 60 min post-injection (Figure 3.7C). In stark contrast, the human apo A-1 found on the rHDL gradually

decreased following rHDL injection; only minute levels were present 240 min post-injection (Figure 3.7B).

Intravenous injection of rHDL can generate PON1-containing α-migrating HDL in ABCA1 knockout mice

Abca1$^{-/-}$ mice secrete normal amounts of pre-β apo A-1-containing HDL particles (47). However, the pre-β-migrating particles in *Abca1*$^{-/-}$ mice do not mature into α-migrating particles and, as a result, are rapidly catabolized by the kidney (5). The increase in endogenous apo A-1 in the rHDL injected *Abca1*$^{-/-}$ mice (Figure 3.8B) suggest that the apo A-1-containing HDL particles are not being hypercatabolized. To determine the types of HDL particles that are present in rHDL injected *Abca1*$^{-/-}$ mice, plasma samples were examined for α- and pre-β-migrating apo A-1-containing HDL. *Abca1*$^{-/-}$ mice intravenously injected with rHDL exhibited an increase in endogenous α- and pre-β-migrating apo A-1-containing particles (Figure 3.9A). In contrast, the human apo A-1 found on rHDL transiently formed α-migrating particles at the 2 min time-point; however, at later time points human apo A-1 α-migrating particles were not found (Figure 3.9B).

PON1 is normally found in plasma associated with α-migrating HDL particles (48). Therefore, given the increase in α-migrating particles following rHDL injection, the α-migrating particles were assessed for the presence of PON1. Indeed, following rHDL injection in *Abca1*$^{-/-}$ mice, PON1 was found as a component of α-migrating HDL (Figure 3.9C). Combined, these data indicate that α-migrating HDL, containing

the atherosclerosis-protective enzyme PON1, can be generated in the absence of ABCA1.

Intravenous injection of D4F:POPC complexes can generate PON1-containing α-migrating HDL in ABCA1 knockout mice

Lecithin:cholesterol acyltransferase (LCAT) is responsible for converting discoidal, pre-β-migrating HDL particles, like the rHDL particles used in our studies, into α-migrating HDL particles (7). Given the surprising increase in α-migrating apo A-1 following rHDL injection in *Abca1*$^{-/-}$ mice, we tested the ability of *Abca1*$^{-/-}$ mice to generate PON1-containing, α-migrating HDL using a different pre-β-migrating complex, which is also capable of activating LCAT. D4F is a synthetic peptide, made up of 18 D-amino acids, that does not have sequence identity to apo A-1, but has properties of the apo A-1 amphipathic helical structure, when folded as an α helix (49). D4F is able to activate LCAT <50% as compared to human apo A-1 (50).

Intravenous injection of D4F:POPC complexes increased plasma PON1 protein levels, as well as endogenous apo A-1 protein levels, 240 min post-injection in *Abca1*$^{-/-}$ mice (Figure 3.10A & B). Surprisingly, this treatment also increased endogenous, α-migrating apo A-1-containing HDL particles (Figure 3.10B).

Discussion

PON1 and CYP7A1 are atherosclerosis-protective enzymes that are highly expressed in the liver (24, 51). In this chapter, bile acids were shown to diminish the expression of PON1 and CYP7A1 in an FXR-FGF15-FGFR4-dependent manner. In addition, by intravenously administrating rHDL or D4F:POPC complexes to *Abca1*$^{-/-}$ mice, we were able to establish the following: 1) functional ABCA1 is not required for the hepatic secretion of PON1 (Figure 3.7 & 3.8), 2) α-migrating HDL can be generated in the absence of ABCA1 (Figure 3.9), and 3) small, pre-β-migrating D4F:POPC complexes are sufficient to be remodeled in plasma to generate α-migrating HDL particles (Figure 3.10).

Bile acids have been shown to alter the expression of many genes (52, 53). However, the mechanism underlying the modulation of PON1 and CYP7A1 expression was not well understood. Bile acids have been shown to modulate the expression of genes through the activation of FXR (26). Our studies further confirm that this occurs in an FXR-dependent process (Figure 3.3 & (34)). The most parsimonious interpretation of the data suggests that bile acids induce the ileal expression of FGF15, in an FXR-dependent manner, which can subsequently can bind to FGFR4 and promote down-stream signaling events that can repress the expression of PON1. Recent studies have also corroborated these findings (54, 55) and suggested the involvement of beta-klotho in the FGFR4-dependent signaling process (56).

In addition to activation of FXR, bile acids can modulate gene expression through the up-regulation and secretion of inflammatory cytokines (35). However, our studies demonstrated that the FXR-FGF15-FGFR4 pathway is an independent pathway from the cytokine-induced pathway (34).

The association of PON1 with HDL, as well as the requirement of ABCA1 to maintain plasma HDL levels, has been extensively studied (22, 48, 57-60). However, the data on the association of PON1 with ABCA1 is limited. Given that both of ABCA1 and PON1 are localized to the plasma membrane and one of the functions of ABCA1 is to mobilize phospholipids on the plasma membrane to allow apo A-1 to interact with the membrane bilayer (61), the requirement of ABCA1 to promote the secretion of PON1 was examined. Total *Abca1*$^{-/-}$ mice had nearly undetectable levels of plasma PON1 (Figure 3.7); however, mice with a liver-specific deletion of ABCA1 had substantially more plasma PON1 compared to the total *ABCA1*$^{-/-}$ mice (Figure 3.5). Further, intravenous injection of rHDL and D4F:POPC complexes increased plasma PON1 protein levels in total *Abca1*$^{-/-}$ mice (Figures 3.9 and 3.12). These results clearly indicate that PON1 is secreted in an ABCA1-independent manner, suggesting that the secretion of PON1 relates instead to the availability of HDL particles.

Previous studies using human PON1-transfected CHO cells found that larger HDL particles, consisting of apo A-1 and phospholipid, are the most suitable vector for the secretion of PON1 (44). Our studies in *Abca1*$^{-/-}$ mice support these findings: only when *Abca1*$^{-/-}$ mice were injected with rHDL, to generate α-migrating particles, was PON1 present in the plasma (Figure 3.9). Indeed, our studies showed that PON1

was associated with α-migrating, apo A-1 containing HDL particles (Figure 3.9). It is interesting to note that plasma PON1 protein levels were maximal at 240 min following rHDL injection (Figure 3.8). Apo A-1, on the other hand, was maximal at 60 min post-injection (Figure 3.8). These data suggests that 1) the secretion of PON1 is a slow occurring process and/or 2) PON1 containing HDL are catabolized at a slower rate than the total apo A-1 pool. We hypothesize that the increased plasma PON1 levels are a result of both of these processes. Previous *in vitro* studies indicated that the secretion of PON1 onto an HDL acceptor was a slow and inefficient process (44). Further, during HDL remodeling, lipid free apo A-1 would be released from α-migrating HDL particles and would subsequently be catabolized by the kidney (62). As PON1 associated exclusively with α-migrating particles in our studies, it suggests that during HDL remodeling, PON1 is retained on the α-migrating HDL particle and would thus have a slower clearance rate than apo A-1.

The process by which PON1 is secreted and associates exclusively with HDL particles is not well understood. Previous studies demonstrated that the N-terminal sequence of PON1 is necessary for its association with HDL particles (23); prior to secretion, the N-terminal sequence is essential in order for PON1 to associate with the plasma membrane (44). Further studies, examining the kinetics of PON1 secretion, demonstrated that PON1 secretion is a saturable process, suggesting that a plasma membrane receptor may be involved in this process (44). One candidate receptor is scavenger receptor B1 (SR-B1), an HDL-binding receptor that is responsible for the uptake and removal of HDL-cholesterol (44, 63). SR-B1 binds to lipoproteins on the

surface of the plasma membrane thereby transiently associating HDL particles to the cell surface-associated PON1 (63, 64). It is interest to note that SR-B1 binds to α-migrating HDL particles, the vesicles that carry PON1 in plasma, but is unable to bind to lipid poor apo A-1 particles (63).

The pre-β migrating reconstituted HDL used in our studies resemble pre-β-migrating particles that can be generated by ABCA1 (65). By infusing human apo A-1-containing rHDL in *Abca1*$^{-/-}$ mice to examine the generation of α-migrating particles, a detailed account of how HDL particles are remodeled in the periphery in the absence of ABCA1 was possible. Following intravenous injection of rHDL in *Abca1*$^{-/-}$ mice, a transient increase in α-migrating human apo A-1 was noted at 2 min post-injection (Figure 2); however, at subsequent time-points, there was an increase in α-migrating endogenous apo A-1 (Figure 2). These results suggest the following model: the rHDL particle acquired cholesterol in the periphery, possibly by ATP binding cassette protein G1 (ABCG1) mediated export (66), and activated LCAT. LCAT subsequently converted the discoidal, pre-β-migrating rHDL particle into an α-migrating particle, containing human apo A-1. As the rHDL particle was further remodeled in plasma, endogenous apo A-1 began to associate with the rHDL particle. As a result of remodeling in the periphery, human apo A-1 came off of the α-migrating particle as lipid free/poor human apo A-1, which are removed from the plasma via filtration by the kidneys (5). Without the generation of nascent, discoidal pre-β HDL, formed by ABCA1, the endogenous apo A-1 α-migrating particles eventually decimate.

Although this model provides intriguing details concerning the generation of α-migrating particles, it is possible that other processes are responsible for the observed phenotype. For example, it is unknown whether the α-migrating HDL particles present following rHDL injection contained the original POPC lipid core or if they contain a lipid core comprised of endogenous lipid. Further studies are necessary to examine this proposition and would provide additional insights into the generation of α-migrating HDL.

The present findings are consistent with the idea that pre-β-migrating particles are quickly remodeled into α-migrating particles independent of ABCA1 (6). In addition, intravenous injection of small, pre-β-migrating D4F:POPC complexes were sufficient to generate α-migrating particles in *Abca1*$^{-/-}$ mice (Figure 3.10). These results are surprising as it indicates that a nascent pre-β-migrating HDL is not necessary to generate an α-migrating particle. Further, the results from this study indicate that the α-migrating particles can maintained in *Abca1*$^{-/-}$ mice for an extended period of time, at least 4 hr. This is the first study to our knowledge that has shown the presence, and maintenance, of α-migrating particles in *Abca1*$^{-/-}$ mice.

The use of apo A-1 mimetic peptides, apo A-1 variants, and rHDL has been of growing interest in the field, providing stimulating discussion over their use as a therapeutic agent (67, 68). Current approaches for increasing HDL levels in individuals are limited. Statins, niacin, and fibric acid derivatives have had modest success in raising HDL cholesterol, increasing HDL cholesterol levels up to 35% (68); however, the effectiveness of these drugs in the context of individuals with extremely

low levels of HDL, especially those that exhibit increased apo A-1 particle catabolization, such as Tangier patients, are unknown. Indeed, infusion of apo A-1 complexes have shown promising results to increase HDL and modulate reverse cholesterol transport (65, 69, 70). The results from our study indicate that infusing rHDL or other HDL-like complexes may be beneficial to individuals with extremely low HDL levels as this process will increase plasma apo A-1 residence time, by slowing apo A-1 catabolism.

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Chapter Three contains material that appears in Atherosclerosis Thrombosis and Vascular Biology 2006 vol. 26, Gutierrez A, Ratliff EP, Andres AM, Huang X, McKeehan WL, Davis RA. The dissertation author was the secondary researcher and the co-authors listed in this publication directed and supervised the research which forms the basis for this chapter.

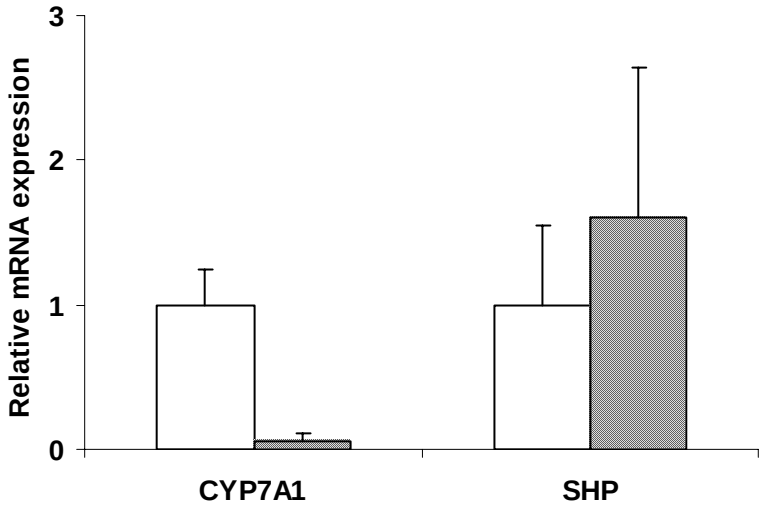


Figure 3.1. Endogenous CYP7A1 expression is repressed via a SHP-independent mechanism in CYP7A1-transgenic, LDLR knockout mice.
Ldlr$^{-/-}$ (open bars) and *Cyp7a1*-transgenic, *Ldlr*$^{-/-}$ (hatched bars) mice were fed a 1.25% cholesterol-containing diet. Hepatic mRNA expression of (A) endogenous CYP7A1 and (B) SHP are shown as the mean ± SD (n = 5). * $P < 0.05$.

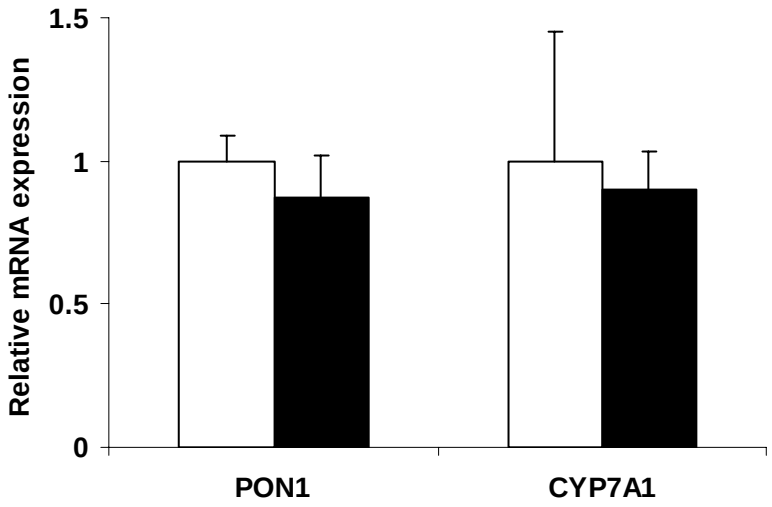


Figure 3.2. FGFR4 is required for the dietary, bile-acid mediated reduction in hepatic expression of CYP7A1 and PON1.
Fgfr4$^{-/-}$ mice were fed either a chow diet (open bars) or a 0.5% taurocholate-containing diet (closed bars). Hepatic mRNA expression of (A) PON1 and (B) CYP7A1 are shown as the mean ± SD (n = 5). * $P < 0.05$.

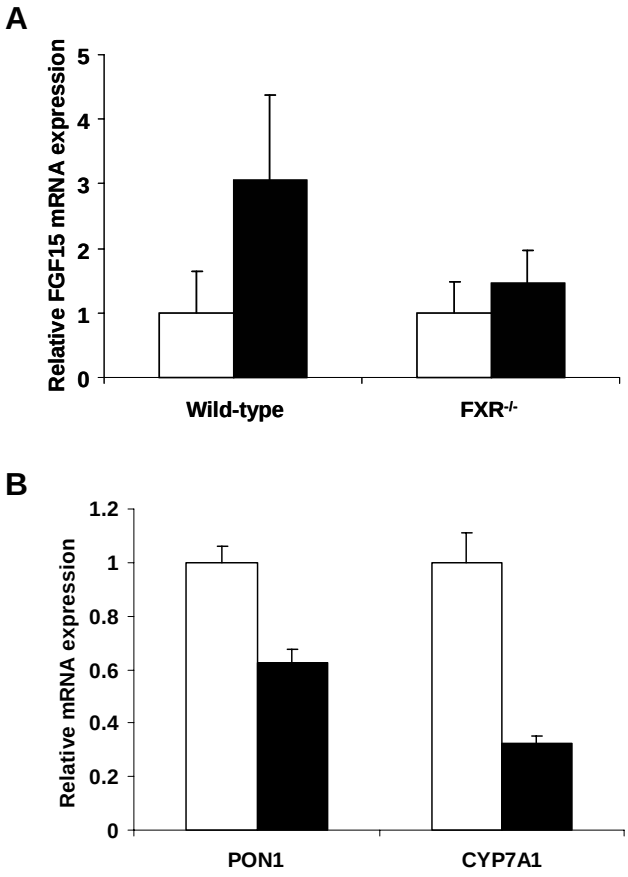


Figure 3.3. FGF19/FGF15 decreases the expression of CYP7A1 and PON1. (A) Wild-type and *Fxr*$^{-/-}$ mice were fed either a chow diet (open bars) or a 0.5% taurocholate-containing diet (closed bars). Ileal expression of FGF15 is shown as the mean in wild-type and *Fxr*$^{-/-}$ mice are shown as the mean ± SD (n = 5). (B) HepG2 cells were given no treatment (open bars) or incubated with 160 ng/mL FGF19 (closed bars). mRNA expression of PON1 and CYP7A1 are shown as the mean ± SD of triplicate dishes. * $P < 0.05$.

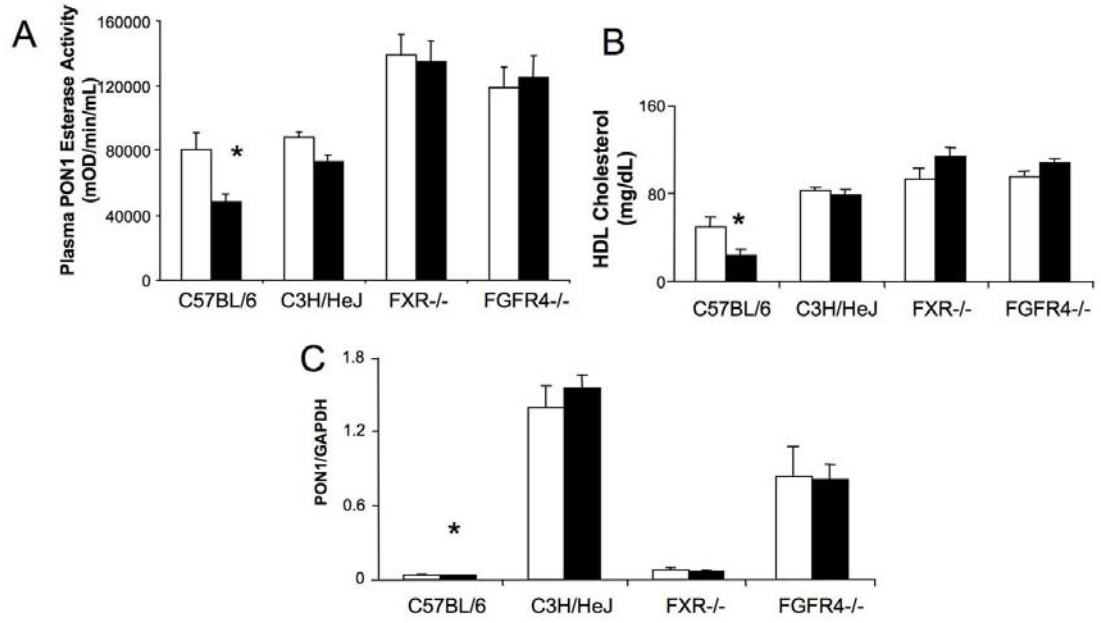


Figure 3.4. Plasma activity of PON1 varies in parallel with HDL levels, but not hepatic mRNA expression.
The indicated strains of mice and *Fxr*$^{-/-}$ (C57BL/6 background) and *Fgfr4*$^{-/-}$ (129 Sv background) were fed either a chow diet (open bars) or a 0.5% taurocholate-containing diet (closed bars). At sacrifice, blood and livers were obtained and used to measure (A) plasma PON1 activity, (B) plasma HDL cholesterol levels, (C) and hepatic PON1 mRNA expression. Each value represents the mean ± SD (n = 6). * $P < 0.05$.

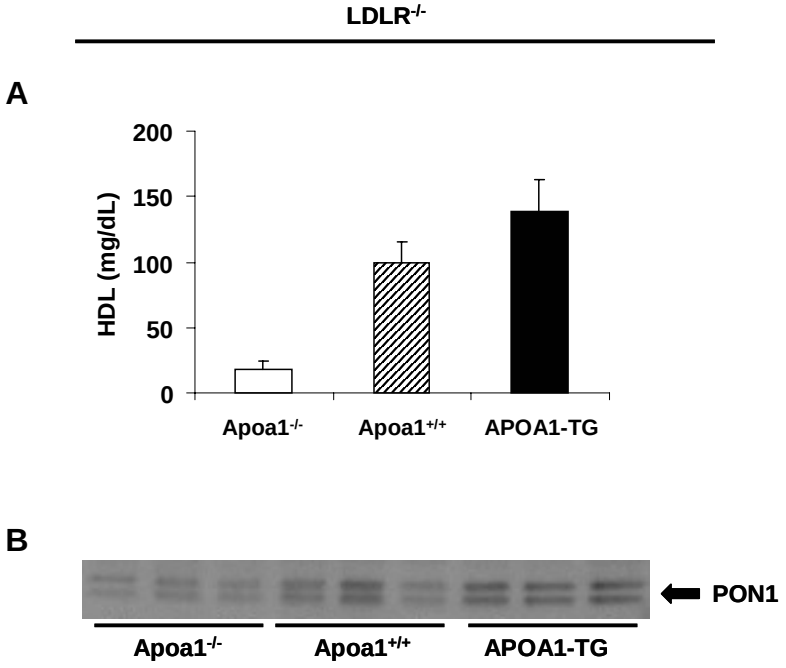


Figure 3.5. Plasma PON1 protein levels correlate with plasma HDL cholesterol levels and the amount of apo A-1 present in plasma.
Apoa1$^{-/-}$/*Ldlr*$^{-/-}$ mice, *Ldlr*$^{-/-}$ mice, and human *APOA1*-transgenic *Ldlr*$^{-/-}$ were maintained on a chow diet. Blood was obtained used to measure (A) plasma HDL cholesterol levels, which are shown as the mean ± SD (n = 3-5). * $P < 0.05$. (B) Plasma PON1 protein levels.

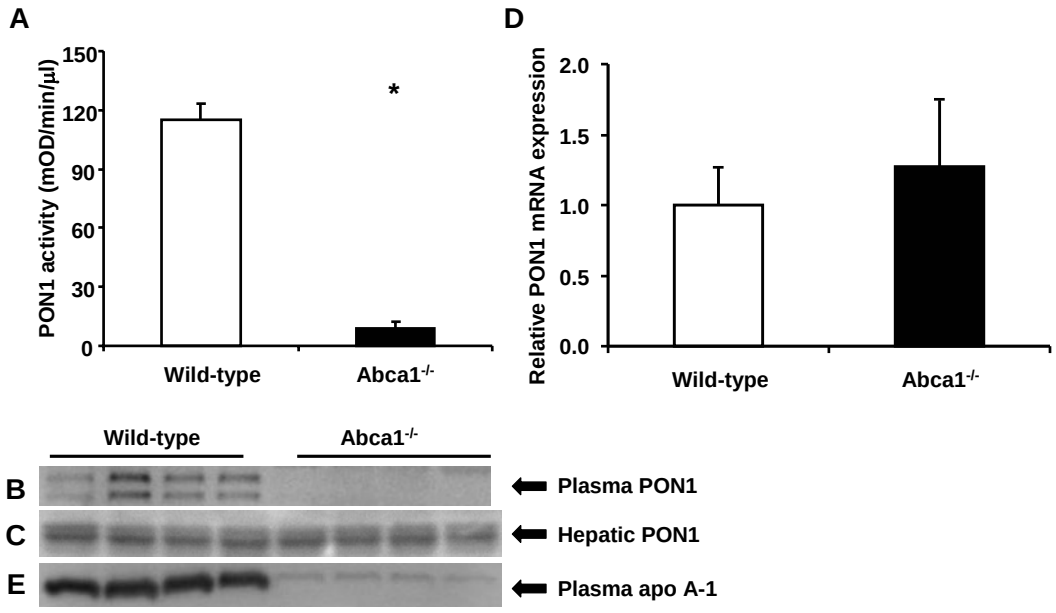


Figure 3.6. Plasma levels of PON1 are markedly reduced in ABCA1 knockout mice in spite of normal hepatic content of PON1 protein.
Wild-type and *Abca1*$^{-/-}$ mice were maintained a chow diet. (A) Plasma PON1 arylesterase activity was determined using phenyl acetate as the substrate. (B & E) Plasma samples were run on SDS-PAGE and probed for (B) PON1 and (E) apo A-1. (C) Hepatic membranes were prepared and run on SDS-PAGE and probed for PON1. (D) Relative hepatic PON1 mRNA levels. For (A) & (D), each value represents the mean ± SD (n = 4). * $P < 0.05$.

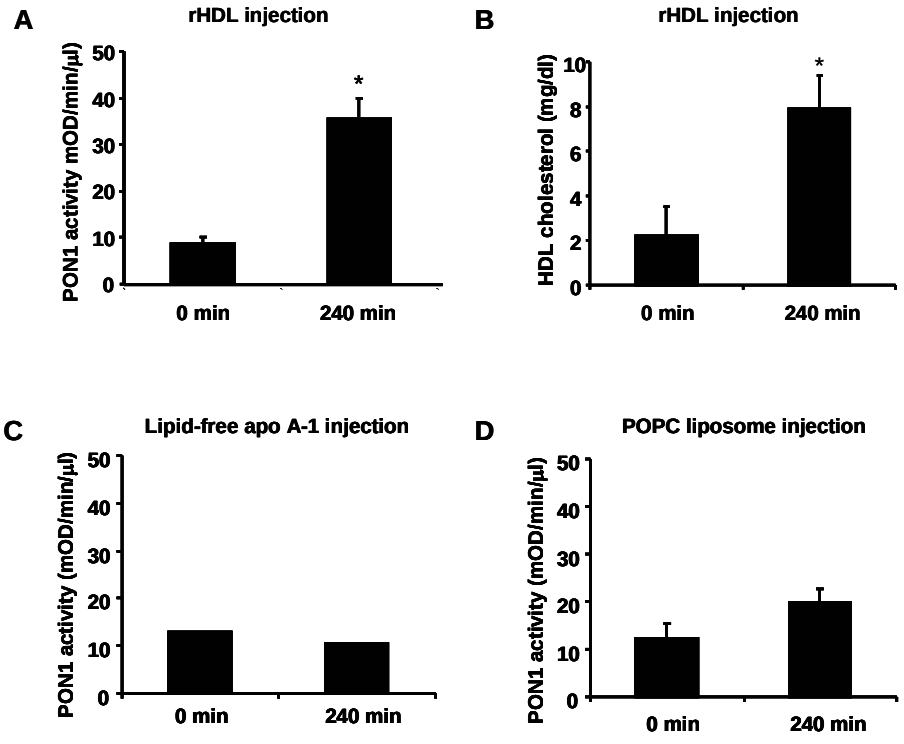


Figure 3.7. Intravenous injection of rHDL increases plasma PON1 activity and HDL levels.

Abca1$^{-/-}$ mice were injected intravenously with (A) rHDL (0.875 mg human apo A-1 protein), (B) lipid-free apo A-1 (1 mg apo A-1 protein), or (C) POPC liposomes. Mice were bled prior rHDL injection (0 min) and 240 min post-injection. (A, C, D) Plasma PON1 activity. (B) Plasma HDL cholesterol levels were measured using a commercially available kit. Each value represents the mean ± S.D. (n = 2-4). * $P < 0.05$.

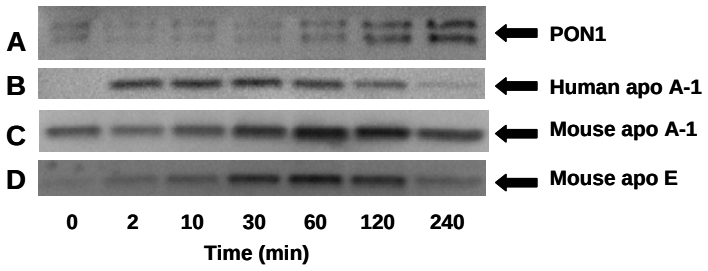


Figure 3.8. Intravenous injection of rHDL alters plasma apolipoprotein content in ABCA1 knockout mice.
Abca1$^{-/-}$ mice were injected intravenously with rHDL (0.875 mg human apo A-1 protein). Mice were bled prior rHDL injection (0 min) and at 2, 10, 30, 60, 120, and 240 min post-injection. Plasma samples were run on SDS-PAGE and probed for (A) PON1, (B) human apo A-1, (C) mouse apo A-1, and (D) mouse apo E, respectively.

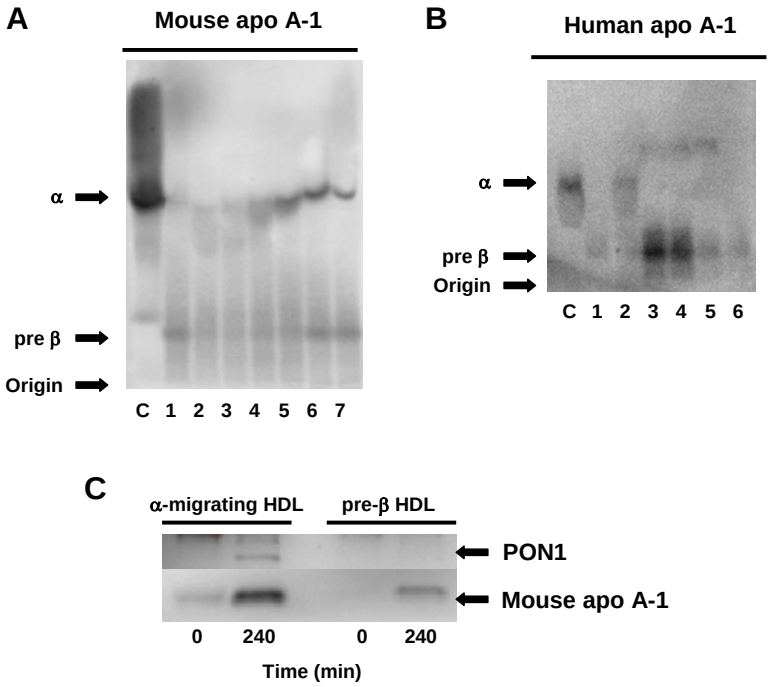


Figure 3.9. rHDL dependent increase in plasma PON1 occurs in parallel with the increase of α-migrating HDL containing endogenous apo A-1.
Abca1$^{-/-}$ mice were injected intravenously with rHDL (0.875 mg human apo A-1 protein). Mice were bled prior rHDL injection (0 min) and at 2, 10, 30, 60, 120, and 240 min post-injection. (A) Plasma samples were run on a 0.75% agarose gel and the blot was probed using an anti-mouse apo A-1 antibody. Lanes are denoted as follows: C: wild-type plasma, 1: 0 min, 2: 2 min, 3:10 min, 4: 30 min, 5: 60 min, 6: 120 min, and 7: 240 min. (B) Plasma samples were run on a 0.75% agarose gel and the blot was probed using an anti-human apo A-1 antibody. Lanes are denoted as follows: C: human *APOA1*-transgenic plasma, 1: 0 min, 2: 2 min, 3: 30 min, 4: 60 min, 5: 120 min, and 6: 240 min. (C) Pooled plasma samples were separated by FPLC. Fractions corresponding to α-migrating and pre-β-migrating HDL were concentrated, run on SDS-PAGE, and probed for PON1 and apo A-1.

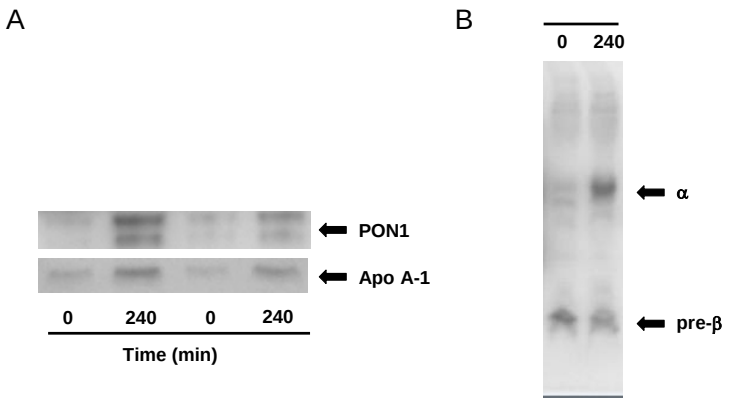


Figure 3.10. Intravenous injection of D4F:POPC complexes increases PON1-containing, α-migrating HDL in ABCA1 knockout mice.
Abca1$^{-/-}$ mice were injected intravenously with D4F:POPC complexes (0.5 mg peptide). Mice were bled prior D4F:POPC injection (0 min) and at 240 min post-injection. (A) Plasma samples were run on SDS-PAGE and probed for PON1 and apo A-1. (B) Plasma samples were run on a Native 4-20% PAGE and probed for apo A-1. Fractions corresponding to α-migrating and pre-β-migrating HDL were concentrated, run on SDS-PAGE, and probed for PON1.

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Chapter 4

Disruption of Thioredoxin-Interacting Protein (Txnip) decreases LPL activity in the heart and skeletal muscle and increases fasting plasma triglyceride levels

Introduction

A critical role for thioredoxin-interacting protein (Txnip) in regulating triglyceride metabolism was established when it was found that the *Txnip* gene was mutated in the *Hyplip1* locus of HcB-19 mice, whom display an abnormal fasting phenotype, notably a significant increase in plasma triglyceride levels (1-3). Further studies have implicated the involvement of Txnip in a myriad of other metabolic diseases including cancer, diabetes, and heart disease (4-8). Clearly, understanding the metabolic perturbations that occur as a result of Txnip deficiency, particularly during the transition into the fasting state, is of critical importance.

In response to fasting conditions, the heart and skeletal muscle are able to alter their preference for fuel consumption (9). As a result, these tissues increase their reliance on the utilization of triglyceride-derived fatty acids and ketones, thereby conserving glucose for use in other tissues, particularly the brain (10). The modulation of lipoprotein lipase (LPL) activity, which is responsible for the hydrolysis of a majority of triglycerides found in plasma, in the heart and skeletal muscle plays a

pivotal role in this process (11). During times of food deprivation, the heart and skeletal muscle exhibit an increase in LPL activity, thereby increasing the supply of fatty acids that can undergo oxidative phosphorylation and be utilized by these tissues to meet their energy requirements (10, 12). Previous studies have demonstrated that increased LPL mRNA expression and post-translational regulation contribute to the increase in LPL activity in the heart and skeletal muscle (12, 13).

In addition to being used as an energy substrate, LPL-derived fatty acids play a significant role in the expression of metabolic genes (14). Specifically, LPL-derived fatty acids have been shown to be ligands for the peroxisome proliferator-activated receptor (PPAR) family, which consists of three subtypes: PPARα, PPARδ, and PPARγ (14, 15). The abundance of each of the PPAR subtypes varies with tissue type; in the heart and skeletal muscle, PPARα and PPARδ are highly expressed (16). Previous studies have shown that PPARα is essential in maintaining a proper fasting response (17). In addition, further studies suggest that PPARα and PPARδ have some overlapping/redundant functions (18). Indeed, ablation of either PPARα or PPARδ in the heart or skeletal muscle results in decreased expression of genes involved in fatty acid metabolism (18-20).

In this study, we demonstrate that Txnip ablation in mice is associated with a diminished capacity to clear a bolus of trigycerides that is coordinate with a reduction in LPL activity in the heart and skeletal muscle. The reduction in LPL activity is due, in part, to decreased LPL mRNA expression in these tissues. In addition, during the

fasting state, Txnip knockout mice (*Txnip*$^{-/-}$) mice exhibit a reduction in the activation of AMPK and a change in the utilization of fuels.

Materials and Methods

Mice and Diets

Txnip knockout mice and tissue-specific *Txnip* knockout mice were generated as described (21). Mice were maintained on a 12 hr light – 12 hr dark cycle and were fed a standard chow (Harlan Teklad no. 5001) diet for the duration of the experiments. All procedures were approved and performed in concordance with the Institutional Animal Care and Use Committee at San Diego State University.

Plasma collection and FPLC analysis

Following an overnight fast (18 hr), wild-type and *Txnip*$^{-/-}$ mice were anesthetized using isofluorane and blood was collected via retro-orbital puncture. Blood was centrifuged at 10,000 rpm at 4^{o}C to obtain plasma. Plasma metabolites were measured using commercially available enzymatic assays: triglycerides and glucose (Wako Chemicals USA) and 3-hydroxybutyrate (Stan-Bio). VLDL, LDL, and HDL fractions were separated by fast-protein liquid chromatography (FPLC); pooled plasma samples were fractioned using a Superose 6 columns (Amersham Pharmacia Biotech).

For the initial time-course experiment examining plasma metabolites, food was removed from *Txnip*$^{-/-}$ mice at 4 PM and plasma was obtained just prior the fast and at 8 PM, 10 PM, 12 AM, and 2 AM. The plasma metabolites were measured as described above.

For the second set of time-course experiments, wild-type and *Txnip*$^{-/-}$ mice were fasted at mid-dark (12:00 AM) and plasma was obtained at various time-points; plasma was also obtained from one set of wild-type and *Txnip*$^{-/-}$ mice at mid-dark, prior to a fast (0 hr time-point). Plasma triglyceride levels were measured as described above.

Western Blot Analysis

Plasma samples were diluted with 0.1 M Tris (pH 6.7), water, and SDS sample buffer (pH 8.0). Tissues were homogenized in ice-cold radio-immunoprecipitation (RIPA) buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 2 mM EGTA, 1 mM EDTA, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 20 mM NaF, 1 mM $NaVO_4$, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. Samples were separated using a 4-12% Tris-Glycine gradient gel (Invitrogen), then electroblotted onto polyvinylidene fluoride membranes (GE Healthcare). Nonspecific binding sites were blocked using non-fat dry milk/Tris Buffered Saline with 0.1% Tween 20. For plasma samples, immuno-detection was performed using rabbit anti-mouse apo B antibodies or anti-mouse Angptl4 antibodies (Abcam). For tissue samples, phospho-AMPK Thr172, total AMPK, phospho-ACC Ser79, and total ACC antibodies were used (Cell Signaling Technology). Proteins were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific).

Hepatic Triglyceride Production

Following an overnight fast, wild-type and *Txnip*$^{-/-}$ mice were given an intraperitoneal injection of Poloxamer 407 (1 g/kg mouse), dissolved in 0.9% NaCl (22). Blood was collected retro-orbitally at baseline and at 1, 2, 4, and 6 hr post-injection; plasma triglyceride levels were subsequently measured.

Intravenous Lipid Loading Test

Wild-type and *Txnip*$^{-/-}$ mice were given an intravenous bolus of 20% Intralipid® triglycerides following an overnight fast (250 μl/mouse; Sigma-Aldrich). Plasma triglyceride levels measured at 5 min post injection was considered 100 percent of the lipid load. Blood was collected at 15, 30, 60, and 120 min post-injection and plasma triglyceride levels were measured. Data is expressed as percent of triglyceride levels remaining, as compared to the plasma triglyceride levels at 5 min post-injection.

LPL Activity in Tissues

LPL activity in tissues was performed as described (23), with a few modifications. Hearts, soleus muscles, and gastrocnemius muscles were removed and homogenized in a buffer containing 25 mM ammonia-HCl pH 8.2, 5 mM EDTA, 0.8% Triton X-100 (w/v), 0.01% SDS (w/v), 5000 IU/l heparin, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aprotinin (9 ml/g tissue). Insoluble material was removed via centrifugation (18,000 X g for 20 min @ 4^{o}C). Tissue lysates were

incubated with an emulsion containing [9,10-^{3}H(N)] triolein (PerkinElmer NEN Radiochemicals) and 10% Intralipid (Sigma-Aldrich) at 25^{o}C for 60 min in a shaking water bath; 2 μCi of [9,10-^{3}H(N)] triolein was used per reaction. The reaction was stopped by the addition of distilled H_2O (0.5 ml) and 2 ml isopropanol/hexane/H_2SO_4 (48:48.3:1 v/v/v). Released fatty acids were extracted from the upper phase with the addition of 1 ml alkaline ethanol (ethanol 95%/H_2O/2 M NaOH, 500:475:25 v/v/v) and 3 ml hexane. Following centrifugation, the hexane layer was removed to rid of unhydrolysed triglycerides. After an additional extraction was performed using 3 ml hexane, 500 μl of the alkaline ethanol phase, containing fatty acids, was added to a scintillation vial containing 10 mL of Ecolite (MP Biomedicals) and counted in a β-scintiallation counter (Beckman Instruments).

RNA isolation, cDNA synthesis, Real-time PCR, and microarray

After euthanization, hearts, soleus muscles, and gastrocnemius muscles were removed and flash frozen in liquid N_2. RNA was isolated from frozen tissue using the PerfectPure RNA Tissue kit (5 PRIME) following the manufacturer's protocol. cDNA was synthesized from the isolated RNA using the iScript cDNA synthesis kit (BioRad).

Quantitative PCR, using SYBR Green detection chemistry, was performed on an iQ-cycler (BioRad). Primer sequences are shown in Table 1. Quantitative measurements were determined using the Pfaffl method and expression of GAPDH was used as the reference gene.

Microarray analysis of genes in the soleus of fasted wild-type and *Txnip*$^{-/-}$ mice was performed in collaboration with Dr. Aldons J. Lusis at UCLA Department of Microbiology, Immunology, and Molecular Genetics.

Ex Vivo Fuel Oxidation Experiments

Oxidation of fuel substrates by isolated soleus muscles were performed following a previously described procedure with slight modifications (21, 24). Following an overnight fast(~18 hr), wild-type and *Txnip*$^{-/-}$ mice, soleus muscles were carefully extracted and immediately placed into a capped 25 ml e-flask containing 3 ml of pre-oxygenated (bubbled 95% oxygen:5% CO_2 mixture for at least 1 min) Krebs Ringer solution, pH 7.4 (10 mM glucose, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 5 mM KCl, 125 mM NaCl and 2.5 mM $CaCl_2 \cdot H_2O$) at 37^{o}C in a shaking water bath for 20 min (pre-incubation step). Tissues were then moved into a new flask with the same buffer and conditions supplemented with either [^{14}C]-glucose (0.5 µCi/ml), [1-^{14}C]-3-hydroxybutyrate (0.5 µCi/ml), or [1-^{14}C]-oleate (0.5 µCi/ml). The atmospheric air of the flasks were then re-equilibrated with 95% oxygen:5% CO_2 mixture and a new stopper with a holding well containing a piece of filter paper was used to seal the flasks. Tissues were then left to incubate in the 37^{o}C shaking water bath for 1 hr. After this time, 350 µl of 1M hyamine hydroxide (Perkin Elmer) was injected through the stopper into the center well and the oxidation reaction was stopped by injecting 400 µl 1M sulfuric acid directly into the media. CO_2 was allowed

to collect overnight, and the filter papers were then transferred to vials for liquid scintillation counting.

Statstical Analysis

Statistical analysis between the groups of mice were performed using Student's t-test (double tailed, unpaired). All values are reported as mean $\pm$ S.D.

Results

Ablation of Thioredoxin-interacting protein (Txnip) in mice causes accumulation VLDL/chylomicron triglycerides

In order to gain insight into the metabolic role of Txnip, our lab generated Txnip knockout (*Txnip*$^{-/-}$) mice via the Cre-loxP gene recombination system (21, 25). Complete ablation of the *Txnip* gene was performed by breeding homozygous floxed (*Txnip*$^{fl/fl}$) mice with zp3-cre mice (26). Under non-fasting conditions, wild-type and *Txnip*$^{-/-}$ mice exhibited similar plasma metabolic profiles in regards to glucose, ketone, and triglyceride levels (data not shown); however, following an 18-hr fast, *Txnip*$^{-/-}$ mice exhibited an abnormal plasma phenotype (Figure 4.1A-C). *Txnip*$^{-/-}$ mice displayed a ~7-fold increase in fasting plasma triglyceride levels and a ~3-fold increase in ketone levels as compared to wild-type mice (Figure 4.1A & B). Concomitantly, *Txnip*$^{-/-}$ mice are hypoglycemic, displaying a ~50% reduction in fasting plasma glucose levels as compared to wild-type mice (Figure 4.1C). The reduction in plasma glucose levels observed in *Txnip*$^{-/-}$ mice was not a result of altered levels of the pancreatic hormones insulin and glucagon (data not shown).

Given the critical role of the liver in the secretion of VLDL triglycerides, ketogenesis, and gluconeogenesis, our lab generated liver-specific Txnip knockout mice (*Txnip*$^{L-/L-}$) by breeding homozygous floxed (*Txnip*$^{fl/fl}$) mice with Alb-cre mice (27). Previous studies in HcB-19 mice implicated the liver as a major site of Txnip action (1-3). In contrast to these findings, the plasma profile of *Txnip*$^{L-/L-}$ mice did not

recapitulate the phenotype observed in the *Txnip*$^{-/-}$ mice (Figure 4.1A-C). These results indicate that the liver is not the primary site for the metabolic action of Txnip, alternatively indicating that the phenotype of *Txnip*$^{-/-}$ mice is primarily due to a deficiency of *Txnip* in extra-hepatic tissues.

Considering that *Txnip*$^{L-/L-}$ mice had an indiscernible phenotype from wild-type mice, our lab hypothesized that the hyperlipidemic phenotype of *Txnip*$^{-/-}$ mice was primarily due to Txnip ablation in extra-hepatic tissues. In wild-type mice, *Txnip* is highly expressed in the skeletal muscle and heart (data not shown), two catabolic tissues that are capable of utilizing each of the following fuels: fatty acids (free or triglyceride-derived), glucose, and ketones (10). Therefore, our lab generated skeletal muscle and heart-specific Txnip knockout mice (*Txnip*$^{M-/M-}$) by breeding homozygous floxed (*Txnip*$^{fl/fl}$) mice with MCK-cre mice (28). Indeed, ablation of Txnip in the skeletal muscle and heart recapitulated the phenotype observed in *Txnip*$^{-/-}$ mice. *Txnip*$^{M-/M-}$ mice exhibited hypertriglyceridemia, hyperketonemia, and hypoglycemia following an overnight fast (Figure 4.1A-C), a phenotype similar to that of *Txnip*$^{-/-}$ mice, albeit to a milder degree.

In order to gain insight into the plasma triglyceride phenotype, pooled plasma samples from wild-type and *Txnip*$^{-/-}$ mice were separated by FPLC; a majority of the plasma triglycerides in *Txnip*$^{-/-}$ mice accumulated in the VLDL/chylomicron fraction (Figure 4.2A). The increase in VLDL/chylomicron triglycerides in *Txnip*$^{-/-}$ mice corresponded to a significant increase in plasma apo B-48 levels; surprisingly, however, plasma apo B-100 levels were diminished in *Txnip*$^{-/-}$ mice (Figure 4.2B).

Impaired triglyceride clearance in Txnip knockout mice

The increase in plasma triglycerides observed in *Txnip*$^{-/-}$ mice could result from increased secretion of triglyceride-rich lipoproteins (VLDL/chylomicrons) and/or diminished clearance of these triglyceride-rich particles (29). Following an intraperitoneal injection with Poloxamer-407, a compound that blocks the actions of lipoprotein lipase (LPL) (30), wild-type and *Txnip*$^{-/-}$ mice accumulated similar levels of plasma triglycerides (Figure 4.3A), suggesting that the hepatic triglyceride production rate is not increased in *Txnip*$^{-/-}$ mice. As *Txnip*$^{L-/L-}$ mice did not exhibit elevated plasma triglyceride levels following an overnight fast (Figure 4.1A), these data are consistent with the idea that Txnip does not exert its metabolic effects through the liver.

In contrast, following an intravenous injection of Intralipid® triglycerides, fasting *Txnip*$^{-/-}$ mice exhibited a severely impaired clearance of the plasma triglycerides as compared to wild-type mice (Figure 4.3B). While wild-type mice were able to clear 50% of the injected Intralipid® load in ~35 min, *Txnip*$^{-/-}$ mice required ~120 min to clear the same load (Figure 4.3B).

Fasting Txnip knockout mice exhibit decreased LPL activity

Our results suggest that ablation of *Txnip* in the heart and skeletal muscle is sufficient to cause hypertriglyceridemia as a result of impaired clearance of plasma triglycerides. The heart and skeletal muscle play a major role in the utilization of fatty acids derived from triglycerides; in fact, the heart obtains 70% of its energy need from

fatty acids (31). Indeed, previous studies have suggested LPL activity in the skeletal muscle and heart could be a major determinant of plasma triglyceride levels (32). Using a [^{3}H] triolein emulsion as a substrate, LPL activity was measured in the heart and skeletal muscle of wild-type and *Txnip*$^{-/-}$ mice after an overnight fast. LPL activity was dramatically reduced in the heart, soleus, and gastrocnemius from *Txnip*$^{-/-}$ mice (Figure 4.4A).

It is interesting to note that LPL mRNA expression was also decreased in the heart, soleus, and gastrocnemius muscle of *Txnip*$^{-/-}$ mice following an overnight fast (Figure 4.4B). These data suggest that decreased LPL mRNA expression could contribute to the lower LPL activity in the heart and skeletal muscle of *Txnip*$^{-/-}$ mice; indeed, previous studies have indicated that reduced expression of LPL was associated with higher plasma triglyceride levels (33). However, it is important to note that LPL activity is primarily controlled at the post-translational level (34).

Possible contributions of Angptl4 and AMPK to modulate LPL activity in Txnip knockout mice

There are many post-translational steps that ultimately regulate LPL-mediated lipolysis (34). These steps include processes such as intracellular degradation, displacement from HSPG binding sites, the abundance of plasma apo C-II, as well as others (34). Recent studies established that two members of the angiopoietin family, angiopoietin-like protein (Angptl) 3 and Angptl4, also modulate lipolysis by inhibiting LPL activity (35). Angptl4 is a secreted protein that is highly expressed in adipose

and liver and, to a lesser extent, in the skeletal muscle and the heart (36). Recent studies demonstrate that Angptl4 converts catalytically active LPL dimers into inactive monomers (37).

Using microarray analysis, the expression of Angptl4 was found to be up-regulated by ~2.7-fold in the soleus muscle of *Txnip*$^{-/-}$ mice during the fasted state (data not shown). To determine if Angptl4 contributes to the hypertriglyceridemic phenotype of *Txnip*$^{-/-}$ mice, mRNA expression of Angptl4 was measured in the skeletal muscle and the heart. Consistent with the microarray results, the expression of Angptl4 was increased in the soleus of *Txnip*$^{-/-}$ mice (Figure 4.5A); however, the expression did not differ in the heart and gastrocnemius (Figure 4.5A). Further studies examining the physiological role of Angptl4 demonstrated the importance of plasma Angptl4 levels: intravenous injection of Angptl4 was shown to increase plasma triglyceride levels (38). Therefore, plasma Angptl4 levels, which have been shown to have three different forms in plasma (39), were measured in wild-type and *Txnip*$^{-/-}$ mice; however, little or no difference in plasma Angptl4 levels were found (Figure 4.5B). Therefore, these data suggest that Angptl4 may not play a predominant role in modulating plasma triglyceride levels in *Txnip*$^{-/-}$ mice.

In addition to disruption of LPL dimers, the availability of functionally relevant LPL can modulate plasma triglyceride levels. AMP-activated protein kinase (AMPK) acts as a metabolic “switch” in tissues by shutting down anabolic processes and activating catabolic processes (40). In order to become activated, AMPK is phosphorylated at Thr 172 by LKB1 (41). Recent studies demonstrate that AMP-

activated protein kinase (AMPK) can facilitate the translocation of LPL to the vascular endothelium in the heart, possibly through the activation of p38 MAPK (42, 43). Further studies demonstrate that activation of AMPK increases the mRNA expression, protein, and activity of LPL in L6 skeletal muscle cells (44).

Following an overnight fast, *Txnip*$^{-/-}$ mice exhibited a significant reduction in phospho-AMPK (Thr 172) in the heart and soleus (Figure 4.6A & C). Similarly, the phosphorylation of ACC (Ser 79), an established downstream target of AMPK, was also diminished in *Txnip*$^{-/-}$ mice in the heart and soleus thereby demonstrating that AMPK activity is impaired in these tissues of *Txnip*$^{-/-}$ mice (Figure 4.6B & D). In contrast, phospho-AMPK levels did not differ between fasting wild-type and *Txnip*$^{-/-}$ mice in the gastrocnemius (Figure 4.6E). These results are intriguing and further studies are necessary to determine the contribution of impaired AMPK signaling to modulating plasma triglyceride levels in *Txnip*$^{-/-}$ mice.

Txnip knockout mice exhibit reduced PPAR expression in the heart

Lipolysis of triglyceride-rich VLDL, through the action of LPL, generates ligands for members of the PPAR family (14). Given that the heart obtains 70% of its energy from fatty acids (31), the impact of reduced LPL activity on the expression of PPARs and their respective target genes was examined in the hearts of *Txnip*$^{-/-}$ mice following a fast. *Txnip*$^{-/-}$ mice exhibited significantly lower expression of PPARα in the heart as compared to wild-type mice (Figure 4.7). Similarly, based upon our microarray experiments, PPARα expression was found to be diminished in the soleus

muscle of *Txnip*$^{-/-}$ mice (data not shown). Furthermore, a subset of PPAR target genes were also found to be decreased following a fast (Figure 4.7). These data suggest that the transcriptional activities of PPAR may be diminished in the hearts of fasting *Txnip*$^{-/-}$ mice.

Impaired mitochondrial function in Txnip knockout mice

Previous studies found that inhibition of mitochondrial fatty acid oxidation by etomoxir resulted in decreased lipolysis (45). Alternatively, the LPL-mediated hydrolysis of triglycerides provides fatty acid ligands for the PPARα (14); PPARα is required for the induction of mitochondrial fatty acid oxidation genes (46). These data suggests that mitochondrial function and the regulation of lipolysis are coordinately linked.

Given the impaired lipolysis observed in *Txnip*$^{-/-}$ mice, the mitochondrial utilization of fatty acids, as well as the utilization of other metabolic fuels, were assessed in the soleus muscle of *Txnip*$^{-/-}$ and wild-type mice. Interestingly, the soleus from *Txnip*$^{-/-}$ mice exhibited a diminished capacity to oxidize [1^{14}C]-oleic acid, [^{14}C]-glucose, and [^{14}C]-3-hydroxybutyrate to $^{14}CO_2$ (Figure 4.8). These results indicate that mitochondrial respiration is impaired in the soleus muscle of *Txnip*$^{-/-}$ mice. It is likely, however, that mitochondrial respiration is not impaired in all the tissues of *Txnip*$^{-/-}$ mice; for example, the hepatic production of ketones requires acetyl-CoA derived from fatty acid oxidation from the liver (47). *Txnip*$^{-/-}$ mice have dramatically increased plasma ketone levels (Figure 4.1B) and urinary ketone levels (data not

shown) suggesting that *Txnip*$^{-/-}$ mice have increased ketogenesis and, thus, hepatic mitochondrial function is not impaired. In order to compensate for the energy deficits present with reduced mitochondrial function, further studies in our lab demonstrated that *Txnip*$^{-/-}$ mice actually have increased glycolysis (data not shown) (21).

Elevated plasma triglyceride levels in Txnip knockout mice occur after changes in other plasma metabolites and correlate with a decrease in LPL activity in the heart and skeletal muscle

To understand the unique metabolic phenotype of *Txnip*$^{-/-}$ mice that is only observed in the fasted state, our lab examined the changes in the plasma metabolites through a fasting time-course experiment. Food was removed from *Txnip*$^{-/-}$ mice at 4 pm (light period), prior to the dark period when mice normally feed. As shown in Figure 4.9, changes in plasma glucose levels and plasma ketone levels in *Txnip*$^{-/-}$ mice were significantly altered within 4 hr after the initial fast, which preceded the changes observed in plasma triglyceride levels (8 hr after the initial fast). As the availability/utilization of each of these fuel sources is interconnected (10), these data suggests that alterations in glucose and/or ketone metabolism could contribute to the changes in fasting plasma triglyceride levels.

In a separate set of experiments, we decided to measure plasma triglyceride levels of *Txnip*$^{-/-}$ mice during the mid-dark period, in which the mice have free access to food and are normally feeding, and at various time-points following a fast; thus, a time-course of the events leading to hypertriglyceridemia can be examined. This set

of experiments was performed to obtain initial data that would ultimately provide mechanistic insight into the accumulation of plasma triglycerides that is observed in *Txnip*$^{-/-}$ mice.

For this experiment, the start of the time-course was chosen based upon phenotypic changes between wild-type and *Txnip*$^{-/-}$ mice that were observed at other time-points (data not shown). Indeed, previous studies have indicated that *Txnip* deficiency disrupts the transition from the fed state to the fasted state in mice (48). During the fed state (mid-dark period), wild-type and *Txnip*$^{-/-}$ mice exhibited similar plasma triglyceride levels (Figure 4.10A). However, following 3 and 6 hours of fasting, *Txnip*$^{-/-}$ mice already exhibited significantly higher plasma triglyceride levels than wild-type mice; the difference in plasma triglyceride levels could be attributed to a decrease in plasma triglyceride levels in wild-type mice, while triglyceride levels in *Txnip*$^{-/-}$ mice were maintained (Figure 4.10A). However, following 9 hours of fasting, plasma triglyceride levels in *Txnip*$^{-/-}$ mice increased significantly and were ~2.5-fold higher than the plasma triglyceride levels observed in wild-type mice (Figure 4.10A).

Interestingly, during the fed state, LPL activity was reduced by 20% in the heart (Figure 4.10B) and by 20% in the soleus (Figure 4.10C) of *Txnip*$^{-/-}$ mice as compared to wild-type mice; no significant change in LPL activity was observed in the gastrocnemius (Figure 4.10D). These results suggest that LPL activity in the heart and skeletal muscle alone is not sufficient to dictate plasma triglyceride levels in *Txnip*$^{-/-}$ mice in the fed state; thus, other factors and/or tissues contribute to plasma triglyceride

levels so that *Txnip*$^{-/-}$ mice maintain similar triglyceride levels as wild-type mice in the fed state.

However, following 9 hours of fasting, when plasma triglyceride levels in *Txnip*$^{-/-}$ mice significantly increase, *Txnip*$^{-/-}$ mice exhibited a ~60% decrease in LPL activity in the heart, a ~70% decrease in the soleus, and a ~60% decrease in the gastrocnemius, as compared to wild-type mice (Figure 4.10B, C, & D). As altered LPL activities in the skeletal muscle and heart contribute to plasma triglyceride levels, these results strongly suggest that reduced LPL activity in the heart and skeletal muscle modulate plasma triglyceride levels in *Txnip*$^{-/-}$ mice.

Discussion

The present findings demonstrate that Txnip expression is essential in maintaining a proper fasting response. *Txnip*$^{-/-}$ mice exhibited diminished plasma glucose levels following an overnight fast, as well as increased plasma ketone levels and triglyceride levels (Figure 4.1); this phenotype was attributed to the ablation of Txnip specifically in the skeletal muscle and heart (Figure 4.1). The alterations in plasma glucose and ketone levels occurred quickly following a fast, while changes in plasma triglyceride levels occurred at a later time-point (Figure 4.9). Normally, the skeletal muscle and heart increase their reliance on fatty-acid derived fuels, specifically triglyceride-derived fatty acids and ketones, following a fast (10). *Txnip*$^{-/-}$ mice exhibited a reduced capacity to oxidize fatty acids and ketones through the mitochondria, suggesting that *Txnip*$^{-/-}$ mice are unable to efficiently switch to the utilization of fatty acids and ketones (Figure 4.8). Surprisingly, however, *Txnip*$^{-/-}$ mice also displayed impaired glucose oxidation during the fasting state (Figure 4.8).

To compensate for the impaired mitochondrial oxidation, *Txnip*$^{-/-}$ mice up-regulate glycolysis (data not shown) (21); in fact, recent studies in our lab demonstrate that the skeletal muscle and heart of *Txnip*$^{-/-}$ mice actually over-compensated for the decreased mitochondrial oxidation and have an increased ATP:AMP ratio, indicating a higher energy state (data not shown). Combined with the diminished oxidative capacity observed in the soleus (Figure 4.8), these findings are reminiscent of the Warburg effect, in which cancer cells increase their utilization of glucose through

glycolysis while diminishing the oxidative capacity of the mitochondria. These data are consistent with previous studies indicating that Txnip acts as a tumor suppressor (5).

Consistent with the higher energy state observed in the heart and soleus of *Txnip*$^{-/-}$ mice (data not shown), AMPK was less activated (phosphorylated (Thr 172)) in these tissues (Figure 4.6). In contrast, the gastrocnemius of *Txnip*$^{-/-}$ mice exhibited little, if any change, in the activation of AMPK as compared to wild-type mice (Figure 4.6). Although the reason for this difference is unknown, it could be due to the metabolic nature of these different tissues. The heart and soleus muscles are known to be highly oxidative, while the gastrocnemius is mainly glycolytic. As previously indicated, glycolysis is up-regulated in *Txnip*$^{-/-}$ mice (21), suggesting that the heart and soleus had to alter their metabolic preference in order to increase glycolysis; in contrast, the gastrocnemius, which is already glycolytic in nature, did not have to change its metabolic nature. Further studies will need to be performed to demonstrate that glycolysis is specifically increased in these tissues.

The hydrolysis of triglyceride-rich lipoproteins, via the action of LPL, generates fatty acids, which are ligands for members of the PPAR family (14). The two most highly expressed isoforms of PPAR in the heart and skeletal muscle are PPARα and PPARδ (16). Our studies demonstrated that *Txnip*$^{-/-}$ mice had diminished PPARα expression in the heart, but not PPARδ; however, there was a reduction in the expression of a subset of PPAR target genes (Figure 4.7). These studies suggest that the transcriptional activities of PPARα and PPARδ may be decreased; further studies,

such as measuring PPARα and PPARδ protein levels and performing an *in vivo* chromatin immuno-precipitation assay in the heart, would provide insight into the contribution of PPARs in modulating gene expression in the hearts of *Txnip*$^{-/-}$ mice.

Both PPARα and PPARδ have been shown to modulate fatty acid oxidation (18-20). In addition, activation of PPARδ has been shown to enhance mitochondrial function (49). Furthermore, activation of AMPK has been shown to promote mitochondrial biogenesis through a PGC-1α-dependent manner (50). Therefore, it is intriguing to speculate that these factors could contribute to the reduced mitochondrial oxidation observed in the *Txnip*$^{-/-}$ mice (Figure 4.8). Further studies are necessary to determine if the impaired mitochondrial oxidation is due to an intrinsic defect in the mitochondria or a signaling defect resulting in impaired mitochondrial function.

Hypertriglyceridemia, as a result of *Txnip* deficiency, appears to be independent of the liver as the rate of triglyceride secretion between wild-type and *Txnip*$^{-/-}$ mice did not differ (Figure 4.2A); additionally, mice with liver-specific ablation of *Txnip*$^{-/-}$ mice did not exhibit elevated fasting plasma triglyceride levels (Figure 4.1A). Alternatively, our studies establish that disruption of *Txnip* results in an impairment of plasma triglyceride clearance (Figure 4.2B), thereby implicating Txnip expression in extra-hepatic tissues as being essential in maintaining plasma triglyceride levels. Indeed, our additional findings that LPL activity was decreased in the heart and skeletal muscle supports this notion (Figure 4.4). Further, mice with *Txnip* specifically deleted in the heart and skeletal muscle exhibited increased fasting

plasma triglyceride levels (Figure 4.1A), suggesting that Txnip acts locally within these tissues to reduce LPL activity.

In contrast to our studies, previous studies using HcB-19 mice, which exhibit a nonsense mutation in the *Txnip* gene resulting in a truncated, non-functional protein, indicated that HcB-19 mice have similar LPL activity levels, based upon post-heparin plasma measurements (2). The reason for this discrepancy could be attributed to the procedure to obtain post-heparin plasma, to the different methods to determine LPL activity, and/or to possible differences between these strains. Recent studies indicate that there are two pools of LPL found on the endothelium, only one of which appears to be responsible for lipolysis of triglyceride-rich particles (51). The lipolysis relevant pool is released quickly after intravenous heparin injection, while the lipolysis irrelevant pool is released into plasma within 15 min (51); the aforementioned studies collected plasma 30 min post-heparin injection (2). While our studies suggests that LPL activity in tissues contribute to plasma triglyceride levels (Figure 4.4A), further studies would be necessary to clearly establish that LPL levels on the endothelium of the heart and skeletal muscle modulate plasma triglyceride levels in *Txnip*$^{-/-}$ mice.

Along with changes in LPL activity, HcB-19 mice differ from *Txnip*$^{-/-}$ mice in other phenotypes as well. For example, HcB-19 mice were reported to have increased VLDL secretion (2); however, *Txnip*$^{-/-}$ mice did not exhibit a change in triglyceride secretion (Figure 4.3). Furthermore, the perturbed metabolic phenotype of HcB-19 mice was attributed to alterations in the liver (1-3); however, metabolic alterations in

the skeletal muscle and heart appear to be responsible for the fasting phenotype of *Txnip*$^{-/-}$ mice (Figure 4.1).

The mechanism by which Txnip ablation in the skeletal muscle and heart causes a reduction in LPL activity is unknown. However, given that *Txnip*$^{-/-}$ mice have a higher energy state in the skeletal muscle and heart than wild-type mice, in spite of impaired mitochondrial function, it is reasonable to propose that these tissues respond to the diminished necessity of triglyceride-derived fatty acids by decreasing LPL activity. Following an overnight fast, *Txnip*$^{-/-}$ mice exhibited a decrease in LPL mRNA expression in the heart and skeletal muscle, which correlated with the reduction in LPL activity in these tissues (Figure 4.4); this observation is consistent with previous studies that indicated LPL mRNA expression and LPL activity are correlated in the heart and skeletal muscle (13). Based upon these observations, it would be interesting to determine if the reduction in LPL mRNA expression led to a similar reduction in LPL protein mass in the skeletal muscle and heart of *Txnip*$^{-/-}$ mice, thereby providing a potential mechanism for the reduction in LPL activity observed in these tissues of *Txnip*$^{-/-}$ mice. During the transition from the fed state to the fasting state the heart and skeletal muscle increase their reliance on the utilization of fatty-acid derived fuels, thereby conserving glucose for use by the brain (10). If *Txnip*$^{-/-}$ mice indeed have decreased LPL protein levels in the fasting state, it is tempting to speculate that, when the heart and skeletal muscle normally increase their reliance of fatty acid derived fuels by releasing additional LPL to the vascular endothelium,

Txnip$^{-/-}$ mice are incapable of doing so due to diminished LPL protein levels, ultimately resulting in increased plasma triglyceride levels.

To gain insights into the modulation of LPL activity, our lab performed various time-course experiments. In our first set of experiments, alterations in glucose and ketone metabolism were shown to precede the increase in plasma triglyceride levels of *Txnip*$^{-/-}$ mice following a fast (Figure 4.9). As the utilization of each of these three fuel sources are interconnected (10), these results suggest that changes in triglyceride metabolism may be a result of the initial changes of glucose and ketone metabolism. For example, *Txnip*$^{-/-}$ mice have a higher energy state in the skeletal muscle and heart, due to increased utilization of glucose; as a result, these events could signal to diminish LPL activity as it would be unnecessary to produce additional amounts of energy through the acquisition of LPL-derived fatty acids. In the second set of time-course experiments, LPL activity was shown to be greatly reduced in the heart and skeletal muscle only after 6-9 hrs of fasting (Figure 4.10B–D). Understanding the specific metabolic changes that occur in the heart and skeletal muscle at these time-points is necessary to provide further insight into the mechanism by which Txnip ablation causes an increase in fasting plasma triglyceride levels. In regards to time-course expression, it is interesting to note that in wild-type mice, *Txnip* expression reaches its maximal level within 3 hr of a fast (data not shown), which is prior to the time-point where changes are in plasma triglyceride levels are observed in *Txnip*$^{-/-}$ mice (Figure 4.10A).

Previous studies have demonstrated that impaired fatty acid oxidation usually leads to an up-regulation of LPL expression, which would provide an increase in fatty acid substrates for the use in the mitochondria (52). However, *Txnip*$^{-/-}$ mice exhibit impaired fatty acid oxidation as well as decreased LPL expression. Based upon the findings in the present studies, two possible contributions to decreased LPL expression come to mind. First, previous studies demonstrate that AMPK and PPARδ act synergistically to up-regulate LPL mRNA expression (53). Our studies demonstrated that *Txnip*$^{-/-}$ mice have diminished AMPK activation and may have decreased PPARδ transcriptional activity, due to the reduced availability of triglyceride-derived fatty acids. This hypothesis could account for the changes observed in the soleus and the heart; however, it can not explain the differences observed in the gastrocnemius. Alternatively, previous studies demonstrate that an increase in glycolytic fibers in muscle lead to diminished LPL mRNA expression (54, 55). The heart and skeletal muscle appear to have increased glycolysis in *Txnip*$^{-/-}$ mice, suggesting that increased glycolysis could be a predominant force in modulating LPL expression.

To specifically discern the role of Txnip in modulating LPL expression and activity requires further research. It would be interesting to speculate a role for thioredoxin in modulating the metabolic changes observed in *Txnip*$^{-/-}$ mice given that the only known function of Txnip is to bind to and inhibit thioredoxin, which has been shown to modulate the activities of many transcription factors (56). Within the context of the skeletal muscle and the heart, FOXO1 and PPARδ have been shown to promote the expression of LPL (57, 58); further studies examining the modulation of

FOXO1 and PPARδ transcriptional activities may be of interest. However, a role for thioredoxin in modulating FOXO1 and PPARδ has yet to be established. Therefore, it may be of interest to examine the transcriptional activities of additional transcription factors that can modulate LPL expression in other tissues (59), including AP-1, whose activity is controlled by thioredoxin (56).

In contrast to the aforementioned proposal, recent studies demonstrate that the ability of Txnip to inhibit glucose uptake is independent of its ability to bind to thioredoxin (48, 60). In addition, our studies demonstrate the alterations in glucose metabolism precede, and could possibly contribute to, the changes in plasma triglyceride levels of *Txnip*$^{-/-}$ mice (Figure 4.9). These findings cast doubt on the role of thioredoxin in modulating plasma triglyceride levels in *Txnip*$^{-/-}$ mice. Thus, it may be of interest to focus future studies on examining the role of Txnip as a member of the alpha-arrestins protein family (60, 61), in regards to modulating plasma triglyceride levels.

Acknowledgements

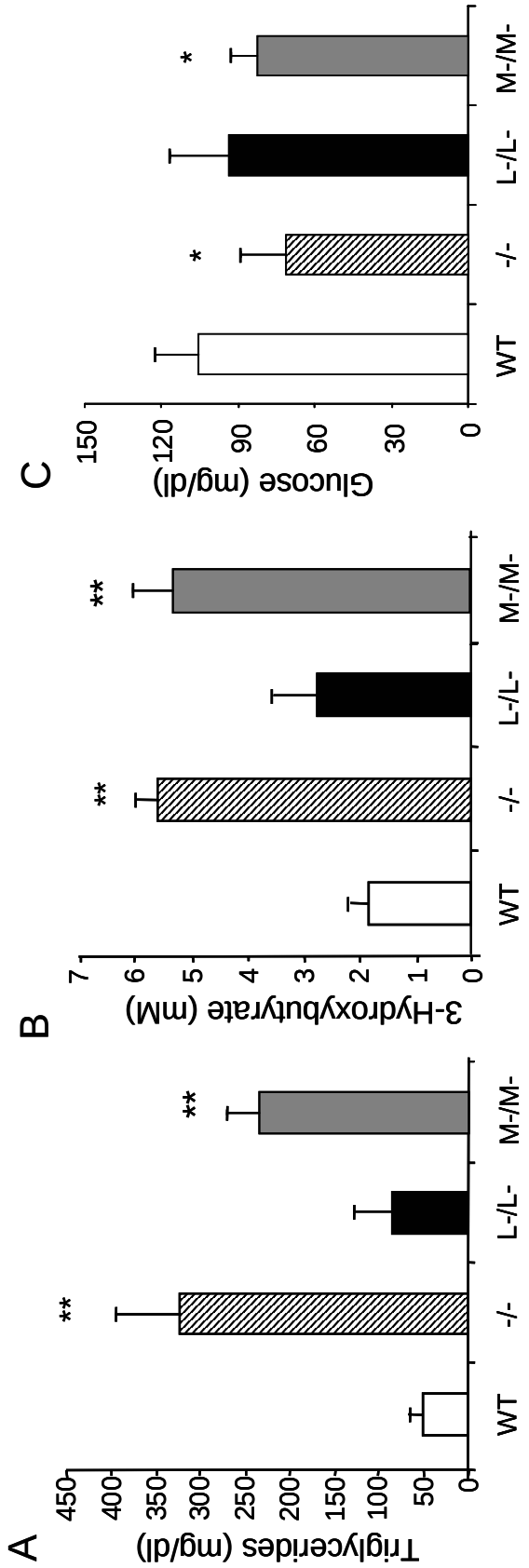
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Chapter Four, in part, is currently being prepared for submission for publication of the material. The dissertation author was the primary researcher and author of this material.

Table 4.1. Primers used for quantitative real-time PCR.

Gene	Forward 5' - 3'	Reverse 5' - 3'
ACOX1	CCAGACTTCCAACATGAGGAC	TGCACAGAGTTTTTAAACCACA
CD36	AGCTGCACCACATATCTACCA	TGAGGAATGGATCTTTGTAACC
LPL	TTATCCCAATGGAGGCACTTT	CACGTCTCCGAGTCCTCTCTCT
PPAR-α	TCTTCACGATGCTGTCCTCCT	GGAACTCGCCTGTGATAAAGC
PPAR-δ	ATCAGTGTGCACGTGTTCTACC	GTCACCTGGTCATTGAGGAAG
UCP-3	CTTCCTCCCTGAACTGAAGC	CCCAGGAACTTCACAACCGT
GAPDH	GTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTG

Figure 4.1. Txnip knockout mice have an impaired fasting response.
Following an overnight fast, plasma was obtained from wild-type (WT), *Txnip* knockout (-/-), liver-specific *Txnip* knockout (L-/L-), and skeletal muscle and heart-specific *Txnip* knockout (M-/M-) mice. Plasma (A) triglyceride, (B) 3-hydroxybutyrate, and (C) glucose levels were measured using commercially available enzyme assays. The mean $\pm$ S.D. (n = 4-8) are shown. * $P < 0.05$, ** $P < 0.01$.


A
Triglycerides (mg/dl)
0
50
100
150
200
250
300
350
400
450
**
**
WT
-/-
L-/L-
M-/M-
B
3-Hydroxybutyrate (mM)
0
1
2
3
4
5
6
7
**
**
WT
-/-
L-/L-
M-/M-
C
Glucose (mg/dl)
0
30
60
90
120
150
*
*
WT
-/-
L-/L-
M-/M-

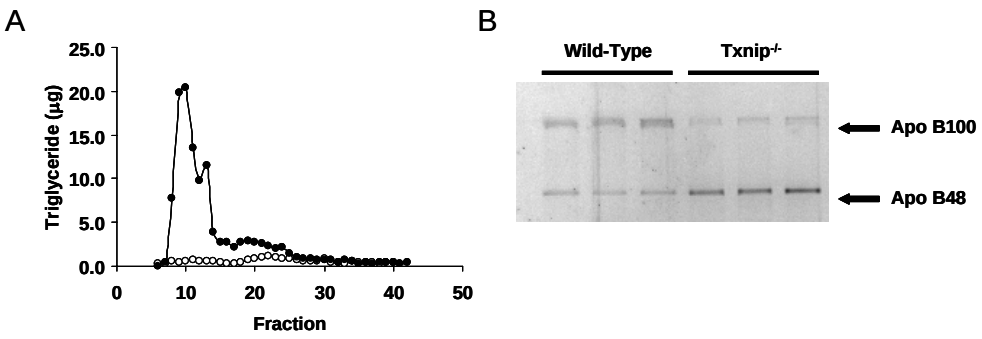


Figure 4.2. Fasting Txnip knockout mice display increased triglyceride content in the VLDL/chylomicron fraction

A) Triglyceride profile of lipoproteins fractionated by fast-protein liquid chromatography of pooled plasma from wild-type (open circles) and *Txnip*$^{-/-}$ mice (closed circles) (n = 4). B) Plasma apolipoprotein B protein levels were determined by Western blot; apo B100 and apo B48 bands are indicated.

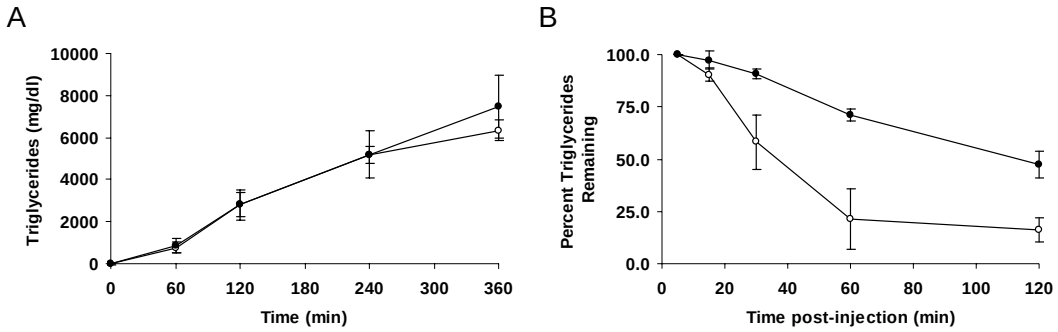


Figure 4.3. Txnip knockout mice display an impaired ability to clear plasma triglycerides.

A) Wild-type (open circles) and *Txnip*$^{-/-}$ mice (closed circles) were intraperitoneally injected with Poloxamer-407. Plasma samples were taken from mice at the indicated time-points and plasma triglyceride levels were measured. B) Wild-type (open circles) and *Txnip*$^{-/-}$ mice (closed circles) were injected intravenously with Intralipid® triglycerides. Plasma samples were taken from mice at the indicated time-points and plasma triglyceride levels were measured. The mean $\pm$ S.D. (n = 4-5) are shown.

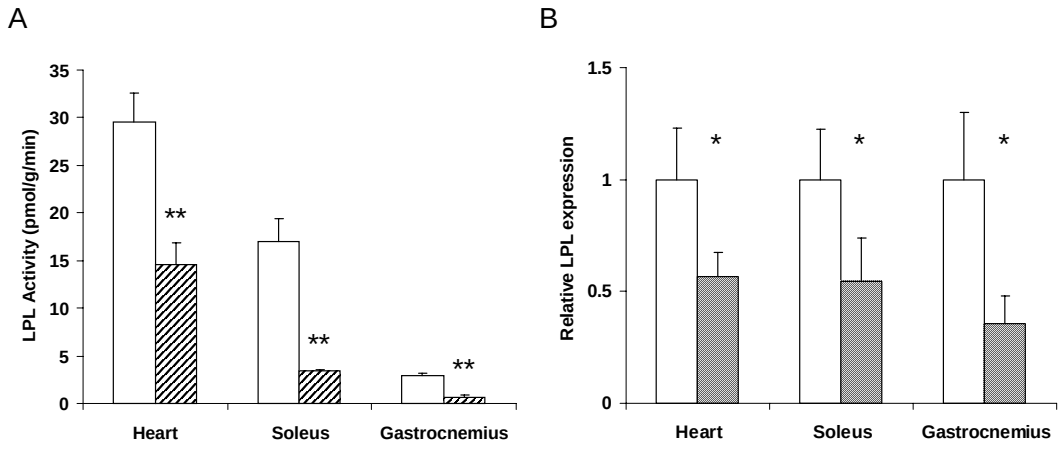


Figure 4.4. Txnip knockout mice have diminished LPL activity and LPL mRNA expression in the heart and skeletal muscle.
Following an overnight fast, the heart, gastrocnemius, and soleus muscle were removed from wild-type (open bars) and *Txnip*$^{-/-}$ mice (hatched bars). A) LPL activity was determined using a [^{3}H] triolein emulsion as the substrate. B) RNA was extracted from the isolated tissues, reverse-transcribed into cDNA and LPL mRNA expression levels were measured using quantitative real-time PCR. The mean $\pm$ S.D. (n = 5) are shown.* $P < 0.01$, ** $P < 0.001$

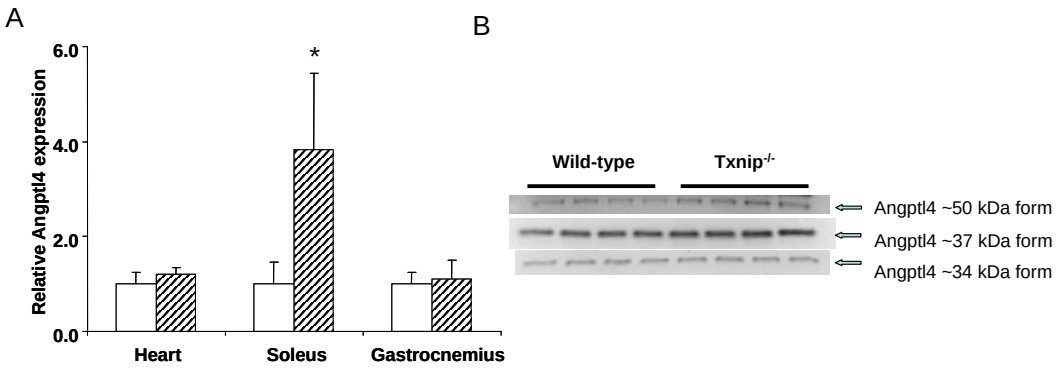
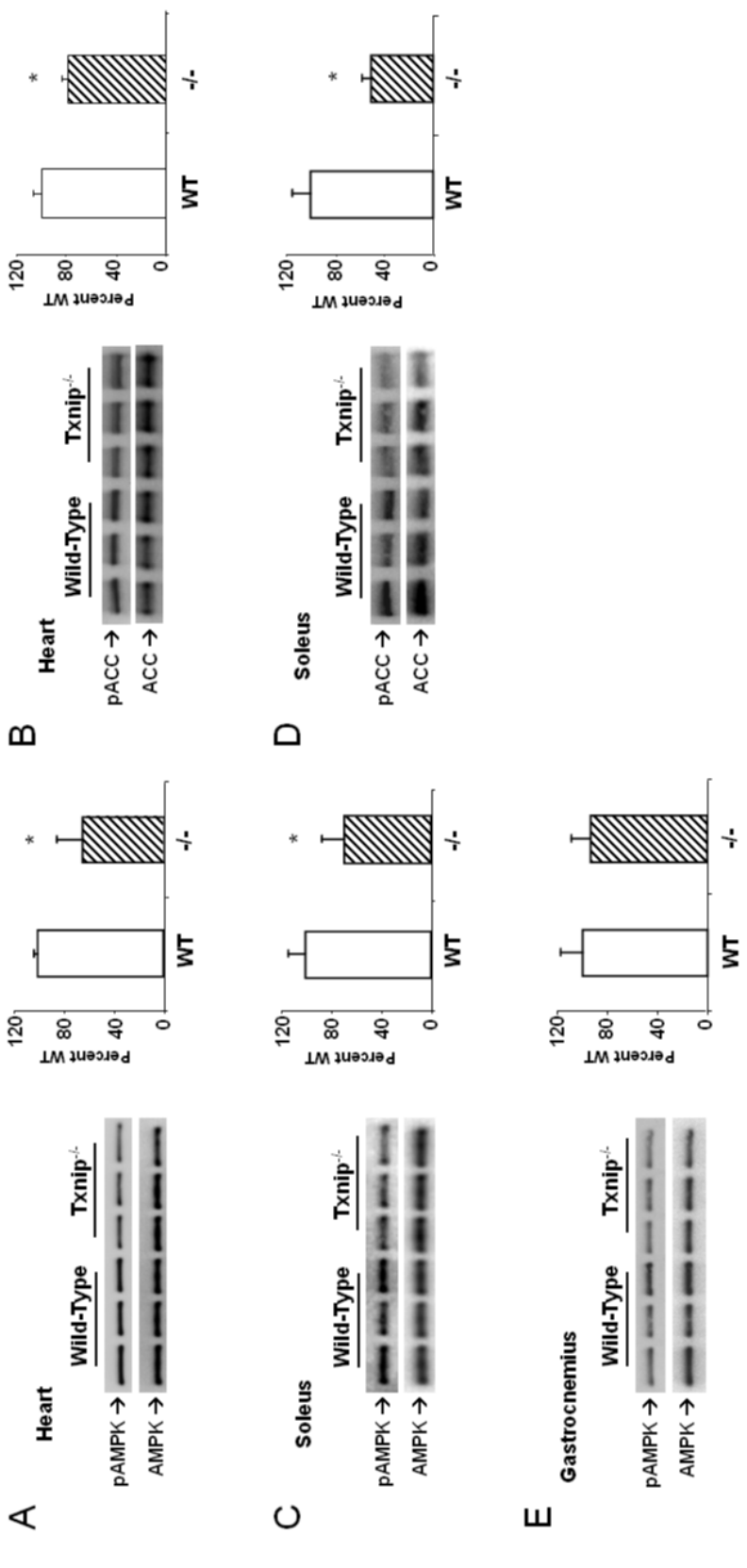


Figure 4.5. Diminished LPL activity in the heart and skeletal muscle of Txnip knockout mice is independent of plasma Angptl4.
Following an overnight fast, the heart, gastrocnemius, and soleus muscle were removed from wild-type (open bars) and *Txnip*$^{-/-}$ mice (hatched bars). A) RNA was extracted from isolated tissues, reverse-transcribed into cDNA and Angptl4 mRNA expression levels were measured using quantitative real-time PCR. The mean $\pm$ S.D. (n = 5) are shown.* $P < 0.05$. B) Plasma Angptl4 protein levels were determined by Western blot.

Figure 4.6. Txnip knockout mice display impaired AMPK activation in the heart and soleus, but not the gastrocnemius.
Following an overnight fast, the heart, gastrocnemius, and soleus muscle were removed from wild-type and *Txnip*$^{-/-}$ mice. Tissues were homogenized using RIPA buffer as described in the materials and methods and 20 μg was separated by SDS-PAGE. Blots were probed using (A, C, & E) phospho-AMPK and AMPK or (B & D) phospho-ACC and ACC antibodies. Quantitations of phosphorylated protein levels are depicted beside each blot.


A
Heart
Wild-Type
Txnip-/-
pAMPK →
AMPK →
Percent WT
120
80
40
0
WT
-/-
*
B
Heart
Wild-Type
Txnip-/-
pACC →
ACC →
Percent WT
120
80
40
0
WT
-/-
*
C
Soleus
Wild-Type
Txnip-/-
pAMPK →
AMPK →
Percent WT
120
80
40
0
WT
-/-
*
D
Soleus
Wild-Type
Txnip-/-
pACC →
ACC →
Percent WT
120
80
40
0
WT
-/-
*
E
Gastrocnemius
Wild-Type
Txnip-/-
pAMPK →
AMPK →
Percent WT
120
80
40
0
WT
-/-

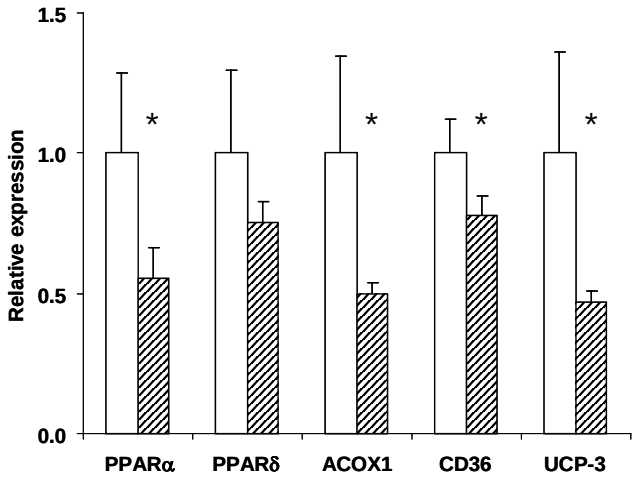


Figure 4.7. Txnip knockout mice have reduced expression of a subset of fatty acid metabolism genes in the heart.
Following an overnight fast, the heart was removed from wild-type (open bars) and *Txnip*$^{-/-}$ mice (hatched bars). RNA was extracted from isolated heart, reverse-transcribed into cDNA and expression levels of PPARα, PPARδ, ACOX1, CD36, and UCP-3 were measured using quantitative real-time PCR. The mean $\pm$ S.D. (n = 5) are shown.* $P < 0.05$.

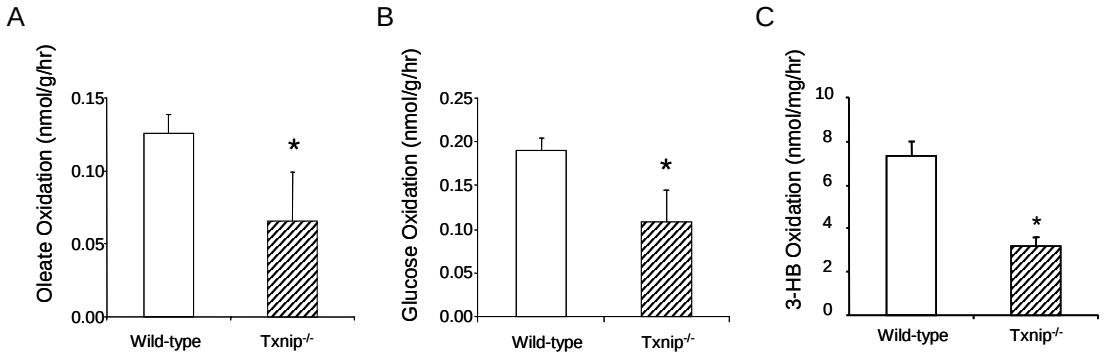


Figure 4.8. Txnip knockout mice have diminished mitochondrial oxidative capacity.

Following an overnight fast, solei were isolated from wild-type (open bars) and *Txnip*$^{-/-}$ mice (hatched bars). overnight fasting mice were incubated for 1 hr at 30^{o}C, with (A) [1-^{14}C]-oleate (0.5 µCi/ml), (B) [^{14}C]-glucose (0.5 µCi/ml), or (C) [1-^{14}C]-3-hydroxybutyrate (0.5 µCi/ml), as described in the materials and methods. The amount of $^{14}CO_2$ generated was measured using a scintillation counter and converted into appropriate units. The mean $\pm$ S.D. (n = 5) are shown.* $P < 0.05$.

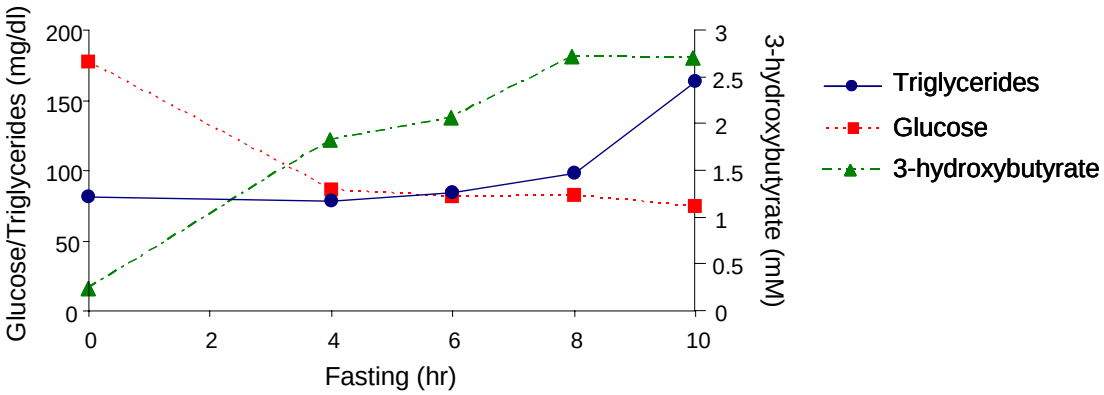
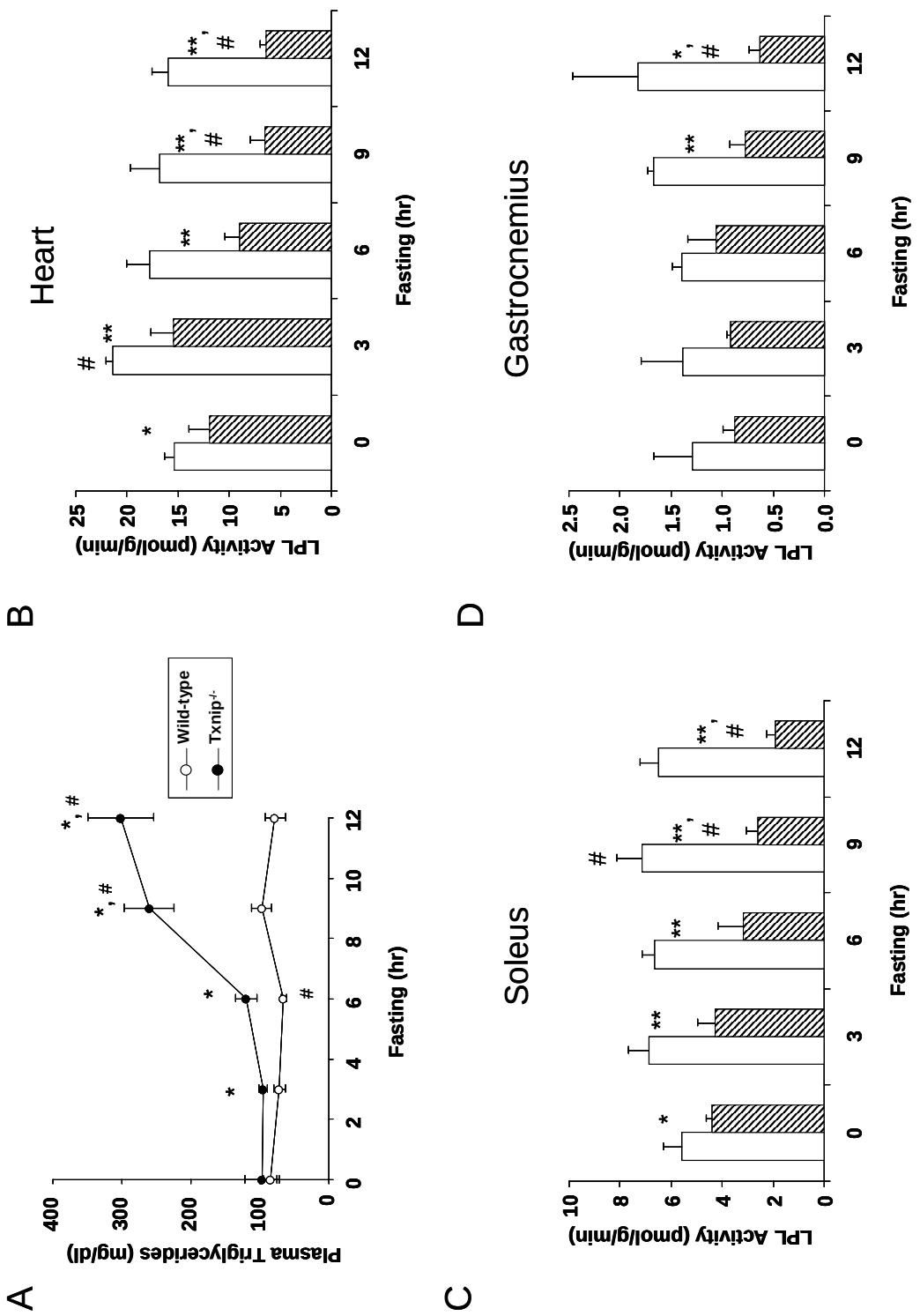


Figure 4.9. Changes in plasma glucose and ketone levels precede an increase in plasma triglyceride levels in Txnip knockout mice.
Txnip$^{-/-}$ mice were fasted prior to the dark cycle (4:00 PM) and bled prior to (0 hr time-point) and at various time-points following the fast. Plasma triglyceride, glucose, and ketone levels were measured as described in the materials and methods.

Figure 4.10. An increase in plasma triglyceride levels correlates with diminished LPL activity in the heart and skeletal muscle of Txnip knockout mice.
Wild-type and *Txnip*$^{-/-}$ mice were fasted at mid-dark (12:00 AM) and sacrificed at various time-points; one set of wild-type and *Txnip*$^{-/-}$ mice were sacrificed prior to a fast (0 time-point). A) Plasma triglyceride levels were measured at the various time-points using commercially available enzyme assays. B-D) LPL activity was determined using a [^{3}H] triolein emulsion as the substrate in the heart (B), soleus (C), and gastrocnemius (D) of wild-type and *Txnip*$^{-/-}$ mice. The mean $\pm$ S.D. (n = 4) are shown. * denotes a difference between wild-type and *Txnip*$^{-/-}$ mice ($P < 0.05$) and $^{\#}$ denotes a difference within a mouse strain at a particular time-point as compared to their respective 0 hr, baseline, time-point ($P < 0.05$).


A
Plasma Triglycerides (mg/dl)
Fasting (hr)
Wild-type
Txnip-/-
B
Heart
LPL Activity (pmol/g/min)
Fasting (hr)
C
Soleus
LPL Activity (pmol/g/min)
Fasting (hr)
D
Gastrocnemius
LPL Activity (pmol/g/min)
Fasting (hr)

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Chapter 5

Summary and Future Directions

Summary

The studies presented in this dissertation attempt to uncover various metabolic roles of CYP7A1, PON1, and Txnip. In Chapter 2, experiments were undertaken to determine if the expression of a CYP7A1 transgene was protective in LDL receptor deficient mice. Indeed, transgenic expression of CYP7A1 exerted many protective effects in LDL receptor-deficient mice, especially when these mice were fed a cholesterol-enriched diet; CYP7A1-transgenic, LDL receptor-deficient mice exhibited reduced plasma cholesterol levels, alterations in the expression of many metabolic genes in the liver and intestine, and reduced cholesterol absorption (Figure 5.1).

In Chapter 3, experiments were performed to determine the mechanism by which bile acid feeding reduces the expression of CYP7A1 and PON1. Using various mouse models, bile acids were shown to diminish the expression of CYP7A1 and PON1 by a similar mechanism involving FXR and the induction of ileal FGF15 (Figure 5.2). In the course of these studies, plasma PON1 levels were found to correlate well with plasma HDL cholesterol levels. Thus, additional experiments were performed to develop an experimental method to increase plasma HDL cholesterol levels and plasma PON1 protein levels in HDL-deficient ABCA1 knockout mice.

In the final set of experiments, Chapter 4, the role of Txnip in modulating the fasting response, in particular maintaining plasma triglyceride levels, was examined. Txnip knockout (*Txnip*$^{-/-}$) mice exhibited an impaired ability to clear plasma triglycerides, which correlated with a reduction of LPL mRNA expression and LPL activity in the skeletal muscle and heart. The role of Angptl4 and AMPK in modulating LPL activity were examined, however, conclusive evidence for a definitive role of either of these proteins was not established. Further studies demonstrated that *Txnip*$^{-/-}$ mice have impaired mitochondrial oxidation, but were able to compensate for their energy needs by increasing glycolysis. These factors, in of themselves, could also contribute to the reduced LPL activity observed in the *Txnip*$^{-/-}$ mice (Figure 5.3).

Future Directions

As the Txnip project was the final set of experiments performed for my thesis, the Txnip project will be the focus for these future directions. Along with LPL activity in the heart and skeletal muscle, LPL activity in adipose plays a significant role in modulating plasma triglyceride levels (1). LPL activity is normally up-regulated in adipose during the post-prandial period promoting the storage of VLDL-derived triglycerides; in contrast, during the fasting state, LPL activity is decreased (1). Interestingly, concurrent with the diminished LPL activity observed in the heart and skeletal muscle of *Txnip*$^{-/-}$ mice in the fasting state, a decrease in LPL activity was also noted in the epididymal fat pad (Figure 5.4). Further time-course experiments

revealed that LPL activity in epididymal fat of *Txnip*$^{-/-}$ mice in the fed state (mid-dark period) is highly variable, but similar, to that observed in wild-type mice; only after 6 hr of fasting was there a significant reduction in LPL activity in the epididymal fat of *Txnip*$^{-/-}$ mice (Figure 5.5). The time-points at which changes in LPL activity in the epididymal fat pad occurred are similar to the time-points drastic changes in LPL activity were observed in the skeletal muscles and hearts of *Txnip*$^{-/-}$ mice (Figure 4.10).

A reduction in adipose LPL activity normally correlates with decreased adipose mass. However, previous findings in our lab (in collaboration with Jason Kim, Pennsylvania State University), using proton magnetic resonance spectroscopy, demonstrated that *Txnip*$^{-/-}$ mice had an increase in fat mass, which correlated with the larger epididymal fat pads observed in these mice (2). Further studies by our lab demonstrated that *Txnip*$^{-/-}$ mice contain larger adipocytes in the epididymal fat (data not shown). Based upon these preliminary findings, it would be interesting to examine the role of Txnip in adipose and to determine if the metabolic alterations in the adipose of *Txnip*$^{-/-}$ mice are due to intrinsic abnormalities controlling fat accumulation or due to signaling events (cytokines, secreted proteins, etc.) from distal tissues. One possibility to further examine this topic is to breed the *Txnip*$^{fl/fl}$ mice (2) with aP2-cre mice (3) to generate adipose tissue-specific *Txnip* knockout mice.

In addition to changes in fat metabolism, there are many avenues of research which are worth exploring regarding energy metabolism. Foremost, one intriguing finding by our studies is that *Txnip*$^{-/-}$ mice exhibit increased glycolysis (data not

shown) and lower mitochondrial oxidation of fuels (Figure 4.8). Therefore, it would be of interest to determine whether the impairment of the mitochondria leads to the up-regulation of glycolysis, or whether the opposite occurs, in *Txnip*$^{-/-}$ mice.

In support of the latter, recent studies by our lab demonstrated that the E1α subunit of the mitochondrial pyruvate dehydrogenase complex (PDC) was in a more inhibited (phosphorylated) state in the solei isolated from *Txnip*$^{-/-}$ mice (Figure 5.6); the phosphorylation of the pyruvate dehydrogenase E1α subunit renders the PDC incapable of facilitating the entry and oxidation of pyruvate by the mitochondria (4). Furthermore, the heart and skeletal muscle of *Txnip*$^{-/-}$ mice have a higher energy state than wild-type mice, as characterized by an increased ATP:AMP ratio; a higher energy state is known to activate pyruvate dehydrogenase kinases (PDKs), which, in turn, phosphorylate and inhibit pyruvate dehydrogenase (4). These results suggest that an up-regulation of glycolysis precedes the impairment in the mitochondria.

Further studies are necessary to examine this hypothesis. Specifically, it would be interesting to inhibit glycolysis in *Txnip*$^{-/-}$ mice and determine whether the mitochondrial oxidative capacity is similar to wild-type mice. In addition, thorough characterization of the mitochondria, examining the quantity and quality, would help in determining whether *Txnip*$^{-/-}$ mice have an intrinsic defect in the mitochondria.

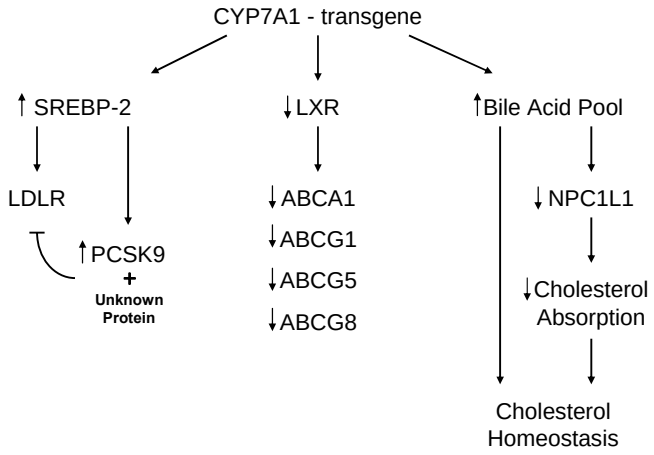


Figure 5.1. Effects of expressing a CYP7A1-transgene in wild-type and LDL receptor-deficient mice.
Transgenic expression of CYP7A1 in the liver causes a robust increase in the expression of SREBP-2 and its target gene, PCSK9. Further studies demonstrated that increased PCSK9 protein levels alone are not sufficient to degrade the LDLR, suggesting that another unknown protein works in concert with PCSK9 to degrade the LDL receptor. In LDL receptor deficient mice, transgenic expression of CYP7A1 leads to reduced hepatic expression of LXR and its target genes ABCA1, ABCG1, ABCG5, and ABCG8. Further, expression of the CYP7A1 transgene increased the bile-acid pool and decreased the jejunal expression of NPC1L1, which contributes to the reduction in cholesterol absorption observed in these mice. Combined, these processes help maintain cholesterol homeostasis in the CYP7A1-transgenic, LDL receptor-deficient mice.

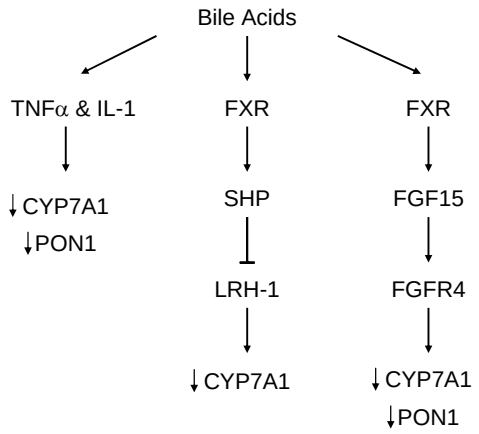


Figure 5.2. Bile acids diminish hepatic expression of CYP7A1 and PON1 through multiple pathways.
Previous studies demonstrated that bile acid feeding increases the expression of inflammatory cytokines, which lead to diminished CYP7A1 and PON1 expression. Additional studies demonstrated that bile acids provide a ligand for FXR, which leads to the up-regulation of the FXR-target gene, SHP. The SHP co-repressor prevents the binding of LRH-1 to the BARE I site on the CYP7A1 promoter thereby decreasing the expression of CYP7A1. The studies presented in Chapter 3 identify a novel pathway for decreasing CYP7A1 and PON1 expression. Bile acids bind to FXR leading to an up-regulation of ileal FGF15, which is eventually carried to the liver where it binds to FGFR4 and diminishes the expression of CYP7A1 and PON1.

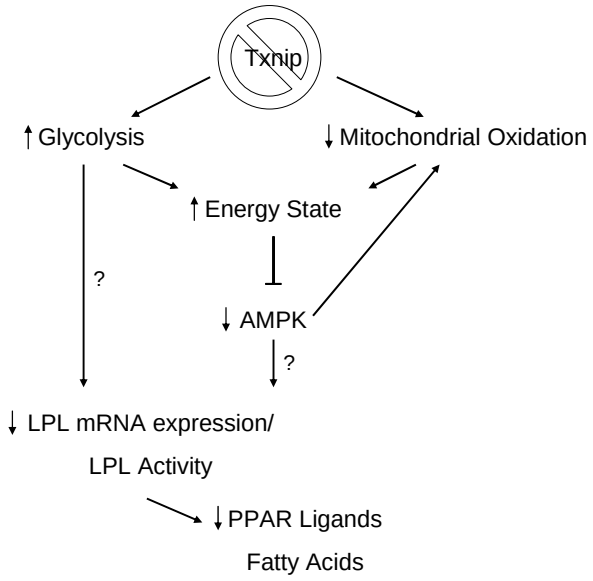


Figure 5.3. Ablation of Txnip alters energy usage and decreases LPL activity in the skeletal muscle and heart.
Txnip exerts its metabolic effects primarily through the skeletal muscle and heart. *Txnip*$^{-/-}$ mice display diminished oxidative capacity; however, *Txnip*$^{-/-}$ mice have increased glycolysis, which actually results in a higher energy state. A high energy state leads to diminished phosphorylation/activation of AMPK, which could contribute to impaired mitochondrial biogenesis. Increased glycolysis and/or diminished activation of AMPK could contribute to the reduced LPL mRNA expression and LPL activity observed in the skeletal muscle and heart of *Txnip*$^{-/-}$ mice. Reduced LPL activity results in a decrease in the availability of fatty acids (PPAR ligands), which correlates with a reduction in a subset of PPAR target genes.

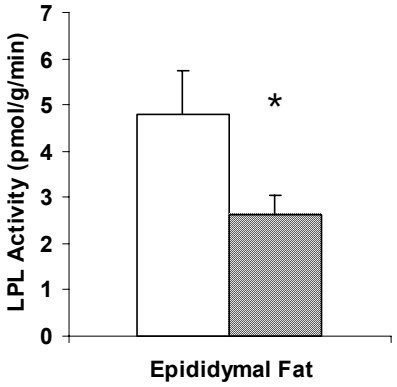


Figure 5.4. Txnip knockout mice have diminished LPL activity in the epididymal fat pad.
Following an overnight fast, the epididymal fat pad was removed from wild-type (open bar) and *Txnip*$^{-/-}$ mice (hatched bar). LPL activity was determined using a [^{3}H] triolein emulsion as the substrate. The mean $\pm$ S.D. (n = 5) are shown.* $P < 0.05$.

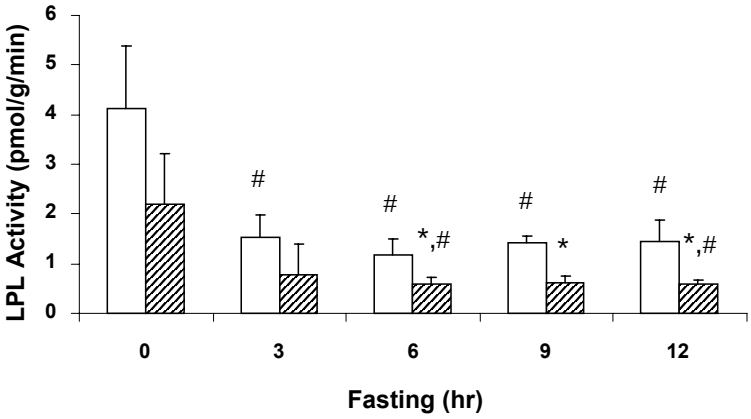


Figure 5.5. Txnip knockout mice display diminished LPL activity in the epididymal fat pad following 6 hr of fasting.
Wild-type (open bars) and *Txnip*$^{-/-}$ mice (hatched bars) were fasted at mid-dark (12:00 AM) and sacrificed at various time-points; one set of wild-type and *Txnip*$^{-/-}$ mice were sacrificed prior to a fast (0 time-point). LPL activity was determined using a [^{3}H] triolein emulsion as the substrate. The mean $\pm$ S.D. (n = 4) are shown. * denotes a difference between wild-type and *Txnip*$^{-/-}$ mice ($P < 0.05$) and $^{\#}$ denotes a difference within a mouse strain at a particular time-point as compared to their respective 0 hr, baseline, time-point ($P < 0.05$).

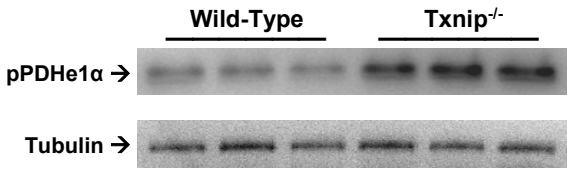


Figure 5.6. Txnip knockout mice display increased phosphorylation of pyruvate dehydrogenase E1α subunit in the soleus.
Following an overnight fast, solei were removed from wild-type and *Txnip*$^{-/-}$ mice. Solei were homogenized using RIPA buffer and separated by SDS-PAGE. Blots were probed using phospho-PDHe1α and tubulin antibodies.

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