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Scp-2/Scp-x Ablation in *Fabp1* Null Mice Differentially Impacts Hepatic Endocannabinoid Level Depending on Dietary Fat

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Abstract

Dysregulation of the hepatic endocannabinoid (EC) system and high fat diet (HFD) are associated with non-alcoholic fatty liver disease. Liver cytosol contains high levels of two novel endocannabinoid binding proteins—liver fatty acid binding protein (FABP1) and sterol carrier protein-2 (SCP-2). While *Fabp1* gene ablation significantly increases hepatic levels of arachidonic acid (ARA)-containing EC and sex-dependent response to pair-fed high fat diet (HFD), the presence of SCP-2 complicates interpretation. These issues were addressed by ablating *Scp-2/Scp-x* in *Fabp1* null mice (TKO). In control-fed mice, TKO increased hepatic levels of arachidonoylethanolamide (AEA) in both sexes. HFD impacted hepatic EC levels by decreasing AEA in TKO females and decreasing 2-arachidonoyl glycerol (2-AG) in WT of both sexes. Only TKO males on HFD had increased hepatic 2-AG levels. Hepatic ARA levels were decreased in control-fed TKO of both sexes. Changes in hepatic AEA/2-AG levels were not associated with altered amounts of hepatic proteins involved in AEA/2-AG synthesis or degradation. These findings suggested that ablation of the *Scp-2/Scp-x* gene in *Fabp1* null mice exacerbated hepatic EC accumulation and antagonized the impact of HFD on hepatic EC levels—suggesting both proteins play important roles in regulating the hepatic EC system.

Keywords

mouse; liver; FABP1; gene ablation; endocannabinoid; high fat diet

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1. Introduction

Worldwide, high fat diets (HFD, about 40% of energy) are increasing in prevalence [1]. HFD not only induces obesity but also non-alcoholic fatty liver (NAFLD)—the most common of all liver disorders [1]. While the biochemical basis for NAFLD is not completely understood, recent studies have established a link between HFD-induced NAFLD and the hepatic endocannabinoid (EC) system. Expression of the cannabinoid receptor-1 (CB1) is low in normal liver (localized primarily in hepatocytes), but is increased by a HFD, and CB1 mRNA (*Cnr1*) is upregulated 34-fold in patients with NAFLD [2–4]. Concomitant to HFD-induced obesity and NAFLD, hepatic arachidonoylethanolamide (AEA), but not 2-arachidonoyl glycerol (2-AG), is selectively increased in males [2, 3]. HFD-induced increases in AEA, together with CB1's higher affinity for AEA than 2-AG, results in hepatic activation of CB1 receptors—a requirement for development of NAFLD in male mice [2, 3, 5]. These findings suggest that peripheral CB1 receptors could be novel targets for drugs against NAFLD as well as obesity.

Until recently, a major unanswered question in the field has been how EC, very poorly aqueous soluble and highly membrane-bound lipidic molecules, are desorbed and trafficked through the cytosol to metabolic sites. Overexpression of other members of the fatty acid binding protein FABP family found in brain (i.e. FABP5, 7) in transformed COS-7 or N18GT2 cells demonstrated their roles as cytosolic AEA binding 'chaperone' protein for trafficking between sites of AEA synthesis through the cytosol to FAAH—the major AEA degradative enzyme in endoplasmic reticulum [6–8]. The novel discovery that the liver fatty acid binding protein (FABP1, L-FABP) has high affinity not only for arachidonic acid (ARA)—the precursor of AEA and 2-AG [9, 10], but also for AEA and 2-AG as well as cannabinoids (e.g. THC, cannabidiol) suggested that FABP1 may fulfill this EC binding 'chaperone' role in liver [11, 12]. As such FABP1 may account for the first-pass high hepatic clearance rate diminishing plasma availability of the EC precursor ARA [11, 12] and cannabinoids [13] as well as the likely first pass removal of plasma EC for intracellular degradation. Consistent with this possibility, FABP1 gene ablation (LKO) markedly increased hepatic levels of AEA and 2-AG in male mice [11]. This finding was consistent for a role for other FABP family members in cytosolic trafficking these EC to intrahepatic sites for degradation analogous to roles for FABP5 and 7 thought to occur in brain [14].

However, several factors complicate clear interpretation of the above findings in mice. For example, hepatic FABP1 is markedly upregulated by *ad libitum* feeding of HFD [15, 16] and in NAFLD [17, 18]. Further, to date our knowledge about cellular and molecular defects in the hepatic EC system and in NAFLD is heavily based on studies of HFD-induced rodent models [19]. However, almost all of these studies fed HFD *ad libitum* to males from HFD-susceptible mouse or rat strains known to exhibit a strong preference for and consume more HFD than normal chow [20]. Thus, it is unclear if the HFD-induced increase in hepatic AEA and NAFLD is due to the higher proportion of fat in the diet or to the increased intake of HFD. In addition, despite the fact that the prevalence of obesity and NAFLD is greater in women, little is known about the impact of HFD on hepatic EC and lipid accumulation in female models of NAFLD [21]. Finally, liver also expresses high levels of sterol carrier protein-2 (SCP-2)—another cytosolic protein that also exhibits high affinity for EC (AEA,

2-AG) and their analogues [11, 22]. Thus, although *Fabp1* gene ablation (LKO) significantly increases hepatic AEA and 2-AG level [11], it is unclear if expression of SCP-2 compensates at least in part for loss of FABP1. Taken together, these observations suggest that not only FABP1, but also SCP-2 may impact hepatic EC levels and/or the hepatic EC response to HFD. Therefore, this possibility was examined in livers of both male and female, wild-type (WT) and *Fabp1* gene ablated (knockout, LKO) mice pair-fed HFD. Pair-fed HFD eliminates the potential complications of mouse preference for and increased consumption of a HFD [23].

2. Materials and Methods

2.1 Materials

The following unlabeled lipids were obtained from Cayman Chemical (Ann Arbor, MI): n-6 arachidonic acid (ARA), n-3 docosahexaenoic acid (DHA), n-6 arachidonoylethanolamine (AEA), oleoylethanolamide (OEA), palmitoylethanolamide (PEA), n-3 docosahexaenoylethanolamide (DHEA), n-3 eicosapentaenoylethanolamide (EPEA), 2-arachidonoylglycerol (2-AG), 2-oleoylglycerol (2-OG), and 2-palmitoylglycerol (2-PG). The corresponding deuterated lipids [ARA-d_8 (20:4n-6-d_8), DHA-d_5 (22:6n-3-d_5), AEA-d_4, OEA-d_2, PEA-d_4, DHEA-d_4, EPEA-d_4, and 2-AG-d_8] were likewise purchased from Cayman Chemical (Ann Arbor, MI). All reagents and solvents were the highest grade commercially available.

2.2 Animal Care

Wild-type (WT) male and female C57BL/6NCr mice were acquired from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). Mice ablated in both the liver fatty acid binding protein gene (*Fabp1*) and the sterol carrier protein-2/sterol carrier protein-x (*Scp-2/Scp-x*) gene (TKO) mice were developed as described earlier [24–26]. For colony maintenance, mice were housed in barrier cages, placed on ventilated racks, maintained at 25°C on a 12-hr light/dark cycle, and permitted *ad libitum* access to water and standard rodent chow mix [5% calories from fat; D8604 Rodent Diet, Teklad Diets (Madison, WI)]. All animal protocols were approved by Texas A&M University's Institutional Animal Care and Use Committee. Quarterly sentinel monitored confirmed the mice to be free of all known rodent pathogens.

2.3 Dietary Study

Seven week old WT male, WT female, TKO male, and TKO female mice of 16 mice per group were individually housed in Tecniplast Sealsafe IVC cages (external water bottles, wire lid holders for food pellets). Mice were then acclimated for 1 week on a defined, 10 kcal% fat, phytol-free and phytoestrogen-free control chow (#B12450b, Research Diets, New Brunswick, NJ). Phytol- and phytoestrogen-free diets were chosen because these molecules may impact sex differences and the effect of TKO [24, 26]. Each group of 16 individually-housed mice was then divided into two groups: The first half of each group was continued for an additional 12 weeks on the above defined diet. The second half of each group was pair-fed high fat diet (HFD, # D12451, Research Diets, New Brunswick, NJ) such that mice pair-fed HFD did not differ in caloric intake from control-fed mice. HFD diet

#D12451 was based on the above control diet (#B12450b) modified to increase fat from 10 kcal % to 45 kcal% at the expense of decreasing carbohydrate from 70 kcal % to 35 kcal % while maintaining protein constant. Analysis of the fatty acid profiles of these diets (DIO FA Profile 11-11.xls, Research Diets, New Brunswick, NJ) indicated that the control chow had relatively low levels of n-6 PUFA such as 18.3 g/kg of 18:2n-6 (precursor of 20:4n-6) and 0.1 g/kg of 20:4n-6, as well as even lower levels of n-3 PUFA such as 2.2 g/kg 18:3n-3, 0 g/kg 20:5n-3, and 0 g/kg 22:6n-3. In contrast, the HFD had >3-fold higher levels of n-6 PUFA such as 56.7 g/kg of C18:2n-6 and 0.5 g/kg of C20:4n-6, but only slightly increased levels of the n-3PUFA such as 4.3 g/kg of 18:3n-3, 0 g/kg of 20:5n-3, and 0.2 g/kg of 22:6n-3. Thus, the HFD was enriched 3-fold in the fatty acid precursors (20:4n-6 and 18:2n-6) from which arachidonic acid (ARA)-derived endocannabinoids are ultimately derived.

At the end of the dietary study mice were fasted overnight, anesthetized 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) and euthanized by cervical dislocation as the secondary form of euthanasia according to the AVMA Guidelines for the Euthanasia of Animals as previously described [27]. Livers were then collected, flash frozen, and stored at −80°C for subsequent analysis of liver triacylglycerol, NAE and 2-MG (LC/MS), and western blotting of proteins in the EC system.

2.4 Liver Triacyglycerol Quantitation

Frozen mouse liver (100–200 mg wet weight) was homogenized in 1.0 mL of ice-cold homogenization buffer as described earlier [28]. Triacylglycerol (TG) was measured in liver homogenate using a diagnostic kit from Wako Chemicals (Richmond, VA) as per the manufacturer's instructions. Measuring triacyglycerol in liver homogenate with the Wako kit versus chemical analysis (i.e. solvent extraction, thin layer chromatography, elution and colorimetric measurement) revealed no significant differences between the two methods [29].

2.5 Liver Lipid Extraction and Liquid Chromatography/Mass Spectrometry (LC/MS) Analysis and Quantitation of N-Acylethanolamide (NAE) and 2-Monoacylglycerol (2-MG)

Lipids were extracted from mouse livers as described earlier [11, 28]. Briefly, frozen mouse liver (100–200 mg wet weight) was homogenized in 1.0 mL of ice-cold homogenization buffer containing each of the deuterated standards AEA-d_4, OEA-d_2, PEA-d_4, DHEA-d_4, EPEA-d_4, and 2-AG-d_8 such that the final amount of each standard was 2000pg. The final lipid residue was redissolved in 100 μL of ice-cold methanol, purged with N_2, and stored at −80°C until liquid chromatography-mass spectrometry (LC-MS) analysis below.

LC/MS analysis of liver NAE and 2-MG was performed as described earlier by our lab [30, 31]. Briefly, identification and quantitation of individual NAE or 2-MG was accomplished through use of: i) Addition of internal standards to each aliquot of liver homogenate including deuterated AEA-d_4, OEA-d_2, PEA-d_4, DHEA-d_4, EPEA-d_4, and 2-AG-d_8 from Cayman Chemical (Ann Arbor, MI); ii) Comparison with external standard curves of n-6 arachidonoylethanolamide (AEA), oleoylethanolamide (OEA), palmitoylethanolamide

(PEA), n-3 docosahexaenoylethanolamide (DHEA), n-3 eicosapentaenoylethanolamide (EPEA), 2-arachidonoylglycerol (2-AG), 2-oleoylglycerol (2-OG), and 2-palmitoylglycerol (2-PG) from Cayman Chemical (Ann Arbor, MI). All reagents and solvents used for extraction and LC/MS were of the highest commercial grade available.

2.6 Extraction of Liver Lipids and Determination of Free and Total ARA and DHA by Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

All procedures for lipid extraction of Free and Total ARA were performed similarly as we described previously in other tissues [30]. Concomitantly, to determine Free and Total DHA, 2 μg of DHA-d_5 internal standard was also added to the liver homogenate prior to extraction. The final extract and washes were combined and dried under N_2 to ~1 mL to facilitate precipitation of residual organic soluble protein followed by sedimentation of the protein residue by centrifugation at 3,250 xg for 15 min at 4 °C. The organic supernatant was removed, placed in a new silanized screw top tube, and sedimented protein retained for subsequent quantification. The supernatant was then dried under N_2 and lipids redissolved in 1 mL of hexane:2-propanol (3:2 by volume). The extracts were then split into two parts to determine Free ARA and Free DHA as well as Total ARA and Total DHA, respectively.

Free (unesterified) ARA and Free (unesterified) DHA were determined by drying down half of the lipid extract under N_2, redissolving in 150 μL of hexane:2-propanol (3:2 by volume), transferred to a silanized microinsert, dried down under N_2, and 20 μL of acetonitrile and 20 μL of H_2O (1:1 by volume) added as described earlier in preparation for LC-MS analysis [30].

Total ARA and Total DHA were determined by drying down the second half of the lipid extract under N_2, saponification in 180 μL methanol and 20 μL of 5 M KOH in water at 60 °C for 1 h, and neutralizing the saponified digest with 20 μL of 5 M HCl. Lipids were then extracted by addition of 780 μL of 0.9% NaCl followed by three extractions with 2 mL hexane. All hexane extracts were combined in a new silanized tube, dried under N_2, 1 mL acetonitrile was added, and 10 μL aliquots of extract transferred to silanized microinserts followed by the addition of 10 μL of acetonitrile and 20 μL of H_2O (1:1 by volume) as described earlier in preparation for LC-MS analysis [30].

Protein content of the sedimented protein after lipid extraction of the liver homogenate above was determined by drying of residual solvent followed by hydrolysis with 0.2 M KOH at 65°C overnight [32]. Samples were then mixed with Bradford dye binding reagent and allowed to equilibrate for 10 min prior to reading on a spectrophotometer at 595 nm using BSA as a standard [33].

The above lipid extracts prepared for analysis of free ARA and DHA or saponified for determination of total ARA and DHA were resolved and quantitated by LC-MS as we described earlier in other tissues [30].

2.7 Western Blotting

Frozen mouse liver samples (0.1 g wet weight) were minced with a razor blade, 0.5 mL of ice cold PBS (pH 7.4) was added, and samples individually homogenized at 2000 rpm with a

motor-driven pestle (Tekmar Co, Cincinnati, OH). For protein quantitation, aliquots of homogenate were placed in Costar 96-well assay plates (Corning, Corning, NY), incubated with Bradford protein micro-assay reagents according to the manufacturer's instructions (Bio-rad, Hercules, CA), and read with a BioTek Synergy 2 micro-plate reader (BioTek Instruments, Winooski, VT).

Aliquots (10 μg protein) of liver homogenates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting as described earlier [30, 31, 34]. The following antibodies for western blotting were purchased as follows: Goat polyclonal anti-fatty acid amide hydrolase (FAAH, sc-26427), anti- fatty acid transport protein 4 (FATP-4; sc-5834), anti-N-acylphosphatidylethanolamide phospholipase-D (NAPE-PLD; sc-163117), and anti-cannabinoid receptor-1 (CB1; sc-10066) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-mouse N-acylethanolamide hydrolyzing acid amidase (NAAA; sc-100470), rabbit polyclonal anti-monoacylglyceride lipase (MGL, sc-134789) and anti- diacylglycerol lipase α (DAGLα; sc-133307) were from Santa Cruz Biotech (Santa Cruz, CA). Specific monoclonal anti-mouse heat shock protein-70 (HSP70; ab2787) was from Abcam (Cambridge, MA). The anti-FABP1 and anti-SCP-2 antibodies were validated by western blots of WT and Fabp1/Scp2/Scpx gene-ablated (TKO) mice which detected the presence of the respective gene products in livers from WT mice, but complete absence from livers of TKO mice. All other antibodies were validated as stated by the respective manufacturer. Relative protein levels were normalized to Ponceau S stains (Supplementary Fig. 2–5) used as gel loading controls as described earlier [35]. With regards to the use of Ponceau staining in lieu of "housekeeper" normalization, the use of this alternative is explained earlier [36] and has been highly recommended by a NIH joint workshop with Nature Publishing Group and Science held in June of 2014 with journal editors representing over 30 basic/preclinical science journals. Values were compared to male WT set to 1 and data presented as mean ± SEM (n = 8).

2.9 Statistical Analysis

Statistical analysis was performed by three-way ANOVA (SigmaPlot 12.5, Systat Software) followed by Student-Newman-Keuls Pairwise Multiple Comparison Procedure. All groups of data passed the Shapiro-Wilk Normality Test and the Equal Variance Test. All values represent the mean ± SEM. Statistical differences of $P < 0.05$ are designated by the use of different lower-case lettering, i.e., a, b, c, d.

3. Results

3.1 Fabp1/Scp-2/Scp-x Gene Ablation (TKO) and Sex Oppositely Impact Hepatic N-arachidonoylethanolamide (AEA) Level in Mice

Feeding HFD *ad libitum* is known to selectively increase liver levels of the arachidonic acid (ARA)-containing arachidonoylethanolamide (AEA) in male mice [2, 3, 37, 38]. However, such *ad libitum* fed HFD did not discriminate effects of: i) increased proportion of fat in HFD versus preference for/increased intake of HFD; or ii) effect of HFD on AEA in females. These issues were resolved by pair-feeding HFD to both male and female wild-type (WT) and *Fabp1/Scp-2/Scp-x* gene ablated (TKO) mice.

TKO alone markedly increased liver AEA level in both male and female control-fed mice by 2.4- and 2.8-fold, respectively (Fig. 1A). HFD alone did not significantly alter wild-type (WT) liver AEA level regardless of sex (Fig. 1A). However, due to control-fed WT females' 3-fold intrinsically lower AEA level, the ratio of AEA in HFD-fed/control-fed livers was 1.3-fold higher in WT females vs males (Supplementary Fig. 1A). In contrast, TKO selectively counteracted HFD's ability to lower hepatic AEA level in male, but not female mice (Fig. 1A). Yet due to a much smaller difference in HFD-fed TKO vs WT females' AEA level, the ratio of AEA in HFD-fed/control-fed liver was significantly lower in TKO females vs TKO males (Supplementary Fig. 1A). Three-way ANOVA indicated a significant interaction between sex and genotype with respect to hepatic AEA levels (sex x genotype, $P = 0.016$).

Taken together, these findings indicated that TKO significantly increased hepatic level of AEA in males regardless of diet and also in control-fed females. The inability of pair-fed HFD to increase AEA in either male or female WT mice suggested that the increased liver AEA level seen in males on an *ad libitum*-fed HFD [2, 3, 37, 38] correlated with increased consumption of HFD, rather than an increased proportion of dietary fat.

3.2 Fabp1 Gene Ablation (LKO) Differentially Impacts the Ability of a Pair-Fed High Fat Diet (HFD) to Alter Hepatic Levels of non-Arachidonic Acid (ARA)-Containing N-Acylethanolamide (NAE) in Male vs Female Mice

TKO alone had little effect on hepatic levels of most NAE [i.e. oleoylethanolamide (OEA, Fig. 1B), palmitoylethanolamide (PEA, Fig. 1C), or docosahexaenoylethanolamide (DHEA, Fig. 1D)] in either male or female control-fed mice. However, TKO significantly increased hepatic levels of EPEA in control-fed females, but not males (Fig. 1E). In contrast, HFD alone had little impact on WT male or female hepatic levels of OEA, PEA, and DHEA (Fig. 1B, C, D), but selectively increased the level of EPEA (Fig. 1E). However, due to the WT mice' intrinsic sex differences in hepatic NAE levels, the ratio of PEA, DHEA, and EPEA in HFD-fed/control-fed livers was significantly higher in WT females than their male counterparts (Supplementary Fig. 1C, D, E). In HFD-fed mice, TKO had no impact on NAE levels in males (Fig. 1B, C, D, E), but markedly decreased that of PEA (Fig. 1C) while increasing that of DHEA (Fig. 1D) in females. Consequently, TKO decreased the ratios of OEA, PEA, and DHEA (but not EPEA) in HFD-fed/control-fed males (Supplementary Fig. 1B, C, D) and ratios of OEA, PEA, and EPEA (Supplementary Fig. 1B, C, E) while increasing that of DHEA (Supplementary Fig. 1D) in females. Three-way ANOVA indicated the following interactions: OEA (genotype x diet, $P = 0.003$), PEA (sex x genotype, $P < 0.001$; genotype x diet, $P < 0.001$), DHEA (sex x diet, $P < 0.001$), EPEA (sex x genotype, $P = 0.012$; sex x diet, $P = 0.035$).

Thus, in general TKO alone had little effect on hepatic NAE levels regardless of sex. In contrast, HFD selectively increased levels of some NAE in males (EPEA) and nearly all NAE in females.

3.3 Sex has Greater Impact than Fabp1/Scp-2/Scp-x Gene Ablation (TKO) on Hepatic Level of the Arachidonoic Acid (ARA)-Containing 2-Arachidonoylglycerol (2-AG) in Mice

Feeding HFD *ad libitum* does not impact liver levels of the most prevalent arachidonic acid (ARA)-containing EC agonist of CB receptors (i.e. 2-arachidonoylglycerol or 2-AG) in male mice [2, 3, 37, 38]. However, nothing is known regarding the impact of HFD, TKO, or both together on liver 2-AG levels in females. These issues were resolved by pair-feeding HFD in both male and female wild-type (WT) and *Fabp1/Scp-2/Scp-x* gene ablated (TKO) mice.

TKO alone had no impact on liver 2-AG level in either male or female mice fed control diet (Fig. 2A). In contrast, HFD alone markedly decreased liver 2-AG level by 2.5- and 4.8-fold in male and female WT mice, respectively (Fig. 2A). Due to control-fed WT females' intrinsically higher 2-AG level, however, the ratio of ratio of 2-AG in HFD-fed/control-fed livers was significantly higher in WT males (Supplementary Fig. 1F). In HFD-fed mice TKO selectively counteracted HFD's inability to lower hepatic 2-AG level in male, but not female mice (Fig. 2A). Further, due to the higher intrinsic 2-AG level in control-fed TKO females, the liver 2-AG ratio in HFD-fed/control-fed TKO mice was over 5-fold higher in males than females (Supplementary Fig. 1F). Three-way ANOVA indicated the following significant interactions with respect to hepatic 2-AG levels: sex x genotype, $P = 0.035$; sex x diet, $P < 0.001$.

Thus, pair-fed HFD decreased hepatic 2-AG in WT males (and even more so WT females). This was opposite to the lack of effect of *ad libitum* fed HFD on 2-AG in WT males reported in the literature [2, 3, 37, 38] and suggested that preference for/increased consumption of HFD significantly complicated interpretation of response due to an increased proportion of dietary fat. Finally, TKO selectively antagonized the HFD-induced increase in hepatic 2-AG in females.

3.4 Impact of Fabp1/Scp-2/Scp-x Gene Ablation (TKO) and Sex on non-Arachidonic Acid (non-ARA)-Containing 2-Monoacylglycerols (2-MG) in Mouse Liver

In control-fed mice, TKO alone differentially impacted 2-MG levels in sex-dependent fashion—selectively increasing 2-PG in males (Fig. 2C) while selectively decreasing that of 2-OG in females (Fig. 2B). Conversely, HFD alone markedly decreased hepatic levels of both 2-OG (Fig. 2B) and 2-PG (Fig. 2C) in male and female WT mice. But, due to the control-fed WT females' higher levels of these 2-MGs, the ratios of 2-OG (Supplementary Fig. 1G) and 2-PG (Supplementary Fig. 1H) in HFD-fed/control-fed in female livers were significantly lower than in males. In HFD-fed mice, TKO counteracted the HFD-induced lowering of 2-OG and 2-PG in males but not in females (Fig. 2B, C). Three-way ANOVA indicated the following significant interactions with respect to hepatic 2-OG and 2-PG levels: 2-OG (sex x genotype, $P < 0.001$; sex x diet, $P < 0.001$; genotype x diet, $P = 0.004$), 2-PG (sex x genotype, $P < 0.001$).

Thus, in control-fed mice TKO selectively increased hepatic levels of some 'entourage' 2-MG such as 2-PG in males but not females. In contrast, HFD alone oppositely impacted the 2-MG levels—decreasing both 2-OG and 2-PG in both males and females—effects antagonized by TKO in HFD-fed males but not females.

3.5 Fabp1/Scp-2/Scp-x Gene Ablation (TKO) and Pair-Fed High Fat Diet (HFD) differentially Alter Hepatic Levels of n-6 Arachidonic Acid (ARA) and n-3 Docosahexaenoic Acid (DHA)

Since endocannabinoids (EC) such as AEA and 2-AG are derived from ARA-containing phospholipids, it was important to determine if the TKO- and/or HFD-induced changes in liver EC are at least partly associated with altered hepatic ARA levels. Therefore, hepatic Free ARA and Total ARA were determined in livers of male and female WT and TKO mice fed control-diet (CO) or high fat diet (HFD). In female WT mice, pair-fed HFD decreased Free ARA (Fig. 3A) by 30% as compared to control-fed while not significantly impacting Free ARA (Fig. 3A) in their male WT counterparts. Free ARA (Fig. 3A) in the livers of control-fed female WT mice was significantly increased by 70% as compared to that of control-fed male WT mice. Total ARA was decreased by 25% (Fig. 3B) in livers of control-fed male TKO as compared to WT mice. In WT mice, Total ARA (Fig. 3B) was significantly decreased in control-fed female mice as compared to control-fed males. In pair-fed HFD male mice, TKO did not significantly alter Free ARA (Fig. 3A) or Total ARA (Fig. 3B). Likewise, in pair-fed HFD-fed female mice, TKO did not significantly impact either Free or Total ARA (Fig. 3A,B). Subtraction of Free from Total ARA showed that TKO (but not HFD or TKO/HFD together) decreased the quantity of esterified ARA from which AEA and/or 2-AG are derived, regardless of sex. Thus, the TKO-induced increase in hepatic AEA (Fig. 1A) was not attributed to altered liver Total ARA content, but that of HFD-induced decrease in hepatic 2-AG (Fig. 2A) was attributable at least in part to decreased liver Total ARA. Three-way ANOVA indicated the following significant interactions with respect to hepatic levels of Free/Total ARA: Free ARA (sex x genotype, $P < 0.001$; sex x diet, $P < 0.001$; genotype x diet, $P < 0.001$), Total ARA (genotype x diet, $P < 0.001$).

Since DHEA is derived from esterified DHA, the possibility that TKO- and/or HFD-induced changes in liver DHEA were associated with altered hepatic DHA levels was examined. While TKO alone slightly decreased Free DHA (Fig. 3C), it decreased Total DHA nearly 40% (Fig. 3D) in livers of control-fed males. Pair-fed HFD alone increased Free DHA (Fig. 3C) but not Total DHA (Fig. 3D) in WT males. In female mice, TKO alone decreased both Free DHA (Fig. 3C) and Total DHA (Fig. 3D). Likewise, pair-fed HFD alone also decreased Free DHA (Fig. 3C) and Total DHA (Fig. 3D). In pair-fed HFD female mice, TKO did not further impact either Free or Total DHA (Fig. 3C, D). Three-way ANOVA indicated the following significant interactions with respect to hepatic Free/Total DHA levels: Free DHA (sex x genotype, $P < 0.001$; sex x diet, $P < 0.001$; genotype x diet, $P = 0.004$), Total DHA (sex x diet, $P < 0.001$; genotype x diet, $P = 0.003$). Taken together, these findings indicated that, in females the HFD-induced increase in DHEA—markedly exacerbated by TKO was not associated with an overall increase in DHA from which DHEA is derived. Instead, TKO decreased Total DHA in both sexes, regardless of sex or pair-fed HFD diet.

3.6 Fabp1/Scp-2/Scp-x Gene Ablation (TKO) selectively Impacts Hepatic Protein Levels of Key Enzymes in Synthesis and Degradation of Arachidonic Acid (ARA)-Containing Endocannabinoids, AEA and 2-AG

Since hepatic AEA level is regulated at least in part by synthesis [39–41], the possibility that the TKO—induced increase in liver AEA (Fig. 1A) and HFD-induced decrease in 2-AG (Fig. 2A) was attributed not only to loss of FABP1 and SCP-2, but at least in part to altered

hepatic levels of synthetic enzymes, was considered. Therefore, SDS-PAGE and western blotting was performed to determine liver protein levels of key synthetic enzymes for AEA (NAPEPLD, N-acylphosphatidylethanolamide phospholipase-D) and 2-AG (DAGLα, diacylglycerol lipase A) as in Methods. TKO alone significantly increased hepatic protein level of DAGLα (Fig. 4B), but not NAPEPLD (Fig. 4A) in both male and female control-fed mice. HFD alone decreased expression of liver DAGLα (Fig. 4B) but not NAPEPLD (Fig. 4A) in WT males, while increasing NAPEPLD (Fig. 4A) but not DAGLα (Fig. 4B) in females. In HFD-fed mice TKO diminished the HFD-induced increase in hepatic protein level of DAGLα in males but not females (Fig. 4B). Three-way ANOVA indicated the following significant interactions with respect to hepatic levels of NAPEPLD or DAGLα: NAPEPLD (sex x genotype, $P < 0.001$; genotype x diet, $P = 0.01$), DAGLα (sex x diet, $P = 0.002$).

Liver levels of AEA and 2-AG are regulated not only by synthesis, but also at least in part by intracellular degradation/hydrolysis [39–41]. Therefore, the possibility that the TKO—induced increase in liver AEA (Fig. 1A) and HFD-induced decrease in 2-AG (Fig. 2A) were due to concomitant down- and up-regulation of degradative enzymes, respectively, was considered. SDS-PAGE and western blotting was performed to determine liver protein levels of key enzymes in the degradation/hydrolysis of AEA (FAAH, fatty acid amide hydrolase; NAAA, N-acylethanolamide-hydrolyzing acid amidase) and 2-AG (MAGL, 2-monoacylglycerol lipase). Neither TKO alone, HFD alone, nor both together significantly altered hepatic protein level of FAAH, the major enzyme that hydrolyzes AEA in endoplasmic reticulum, regardless of sex (Fig. 5A). Three-way ANOVA did not indicate any significant sex, genotype, or diet interactions. Neither TKO alone nor HFD alone also did not affect hepatic protein level of NAAA, a secondary enzyme that hydrolyzes AEA in lysosomes, in either sex (Fig. 5B). However, TKO together with HFD modestly reduced hepatic protein level of NAAA in both male and female mice (Fig. 5B). Three-way ANOVA indicated the following significant interactions with respect to hepatic levels of NAAA: sex x genotype, $P = 0.007$; genotype x diet, $P < 0.001$ In contrast, TKO alone affected hepatic protein level of MAGL, the major enzyme hydrolyzing 2-AG in cytosol and at the lipid droplet surface, in control-fed male and female mice. HFD alone also decreased MAGL protein level in males and less so females (Fig. 5C). Three-way ANOVA indicated the following significant interactions with respect to hepatic levels of MAGL: sex x diet, $P < 0.001$; genotype x diet, $P < 0.001$.

Taken together, these findings suggest that neither the TKO-induced increase in liver AEA (Fig. 1A) nor the HFD-induced decrease in 2-AG (Fig. 2A) were accompanied by altered hepatic protein level of NAPEPLD or DAGLα, respectively, or marked decrease in AEA hydrolytic enzymes (FAAH, NAAA), or marked increase in 2-AG hydrolytic enzyme MAGL. It is important to note, however, that western blotting is a semi-quantitative technique and only reflects the total protein amounts that could miss changes in the activities of the synthetic and catabolic enzymes.

3.7 Effect of Fabp1/Scp-2/Scp-x Gene Ablation (TKO) and HFD on Liver Protein Levels of Cannabinoid Receptor-1 (CB1)

While liver protein levels of the major hepatic cannabinoid receptor, i.e. CB1, is normally low, it is significantly upregulated by *ad libitum* fed HFD in male mice [2–4]. Therefore, the impact of *Fabp1/Scp-2/Scp-x* gene ablation and HFD on hepatic levels of CB1 protein level was examined in male and female mice. TKO alone did not significantly alter hepatic protein level of CB1 in either sex on control diet (Fig. 6A). While HFD alone only slightly altered CB1 level in males, it markedly increased that in females (Fig. 6A). TKO decreased/diminished the ability of HFD to increase hepatic CB1 protein (Fig. 6A). Thus, TKO had little effect on hepatic CB1 protein in control-fed mice—in contrast to earlier studies with male mice *ad libitum* fed HFD showing increased hepatic CB1 [2–4]. This suggested likely involvement of increased appetite/food intake rather than higher proportion of dietary fat accounted for increased hepatic CB1 under *ad libitum* HFD conditions. Interestingly, however, pair-fed HFD significantly increased hepatic CB1 level in females—an effect blocked by TKO. Three-way ANOVA indicated the following significant interactions with respect to hepatic CB1 levels: sex x genotype, $P = 0.005$; sex x diet, $P < 0.001$; genotype x diet, $P < 0.001$.

3.8 Effect of Fabp1/Scp-2/Scp-x Gene Ablation (TKO) on Concomitant Upregulation of other Liver EC ‘Chaperone’ Proteins in Mice Fed HFD

Liver also expresses a third cytosolic binding ‘chaperone’ protein heat shock protein-70 (HSP70), which bind AEA [42]. Since *Fabp1/Scp-2/Scp-x* gene ablation (TKO) deletes FABP1 and SCP-2, the impact of TKO on potential compensatory upregulation of HSP70 was examined. In control-fed mice, TKO alone elicited sex-dependent alteration in liver HSP70 protein level—decreasing that in males while increasing that in females (Fig. 6B). HFD alone selectively decreased HSP70 in WT male but not female mice (Fig. 6B). TKO had no further effect on the impact of HFD on HSP70 protein level in livers of either sex (Fig. 6B). Taken together, these data indicated that TKO did not or only slightly elicited concomitant upregulation of the other major cytosolic AEA binding ‘chaperone’ protein HSP70 in liver. Females had significantly higher level of HSP70 than males, but again neither TKO, HFD, nor did both together have much effect on HSP70 in female livers. Three-way ANOVA indicated a significant interaction between sex and genotype with respect to hepatic HSP70 levels: sex x genotype, $P = 0.005$.

3.9 Impact of Fabp1/Scp-2/Scp-x Gene Ablation (TKO) on the Ability of Pair-Fed HFD to Induce Hepatic Triacyglycerol

Ad libitum-fed HFD diet increases not only hepatic endocannabinoid levels (e.g. AEA) and CB1 receptor, but also triacylglycerol—the hallmark of non-alcoholic fatty liver disease (NAFLD) liver [38] [2, 3]. Therefore, the impact of pair-fed HFD and TKO on liver triacylglycerol was examined as described in Methods. TKO alone significantly reduced liver triacylglycerol level in both male and female control-fed mice (Table 1). Pair-fed HFD alone did not induce but instead reduced hepatic triacylglycerol accumulation in control-fed male and female mice (Table 1). This was in marked contrast to prior studies with *ad libitum* fed HFD which increased hepatic triacylglycerol. Finally, TKO abolished the ability of pair-

fed HFD to further reduce liver triacylglycerol level (Table 1). Thus, TKO significantly reduced liver triacylglycerol levels and blocked the ability of pair-fed HFD to reduce liver triacylglycerol. Three-way ANOVA indicated the following significant interaction with respect to hepatic triacylglycerol levels: genotype x diet, $P < 0.001$.

4. Discussion

Upregulation of hepatic endocannabinoids (EC) such as AEA [2, 3] and increased hepatic expression of the major cannabinoid receptor [i.e. CB1 [2–4]] are strongly linked to non-alcoholic fatty liver disease (NAFLD). This is especially the case in both human and animal models of NAFLD induced by *ad libitum* fed HFD [2–4]. While the individual enzymes for EC synthesis and degradation as well as their receptors (e.g. CB1) are increasingly well understood, a major conundrum in understanding the regulation of hepatic EC metabolism has been how very poorly aqueous-soluble and highly membrane-bound lipidic molecules such as EC traffic from the plasma membrane through the cytosol to intracellular organelles for metabolism. Studies with non-selective inhibitors of fatty acid binding proteins [6] and with cultured transformed cells overexpressing other FABP family members (FABP5 or 7), determined that these cytosolic proteins function in binding 'chaperoning' AEA for intracellular degradation by FAAH [6–8, 14]. In contrast, until recently almost nothing was known regarding the existence of functional equivalent 'chaperones' for AEA degradation/hydrolysis, much less that of 2-AG, in liver. Although liver expresses only barely detectable amounts of FABP5 and/or 7, nevertheless liver cytosol contains at least an order of magnitude higher level (3–10% of liver cytosolic protein) of the liver fatty acid binding protein (FABP1, L-FABP), which has broad specificity for a variety of other types of lipidic ligands including fatty acids, cholesterol, bile acids, and xenobiotics (e.g. fibrates, plasticizers) [43–47]. More importantly, FABP1 also has high affinity for esterified ligands such as fatty acyl-CoA, fatty acyl-carnitine, lysophospholipid, and non-ARA containing monoacylglycerol [9, 48–52]. Examination of the structure of AEA and 2-AG reveals that these molecules are also esterified fatty acids—i.e. arachidonic acid (ARA) esterified to ethanolamine and glycerol, respectively. This suggested that FABP1 may also bind 'chaperone' not only AEA (analogous to FABP5 and 7) but also 2-AG whose cytosolic binding 'chaperone' protein remained elusive. A breakthrough came with the very recent discovery that FABP1 has high affinity for AEA and even more so 2-AG [11, 12, 53]. Furthermore, ablation of the *Fabp1* gene (LKO) significantly increased hepatic levels of AEA and 2-AG as well as altered the impact of HFD thereon [11]. However, liver not only expresses FABP1, but liver cytosol also contains high levels of sterol carrier protein-2 (SCP-2)—another major AEA and 2-AG binding 'chaperone' protein [11, 22, 54]. Thus, the concomitant presence and/or compensatory upregulation of SCP-2 may potentially complicate interpretation of the impact of LKO on the hepatic EC system. The studies described herein with pair-fed HFD, male and female mice, ablated in both the *Fabp1* and *Scp-2/Scp-x* genes (TKO) provided several new insights:

First, ablating the *Scp-2/Scp-x* gene in *Fabp1* null mice (TKO) dramatically increased hepatic AEA by >2- and >3-fold in control-fed male and female mice, respectively. In contrast, the impact of singly ablating only the *Fabp1* gene (LKO) much more modestly increased liver AEA (20 and 40% in male and female, respectively) and 2-AG (40% in male

but not female) in control-fed mice [11]. Thus, while TKO and LKO both exhibited similar qualitative sex-dependent impact on liver AEA, the effect of TKO was an order of magnitude greater increase in hepatic AEA (both males and females) and 2-AG (males only) in liver. Importantly, neither TKO described herein nor LKO [11] induced concomitant upregulation of hepatic HSP70. While HSP70 is a cytosolic protein that also binds AEA, albeit with much lower affinity than either FABP1 or SCP-2 [42], its functional significance in determining hepatic AEA and 2-AG level is not known. Finally, it is important to note that the much more dramatic TKO-induced increases in hepatic AEA level were not accompanied with concomitant upregulation of the key synthetic enzyme NAPEPLD or downregulation of the key degradative/hydrolytic enzymes FAAH and NAAA. In contrast, concomitant upregulation of the key synthetic enzyme DAGLα and the downregulation of the key degradative/hydrolytic enzyme MAGL in TKO mice were associated at least in part with the observed TKO-induced increase in 2-AG. It is important to note, however, that western blotting is a semi-quantitative technique and only reflects the total protein amounts that could miss changes in the activities of the synthetic and catabolic enzymes. Thus, the presence of SCP-2 in LKO liver may have largely compensated for the effect of loss of FABP1 on hepatic AEA and, at least in part 2-AG levels.

Second, TKO conferred on pair-fed HFD the ability to increase hepatic AEA and 2-AG in males, but not females. It is important to note that pair-feeding HFD alone did not increase hepatic AEA and even decreased 2-AG in male mice—in marked contrast to *ad libitum* fed HFD which increased hepatic AEA without altering 2-AG level in males [2, 3, 37, 38]. Taken together with earlier findings [28], these data suggested that hepatic AEA and/or 2-AG accumulation induced by *ad libitum* fed HFD was attributable primarily to the known preference/hyperphagia for HFD [20, 23] rather than to increased dietary proportion of fat. Finally, it is important to note that while LKO also conferred on pair-fed HFD the ability to increase hepatic AEA in males, LKO did not or only slightly increased 2-AG in males or females [28]. This suggested that the loss of both FABP1 and SCP-2 modestly exacerbated the impact of HFD on hepatic EC levels more than loss of only FABP1.

Third, TKO had no effect on protein level of the major liver cannabinoid receptor (CB1) in either male or female control-fed mice. Increased AEA and activation of CB1 in male mice is known to induce hepatic accumulation of triacylglycerol—a hallmark of NAFLD [2–4]. Despite the lack of effect of TKO alone on liver CB1 and even increased AEA in TKO control-fed mice, however, TKO alone actually decreased hepatic triacylglycerol accumulation. Likewise, LKO had little effect on hepatic CB1 and also increased AEA (albeit less so than in TKO), but LKO alone also decreased triacylglycerol in control-fed male mice [28]. Taken together, these data would indicate that loss of FABP1 rather than SCP-2 had the major inhibitory impact on hepatic triacylglycerol and loss of FABP1 antagonized the ability of increased AEA to induce hepatic triacylglycerol accumulation. Loss of FABP1 likely elicited these effects not only by loss of AEA binding ‘chaperoning’ capacity to stimulate SREBP1-mediated triacylglycerol synthesis [2, 38], but also by reducing fatty acyl-CoA binding ‘chaperoning’ capacity and targeting of bound fatty acyl-CoA to endoplasmic for synthesis of phosphatidic acid—the major precursor of hepatic triacylglycerol synthesis [55–57].

Fourth, ablating the *Scp-2/Scp-x* gene in *Fabp1* null mice (TKO) diminished/abolished the effect of pair-fed HFD on hepatic CB1 protein level. Pair-fed HFD alone only slightly increased hepatic CB1 and even decreased triacylglycerol in WT males—in marked contrast to the increased CB1 and triacylglycerol observed in livers of WT males fed HFD *ad libitum* [3, 5, 23, 58]. TKO also prevented any increase in triglyceride in mice pair fed HFD. In contrast, LKO alone had no effect on hepatic level of triacylglycerol in male mice pair-fed HFD [28]. This again suggested that the continued presence of SCP-2 in LKO mice pair-fed HFD also compensated in part for the loss of FABP1.

Fifth, it is important to note that the TKO-induced selective increase in hepatic AEA in male and female mice was not accompanied by any increase in esterified ARA—the precursor from with AEA is derived. This suggested that loss of both FABP1 and SCP-2 may have decreased hepatic uptake of ARA. Consistent with this possibility, both FABP1 and SCP-2 have high affinity for ARA and fluorescent ARA analogues and stimulate their esterification [9, 57, 59–63]. Furthermore, overexpression of FABP1 in cultured cells increases uptake of a fluorescent ARA analogue [63]. The finding that SCP-2 overexpression increases the uptake of other types of fatty acids and their analogues [64, 65], suggests that loss of SCP-2 would exacerbate the impact of *Fabp1* gene ablation on uptake of ARA. Finally, in contrast to the TKO-induced increase in AEA, the HFD-induced selective decrease in hepatic 2-AG was accompanied by decreased: i) dietary quantity of ARA and its precursors—which instead where higher in high fat diet than control diet; ii) levels of hepatic esterified ARA. Since hepatic 2-AG levels were at least an order of magnitude higher than those of AEA, these findings suggested that esterified ARA may have been preferentially spared/targeted for synthesis of AEA vs 2-AG by an as yet unresolved mechanism.

Sixth, the selective TKO-induced increase in hepatic DHEA in pair-fed HFD female mice was not accompanied by increased hepatic level of esterified DHA—the precursor from with DHEA is derived. On the contrary, TKO significantly reduced the hepatic level of esterified DHA. Since FABP1 binds DHA [59, 63] and FABP1 overexpression increases DHA uptake [63], the absence of FABP1 in TKO mice would be expected to lower hepatic DHA as was observed. Likewise, since SCP-2 overexpression also increases the uptake of fatty acids [64, 65], loss of SCP-2 in TKO mice would also be expected to be accompanied by decreased hepatic DHA. Neither the TKO-induced increase in DHEA nor the HFD-induced selective increase in hepatic DHEA in female WT mice was accompanied by any large increase in DHA. Finally, it is interesting to note that the impact of TKO on hepatic levels of esterified ARA was less than on DHA—suggesting that liver esterified ARA was preferentially retained over esterified DHA in response to loss of FABP1 and SCP-2. This finding was not accompanied by any difference in affinity of FABP1 for ARA vs DHA [63].

Seventh, TKO, HFD or both together also elicited significant changes (especially in females) in hepatic levels of non-endocannabinoid N-acylethanolamides and 2-monoacylglycerols that may also affect signaling pathways that impact hepatic lipid levels. At least two signaling pathways have been proposed to be impacted by non-endocannabinoid NAEs or 2-MGs: i) AEA-induced CB1 signaling to induce SREBP1c and other proteins inducing transcription of multiple genes involved in in lipogenesis [2, 38]. CB1 activation upregulates SREBP1c and thereby increases levels of ACC1 and FASN to increase de novo lipogenesis

while concomitantly decreasing expression of CPT-1 and fatty acid oxidation [38]. In HepG2 cells, CB1 receptors regulated not only SREBP1c, but also ChREBP, and LXR—all of which were induced by medium containing higher level of oleic and palmitic acid, thereby increasing fat accumulation [5, 58]. Some investigators have suggested that several non-endocannabinoid N-acylethanolamides and 2-monoacylglycerols act as 'entourage' molecules that may potentiate (OEA, PEA, 2-OG) or downregulate (EPEA, DHEA) the ability of AEA or 2-AG to activate the cannabinoid receptor CB1 [1, 66–73]. The fact that FABP1[11] and SCP-2 [11, 22, 54, 74] binds non-endocannabinoid NAEs and 2-MGs suggests potential roles in their targeting to intracellular organelles such as endoplasmic reticulum from which SREBP1 is released. Despite this possibility, however, there is not general agreement on this putative 'entourage' role for the non-endocannabinoid NAEs and 2-MGs. ii) Ligand induced activation of PPARs transcription of genes involved in fatty acid oxidation—thereby potentially antagonizing the actions of AEA/CB1 on nuclear receptors involved in de novo lipogenesis [23]. OEA and PEA target PPARα, which induces transcription of multiple genes in fatty acid oxidation [23]. OEA, does not bind CB1 or CB2, but is an endogenous PPARα agonist that lowers body weight and decreases NAFLD, while PEA is less potent [75, 76]. OEA induces PPARα target genes including PPARα itself, FAT/CD36, L-FABP, UCP2, ACS [75, 76]. Interestingly, at high concentrations AEA itself has been reported to target PPARγ [23] and PPARα [77] receptors involved in lipid storage and fatty acid oxidation respectively. FABP1 (but not SCP-2) is known to facilitate ligand (e.g. fatty acids, fibrates) trafficking into the nucleus, interaction with PPARα therein, and activating PPARα transcription of fatty acid oxidative genes [78]. By analogy, FABP1 binding the endocannabinoids as well as non-endocannabinoid NAEs and 2-MGs, FABP1[11] may similarly act to facilitate their signaling action in the nucleus. Finally, in terms of functional significance, the overall impact of altered endocannabinoids (AEA, 2-AG), together with the complex pattern of changes in non-endocannabinoid NAEs and 2-MGs was associated with hepatic triacylglycerol levels that were decreased by TKO alone, decreased by pair-fed HFD alone, but increased modestly in HFD-fed TKO mice.

In summary, ablation of *Scp-2/Scp-x* in *Fabp1* gene ablated mice (TKO) markedly exacerbated the impact of only singly ablating *Fabp1* (LKO) on the hepatic endocannabinoid system in control-fed mice. Furthermore, TKO diminished/abolished many of the effect of pair-fed HFD on the hepatic endocannabinoid system—again to a greater extent than LKO alone. Taken together these effects on the hepatic EC system indicated that although hepatic SCP-2 levels are several-fold lower than those of FABP1 in mice, continued expression of SCP-2 in LKO mice may have compensated at least in part for the loss of FABP1. Further loss of SCP-2 in TKO mice dramatically exacerbated the impact of losing only FABP1 on the hepatic endocannabinoid system and diminished the potential impact of HFD diet for inducing NAFLD. Conversely, liver FABP1 level is increased in both animal and human models of NAFLD [17, 18, 79]. In addition, expression of the highly common human FABP1 T94A variant is highly associated with NAFLD in human subjects [12, 80, 81].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AEA	n-6 arachidonoylethanolamide (anandamide)
2-AG	2-arachidonoyl glycerol
ARA	arachidonic acid
CB1	cannabinoid receptor-1
DAGLα	diacylglycerol lipase A
DHA	n-3 docosahexaenoic acid
DHEA	n-3 docosahexaenoylethanolamide
EC	endocannabinoid
EPEA	n-3 eicosapentaenoylethanolamide
FAAH	fatty acid amide hydrolase
FABP1	liver fatty acid binding protein-1 (L-FABP)
HFD	high fat diet
HSP70	heat shock protein 70
LCFA	long chain fatty acid
LCFA-CoA	long chain fatty acyl CoA
LC/MS	liquid chromatography/mass spectrometry
LKO	FABP1 gene ablated mouse on C57BL/6NCr background
2-MG	2-monoacylglycerol
MAGL	2-monoacylglycerol lipase
NAAA	N-acylethanolamide-hydrolyzing acid amidase
NAE	N-acylethanolamides
NAFLD	non-alcohol fatty liver disease
NAPE	N-acylphosphatidylethanolamide
NAPEPLD	N-acylphosphatidylethanolamide phospholipase-D
OEA	oleoylethanolamide

2-OG	2-oleoyl glycerol
PEA	palmitoylethanolamide
2-PG	2-palmitoyl glycerol
SCP-2	sterol carrier protein-2
SCP-x	sterol carrier protein-x
TKO	Scp-2/Scp-x gene ablated mouse on C57BL/6NCr background; wild-type C57BL/6NCr mouse.

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- *Scp-2*/*Scp-x* gene ablation in *Fabp1* null mice (TKO) reduced impact of high fat diet
- TKO exacerbated hepatic increase in AEA in males and females
- High fat diet (HFD) decreased hepatic AEA and 2-AG in males and females
- Overall hepatic arachidonic acid levels were not affected by TKO and/or HFD

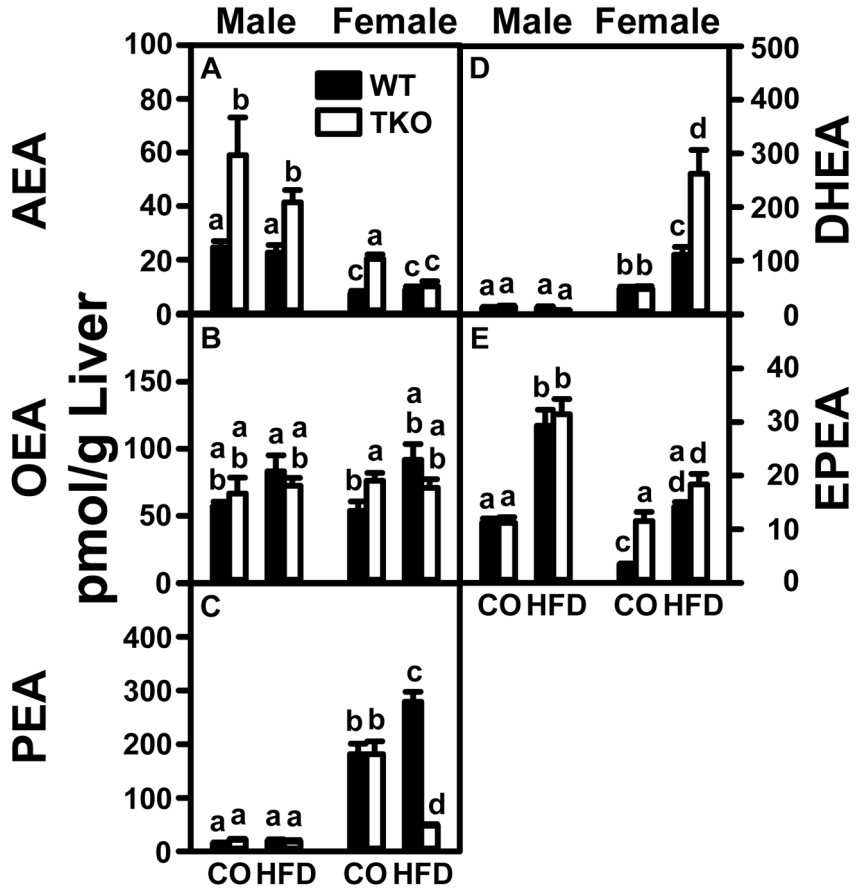

Fig. 1. Effect of *Fabp1/Scp-2/Scp-x* gene ablation (TKO) and sex on hepatic levels of N-acylethanolamides in mice fed high-fat diet (HFD)

Male (M) and female (F) wild-type (WT, **black bars**) and *Fabp1/Scp-2/Scp-x* gene ablated (TKO **open bars**) mice were pair-fed a control diet (CO) or high-fat diet (HFD) as described in Methods. At the end of the dietary study, the mice were fasted overnight, livers removed/flash frozen and stored at −80°C. Lipids were then extracted for analysis and quantitation of (A) AEA, (B) OEA, (C) PEA, (D) DHEA, (E) EPEA by LC-MS analysis using deuterated internal standards (Cayman Chemical) as described in Methods. Data represent the mean ± SEM (n = 8). By ANOVA, statistically different values ($P < 0.05$) are designated by different lower-case letters (a, b, c, d).

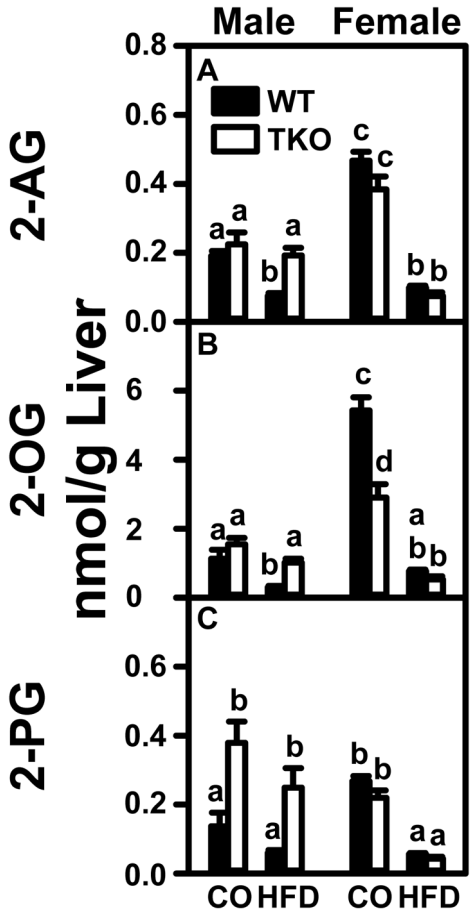

Fig. 2. Impact of *Fabp1/Scp-2/Scp-x* gene ablation (TKO) and sex on hepatic levels of 2-monoacylglycerols (2-MG) in mice fed high-fat diet (HFD)

Male (M) and female (F) wild-type (WT, **black bars**) and *Fabp1/Scp-2/Scp-x* gene ablated (TKO **open bars**) mice were pair-fed a control diet (CO) or high-fat diet (HFD) as described in Methods. At the end of the dietary study, the mice were fasted overnight; livers removed/flash frozen and stored at −80°C. Lipids were then extracted for analysis and quantitation of (A) 2-AG, (B) 2-OG, (C) 2-PG by LC-MS analysis using deuterated internal standards (Cayman Chemical) as described in Methods. Data represent the mean ± SEM (n = 8). By ANOVA, statistically different values ($P < 0.05$) are designated by different lower-case letters (a, b, c, d).

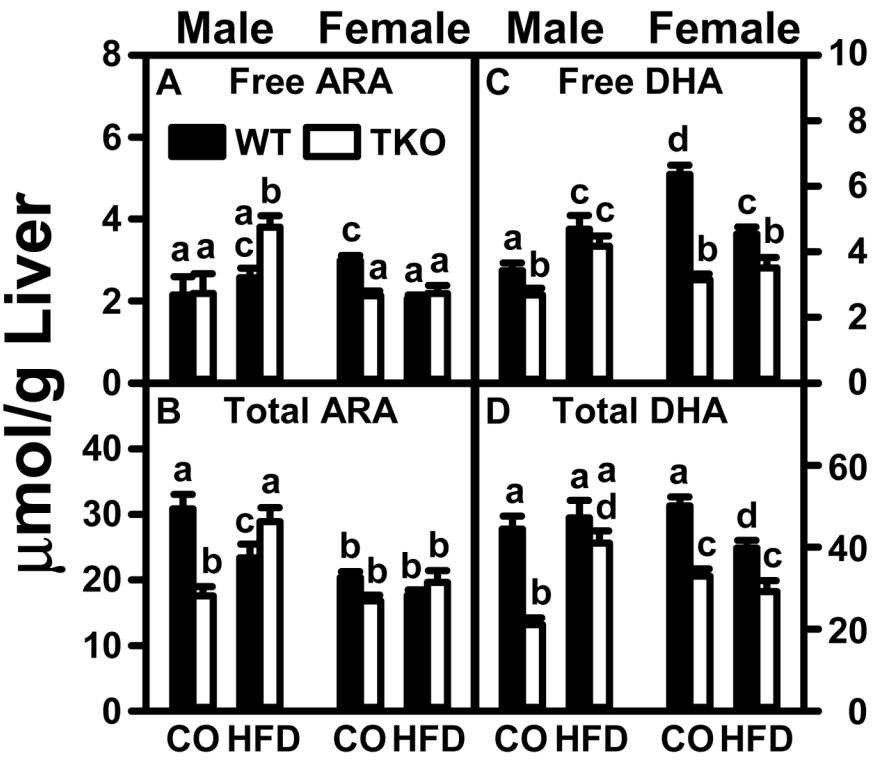

Fig. 3. *Fabp1/Scp-2/Scp-x* gene ablation (TKO) and pair-fed high fat diet (HFD) impact liver levels of unesterified Free and Total arachidonic acid (ARA) and docosahexaenoic acid (DHA)

WT (**black bars**) and TKO (**open bars**) mice (8 wk old) were fed phytol-free, phytoestrogen-free control chow (CO) or pair-fed high fat diet (HFD), overnight fasted, livers collected/flash frozen, and stored at −80°C as described in Methods. Liver levels of (A) Free ARA, (B) Total ARA, (C) Free DHA, (D) Total DHA were determined after lipid extraction and LC-MS using deuterated ARA-d_8 and DHA-d_5 internal standards as described in Methods. Data represent the mean ± SEM (n = 6–7). By ANOVA, statistically different values ($P < 0.05$) are designated by different lower-case letters (a, b, c, d).

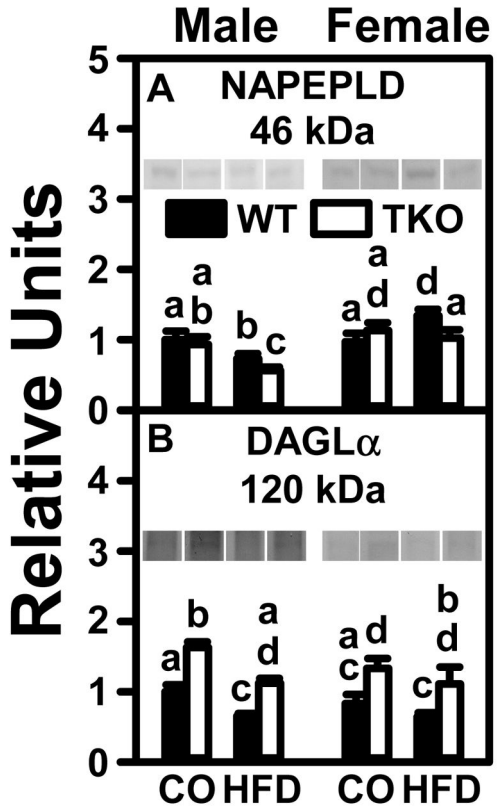

Fig. 4. *Fabp1/Scp-2/Scp-x* gene ablation (TKO) selectively increases hepatic protein level of key enzyme in synthesis of 2-AG, but not the other arachidonic acid (ARA)-containing endocannabinoid, AEA

Male (M) and female (F) wild-type (WT, **black bars**) and *Fabp1/Scp-2/Scp-x* gene ablated (TKO **open bars**) mice were pair-fed a control diet (CO) or high-fat diet (HFD) and at the end of the dietary study, the mice were fasted overnight, livers removed/flash frozen and stored at −80°C as described in Methods. Aliquots of liver homogenate were resolved by SDS-PAGE, individual proteins identified by western blotting, scanned, and quantitated using Ponceau stain (Supplemental Fig. 2, 3) as loading control to normalize respective western blotted proteins as described in Methods. (A) N-acylphosphatidylethanolamide phospholipase-D (NAPEPLD) (46 kDa); (B) diacylglycerol lipase A (DAGLα) (120 kDa). Values represent the mean ± SEM (n = 8). By ANOVA, statistically different values ($P < 0.05$) are designated by different lower-case letters (a, b, c, d).

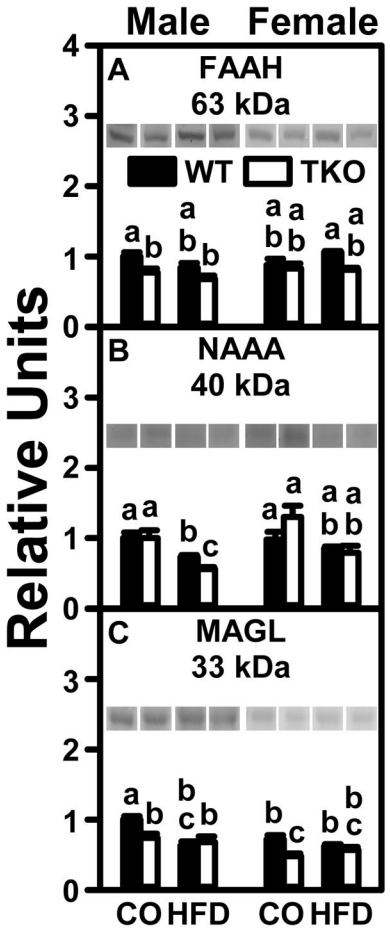

Fig. 5. *Fabp1/Scp-2/Scp-x* gene ablation (TKO) has little impact on hepatic protein levels of endocannabinoid (EC) degradative/hydrolase enzymes

Male (M) and female (F) wild-type (WT, **black bars**) and *Fabp1/Scp-2/Scp-x* gene ablated (TKO **open bars**) mice were pair-fed a control diet (CO) or high-fat diet (HFD) and at the end of the dietary study, the mice were fasted overnight, livers removed/flash frozen and stored at −80°C as described in Methods. Aliquots of liver homogenate were resolved by SDS-PAGE, individual proteins identified by western blotting, scanned, and quantitated using Ponceau stain (Supplemental Fig. 3, 4) as loading control to normalize respective western blotted proteins as described in Methods. (A) fatty acid amide hydrolase (FAAH) (63 kDa); (B) N-acylethanolamide-hydrolyzing acid amidase (NAAA) (40 kDa); (C) 2-monoacylglycerol lipase (MAGL) (33 kDa). Values represent the mean ± SEM (n = 8). By ANOVA, statistically different values are designated by different lower-case letters (a, b, c).

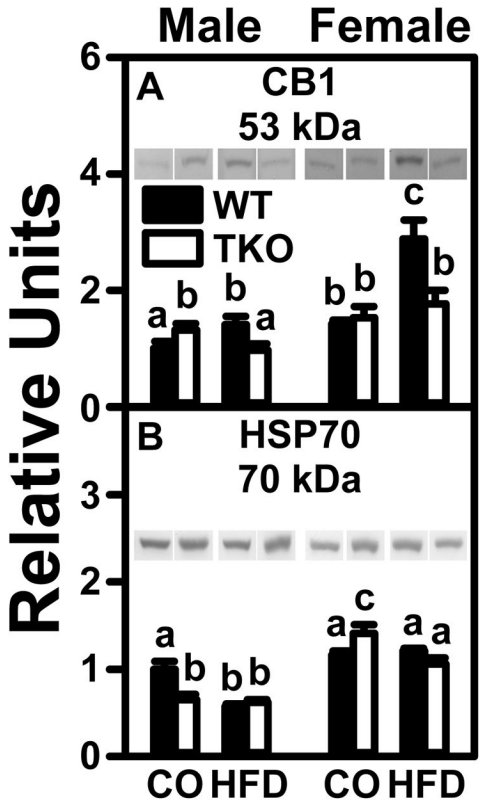

Fig. 6. Sex and HFD have greater impact than *Fabp1/Scp-2/Scp-x* gene ablation (TKO) on liver protein levels of cannabinoid receptor-1 (CB1) and endocannabinoid (EC) chaperone, HSP70

Male (M) and female (F) wild-type (WT, **black bars**) and *Fabp1/Scp-2/Scp-x* gene ablated (TKO **open bars**) mice were pair-fed a control diet (CO) or high-fat diet (HFD) and at the end of the dietary study, the mice were fasted overnight, livers removed/flash frozen and stored at −80°C as described in Methods. Aliquots of liver homogenate were resolved by SDS-PAGE, individual proteins identified by western blotting, scanned, and quantitated using Ponceau stain (Supplemental Fig. 2,5) as loading control to normalize respective western blotted proteins as described in Methods. (A) cannabinoid receptor-1 (CB1) (53 kDa); (B) heat shock protein 70 (HSP70) (70 kDa). Values represent the mean ± SEM (n = 8). By ANOVA, statistically different values are represented by lower-case letters (a, b, c).

Table 1

Impact of Fabp1/Scp-2/Scp-x gene ablation (TKO) and pair-fed high fat diet (HFD) on liver triacyglycerol

Male (M) and female (F) wild-type (WT) and *Fabp1/Scp-2/Scp-x* gene ablated (TKO) mice were pair-fed a control diet (CO) or high-fat diet (HFD) as described in Methods. At the end of the dietary study, the mice were fasted overnight; livers removed/flash frozen and stored at −80°C. Liver triacylglycerol (nmol/mg protein) was determined as described in Methods. Values represent the mean ± SEM (n = 7). By ANOVA, statistically significant differences ($P < 0.05$) are designated by different lower-case letters (a, b, c).

Genotype	Diet	Sex	Triacylglycerol (nmol/mg)
Wild-type	Control	Male	93±3a
Wild-type	Control	Female	94±3a
Wild-type	High-Fat	Male	59±5bc
Wild-type	High-Fat	Female	47±5b
TKO	Control	Male	54±2b
TKO	Control	Female	47±4b
TKO	High-Fat	Male	70±8c
TKO	High-Fat	Female	67±9c