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ABSTRACT OF DISSERTATION

Nadezhda Steliyanova Sabeva

The GRADUATE SCHOOL

UNIVERSITY OF KENTUCKY

2011

REGULATION OF ABCG5 AND ABCG8 STEROL TRANSPORTERS IN BILIARY CHOLESTEROL ELIMINATION, REVERSE CHOLESTEROL TRANSPORT AND DYSLIPIDEMIA

ABSTRACT OF DISSERTATION

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

By

Nadezhda Steliyanova Sabeva

Lexington, Kentucky

Director: Dr. Gregory Graf, Associate Professor, Pharmaceutical Sciences

Lexington, Kentucky

2011

ABSTRACT OF DISSERTATION

REGULATION OF ABCG5 AND ABCG8 STEROL TRANSPORTERS IN BILIARY CHOLESTEROL ELIMINATION, REVERSE CHOLESTEROL TRANSPORT AND DYSLIPIDEMIA

ATP-binding cassette transporters ABCA1 and ABCG1 initiate reverse cholesterol transport generating HDL particles, whereas ABCG5/G8 promote biliary cholesterol secretion thereby facilitating the last step of reverse cholesterol transport. Mutations in the leptin axis result in obesity and dyslipidemia in ob/ob and db/db mice. These mice have defective HDL clearance, increased plasma cholesterol and decreased biliary cholesterol elimination. My studies demonstrate that ABCG5/G8 protein is low in these animals and can be restored with caloric restriction or leptin replacement. To directly test whether ABCG5/G8 alone is able to correct reverse cholesterol transport defect, liver specific ABCG5/G8 expression was achieved in db/db mice by administration of adenoviral ABCG5 and ABCG8. Restoration of biliary cholesterol is able partially to correct dyslipidemia in obese mice, but only in the presence of ezetimibe, an inhibitor of cholesterol absorption.

ABCG5/G8 is the body's primary defense against toxic effects of plant sterols. Plant sterols are used as cholesterol lowering food supplements. However, increased plasma plant sterol concentrations are associated with vascular lesions in dyslipidemic patients and animals. My *in vitro* studies demonstrate that individual plant sterol alter ABCA1 and ABCG1 abundance, cholesterol efflux and inflammatory cytokine secretion in macrophage foam cells at levels found in humans that consume plant sterol supplements.

KEY WORDS: Reverse cholesterol transport, Dyslipidemia, Leptin, ABC-transporters, Macrophage

Nadezhda Sabeva

June 17, 2011

REGULATION OF ABCG5 AND ABCG8 STEROL TRANSPORTERS IN BILIARY CHOLESTEROL ELIMINATION, REVERSE CHOLESTEROL TRANSPORT AND DYSLIPIDEMIA

Ву

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> June 17, 2011 Date

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DATE

DISSERTATION

Nadezhda Steliyanova Sabeva

The Graduate School

University of Kentucky

2011

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Chapter 1: GENERAL INTRODUCTION

Sterols play indispensable roles in eukaryotic organisms. As a component of plasma and organelle membranes and as a precursor of essential molecules like steroid hormones, vitamin D and bile acids, cholesterol is the predominant sterol in mammals. Mammalian cells produce their own cholesterol and receive cholesterol by uptake from lipoproteins. Cells also continuously lose cholesterol to the outside circulation. Defects in synthesis, influx and efflux of cholesterol have devastating consequences for the individual. Genetic diseases such as desmosterolosis, Smith-Lemli-Opitz syndrome, Niemann-Pick C1 disease, Familial hypercholesterolemia, Tangier disease and sitosterolemia are examples of disorders caused by mutations in genes involved in cholesterol biogenesis, intracellular trafficking, cellular uptake and efflux. Conversely, excess cholesterol is the initial factor for the development of atherosclerosis and cardiovascular diseases (CVD). Hence, cellular and plasma cholesterol levels require strict regulation.

Cholesterol structure, synthesis and metabolism

Cholesterol is an isoprenoid which contains 4 fused hydrocarbon rings and a hydrocarbon side chain at C17 (Figure 1.1.). Cholesterol is a highly hydrophobic molecule with limited polarity due to the C3 hydroxyl group that determines cholesterol positioning within the lipid bilayer. The hydrophobic cholesterol property not only influences cell membrane fluidity, but also requires specific transport mechanisms in order to extrude cholesterol to the aqueous intra- and extracellular environment of the body [1].

Cholesterol synthesis is initiated by the formation of 3-hydroxy-3 methylglutaryl CoA (HMG-CoA) from acetyl CoA and acetoacetyl CoA and is mediated by HMG-CoA synthase (HMGS). HMG-CoA is reduced to mevalonate via HMG-CoA reductase (HMGR), the

enzyme responsible for the irreversible and rate limiting reaction in cholesterol synthesis [2]. Subsequently, mevalonate is converted to isoprenyl pyrophosphate, followed by a cascade of condensation reactions deriving the 30-carbon linear molecule of squalene that is finally cyclized to the steroid ring structure of lanosterol. The precursor of cholesterol, lanosterol, undergoes an additional 19 reaction steps in order to yield cholesterol.





Most of the cholesterol present in the body resides in cell membranes. The amphipathic molecule of cholesterol is placed in parallel to the fatty acid chains of the phospholipids and intercalates its polar hydroxyl group close to the phospholipid head group [1]. Due to its rigid structure, cholesterol increases lipid order and decreases fluidity of the cell membrane. Thus, membrane properties like permeability and stability, lipid raft formation and signal transduction are strongly influenced by cholesterol.

Bile acid synthesis and metabolism

Approximately 90% of the metabolized cholesterol in the body is converted to bile acids (~500 mg/day) [3]. Bile acid (BA) synthesis takes place in the liver and involves 17

different enzymatic reactions. The immediate products of bile acid synthesis are termed primary Bas. In humans these are predominantly cholic acid (CA) and chenodeoxycholic acid (CDCA) (Figure 1.2.), whereas in mice CA and muricholic acid are most abundant. Secreted from the bile into the intestine, primary BAs are converted by the anaerobic gut bacteria to secondary BAs – deoxycholic (DCA) and lithocholic (LCA) [4]. In contrast to hydrophobic cholesterol, the more hydrophilic BAs act as biological detergents and contribute to the absorption of dietary lipids and fat-soluble vitamins in small intestine. Individuals harboring mutations that result in BA deficiency suffer from lipid malabsorption and fat soluble vitamin deficiency [3].



FIGURE 1.2. Primary bile acids synthesized in murine liver. Cholic acid is the predominant product of the classical pathway, and muricholic acid is the prevalent derivate from the alternative pathway.

The production of BAs from cholesterol involves more than one route. In the classical or neutral pathway, cholesterol 7α -hydroxylase (Cyp7a1) is the rate controlling enzyme converting cholesterol to 7α -hydroxycholesterol (7α -OH-C) [5-7]. The subsequent critical step of this route is the reaction mediated by 12α -hydroxylase (Cyp8b1). Since this enzyme is responsible for cholate production, the activity of Cyp8b1 determines the ratio between the cholate and muricholate [3].

The alternative pathway is associated with the conversion of cholesterol to oxysterols. In the liver, sterol 27 hydroxylase (Cyp27a1) accounts for the synthesis of 27 hydroxycholesterol (27-OH-C) [5]. Studies in Cyp7a1 deficient mice revealed that approximately 25% of the BA pool originates from the alternative route [8]. By-products of this step are other oxysterols, 24- and 25-hydroxysterols (24-OH-C, 25-OH-C), which together with 27-OH-C are predominantly metabolized to muricholate.

BA amphipathicity is increased and solubility is enhanced via conjugation to amino acids, *e.g.* taurine or glycine [9]. In the gut, surrounded by the intestinal flora, primary BAs undergo structural modification where CA is metabolized to LCA and CDCA is converted to DCA (secondary BAs) [4]. Approximately 95% of the BAs are recovered and enter the enterohepatic circulation, and the lost 5% are restored by the new synthesis in the liver.

Plant sterols structure and metabolism in mammals

Structurally similar to cholesterol, phytosterols (PS) are membrane constituents in plants and precursors for plant hormones and other secondary metabolites, i.e. sterol derivatives that play protective roles against pathogen and insect attack [10-12]. Whereas in mammals cholesterol is the prevalent sterol, in plants PS are always present in a mixture. The most prevalent PS are sitosterol and campesterol, which are identical to cholesterol in ring structure with a double bond between C5 and C6, but differ in their side chain by a methyl or ethyl group attached on the side chain at C24, respectively [13]. As demonstrated on Figure 1.3., brassicasterol and stigmasterol complicate their structure with a double bond at C22. Plant stanols vary from the corresponding sterols by the absence of a double bond between C5 and C6.

PS are naturally present in the diet. In the typical Western type diet, plant sterols are almost equal to that of cholesterol (400mg/day). However, their poor absorption in the intestine (0.4-3.5%) compared to cholesterol (35-70%) ensures low concentrations of PS

in the plasma (0.2-1.0 mg/dL) [14]. PS have been recognized as dietary components with low density lipoprotein cholesterol (LDL-cholesterol) lowering properties in various populations and patient groups as early as 1951 [15, 16]. In the intestinal lumen PS compete with cholesterol for micellar solubilization and thereby interfere with cholesterol absorption and reduce plasma cholesterol levels [17].



FIGURE 1.3. Chemical structure of prevalent plant sterols compared to cholesterol. Plant sterols differ from cholesterol on the six carbon side chain by the group attached at C24 position and the double bond at C22.

Various studies in humans and animal models have shown that high levels of PS supplementation reduce plaque formation [18-22]. The reduction of atherosclerosis has been correlated with a decrease in plasma cholesterol levels in response to PS supplementation. However patients with sitosterolemia, a rare autosomal recessive disorder characterized by defective ATP-binding cassette (ATP) transporter ABCG5 and/or ABCG8 and ~50 fold increased serum PS concentrations, often develop premature coronary heart disease (CHD) and accelerated atherothrombosis [23, 24]. Moreover, recent studies reported increased risk for CHD in non-sitosterolemic individuals with high plasma PS levels [25, 26]. In fact, it has been confirmed that different sterols provoke various physiological effects [27-30]. Chapter 4 discusses different actions of PS on macrophage functionality.

Excess plant and mammal sterol in peripheral tissues, including macrophage foam cells in the vascular lesions, is moved through the plasma compartment to the liver. Hepatic cholesterol makes its way out of the body by conversion to primary BAs or is secreted as a free form. Each of these steps in cholesterol homeostasis is mediated by ABC transporters.

ATP-BINDING CASSETTE (ABC) TRANSPORTERS

The ATP-binding cassette (ABC) transporters are a large family of proteins that hydrolyze adenosine triphosphate (ATP) and use this energy to transfer a large variety of substrates (metal ions, peptides, sugar, cholesterol and plant sterols, bile acids and phospholipids) across cellular membranes in bacterial and eukaryotic cells. Most mammalian ABC transporters harbor an ATP-binding region of approximately 100 amino acids where the Walker A and Walker B motifs are connected by a dodecapeptide sequence recognized as Linker region (C region) [31]. The Walker motifs are highly conserved and recognized as nucleotide binding domain (NBD). ABC transporters are associated with transmembrane (TM) domains containing six TM helices that are highly diverse among family members. Transporters that contain one ATP-binding site and one TM spanning domain are called half-transporters. However, functional transporters are composed of two NBDs and two TMs. Therefore, the ABC half-transporters act as homodimers (assembly with identical half-transporters) or heterodimers (assembly with different half-transporters) [32].

The human genome contains 48 ABC transporters, which are based on structure and homology. Classified into 7 subfamilies (A-G), in the following sections, transporters that have been implicated in the studies described in this thesis will be discussed.

The ABCA-family

ABCA1 (A1) is the most studied protein of the all 12 members of the ABCA-family. It is ubiquitously expressed, but is most prominent in enterocytes, hepatocytes and macrophages [33]. Mutations in ABCA1 cause the recessive disorder Tangier disease (TD) associated with severe high density lipoprotein (HDL) deficiency and cholesteryl ester accumulation in many tissues including tonsils, lymph nodes, liver, spleen and intestine [34, 35]. Hepatic and intestinal ABCA1 facilitates the transfer of phospholipids and cholesterol to lipid poor nascent HDL (pre-β-HDL) and it is essential for the formation of "mature" or "spherical" HDL. This was best demonstrated in liver and intestinal-specific ABCA1 deficient mouse models, where a synthetic LXR agonist elevated HDL cholesterol levels regardless of the deletion [36, 37]. ABCA1 mediates lipid efflux to apoprotein A-I (apoA-I) in a two-step mechanism in which there is initial formation of an apoA-I complex, followed by lipidation of the bound apoprotein and dissociation of the resultant apoA-I /lipid complex [38, 39]. Hepatic and intestinal levels of apoA-I mRNA are normal in Tangier patients and ABCA1 deficient mouse models [40, 41]. Studies demonstrate that exceptionally rapid catabolism but not defective synthesis of the apoprotein could explain the extremely low levels of HDL and apoA-I.

Although liver and intestinal ABCA1 are responsible for the main production of HDL in the body, ABCA1 is critical for the efflux of excess cholesterol from macrophages and prevents the formation of foam cells, fatty streaks, and atherosclerotic plaques [42-44]. Hence, in Chapter 4 we evaluate ABCA1 expression, protein stability and function in mouse peritoneal and human macrophages after treatment with cholesterol lowering plant sterols.

The ABCB-family

The ABCB-family is also known as the multi-drug resistance/P-glycoprotein family. Two members of the ABCB-family, ABCB4 (B4) (Mdr2/MDR3) and ABCB11 (B11) (BSEP), are critical for lipid metabolism. They are predominantly expressed on the apical surface of hepatocytes and participate in bile formation.

ABCB4 mRNA is found in spleen, adrenal gland, heart and muscle but its predominant role is in the liver [45]. ABCB4 P-glycoprotein is involved in the secretion of phospholipids from the canalicular membrane of the hepatocytes into the bile. The physiological role of the phospholipids is not only to solubilize biliary cholesterol in the gallbladder but also to protect canalicular structures against the detergent action of bile salts. The Mdr2^{-/-} mice which were used to study the functionality of the transporter, have normal secretion of BAs but severely damaged and inflamed bile duct [46, 47]. Patients with mutation in ABCB4 develop progressive familial intrahepatic cholestasis (PFIC) type 3 [48]. Moreover, the Mdr2^{-/-} mouse model revealed the coupled secretion of phospholipids and cholesterol into the bile [49, 50].

ABCB11, known as bile salt export pump (BSEP), is expressed on the apical surface of hepatocytes and transports BAs across the canalicular membrane [51, 52]. BA secretion is the main driving force for the secretion of phospholipids and cholesterol into the bile [53]. As the predominant component in bile, BAs form BA micelles, which are suitable acceptors for cholesterol secreted into the bile [54]. In humans disruption of ABCB11 causes PFIC type 2 [55]. ABCB11 is a FXR/RXR target gene positively regulated by rising concentrations of bile salts in the liver, a protective mechanism against the toxic accumulation of bile salts [56, 57]. Gallbladder bile contains mixed micelles, which act as biological detergent in the lumen of the small intestine facilitating dietary cholesterol and fatty acid absorption in the gut. This is also the route used by cholesterol and bile acids to make its way out of the body.

The ABCG-family

Members of the ABCG-family are half-transporters that either for a heterodimer with each other like ABCG5 and ABCG8, or form homodimers like ABCG1, ABCG2, and ABCG4 in order to become functionally active.

ABCG1 is predominantly expressed in macrophages where it facilitates the efflux of excess cholesterol [58, 59]. As a liver X-receptor (LXR) target gene, ABCG1 mRNA and protein levels are upregulated upon cholesterol loading [58]. Conversely, cholesterol depletion and statin treatment downregulate expression of this transporter [60].

Various studies demonstrate that ABCG1 stimulates cholesterol export to mature HDL, but not to lipid free apoA-I (like ABCA1) [61, 62]. Whereas, ABCA1 is responsible for the lipidation of lipid-poor apoA-I, ABCG1 participates in the lipid transfer to mature HDL [63, 64]. Deletion of ABCG1 leads to lipid accumulation in certain tissue macrophages, highlighting the important role of ABCG1 in cholesterol efflux [65]. However, independent studies have demonstrated that Abcg1^{-/-} mice, in LDLR^{-/-} background, have either decreased or increased atherosclerotic plaque formation [66-68]. In Chapter 4 we evaluate phytosterol effect on ABCG1 expression, protein stability and function in mouse peritoneal and human macrophages.

ABCG5 and ABCG8 are expressed predominantly in liver, intestine and gallbladder [69, 70]. Located at chromosome 2p21, Abcg5 and Abcg8 genes are situated in a head-tohead orientation. In humans, their bidirectional common promoter region contains 374 bp and assures their coordinate gene regulation [69, 71]. Mutations in either ABCG5 or ABCG8 result in sitosterolemia, an autosomal recessively inherited lipid metabolic disorder associated with increased deposition of PS and cholesterol in the coronary artery wall and in the skin as xanthomas [69, 70, 72, 73]. ABCG5 and ABCG8 are glycoproteins synthesized in the endoplasmic reticulum (ER) [74]. The lectin chaperone calnexin (CNX) associates with both half-transporters and mediates their proper protein folding [75]. Co-expression of both ABCG5 and ABCG8 in cultured cells or in the liver of ABCG5/G8 deficient mice results in ABCG5 and ABCG8 heterodimerization (ABCG5/G8), which is required for trafficking through the Golgi apparatus (GA) to the apical surface of hepatocytes or enterocytes [74, 76]. In intestine, ABCG5/G8 opposes the absorption of sterols. Free sterols are pumped from the intestinal epithelium into the gut lumen by ABCG5/G8, allowing predominant incorporation of cholesterol esters into chylomicrons (CMs) [69]. However, the selective elimination of PS by ABCG5/G8 is determined from acyl-CoA:cholesterol acyltransferase 2 (ACAT2). ACAT2, the main enterocyte cholesterolesterifying enzyme, has a lower affinity for PS than cholesterol [77]. In liver, ABCG5/G8 mediates the excretion of free cholesterol into the bile. Hence, mice deficient in ABCG5,

ABCG8, or both transporters have an increase in dietary sterol absorption and a reduction in biliary cholesterol secretion [78-80]. Conversely, expression of a human transgene in wild type mice results in a 6–8 fold increase in biliary cholesterol concentration and supersaturation of bile [81].

Activation of LXR α by cholesterol feeding or administration of LXR-agonists increases ABCG5 and ABCG8 mRNA as well as biliary cholesterol concentrations [82]. In addition, these effects are absent in mice lacking LXR α/β , suggesting that LXR is the principal regulator of ABCG5 and ABCG8 expression in response to dietary cholesterol [83]. Moreover, liver receptor homolog-1 (LRH-1), hepatocyte nuclear factor 4 α (HNF4 α), GATA, FOXO1 and FXR transcription factors control ABCG5 and ABCG8 transcriptional regulation [84-87]. Although the main steps involved in Abcg5 and Abcg8 nuclear regulation have been elucidated, little is known about ABCG5/G8 posttranscriptional control.

MOLECULAR REGULATION OF CHOLESTEROL METABOLISM BY NUCLEAR RECEPTORS

Nuclear hormone receptors are transcription factors that regulate diverse physiological processes such as reproduction, development and whole body metabolism [88]. These receptors have a conserved DNA-binding domain (DBD), which recognizes corresponding DNA core sequences, called hormone response elements (HRE) in the gene promoter. The C-terminus of the receptor harbors the ligand binding domain (LBD) and participates in dimerization. The N-terminus contains a transcriptional activation function (AF-1) domain and mediates the association with co-repressors and co-activators [89]. Generally, nuclear receptors are inactive when bound to co-repressors [89]. Following ligand binding, they undergo a conformational change, dissociate from the co-repressor, associate with a co-activator, dimerize with a partner and initiate transcription of the target gene. A number of nuclear receptors regulate key proteins and pathways in cholesterol absorption and elimination.

The liver-X-receptors (LXR)

The predominant nuclear receptor regulating cholesterol homeostasis is liver-X-receptor, LXR. There are two LXR genes, LXRα expressed mainly in liver, macrophages, adipose tissue, lung, spleen and kidney, and the ubiquitously expressed LXRβ [90-92]. Oxysterols are the natural ligands of LXR. They are intermediates generated mainly from endogenous enzymatic reactions during cholesterol (24, 25-epoxycholesterol, 24, 25-EC) or steroid hormone biosynthesis (22-hydroxycholesterol, 22-OH-C), or compounds produced from cholesterol by different isoforms of cytochrome P450 (CYP) (24, 25, 27-OH-C) [93].

Cholesterol loading of the cell results in increased production of oxysterols, which stimulates LXR and upregulates the expression of ABCA1 and ABCG1 sterol transporters, enhancing cholesterol efflux from the plasma membrane to lipid-poor apoA-I and HDL, respectively, thus providing a regulatory feedback mechanism maintaining a constant cholesterol content [94]. Multiple studies in various cell lines have demonstrated that LXR agonists (T0901317 and GW3965) increase ABCA1 and ABCG1 expression and accelerate cholesterol efflux [95-97].

In liver, LXR target genes drive hepatic cholesterol elemination with cholesterol transport into the bile via ABCG5/G8 and stimulate bile acid synthesis via Cyp7a1 [82, 98]. LXRα deficient mice do not activate Cyp7a1 or ABCG5/G8 expression when maintained on a cholesterol-rich diet [82, 99]. Conversely, T0901317 fails to increase biliary cholesterol secretion in ABCG5/G8 double knockout mice [83]. LXR agonists stimulate lipogenesis in hepatocytes. This effect is mediated by the sterol response element binding protein (SREPB) -1c transcription factor which harbors two LXR response elements within the promoter [100]. Subsequently, SREBP-1c binds to the SREBP response element (SRE) of genes involved in fatty acid synthesis. Independent from SREBP-1c, LXR directly regulates the expression of lipogenic enzymes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD-1) [101-103]. In addition, P. Tontonoz and colleagues demonstrated that LXR also monitors the delivery of cholesterol into the cell. LXR initiates degradation of LDLR through Idol (inducible degrader of LDLR)-dependent ubiquitination [104].

In intestine, LXR directly and indirectly opposes intestinal cholesterol absorption by ABCG5/G8 upregulation. LXR triggers a decrease in NPC1L1 localization on the apical surface of enterocytes and increased expression of ABCG5/G8 [82, 105]. Beyond its regulatory role in RCT, LXR may have a similar contribution in transintestinal cholesterol elimination (TICE) (discussed below). Indeed, GW3965 does not stimulate biliary cholesterol secretion, but increased fecal neutral sterol (FNS) loss in ABCB4 deficient mice [106]. The same compound was shown to increase radiolabelled cholesterol from plasma to the feces in the same model.

LXR also plays a role as a regulator of inflammation. By exerting anti-inflammatory effects, both LXR-agonists, T0901317 and GW3965 reduce LPS-induced expression of interleukin (IL)-6 and IL-1 β in mouse peritoneal macrophages [107]. Conversely, LXR α/β deficient mice demonstrated greater susceptibility to lipopolysaccharide (LPS) administration increasing synthesis and circulation of tumor necrosis factor (TNF) α , IL-6 and IL-1 β compared to wild-type controls. Thus, when a macrophage engulfs cholesterol rich-material, LXRs are activated protecting the cell from lipid overload. Conversely, ingestion of material poor in cholesterol such as bacteria, LXRs remain inactive allowing inflammatory reactions to develop [107].

The farnesoid-X-receptors (FXR)

Upon BA binding and activation, the BA sensor FXR heterodimerizes with RXR thereby controlling cellular BA levels [108-110]. FXR is expressed predominantly in liver and intestine, the two key organs involved in BA homeostasis [111]. BAs control their own synthesis by negative feedback via three independent pathways. Reabsorption of BAs from the ileum and their delivery to the liver activates hepatic FXR and elevates the expression of the FXR target gene SHP, which in turn inactivates liver receptor homolog 1 (LRH-1), a transcription factor involved in Cyp7a1 expression. The result is SHP-dependent transcriptional suppression of Cyp7a1 [112, 113]. In the second pathway, intestinal FXR activation increases the expression of fibroblast growth factor 15 (FGF15) in enterocytes. Secreted FGF15 binds to hepatic fibroblast growth factor receptor 4 (FGFR4), activating c-Jun N-terminal kinase pathway (JNK) and ERK1/2 pathway and subsequently repressing Cyp7a1 [114]. Lastly, direct activation of JNK pathway via bile acids in the liver contributes to inactivate Cyp7a1 expression [115].

Hepatic FXR regulates the transcription of transporters involved in liver BAs uptake and excretion. Transcription of Na/taurocholate co-transporting polypeptide (NTCP), protein

responsible for uptake of bile acids from the blood compartment is suppressed by FXR via SHP [57]. Conversely, FXR upregulates transcription of proteins involved in bile acid export (ABCB11) including the phospholipid flippase, ABCB4, thereby protecting hepatocytes from bile salt toxicity and progression of intrahepatic cholestasis [56, 116].

The liver receptor homolog (LRH-1)

LRH-1 belongs to the nuclear receptor family 5A along with steroidogenic factor 1. Unlike other nuclear receptors, It binds to DNA as a monomer and does not require a ligand [117]. LRH-1 is found to be essential for embryogenic development [118]. It is expressed predominantly in the liver and intestine, but also in exocrine pancreas and ovaries [119, 120].

LRH-1 participates in regulation of BA homeostasis. LRH-1 binding sites are found in the promoter of Cyp7a1 and Cyp8b1, enzymes controlling the initial steps in BA synthesis [112, 113, 121]. Lack of LRH-1 in the intestine represses FGF15 expression and sustains Cyp7a1 expression [118]. In addition, genes involved in cholesterol metabolism and transport, such as apoA-I, scavenger receptor class B type 1 (SR-B1) and ABCG5/G8 are positively regulated by LRH-1 in the enterohepatic axis [84, 122, 123].

ADDITIONAL TRANSCRIPTION REGULATORY FACTORS INVOLVED IN CHOLESTEROL METABOLISM

Sterol regulatory element binding proteins (SREBPs)

Additional group of oxysterols and sterol sensors (INSIG and SCAP) in the cell control the transcription of sterol regulatory element binding proteins (SREBPs). SREBPs are a family of membrane bound transcription factors that regulate the expression of genes involved in cholesterol and fatty acid synthesis. Three SREBPs have been described in mammals, SREBP-1a, -1c, and -2, where SREBP-1a and -1c are encoded by a single gene [124, 125]. SREBP-1c is highly expressed in mouse and human liver, white adipose tissue, adrenal gland and brain, whereas SREBP-1a and SREBP-2 are found in all tissues and in cultured cell lines [126, 127].

SREBPs belong to the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors. In contrast to the bHLH-Zip members, SREBPs are synthesized as inactive precursors bound to the endoplasmic reticulum (ER) [128, 129]. Each SREBP precursor harbors an NH₂-terminal domain, which contains the bHLH-Zip region for binding DNA; two hydrophobic membrane-spanning segments connected by a short 30 AA loop that projects into the ER lumen; and a COOH-terminal domain, which has regulatory activity and binds to an escort and sterol sensing protein designated as SCAP (SREBP cleavage-activating protein) [130]. When cells become cholesterol depleted, the SCAP/SREBP complex exits the ER and relocates in GA, where two membrane bound proteases, site 1 protease (S1P) and site 2 protease (S2P), cleave the precursor protein releasing the mature nSREBP into the cytoplasm. nSREBP translocates to the nucleus, where it activates transcription by binding to sterol response elements (SRE) in the promoter region of genes involved in cholesterol biosynthesis (HMGR) and cholesterol uptake (LDLR) [124, 125, 131]. When the cholesterol content of cells rises, cholesterol binds to SCAP and triggers conformational changes that induce the binding of SCAP to

ER residing protein INSIG-1 (insulin-induced-gene). Therefore, INSIG-1 controls cholesterol concentration in the cell through SREBP-2 [132].

SREBP-1 transcription factors undergo similar processing mechanisms. However, SREBP-1c transcription and processing of the mature precursor are controlled by insulin. In fasted state, where insulin is depleted, INSIG-2 is highly expressed, retaining SREBP-1c in the ER. In the presence of insulin, the fed state, INSIG-2 is strongly repressed by insulin [133]. SREBP-1c is actively processed to its nuclear form and converts excess carbohydrates to fatty acids activating genes involved in lipogenesis (ACC, FAS, SCD-1). In addition, nSREBP-1c recognizes SRE in INSIG-1 promoter and triggers gene expression, regulating indirectly cholesterol biosynthesis [134].

CHOLESTEROL TRAFFICKING IN THE BODY

Cholesterol is essential for cellular homeostasis, but dietary intake and cellular demand widely vary. Although, most cells have the capacity to make the cholesterol they need for membrane structural functions, some cells (steroidogenic cells and hepatocytes) that use cholesterol as a precursor for other molecules (steroid hormones and bile acids) and need an exogenous cholesterol supply [135]. Dietary lipids are delivered to the peripheral tissues by the TG-rich chylomicrons (CMs) upon remodeling of CMs by lipoprotein lipase (LPL) to CM remnant (CMR). Liver plays a central role in the clearance of cholesterol from the circulation. Further, liver is recognized as the main organ responsible for cholesterol distribution in the body. Several distinct pathways that can be distinguished are discussed below.

Cholesterol flux in the enterocyte

The intestinal lumen is exposed to exogenous dietary cholesterol (Western diet, 300-450 mg/day) and endogenous biliary cholesterol (800 – 1400 mg/day) daily. Localized in the brush-border membrane of enterocytes, Niemann-Pick C1-like 1 (NPC1L1) is considered to be the main protein involved in intestinal cholesterol absorption [136]. Deletion of NPC1L1 or inhibition with ezetimibe (EZ) decreased total fractional sterol absorption and revealed that NPC1L1 does not distinguish dietary from biliary cholesterol and/or PS [137]. Nevertheless, NPC1L1 knockout mice display only partial reduction of cholesterol absorption, indicating an alternative pathway and suggesting more than one transporter may be involved. Placed on both apical and basolateral surface of enterocytes, scavenger receptor, class B, type 1 (SR-BI) was thought to contribute to the process [138]. However, disruption of the gene appears to have a negligible effect on intestinal cholesterol absorption in mice [139]. In addition, ABCA1 is also present on the basolateral membrane of the enterocytes and accounts for ~30% of the HDL biogenesis by transferring cholesterol to lipid poor pre- β -HDL [43].



FIGURE 1.4. Schematic description of the main routes of cholesterol trafficking in enterocytes.

FC, free cholesterol; CE, cholesteryl ester; PL, phospholipid; PS, plant sterol; ABCA1, ABCG5, ABCG8, ATP binding cassette A1, G5, G8; ACAT, acyl-coenzyme A: cholesterol acyltransferase; HDL, high density lipoprotein; LDL, low density lipoprotein; LDLR, LDL receptor; LRP, LDLR related protein; SR-BI, scavenger receptor class B type 1; NPC1L1, Niemann-Pick disease type C1 gene-like 1; MTTP, microsomal triglyceride transfer protein; apoB-48, apolipoprotein B-48.

In the enterocyte, acyl-coenzyme A: cholesterol acyltransferase 2 (ACAT2) targets the 3'–OH (hydroxyl) group of cholesterol and esterifies fatty acids to form cholesteryl ester [140]. Approximately 50-60% of the intestinal cholesterol is esterified, packaged into chylomicrons (CMs), secreted into the mesenteric lymph and ultimately reaches the liver via plasma. PS are poor substrates of ACAT2. Consequently, they are poorly esterified and fail to be efficiently incorporated into CMs; therefore less than 1% enter into the circulation [141, 142]. Unesterified cholesterol and PS are recognized by ABCG5/G8 sterol transporters and excreted back into the intestinal lumen [69, 143]. In

addition, disruption of ABCG5/G8 diminishes excretion of cholesterol into the intestinal lumen and elevates fractional cholesterol absorption [78].

CMs are the major lipoprotein secreted by the intestine. CMs contain cholesterol ester (CE) and a predominantly triglyceride rich core surrounded by a monolayer of phospholipids, unesterified cholesterol and apolipoproteins (apoB-48, apoA-I, apoA-IV) [144]. CMs are secreted into the mesenteric lymph and reach the blood stream via the thoracic duct. In plasma, CMs incorporate additional apoproteins (apoE, apoCI, apoCIII). The CM triglyceride-rich core is hydrolyzed by LPL in the capillary endothelium, which releases free fatty acids for energy production or storage in the form of triglycerides in peripheral tissues (muscle, adipose) [145]. Consequently, triglyceride-rich CMs particles are transformed to CE-rich CMR, which are cleared by the liver by binding of apoE to LDLR or LDLR related protein 1 (LRP-1) [146, 147]. Therefore, biliary cholesterol absorbed from the intestine is returned to the liver.

Hepatocytes cholesterol flux

The liver is the principal organ involved in cholesterol regulation and metabolism in mammals. Cholesterol is delivered to the liver by apoE/apoB-48–containing CMR and LDL by LDLR and/or LRP-1 mediated endocytosis [146, 147]. Moreover, SR-BI found on the basolateral membrane of hepatocytes is involved in the selective uptake of cholesterol esters from HDL [148]. Cholesterol synthesis in the liver comprises a significant source of cholesterol in the hepatocytes.

In the liver, cholesterol becomes part of different metabolic pathways (Figure 1.5.). Excess cholesterol in the liver can be stored as cholesterol ester after esterification by ACAT. Alternatively, cholesterol can be secreted into the blood stream in the free or esterified form via two apolipoprotein dependent routes - as HDL (discussed above) or VLDL, respectively. The VLDL particle is the predominant shuttle responsible for the

delivery of fatty acids and cholesterol from the liver to peripheral tissues. VLDL biogenesis is initiated by microsomal TG transfer protein (MTTP), an ER resident protein that mediates the transfer of lipids (triglycerides) to apoB-100 [149, 150]. Prior to a secretion into the blood, VLDL is enriched with triglyceride with a small amount of cholesterol ester in the core. Upon entering the circulation, the VLDL particles associate with apoE and apoC I-III. Similar to CMs, LPL hydrolyzes VLDL triglycerides forming smaller intermediate density lipoprotein (IDL). Further hydrolysis of IDL generates LDL, which is taken up by the liver or peripheral tissues via LDLR [146].

Enterohepatic circulation

As discussed above, free cholesterol in the liver is transported to the bile by the ABCG5/G8 heterodimer. In addition, cholesterol is converted to bile acids by Cyp7a1 and excreted to the bile via ABCB11. The last pathways have been demonstrated to be coupled with phospholipid secretion into the bile by ABCB4. Deletion of the phospholipid transporter abolishes biliary cholesterol secretion [50]. Conversely, increased bile salt flux stimulates phospholipid and cholesterol translocation into the bile [46, 54]. Produced by hepatocytes, the bile contains BAs, cholesterol and phospholipids, organized into mixed micelles. Between meals, bile accumulates in the gallbladder. In response to a fatty meal, the gallbladder contracts and releases its components into the duodenum, promoting the solubilization, digestion and absorption of fat. Cholesterol and bile acids are absorbed in the intestine and re-enter the body, a cycle known as *enterohepatic circulation*. Whereas reabsorption of cholesterol occurs in duodenum, bile acids re-enter mainly in the ileum and return to the liver via the portal vein. Only a small part of the bile acids escapes reabsorption and is excreted into the feces. Cholesterol undergoes bacterial transformation in the intestine and is absorbed or excreted with feces [151].



FIGURE 1.5. Overview of main routes of cholesterol in hepatocyte.

FC, free cholesterol; CE, cholesteryl ester; BA, bile acid; PL, phospholipid; ABCA1, ABCB4, ABCB11, ABCG5, ABCG8, ATP-binding cassette A1, B4, B11, G5, G8; ACAT, acyl-coenzyme A: cholesterol acyltransferase; CEH, CE hydrolase; HDL, high density lipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LDLR, LDL receptor; LRP, LDLR related protein; SR-BI, scavenger receptor class B type 1.

Reverse cholesterol transport (RCT)

RCT is the process by which excess cholesterol from the peripheral tissues, including macrophages from the vessel walls, is transported to the liver for biliary excretion and eventually removed out of the body by the feces. HDL acts as cholesterol acceptor and carrier in RCT pathway by delivering its cholesterol the liver. The predominant HDL apoprotein, ApoA-I, is synthesized in the liver and intestine [152] and released as a discoidal lipid poor pre- β HDL particle which contains 2 copies of apoA-I [153]. ApoA-I concentrations correlate with HDL cholesterol levels in plasma. That was best demonstrated in ABCA1 transgenic mice, where overexpression of human apoA-I raised
HDL-cholesterol concentrations and provided protection against atherosclerosis [154]. Ubiquitously expressed ABCA1 facilitates efflux of free cholesterol and phospholipids from peripheral cells and macrophages to apoA-I, forming larger and more mature HDL [34, 155, 156]. Lecithin-cholesterol acyltransferase (LCAT) is an enzyme secreted from the liver, circulates in blood and associates with lipid free lipoproteins. LCAT reacts with free cholesterol and catalyzes the transfer of the 2-acyl group from lecithin to 3- β -OH of cholesterol to generate cholesteryl esters [157]. Cholesteryl esters are retained in the HDL core, forming the mature HDL particle. HDL metabolism is highly dependent on LCAT activity. LCAT deficiency in humans is associated with reduced HDL and ApoA-I levels and rapid catabolism of cholesterol ester-poor ApoA-I [158, 159].

Mature HDL undergoes further remodeling by lipid transfer proteins, which are important determinants of HDL function and clearance. Cholesteryl ester transfer protein (CETP) promotes exchange of cholesteryl esters from HDL to and triglycerides within the lipoprotein core between HDL and ApoB containing particles (LDL, IDL, VLDL, CMs, and CMR) [160]. Thus, HDL is depleted of cholesterol and is enriched in triglycerides, whereas ApoB-lipoproteins incorporate cholesteryl esters. CETP-deficient patients are characterized by extremely elevated levels of HDL-cholesterol [161]. Unlike humans, mice lack the CETP gene. However, expression of a human CETP transgene significantly reduces HDL [162]. Phospholipid transfer protein (PLTP) delivers phospholipids to HDL from apoB-containing particles [163]. Disruption of PLTP in mice causes enhanced clearance of HDL and apoA-I and results in significant reduction of their levels [164].

Cholesterol delivery from HDL to the liver is another important step in RCT [165]. SR-BI, a member of the scavenger receptor superfamily of proteins, has been shown to play a major role in selective cholesterol uptake [166]. Mice deficient in SR-BI have elevated levels of HDL [167]. Conversely, overexpression of the receptor results in reduced plasma concentrations, not only of HDL, but also of apoA-I, due to accelerated clearance of the particles after interaction with SR-BI [168-170].



FIGURE 1.6. Overview of Reverse cholesterol transport (RCT).

FC, free cholesterol; CE, cholesteryl ester; PL, phospholipid; ABCA1, ABC-transporter A1, HDL, high density lipoprotein; SR-BI, scavenger receptor class B type 1, CMs, chylomicrons; VLDL, very low density lipoprotein; CETP, cholesterol ester transfer protein; PLTP, phospholipid transfer protein; LPL, lipoprotein lipase.

In addition to the hepatobiliary route, a body of literature has emerged within the last year describing an alternative pathway. Transintestinal cholesterol excretion (TICE) is now thought to play an important role for cholesterol elimination from the body [171]. Most of the studies were conducted in mouse models with impaired biliary cholesterol secretion. ABCB4 and ABCG5/8 knockout mice were characterized with a 6 fold decrease in biliary cholesterol concentrations, but no change in fecal sterol output compared to wild-type controls [106, 172]. Moreover, when challenged with the LXR agonist T0901317, ABCB4 deficient mice had an increase in fecal neutral sterol excretion but no induction of biliary cholesterol concentrations [173]. Conversely, NPC1L1 liver-specific transgenic mice have 4-20 fold decrease in biliary cholesterol but no change in fecal neutral sterols [174]. Although, there is strong evidence for the existence of TICE in mice, little is known about the molecular mechanisms involved in this alternative cholesterol elimination pathway [174, 175].

HARMFUL EFFECTS OF CHOLESTEROL

The hydrophobic characteristics of cholesterol obligate its incorporation into the hydrophobic core of water-soluble lipoprotein particles, which allows its trafficking through the aqueous environment of blood. Lipoproteins differ in their size, lipids and protein content, and the ratio of cholesteryl esters to triglycerides. CMs and VLDLs are large triglyceride-rich particles with very low density. The hydrolytic activity of LPL modifies VLDL to IDL and LDL, particles that become enriched in cholesterol as triglycerides are hydrolyzed. HDL is the smallest and most dense lipoprotein, which contains a cholesteryl ester-rich core with very little triglycerides.

Disruptions of cholesterol metabolism cause increased plasma cholesterol concentrations. Accumulation of cholesterol in plasma is an independent predictor and

a risk factor for development of atherosclerosis and coronary heart disease (CHD) [176, 177]. High levels of HDL have been associated with protective and beneficial effects, whereas high levels of LDL are associated with CVD [178]. When present in excess in the blood stream, cholesterol accumulates in the macrophages in the vessel wall, which causes fatty steaks formation that narrow and harden the arterial wall. As the build-up progresses, atherosclerotic lesions develop plaques, which can block the vessel or rupture and subsequently obstruct capillaries in the heart or in the brain [179]. Therefore, treatment of dyslipidemia is essential for prevention of CVD, a leading cause of death in the developed world.

Cholesterol-lowering therapies

Five main classes of therapeutic agents have been used for the treatment of dyslipidemia. Their primary focus is reduction of LDL-cholesterol and TGs, and normalizing the levels of HDL-cholesterol. Fibrates and niacin primarily target TGs and HDL-cholesterol, whereas bile acids sequestrants, statins and ezetimibe (EZ) focus on LDL-cholesterol.

<u>Bile acid sequestrants</u> were one of the first classes of drugs used as cholesterol-lowering therapy and had effectively decreased the risk of cardiovascular disease (CAD) [180]. Bile acid sequestrants act in the small intestine, where they bind BAs and prevent their absorption thereby inhibiting the BAs enterohepatic circulation. This causes a significant increase of BAs bound to bile acid sequestrants in feces. Reduction of BAs in the liver upregulates the rate-limiting enzyme for conversion of cholesterol to BAs, Cyp7a1. The decline in cholesterol levels in liver increases the hepatic LDLR, which binds circulating LDL-cholesterol and results in a decrease in the level of circulating LDL-cholesterol [181]. However, cholesterol depletion in liver initiates cholesterol biosynthesis reducing the efficacy of this therapeutic. Bile acid sequestrants also inhibit cholesterol absorption by

preventing formation of micelles composed of BAs in the intestinal lumen, which may also contribute to the LDL-cholesterol lowering effect.

<u>Statins</u> are by far the most powerful cholesterol lowering agents and reduce LDLcholesterol and prevent cardiovascular events. Statins inhibit the enzyme HMG-CoA reductase, thereby reducing endogenous cholesterol synthesis, causing a compensatory increase in LDLR expression [182]. Hepatic LDLR upregulation correlates with accelerated uptake and clearance of LDL [146]. Reduced free cholesterol content in hepatocytes activates SREBP-2 processing as discussed above. Numerous trials demonstrate their powerful efficacy in preventing cardiovascular outcomes [183].

<u>Fibrates (or fibric acid derivatives)</u> are agonists of peroxisome proliferator-activated receptor (PPAR) α , a transcriptional factor predominantly expressed in liver and muscle and highly involved in lipoprotein metabolism. PPAR α activation targets the transcription of apoA-I and apoA-II, enhancing HDL biogenesis [184, 185]. Fibrate therapy has been associated with increased expression of acyl-coenzyme A synthetase (ACS) and fatty acid transport protein (FATP), which facilitate transport, acylation and subsequent β -oxidation of fatty acids and decreased triglyceride synthesis in the liver [186]. Fibrate treatment increases LPL expression and reduces LPL inhibitor, apoC-III, thereby increasing the hydrolysis of triglycerides in CMs and VLDL and improving their clearance from circulation by LDLR [187, 188].

<u>Niacin, nicotinic acid</u>, is a water soluble B-complex vitamin used to treat dyslipidemia as early as 1955 [189]. Niacin is a hormone sensitive lipase (HSL) inhibitor in adipose tissue that reduces the transport of free fatty acid to the liver and reduces hepatic triglyceride synthesis [190]. In liver, niacin inhibits fatty acid-synthesis and esterification. The net effect is decreased triglyceride synthesis and increased ApoB-degradation [191]. VLDL production is reduced, which accounts for a reduction in LDL levels. In addition, niacin elevates HDL by reducing hepatic apoA-I decay, but does not reduces cholesteryl ester clearance, thereby enhancing RCT [192].

<u>Ezetimibe</u> (EZ, SCH 58235) is a cholesterol absorption inhibitor and blocks cholesterol absorption from the intestinal lumen into enterocytes [193]. The molecular target of ezetimibe is the sterol transporter, Niemann-Pick C1-like 1 protein (NPC1L1) [136, 194]. EZ lowers LDL-cholesterol by blocking the intestinal absorption of dietary and biliary cholesterol without affecting TG or fat-soluble vitamins uptake [195]. Inhibition of NPC1L1 also impairs plant sterols intestinal absorption. EZ treatment of sitosterolemic patients produces significant and progressive reduction of plasma plant sterols concentrations [196].

<u>CETP-inhibitors</u> (torcetrapib, anacetrapib, dalcetrapib) prevent the exchange of cholesteryl esters of HDL with triglycerides of apoB-containing particles, blocking the CETP action. Thus, the amount of mature HDL particles is increased, while LDL-cholesterol is reduced. Although Japanese subjects with homozygous CETP-deficiency have reduced incidence of CHD and increased life expectancy, the CETP inhibitor - torcetrapib did not prevent progression of carotid or coronary atherosclerosis more effectively than statins in clinical trials [197, 198]. Moreover, the same CETP-inhibitor induced side effects as increased blood pressure which possibly caused excess of cardiovascular and overall mortality [199]. Thus, it remains unclear what it will be the future of CETP-inhibitor for prevention and treatment of CVD.

In the last two years a combinational therapy for hypercholesterolemia treatment began. Supplementation of two lipid-lowering drugs in a decreased dose regimen had a beneficial outcome on the side effects and targets different pharmacokinetic mechanisms which overall increases the efficacy of the therapeutics and has additive effects on plasma cholesterol lowering [200]. However, the hypercholesterolemic drug combinational therapy needs more examination in order to provide consistent normalization of serum lipid profile and reduce the risk for CVD.

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Chapter 2: ROLE OF HEPATIC LEPTIN RECEPTOR SIGNALING IN REVERSE CHOLESTEROL TRANSPORT

INTRODUCTION

ABCG5 and ABCG8 play a major role in the elimination of dietary and endogenously synthesized sterols in humans and mice [69, 78-80, 201]. In intestine, the ABCG5/G8 heterodimer opposes the absorption of biliary and dietary sterols [78, 143]. In liver, the ABCG5/G8 sterol transporter is the major route for cholesterol excretion into bile [78-80]. Although biliary cholesterol excretion generally correlates with expression levels of ABCG5 and ABCG8 mRNAs, exceptions include increases in biliary cholesterol excretion following treatment with diosgenin or tauroursodeoxycholate (TUDCA) and in liver transplant patients following surgery [202-205]. The uncoupling of biliary cholesterol excretion from ABCG5/G8 expression levels suggests that other pathways may contribute to biliary cholesterol excretion. However, it should be noted that the effects of both diosgenin and TUDCA were dependent on the presence of G5G8 [205, 206], suggesting that post-transcriptional regulation of ABCG5/G8 may also influence ABCG5/G8 sterol transporter abundance and activity or that the pathways which supply cholesterol to the ABCG5/G8 sterol transporter can be regulated.

Given the role of the ABCG5/G8 sterol transporter in biliary cholesterol excretion, perhaps it is not surprising that quantitative trait locus (QTL) mapping studies have identified *Abcg5/Abcg8* as a lithogenic locus [207, 208]. However, the *Abcg5/Abcg8* locus is only one of over twenty QTLs associated with increased or decreased cholesterol gallstone susceptibility, indicating that many genetic factors are involved in lithogenesis [209]. Furthermore, ABCG5/G8 transgenic mice do not develop cholesterol gallstones despite supersaturation of bile [81]. In humans, obesity is considered a major

risk factor for cholesterol gallstone formation [210-213]. Surprisingly, two mouse models of obesity, leptin receptor defective (db/db) and leptin deficient (ob/ob), are protected from cholesterol gallstone formation when fed lithogenic diets that contain cholesterol and cholic acid [214]. Indeed, db/db and ob/ob mice have lower levels of biliary cholesterol than their wild-type counterparts [214, 215]. Replacement of leptin in ob/ob mice results in rapid weight loss, reductions in HDL-C, increases in biliary cholesterol concentrations and restoration of cholesterol gallstone susceptibility [216, 217]. Although the same characteristics are also observed in mice that are pair-fed to match caloric intake, the control mice are still resistant to cholesterol gallstone formation, indicating that the 2-3 fold increases in biliary cholesterol concentrations are insufficient to promote gallstone formation.

Leptin signaling-deficient animal models are characterized by dyslipidemia, insulin resistance, steatosis and inflammation. These metabolic disorders compromise liver function and may be responsible for reduced biliary cholesterol secretion. Within the liver, evidence of ER stress has been reported in both diet-induced obesity and in ob/ob mice [218]. Further, alleviation of endoplasmic reticulum (ER) stress by administration of chemical chaperones restores insulin sensitivity in ob/ob mice [219]. The assembly of the ABCG5/G8 sterol transporter occurs in the ER and is an inefficient process, whereby most of the ABCG5 and ABCG8 monomers fail to find their respective partners and are rapidly degraded [74]. Moreover, mutations in either ABCG5 or ABCG8 result in failures in the assembly and trafficking of the ABCG5/G8 sterol transporter and causes sitosterolemia [75]. The assembly of the complex is dependent upon N-linked glycans that interact with the ER chaperone Calnexin (CNX) [75]. Under conditions of ER stress, it is plausible that the assembly of the ABCG5/G8 sterol transporter is compromised.

Experimental Rationale

Leptin-deficient (ob/ob) and leptin receptor defective (db/db) mice are animal models of obesity and dyslipidemia. In contrast to obese humans, db/db and ob/ob mice maintain low biliary cholesterol concentrations and are not susceptible to gallstones. We hypothesized that this phenotype correlates with a reduction in hepatic ABCG5/G8. ABCG5/G8 protein abundance was expected to be restored upon leptin or TUDCA administration, therapies known to accelerate biliary cholesterol content.

EXPERIMENTAL PROCEDURES

Reagents and Buffers: General chemical reagents were obtained from Sigma (St. Louis, MO). Recombinant murine leptin was obtained from Biomyx Technology (San Diego, CA). Protein Sample Buffer (PSB, 30 mM Tris base, 10 mM EDTA, pH 6.8, 3% SDS, 20% glycerol, 0.00625% bromophenol blue). Membrane Buffer (250 mM sucrose, 2 mM MgCl₂, 20 mM, pH 7.5). Buffer A (20 mMTris, pH 7.6, 137 mM NaCl, 0.2% Tween 20, 5% milk). Buffer B (20 mM Tris, pH 7.6, 137 mM NaCl, 0.2% Tween 20). CNX, GRP78 were purchased from StressGen, Nventa (San Diego, CA). Secondary antibodies and enhanced chemiluminescence reagents were purchased from Pierce (Rockford, IL). Rabbit anti-ABCG5 and mouse anti-ABCG8 antibodies have been previously reported [76, 81].

Animals and Treatments: In the first experiment, male and female db/db mice on the C57BLKS (000642) background were used at 16 weeks of age. In all subsequent experiments, db/db (000697) and ob/ob (000632) mice on the C57BL/6J background were used between 8 and 12 weeks of age. All mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in a temperature-controlled room with 12:12 light:dark cycle (6:00 am to 6:00 pm). Upon arrival, mice were allowed to adapt to the environment for 1 week. All mice were maintained on standard rodent chow (Harlan Teklad 2014S). Analysis of the data from the first experiment indicated no

differential effects due to sex. In all subsequent experiments, male mice were used. Tissues from type I diabetic mice were generously provided by Dr. Ming Gong (University of Kentucky). STZ treatment was conducted as previously described [220]. Ob/ob mice were injected once daily with equal volumes of saline or leptin (10 µg/g bw, ip) for 7 days as previously described [217]. To achieve isocaloric intake, mice treated with saline were pair-fed with mice treated with leptin [217]. Mice treated with tauroursodeoxycholic acid (TUDCA) received twice-daily injections (ip, 7:00 am & 7:00 pm) of 250 mg/kg (500 mg/kg/day) as previously described [219].

Blood glucose levels were measured using a standard glucometer from a drop of blood obtained by tail-vein prick. Control and STZ mice were killed by exsanguination under ketamine xylazine anesthesia. All others were euthanized with CO₂ following a four hour fast beginning shortly after "lights on". Blood was collected from the right ventricle with a 1 cc syringe fitted with a 20 ga hypodermic needle. Gall-bladder bile was collected with an insulin syringe fitted with a 26 ga needle. Bile samples were stored at -20°C until analysis. Serum was separated by centrifugation and stored at 4°C for fast protein liquid chromatography (FPLC) fractionation. Livers and other tissues were excised, rinsed with PBS to remove blood and snap frozen in liquid nitrogen. Tissue samples were stored at -80°C until analyses.

Lipid Analyses: Serum was fractionated by FPLC using Superose 6 HR10/30 column to separate lipoprotein fractions. Serum cholesterol concentrations and cholesterol content in FPLC fractions were determined by colorimetric-enzymatic assays (Wako Chemicals, Richmond, VA). The concentration of cholesterol in gallbladder bile was measured as previously described by GC-MS [221].

Membrane proteins analysis: A total of 100-200 mg of liver or intestine (duodenum, jejunum and ileum) was homogenized by a polytron in 1.2 ml Membrane Buffer containing protease inhibitors (Roche Diagnostics). The crude preparation was

centrifuged at 2000 x g for 10 min at 4°C. The supernatant was collected and centrifuged at 100,000 x g for 45 min at 4°C. The membrane pellet was suspended in PSB. Protein concentrations were determined by BCA assay (Pierce). PSB was added to samples to achieve uniform concentrations, β -mercaptoethanol was added to a final concentration of 1.2% (v/v) and samples were heated to 95°C for 5 min. Proteins were size fractionated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated in Buffer A for 30 min at 22°C. Primary antibodies were diluted in Buffer A and incubated with membranes for 60 min at 22°C. Membranes were washed three times for 5 min in Buffer B. Horseradish peroxidaseconjugated goat anti-rabbit IgG and goat anti-mouse IgG (Pierce) were diluted (1:20 000) and incubated with membranes for 30 min at 22°C. Membranes were washed three times for 5 min in Buffer B and visualized by enhanced chemiluminescence (Pierce). Protein loading was assessed by visual inspection of Ponceau S-stained membranes and by re-blotting with GRP78 and CNX. Expression levels were semiquantified by densitometry. Radiography films were scanned as Tiff images and sum intensities quantified using KODAK Molecular Imaging software (V 4.0). Film background was subtracted from all bands of interest. Expression levels for ABCG5 and ABCG8 were normalized to signals for GRP78 obtained by re-blotting of stripped membranes.

Quantitative Real-time PCR: Total RNA was extracted from tissues using RNeasy Mini Kit (QIAGEN, Valencia, CA). Samples of total RNA (1.0 μ g) from mouse livers were transcribed using SuperScriptTMFirst Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) using random hexanes in a final total reaction volume 20 μ l. Primers (Table 1) have been previously described [222], but were validated to ensure amplification of single transcripts and covariance of amplification efficiencies with the invariant control (Cyclophilin) according to the manufacturer's instructions (Applied Biosystems, Guide and User Bulletin 2). Quantitative RT-PCR was performed on an Applied Biosystem 7700 Sequence Detection System. Standard reaction volume was 30 μ l containing 1 x SYBR Green PCR master mix (Applied Biosystems), 1 μ l of cDNA template 150 nM of each

oligonucleotide. Initial steps of RT-PCR were 10 min at 95°C. Cycles (n=40) consisted of a 15 sec melt at 95°C, followed by a 1 minute annealing/extension at 60°C. All reactions were performed in triplicate. Means of the differences in threshold cycle (C_T) values from cyclophilin and their standard deviations were calculated for each treatment group (ΔC_T). The relative abundance of each transcript within treatment groups was determined by subtracting the control group mean difference from the remaining treatment groups ($\Delta\Delta C_T$) and calculated according to the expression 2^{- $\Delta\Delta C_T$}. The standard deviations of the difference between control and each treatment group were calculated as the square root of the sums of squares for the standard deviation of the ΔC_T means.

Target	Forward	Reverse
ABCG5	TGGATCCAACACCTCTATGCTAAA	GGCAGGTTTTCTCGATGAACTG
ABCG8	TGCCCACCTTCCACATGTC	ATGAAGCCGGCAGTAAGGTAGA
LXRα	TCTGGAGACGTCACGGAGGTA	CCCGGTTGTAACTGAAGTCCTT
SREBP-1c	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT
Cyp7A1	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA

TABLE 2.1: Oligonucleotides used for quantative RT-PCT

Statistical Analysis: Data were analyzed by two-way, one-way, repeated measures oneway analysis of variance (ANOVA) and Student's *t*-test as indicated in text. Differences due to main effects and their interactions were determined by F-tests. Post-hoc analyses were conducted using Bonferroni or Dunnett's Multiple Comparison Tests where appropriate.

RESULTS

Steady-state levels of immunoreactive ABCG5 and ABCG8 were evaluated in livers of male and female db/db mice and their heterozygous controls (Figure 2.1). Db/db mice were significantly heavier (48 \pm 5 vs. 28 \pm 3 g) and had elevated fasting glucose levels $(185 \pm 18 \text{ vs.} > 475 \text{ mg/dl}; \text{ four had values in excess of 500 mg/dl, the limit of the}$ glucometer). Membrane proteins were prepared from livers of obese and control male and female mice (n=10 per genotype, 5 per sex) and subjected to SDS-PAGE and immunoblot analysis using antibodies directed against ABCG5 and ABCG8. For the purpose of presentation, equal amounts of membrane protein from each animal were pooled (Figure 2.1A). Two bands are routinely observed for both ABCG5 and ABCG8. The higher molecular weight, diffuse bands correspond to the post-Golgi forms of each protein whereas the lower molecular weight bands correspond to the reticular forms that have yet to heterodimerize and traffic to the cell surface. It should be noted that for ABCG5, the lower molecular weight band contains both specific and non-specific signals since it is also observed in ABCG5/G8 deficient mice [78]. GRP78 (Bip) expression was used to verify equal loading of proteins among lanes. Expression of CNX and CRT was also evaluated since previous reports indicate both ER lectins may be involved in the dimerization and folding of ABCG5/G8 (Figure 2.2.). Although no differences were observed for CRT, there appeared to be a modest reduction in the abundance of CNX.

Additionally, ABCG2 (G2) and ABCA1 (A1) expression was also evaluated. ABCG2 is the most closely related member of the G-subfamily to ABCG5 and ABCG8. Like ABCG5 and ABCG8, it is a glycoprotein that forms a dimer within the ER prior to the transport of the functional complex to the apical surface, where it mediates excretion of dietary compounds into the biliary space [223, 224]. However, it is not involved in hepatobiliary transport of cholesterol and does not interact with either ABCG5 or ABCG8 [76, 225]. The expression of ABCG2 appears to be greater in male control mice than in the remaining three groups, but there were no differences between female control and

db/db mice, indicating that unlike reductions in ABCG5/G8, differences in ABCG2 could not be independently attributed to the presence of the db mutation. Like G5 and G8, ABCA1 expression is regulated by LXR. We evaluated the expression of ABCA1 by immunoblotting (Figure. 2.2.). In both male and female mice, ABCA1 levels were reduced when compared to lean controls.



FIGURE 2.1. Expression of ABCG5 and ABCG8 and biliary cholesterol concentrations in wild-type (con) and db/db mice. A) ABCG5 (G5) and ABCG8 (G8) protein expression. The immature (reticular) and mature (post-Golgi) are noted by brackets. (*) denotes non-specific signals present in the ABCG5 immunoblot. Bip and ABCG2 were used as controls for equal protein loading and general effects on protein processing, n=5/group. B) Densitometric analysis of the signal intensities for ABCG5 and ABCG8 were normalized to the values obtained for Bip. C) The relative abundance of ABCG5 and ABCG8 mRNAs were quantified by RT-PCR. D) Cholesterol concentrations in gall-bladder bile. Data represent the mean \pm SD. (†) denote significant differences from control group at P < 0.05.



FIGURE 2.2. ABCA1, Calnexin and Calreticulin expression in db/db mice. Pooled membrane preparations from livers of male and female db/db and their lean controls were analyzed by SDS-PAGE (50 μg/lane) and immunoblotting.

Densitometric analysis of signal intensities for ABCG5 and ABCG8 was conducted for each mouse as described in methods. The data are expressed as the ratio of signal intensities for ABCG5 and ABCG8 to Bip (Figure 2.1B). The effect of genotype was significant for both ABCG5 and ABCG8 (p<0.01). However, there was no effect of sex or a genotype by sex interaction. In both male and female mice, levels of ABCG5 and ABCG8 were reduced between 50 and 60% in db/db mice compared to controls. ABCG5 and ABCG8 protein levels were also determined in three segments of the small intestine. While there was a tendency for reduced protein levels in each segment, none reached statistical significance (not shown). To determine if the reduction in ABCG5 and ABCG8 protein was associated with a reduction in their respective mRNAs, total RNA was isolated and analyzed by quantitative RT-PCR (Figure 2.1C). The analysis indicates that there was no change in ABCG5 or ABCG8 mRNA levels. The data show that the reduction in ABCG5 and ABCG8 protein occurred in the absence of changes in mRNA for each half-transporter, indicating that differences in ABCG5 and ABCG5 and ABCG8 protein levels were not associated with a decrease in transcription. To determine if the reduction in ABCG5 and ABCG8 protein correlated with reduced biliary cholesterol concentrations, gall bladder bile was analyzed by GC-MS (Figure 2.1D). Bile was collected from control and db/db mice following a 4 hour fast beginning shortly after "lights on". Cholesterol concentrations were reduced by 45% in db/db mice compared to controls (2.2 ± 0.53 vs. 0.9 ± 0.56 mM).

Like db/db mice, ob/ob mice harbor a deficiency in the leptin axis, are hyperphagic and obese. However, the defect in the leptin axis can be overcome by daily injections of recombinant leptin. For these experiments, I used a model of leptin replacement that has been shown to induce weight loss, restore plasma lipoprotein profiles and biliary cholesterol concentrations to wild-type levels [216, 217]. Four groups of mice (n = 3 per group) were compared. Wild-type control and ob/ob control mice were not treated. A second group of ob/ob mice received daily injections of recombinant leptin (10 μ g/g/day) for each of seven days. Because of leptin's profound effect on food intake in ob/ob mice, a third group of ob/ob mice was treated with daily injections of the carrier (saline) for 7 days. Mice assigned to the saline group were pair-fed to mice receiving daily injections of leptin in order to achieve similar caloric intake and degrees of weight loss. Body weight was monitored daily in the ob/ob mice assigned to the leptin-treated and their pair-fed controls (Figure 2.3A). Body weight declined to a similar extent over the seven day period in these two groups of ob/ob mice indicating that caloric intake was similar in the two groups.

Following a four hour fast after the last injection on day 7, mice were killed and tissues harvested for analysis. Serum cholesterol concentrations were greater in untreated ob/ob than in wild-type mice (Figure 2.3B, p<0.05). Leptin treatment reduced serum cholesterol concentrations in ob/ob mice (p<0.05) to levels similar to those observed in wild-type mice. There was also a tendency for reduced serum cholesterol in pair-fed mice, but it failed to reach statistical significance. FPLC analysis of pooled plasma

indicates that the reduction in serum cholesterol concentrations in leptin- treated and pair-fed controls was confined to the LDL containing fractions (13-18) and was slightly offset by modest increases in the HDL containing fractions (18-22, Figure 2.3C).



FIGURE 2.3. Body weight and serum cholesterol levels in wild-type mice and ob/ob control and treated mice. A) Body weight was monitored over the seven day treatment period. B) Total serum cholesterol and C) relative cholesterol content in pooled, FPLC-

fractionated serum from each group of mice, n=3/group. Data represent the mean \pm SD. (†) denote significant differences from control group at *P* < 0.05.

Immunoblot analysis of liver membrane preparations from wild-type and ob/ob mice confirmed results in db/db mice that indicate a reduction in ABCG5 and ABCG8 protein in mice harboring defects in the leptin axis (Figure 2.4A, densitometry not shown). Administration of leptin increased ABCG5 and ABCG8 protein in ob/ob mice to levels greater than those observed in wild-type mice. Immunoreactive ABCG5 and ABCG8 levels were restored in ob/ob mice that were pair-fed to leptin-treated mice. Immunoreactive levels of ABCG2 were constant across all groups, indicating that global changes in protein synthesis and processing are insufficient to explain the reduction of ABCG5 and ABCG8 in ob/ob mice and their stabilization in leptin-treated and pair-fed mice. In addition, no differences were appreciated among treatment groups for CNX, CRT or ABCA1 (Figure. 2.5). While it is plausible that this difference could be due to differences between the two genotypes, it should be noted that the db/db mice were substantially older and had higher body weights and fasting glucose levels than the ob/ob mice.

A one-way ANOVA indicated differences among treatment groups for biliary cholesterol concentrations. The reduction in immunoreactive ABCG5 and ABCG8 in untreated ob/ob mice relative to wild-type mice correlated with a reduction in biliary cholesterol concentration (Figure 2.4B). Conversely, the restoration of ABCG5 and ABCG8 either by leptin-treatment or calorie restriction in their pair-fed controls increased biliary cholesterol concentrations. However, the additional increase in ABCG5 and ABCG8 protein levels following leptin treatment was not associated with a further increase in biliary cholesterol concentration. Finally, we evaluated the levels of ABCG5 and ABCG8 mRNA (Figure 2.4C). As in db/db mice, there was no reduction in mRNA levels for either ABCG5 or ABCG8 in ob/ob mice compared to wild-type controls, indicating that the

reduction in ABCG5/G8 occurred post-transcriptionally. If anything, there is a modest increase, although these differences were not significant.



Figure 2.4. Expression of ABCG5 and ABCG8 and biliary cholesterol in wild-type and ob/ob control and treated mice. A) ABCG5 (G5), ABCG8 (G8), ABCG2 (G2) and Bip protein immunoblotting analysis (n=3/group). **B)** Cholesterol concentrations in gall-bladder bile. **C)** The relative expression of mRNAs as determined by RT-PCR, n=3/group. (*) denotes nonspecific band. Data represent the mean ± SD.

The expression of LXRα, SREBP-1c and Cyp7a1 was also evaluated. There is a modest, but consistent increase in LXRα, SREBP-1c, ABCG5 and ABCG8 mRNAs in ob/ob mice when compared to wild-type controls regardless of treatment. There was no change in Cyp7a1. When compared to untreated ob/ob mice, neither leptin treatment nor calorie restriction resulted in a significant increase in message levels for either ABCG5 or ABCG8. In conclusion, ABCG5 and ABCG8 protein levels are reduced in ob/ob mice and can be restored with either leptin replacement or dietary restriction, both of which increase biliary cholesterol content. However, changes in ABCG5 and ABCG8 protein levels and biliary cholesterol concentrations do not correlate with changes in mRNA levels for either transcript.



Figure 2.5. ABCA1, Calnexin and Calreticulin expression in wild-type and ob/ob control and treated mice. Pooled membrane preparations from livers of male wild-type and ob/ob (control and treated) mice were analyzed by immunoblotting.

Both db/db and ob/ob mice are obese, insulin resistant and routinely studied as a model of type 2 diabetes. Leptin treatment and dietary restriction are associated with increased insulin sensitivity. To determine if a lack of insulin signaling in the absence of obesity could explain the post-transcriptional reductions in ABCG5/G8, we evaluated levels of ABCG5 and ABCG8 in a mouse model of type I diabetes. Livers from control and streptozotocin (STZ) treated mice were generously provided by Dr. Ming Gong (University of Kentucky). Membranes were prepared from livers and analyzed as in Figure 2.1 (Figure 2.6A). No differences in weights were detected between control and STZ treated mice; however, STZ treated mice were hyperglycemic (Figure 2.6B, C). Despite insulin insufficiency, there was no change in immunoreactive ABCG5 or ABCG8 nor was there a compensatory increase in ABCG5 or ABCG8 mRNA (not shown). The results indicate that hyperglycemia and a lack of insulin signaling are insufficient to cause post-transcriptional reductions in the ABCG5/G8 sterol transporter.



FIGURE 2.6. Expression of ABCG5 and ABCG8, body weight and blood glucose levels in control and diabetic (STZ) mice. A) ABCG5 (G5), ABCG8 (G8) and Bip immunoblotting analysis, (n=3/group). B) Body weights and C) blood glucose levels at the termination of the experiment. (*) denotes nonspecific band. Data represent the mean \pm SD. (†) denote significant differences from control group at p < 0.05.

Tauroursodeoxycholate (TUDCA) has been shown to increase the biliary excretion of cholesterol in the absence of changes in ABCG5 and ABCG8 mRNA levels [205]. Secondly, TUDCA has been shown to function as a molecular chaperone and was

recently reported to alleviate ER stress and hyperglycemia in ob/ob mice [219, 226]. Given that the ABCG5/G8 heterodimer is formed in the ER in a glycan-dependent manner that involves CNX, it has been hypothesized that TUDCA might increase biliary cholesterol excretion by stabilizing the ABCG5/G8 heterodimer in mice with defects in the leptin axis. To test this hypothesis we treated wild-type and db/db mice with either PBS (control) or TUDCA.



Figure 2.7. Body weight, blood glucose and serum cholesterol levels in control and TUDCA treated wild-type and db/db mice. A) Body weight, B) blood glucose, and C) serum cholesterol levels. D) Relative cholesterol content in pooled, FPLC-fractionated serum from each group. Data represent the mean \pm SD. (†) denote significant differences at P < 0.05.

Body weight and plasma glucose concentrations were analyzed using two-way repeated measures ANOVAs. For body weight, there were no differences between weights on Day 1 and Day 10 within any of the treatment groups (Figure 2.7A). For blood glucose, the treatment by time interaction tended to be significant (p=0.07). A post-hoc analysis indicated that plasma glucose levels were lower on day 10 than on Day 1 in db/db mice treated with TUDCA, but not in db/db control mice (Figure 2.7B). However, it should be noted that recorded plasma glucose levels declined slightly in PBS treated mice, elevating the P-value for the treatment by time interaction. Plasma cholesterol data was analyzed using a two way ANOVA comparing genotype, treatment with TUDCA and their interaction (Figure 2.7C). Although genotype was associated with a modest, but significant increase in plasma cholesterol levels (p<0.05), significant differences were not detected for treatment or the treatment by genotype interaction. FPLC fractionation of pooled serum confirmed the elevations in serum cholesterol concentrations were largely due to increases in LDL containing fractions, although HDL containing fractions also had increased cholesterol content (Figure 2.7D).

Immunoblot analysis indicates that TUDCA increased ABCG5 and ABCG8 protein levels in both wild-type and db/db mice (Figure 2.8A). Consistent with previous observations, biliary cholesterol concentrations were lower in control, db/db mice compared to wildtype controls (Figure 2.8B). TUDCA treatment was associated with increased biliary cholesterol concentrations regardless of genotype; however, the genotype by treatment interaction was not significant, indicating that TUDCA increased biliary cholesterol concentrations irrespective of genotype. Interestingly, the expression of ABCG2 appears to be modestly reduced in db/db mice when compared to controls, an observation that is consistent with that observed in Figure 2.1. However, levels of ABCG2 were not affected in either wild-type or db/db mice by treatment with TUDCA, indicating that the effects of TUDCA show some degree of specificity. CNX, CRT and A1 were neither affected by genotype nor treatment with TUDCA (Figure 2.9).



Figure 2.8. Expression of ABCG5 and ABCG8 and biliary cholesterol concentrations in control and TUDCA-treated wild-type and db/db mice. A) ABCG5 (G5), ABCG8 (G8), ABCG2 (G2) and Bip immunoblotting analysis. **B)** Biliary cholesterol concentrations. **C)** The relative expression of mRNAs as determined by RT-PCR. (*) denotes nonspecific band. Data represent the mean ± SD.

Lastly, I evaluated mRNAs for ABCG5, ABCG8, LXR α , SREBP-1C and Cyp7A1 (Figure 2.8C). Consistent with earlier studies, there were no changes in ABCG5 and ABCG8 mRNAs between wild-type and db/db mice, nor were there differences in LXR α or Cyp7A1. As expected, db/db mice have elevated mRNAs encoding SREBP-1c; treatment with TUDCA suppressed Cyp7A1 mRNA in both genotypes. In conclusion, TUDCA increased ABCG5 and ABCG8 protein levels and biliary cholesterol concentrations in both wild-type and db/db mice independently of changes in mRNA levels for either protein.



Figure 2.9. ABCA1, Calnexin and Calreticulin expression in control and TUDCA-treated wild-type and db/db mice. A) Membrane preparations from livers of male untreated (PBS) and TUDCA-treated wild-type and db/db mice were analyzed by immunoblotting.

DISCUSSION

The major findings of this study are that defects in the leptin axis are associated with reductions in the levels of immunoreactive ABCG5/G8 sterol transporter. These reductions are not associated with decreased levels of mRNAs encoding either subunit. The reduction in ABCG5/G8 sterol transporter levels can be restored with caloric restriction and further enhanced by leptin replacement. Similarly, administration of TUDCA can restore immunoreactive ABCG5 and ABCG8 in db/db mice, but also increases ABCG5/G8 in wild-type mice. For caloric restriction, leptin-replacement and TUDCA-treatment, the increases in ABCG5/G8 abundance are associated with increased biliary cholesterol concentrations.

Beyond the absence of a functional leptin axis, the molecular mechanism for the posttranscriptional reduction in ABCG5/G8 transporter levels in db/db and ob/ob mice is not known. A number of possibilities exist and include a decrease in the rate of translation, assembly of the complex within the ER, and stability of the post-Golgi complex. Studies in cell culture indicate that the bulk of ABCG5 and ABCG8 monomers fails to find their respective partners and are rapidly degraded [74]. This may also be true in vivo, since the reticular forms of both ABCG5 and ABCG8 have half-lives of approximately two hours and are generally visible in immunoblots of hepatic and intestinal membrane preparations. A recent report indicates that expression of CRT can facilitate the trafficking of ABCG5/G8 transporters to the cell surface, suggesting that chaperone activity can be both limiting and overcome experimentally [227]. I evaluated the expression of CNX and CRT in these experiments. In the first experiment, CNX levels were modestly reduced. However, in subsequent experiments this finding was not reproducible. There are significant differences between the db/db mice used in the experiment presented in Figure 2.1 and the remainder of the studies that may account for the differences in CNX expression. First, the mice are on the KS strain of C57BI/6 mice. Secondly, they were considerably older and had greater fasting glucose levels.

Regardless of these differences, reductions in ABCG5/G8 abundance and biliary cholesterol concentrations are consistent across all experiments, indicating that reduced CNX expression in older db/db mice is insufficient to explain the reduction in ABCG5/G8 abundance and activity.

Although diabetes in the absence of leptin deficiency failed to reduce ABCG5/G8 transporter levels, calorie restriction in ob/ob mice largely restored levels of the complex. This effect is presumably metabolic in nature and may be due to a reduction in hepatic ER stress. A recent study indicates that alleviation of ER stress is sufficient to correct hyperglycemia in ob/ob mice [219]. In the present study, we treated db/db mice with TUDCA using the same treatment paradigm previously reported. We did see a reduction in plasma glucose levels in db/db mice when comparing Day 1 to Day 10 of treatment in post-hoc analysis. In addition, we saw an increase in ABCG5/G8 transporters compared to control, db/db mice indicating that TUDCA treatment was sufficient to correct the effect of leptin deficiency on ABCG5/G8 levels. Interestingly, the effect of TUDCA was also observed in wild-type mice. This observation suggests that chaperone activity may be limiting in wild-type mice and supports in vitro data suggesting that a significant number of ABCG5 and ABCG8 monomers fail to reach their final destination. However, the interpretation of the effect of TUDCA on ABCG5/G8 is limited by the fact that the mechanism by which TUDCA acts as a molecular chaperone is not known and its ability to suppress Cyp7a1, presumably via FXR, indicates that the molecule elicits responses beyond its effects as a molecular chaperone. Further, TUDCA has been shown to acutely increase biliary cholesterol secretion when infused into mice [228]. It is unlikely that this effect involved alterations in the assembly of ABCG5/G8 transporters via chaperone activity, although these experiments cannot preclude this possibility. Furthermore, feeding cholate to mice has been shown to increase both ABCG5/G8 abundance and mRNA, an effect lost in FXR deficient mice [202]. A careful study of the effects of individual bile acids on complex synthesis, assembly and transport will be required to elucidate the mechanisms by which they influence activity of the ABCG5/G8 transporter.

The effect of leptin on ABCG5/G8 is not entirely explained by caloric restriction and weight loss. The dose of leptin used in the present study increased ABCG5/G8 to levels greater than those observed in wild-type mice. However, this increase was not associated with a further increase in biliary cholesterol concentrations. This observation is largely consistent with those of Cohen and his colleagues which show that while leptin infusion increases biliary cholesterol concentrations relative to untreated ob/ob mice, pair-feeding mice to match caloric intake with those receiving leptin also increases biliary cholesterol secretion [216, 217]. In these studies, biliary cholesterol concentrations in pair-fed mice were greater than those observed in leptin-treated mice evaluated after 14 days of leptin replacement on control or lithogenic diets. The uncoupling of ABCG5/G8 protein levels with biliary cholesterol concentrations suggests that ABCG5/G8 transporter activity is regulated beyond abundance of mRNA and protein. These may include pathways that supply cholesterol to the transporter, alter the subcellular localization of ABCG5/G8 or directly affect transport activity within the canalicular membrane. Although such mechanisms have yet to be reported, the present study cannot preclude their involvement.

Chapter 3: LIVER SPECIFIC EXPRESSION OF ABCG5/G8 REQUIRES INHIBITION OF CHOLESTEROL ABSORPTION TO REDUCE PLASMA CHOLESTEROL IN DYSLIPIDEMIC DB/DB MICE

INTRODUCTION

Metabolic syndrome is a constellation of risk factors that includes obesity, hypertriglyceridemia, insulin resistance, and low high density (HDL) cholesterol that collectively contribute greater increased risk for cardiovascular diseases (CVD) than their sum [229]. Virtually all of these are also independent risk factors of gallbladder disease [230]. Mice that are homozygous for spontaneous mutations in either the gene encoding leptin (ob/ob) or its receptor (db/db) are obese, insulin resistant and hypertriglyceridemic [231]. However, these strains have a unique dyslipidemia characterized by the accumulation of large, cholesterol enriched HDL [232]. While the phenotype is leptin-dependent, the mechanisms responsible for the development of this unique dyslipidemia remain unclear.

HDL is considered to be atheroprotective based on a variety of beneficial effects on vascular function, plasma lipids and its multiple roles in the reverse cholesterol transport (RCT) pathway [233]. HDL acts as an acceptor for cholesterol in peripheral tissues as well as a donor of cholesterol to the liver, either directly or through other plasma lipoproteins. SR-BI is a class B scavenger receptor that binds HDL, mediates the selective delivery of cholesterol esters to cells, and is a major determinant of plasma HDL levels in both humans and mice [165, 167, 234, 235]. SR-BI also plays a central role in biliary excretion of cholesterol. The absence of SR-BI reduces biliary cholesterol concentrations; whereas adenoviral mediated overexpression of SR-BI increases biliary cholesterol excretion and lowers plasma HDL [168, 170, 236]. Using polarized

hepatocytes, McPherson and colleagues showed that SR-BI could bind HDL and deliver it to the subcanalicular domain [237]. The excretion of cholesterol into bile is largely dependent upon the activity of the ABCG5/G8 sterol transporter [78, 238]. This complex is thought to function at the apical surface of hepatocytes and promote the enrichment of cholesterol in the outer leaflet of the canalicular membrane [74, 239]. ABCG5/G8 is also expressed in the small intestine where it opposes the absorption of dietary sterols [78, 238]. Effective opposition to accumulation of dietary cholesterol appears to require both hepatic and intestinal ABCG5/G8. Mice harboring a human ABCG5/G8 transgene under the control of the endogenous promoter is expressed in both liver and intestine and opposes cholesterol accumulation in LDLR deficient mice, whereas a liver specific transgenic ABCG5/G8 fails to prevent cholesterol accumulation unless cholesterol absorption is inhibited [78, 240].

The unique dyslipidemia associated with leptin deficiency may be caused by alterations in HDL structure, SR-BI function or impaired hepatobiliary cholesterol transport by ABCG5/G8. HDL particles obtained from ob/ob mice are larger, enriched in apoA-II, and demonstrate delayed clearance in the plasma of obese compared to lean mice [232]. SR-BI is either reduced or unchanged in leptin deficient mice [232, 241]. In Chapter 2 it was demonstrated that ABCG5/G8 expression is post-transcriptionally reduced in both ob/ob and db/db mice. Leptin replacement in ob/ob mice restores biliary cholesterol elimination, increases both SR-BI and ABCG5/G8 protein, and normalizes plasma HDL [216, 241, 242].

Experimental rationale

Ob/ob and db/db mice are models of obesity and dyslipidemia, known to have defects in the leptin axis, defective HDL clearance, increased plasma cholesterol and decreased biliary cholesterol concentrations associated with reduction in ABCG5/G8 proteins. I hypothesized that the disruption in hepatobiliary cholesterol excretion may impact SR-BI levels or function, and consequently plasma HDL levels. To test this hypothesis, I restored biliary cholesterol excretion with adenoviral vectors encoding ABCG5 and ABCG8 in db/db mice. Based on the possible concern that a significant portion of the excreted cholesterol would be reabsorbed in the intestine and mitigate the effects of hepatic ABCG5/G8, the experiment was conducted in the presence and absence of EZ. Expression of ABCG5/G8 was expected to increase biliary cholesterol and fecal neutral sterol (FNS) excretion, and normalize plasma cholesterol levels.

EXPERIMENTAL PROCEDURE

Reagents and Buffers: General chemical reagents were purchased from Sigma (St. Louis, MO). Serum, triglycerides and phospholipids measurements used colorimetricenzymatic assay kits (Wako Chemicals USA Inc., Richmond, VA). MBST/OG was used in hepatic lipid extraction (0.97% 2-(*N*-morpholino) ethanesulfonic acid, 1.7% NaCl, 2% Triton X-100 and 3.5% Octylglucopyrannoside). The rest of the reagents and buffers are as reported in Chapter 2.

Animals and Treatments: Male db/db mice on C57BL/6J background at 32 weeks of age were used in these studies. All mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained in the same conditions as specified in Chapter2. Mice were maintained on standard rodent chow diet (Harlan Teklad 2014S) or chow diet supplemented with EZ (0.005% w/w), where specified. Diets were formulated by Research Diets Inc. (New Brunswick, NJ).

Adenovirus treatment: Recombinant adenoviral vectors containing cDNAs for ABCG5 and ABCG8 have been described previously [74, 76]. Briefly, the viruses were propagated in HEK-293 cells, purified using AdEAsy Virus Purification Kits, Stratagene (La Jolla, CA). Viral titer was quantified by Adeno-X Rapid Titer Kit, Clontech (Mountain View, CA). On Day -4 of the experiment, two db/db groups (n=3) were provided standard chow diet or chow supplemented with ezetimibe (0.005%). On Day 0, mice were placed in clean cages and injected (i.v., tail vein) with a cocktail of adenoviruses encoding both ABCG8 and ABCG5 in a 2:1 ratio (5×10^8 particles/kg total virus). Pilot studies indicate that this ratio optimizes the appearance of the mature, post-Golgi forms of both ABCG5 and ABCG8 and is the minimal dose capable of increasing biliary cholesterol concentrations (not shown). After 72 hours (Day 3), the animals were anesthetized with ketamine:xylazine anesthesia after a 4 hr fast beginning shortly after "lights on". Blood, gallbladder bile, tissues and feces were collected and analyzed. Tissues were snap frozen in liquid nitrogen and stored at -80°C until processing for membrane preparation, SDS-PAGE, immunoblot analysis and qRT-PCR.

Lipid analysis: The total concentration of cholesterol in gallbladder bile was analyzed by GC-MS/FID. Briefly, 10 μ l of bile were placed into a glass tube together with 5 α cholestane. Lipids were saponified for 2 hrs at 90-100°C in hydrolysis solution (6% KOH 10 M stock solutions dissolved in 100% ethanol). After cooling to room temperature, 2 ml petroleum ether and 1 ml water were added. The solution was vigorously vortexed and centrifuged at 2800 rpm for 15 min. The ether phase was separated and dried under a steam of nitrogen. A 1:1 mixture -N,O-Bis(trimethylsilyl)trifluoroacetamide and Pyridine was added to the test tube, transferred to GC vials and heated at 75°C for 20 min. One microliter of the sample was injected into the GC (Agilent 6890 GC G2579A system; Palo Alto, CA) equipped with Agilent Column J&W 122-5711, DB5ht, and flame ionization detector (FID). An Agilent 5973 network mass selective detector was used to identify target peaks. The GC program was as follows: injector: 1 μ l at 10:1 split, 360°C; detector: FID, 280°C; oven: 150°C (5 min) to 280°C at 8°C/min; carrier: helium, 1.0 ml/min. Additional serum analysis was performed by FPLC fractionation using a Superose 6 HR10/30 column to separate lipoprotein fractions. Total cholesterol content in FPLC fractions was determined by colorimetric-enzymatic assays. Hepatic lipids were

determined by colorimetric assay following Folch extraction. Approximately 100 mg of liver was homogenized using dounce pestle. The homogenate was placed in 1 ml MBST/OG for 30 min in ice. Lipids were extracted in 2:1 CHCl₃: MeOH at room temperature. The chloroform phase was dried under a stream of N₂, solubilized in 1 volume of Triton X-100/CHCl₃, dried and resolubilized in 2 volumes of water. The measured values were normalized to the sample weight.

Fecal neutral sterol analysis: Total feces from the 72 hr period, following infection (Day 0), was collected, dried at 37°C, weighed and ground to powder. An aliquot of 0.125 g feces was placed into a glass tube with 1.25 ml ethanol and 0.25 ml 10 N NaOH. Lipids were saponified at 72°C in a water bath for 2 hrs and extracted (water, ethanol and petroleum ether, 1:1:1 v: v: v). 5 α -cholestane (0.150 mg) was used as internal standard. Following extraction, the organic phase was dried under a steam of N₂, and solubilized in hexane. The amount of neutral sterols (cholesterol, coprostanol and cholestanol) was quantified by GC-MS/FID.

Membrane proteins and qRT-PCR analysis: The preparations of membrane proteins, SDS-PAGE and immunoblotting were conducted as previously described in Chapter 2. Total RNAs were extracted from liver followed by cDNA synthesis using the same procedures as in Chapter 2. To determine relative abundance, quantitative RT-PCR was conducted using SYBR Green as detector on Applied Biosystem 7900 HT fast-Real Time PCR System (Carlsbad, CA). Oligonucleotides used for qRT-PCR are listed in Table 3.1 or as reported in Chapter 2.

Statistical analysis: Data are expressed as means \pm SD. Data were analyzed by 1 way ANOVA. Post-hoc comparisons were conducted with a Dunnett's Multiple Comparison test with obese control (Con) mice serving as the control. Differences were considered significant at p < 0.05.

Target	Forward	Reverse
SREBP-2	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA
HMGR	CTTGTGGAATGCCTTGTGATTG	AGCCGAAGCAGCACATGAT
HMGS	GCCGTGAACTGGGTCGAA	GCATATATAGCAATGTCTCCTGCAA
LDLR	AGGCTGTGGGCTCCATAGG	TGCGGTCCAGGGTCATCT
LRP1	TCTCGTCCGTGCTGGCCAGG	GGCTGCTGATGGCTCCCGAC
SR-BI	TCCCCATGAACTGTTCTGTGAA	TGCCCGATGCCCTTGA
VLDLR	ACCTGTTCCTGTCCCAATGG	TCACTGTAAGTCACAGGAGTTGAAGTAC
NPC1L1	TGGACTGGAAGGACCATTTCC	GCGCCCCGTAGTCAGCTAT
FXR	TGAGAACCCACAGCATTTCG	GCGTGGTGATGGTTGAATGTC
LRH-1	TGGGAAGGAAGGGACAATCTT	CGAGACTCAGGAGGTTGTTGAA
Cyp7a1	GCTGTCTGGGTCACGGAAGG	AAGTGAATAGGGACGCCCGC
Cyp8b1	GCCTTCAAGTATGATCGGTTCCT	GATCTTCTTGCCCGACTTGTAGA
Apo-A1	CTCCTCCTTGGGCCAACA	TGACTAACGGTTGAACCCAGAGT
ABCA1	CGTTTCCGGGAAGTGTCCTA	GCTAGAGATGACAAGGAGGATGGA

 TABLE 3.1: Oligonucleotides used for quantitative RT-PCR

RESULTS

To restore biliary cholesterol elimination in obese (db/db) mice, I utilized adenoviral vectors encoding ABCG5 and ABCG8. Previous reports from liver-specific ABCG5/G8 transgenic mice suggest that a significant portion of biliary cholesterol is reabsorbed and mitigates the efficacy of enhanced hepatic ABCG5/G8 activity [240]. Therefore, I expressed recombinant ABCG5/G8 in the presence and absence of the cholesterol absorption inhibitor, EZ. As an additional control, lean mice were maintained on standard chow and treated with the control virus. On the day of infection, mice were placed in clean cages and injected with either control adenovirus (Con) or a cocktail of adenoviruses encoding ABCG5 and ABCG8 (G5G8). Three days after adenoviral delivery, mice were killed and feces and tissues collected.

Recombinant proteins were detected in pooled hepatic membrane preparations from G5G8 infected mice by immunoblot analysis (Figure 3.1A). Both the immature, reticular forms and mature, post-Golgi forms of G5 and G8 were present. Although there appears to be less ABCG5 and ABCG8 expressed in the EZ treated group, densitometric analysis of the individual samples and qRT-PCR of mRNAs for ABCG5 and ABCG8 indicated no statistical differences between these two groups (Figure 3.2). In addition, ABCG5/G8 increased biliary cholesterol concentrations in obese (db/db) mice by 7.3 (Chow) and 9.3 (EZ) fold compared to obese controls, suggesting that the level of ABCG5/G8 expression was functionally similar (Figure 3.1B).

Although there is controversy in the literature, SR-BI levels are thought to be lower in leptin deficient mice and may contribute to the accumulation of large HDLs and reduced biliary cholesterol excretion. Therefore, hepatic levels of SR-BI were determined. In this cohort we did not observe differences in SR-BI between lean and db/db mice. Surprisingly, there was modest, but significant reduction in SR-BI in ABCG5/G8 expressing mice (Figure 3.3A, B).


FIGURE 3.1. Protein expression and biliary cholesterol concentrations in lean and obese mice infected with control adenovirus (Con) or ABCG5 and ABCG8 (G5G8) in the presence and absence of ezetimibe (EZ). A) Immunoblotting analysis for ABCG5, ABCG8 and SR-BI. Asterisk (*) denotes detection of a non-specific signal which overlaps with the lower molecular band weight of G5. B) Cholesterol concentrations in bile (mM). *** p<0.001.

In a second cohort of db/db mice examined at 16 weeks of age significant reductions in both SR-BI protein and mRNA compared to their lean littermates was observed (Figure 3.3C-E). Total plasma cholesterol and the FPLC profiles in these independent cohorts of db/db mice were virtually identical (Figure 3.5A, B, 3.3F, G). This indicates that while hepatic SR-BI levels may be reduced in db/db or ob/ob mice of different ages, the

dyslipidemic phenotype characteristic of these strains is not dependent upon a reduction in the abundance of SR-BI protein.



Figure 3.2. Quantitative RT-PCR and densitometric analysis of ABCG5 and ABCG8 from liver in lean and obese mice infected with control adenovirus (Con) or ABCG5 and ABCG8 (G5G8) in the presence and absence of ezetimibe (EZ). The relative abundance of A) ABCG5 and B) ABCG8 were evaluated upon hepatic mRNA isolation by qRT-PCR. Membrane preparations for ABCG5 and ABCG8 were analyzed individually and also blotted for the loading control CNX. Relative abundance of C) ABCG5 and D) ABCG8 protein was determined by densitometry. * p<0.05.



Figure 3.3. SR-BI protein and mRNAs in lean and obese (db/db) mice at 32 (A,B) and 16 (C-G) weeks of age. Densitometric **(A, D)** and qRT-PCR analysis **(B, E)** of SR-BI from liver. Total and FPLC fractionated plasma cholesterol levels **(F,G)** were also determined. Samples were prepared and analyzed as described in methods. * p<0.05, ** p<0.01, ***p<0.001.

As previously reported, FNS were lower in db/db mice compared to lean controls (Figure 3.4 A). Hepatic ABCG5/G8 expression tended to increase FNS, but this difference failed to reach statistical significance. The inclusion of EZ in the diet increased FNS by 4.6 fold, but there was no further elevation in FNS in mice treated with both EZ and ABCG5/G8. Mice deficient in NPC1L1 exhibit an unexplained reduction in total stool output as well as alterations in the cholesterol to coprostanol ratio [243]. The inhibition of NPC1L1 with EZ also reduces stool output in obese (db/db) mice (Figure 3.4 B). Because the reduction in FNS in obese controls comes predominately at the expense of cholesterol, there is a dramatic decreased in the cholesterol to coprostanol ratio in db/db compared to lean

controls (Figure 3.4 C). The apparent increase in this ratio following EZ treatment alone failed to reach statistical significance. However, a 2 way ANOVA of the data from only db/db mice revealed an overall effect of EZ.



FIGURE 3.4. Sterol balance in lean and obese mice infected with control adenovirus (Con) or ABCG5 and ABCG8 (G5G8) virus in the presence and absence of EZ. A) Total FNS output over 3 days following viral administration. B) Total stool output (dry weight) over 72 hrs. C) Ratio of fecal cholesterol to coprostanol. * p<0.05, ** p<0.01, *** p<0.001.

Total serum cholesterol levels were elevated in obese (db/db) mice, but were unaffected by ABCG5/G8 expression or the inclusion of EZ in the diet (Figure 3.5 A). In the presence of EZ, hepatic expression of ABCG5/G8 reduced serum cholesterol, but failed to fully normalize total cholesterol to levels observed in lean controls. Serum from mice within each of the five groups was subjected to FPLC analysis (Figure 3.5 B). The reduction in plasma cholesterol in mice treated with ABCG5/G8 and EZ occurred in both the HDL and LDL/HDL₁ fractions. Treatment with ABCG5/G8 or EZ alone had no effect on the FPLC profile of obese mice (not shown).

The inability of hepatic ABCG5/G8 to lower plasma cholesterol in the absence of EZ suggests the increase in biliary cholesterol excretion was offset by reabsorption. Increasing dietary cholesterol modulates intestinal expression of genes involved in cholesterol absorption and synthesis [137]. Quantitative RT-PCR was used to determine if adenoviral G5G8 promoted a sufficient increase in intestinal cholesterol delivery to modulate expression in these pathways (Table 3.2.). Neither ABCG5/G8 expression nor the inclusion of EZ in the diet had an effect on intestinal levels of NPC1L1, ABCA1 and many other gene products involved in intestinal cholesterol absorption or synthesis. However, there was a significant reduction in HMGS in mice infected with ABCG5/G8 in the presence and absence of EZ. Intestinal lipid content was also measured (Table 3.3). While db/db mice have elevated intestinal lipids, compared to lean controls, the expression of ABCG5/G8 alone did not result in an accumulation of cholesterol. However, there was an increase in intestinal lipids in the presence of EZ. These data suggest that the increase in biliary cholesterol concentrations following ABCG5/G8 expression had little impact on intestinal cholesterol delivery, but do not exclude a role for cholesterol reabsorption and intestinally derived lipoproteins in limiting the effectiveness of ABCG5/G8 expression in cholesterol lowering.



FIGURE 3.5. Serum cholesterol and lipoprotein profiles in lean and db/db mice infected with control adenovirus (Con) or ABCG5/G8 in the presence and absence of EZ. A) Total serum cholesterol levels in lean and db/db mice. * p<0.05, *** p<0.001. B) Relative cholesterol content in FPLC fractionated serum in individual samples and expressed as the average in each group. Lean (Con, \blacktriangle) and obese (Con, \bullet) mice were infected with control virus and mice treated with both ABCG5/G8 and EZ (G5G8/EZ, O).

In order to determine the source of biliary cholesterol in ABCG5/G8 infected animals I measured hepatic lipids and gene expression. Not surprisingly, hepatic triglycerides and phospholipids were elevated in db/db mice, but there was no accumulation of hepatic cholesterol and no decrease following ABCG5/G8 expression in the presence or absence

of EZ (Table 3.2). Hepatic G5G8 expression increased HMG-CoA reductase (HMGCR) and synthase (HMGCS) mRNA levels in *db/db* mice in the presence or absence of EZ (p < 0.05), suggesting up-regulation of cholesterol synthesis (Figure 3.6). VLDLR, LDLR and LRP1 mRNAs were not affected by G5G8 expression and increased biliary cholesterol secretion. Additional genes involved in bile acid metabolism and HDL biogenesis were also unaffected (Table 3.4). These data indicate that the increase of ABCG5/G8 activity and mobilization of cholesterol was detected by hepatocytes, but rather than promoting uptake of plasma lipoproteins, the net loss of cholesterol to bile was offset by an increase in synthesis.

Phenotype	lean	obese				
Diet	chow			EZ		
Virus	control		G5G8	control	G5G8	
ABCG5	0.9±0.1	1.0±0.1	1.4±0.3	0.8±0.4	1.3±0.3	
ABCG8	1.0±0.1	1.0±0.1	1.2±0.2	0.8±0.2	1.3±0.3	
ABCA1	0.7±0.1	1.0±0.6	0.9±0.2	0.8±0.4	1.0±0.3	
LXRα	0.9±0.1	1.0±0.2	0.7±0.2	0.7±0.3	0.8±0.3	
SREBP2	1.6±0.3	1.0±0.4	0.6±0.2	0.8±0.4	0.6±0.2	
HMGCR	2.7±0.7 ^a	1.0±0.7	1.0±0.3	1.2±0.4	1.1±0.5	
HMGCS	1.0±0.3	1.0±0.8	0.3±0.1 ^ª	0.5±0.1	0.3±0.1 ^ª	
NPC1L1	1.0±0.1	1.0±0.1	0.7±0.2	0.7±0.1	0.9±0.3	
SR-BI	1.3±0.2	1.0±0.1	1.1±0.2	1.4±0.5	0.1±0.3	

TABLE 3.2: Intestinal gene expression (mRNA) in lean and obese mice following infection with ABCG5/G8 in the presence and absence of ezetimibe (EZ)

TABLE 3.3: Hepatic and intestinal lipids in lean and obese mice following infectionwith G5G8 in the presence and absence of ezetimibe (EZ)

Phenotype	lean	obese				
Diet	chow			EZ		
Virus	control		G5G8	control	G5G8	
Intestine (mg/g)						
TC	0.56±0.1	1.25±0.5	1.2±0.6	1.6±0.2	2.83±1.2 ^a	
TG	3.78±0.2	7.53±2.9	7.03±1.5	9.22±2.7	14.79±4.2 ^a	
PL	6.22±2.0	8.74±0.6	8.94±3.4	14.3±1.8	16.8±1.8 ^a	
Liver (mg/g)						
TC	1.88±0.2	2.04±0.3	2.63±0.2	1.8±0.4	1.93±0.7	
TG	5.22±0.4 ^a	17.3±2.7	21.03±0.9	21.3±1.6	19.62±2.3	
PL	4.1±0.4 ^a	12.5±1.4	15.35±0.4	11.1±1.8	12.86±2.2	

TABLE 3.4: Hepatic expression of genes (mRNA) involved in HDL biogenesis and bile
acid homeostasis

Phenotypes	obese				
Diet	cł	now	EZ		
virus	control	G5G8	control	G5G8	
ABCA1	1.0±0.3	1.6±0.2	1.6±0.1	1.0±0.3	
Apo-A1	1.0±0.4	0.9±0.2	0.7±0.4	0.8±0.4	
LXRα	1.0±0.1	1.2±0.2	0.9±0.2	1.0±0.1	
FXR	1.0±0.2	0.8±0.1	1.0±0.3	1.0±0.2	
LRH-1	1.0±0.5	1.4±0.1	1.5±0.3	1.0±0.5	
Cyp7A1	1.0±0.1	1.1±0.8	0.7±0.4	1.1±0.8	
Cyp8b1	1.0±0.3	0.5±0.5	0.8±0.6	0.6±0.3	





DISCUSSION

Mice lacking functional leptin or its receptor have decreased levels of hepatic ABCG5/G8, reduced biliary cholesterol excretion and increased HDL cholesterol associated with a delayed clearance of these particles. In the present study, I tested the hypothesis that accelerating biliary cholesterol excretion by adenoviral expression of ABCG/5G8 could correct dyslipidemia in db/db mice. These results demonstrate that accelerating biliary cholesterol excretion can reduce both HDL and large HDL₁ particles characteristic of db/db mice when cholesterol absorption is inhibited with EZ. However, the combination of these treatments failed to fully correct dyslipidemia. This may be due to the acute nature of the experiment; perhaps sustained expression of ABCG5/G8 in the presence of EZ could fully restore the lipoprotein profile in db/db mice.

The role of enhanced ABCG5/G8 activity in opposing plasma accumulation of dietary cholesterol in ApoB containing lipoproteins is well established [244]. In the present study, hepatic ABCG5/G8 partially corrected a dyslipidemia characterized by increased cholesterol in ApoA containing lipoproteins in mice maintained on low cholesterol diets. The classical view of RCT presumes that cholesterol elimination requires delivery of cholesterol from peripheral tissues to the liver. However, an emerging body of literature supports a significant role for transintestinal excretion of cholesterol and recent studies have produced conflicting results on the necessity of biliary cholesterol excretion on macrophage RCT [172, 174, 245-247]. The relative contribution of this alternative pathway on overall RCT and in opposing the accumulation of endogenously synthesized cholesterol in plasma is not known.

The data are consistent with previous reports that EZ promotes transintestinal elimination of cholesterol. Despite a five-fold increase in fecal sterol loss, EZ treatment alone failed to lower serum cholesterol in db/db mice. My analysis of hepatic and intestinal gene expression as well as lipids in these tissues and bile did not reveal the

source for increased fecal sterols in EZ treated mice. Similarly, biliary cholesterol excretion was increased almost eight-fold in ABCG5/G8 infected mice in the absence of EZ without a reduction in plasma cholesterol. Clearly, this level of excretion reduced the regulatory pool of cholesterol in hepatocytes which unregulated the expression of cholesterol synthetic genes. However, I saw no increase in hepatic SR-BI or LDLR mRNAs. This observation suggests that biliary cholesterol excretion and hepatic uptake of HDL are functionally uncoupled. Whether this is a common feature or unique to db/db mice it is not known.

Although leptin has been shown to increase SR-BI levels in ob/ob liver, there are conflicting reports on whether the abundance of the HDL receptor is reduced [232, 241]. Unfortunately, our study does nothing to resolve this issue. In independent cohorts of db/db mice I obtained different results for SR-BI levels in the liver. Housing, diet, light: dark cycle and other conditions of animal husbandry were not different between these two groups. Although these mice were of different ages, this explanation is unsatisfying. What can be said is that there is no correlation between HDL levels in plasma and alterations in SR-BI abundance in db/db mice. It is also important to note that I did not determine HDL clearance rates or other measures of SR-BI function in the studies.

In conclusion, effective cholesterol lowering via enhanced biliary excretion was entirely dependent on EZ treatment. Put another way, reducing plasma cholesterol by inhibition of cholesterol absorption was entirely dependent on enhanced biliary cholesterol excretion. These observations reveal the requirement for a cooperative relationship between the liver and intestine for reducing plasma cholesterol in db/db mice.

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Chapter 4: PLANT STEROLS INFLUENCE ABC TRANSPORTER EXPRESSION, CHOLESTEROL EFFLUX AND INFLAMMATORY CYTOKINE SECRETION IN MACROPHAGE FOAM CELLS

INTRODUCTION

A number of functional foods contain added phytosterols, a mixture of commonly consumed non-cholesterol sterols (sitosterol, campesterol and stigmasterol) found in the oils of the seeds, beans, and legumes of the plants from which they are extracted [18]. When supplied at a dose of 2-4 g per day, phytosterol-esters and their fully hydrogenated stanol-ester derivatives reduce LDL cholesterol by approximately 10%, even when added to statin therapy [248-250]. However, patients consuming phytosterols in the form of supplements and functional foods have increased phytosterols in plasma and tissues [18, 248-251]. It is not known if this increase in plasma phytosterols is required for their cholesterol lowering effect, nor is it known if this level of accumulation confers cardiovascular risk or benefit.

There is considerable controversy in the literature concerning the association between plasma levels of plant sterols and the incidence of cardiovascular disease [248, 250, 252]. As with the clinical data, studies in mouse models of atherosclerosis have generated mixed results. Phytosterol supplementation in mice lacking one copy of the LDL receptor resulted in a reduction in both plasma cholesterol and vascular lesion area [22]. However, a more recent study in ApoE deficient mice showed that phytosterol supplementation impaired endothelial function, increased lesion size following cerebral artery occlusion, and increased atherosclerotic lesion area compared to mice treated with the cholesterol absorption inhibitor, EZ [251]. In fact, patients that reported regular use of phytosterol supplements had the highest phytosterol concentrations in both

plasma and lesions [251]. However, no conclusions can be made concerning the role of phytosterols in disease progression.

Studies addressing cardiovascular phenotypes in both humans and rodents have generally been limited to commercially available mixtures of phytosterols. However, it is clear from a variety of in vitro studies that individual phytosterols have distinct biological activities that include the modulation of signaling pathways and activation of cellular stress responses, growth arrest, and death mechanisms [253-255]. Many of these have implications for lipid metabolism, inflammation and the development of cardiovascular disease. The pro-apoptotic characteristics of sitosterol recognize this plant sterol as cytotoxic and chemotherapeutic-sensitizing agent to cancer cell lines [255, 256]. Stigmasterol and campesterol, but not sitosterol, interfere with SREBP processing and reduce the expression of genes in the cholesterol biosynthetic and uptake pathways in Y1 adrenal cells [257]. Independently of SREBP processing, stigmasterol is shown to be an LXR ligand that promotes the expression of ABCA1 and ABCG1, two transporters involved in the reverse cholesterol transport pathway that opposes cholesterol accumulation in tissues [257, 258]. Conversely, stigmasterol had no effect on LXR dependent gene expression, and antagonized FXR and PXR activity in hepatocytes [259]. Collectively, these observations indicate that the biological activity of phytosterols is both cell-type and sterol specific.

Campesterol, sitosterol and stigmasterol individually have the potential to decrease apoB hepatic levels and retard the production of atherogenic lipoproteins in liver (HepG2) and intestinal (CaCo-2) cell lines [27]. Whereas stigmasterol has the ability to suppress hepatic HMGR and CYP7A1 activity, reduce cholesterol absorption and decrease hepatic cholesterol content when fed to rats, sitosterol accelerates cholesterol synthesis and induces cell death in mouse peritoneal macrophages [28-30].

Experimental Rationale

There is inconsistency in the literature regarding the beneficial or deleterious effects of plant sterols. Although phytosterols accumulate in vascular lesions, the actions of phytosterols on macrophage function are poorly understood. Clearly, plant sterols confer LXR agonist activity or/and interfere with SREBP processing influencing cholesterol homeostasis. We hypothesized that individual phytosterols would differentially influence macrophage ABC transporter abundance, cholesterol efflux and inflammatory cytokine secretion.

EXPERIMENTAL PROCEDURES

Reagents and Buffers: Stigmasterol, 22(R)-dehydrocholesterol, and 5µ-cholestane were purchased from Steraloids (Newport, RI). Cholesterol, β-sitosterol, campesterol, brassicasterol, Phorbol 12-myristate 13-acetate (PMA) and 1α , $2\alpha I^{3}H$]-cholesterol were purchased from Sigma (St. Louis, MO). Sterols were solubilized in 100% ethanol at a final concentration of 5 mg/ml. RPMI 1640 medium, Fetal Bovine Serum (FBS), and Lglutamine were purchased from Atlanta Biologicals (Lawrenceville, GA). Penicillin/Streptomycin was obtained from Invitrogen/Gibco (Carlsbad, CA). Human Apo A-I was purchased from Biodesign International (Saco, ME). Anti-ABCG1 antibody was purchased from GeneTex (San Antonio, TX). Anti-ABCA1 antibody was a kind gift from Mason Freeman (Harvard Medical School, Boston, MA). Quantitative real-time PCR, the preparation of membrane proteins, SDS-PAGE, and immunoblotting were conducted as described in CHAPTER 2.

Animals and Cell Culture: All animal procedures were conducted in accordance with the University animal care and use committee. C57BL6/J male mice (Jackson Laboratories, 8-10 weeks) were injected intraperitoneally with 2 ml of sterile 10% Brewer's thioglycollate medium. Five days after injection, macrophages were collected by

peritoneal lavage using sterile phosphate-buffered saline (PBS). Mouse peritoneal macrophages (MPMs) were washed with PBS, recovered by centrifugation at 500 x g (10 min, 22°C), suspended in <u>Medium A</u> (RPMI 1640 containing 10 mM HEPES buffer, gentamicin (50 µg/ml), streptomycin (100 µg/ml), penicillin (100 IU/ml), and sodium bicarbonate (2 g/L), 7.5% FBS). Cells (9 x 10^6) were plated in 10 cm dishes for 4 hours. Cells were washed once, fed Medium A and cultured for 24 hr prior to initiation of experiments. For treatment with sterols, cells were incubated in <u>Medium B</u> (RPMI 1640 containing 10 mM HEPES buffer, gentamicin (50 µg/ml), streptomycin (100 µg/ml), streptomycin (100 µg/ml), penicillin (100 IU/ml), and sodium bicarbonate (2 g/L), 2 mg/ml fatty acid free BSA). <u>Medium C</u> consisted of Medium B supplemented with sodium compactin (5 µM) and mevalonate (50 µM).

Human monocyte/macrophages (THP-1) were obtained from the American Type Culture Collection (ATCC) and maintained in <u>Medium D</u> (RPMI 1640 containing 10 mM HEPES buffer, gentamicin (50 μ g/ml), streptomycin (100 μ g/ml), penicillin (100 IU/ml), and sodium bicarbonate (2 g/L), 5% FBS) according to the suppliers instructions. For studies of THP-1 macrophages, monocytes were seeded at a density of 1.5 x 10⁶ cells per well in 6-well plates in Medium D containing 50 ng/ml phorbol myristate acetate (PMA) and allowed to differentiate into macrophages for 72 hrs. Following differentiation, the medium was removed, the cells were washed twice with Medium B, and treatments applied as in MPMs as indicated.

Lipoproteins: Low density lipoprotein (LDL; d=1.020-1.063 g/ml) and HDL (d=1.063-1.21 g/ml) were isolated as previously described and generously provided by Dr. Marcielle de Beer (Cardiovascular Research Center, University of Kentucky) [260]. Aggregated LDL (agLDL) preparation: isolated LDL (1 mg/ml protein) was aggregated by vortexing for 1 min. To break large aggregates, the solution was sonicated for 10 min (70% duty cycle) on ice using a Branson Sonifier and passed through a 0.45 μ m filter. Measurement of thiobarbituric acid-reactive substances (TBARS) was conducted to confirm the absence

of oxidation during the aggregation procedure. For the incorporation of phytosterols into agLDL, aggregation was conducted in the presence of the indicated sterol. Partitioning of exogenously added sterols into agLDL was confirmed using [³H]-cholesterol and [³H]-sitosterol. Greater than 99% of labeled sterols were TCA precipitable under these conditions (not shown).

Cholesterol Loading and Analysis: To measure cholesterol loading, macrophages were incubated for 48 hr at 37°C in Medium B alone, in the presence of the indicated sterols delivered in ethanol, or in 100 µg protein/ml agLDL containing the indicated sterols and their concentrations. Following extensive washing, total cellular lipids were extracted twice with 2 ml of hexane:isopropanol (3:2), dried under nitrogen gas and suspended in 1 ml of 33% KOH (in ethanol) containing 5 µg of 5 α -cholestane as an internal standard. Samples were saponified at 70°C for 2 h. Water (1 ml) and petroleum ether (2 ml) were added to each sample. Samples were vigorously vortexed for 2 min, centrifuged (2000 x g, 10min, and 22°C), and the organic phase collected, and dried under nitrogen gas. Sterols were derivatized using N,O-Bis(trimethylsilyl)trifluoroacetamide:pyridine (1:1) (Sigma) and assayed by gas chromatography-mass spectroscopy (GC-MS) as previously described [261]. Cell proteins were solublized in 1N NaOH overnight and total protein determined by BCA assay (Pierce). Total cellular sterol content was expressed as µg sterol per mg total cell protein after normalization to the internal standard. The limit of detection for sterols by GC-MS is 50 ng/mg total cell protein.

Cholesterol Efflux: THP-1 and mouse peritoneal macrophages were loaded with agLDL (100 µg/ml protein) containing the indicated sterols and 1 µCi/ml [³H]-cholesterol for 24 hours in <u>Medium E</u> (RPMI 1640 containing 10 mM HEPES buffer, gentamicin (50 µg/ml), streptomycin (100 µg/ml), penicillin (100 IU/ml), and 2 g/L sodium bicarbonate, 1% FBS). [³H]-cholesterol (10 µCi/mg LDL protein) was added to LDL prior to aggregation. Cells were washed and allowed to equilibrate in <u>Medium F</u> (RPMI 1640, 10 mM HEPES buffer, 0.2 mg/ml fatty acid free BSA) for 1 hr. Cells were washed and cholesterol efflux was

determined in the presence or absence of Apo AI (30 μ g/mI) or human HDL (100 μ g/mL) in Medium B for 4 hr. [³H]-cholesterol present in the medium and cells was determined by liquid scintillation counting, normalized to total cell protein and percent efflux calculated. Percent efflux from cells incubated in medium containing neither acceptor was subtracted as background.

Inflammatory Cytokine Measurement: Inflammatory cytokine production was measured in the supernatants of THP-1 macrophages cultured in medium (control), agLDL (100 μ g/ml) prepared in the presence of carrier or the indicated sterol (10 μ g/ml) for 48 hours. As a positive control for activation of macrophages, cells were incubated for 24 hr in the presence of IFN γ (20 ng/ml) followed by LPS (100 ng/ml). The culture media were collected and centrifuged to remove non-adherent cells. Cytometric Bead Array (CBA) Kits (BD Pharmingen, San Jose, CA) were utilized to simultaneously quantify the following cytokine concentrations: TNFα, interleukin (IL) -1β, IL-6, IL-8, IL-10 and IL-12 as previously described [262, 263]. These cytokines, with the exception of IL-10, are indicative of the inflammatory function of macrophages produced at high levels through classical activation. Bead populations with distinct fluorescence intensities coated with capture antibodies specific for each cytokine were incubated with fluorochrome conjugated detection antibodies along with 50 μ l of a two-fold dilution of each sample for 3 hours at room temperature. Fluorescence intensities were assayed by flow cytometry and compare to a standard curve generated for each cytokine to determine the concentration in each sample.

Statistical Analysis: Data were analyzed by one-way ANOVA to determine if there were differences among treatment groups. A post-hoc Dunnett's multiple comparison test was conducted to compare each treatment to the control.

RESULTS

Previous reports indicate that the effects of phytosterols on LXR and SREBP-2 target genes are cell type dependent. To determine their effects in macrophages, I evaluated ABCA1 and LDLR protein abundance in elicited MPMs following treatment with individual non-cholesterol sterols differing at the 22 and 24 carbon positions within the cholesterol side chain (Figure 4.1).



FIGURE 4.1. Effect of 22 and 24 substitution of the cholesterol side-chain on expression of ABCA1 and LDLR in MPMs. A) Immunoblot analysis for ABCA1 and LDLR levels from MPMs. B) Densitometric analysis of the signal intensities for ABCA1 and LDLR were normalized to the values obtained for Calnexin. Data represent the mean \pm SD. (*) denote significant differences from control treated cells at p < 0.01.

Cells were harvested, cultured for 24 hr and incubated in the presence of serum free medium containing carrier (Control, ethanol) or medium supplemented with 50 µg/ml of the indicated sterol for 48 hr. Consistent with previous reports in Y1 adrenal cells, stigmasterol and 22(R)-dehydrocholesterol increased ABCA1 expression and decreased LDLR abundance whereas the remaining sterols had no effect. One potential explanation for the differences in responses among the sterols is their entry and accumulation in macrophages.

The concentrations of phytosterols used in this and previous studies are substantially greater than what are typically observed in plasma of individuals consuming phytosterol supplements. To determine if phytosterols affected the expression of ABCA1 and LDLR at concentrations that are observed in vivo (4-20 μ g/ml [30]), I conducted a time-course experiment using 10 µg/ml phytosterol (Figure 4.2A). Among the commonly consumed phytosterols, only stigmasterol increased expression of ABCA1 and decreased expression of LDLR. The effects of stigmasterol on ABCA1 and LDLR were discernable by 4 hours and persisted up to 48 hours. However, LDLR expression decreased in control cells after 24 hours, suggesting that reductions over this period were unrelated to the presence of stigmasterol. This result also implies that the cellular content of cholesterol is dynamic over the 72 hr culture period and that the effects of stigmasterol may be dependent upon, or secondary to changes in endogenous cholesterol synthesis, a known source of LXR ligands [258]. To address this concern and to determine the minimal concentration of stigmasterol required to elicit changes in ABCA1 and LDLR abundance, a dose-response experiment was conducted in the presence of the HMGCR inhibitor, compactin (Figure 4.2B). The presence of compactin suppresses ABCA1 and upregulates LDLR. Therefore, 1 μ g/ml 25-hydroxycholesterol (25-OH-C) was used as a positive control since it is known to be both an LXR agonist and a suppressor of SREBP processing. Stigmasterol treatment (0 to 50 μ g/ml for 24 h) resulted in an increase in ABCA1 that was detectible at 1 μ g/ml, increased substantially at 10 μ g/ml, and continued to increase, albeit to a lesser extent from 10 to 50 μ g/ml. For LDLR, a modest suppression was observed at 0.5 μ g/ml and further increases in stigmasterol resulted in complete suppression of immunoreactive LDL receptor. These results demonstrate that the effects of stigmasterol on LDLR and ABCA1 in macrophages are dose-dependent and not due to accumulation of cholesterol biosynthetic intermediates.



FIGURE 4.2. Effect of time and concentration of stigmasterol on immunoreactive ABCA1 and LDLR in elicited MPMs. ABCA1, LDLR and Calnexin protein expression analyzed after A) Time-course experiment with MPMs cultured in Medium B containing stigmasterol (10 μ g/ml) for up to 48h. B) Concentration dose dependent effect of stigmasterol on MPMs incubated in Medium C. 25-OH-C was used as a positive control for 24 hrs.

Next I determined if the effect of stigmasterol on ABCA1 abundance was associated with changes in mRNA levels for this and other LXR target genes (Figure 4.3). Each of the LXR target genes was increased by stigmasterol, but not sitosterol.



FIGURE 4.3. Effect of stigmasterol on the expression of LXR (A) and SREBP1 (B), SREBP2 (C) and selected target genes in MPMs. Macrophages were incubated in Medium C

(Control) or Medium C supplemented with LXR agonist, TO901317 (1 μ M) was used as a positive control, sitosterol (10 μ g/ml), stigmasterol (10 μ g/ml), or 25-OH-C (1 μ g/ml) for 24 hrs. Total RNA was isolated and processed for quantitative RT-PCR. Data represent the mean ± SD. (*) denote significant differences from control treated cells at *p* < 0.01.

Similarly, campesterol did not alter LXR target gene expression. We also evaluated expression of SREBPs and selected targets. Not surprisingly, SREBP-1c was also upregulated by stigmasterol as well as its downstream targets fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC1). However, stigmasterol had no effect on SREBP2 or its target genes, suggesting the mechanism by which stigmasterol suppresses LDLR protein is post-transcriptional, distinct from that of 25-OH-C, and independent of interference with SREBP processing.

Although plant sterols may affect gene expression and cholesterol trafficking when added directly to the culture medium, macrophage foam cells acquire plant sterols from modified lipoproteins *in vivo*. Moreover, increased atherogenicity is suggested to occur when lipid stores are accumulated in the lysosomes and are less available for efflux from the macrophages compared to cytoplasmic occlusions [264]. Commonly used ox-LDL and acetylated LDL (ac-LDL) when incubated with MPMs accumulate cholesterol predominantly in cytoplasmic occlusions [265]. In addition, LDL aggregates are poorly processed by MPMs [266]. Therefore we selected THP-1 cells since these cells are an established model of macrophage foam cells that readily internalize and process agLDL in lysosomes [267]. First, I confirmed that the effects of individual phytosterols on ABC transporter expression would persist in lipid loaded cells and that they were not unique to MPMs. Following differentiation, THP-1 macrophages were cultured with medium supplemented with agLDL (Control) or agLDL prepared in the presence of the indicated sterol. As a positive control, cells were incubated in medium containing both agLDL and an LXR agonist (TO901317).



FIGURE 4.4. Effect of major phytosterols on expression of ABCA1 and ABCG1 and cholesterol efflux to ApoAI and HDL in agLDL loaded THP-1 macrophages. A) Immunoblotting analysis for levels of ABCA1 and ABCG1 after 48 hr incubation in Media E containing carrier (agLDL, 100 mg/g protein), T0901317 (10 μ M) and designated sterols (10 μ g/ml). B) Percent cholesterol efflux assay measured in a parallel experiment. [³H]-cholesterol (10 μ Ci/mg LDL protein) was added to LDL prior to aggregation. Data are the mean ± SD of three replicates. (*) denote significant differences (*P*< 0.05) compared to control cells.

As an additional control, agLDL was prepared in the presence of cholesterol to maintain equality of total added sterols. Following 48 hr of treatment, membrane proteins were prepared and analyzed by SDS-PAGE and immunoblotting (Figure 4.4 A). Incubation of THP-1 macrophages with agLDL in the absence of additional sterols increases ABCA1 and suppresses LDLR below the limits of detection (not shown). The addition of the LXR agonist further increased ABCA1 protein in agLDL loaded macrophages. ABCG1 was also increased in THP-1 loaded macrophages, the measurements of which proved difficult in mouse macrophages using commercially available antibodies. The incorporation of cholesterol, sitosterol and campesterol in agLDL had no effect on ABCA1 or ABCG1 abundance. Consistent with mRNA data in MPMs, the inclusion of stigmasterol in agLDL increased both transporters in human macrophages, whereas other phytosterols had no effect.

Sterol	agLDL +					
	Control	Carrier	Cholesterol	Stigmasterol	Sitosterol	Campesterol
Cholesterol	7.89	42.96	47.36	49.00	45.21	44.68
	± 0.74	± 5.74	± 6.52	± 6.18	± 10.18	± 1.92
Stigmasterol				6.41		
				± 2.43		
Sitosterol					5.28	
					± 1.22	
Campesterol						14.16
						± 1.01
Total Sterols	7.89	42.96	47.36	55.41	50.49	58.84
	± 1.04	± 5.74	± 6.52	± 5.14	± 3.65	± 3.37

TABLE 4.1: Cellular sterol content (μ g/mg total cell protein) following incubation with agLDL prepared in the presence (+) of the indicated sterol

Values represent means ±SD. Empty cells indicate that levels were below the limits of detection by GC-MS (50 ng/mg total cell protein).

Cellular sterol content was determined before and after incubation with agLDL (Table 4.1). Lipids were extracted and analyzed by GC-MS. Incubation of THP-1 macrophages with agLDL resulted in a 5-6 fold increase in total cellular sterol content. The addition of phytosterols collectively and individually had no effect on the extent of cholesterol accumulation or total cellular sterol content when compared to the cholesterol control, indicating that changes in ABC transporter expression are not merely a function of total cholesterol or sterol content of THP-1 macrophages.

Next I determined if phytosterols altered efflux of cholesterol from agLDL loaded THP-1 macrophages to apoA-I and HDL (Figure 4.4 B). THP-1 monocytes were differentiated into macrophages and incubated with agLDL prepared in the presence of [³H]cholesterol and the indicated sterol for 48 hours (100 µg/ml LDL, 10 µg/ml sterol, 1 μ Ci/ml [³H]-cholesterol). Following the loading phase, the cells were washed and allowed to equilibrate in serum free medium for 1 hr. The equilibration medium was removed, the cells were washed and the medium replaced in Medium B containing apoA-I (30 μ g/ml) or HDL (100 μ g/ml) for 4hr. Relative to control cells, in which no additional sterols were added to the LDL aggregates, TO901317 enhanced efflux of [³H]cholesterol to both apoA-I and HDL. Sitosterol had no effect on efflux to either acceptor, although there was a tendency for a decrease to HDL. The presence of stigmasterol increased efflux to apoA-I by 25% and tended to increase efflux to HDL. Campesterol resulted in a modest, but significant decrease in efflux to HDL, but did not alter efflux to apoA-I. These results indicate that the effects of individual phytosterols on ABC transporter expression and cholesterol efflux are directly correlated in human cultured macrophages.



Figure 4.5. Sterol loading of THP-1 cells induces the synthesis and secretion of TNF α , IL-6 and IL-1 β . THP-1 macrophages were incubated in medium (control), agLDL (carrier) or agLDL with the indicated sterol (10 µg/ml) for 48 h. As a positive control for activation of macrophages, cells were incubated for 24 hr in the presence of IFN γ ng/ml) followed by LPS (100 ng/ml). The amount of **A**) TNF α , **B**) IL-6 and **C**) IL-1 β released to the media were analyzed by CBA assay. Data are the mean ± SEM of six replicates. ** *p* < 0.01 vs. untreated control, * *p* < 0.05 vs. untreated control, † *p* < 0.05 vs. AgLDL + carrier.

Beyond the accumulation of lipid, macrophages contribute to the inflammatory state of the atherosclerotic lesion. To determine if phytosterols alter the inflammatory response to agLDL loading, I evaluated the secretion of inflammatory cytokines in the culture medium using a commercially available cytometric bead assay (CBA) inflammation panel (Figure 4.5). First, I used pretreatment with interferon (IFN)-y followed by lipopolysaccharide (LPS) as a control for classical activation of macrophages. Following pretreatment with IFN- γ , LPS dramatically increased the secretion of TNF α , IL-6 and IL- 1β . Compared to untreated cells, incubation with agLDL increased the expression of each of these proinflammatory cytokines. The effect of phytosterols on the response to agLDL loading was assessed by comparing the levels of secreted cytokines to cells treated with agLDL prepared in the presence of the carrier (ethanol). The presence of stigmasterol decreased agLDL-induced secretion of TNF α , IL-6 and IL-1 β . The presence of sitosterol increased the secretion of TNF α and IL-1 β , but not IL-6. Campesterol had no effect on the inflammatory response to agLDL. Levels of IL-8 were unaffected by treatments. IL-10 and IL12p70 were below the limits of detection in our assay (not shown).

DISCUSSION

These studies report that stigmasterol increases expression of ABCA1 and ABCG1, enhances cholesterol efflux, and decreases the inflammatory response to uptake of modified lipoproteins in multiple models of macrophage foam cells. Conversely, sitosterol exacerbated the inflammatory response of agLDL loading and tended to decrease cholesterol efflux. Although campesterol had no effect on the abundance of ABC transporters or secretion of cytokines, it had a modest inhibitory effect on cholesterol efflux from agLDL loaded macrophages to HDL.

The increase in efflux to apoA-I and HDL in the presence of stigmasterol is presumably mediated by the increase in ABCA1 and ABCG1 protein, respectively. However, a role for other sterol transporting proteins such as SR-BI, CD36 and ABCG4 cannot be excluded. Further complicating matters is the fact that LXR agonists have been shown to enhance efflux of cholesterol by promoting the transport of cholesterol to the cell surface in human macrophages [268]. Given that stigmasterol interacts with at least two independent sterol sensing mechanisms in other cell types, the precise mechanism(s) by which stigmasterol enhances cholesterol efflux to apoA-I and HDL in macrophages remains difficult to definitively establish. Similarly, the mechanism for suppression of inflammatory cytokine secretion remains unknown, but is likely related to activation of LXR signaling based on the increase in LXR target genes by this sterol and emerging role of this nuclear hormone receptor in the suppression of inflammation [269].

The suppression of LDLR by stigmasterol appears to be independent of disruptions in SREBP2 processing since target genes for this transcription factor are unaffected by this phytosterol. It is tempting to speculate that the mechanism is also LXR dependent based on recent reports of LXR-mediated LDLR degradation [104]. However, I did not pursue this effect of stigmasterol in macrophage foam cells since lipid loading in the absence of

stigmasterol or the synthetic LXR ligand is sufficient to suppress LDLR levels below the limits of detection.

In general terms, the effects of sitosterol were opposite of stigmasterol. Sitosterol increased the inflammatory response of agLDL loaded macrophages and tended to reduce cholesterol efflux. However, sitosterol had no effect on immunoreactive levels of ABC transporters or mRNA levels of any of the transcripts examined. Previous reports in macrophages and other cell types indicate that sitosterol inhibits cell growth, activates components of the integrated stress response and at sufficient concentrations is toxic to cells [254, 255]. I did not observe cytotoxicity with sitosterol treatment in these studies, but it is important to note that it did not quantify direct measures of cellular stress since these effects were beyond the scope of our study.

These studies add to a growing body of literature demonstrating that individual phytosterols affect a number of signaling, trafficking, and enzymatic mechanisms with implications in the development and progression of cardiovascular disease. The relative abundance of stigmasterol in commercially available phytosterol supplements and functional foods compounded with its limited absorption make it unlikely that the levels of this individual phytosterol accumulate in sufficient quantities to have a significant positive impact on the reverse cholesterol transport or inflammatory pathways within macrophages. However, supplying stigmasterol as the sole source of phytosterol in the diet increased its levels to 20 μ g/ml in serum and reduced cholesterol absorption, plasma cholesterol and hepatic HMGCR activity, suggesting that the beneficial effects of this phytosterol are achievable [30].

A critical question with respect to the use of phytosterols as supplements and within functional foods is whether the benefits of cholesterol lowering are greater than potential risk associated with the accumulation of plant sterols in plasma and tissues. It is important to note that humans consume significant amounts of phytosterols depending on their diet and phytosterol supplements are generally regarded as safe. However, cholesterol lowering therapies persist for decades. Increasing phytosterol consumption to levels sufficient for cholesterol lowering, particularly in patients that harbor polymorphisms in *ABCG5/ABCG8*, may limit cardiovascular benefit depending on sterol composition. On the other hand, added benefit may be achievable through the use of supplements enriched in stigmasterol or other 22-dehydrosterols. Additional studies of individual phytosterols are required to determine if sterol composition can be optimized to achieve added cardiovascular benefit beyond cholesterol lowering.

Chapter 5: SUMMARY OF RESEARCH

The clinical and epidemiological importance of CVD related to vascular lesion development has been growing rapidly in the recent decades. Obesity, insulin resistance and hypercholesterolemia are frequent metabolic derangements found in patients with progressing atherosclerosis, and are often linked to impaired reverse cholesterol transport (RCT). RCT is an atheroprotective process whereby ABC cholesterol transporters (ABCA1, ABCG1 and ABCG5/G8) play a crucial role in initiating the movement of cholesterol from peripheral tissues and vascular lesion macrophages to the liver as well as promoting hepatic cholesterol excretion out of the body. The major findings of these studies are the post-transcriptional regulation of the ABCG5/G8 complex and its cooperate role in the liver and intestine in the process of maintaining cholesterol homeostasis. My data demonstrated that a defect in the leptin axis is associated with reductions in ABCG5/G8 protein. Leptin replacement and caloric restriction restored ABCG5/G8 abundance which were further associated with increased biliary cholesterol excretion and corrected hypercholesterolemia in ob/ob mice. Although TUDCA treatment and liver specific ABCG5/G8 adenoviral replacement increased ABCG5/G8 protein expression and accelerated biliary cholesterol secretion, it failed to correct dyslipidemia in obese db/db mice. Further, adenoviral ABCG5/G8 ameliorated high cholesterol levels only when cholesterol reabsorption was inhibited, underscoring the collaborative role between hepatic and intestinal ABCG5/G8. In the last chapter, my in vitro studies reported that phytosterols influence the initial step of RCT and had diverse effects on cholesterol efflux and pro-inflammatory response in macrophage foam cells.

Beyond the fact that ABCG5 and ABCG8 are N-linked glycoproteins which reside in the ER as monomers and require CNX or CRT for proper protein folding for heterodimer complex formation and trafficking to the cell surface, little is known about the post-transcriptional regulation of these cholesterol transporters. To gain insight into the

process, series of in vivo experiments were conducted in leptin-axis defective mice (db/db and ob/ob). These strains exhibit pronounced obesity, dyslipidemia and reduced biliary cholesterol secretion. In fact, the mice remain resistant to gallstone formation while maintained on high cholesterol and cholate diet, suggesting an effect of leptin deficiency on hepatobiliary regulation [214]. Hepatic immunoblot analysis from db/db and ob/ob mice revealed that impaired cholesterol excretion into the bile is associated with a reduction in ABCG5/G8 protein. This decrease did not correlate with a reduction in messenger RNA of the sterol transporters. Cell culture studies indicated that the halflife of the immature forms of ABCG5 and ABCG8 are significantly shorter compared to the mature forms, but the two forms of the proteins are present in similar amount [74]. My animal studies revealed that there was a difference between the processed and unprocessed form of ABCG5 and ABCG8 in wild type control mice, suggesting efficient assembly of the complex in the ER and/or prolonged stability of the post-Golgi complex. in vivo. Caloric restriction and leptin replacement increased the stability of the unprocessed protein form and restored the mature ABCG5/G8 form in ob/ob mice, repairing biliary cholesterol secretion. In fact, leptin administration alone increased ABCG5/G8 levels greater than those detected in wild type and pair-fed mice. However, there was no correlation between cholesterol transporter expression and biliary cholesterol concentrations, which possibly has a gallstone protective function. Cholesterol transport from the hepatocyte into the bile is a complex process which is still under investigation. Expressed in locations where bile acids are present in high concentrations, ABCG5/G8 acts as a floppase, transferring cholesterol from the inner to the outer leaflet of the plasma membrane [239]. In cell models, ABCG5/G8 cholesterol efflux is greatly dependent on mixed bile salt micelles which serve as a cholesterol acceptor [54]. Lastly, Harvey et al. have demonstrated that bile acids stimulate ATP hydrolysis in ABCG5/G8, possibly promoting an active confirmation of the complex.

The exact mechanism by which leptin signaling normalized abundance of the transporters remains unknown. The uncoupling of biliary cholesterol concentrations

from ABCG5/G8 protein levels suggest that ABCG5/G8 transporter activity is regulated beyond abundance of mRNA and protein. Indeed, Cohen et al have reported that intracerebroventricular leptin administration in rats exerts the same effect in liver metabolism as intravenously administration, indicating that the hepatic response to leptin is conducted through the central nervous system [270]. However, direct activation of hepatic leptin signaling is not excluded since many of the leptin receptor (ObR) spliced forms were found also in liver. Additional experiments need to be conducted in order to be determined whether hepatic ObR-s independently triggers leptin signaling activation.

Obesity and insulin resistance have been associated with ER-stress. It has been demonstrated that obesity creates ER-stress and initiates the unfolded protein response signaling pathways not only in peripheral tissues but in the hypothalamus, which sequentially leads to inhibition of leptin receptor signaling and promotes leptin resistance [219, 271]. Dietary restriction has been shown to have beneficial effects on obesity and insulin resistance, indicating possible ER-stress amelioration. Alleviation of ER-stress with the chemical chaperons PBA and TUDCA sensitized leptin receptor signaling in the hypothalamus and corrected hyperglycemia in ob/ob mice [219, 271]. However, this mechanism is not dependent entirely on CNS since PBA-treatment in db/db mice did not exert leptin-sensitizing effect in hypothalamus but decreased blood glucose levels, indicating direct effect of the molecular chaperone in liver. My results revealed that long term treatment (10 days) with TUDCA improved glycemic control and increased ABCG5/G8 transporter in db/db mice. However, the TUDCA effect was not genotype specific, since transporter protein levels were increased in wild-type mice, suggesting that the ER may possess a significant number of ABCG5 and ABCG8 monomers, which shortly after synthesis are degraded and never become functionally active. However, little is known about TUDCA mechanism of action as a molecular chaperone. In addition, TUDCA belongs to the hydrophilic class of bile acids and although it is a very weak agonist it could possibly activate FXR suppressing Cyp7a1.

Moreover, acute liver perfusion with TUDCA increases biliary cholesterol secretion [239]. A similar bile acid, cholic acid, elevates ABCG5/G8 protein and mRNA abundance in wild-type mice and loses its effect in FXR knockouts [202]. Recently, an FXR response element was identified in the promoter unit of ABCG5 and ABCG8 [87]. However, in the current research increased ABCG5 and ABCG8 transcription upon TUDCA treatment was not detected. It could be speculated that TUDCA could act via the membrane bound bile acid receptor TGR5 since the activation and translocation of CFTR (ABCC7) is upregulated via TGR5 agonists [272]. Although biliary cholesterol composition was no different compared to wild type controls, TGR5 deficient mice, similar to ob/ob and db/db mice, had low biliary cholesterol saturation index and were resistant to gallstones when fed on lithogenic diet [273]. The existence of such mechanism regulating ABCG5/G8 complex requires further investigation.

Both db/db and ob/ob develop premature insulin resistance and are a useful model in type 2 diabetes studies. To investigate whether a lack of insulin signaling on ABCG5/G8 stability and exclude a possible influence of obesity, protein levels of the sterol transporters where evaluated in STZ-induced type 1 diabetic lean mice. Besides the differences in blood glucose levels, STZ mice demonstrated similar immunoreactive ABCG5/G8 levels, indicating that hyperglycemia is not sufficient to disrupt ABCG5/G8 post-translational regulation. Conversely, insulin resistant and predisposed to gallstone formation LIRKO mice are characterized with increased expression of ABCG5 and ABCG8 mRNA levels in a FOXO1 dependent manner resulting in accelerated biliary cholesterol concentration [86]. The discrepancy in the results could only be explained with the genetic background of the animal models and requires further investigation.

Hypercholesterolemia and obesity in leptin axis defective db/db and ob/ob mice is fundamentally different from that seen in high cholesterol diet models, which is characterized by increased VLDL and LDL cholesterol. However, this model provides the opportunity to investigate the development of obesity and dyslipidemia on low cholesterol diets. Ob/ob and db/db mice are one of the few mouse models of genetically predisposed hypercholesterolemia associated with leptin deficiency, where cholesterol accumulates in both HDL and a large HDL (HDL₁) fractions. Leptin replacement in ob/ob mice restored biliary cholesterol elimination, increased both SR-BI and ABCG5/G8 proteins, and normalized plasma HDL [216, 241, 242]. Because the molecular mechanism of action initiated by the hormone remained elusive, it has been suggested that impaired apoprotein clearance is due to reduction in HDL receptor (SR-BI) protein and mRNA abundance [241]. However, there is controversy as to whether SR-BI is changed [232]. In order to clarify the ambiguous data regarding protein and transcript SR-BI abundance in ob/ob and db/db mice, I used 2 cohorts of mice of different ages. Whereas older db/db mice had no change in HDL receptor expression compared to lean controls, the younger cohort had significant reductions in both SR-BI protein and mRNA. Unfortunately, my studies cannot provide sufficient explanation for the differences in SR-BI. It can only be concluded that there is no correlation between HDL levels in plasma and alterations in SR-BI abundance in db/db mice.

Chapter 3 addresses the question as to whether acceleration of biliary cholesterol excretion by liver-specific adenoviral expression of ABCG5/G8 could correct dyslipidemia in db/db mice. In order to prevent possible reabsorption of cholesterol from the small intestine, db/db mice were supplemented with EZ, which blocks fractional absorption via the NPC1L1 dependent pathway. Consistent with the observations in Chapter 2, expression of adenoviral ABCG5/8 in liver accelerated biliary cholesterol secretion. However, ABCG5/G8 did not have an effect on plasma cholesterol levels. As a compensatory effect, the reduced regulatory pool of cholesterol in hepatocytes resulted in upregulated expression of cholesterol synthetic genes. However, I saw no increase in hepatic SR-BI or LDLR mRNAs. This observation suggests that biliary cholesterol excretion and hepatic uptake of HDL are functionally uncoupled. Whether this is a common feature or unique to db/db mice it is not known. Similarly, EZ treatment alone increased FNS drastically but failed to lower serum cholesterol in db/db mice. Only in

combination with EZ, ABCG5/G8 could partially correct the dyslipidemic profile in db/db mice maintained on low cholesterol diet. These observations underscore the role of biliary cholesterol secretion and fractional intestinal absorption on plasma cholesterol. Undoubtedly, plasma cholesterol regulation requires a cooperative relationship between the liver and intestine.

Beyond its role in liver, ABCG5/G8 in the intestine is the primary defense for PS accumulation in the body. Sharing similar properties with cholesterol, dietary PS compete with cholesterol for micellar solubilization and thereby interfere with cholesterol absorption. PS dietary supplementation has been used as a cholesterollowering therapeutic reducing LDL-cholesterol level. However, the action of PS goes beyond a simple replacement of cholesterol. PS are bioactive molecules with both celltype and sterol specific effects [255, 257, 258]. Conflicting research reports in humans and mice discuss the beneficial and deleterious effects of plant sterols on dyslipidemia and atherosclerosis [22, 251]. In fact, various reports demonstrate that different dietary sterols have diverse effects on SREBP-processing and LXR-target genes involved in cholesterol metabolism [257, 258]. ABCA1 and ABCG1, two cholesterol transporters involved in the initial step of RCT and oppose cholesterol accumulation in tissues, have been demonstrated to be influenced by PS. The studies in Chapter 4 report the effect of agLDL loaded with commonly encountered dietary sterols on mouse peritoneal and human macrophages. Stigmasterol increased expression of ABCA1 and ABCG1 and enhanced cholesterol efflux. Stigmasterol decreased the inflammatory response to uptake of modified lipoproteins, whereas sitosterol impaired the inflammatory response of agLDL loading. Conversely, sitosterol and campesterol did not change ABCA1 and ABCG1 abundance but tended to decrease cholesterol efflux. Campesterol had a modest inhibitory effect on cholesterol efflux from agLDL loaded macrophages to HDL. The increase in efflux to apoA-I and HDL in the presence of stigmasterol perhaps was predominantly facilitated by the upregulation of ABCA1 and ABCG1. However, the exact mechanism by which stigmasterol promoted cholesterol efflux from macrophages is
difficult to determine since stigmasterol interacts with at least two independent sterol sensing mechanisms (LXR and SREBP-2 dependent gene regulation). Likewise, the mechanism for suppression of inflammatory cytokine secretion remained unclear. This possibly is likely related to LXR activation based on the increase in LXR target genes by this sterol and the emerging role of LXR in the suppression of inflammation [269]. In contrast to other reports, stigmasterol did not have an effect on SREBP-2 transcription factor. Therefore, the suppression of LDLR by stigmasterol possibly relies on LXR dependent mechanism based on recent reports of LXR-mediated LDLR degradation [104]. Although my *in vitro* studies demonstrated beneficial effects of stigmasterol on macrophage function, the relative absorption and abundance of stigmasterol will accumulates in sufficient quantities to have a significant positive impact on RCT or inflammatory pathways within macrophages.

This research demonstrated that leptin signaling corrected hypercholesterolemia and restored hepatic ABCG5/G8 abundance post-transcriptionally. However, neither hepatic-specific ABCG5/G8 expression, nor inhibition of intestinal cholesterol absorption can normalize elevated plasma cholesterol in db/db mice. This finding identified not only distinct role for hepatic and intestinal ABCG5/G8 in modulating sterol metabolism but underscored the obligate cooperative relationship between liver and intestine in regulating plasma cholesterol content. Conversely, ABCG5/G8 in the intestine acts as a primary defense against PS absorption. Very low concentrations of biologically active PS influence the initial step of RCT pathway and the proinflammatory response in macrophage foam cells in vascular lesions. Understanding the nature of the interactions between cholesterol, PS, nuclear hormone receptors and ABC transporters involved in RCT is critical for the development of novel therapies for regulation of lipid homeostasis.

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APPENDICES

ABBREVIATIONS

ABCA1, ABCG1, ABCG5, ABCG8 ATP-binding cassette (ATP) transporter A1, G1, G5; G8; ACAT, acyl-CoA: cholesterol acyltransferase; apo-A1, apoB-48, apoB-100, apolipoprotein A1, B48, B100; CA, BA, bile acid; BS, bile salt; cholic acid; CDCA, chenodeoxycholic acid; CYP7A1, cholesterol 7 α -hydroxylase; CYP8B1, sterol 12 α -hydroxylase; CYP27A1, sterol 27 hydroxylase; DCA, deoxycholic acid; ER, endoplasmic reticulum; FH, familial hypocholesterolemia; FC, free cholesterol; FNS, fecal neutral sterols; FXR, farnesoid-xreceptors; HDL, high density lipoprotein; HMGS, HMG CoA synthase; HMGR, HMG-CoA reductase; IDL, intermediate density lipoprotein; LCA, lithocholic acid; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LRH-1, liver receptor homolog-1; LRP, LDLR related protein; LXR, liver-X-receptors; MTTP, microsomal triglyceride transfer protein; NPC1L1, Niemann-Pick disease type C1 gene-like 1; PL, phospholipids; PS, plant sterol; PBA, 4-phenyl butyric acid; SREBP, sterol response element binding protein; SRE, SREBE response element; SR-BI, scavenger receptor class B type 1; TD, Tangier disease; TUDCA, tauroursodeaxycholic acid; VLDL, very low density lipoprotein; UPR, unfolded protein response; 7 α -OH-C, 7 α -hydroxycholesterol; 25-OH-C, 25-OH cholesterol;

EXPERIMENTAL PROCEDURE PROTOCOLS

A: PREPARATION OF MEMBRANE PROTEINS FROM TISSUES

Solutions:	
Membrane Buffer:	<u>500 ml</u>
20 mM Tris-Cl (pH 7.5)	10 ml 1 M Tris
2 mM MgCl ₂	1 ml 1 M MgCl ₂
0.25 M sucrose	42.8 g
Add Tris and $MgCl_2$ to 400 ml water, pH to	7.5. Add sucrose and qs to 500 ml. Store at
4°C for up to 1 month. Add protease inhibit	ors immediately prior to use.
1:100 Protease Inhibitor Cocktail	
4X Protein Sample Buffer:	<u>500 ml</u>
120 mM Tris base	60 ml 1M Tris
40 mM EDTA	40 ml 1M EDTA
4% SDS	20 g
20% glycerol	100 ml
0.025% bromophenol blue	125 mg
Add Tris, EDTA , SDS and 25 ml water a	nd pH to 6.8. Add bromophenol blue and
glycerol and qs to 50 ml. Membrane protein	ns are typically solubilized in 3% SDS.

1X Protein Sample Buffer:

4X Buffer	2.5 ml
10% SDS	2 ml
Water	5.5 ml

Protocol:

- Weigh out tissue (pulverized) 0.2 g for everything except fat (for fat use 0.4 g) into 14 ml Falcon (2059) tubes containing 1.2 ml of membrane buffer.
- 2. Homogenize approx. 1 min and put on ice (Turax Homogenizer)
- 3. Spin for 10 min., 3500 rpm @ 4 °C (~2000 x g)

- 4. Remove 1 ml of supernatant. Do not take any of the pellet or fat/junk that may be on top of supernatant.
- 5. Place in centrifuge tube for Ti 50.4 (3 ml) rotor.
- 6. Centrifuge at 100,000 x g (30, 500 rpm), 60 min, 4°C.
- 7. Decant supernatant. Leave tube upside down to drain for 1 min.
- Suspend pellet in 1X sample buffer (100 μl 250μl). Use pipette or insulin syringe (Baxter 59505-1) to completely resuspend pellet.
- 9. Transfer to 1.5 ml eppendorf tube. Store at -20 °C. Do not add β -ME until preparing samples for gel.
- 10. Determine protein concentration with BCA. Use 2-5 µl in BCA protein assay
- 11. Immediately prior to SDS-PAGE, add β -ME to a final concentration of 1.2% and heat at 95°C for 5 min.
- 12. Load 50 ug protein/ lane.

B: SDS PAGE

Solutions:

Acrylamide: bisacrylamide – 37.5:1

Lower Tris

	500 ml
Tris-base (1.5 M)	90.75 g
SDS (0.4 %)	20 ml (10%)
ddH ₂ O	qs to 500ml

pH to 8.8 with HCl and store at 4°C

Upper Tris

	200 ml
Tris-base (0.5 M)	29.2 g
SDS (0.4 %)	0.8 g
ddH₂O	qs to 200ml

pH to 6.8 with HCl and store at 4°C

10X Running Buffer

	1.0 L
Tris-base (1X: 25 mM)	30 g
Glycine (1X: 192 mM)	144 g
SDS (1X: 0.1% w:v)	10 g
ddH₂O	qs to 1.0 L

10X Transfer Buffer

1.0 L
30 g
144 g
qs to 1.0 L

No need to pH; pre-chill 1X to 4°C.

5X Sample Buffer

	50 mL
Tris Base (0.3125 M)	1.89 g
SDS (2.5%)	12.5 ml of 10% stock
glycerol (50%)	25 ml
bromophenol blue (0.125%)	62.5 mg
ddH₂O	qd to 50 ml

Protocol:

- 1. Wash glass spacers and combs in EtOH, and assemble plates.
- 2. Pour resolving gel to 0.5 cm below comb.

Mini Gels (18ml/2 gels)

Percent Gel	10 %
ddH₂O	9.0 ml
lower tris	4.5 ml
40% acryl-	4.5 ml
10% aps	180 µl
Temed	20 µl

- 1. Overlay with water saturated isobutanol and allow to polymerize.
- 2. Rinse top of gel with distilled H_2O . Remove remaining water with filter paper.
- 3. Insert comb and pour stacking gel.

Mini (6 ml)

	3.0%	4.0%
ddH ₂ O	4.0 ml	3.9 ml
upper Tris	1.5 ml	1.5 ml
acryl-bis	450 μl	600 μl
10% APS	60 μl	60 µl
TEMED	10 µl	20 µl

- 1. Allow to polymerize and submerge in 1X Running Buffer until use.
- Heat samples to 95°C for 5 min. and quick spin. While samples are heating, remove comb and rinse wells with running buffer.
- 3. Run at 50 mAmps (constant current)
- 4. Stain gels overnight with coomassie and distain.

Transfer to nitrocellulose membrane for western blot

- 1. Remove stacking gel and soak in 1X Transfer Buffer for 5 min.
- 2. Cut two pieces of filter paper and 1 piece of nitrocellulose membrane for each gel.
- 3. Pre-wet nitrocellulose membrane in 1X Transfer Buffer for 5 min.
- Build sandwich at 4°C Transfer Buffer, and run at 200 mAmps (constant current) for 2hrs.
- 5. After transfer, trim excess membrane and allow drying at room temp.

Transfer to nitrocellulose membrane for western blot

- 1. Remove stacking gel and soak in 1X Transfer Buffer for 5 min.
- 2. Cut two pieces of filter paper and 1 piece of nitrocellulose membrane for each gel.
- 3. Wet in 1X Transfer Buffer for 5 min.

- 4. Build sandwich in cold (4°C) Transfer Buffer, and run at 200 mAmps (constant current) for 2 hrs.
- 5. After transfer, trim excess membrane and <u>do not</u> allow nitrocellulose to dry

C: WESTERN BLOTTING

Solutions:	
<u>10X TBS</u>	<u>1L</u>
Tris-base (200 mM)	24.2 g
NaCl (1.37 M)	80.0 g
ddH ₂ O	qs to 1 L
pH to 7.6 with HCl	
<u>10% Tween 20</u>	<u>500 ml</u>
Tween 20	50 ml
TBS	450 ml. Store at 4°C
<u>TBST (Wash Buffer)</u>	<u>1 L</u>
10% Tween 20 (0.2 %)	20 ml
10X TBS	100ml. qs to 1 L
Blotting Buffer	<u>100 ml</u>
Carnation dry milk (5 %)	5.0 g
10% Tween 20 (0.2 %)	2 ml
1X TBS	qs to 100 ml
Alternatively, add 5 g dry milk to 10	00 ml Wash buffer.
Stripping Buffer	<u>1L</u>
Tris-base (62.5 mM)	1M 62.5 ml

SDS (2.0 %)20 ml 10% SDSβ-mercaptoethanol (100 mM)78 mlAdd Tris to 750 ml water and pH to 6.7. Add SDS, β-ME and qs to 1L.

<u>Protocol:</u>

- 1. Block membrane with Blotting Buffer for 30 min at room temp.
- Pour off Blotting Buffer and incubate with 1° antibody for 1 hr at room temp., or 4°C overnight.
- Remove 1° antibody and wash 15 min at room temp. Change Wash Buffer every 5 min.
- 4. Incubate with 2[°] antibody for 30 min at room temp.
- Remove 2° antibody and wash 15 min at room temp. Change Wash Buffer every 5 min.
- 6. Wash with TBS for 5 min.
- 7. Develop with ECL reagent
- 8. Immediately prior to use combine 3 ml of reagent A with 3 ml reagent B for each membrane and vortex. Place membrane in a clean dish, add ECl reagent and incubate for 5 min. at room temp. Wrap membrane in food service film and immediately expose to film (typically 30 s, 60 s, 3 min. and 5 min.; however, some reactions may take longer to develop).

D: STEROLS ANALYSIS IN BILE/PLASMA

- 1. Put 100 µl ISTD (internal standard, 100µl \times 100 µg/ml 5 α -cholestane =10 µg 5 α cholestane. ISTD is 100 µg/ml 5 α -cholestane) in culture tubes (10 µg 5 α -cholestane).
- 2. Pipette 10 μl bile/plasma in tubes.
- Put freshly prepared 1 ml hydrolysis solution in each tube. KOH stock solution: 10 mol/L. Every 6 ml KOH stock solution mixed with 94 ml Ethanol.

- Heat at 90-100°C for two hours for saponification. (During saponification, vortex tubes)
- 5. Cool down to room temperature.
- 6. Put 1 ml of distilled water in each tube and vortex.
- 7. Put 2 ml petroleum ether in each tube and vortex vigorously for 10 to 15 seconds.
- 8. Centrifuge at 2800 rpm for 15-20 min.
- 9. Remove the upper phase (petroleum phase) to a test tube.
- 10. Put 2 ml petroleum ether in each tube and vortex vigorously for 10 to 15 seconds.
- 11. Centrifuge at 2800 rpm for 15-20 min.
- 12. Remove the upper phase (petroleum phase) to the same test tube and dry down under nitrogen stream.
- 13. Add 100 μ l Tri-sil reagent in each test tube and mix it.
- 14. Transfer it to GC vial and heat at 75°C for 15-30 min.
- 15. Cool down to room temperature and transfer the solution into GC insert and put the insert back to GC vial.
- 16. Run samples on GC-MS.

E: HEPATIC LIPIDS EXTRACTION PROTOCOL

Folch reagent/BHT: 280 ml chloroform + 140 ml methanol (2:1) + 42mg BHT (to 100ug/ml)

- 1. Prepare Folch reagent/BHT.
- Cut 100 mg of liver (0.1 g) and put in dounce test tube. Homogenize using dounce pestle.
- After three/four ups and downs, insert 1 ml of MBTS/OG and homogenize a little more. Put the dounce test tube in ice and wait 30 min for extraction.
- 4. Add 2 ml of Folch/BHT reagent. Vortex for 3 x 10 sec (vortex all samples once, then twice, then three times. Leave 5 min. waiting time between each vortex).

- 5. The solution should look like *floating milky-powders* right now. Centrifuge at 2000 rpm for 10 min at RT. (RPM = 2000, RCF = 850, Rotor (*the big wheel and its name*) = S-5.1, Time = 10 min, Temp = 22°C).
- 6. After centrifugation, the solution should have three phases
 - methanol phase
 - protein & nucleic acid phase
 - chloroform and FA phase at bottom
- 7. Move majority of LOWER PHASE to reaction tube with glass pipet, dry under N_2 to about 100 $\mu l.$
- 8. Add 1 volume (200 μ l) of 1% Triton x 100 in CHCl_{3.}
- 9. Dry down by N_{2.}
- 10. Add 0.5 volume (100 μ l) of H₂O.
- 11. Measure cholesterol and TG, and PL with Wako Kit.

F: LIPID EXTRACTION FROM CELLS

Protocol:

- 1. Rinse plates 2X with 3 mL PBS (4° C).
- 2 Scrape dishes in 3 mL of PBS and transfer to a 12x75mm glass tube.
- 3. Vortex and take a 100 μ L aliquot for protein determination.
- 4. Centrifuge at 1000 x g for 5 min. (4°C), and aspirate PBS.
- 5. Add 2 mL 3:2 hexane: isopropanol. Vortex!
- 6. Incubate and for 10 min. (20^oC).
- 7. Centrifuge at 1000 x g for 5 min. (4° C), and transfer supernatant to a fresh 12x75 mm tube
- 8. To pellet, add 2 mL 3:2 hexane: isopropanol. Vortex!
- 9. Incubate and for 10 min. (20°C).
- 10. Centrifuge at 1000 x g for 5 min. (4°C), and transfer supernatant to 12x75 mm tube
- (7).

11. Evaporate to dryness under N₂ (g).

12. Solubilize the extracted lipids in a suitable solvent.

For Storage: Suspend in 500 μ L of CHCl₃, flush with N₂ (g) and store at -20^oC

For TLC: Suspend in 200 µL of 1:1 CHCl₃:CH₃OH (must be used immediately)

For Enzymatic Cholesterol Assays: Suspend in 2 mL 1% Triton in CHCl₃. Solution can be stored at -20° C.

Notes: If the cells are in 6-well or 12-well plates, Hexane: Isopropanol 1mL or 0.5mL, respectively can be added directly to the plate. After extraction, aspirate the solvent and proceed with step 11.

G: FECAL NEUTRAL STEROL ASSAY

- 1. Let samples dry on 37°C for couple days. Grind them.
- Weight out exactly 0.25g aliquot of dried, ground stool and place in a f. a. tube. Add 2.5ml ethanol, vortex, then add 0.5ml of 10N NaOH and vortex again.
- Add 2.5ml water and 2.5ml ethanol to the dried residue, place tubes in water bath at about 45-50°C for a few min. Remove tubes, vortex, then add 2.5ml PE (petroleum ether) and 0.5ml of 5-cholestene standard in hexane (1.0mg/ml). Shake tubes vigorous for about one min. Centrifuge tubes after balancing. Spin for 10min @ 1500rpm until the phases fully separate.
- 4. If the sample were derived from animals fed diet with no added cholesterol than take 1 ml aliquot of PE phase for GC. For samples from cholesterol-fed animals take 1 ml PE aliquot.
- 5. Pipette PE aliquot into 12×75 mm glass disposable tubes, dry under air, and then redissolve sterols in 400 µl hexane. Transfer 2 x 200µl to GC vial.
- Run samples on GC at a temperature of 280°C and using a 10 min run time. Run duplicates
 of the following standard mixtures before and after the samples.
- a) Cholesterol: 5-cholesten (1:1). Ratio of peak areas should be 0.88-0.90
- b) β-coprostanol: 5-Cholestene (1:1). Ratio should be 0.90-0.91
- c) α -Cholestanone: 5-Cholestene (1:1). Ratio should be 0.95-0.96.
- 7. Each of these standards mixtures is prepared using 20mg of each sterol per 100ml hexane.

The peak area for 5-cholestene should be about the same for all samples. If not, shake tubes again vigorously, centrifuge again and repeat the assay on another aliquot of the upper phase.

$$\frac{sample}{std} = \frac{x}{1}$$

8. Calculate the amount of cholesterol, coprostanol and cholestanone and sum these to obtain the total neutral sterol content of the stool sample.

Sterol standards for Fecal Neutral Sterol

5-cholestene	Sigma
5α-cholestan-3-one (Cholestanone)	Steraloids
5α-cholestan-3β-ol (coprostan-3-ol) coprostanol	Sigma

H: CHOLESTEROL LOADING AND ANALYSIS IN THP1 CELLS

Culture medium: RPMI 1640 + 10% FBS + 1%P/S

Assay Medium: RPMI 1640 + 1%P/S+ 1% FBS alone and either 100 μg protein/ml AgLDL +/- plant sterols.

<u>Day0</u>

- Culture THP 1 cells @ 37 C in 10 cm dish / 9x106. (Control; AgLDL + 20 μg/ml cholesterol; AgLDL + 20 μg/ml stigmasterol; AggLDL+20 μg/ml sitosterol)
- 2. Use RPMI 1640 medium +10% FBS +1%P/S+ 200 μg/ml PMA

<u>Day3</u>

- To measure the sterol loading, macrophages were incubated for 3-4 days at 37C in culture medium containing 1% FBS alone and either 100 µg protein/ml AgLDL +/plant sterols.
- 2. Change the sterol medium every3-4 days

Aggregation of LDL

LDL stock 5.06 mg/ml

- Add 3mg LDL /30 ml assay medium (5ml in each dish)
- Split this volume in 3, 50 ml polypropylene conical tubes (30 X 115 mm) Falcon.
- Add the appropriate amount of sterol in the desired concentration.
- Aggregate by vortex 1 min on ice to break up large aggregates and passed through a 0.45 μm filter. This produces small (~ 30-75 nm) aggregates that produce maximal uptake and lysosomal delivery.

<u>Day 6</u>

- 1. Aspirate the medium.
- 2. Wash with 2 x PSB.
- 3. Add 1 ml Membrane buffer.
- 4. Prepare membrane samples for western blot
- 5. Blot for AbCG1, ABCA1.

I: CELLULAR CHOLESTEROL EFFLUX ASSAY ([³H]-CHOLESTEROL LABELED CELLS)

The following protocol was developed for the efflux of cellular cholesterol from THP1 cells to HDL. Other extra-cellular cholesterol acceptors may be substituted for HDL. Many cell types have been examined for cellular cholesterol efflux. For other cell types simply substitute their respective media for RPMI.

I. Reagents

Growth Medium:	RPMI 1640 + 10% FBS + 1% P/S + 10ng/ml PMA
Labeling Medium:	RPMI 1640 + 10% FBLPPS + 1% P/S + 1 μ Ci/ml [³ H]-Cholesterol
(NEN #NE	Γ 725) +/- 100μg/ml agLDL+/- 20 μg/ml plant sterols

STOCK [³H]-Cholesterol 1.0 µCi/ml

57.6 μCi /mmol

FW 386.7

Assay Medium: RPMI 1640 + 0.2% FAF-BSA (Roche #100 377)

PBS: Dulbecco's PBS without Mg²⁺/Ca²⁺

PBS-FAF BSA: PBS + 0.02 % FAF-BSA

Solubilization Solution: 0.1N NaOH + 0.1% SDS

II. Cell Culture (Day 0)

All assays are done in triplicate. Seed cells at 7×10^6 cells/well in 12 well plates in Growth Medium (37°C, 8.8% CO₂)

- III. Labeling of Cells (Day 3)
- a. Aspirate growth medium
- b. Wash cells 2X with PBS (37°C)
- c. Add 1 ml Labeling Medium

Row #	Labeling Medium	AgLDL	Plant sterols
#1	+	+	-
#2	+	+	T comp
#3	+	+	Stigmasterol
#4	+	+	B-Sitosterol

- d. Incubate 12-16 h (37°C, 8.8% CO₂)
- IV. Efflux Assay (Day 4)
- Aspirate Labeling Medium (Store all liquid radioactive waste in appropriate container)
- b. Wash 2X with Assay Medium (37°C)
- c. Add 1 ml Assay Medium
- d. Incubate for 1 h (37°C, 8.8% CO₂)
- e. Aspirate medium
- f. Add 0.7 ml of Assay Medium containing desired amount of 30 $\mu g/ml$ apoA-I or other acceptor

- g. Incubate 100 μ l from each well and pool in the appropriate 1.5 ml tube for desired time (37°C, 8.8% CO₂).
- 15 min; 30 min; 60 min (1 hour); 120 min (2 hours); 240 min (4 hours); 480 min (8 hours)

All subsequent steps should be done on ice or in the cold room with pre-chilled

<u>solutions</u>

- h. Transfer Assay Medium to 1.5 ml tube
 - i. Centrifuge at 1000 x g for 10 min
 - ii. Transfer supernatants to scintillation vials containing 10 ml scintillation cocktail
- i. Wash cells 3X with PBS-FAF BSA (4°C)
- j. Wash cells 2X with PBS (4°C)
- k. Add 0.5 ml Solubilization Solution to cells and incubate on rotator (30 min, RT).
- I. Transfer solubilized cells to 1.5 ml centrifuge tube
- V. Data analysis
- a. Determine protein concentration of solubilized cells
- After protein assay is complete, transfer solubilized cells to scintillation vials containing 10 ml scintillation cocktail
- c. Count on beta counter (Program #44: 2 min, Sigma, 2%, H#, SL DPM)
- d. Express counts from medium and cells in *p*mol cholesterol /mg total cell protein to normalize for cell numbers
- e. Calculate Efflux as percent of total cellular cholesterol (Medium/ Cells + Medium)

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