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The effect of fibroblast growth factor 15 deficiency on the development of high fat diet induced non-alcoholic steatohepatitis

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ABSTRACT

Non-alcoholic steatohepatitis (NASH) is a form of non-alcoholic fatty liver disease (NAFLD) characterized by steatosis, inflammation, and fibrosis often associated with metabolic syndrome. Fibroblast growth factor 15 (FGF15), an endocrine factor mainly produced in the distal part of small intestine, has emerged to be a critical factor in regulating bile acid homeostasis, energy metabolism, and liver regeneration. We hypothesized that FGF15 alters the development of each of the listed features of NASH. To test this hypothesis, four-week old male *Fgf15*$^{-/-}$ and their corresponding wild-type (WT) mice were fed either a high fat diet (HFD) or a control chow diet for six months. The results confirmed that HFD feeding for six months in WT mice recapitulated human NASH phenotype, including macrovesicular steatosis, inflammation, and fibrosis. Whereas FGF15 deficiency had no effect on the severity of liver steatosis or inflammation, it was associated with decreased liver fibrosis. Furthermore, FGF15 deficiency resulted in abnormal bile acid homeostasis, increased insulin resistance, increased HFD-induced serum triglycerides, decreased inductions of hepatic cholesterol content by HFD, and altered gene expression of lipid metabolic enzymes. These data suggest that FGF15 improves lipid homeostasis and reduces bile acid synthesis, but promotes fibrosis during the development of NASH.

1. Introduction

Non-alcoholic steatohepatitis (NASH) is a more severe stage within the spectrum of non-alcoholic fatty liver disease (NAFLD) that is often associated with metabolic syndrome, characterized by steatosis, inflammation, and fibrosis. NASH can progress in severity leading to the development of end-stage liver diseases such as cirrhosis and hepatocellular carcinoma (HCC). The prevalence of NASH is currently on the rise and in 2011, it was estimated that NASH afflicts 3-5% of people in the United States (Vernon et al., 2011). Unfortunately, there is currently no approved therapeutics for the treatment of NASH. To address this problem, intense research efforts are ongoing with a few compounds currently in clinical trials. Several of these compounds modulate the functions of a nuclear receptor, farnesoid X receptor (FXR), and its response gene, *fibroblast growth factor 19* (*FGF19*).

Fibroblast growth factor 15 (FGF15) is a member of the family of fibroblast growth factors (FGFs) and its human orthologue is FGF19. Though FGF15 and FGF19 are orthologues they share only 50% amino acid sequence homology (Nishimura et al., 1999; Xie et al., 1999). Unlike most other FGFs, FGF15 does not bind extracellular heparin sulfate and can travel through systemic circulation to affect functions in distal organs. For this reason, FGF15 is known as an endocrine FGF (Ornitz and Itoh, 2015). Upon the activation of FXR in the ileum, FGF15 is induced in enterocytes and secreted into the portal circulation. In the liver, FGF15 binds to its predominant receptor, fibroblast growth factor receptor 4 (FGFR4), a tyrosine kinase receptor, which then activates the

Abbreviations: Acss2, Acyl-CoA synthetase short-chain 2; Akt, Protein kinase B; ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; Bsep, Bile salt export pump; Creb-Pgc-1α, cAMP regulator element binding-peroxisome proliferator-activated receptor γ coactivator 1α; Ctgf, Connective tissue growth factor; Cyp4a10, Cytochrome p450 4a10; Cyp7a1, Cytochrome p450 7a1; Cyp8b1, Cytochrome p450 8b1; Fas, Fatty acid synthase; Fgf15, Fibroblast growth factor 15; Fgf19, Fibroblast growth factor 19; Fgf21, Fibroblast growth factor 21; Fgfr4, Fibroblast growth factor receptor 4; Fgfr1c, Fibroblast growth factor receptor 1c; HCC, Hepatocellularcarcinoma; HE, Hematoxylin and Eosin; Ibabp, Intestinal bile acid binding protein; Icam1, Intercellular adhesion molecule 1; HFD, High fat diet, HSCshepatic stellate cells; MAPK, Mitogen-activated protein kinases; MCDD, Methionine choline deficient diet; Mtp, microsomal triglyceride transfer protein; NASH, Non-alcoholic steatohepatitis; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K, Phosphoinositide 3-kinase; PLC, Phospholipase C-γ; RAS, Rat sarcoma viral oncogene homolog; STAT, Signal transducer and activator of transcription; Tgfβ1, Transforming growth factor β1; Tnfα, Tumor necrosis factor α; WT, Wild type.

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mitogen-activated protein kinases (MAPK) signaling pathway. This results in down regulation of the expression of the Cytochrome P450 7a1 (*Cyp7a1)* gene which encodes a rate-limiting enzyme for bile acid synthesis (Inagaki et al., 2005; Song et al., 2009; Kong et al., 2012; Li et al., 2014). Therefore, FGF15 acts as a negative feedback factor maintaining bile acid homeostasis. Activation of FGFR4 by FGF15 is dependent upon the presence of β-Klotho, a binding partner of FGFR4, which serves as an obligate co-receptor for FGF15 (Lin et al., 2007; Ornitz and Itoh, 2015). In addition to MAPK signaling, FGFR4 activation induces other signaling pathways including phosphoinositide 3-kinase-Protein Kinase B (PI3K-AKT), Rat sarcoma viral oncogene homolog (RAS), signal transducer and activator of transcription (STAT), and phospholipase C-γ (PLC) pathways (Ornitz and Itoh, 2015). FGFR4 has also been shown to antagonize the pathways mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Drafahl et al., 2010) and cAMP regulator element binding-peroxisome proliferator-activated receptor γ coactivator 1α (CREB-PGC-1α) (Potthoff et al., 2011). FGF15 may also bind the FGFR1c/β-klotho dimer, but to a lesser extent (Kurosu et al., 2007).

In addition to maintaining bile acid homeostasis, FGF15/19 signaling plays a role in a number of other metabolic functions. For example, in transgenic gain-of-function studies, FGF19 increases insulin sensitivity, decreases serum cholesterol and triglyceride levels, and aids in weight loss (Tomlinson et al., 2002; Fu et al., 2004). FGF15/19 also regulates cellular energy homeostasis by decreasing gluconeogenesis (Potthoff et al., 2011), while increasing protein and glycogen synthesis (Kir et al., 2011). Additionally, FGF15 strongly enhances cell proliferation, stimulating HCC development (Uriarte et al., 2015) and liver regeneration (Uriarte et al., 2013; Kong et al., 2014). Recent studies have also shown that FGF15 may play a detrimental role in the development of carbon tetrachloride induced liver fibrosis (Uriarte et al., 2015). Thus, liver fibrosis was reduced in carbon tetrachloride-treated *Fgf15* knockout (KO) mice when compared to wild type (WT) mice. *In vitro* treatment of hepatocytes with FGF15 induced the expression of connective tissue growth factor (*Ctgf*), which may enhance fibrosis via activating hepatic stellate cells (HSCs) (Uriarte et al., 2015). However, the role of FGF15 in NALFD and NASH development has not been clarified.

In the current study, we aimed to analyze the effects of FGF15 deficiency in mice on the development of NASH with a focus on steatosis, inflammation, and fibrosis. A long-term high fat diet (HFD) feeding model was selected as it best recapitulates symptoms of metabolic syndrome in humans.

2. Methods

2.1. Animals and treatment

A whole body *Fgf15* KO mouse strain (*Fgf15*$^{-/-}$) was used with a mixed 75% A129/25% C57BL/6J background (Kong et al., 2014). WT mice with the same genetic background were used as controls. Briefly, homozygous KO and wild-type (WT) colonies were littermates obtained from heterozygous breeders (Fgf15$^{+/-}$) on a mixed 75% A129/25% C57BL/6J background. The established homozygous KO and WT colonies were expanded with similar genetic background. Four week old male KO and WT mice were fed a HFD (60% calories from lard, 0.2796% cholesterol, 20% calories from carbohydrate, Research Diets catalog #D12492, New Brunswick, NJ; n = 5 for WT and n = 4 for KO) or purified control chow diet (10% calories from lard, 0.00136% cholesterol, 70% calories from carbohydrates, Research Diets catalog # D12450J; n = 4 for both WT and KO). Mice were group-housed and provided food and water *ad libitum*. Total body weights were measured weekly. Five months after commencing the designated diet, an oral glucose tolerance test was performed. Animals were euthanized at the end of the sixth month of feeding. Blood, liver and ileum samples were collected as previously described (Kong et al., 2009). The animal protocols conducted in this study were approved by the Rutgers Institutional Animal Care and Use Committee.

2.2. Serum biochemical parameters and hepatic lipid composition

Serum levels of triglyceride, cholesterol, bile acids, alanine aminotransferase activity (ALT), and alkaline phosphatase activity (ALP) were measured using commercially available kits according to the manufacturers' instructions (triglycerides, cholesterol, ALT, and ALP kits - Pointe Scientific, Canton, MI; total bile acids kit - Bioquant, San Diego, CA). Hepatic lipid content was measured as previously described (Li et al., 2012). In brief, tissue lipid extracts were generated by homogenizing 100 mg of tissue in a buffer containing 18 mM Tris (pH 7.5), 300 mM mannitol, 50 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was incubated overnight in 2:1 chloroform-methanol solution with overhead shaking. Water was added to the mixture and the samples were centrifuged to separate the aqueous and lipid phases. The lipid phase was collected and dried via vacuum. The concentrated lipids were dissolved in 60% tert-butanol and 40% Triton X-114/methanol (2:1) mix and analyzed for triglyceride and cholesterol using the previously described kits.

2.3. Histology

Frozen liver samples were sectioned and stained with hematoxylin and eosin (H&E) or Sirius Red and severity scored for histomorphological characteristics of NAFLD. Fibrosis was represented as the percent of tissue area positive for Sirius Red staining determined using ImageJ software (Schneider et al., 2012). Liver sections were immunohistochemically stained for desmin (PIPA519063; Thermo Fisher Scientific, Waltham, MA) with percent positive stained cells calculated with Image Pro Plus (Media Cybernetics Inc., Rockville, MD). Images were captured with a VS120 Slide Scanner (Olympus, Center Valley, PA).

2.4. Gene expression

Total RNA was extracted from homogenized frozen tissue samples in TRIzol Reagent (Thermo Fisher Scientific; Waltham, MA) and reverse transcribed to cDNA. Relative expression of genes of interest was determined via RT q-PCR with Sybr Green chemistry. Primer sequences are listed in Table 1. Expression of *β-actin* was used to normalize mRNA levels.

2.5. Protein analysis

Crude membrane fractions were prepared to allow for the determination of relative BSEP protein levels in the liver by western blot. Crude membrane fractions were prepared as previously described (Csanaky et al., 2009). Protein concentration of the crude membrane faction was measured by BCA assay (Pierce Biotechnology, Rockford, IL) and 50 μg of protein was resolved in a 10% polyacrylamide gel. Protein was transferred to a PVDF membrane and blocked in 5% nonfat dry milk for 2 hours. Membranes were incubated with anti-BSEP primary antibodies (K44, 1:3000) overnight followed by a 1 hour incubation in a species-specific secondary antibody conjugated to horseradish peroxidase. Bands were detected using Pierce ECL western blotting substrate (Pierce Biotechnology, Rockford, IL). Membrane were stripped (Restore Western Blot Stripping Buffer; Thermo Scientific, Waltham, MA) and re-probed with anti-β-actin antibody (1:1000; JLA20, EMD Millipore, Temecula, CA) as a loading control.

2.6. Statistical analysis

The data are expressed as mean ± standard deviation (SD). Comparison of the multiple treatment groups was performed using two-way