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Ablating both *Fabp1* and *Scp2/Scpx* (TKO) induces hepatic phospholipid and cholesterol accumulation in high fat-fed mice

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ABSTRACT

Although singly ablating *Fabp1* or *Scp2/Scpx* genes may exacerbate the impact of high fat diet (HFD) on whole body phenotype and non-alcoholic fatty liver disease (NAFLD), concomitant upregulation of the non-ablated gene, preference for ad libitum fed HFD, and sex differences complicate interpretation. Therefore, these issues were addressed in male and female mice ablated in both genes (*Fabp1/Scp2/Scpx* null or TKO) and pair-fed HFD. Wild-type (WT) males gained more body weight as fat tissue mass (FTM) and exhibited higher hepatic lipid accumulation than WT females. The greater hepatic lipid accumulation in WT males was associated with higher hepatic expression of enzymes in glyceride synthesis, higher hepatic bile acids, and upregulation of transporters involved in hepatic reuptake of serum bile acids. While TKO had little effect on whole body phenotype and hepatic bile acid accumulation in either sex, TKO increased hepatic accumulation of lipids in both, specifically phospholipid and cholesteryl esters in males and females and free cholesterol in females. TKO-induced increases in glycerides were attributed not only to complete loss of FABP1, SCP2 and SCPx, but also in part to sex-dependent upregulation of hepatic lipogenic enzymes. These data with WT and TKO mice pair-fed HFD indicate that: i) Sex significantly impacted the ability of HFD to increase body weight, induce hepatic lipid accumulation and increase hepatic bile acids; and ii) TKO exacerbated the HFD ability to induce hepatic lipid accumulation, regardless of sex, but did not significantly alter whole body phenotype in either sex.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common of all liver disorders, occurring in 10–58% of the US population [33,55,95,115,120]. High fat diets (HFD) induce obesity and NAFLD [33,53,55,115,120,124]. However, despite the fact that NAFLD is more prevalent in women [23,24], most HFD-induced rodent models of NAFLD have focused only on males fed HFD ad libitum (rev. in [62]. Under ad libitum feeding conditions, such male rodents strongly prefer and eat more HFD as compared to normal chow (rev. in [62]. Thus, there is a need for controlled, pair-fed HFD studies not only of male but even more so female rodents to study sexual dimorphism of NAFLD independent of increased total intake of HFD.

While the biochemical basis for NAFLD is not completely

Abbreviations: 3αHSD, 3alpha-hydroxysteroid reductase; ABCA1, ATP-binding cassette sub-family A member 1; ABCG1, 4, 5 or 8, ATP-binding cassette sub-family G member 1,4, 5 or 8; ACAT2, acetyl-CoA acetyltransferase (*Soat2* gene); ACC1, acetyl-CoA carboxylase (*Acaca gene*); ACOX1, acyl-CoA oxidase-1 (*Acox1* gene); AGPAT, 1-acylglycerol-3-phosphate-O-acyltransferase (*Agpat2* gene); APOA1, apolipoprotein A-I; APOB, apolipoprotein B; BA, β-actin; β-HOB, β-hydroxybutyrate; BSEP, bile salt export pump (*Abcb11* gene); C, free cholesterol; CE, cholesteryl ester; COX4, cytochrome *c* oxidase subunit IV; CPT1a or 2, carnitine palmitoyltransferase 1a or 2 (*Cpt1a* or *2* gene); CYP7A1, cytochrome P7A1 (*Cyp7a1* gene); DEXA, dual-energy X-ray absorptiometry; DGAT, diacylglycerol acyltransferase (*Dgat2* gene); FABP1/L-FABP, liver fatty acid binding protein; FASN, fatty acid synthase (*Fasn* gene); FTM, fat tissue mass; FXR, farnesoid x receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase (*Gpam* gene); GST, glutathione s-transferase; HDL-C, high density lipoprotein cholesterol; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase); HFD, high fat diet; HMGCS1, cytosolic 3-hydroxy-3-methylglutaryl-CoA synthase; HSL/CEH, hormone sensitive lipase/cholesteryl ester hydrolase; LDL-R, low density lipoprotein receptor; Lipin 2, phosphatidate phosphatase (*Lpin2* gene); LTM, lean tissue mass; LXR, liver x receptor; MDR2, multidrug resistance protein 2; MRP2, multidrug resistance associated protein; MTP, microsomal triglyceride transfer protein; NEFA, non-esterified fatty acid; non-HDL-C, non-HDL cholesterol; OATP1 or 2, organic anion-transporting polypeptide 1 or 2 (*Slco1a1* or *Slc22a7* genes, respectively); PCTP, phosphatidylcholine transfer protein; PL, phospholipid; PPARα, peroxisome proliferator activated receptor alpha; qRT-PCR, quantitative real-time polymerase chain reaction; RXRα, retinoid x receptor α; SCP2, sterol carrier protein 2; SCPx, sterol carrier protein x; SEM, standard error of the mean; SHP, short heterodimer partner; SR-B1, scavenger receptor class B member 1; SREBP1 and 2 (*Srebf2* gene for SREBP2), sterol regulatory element-binding protein 1 and 2; TC, total cholesterol; TG, triglyceride/triacylglycerol; TKO, fatty acid binding protein/sterol carrier protein-2/sterol carrier protein-x null mouse on C57BL/6NCr background (*Fabp1/Scp2/Scpx* null or gene ablated); WT, wild-type C57BL/6NCr mouse

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understood, two potential liver cytosolic lipid binding/chaperone proteins have been identified as potential candidates: First is the hepatic fatty acid binding protein (FABP1). Genomic studies suggest that a very prevalent SNP in human FABP1 resulting in a T94A amino acid substitution is highly associated with NAFLD [77,96]. FABP1 is the single most prevalent liver cytosolic protein that binds, transports, and targets bound lipidic ligands such as long chain fatty acids and their CoA thioesters [46,74], endocannabinoids [45], bile acids [63], cholesterol [65], and lipidic xenobiotics [47,73,77] to intracellular organelles for storage, oxidation, receptor regulation, or gene regulation. FABP1 is upregulated in human NAFLD and in NAFLD animal models [10,17,38,124]. Gene targeting studies indicate that *Fabp1* gene ablation decreases hepatic lipid accumulation in control-chow fed mice [18,65,91–93,123]. While *Fabp1* silencing ameliorates hepatic steatosis, inflammation, and oxidative stress in HFD fed male mice with NAFLD [82], the impact of sex differences in the context of HFD has not been addressed. Second, protein sterol carrier protein-2 (SCP2, encoded by the *Scp2/Scpx* gene) also binds/chaperones such lipidic ligands. SCP2 binds, transports, and targets bound lipidic ligands such as long chain fatty acids and their CoA thioesters [19,27,28], endocannabinoids [61,70], bile acids [32], cholesterol [101,111], and phospholipids [20,31,105] to intracellular organelles for storage, oxidation, excretion, or receptor regulation. Although ablating the *Scp2/Scpx* gene decreases hepatic lipid accumulation in control rodent chow-fed male mice [56], the impact of sex differences especially in the context of HFD are unclear.

Finally, it is important to note that while studies with mice singly ablated in either the *Fabp1* or *Scp2/Scpx* gene have proven useful in resolving the roles of these genes in determining whole body phenotype and hepatic lipid metabolism, simple interpretation of findings is complicated by compensatory upregulation of the non-ablated gene. For example, individually ablating *Scp2/Scpx* or *Scpx* elicits compensatory upregulation of FABP1 which may have obscured at least in part the impact of the loss of SCP2 [4,30,107]. Therefore, the current study examined the effect of ablating both the *Fabp1* and *Scp2/Scpx* genes (i.e. *Fabp1/Scp2/Scpx* ablation or TKO) on whole body and liver lipid phenotype in both male and female mice pair-fed HFD.

2. Materials and methods

2.1. Materials

Triacylglycerol (L-type Triglyceride M, TG), free cholesterol (free cholesterol, C), total cholesterol (cholesterol E, TC), phospholipid (phospholipid, PL) and non-esterified fatty acid (HR Series NEFA-HR, NEFA) diagnostic kits from Wako Chemicals (Richmond, VA) were used to determine levels of the respective lipids. β-Hydroxybutyrate (β-hydroxybutyrate LiquiColor, β-HOB) and high density lipoprotein cholesterol (Direct HDL-Cholesterol, HDL-C) diagnostic kits from Stanbio Laboratory (Boerne, TX) were used to determine levels of β-HOB and HDL-C. Apolipoprotein B (APOB) and apolipoprotein A-I (APOA1) levels were measured using diagnostic kits from Diazyme Labs (Poway, CA). The Bradford protein micro-assay (Cat # 500-0001, bovine gamma globulin) from Bio-Rad (Hercules, CA) was used to determine protein levels. All reagents and solvents used were of the highest grade available.

2.2. Mice

Wild-type (WT) C57BL/6NCr mice were purchased from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). *Fabp1/Scp2/Scpx* null (TKO) mice backcrossed > 10 generations to the C57BL/6NCr background were generated by our laboratory as in [112]. The term TKO refers to loss of all three proteins even though they are encoded by only two genes (i.e. *Fabp1* and *Scp2/Scpx*). Mice were housed in controlled conditions (T = 25 °C, H = 60–70% added humidity) and 12:12 h light/dark cycle. Mouse protocols were approved by the Texas A&M Institutional Animal Care and Use Committee in compliance with the Guide for the Care and Use of Laboratory Animals. Mice were monitored daily for injury or disease, sentinel monitored quarterly, and were shown free of all known rodent pathogens.

2.3. Dietary high fat

Two groups of 16 each WT male, WT female, TKO male, and TKO female mice aged 7 weeks were individually housed in Tecniplast Sealsafe IVC cages with external water bottles and wire lid holders for food pellets. Mice were acclimated for 1 week on a defined, 10 kcal% fat control chow (#D12450B, Research Diets, New Brunswick, NJ) known to be free of phytol and phytoestrogen. Phytol and phytoestrogen were avoided because they may impact sex differences and FABP1 expression as described earlier [62]. In each group of 16 individually-housed mice, 8 mice were continued an additional 12 weeks on this same defined diet while the other 8 mice were pair-fed based upon food weight an isocaloric high fat diet (HFD, # D12451, Research Diets, New Brunswick, NJ). The high fat diet was formulated by modifying the defined control diet by increasing fat from 10 kcal% to 45 kcal% while decreasing carbohydrate from 70 kcal% to 35 kcal% while keeping protein constant as described [2].

2.4. Whole body phenotype by dual-energy X-ray absorptiometry (DEXA) to determine fat tissue mass (FTM) and lean tissue mass (LTM)

Mice were anesthetized at the beginning (day 0) and end (day 84) of the dietary study using a ketamine/xylazine mixture (0.01 mL/g body weight; 10 mg ketamine/mL and 1 mg xylazine/mL in 0.9% saline solution) as previously described [3]. Dual-energy X-ray absorptiometry (DEXA) images of each mouse were obtained using a Lunar PIXImus densitometer (Lunar Corp., Madison, WI) as previously described [8] after calibration using a phantom mouse with known bone mineral density and fat tissue mass as described [8,88]. Whole body fat tissue mass (FTM) and bone-free lean tissue mass (LTM) were obtained by exposing the entire mouse, minus the head region, to sequential beams of high- and low-energy X-rays and taking X-ray images on a luminescent panel. Soft tissue mass was differentiated from bone mass by measuring the ratios of attenuation at different energies followed by separating soft tissue mass into FTM and LTM as previously described [8].

2.5. Tissue collection

At the end of the dietary study and after overnight fast, mice were anesthetized as above, blood was collected via cardiac puncture followed by cervical dislocation as the secondary form of euthanasia according to the AVMA Guidelines for the Euthanasia of Animals. The blood was coagulated overnight at 4 °C, followed by centrifugation at 14,000 rpm for 20 min at 4 °C; the serum fraction was removed for storage at − 80 °C for subsequent lipid and protein analysis. Likewise, livers were collected, flash frozen, and stored at − 80 °C for subsequent analysis of lipids, Western blotting, and/or qRT-PCR as described in the following sections.

2.6. Liver homogenization and protein analysis

Liver samples (~0.1 g) were minced extensively followed by the addition of 0.5 mL PBS (pH 7.4) and homogenization with a motor-driven pestle (Tekmar Co, Cincinnati, OH) at 2000 rpm. The Bradford protein micro-assay (Bio-rad, Hercules, CA) was used to determine protein levels in the liver homogenates according to the manufacturer's instructions. Protein levels were determined on aliquots of homogenates in Costar 96-well assay plates (Corning, Corning, NY) and read