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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.

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

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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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 up-regulated 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