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G protein-coupled bile acid receptor plays a key role in bile acid metabolism and fasting-induced hepatic steatosis

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Abstract

Bile acids are signaling molecules that play a critical role in regulation of hepatic metabolic homeostasis by activating nuclear farnesoid X receptor (Fxr) and membrane G-protein-coupled receptor (*Tgr5*). The role of FXR in regulation of bile acid synthesis and hepatic metabolism has been studied extensively. However, the role of TGR5 in hepatic metabolism has not been explored. The liver plays a central role in lipid metabolism and impaired response to fasting and feeding contributes to steatosis and non-alcoholic fatty liver and obesity. We have performed a detailed analysis of gallbladder bile acid and lipid metabolism in *Tgr5*^{-/-} mice in both free-fed and fasted conditions. Lipid profiles of serum, liver and adipose tissues, bile acid composition, energy metabolism, and mRNA and protein expression of the genes involved in lipid metabolism were analyzed. Results showed that deficiency of the *Tgr5* gene in mice alleviated fasting-induced hepatic lipid accumulation. Expression of liver oxysterol 7 α -hydroxylase (Cyp7b1) in the alternative bile acid synthesis pathway was reduced. Analysis of gallbladder bile acid composition showed marked increase of tauro-cholic acid and decrease of tauro- α and β -muricholic acid in *Tgr5*^{-/-} mice. *Tgr5*^{-/-} mice had increased hepatic fatty acid oxidation rate and decreased hepatic fatty acid uptake. Interestingly, fasting induction of fibroblast growth factor 21 (*Fgf21*) in liver was attenuated. In addition, fasted *Tgr5*^{-/-} mice had increased activation of hepatic growth hormone-signal transducer and activator of transcription 5 (GH-Stat5) signaling compared to wild type mice.

Conclusion—This study suggests that TGR5 may play a role in determining bile acid composition and in fasting-induced hepatic steatosis through a novel mechanism involving activation of the GH-Stat5 signaling pathway.

Keywords

Bile acids; *Tgr5*; *Cyp7b1*; *Stat5*; growth hormone

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Introduction

Bile acids are signaling molecules that play a key role in regulation of metabolic homeostasis via activation of a nuclear bile acid receptor farnesoid- X- receptor (*Fxr*) and membrane G protein-coupled bile acid receptor (*Gpbar-1*, aka *Tgr5*)^{1, 2}. Bile acids are synthesized from cholesterol exclusively in the liver, secreted into bile and stored in the gallbladder and released to the intestinal tract for emulsifying fats and lipid-soluble vitamins. The classic bile acid synthesis pathway is initiated by cholesterol 7 α -hydroxylase (*Cyp7a1*) for synthesis of two primary bile acids, cholic acid (CA), and chenodeoxycholic acid (CDCA). Sterol 12 α -hydroxylase (*Cyp8b1*) is required for synthesis of CA and sterol 27-hydroxylase (*Cyp27a1*) is involved in steroid side-chain oxidation³. In mouse liver, CDCA is 6 β -hydroxylated to α -muricholic acid (α -MCA), which is epimerized to β -muricholic acid (β -MCA). The alternative bile acid synthesis pathway is initiated by *Cyp27a1* and involved oxysterol 7 α -hydroxylase (*Cyp7b1*). Bile acids are conjugated to the amino acids taurine or glycine before biliary secretion. In the intestine, conjugated CA and CDCA are de-conjugated by bacterial bile salt hydrolases and then de-hydroxylated by bacterial 7 α -dehydroxylase to form deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. Accumulation of high amounts of toxic bile acids could cause liver inflammation and injury and cholestatic liver diseases. Thus, bile acid synthesis is tightly regulated by bile acids returning from the ileum to hepatocytes to inhibit *Cyp7a1* and *Cyp8b1* gene transcription by two mechanisms. In the intestine, bile acids activate FXR to induce fibroblast growth factor 15 (*Fgf15*), which is circulated to the liver to activate the membrane Fgf receptor 4 signaling to inhibit *Cyp7a1* and *Cyp8b1* gene transcription. In the liver, bile acids activate FXR to induce the negative receptor small heterodimer partner (*Shp*) to inhibit *Cyp7a1* and *Cyp8b1* gene transcription.

The liver plays a central role in lipid metabolism during fasting and re-feeding cycles. During the postprandial state, the liver uptakes dietary fats to synthesize triglycerides for VLDL-mediated secretion and transport to adipose tissue for storage and to muscles for energy metabolism. During fasting, fatty acids are mobilized from adipocytes to the liver for energy metabolism. Prolonged fasting is known to induce hepatic steatosis and changes in endocrine and paracrine factors such as thyroid hormone, insulin, glucagon, growth hormone (GH) and fibroblast growth factor 21 (*Fgf21*) and these hormones regulate hepatic glucose, lipid and energy metabolism and maintain metabolic homeostasis⁴⁻⁶. The role of *Fxr* in regulation of metabolic homeostasis has been studied extensively. However, the role of *Tgr5* in liver metabolism has not been explored. *Tgr5* is expressed in many tissues including intestine, gallbladder, liver, and brain⁷⁻¹¹. In the liver, *Tgr5* is expressed in Kupffer cells and sinusoidal endothelial cells, but not in hepatocytes^{10, 11}. In the gastrointestinal tract, activation of *Tgr5* by its specific agonists protects intestinal barrier function and inflammation, stimulates gallbladder refilling, stimulates glucagon like peptide-1 (GLP-1) secretion from enteroendocrine L-cells¹², which stimulates insulin secretion from pancreatic β cells¹³. In brown adipocytes, activation of *Tgr5* induces thyroid hormone deiodinase 2 (*Dio2*), which activates thyroid hormone to stimulate energy metabolism¹⁴. Secondary bile acids LCA and DCA are efficacious activators of *Tgr5* to induce cAMP and activate protein kinase A signaling pathways^{7, 8}. Activation of *Tgr5* by specific *Tgr5*

agonists has been shown to alleviate obesity and hepatic steatosis by maintaining glucose homeostasis in diet-induced obese mice¹⁵. However, *Tgr5* deficient (*Tgr5*^{-/-}) mice do not have abnormal metabolic phenotypes¹⁶ and are protected from lithogenic diet-induced cholesterol gallstone formation¹⁷. Similar to *Fxr*^{-/-} mice, *Tgr5*^{-/-} mice have impaired liver regeneration suggesting that both bile acid receptors are important in lipid metabolism during liver regeneration¹⁸.

To understand the role of *Tgr5* in liver lipid metabolism, we have performed a detailed analysis of bile acid and lipid metabolism in *Tgr5*^{-/-} mice in both free-fed and fasted conditions. We hypothesized that *Tgr5* might play a key role in bile acid synthesis and lipid homeostasis. Our data show that gallbladder bile acid composition of *Tgr5*^{-/-} mice was markedly altered. Surprisingly, we found that *Tgr5*^{-/-} mice were protected from fasting-induced hepatic steatosis by reducing hepatic fatty acid uptake, and increasing hepatic fatty acid oxidation to ameliorate hepatic steatosis, likely through activation of a GH-signal transducer and activator of transcription (Stat5) signaling.

Materials and Methods

Animal experiments

Wild type male C57BL/6J (WT) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were maintained on regular chow diet and water *ad libitum* (unless specified) in a temperature-controlled facility with a 12 h light/12 h dark schedule (6 am to 6 pm light). *Tgr5* knock out (*Tgr5*^{-/-}) mice were obtained from Merck Research Laboratories (Kenilworth, NJ) and bred to C57BL/6J background. Twenty to twenty-one week old WT mice (n=12) or *Tgr5*^{-/-} mice (n=12) were separated to two groups (6 WT and 6 *Tgr5*^{-/-} in each group). One group was free-fed chow diet and the other group was fasted for 24 h. Mice were euthanized at 12 pm and tissues were collected and stored in -80°C until analysis. To study the role of the Jak2/Stat pathway on hepatic lipid metabolism, mice were given a Jak2 inhibitor, tyrphostin Ag 490 (5mg/Kg) (Sigma-Aldrich, St. Louise, MO) or vehicle (phosphate buffered saline, PBS), intra-peritoneally. Mice were fasted for 21 h and were given a second dose of Ag490 (5mg/kg) or vehicle and sacrificed 3 h later. All animal experiments were performed at Northeast Ohio Medical University (NEOMED) and protocols approved by the Institutional Animal Care and Use Committee.

Hepatic β -oxidation rate analysis

WT and *Tgr5*^{-/-} male mice were fasted for 24 h, euthanized and livers were collected. Hepatic fatty acid oxidation rate was assessed using [1-¹⁴C] palmitic acid¹⁹. Intact mitochondria were isolated from mouse liver tissue using sucrose Tris-EDTA buffer. [1-¹⁴C] Palmitic acid (0.4 μ Ci) was suspended in 40 μ l of 7% BSA solution at 37°C and added to 330 μ l incubation mixture (100 mM sucrose, 10 mM Tris HCl, pH 7.4, 5 mM KH₂PO₄, 0.2 mM EDTA, pH8, 80 mM KCl, 1 mM MgCl₂, 2 mM L-carnitine, 0.1 mM Malate, 0.05 mM Coenzyme A, 2 mM ATP, 1 mM DTT, pH 8.0) and 30 μ l of mitochondria protein solution. The mixture were incubated at 37°C for 30 min, transferred to a new micro centrifuge tube with 200 μ l of perchloric acid (1 M) and pre-incubated Whatman filter paper discs in the cap. Samples were incubated for 1 h to entrap CO₂ released from reaction mixture. After

incubation, discs were transferred to scintillation vials and CO₂ released was analyzed using a scintillation counter. Micro centrifuge tubes were centrifuged at 14,000 rpm for 10 min and 400 µl supernatant was used to determine acid soluble metabolite (ASM) levels formed during reaction. Data generated was expressed in nmole/mg/min of palmitic acid converted to CO₂ and ASM.

Hepatic fatty acid uptake analysis

Hepatic fatty acid uptake assay was performed as described^{20, 21}. WT and *Tgr5*^{-/-} male mice were anesthetized with ketamine and xylazine following overnight fasting. ³H-labeled fatty acids (30 µCi, ³H-oleic acid, Perkin-Elmer, Waltham, MA) were dissolved in 200 µl of bovine serum albumin solution (2 mg/ml in saline), which was injected to mice via tail vein. At 1 and 10 min post injection, mice were sacrificed and liver, skeletal muscle, adipose tissue and blood were collected and radioactivity was determined using a scintillation counter. Radioactivity of liver was corrected for radioactivity present in serum during sampling and was normalized with tissue weight, whereas for skeletal muscle radioactivity of tissue was normalized with tissue weight, and expressed as percent of total radioactivity injected to each mice.

Comprehensive Laboratory Animal Monitoring System (CLAMS)

Comprehensive metabolic profiling of 20-21 week old male WT and *Tgr5*^{-/-} mice during 24 h fasting was performed using an Oxymax lab animal monitoring system (Columbus Instruments, Columbus, OH). Respiratory exchange ratio, O₂ consumption and CO₂ production, activity and energy expenditure of mice were measured during 24 h fasting.

Statistics

The statistical significance between groups was determined using one-way ANOVA followed by a Turkey *post hoc* test or t-test, using GraphPad Prism® software (GraphPad Software Inc., CA). Data are presented as mean ± SE, with *p* < 0.05 considered statistically significant.

Results

Tgr5^{-/-} mice were resistant to fasting-induced hepatic steatosis

Prolonged fasting is known to induce hepatic steatosis by accumulation of adipose tissue-derived free fatty acids (FFA) in hepatocytes²². Extended fasting for 24 h significantly decreased body weights in both WT and *Tgr5*^{-/-} mice, but no significant change was observed between WT and *Tgr5*^{-/-} mice after fasting (Supplemental **Fig.1A**). No significant changes in liver weight were observed between free-fed and fasted WT mice, whereas liver weight in *Tgr5*^{-/-} mice was significantly decreased after fasting (Supplemental **Fig.1A**). White adipose tissue (epididymal fat) weight decreased in both fasted WT and *Tgr5*^{-/-} mice (Supplemental **Fig.1A**). Fasting did not affect serum triglyceride levels between WT and *Tgr5*^{-/-} mice. Fasting increased serum FFA by 3-fold in WT mice, but *Tgr5*^{-/-} mice had significantly lower circulating FFA compared to WT mice (Supplemental **Fig.1B**). Serum cholesterol levels in *Tgr5*^{-/-} mice were significantly higher compared to WT mice in both free-fed and fasted conditions (Supplemental **Fig.1B**). Fasting induced serum β-

hydroxybutyrate levels in both WT and *Tgr5*^{-/-} mice by ~10-fold compared to free-fed mice (Supplemental **Fig.1B**).

Fasting significantly increased liver triglycerides and FFA levels in both WT and *Tgr5*^{-/-} mice (**Fig. 1A**). This effect was attenuated in fasted *Tgr5*^{-/-} mice compared to fasted WT mice. Hepatic cholesterol levels show a small but significant increase in fasted WT mice, but no significant change in *Tgr5*^{-/-} mice. Hepatic glycogen content was significantly higher in free-fed *Tgr5*^{-/-} mice compared to WT mice indicating increased glucose storage as glycogen in *Tgr5*^{-/-} mice, but no difference in fasted mice. Fasting significantly decreased hepatic glycogen content in both WT and *Tgr5*^{-/-} mice. Oil-red-O staining for neutral lipids (**Fig.1B**) showed fasting markedly increased lipids in WT mice but *Tgr5*^{-/-} mice had significantly lower lipid content. These results are surprising and may suggest that lack of Tgr5 protects the liver from fasting-induced hepatic steatosis. In white adipose tissue and skeletal muscle, triglyceride levels were decreased in fasted *Tgr5*^{-/-} mice, but not WT mice (**Fig. 1C & 1D**). FFAs were significantly decreased in skeletal muscle (**Fig. 1D**) but not white adipose tissue (**Fig. 1C**) of fasted *Tgr5*^{-/-} mice.

***Tgr5*^{-/-} mice switched to the classic bile acid synthesis pathway to generate a hydrophobic bile acid pool**

To understand the role of Tgr5 in bile acid metabolism, we analyzed hepatic bile acid synthesis gene expression and bile acid composition in gallbladder bile. **Fig. 2A** shows that *Cyp7a1* mRNA levels were the same in the fasted and free-fed states in both WT and *Tgr5*^{-/-} mice. Feeding should induce *Cyp7a1* mRNA expression²³. Unchanged *Cyp7a1* expression in free-fed and fasted mice may be because mice were sacrificed at 12 pm, the zenith of the *Cyp7a1* circadian rhythm. In free-fed mice, *Cyp7b1* and *Cyp27a1* mRNA levels were significantly decreased in *Tgr5*^{-/-} mice compared to WT mice, and fasting significantly decreased *Cyp7b1* and *Cyp27a1* expression in both WT and *Tgr5*^{-/-} mice (**Fig. 2A**). Fasting induced *Cyp8b1* mRNA in WT mice as expected²⁴. However, fasting induction of *Cyp8b1* was significantly attenuated in *Tgr5*^{-/-} mice compared to WT mice (**Fig. 2A**). We performed immunoblot analysis to study if changes of mRNA expression levels of *Cyp7b1* and *Cyp8b1* are consistent to their protein expression levels. **Fig 2B** showed that *Cyp8b1* protein levels were induced by fasting in both wild type and *Tgr5*^{-/-} mice. Expression of *Cyp7b1* is known to exhibit sexual dimorphisms, with higher expression in male than female rodents²⁵. Fasting reduced *Cyp7b1* protein levels in both wild type and *Tgr5*^{-/-} mice (**Fig 2B**), consistent with reduced *Cyp7b1* mRNA levels by fasting. During fasting, bile acids are stored in the gallbladder and bile acid composition in bile represents *de novo* synthesized bile acids and bile acids circulated back to the liver via enterohepatic circulation. We therefore analyzed bile acid composition in gallbladder bile. Data showed a marked increase of TCA and TDCA and decrease of Tα-MCA and Tβ-MCA in both free-fed and fasted *Tgr5*^{-/-} mice compared to WT mice (**Fig. 2C**). TCA, TDCA, and Tα-MCA and Tβ-MCA account for more than 90% of total bile acids in mouse bile. Other minor bile acid species were also increased in both free-fed and fasted *Tgr5*^{-/-} mice. These changes in bile acid composition are consistent with decreased expression of *Cyp7b1* in *Tgr5*^{-/-} mice, indicating that the alternative bile acid synthesis was suppressed and the classic bile acid synthesis pathway was stimulated to produce more CA and less CDCA derived Tβ-MCA.

As the result, the hydrophobicity index of bile acid pool increases from -0.418 in WT mice to -0.22 in *Tgr5*^{-/-} mice. The ratio of 12 α -hydroxylated bile acids (TCA and TDCA) to non-12 α -hydroxylated bile acids (TCDCA and T α -MCA and T β -MCA) increases from 0.71 in WT mice to 1.68 in *Tgr5*^{-/-} mice. It is interesting to point out that bile acid composition seems to dependent on genotype, and fasting/feeding does not affect bile acid composition except TDCA (%) increased two-fold by fasting in both wild type and *Tgr5*^{-/-} mice. TDCA is a preferred ligand for TGR5⁸ indicating that TGR5 signaling may be stimulated in fasted mice.

***Tgr5*^{-/-} mice had increased fatty acid oxidation**

Fasting significantly decreased expression of lipogenic genes, such as sterol regulatory element-binding protein-1 (*Srebp-1*), acetyl-CoA carboxylase alpha (*Acc- α*), fatty acid synthase (*Fasn*) and stearoyl-CoA decarboxylase-1 (*Scd-1*) in both WT and *Tgr5*^{-/-} mice compared to free-fed mice (**Fig. 3A**). Hepatic mRNA expression of *Acc- α* was increased 3-fold in free-fed *Tgr5*^{-/-} mice compared to WT mice. Fasting increased hepatic carnitine palmitoyltransferase 1 α (Cpt1 α), involved in fatty acid β -oxidation, and decreased lipoprotein lipase (*Lpl*) expression in both WT and *Tgr5*^{-/-} mice compared to free-fed mice (**Fig. 3B**). Protein analysis showed significant increase in Cpt1 protein levels only in *Tgr5*^{-/-} mice (**Fig. 3B**) indicating increased hepatic fatty acid oxidation. Peroxisome proliferator-activated receptor α (*Ppar- α*) levels were not altered in either free-fed or fasted WT and *Tgr5*^{-/-} mice. Ppar-gamma co-activator-1 α (*Pgc-1 α*) levels were induced in fasted WT mice, and further increased in fasted *Tgr5*^{-/-} mice (**Fig. 3B**). *Tgr5*^{-/-} mice had significantly increased rate of palmitic acid oxidation to CO₂ and acid soluble material (ASM) compared to WT mice in the fasted condition (**Fig. 3C**). Furthermore, expression levels of liver *Fgf21* mRNA and serum *Fgf21* were drastically induced during fasting in WT mice⁵, but were significantly lower in *Tgr5*^{-/-} mice (**Fig. 3D**). Since hepatic autophagy plays an important role in lipolysis during fasting²⁶, we analyzed expression of the hepatic autophagy marker microtubule-associated protein 1 light chain 3 II (*Lc3II*), which increased 3.5-fold in fasted *Tgr5*^{-/-} mice (**Fig. 3E**).

***Tgr5*^{-/-} mice had altered energy expenditure**

As *Tgr5* is known to regulate energy metabolism and hormones levels, we analyzed serum hormone levels in fed and fasted mice. *Tgr5*^{-/-} mice have increased serum insulin levels only during free-fed conditions and decreased thyroxine (T₃) hormone levels only during fasting conditions compared to WT mice (Supplementary **Fig. 2A** and **2B**). No significant difference in fasting glucagon levels were observed between WT and *Tgr5*^{-/-} fasted mice (Supplementary **Fig. 2C**).

To understand energy metabolism involved in attenuation of hepatic steatosis in *Tgr5*^{-/-} mice, we examined respiratory exchange ratio (RER), energy expenditure and physical activity using CLAMS. Oxygen consumption rate (VO₂) and carbon dioxide production rate (VCO₂) were significantly increased in fasted *Tgr5*^{-/-} mice during both day and night compared to WT mice (**Fig. 4A**). Although both VO₂ and VCO₂ are increasing in *Tgr5*^{-/-} mice at both day and night times, RER (VCO₂/VO₂) increased significantly only in daytime and significantly decreased in nighttime in *Tgr5*^{-/-} mice compared to WT mice (**Fig. 4B**).

Energy expenditure was increased in fasted *Tgr5*^{-/-} mice only during the night (**Fig. 4C**). Physical activity was increased during both day and night in fasted *Tgr5*^{-/-} mice compared to WT mice (**Fig. 4D**). These data indicate that fasted *Tgr5*^{-/-} mice are more active and used more lipids as an energy source (decreased RER) during the active period (night) compared to WT mice. These results are consistent with decreases in hepatic FFA and triglyceride levels and circulating FFA in fasted *Tgr5*^{-/-} mice.

Lack of Tgr5 signaling impaired hepatic fatty acid uptake

To determine whether attenuation of fasting-induced steatosis could be due to altered hepatic lipid uptake in *Tgr5*^{-/-} mice, we analyzed hepatic fatty acid translocase (*Cd36*) and fatty acid transport protein (*Fatp*) mRNA levels. Fasting significantly increased hepatic *Cd36* and *Fatp4* mRNA levels in WT mice (**Fig. 5A**). In *Tgr5*^{-/-} mice, *Cd36* mRNA expression was reduced compared to wild type mice in free fed state. Interestingly, hepatic *Cd36* and *Fatp4* mRNA levels were significantly down regulated in fasted *Tgr5*^{-/-} mice compared to fasted WT mice (**Fig. 5A**). *Fatp5* and *Fabp1* mRNA levels were significantly decreased in fasted *Tgr5*^{-/-} compared to WT mice. Western blot analysis showed *Tgr5*^{-/-} mice had significantly decreased hepatic *Cd36* protein levels in both fed and fasted conditions (**Fig. 5B**). We further determined hepatic VLDL secretion and lipid uptake. Hepatic VLDL secretion was only significantly increased after 3 h in *Tgr5*^{-/-} mice compared to WT mice (**Fig. 5C**). Fatty acid uptake assay showed significant decrease in hepatic fatty acid uptake in *Tgr5*^{-/-} mice compared to WT mice at 1 min and 10 min, but increased fatty acid uptake in skeletal muscle. (**Fig. 5D**). These results suggest that *Tgr5*^{-/-} mice may have impaired hepatic fatty acid uptake, but increased fatty acid oxidation and VLDL secretion and increased fatty acid uptake into skeletal muscle to reduce hepatic triglyceride and FFA during fasting.

Adipose tissue lipolysis in *Tgr5*^{-/-} mice

Prolonged fasting activates lipolysis in white adipose tissue resulting in increased circulating FFA and hepatic steatosis; therefore, we analyzed genes involved in lipid metabolism in white adipose tissue. Fasting significantly induced adipose triglyceride lipase (*Atgl*) mRNA expression, but did not change hormone sensitive lipase (*Hsl*) and monoglyceride lipase (*Mgl1*) mRNA levels in WT and *Tgr5*^{-/-} mice (**Fig. 6A**). Fasting significantly induced uncoupling protein-1 (*Ucp-1*) and *Ucp-3* mRNA levels in WT mice compared to free-fed mice (**Fig. 6A**). In *Tgr5*^{-/-} mice, fasting significantly increased *Ucp-2* mRNA levels compared to free-fed mice. In both WT and *Tgr5*^{-/-} mice, fasting did not affect adipose tissue *Ppar-γ* or its target genes *Cd36*, *Pgc-1α*, fatty acid binding protein 4 (*Fabp4*) expression (**Fig. 6A**). In fasted wild type mice, *Ucp-1*, 2 and 3, *Cpt1a* and *Dio2* mRNA levels were increased in brown adipose tissue indicating increase thermogenesis. However, fasting-induced increases of thermogenesis gene expression was lowered in fasted *Tgr5*^{-/-} mice compared to fasted wild type mice (**Fig. 6B**).

Tgr5^{-/-} mice had increased GH-Stat5 signaling

It has been reported that *Cd36* and hepatic steatosis are negatively regulated by GH-Stat5 signaling²⁷. We analyzed p-Stat5 and Stat5 levels in WT and *Tgr5*^{-/-} mice in fed and fasted conditions. Fasting significantly decreased the p-Stat5/Stat5 ratio in both WT and *Tgr5*^{-/-}

mice compared to free-fed mice. However, the p-Stat5/Stat5 ratio was significantly higher in *Tgr5*^{-/-} mice compared to WT mice in fasting state (**Fig. 7A**). Fasted *Tgr5*^{-/-} mice have significantly higher nuclear Stat5 levels compared to WT fasted mice (**Fig. 7B**). We also analyzed *Ppar-γ* target genes involved in lipid droplet formation since Stat5 activation is known to decrease *Ppar-γ* target gene expression²⁷. Hepatic *aP2 (Fabp4)* and cell death-inducing DEFA-like effector c (*Cidec*) mRNA levels were significantly down regulated in fasted *Tgr5*^{-/-} mice compared to WT mice (**Fig. 7C**). We also analyzed serum GH levels in these mice. **Fig. 7D** shows that serum GH levels were markedly increased in both fasted WT and *Tgr5*^{-/-} mice. However, *Tgr5*^{-/-} mice have significantly higher serum GH levels than WT mice (**Fig. 7D**). These results indicate that TGR5 signaling affect GH levels in both fasting and free fed state. However, *Tgr5*^{-/-} mice may have activated hepatic GH-Stat5 signaling to reduce fatty acid transport to hepatocytes, thus reduces lipid droplets accumulation in *Tgr5*^{-/-} mice during fasting. GH is known to activate the Janus Kinase 2 (Jak2)/Stat pathway. To further study the GH-Jak2/Stat pathway in hepatic lipid metabolism, we injected mice with a Jak2 inhibitor, Ag490, and fasted for 24 h. **Fig. 7E** showed that Ag490 significantly decreased hepatic triglycerides and free fatty acids in fasted WT mice, but did not further reduce hepatic lipids in fasted *Tgr5*^{-/-} mice. Ag490 reduced p-Stat5 in hepatocytes (**Suppl Fig 3A**), tend to increase hepatic Cd36, Fgf21 and Cidec mRNA expression (**Supplemental Fig. 3B**), and increased phosphorylation of adipocyte hormone-sensitive lipase (p-HSL) (**Suppl Fig 3C**). Thus Ag490 may reduce adipocyte lipolysis, consistent with a report that deficiency of Jak2 impaired adipose lipolysis²⁸. These results suggest that similar to adipose-specific *Jak2*^{-/-} mice, increased GH in *Tgr5*^{-/-} mice may also improve hepatic lipid metabolism in fasting.

Discussion

Detailed analysis of bile acid composition in *Tgr5*^{-/-} mice revealed a significant increase in TCA and decrease in T-MCAs in bile, suggesting stimulation of the classic bile acid synthesis pathway and suppression of the alternative pathway. This is consistent with decreased expression of the key regulatory genes *Cyp27a1* and *Cyp7b1* in the alternative bile acid synthesis pathway. Increasing TCA and decreasing TMCAs increases the bile acid hydrophobicity indices in *Tgr5*^{-/-} mice. In mice, TCA is the major *Fxr* ligand and tauro α/β-MCAs are potent *Fxr* antagonists²⁹. This study suggests that Tgr5 may play an important role in altering bile acid composition in bile acid pool and lowering of 12α-hydroxylated bile acids, the levels of which have been linked to insulin resistance in diabetic patients³⁰ and improving glucose sensitivity after vertical sleeve gastrectomy by modulating bile acid composition in circulating bile acids³¹.

Recent studies show that activation of *Fxr* decreases fasting-induced hepatic autophagy, a key pathway involved in maintaining nutrient homeostasis during fasting³². The increase of hepatic Lc3II levels in *Tgr5*^{-/-} mice may indicate increase of lipophagy to decrease hepatic triglyceride levels²⁶ and increased Lc3II levels are correlated to increased GH-Stat5 activation³³. During fasting, *Tgr5*^{-/-} mice have increased hepatic *Pgc-1α* and Cpt1 and increased palmitic acid oxidation. Fasting increases FFA to stimulate Ppar-α activity and induce hepatic *Fgf21* production. This study demonstrates a significant increase of *Pgc-1α* and decrease of hepatic *Fgf21* production in *Tgr5*^{-/-} mice. *Pgc-1α* is known to inhibit

hepatic *Fgf21* production³⁴ and a recent study reports that *Fgf21* inhibits hepatic GH-Stat5 signaling³⁵. Therefore, reducing hepatic *Fgf21* production stimulates hepatic Stat5 signaling in fasted *Tgr5*^{-/-} mice.

The liver is a major target of GH action, and Stat5 signaling activates Jak2/Stat5 signaling to regulate fatty acid oxidation and steroid metabolism²⁷. Mice expressing mutant GH receptor or deletion of Stat5 had increased hepatic *Cd36* expression and hepatic steatosis when challenged with high-fat diet²⁷. Our study shows that *Tgr5*^{-/-} mice have increased serum GH levels compared to WT mice. Chronic infusion of GH activates *Ppar-α* and prevents hepatic steatosis in diabetic rats³⁶. It is possible that this GH-Stat5 mechanism may be activated in *Tgr5*^{-/-} mice to stimulate hepatic lipolysis. Moreover, plasma GH levels show pulsatile secretion patterns in male rats, mice and humans, but GH is constantly secreted at much lower levels in females. Pulsatile GH secretion results in sexual dimorphic expression of many male-specific liver genes including *Cyp7b1*²⁵. In hepatocyte-specific *Stat5*-deficient mice, sexual dimorphic *Cyp7b1* expression is reduced and hypophysectomy reduces *Cyp7b1* expression²⁵. Pulsatile infusion of GH to hypophysectomized mice increases *Cyp7b1* expression, but continuous GH infusion for 5-7 days or disruption of GH pulsatile patterns decreases *Cyp7b1* expression in WT male mice^{25, 37}. It is interesting to note that high-fat diet-fed female *Tgr5*^{-/-} mice accumulated fat, gained weight and had impaired insulin sensitivity, and male mice are not obese but had increased steatosis^{16, 38}. It is possible that GH signaling may play a role in gender-specific regulation of bile acid synthesis by Tgr5. Since TGR5 is not expressed in hepatocytes¹⁰, TGR5 may regulate hepatic metabolism through paracrine or endocrine mechanisms. Bile acids have been detected in the brain³⁹. TGR5 is expressed in the hypothalamus and may regulate neuroendocrine hormone secretions in cholestasis^{40, 41}. It has been reported that pituitary controls bile acid metabolism⁴² and GH plays a role in regulation of bile acid synthesis⁴³. Thus bile acids may activate pituitary TGR5 to regulate GH secretion in hypothalamus. Further studies are needed to explore the brain to liver axis in TGR5 regulation of liver bile acid metabolism.

It is interesting to point out that TGR5 has both positive and negative effect in hepatic metabolism in fasting and feeding response and hepatic steatosis. Our current study shows *Tgr5*^{-/-} mice are protected against fasting-induced hepatic steatosis. Previous studies show *Tgr5*^{-/-} mice are protected against lithogenic diet-induced gallstone and insulin tolerance is improved in male *Tgr5*^{-/-} mice on chow diet but impaired insulin sensitivity when fed a high fat diet^{17, 38}. *Tgr5*^{-/-} mice have increased serum GH, similar to adipose-specific *Jak2*^{-/-} mice with high GH and reduced hepatic fatty liver²⁸. On the other hand, postprandial activation of TGR5 by bile acids is known to improve insulin secretion from β-cells via stimulating glucagon like peptide-1 (GLP-1) secretion in enteroendocrine L cells¹², whereas growth hormone counteracts insulin action in fasting. In this study we report altered lipid metabolism in a model, which has no or low insulin signaling. Beneficial effects we observe during fasting condition are due to lack of insulin signaling and increasing GH-Stat5 signaling. Lack of insulin signaling in fasting condition is similar to diabetic condition.

Fig 8 illustrates a mechanistic link of Tgr5 signaling to regulation of bile acid synthesis and lipid metabolism. Pulsatile GH secretion is known to activate Stat5 to induce male-specific Cyp7b1 expression in mice. High levels of serum GH in fed *Tgr5*^{-/-} mice may inhibit *Cyp7b1* in the alternative bile acid synthesis pathway and results in reducing TMCAs and increasing CA in bile acid pool. GH is known to stimulate lipolysis in adipose tissues and mobilize FFA. In hepatocytes, GH activates GH receptor, which recruits JAK2 to activate Stat5 by phosphorylation. In fasted *Tgr5*^{-/-} mice, increased GH activates Stat5 signaling to decrease *Cd36* expression and thus reduce FFA uptake into hepatocytes. FFA induces *Pgc-1α*, which increases fatty acid oxidation, but increase of *Pgc-1α* in *Tgr5*^{-/-} mice dampens fasting-induced production of *Fgf21*, which is known to reduce hepatic Stat5 signaling and lipophagy to reduce hepatic steatosis in *Tgr5*^{-/-} mice. Thus lower Fgf21 levels may also stimulate hepatic Stat5 activation during fasting. This Cd36-Fgf21-Stat5 regulatory loop may be induced in fasting to regulate lipid metabolism.

In conclusion, deficiency of *Tgr5* in mice alleviates fasting-induced hepatic steatosis by increasing hepatic lipolysis and GH-Stat5 signaling. This study uncovered a novel mechanism that bile acid-activated *Tgr5* signaling may alter GH signaling and bile acid synthesis to regulate hepatic bile acid and lipid metabolism. Further study of GH-Stat5 signaling in *Tgr5*^{-/-} mice is needed to understand the underlying mechanisms for increasing serum GH-Stat5 signaling during fasting, and for GH-Stat5 signaling in modulation of *Cyp7b1* expression levels and bile acid metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Acc-α	acetyl-CoA carboxylase α
aP2	adipocyte 2 (or fatty acid binding protein 4, <i>Fabp4</i>)
ASM	acid soluble metabolite
Cpt1α	carnitine palmitoyltransferase 1α
CDCA	chenodeoxycholic acid
CA	cholic acid
Cd36	cluster of differentiation 36, (or FAT, fatty acid translocase)
CLAMS	comprehensive laboratory animal monitoring system
Cyp7a1	cholesterol 7α-hydroxylase
Cyp8b1	sterol 12α-hydroxylase

Cyp27a1	sterol 27-hydroxylase
Cyp7b1	oxysterol 7 α -hydroxylase
Dio2	Deiodinase 2
DCA	deoxycholic acid
Fatp	fatty acid transport proteins
Fas	fatty acid synthase
FFA	free fatty acid
Fgf15	fibroblast growth factor 15 (human ortholog Fgf19)
Fgf21	fibroblast growth factor 21
Fxr	farnesoid- X- receptor
GH	growth hormone
GLP-1	glucagon like peptide-1
LCA	lithocholic acid
α/β-MCA	α - and β -muricholic acids
Ppar-α	peroxisome proliferator-activated receptor α
Pgc-1α	peroxisome proliferator-activated receptor γ co-activator 1- α
Shp	small heterodimer partner
Stat5	signal transducer and activator of transcription 5
Socs2	suppressors of cytokine signaling 2
T₃	thyroxine
Tgr5	Takeda G protein-coupled receptor 5 (<i>aka</i> G protein coupled bile acid receptor (Gpbar-1))
Ucp	uncoupling protein

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Fig. 1. Effect of *Tgr5* on fasting-induced lipid metabolism

(A) Hepatic triglycerides, free fatty acids, cholesterol and glycogen levels in free-fed (FF) and fasted WT and *Tgr5*^{-/-} mice. (B) Oil-Red-O staining and quantification of liver neutral lipids of fed and fasted WT and *Tgr5*^{-/-} mice. (C) White adipose tissue triglyceride and free fatty acid (FFA) levels in fed and fasted mice. (D) Skeletal muscle triglyceride and FFA levels in FF and fasted mice. An “*” indicates significant difference between FF and fasted condition ($p < 0.05$). A “#” indicates significant difference between WT and *Tgr5*^{-/-} mice in either FF or fasted condition ($p < 0.05$).

Fig. 2. Effect of *Tgr5* on hepatic bile acid metabolism

(A) Relative mRNA expression of hepatic bile acid synthesis gene in free-fed (FF) and fasted WT and *Tgr5*^{-/-} mice. (B) Immunoblot analysis and quantification of Cyp8b1 and Cyp7b1 protein levels in FF and fasted male mice. Each lane represents one mouse (n=3 per group). (C) Bile acid composition of gallbladder bile in FF and fasted mice. Bile acids are quantified as % of total bile acids analyzed (100%), < 0.5% is rounded to 0. An “*” indicates significant difference between FF and fasted condition ($p < 0.05$). A “#” indicates significant difference between WT and *Tgr5*^{-/-} mice in either FF or fasted condition ($p < 0.05$). A “†” indicates significant difference compared to WT male mice.

Fig. 3. Effect of *Tgr5* on fasting-induced hepatic lipid metabolism

(A) Relative mRNA expression of hepatic fatty acid synthesis genes in free-fed (FF) and fasted WT and *Tgr5*^{-/-} mice. (B) Relative mRNA expression of hepatic lipolysis genes in FF and fasted mice, and western blot analysis and quantification of *Cpt1* protein levels in FF and fasted mice. Each lane represents one mouse (n=3 per group). (C) Hepatic β-oxidation rates in fasted mice. Data was presented as nmoles of ¹⁴C-palmitic acid converted into CO₂ and acid soluble material (ASM). (D) Relative expression of hepatic *Fgf21* mRNA and Western blot analysis of serum *Fgf21* protein levels in FF and fasted mice. (E) Western blot analysis of hepatic autophagy *Lc3 I* and *Lc3 II* protein and quantitation of Lc3II levels in FF and fasted mice. Each lane represents one mouse (n=3 per group). An “*” indicates significant difference between FF and fasted condition ($p < 0.05$). A “#” indicates significant difference between WT and *Tgr5*^{-/-} mice in either FF or fasted condition ($p < 0.05$).

Fig. 4. Effect of *Tgr5* on oxygen consumption and physiological activity during fasting condition (A) VO₂ and VCO₂ levels during fasting in WT and *Tgr5*^{-/-} mice. (B) Respiratory exchange ratio during fasting in WT and *Tgr5*^{-/-} mice. (C) Energy expenditure during fasting in WT and *Tgr5*^{-/-} mice. (D) Activity levels during fasting in WT and *Tgr5*^{-/-} mice. A “#” indicates significant difference between WT and *Tgr5*^{-/-} mice in fasted condition ($p < 0.05$).

Fig. 5. Effect of *Tgr5* on hepatic lipid uptake and disposition

(A) Relative mRNA expression of hepatic fatty acid uptake genes in free-fed (FF) and fasted mice. (B) Western blots analysis and quantification of hepatic *Cd36* protein levels in FF and fasted mice. Each lane represents one mouse (n=3 per group). (C) Hepatic VLDL secretion in WT and *Tgr5*^{-/-} mice. (D) Hepatic and muscle lipid uptake in WT and *Tgr5*^{-/-} mice injected ³H-oleic acid. An “*” indicates significant difference between FF and fasted condition ($p < 0.05$). A “#” indicates significant difference between WT and *Tgr5*^{-/-} mice in either FF or fasted condition ($p < 0.05$).

Fig. 6. Effect of *Tgr5* on fasting-induced adipose tissue lipolysis

(A) Relative mRNA expression of the genes involved in fatty acid metabolism in white adipose tissue. (B) Relative mRNA expression of brown adipose-specific genes in free-fed (FF) and fasted mice. An “*” indicates significant difference between FF and fasted condition ($p < 0.05$). A “#” indicates significant difference between WT and *Tgr5*^{-/-} mice in either FF or fasted condition ($p < 0.05$).

Fig. 7. Effect of *Tgr5* on growth hormone signaling and growth hormone levels during Free-fed (FF) and fasted conditions

(A) Western blot analysis and quantification of hepatic p-Stat5 and Stat5 protein levels in both WT and *Tgr5*^{-/-} mice during FF and fasting conditions. Each lane represents one mouse (n=3 per group). (B) Western blot analysis and quantification of Stat5 hepatic nuclear protein expression in fasted WT and *Tgr5*^{-/-} mice. Each lane represents one mouse (n=4 per group). (C) Relative mRNA expression of hepatic *Ppar-γ* target gene in WT and *Tgr5*^{-/-} FF and fasted mice. (D) Serum growth hormone levels in both WT and *Tgr5*^{-/-} mice during FF and fasted conditions. (E) Hepatic triglyceride and free fatty acids levels in fasted WT and *Tgr5*^{-/-} mice treated with either vehicle or Ag 490. An “*” indicates significant difference between FF and fasted condition ($p<0.05$). A “#” indicates significant difference between WT and *Tgr5*^{-/-} mice in either FF or fasted condition ($p<0.05$). A “\$” Indicates significance between vehicle and Jak2 inhibitor treatment ($p<0.05$). A “+” indicates significance between WT and *Tgr5*^{-/-} mice treated with either vehicle or Jak2 inhibitor ($p<0.05$).

Fig. 8. Deletion of *Tgr5* alters bile acid metabolism and alleviates fasting-induced hepatic steatosis via altering hepatic GH-Stat5 signaling

Pulsatile release of GH may activate Stat5 to induce male-specific *Cyp7b1* expression and the alternative bile acid synthesis pathway. In *Tgr5*^{-/-} mice, high levels of non-pulsatile GH secretion (GH resistance) may inhibit *Cyp7b1* and *Cyp27a1* to reduce CDCA and MCA synthesized by the alternative bile acid synthesis pathway and increase CA synthesis. In fasted *Tgr5*^{-/-} mice, GH resistance activates Stat5 signaling to decrease *Cd36* expression and uptake of free fatty acids into hepatocytes. Free fatty acids induce *Pgc-1α*, which increases fatty acid oxidation, but increase of *Pgc-1α* in *Tgr5*^{-/-} mice dampens fasting-induced *Fgf21* production. Lower *Fgf21* levels stimulate hepatic Stat5 activation and reduce hepatic steatosis. This *Cd36*-*Fgf21*-Stat5 regulatory loop may regulate lipid metabolism during fasting and feeding cycles.