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Importance of Large Intestine in Regulating Bile Acids and Glucagon-Like Peptide-1 in Germ-Free Mice[§]

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ABSTRACT

It is known that 1) elevated serum bile acids (BAs) are associated with decreased body weight, 2) elevated glucagon-like peptide-1 (GLP-1) levels can decrease body weight, and 3) germ-free (GF) mice are resistant to diet-induced obesity. The purpose of this study was to test the hypothesis that a lack of intestinal microbiota results in more BAs in the body, resulting in increased BA-mediated transmembrane G protein-coupled receptor 5 (TGR5) signaling and increased serum GLP-1 as a mechanism of resistance of GF mice to diet-induced obesity. GF mice had 2- to 4-fold increased total BAs in the serum, liver, bile, and ileum. Fecal excretion of BAs was 63% less in GF mice. GF mice had decreased secondary BAs and increased taurine-conjugated BAs, as anticipated. Surprisingly, there was an increase in non-12 α -OH BAs, namely, β -muricholic acid, ursodeoxycholic acid (UDCA), and their taurine conjugates, in GF mice. Further, *in vitro* experiments confirmed that UDCA is a primary

BA in mice. There were minimal changes in the mRNA of farnesoid X receptor target genes in the ileum (Fibroblast growth factor 15, small heterodimer protein, and ileal bile acid-binding protein), in the liver (small heterodimer protein, liver receptor homolog-1, and cytochrome P450 7a1), and BA transporters (apical sodium dependent bile acid transporter, organic solute transporter α , and organic solute transporter β) in the ileum of GF mice. Surprisingly, there were marked increases in BA transporters in the large intestine. Increased GLP-1 levels and gallbladder size were observed in GF mice, suggesting activation of TGR5 signaling. In summary, the GF condition results in increased expression of BA transporters in the colon, resulting in 1) an increase in total BA concentrations in tissues, 2) a change in BA composition to favor an increase in non-12 α -OH BAs, and 3) activation of TGR5 signaling with increased gallbladder size and GLP-1.

Introduction

Bile acids (BAs) are amphipathic cholesterol metabolites that aid in the absorption of fats and fat-soluble vitamins from the diet. Primary BAs are synthesized in the liver, secreted into bile, and further metabolized

by intestinal bacteria to produce secondary BAs. Most of the BAs are actively reabsorbed by the ileum and enter the portal vein. Therefore, the diversity of BAs in the circulation is influenced by their metabolism by the liver as well as intestinal bacteria.

The BA profile is important because BAs also regulate host physiology. Individual BAs have physicochemical properties based on their structure. An increase in BAs that have a –OH group in the 12 position, such as cholic acid (CA) and deoxycholic acid (DCA) (Supplemental Fig. 1), increases cholesterol absorption across the intestine (Li-Hawkins et al., 2002). Taurine conjugation lowers the pK_a of BAs, increases their aqueous solubility, and promotes a high intraluminal BA concentration, leading to efficient solubilization of lipids and lipid-soluble vitamins (Setchell et al., 2013). BAs synthesized by the host modify the intestinal bacterial composition, and intestinal bacteria modify the circulating BA composition in the host. Thus, BAs are one of the host factors that help shape the intestinal bacterial community, and thus feeding BAs can alter intestinal microbial composition (Islam et al., 2011).

BAs act as hormones, binding to and activating their receptors: farnesoid X receptor (FXR) and transmembrane G protein-coupled receptor 5 (TGR5) (official name is GPCR). BAs regulate their own synthesis and transport by binding to and activating FXR (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). BAs also activate the membrane-bound G protein-coupled receptor TGR5, leading to an increase in intracellular cAMP production (Maruyama et al., 2002).

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TGR5 activation in brown adipose tissue increases type 2 iodothyronine deiodinase, which converts T_4 to the active T_3 , which increases energy expenditure (Watanabe et al., 2006). TGR5 activation in the enteroendocrine cells of the intestine increases glucagon-like peptide-1 (GLP-1) secretion (Katsuma et al., 2005; Thomas et al., 2009). Patients treated with a BA-binding resin to reduce cholesterol levels also have improved glycemic control, and these BA-binding resins also improved hyperglycemia in rodent models of obesity (Kobayashi et al., 2007; Chen et al., 2010). TGR5 agonists, such as 6 α -ethyl-23(S)-methyl-cholic acid (EMSA or INT-777), are promising drug candidates to treat metabolic disorders, such as type 2 diabetes, obesity, and steatohepatitis, as they improve glucose tolerance and reduce inflammation and steatosis in mice (Keitel and Haussinger, 2012).

Intestinal bacteria contain enzymes that metabolize BAs and change the BA composition in the intestinal content and thus also in the circulation of the host (Narushima et al., 2000, 2006). The capacity of intestinal microbiota to metabolize BAs not only provides intestinal bacteria cellular carbon, nitrogen, and sulfur, but also enables them to be bile resistant and grow in the presence of BAs. Intestinal bacteria perform the following modifications of BAs in the gut lumen: deconjugation of taurine or glycine-conjugated BAs (by bile acid hydrolase), oxidation and epimerization of the 3-, 7-, and 12-hydroxy groups of BAs (by hydroxysteroid dehydrogenases), and 7-dehydroxylation (by 7-dehydratase). Bile acid hydrolase and hydroxysteroid dehydrogenase are present in a broad spectrum of intestinal bacteria, whereas enzymes for 7-dehydratase required to produce DCA and lithocholic acid are restricted to a small number of intestinal anaerobes, such as *Clostridium* (Ridlon et al., 2006).

Germ-free (GF) mice, which lack intestinal bacteria, are resistant to high-fat diet-induced obesity (Backhed et al., 2007; Rabot et al., 2010), and, in contrast, colonization of GF mice with intestinal bacteria results in increased body fat content and insulin resistance (Backhed et al., 2004). Surgical therapies to treat obesity, such as vertical sleeve gastrectomy, result in weight loss and are associated with elevated circulating BAs (Myronovych et al., 2014; Ryan et al., 2014). It is understood that intestinal bacteria regulate BAs in circulation (Zhang et al., 2014), and elevated BAs in circulation are associated with weight loss. BAs activate TGR5, increase secretion of GLP-1, and improve insulin resistance and weight loss. Thus, it might be possible that GF mice have increased concentrations of bile acids that activate TGR5 and increase GLP-1 levels, which might add in the resistance of GF mice to diet-induced obesity. Therefore, in this study, alterations in BA composition and BA signaling in GF mice were examined to test this hypothesis.

Materials and Methods

Animals. All mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility at the University of Kansas Medical Center, with a 14-hour light/10-hour dark cycle, in a temperature- and humidity-controlled environment with ad libitum access to

feed and water. The initial breeding colony of GF C57BL/6J/UNC mice was established with mice obtained from the National Gnotobiotic Rodent Resource Center (University of North Carolina, Chapel Hill, NC). GF mice were housed in vinyl flexible film isolators (Class Biologically Clean Ltd., Madison, WI), with autoclaved, irradiated Harlan Sani chips (#7990.BG Irradiated Teklad Sani Chips; Harlan Teklad, Madison, WI) as bedding and autoclaved paper towels or autoclaved nestlets as enrichment. All conventional (CV) C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice had free access to the autoclaved rodent diet #5K67 (Labdiet, St. Louis, MO) and autoclaved water. The sterility of the isolator was tested routinely by culturing and performing wet mounts and gram stains of feces. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

Chemicals and Reagents. The sources of individual BA standards and internal standards were described previously (Zhang and Klaassen, 2010; Zhang et al., 2011a). A GLP-1 total enzyme-linked immunosorbent assay kit was purchased from EMD Millipore (St. Charles, MO). Oasis-HLB SPE cartridges for BA extraction were purchased from Waters (Milford, MA). All other chemicals and reagents, unless indicated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO).

Bile Collection. The protocol for bile-duct cannulation and bile collection has been described previously (Csanaky et al., 2011). Briefly, mice were anesthetized intraperitoneally with a ketamine/midazolam mixture (100 and 5 mg/kg, respectively), and the common bile duct of each mouse was cannulated through a high-abdominal incision with the shaft of a 30-gauge needle attached to PE-10 tubing. Bile was collected in the dark for 40 minutes into preweighed microcentrifuge tubes on ice. The volume of bile was determined gravimetrically using the value 1 for specific gravity.

Tissue and Feces Collection. Mice were sacrificed with an overdose of pentobarbital and opening the abdominal cavity. Blood was collected from suborbital veins into Microtainer plasma-separating tubes (BD Biosciences, San Jose, CA). Tissues were collected, frozen in liquid nitrogen, and stored at -80°C until further analysis. The small intestine contents were removed, and the tissue was divided into three equal segments, namely, the duodenum, jejunum, and ileum. In this study, the large intestine indicates only the colon and not the cecum. All animal sacrifices and tissue collections were performed between 9:00 AM and noon to minimize variations in BA metabolism due to circadian rhythm (Zhang et al., 2009, 2011b). CV and GF mice were housed individually in metabolic cages, and feces were collected over 24 hours.

Bile Acid Extraction and Quantification by Ultra-Performance Liquid Chromatography–Tandem Mass Spectrometry. Internal standards (40 $\mu\text{g/ml}$ d4-G-CDCA and 20 $\mu\text{g/ml}$ d4-CDCA in MeOH) were added to the samples, and BAs were extracted from the serum, gallbladder bile, livers, biliary bile, ileum, and feces using methods reported previously by our laboratory (Alnouti et al., 2008; Zhang and Klaassen, 2010; Zhang et al., 2011a, 2012). The BAs quantified include taurine-conjugated cholic acid (TCA), taurine-conjugated deoxycholic acid (TDCA), taurine-conjugated chenodeoxycholic acid (TCDCA), taurine-conjugated lithocholic acid (TLCA), taurine-conjugated ursodeoxycholic acid (TUDCA), taurine-conjugated α -muricholic acid, (α MCA), TMDCA taurine-conjugated β -muricholic acid (β MCA), taurine-conjugated ω -muricholic acid (ω MCA), cholic acid (CA), deoxycholic acid (DCA) chenodeoxycholic acid (CDCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), α muricholic acid β MCA, and ω MCA. α MCA and β MCA were summed together and expressed as α + β MCA due to a peak separation issue. The concentrations of individual BAs were summed to derive the concentrations of total conjugated, total unconjugated, and total BAs in each tissue compartment.

ABBREVIATIONS: Abc, ATP-binding cassette; Abcg5/8, ATP-binding cassette sub-family G member 5/8; Asbt, apical sodium dependent bile acid transporter; BA, bile acid; BAAT, Bile acid-CoA:amino acid N-acyltransferase; BAL, bile acid-coenzyme A ligase; Bsep, Bile salt export pump; CA, cholic acid; CDCA, chenodeoxycholic acid; CV, conventional; Cyp, cytochrome P450; DCA, deoxycholic acid; FFAR, free fatty acid receptor; Fgf15, Fibroblast growth factor 15; Fgfr4, Fibroblast growth factor receptor 4; Ibabp, ileal bile acid binding protein; FXR, farnesoid X receptor; GF, germ free; GLP, glucagon-like peptide; LCA, Lithocholic acid; L.Int, Large intestine (Colon); Lrh-1, Liver receptor homolog-1; MCA, muricholic acid; Mrp, multidrug resistance-associated protein; Ntcp, sodium taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; Ost, organic solute transporter; PCR, polymerase chain reaction; SCFA, short-chain fatty acid; SHP, small heterodimer protein; α MCA, Taurine-conjugated α -muricholic acid; TCA, taurine-conjugated cholic acid; TCDCA, taurine-conjugated chenodeoxycholic acid; TDCA, Taurine-conjugated deoxycholic acid; TGR5, transmembrane G protein-coupled receptor 5; β MCA, taurine-conjugated β -muricholic acid; ω MCA, Taurine-conjugated ω -muricholic acid; TLCA, Taurine-conjugated lithocholic acid; TUDCA, taurine-conjugated ursodeoxycholic acid; UDCA, ursodeoxycholic acid.

Preparation of Hepatic Microsomal Fractions. Hepatic microsomes were prepared following a previously published protocol (MacGeoch et al., 1984). Briefly, livers were homogenized in a buffer (sucrose 250 mM, Tris-HCl 50 mM, pH 7.4, EDTA 1 mM, and Phenylmethanesulfonyl fluoride (PMSF) 0.2 mM) with a glass-dounce homogenizer on ice and centrifuged at 10,000g for 25 minutes at 4°C. The supernatant was decanted and centrifuged at 100,000g for 60 minutes at 4°C. The pellet was rinsed with a KCl buffer (KCl 150 mM, Tris-HCl 10 mM, pH 7.4, and EDTA 1 mM) and centrifuged at 100,000g for 60 minutes at 4°C. The microsomes were resuspended in glycerol buffer (Tris-HCl 10 mM, pH 7.4, EDTA 1 mM, phenylmethylsulfonyl fluoride (PMSF) 0.2 mM, and glycerol 20% w/v). Protein concentrations were determined by a BCA assay according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL).

Microsomal Biotransformation Assay. Final reaction mixtures contained CDCA (200 μ M), 0.5 mg of liver microsomal protein, 100 mM potassium phosphate buffer, pH 7.4, and 2 mM NADPH in a final volume of 250 μ l. After preincubation of the microsomal protein and CDCA for 10 minutes at room temperature, reactions were initiated with NADPH and incubated for 60 minutes at 37°C. Reactions were terminated by adding 1 ml of ice-cold acetonitrile, dried under a vacuum, and resuspended in 80 μ l of 50% methanol. Contents were mixed and then centrifuged at 20,000g for 10 minutes. The bile acids were quantified as mentioned above.

GLP-1 Quantification. Total serum GLP-1 was quantified using an enzyme-linked immunosorbent assay kit (EZGLP1T-36K) from EMD Millipore (Billerica, MA) following the manufacturer's protocol.

RNA Isolation. Total RNA was isolated from tissues using RNA Bee reagent (Tel-Test Inc., Friendswood, TX) following the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. The quality of RNA was assessed by running the sample on a denaturing agarose gel and visualizing two discrete 18S and 28S ribosomal RNA bands, with the intensity of the 28S band double that of the 18S band.

Multiplex Suspension Assay. The mRNAs of BA biosynthetic enzymes and BA transporters in the liver and ileum were quantified using Panomics 2.0 QuantiGene Plex technology (Panomics/Affymetrix Inc., Fremont, CA) following the manufacturer's protocol. Individual gene information can be found on the Panomics web site (<http://www.panomics.com>), with panel numbers 21330 and 21383. Fluorescence was analyzed using a Bio-Plex 200 system array reader with Luminex 100 X-MAP technology (Bio-Rad, Hercules, CA), and data were acquired using Bio-Plex data manager software 5.0 (Bio-Rad). The mRNAs of the target genes were normalized to the housekeeping gene Rpl13a.

Quantitative Polymerase Chain Reaction Analysis. Total RNA was transcribed to single-stranded cDNA using the High Capacity cDNA Reverse Transcription Kit 1001073 (Applied Biosystems, Foster City, CA). The resulting cDNA products were amplified by polymerase chain reaction (PCR) using Power SYBR Green PCR Master Mix in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The primers for all real-time PCR reactions were synthesized by Integrated DNA Technologies (Coralville, IA).

cDNA Library Preparation and RNA Sequencing. The cDNA library preparation and sequencing of the transcriptome were performed with the help of the Kansas University Medical Center Genome Sequencing Facility as described previously (Cui et al., 2012). The cDNA libraries from total RNA samples ($n = 3$ per group) were prepared using an Illumina TruSeq RNA sample prep kit (Illumina, San Diego, CA). The average size of the cDNAs was approximately 160 bp (excluding the adapters). The cDNA libraries were validated for RNA integrity and quantity using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) before sequencing. The cDNA libraries were clustered onto a TruSeq paired-end flow cell and sequenced (2×50 bp) using a TruSeq SBS kit (Illumina) on the Illumina HiSeq2000 sequencer (Genome Sequencing Facility, Kansas University Medical Center), with a multiplex strategy of four samples per lane.

RNA-Seq Data Analysis. After the sequencing platform generated the sequencing images, the pixel-level raw data collection, image analysis, and base calling were performed by Illumina's real-time analysis software on a personal computer attached to a HiSeq2000 sequencer. The base call files (*.BCL) were converted to qseq files by Illumina's BCL converter, and the qseq files were subsequently converted to FASTQ files for downstream analysis. The RNA-Seq reads from the FASTQ files were mapped to the mouse mm10 reference genome, and the splice junctions were identified by TopHat. The output files in the binary sequence alignment format were analyzed by Cufflinks to estimate the transcript

Fig. 1. Body and liver weight of GF mice. Data are presented as mean $\pm$ S.E.M., $n = 10$ per group. Asterisks (*) represent a statistically significant difference between CV and GF mice ($P < 0.05$) by Student's t test. Dark blue and light blue bars represent CV and GF male mice, respectively, and red and pink bars represent CV and GF female mice, respectively. F, female; M, male.

abundance and differential expression (Cuffdiff, False discovery rate (Benjamini Hochberg) < 0.05). The mRNA abundance was expressed in fragments per kilobase of exon per million reads mapped.

Statistical Analysis. Data are presented as mean $\pm$ S.E.M. Asterisks (*) represent significant differences between CV and GF mice determined by Student's t test ($P < 0.05$).

Results

General Characterization of GF Mice

The body weights of adult (3 months old) GF male mice were comparable to the body weights of adult CV mice. However, adult GF female mice were 18% heavier than CV mice at the same age (Fig. 1). Both male and female GF mice had similar liver weights as CV mice (Fig. 1). The liver weight to body weight percentage was similar in male CV and GF mice, but was lower in female GF mice compared with CV mice (5.1% in CV and 4.6% in GF female mice) (data not shown).

BAs in Serum, Liver, Bile, and Ileum of CV and GF Mice

Serum. GF male and female mice had a 4-fold increase in total BAs in the serum compared with CV mice (Fig. 2A). The increase in total BAs in the serum of GF mice was due to an increase in primary BAs (11-fold increase in male GF mice and 7-fold increase in female GF mice). Secondary BAs were not detected in GF mice, confirming that intestinal bacteria were absent in these mice. Male and female GF mice had a 3-fold increase in total 12 α -OH BAs (CA, DCA, and their taurine conjugates; Fig. 2A) and a 4-fold increase in non-12 α -OH BAs (BAs other than CA, DCA, and their taurine conjugates; Fig. 2A) compared with their respective CV mice. Taurine-conjugated BAs increased 15- and 12-fold in GF male and female mice, respectively, compared with CV mice, whereas unconjugated BAs were almost absent in male and female GF mice. This increase in taurine-conjugated BAs was anticipated because of the absence of intestinal bacteria, which are known to deconjugate taurine-conjugated BAs (Fig. 2A).

Among individual BAs, taurine-conjugated α + β muricholic acid ($T\alpha$ + β MCA) $T\alpha$ + β MCA increased 45- and 14-fold, respectively, in male and female GF mice compared with CV mice (Fig. 2B). Concentrations of TCA were also 7- and 4-fold higher in the serum of male and female GF mice compared with CV mice (Fig. 2B). Female GF mice also had a 13-fold increased concentration of TCDCA and a 68-fold increased concentration of TUDCA compared with CV mice. The concentrations

Fig. 2. Concentrations of BAs in the serum of CV and GF mice. (A) Concentrations of total BAs (Σ), total 1° (primary) and 2° (secondary) BAs, total 12 α -OH BAs [CA, TCA, DCA, and taurine-conjugated deoxycholic acid (TDCA)] and non-12 α -OH BAs (all other BAs), and total taurine-conjugated (T-Conj) and unconjugated (Unconj) BAs. (B) Concentrations of individual T-conj BAs. (C) Concentrations of individual Unconj BAs. Data are presented as mean $\pm$ S.E.M., $n = 7-10$ mice/group. (D) Proportion of individual BAs in the serum of CV and GF mice. Asterisks (*) represent statistically significant differences between CV and GF mice ($P < 0.05$) by Student's t test. Dark blue and light blue bars represent CV and GF male mice, respectively, and red and pink bars represent CV and GF female mice, respectively. F, female; M, male.

of all unconjugated BAs were lower in the serum of both male and female GF mice compared with CV mice (Fig. 2C).

The proportions of individual BAs in the serum of mice were altered markedly in the GF condition (Fig. 2D). T α + β MCA made up 7% of BAs in the serum of CV male mice but constituted 77% of BAs in the serum of GF male mice. Similarly, in females, T α + β MCAs made up 19% of BAs in the serum in CV mice but 71% of BAs in the serum of GF mice.

Liver. The BA concentrations in the liver are known to increase in pathologic states, such as cholestasis. Interestingly, the absence of intestinal bacteria resulted in a 2-fold increase in total BAs in the livers of male and female mice (Fig. 3A), with no liver damage (data not shown). The increase in total BAs was due to a 3-fold increase in primary BAs and a 3-fold increase in non-12 α -OH BAs in both male and female GF mice compared with CV mice. As expected, there was a decrease in secondary BAs in both male and female GF mice. Both taurine-conjugated and unconjugated BAs were 2-fold higher in the livers of GF male and female mice compared with their respective CV mice (Fig. 3A).

Among conjugated BAs (Fig. 3B), T α + β MCA increased 6-fold in GF male and female mice compared with CV mice (Fig. 3B). In addition, the livers of GF mice have a 1.7- and 1.4-fold increased TUDCA (although not significant) compared with CV male and female mice, respectively. In contrast, the concentrations of TCA, the other major primary BA in mice, are similar in the livers of GF and CV mice (Fig. 3B).

Interestingly, the concentration of TUDCA, a known therapeutic BA, was increased 3-fold in the livers of both male and female GF mice compared with CV mice (Fig. 3B). This suggests that in mice, TUDCA can be synthesized by the liver and therefore is a primary BA, which is in agreement with a recent report (Sayin et al., 2013). Incubating CDCA with hepatic microsomal protein from GF mice resulted in the appearance of a detectable peak, with the same retention time and mass as the UDCA standard (Supplemental Fig. 2).

Among unconjugated BAs (Fig. 3C), β MCA was increased 5-fold in the livers of male and female GF mice compared with CV mice. In contrast, other unconjugated primary BAs (CA, CDCA, and α MCA) are at similar concentrations in the livers of CV and GF mice.

Fig. 3. Concentrations of BAs in the livers of CV and GF mice. (A) Concentrations of total BAs (Σ), total 1° (primary) and 2° (secondary) BAs, total 12 α -OH BAs [CA, TCA, DCA, and taurine-conjugated deoxycholic acid (TDCa)] and non-12 α -OH BAs (all other BAs), and total taurine-conjugated (T-Conj) and unconjugated (Unconj) BAs. (B) Concentrations of individual T-conj BAs. (C) Concentrations of individual Unconj BAs. Data are presented as mean $\pm$ S.E.M., $n = 7$ –10 mice/group. (D) Proportion of individual BAs in the livers of CV and GF mice. Asterisks (*) represent statistically significant differences between CV and GF mice ($P < 0.05$) by Student's t test. Dark blue and light blue bars represent CV and GF male mice, respectively, and red and pink bars represent CV and GF female mice, respectively. F, female; M, male.

The proportions of individual BAs were altered in the livers of GF mice (Fig. 3D). T α + β MCA, which was the predominant BA in the livers of GF mice, was 24% of the total BAs in CV male mice but 71% in GF male mice. Similarly T α + β MCA were 29% of the total BAs in female CV mice but 69% in female GF mice.

Bile. Biliary excretion of total BAs was 3-fold higher in male GF mice and 2-fold higher in female GF mice compared with their respective CV mice (Fig. 4A). The increase in total BAs in the bile of GF mice was due to a 3-fold increase in primary BAs in male GF mice and a 2-fold increase in female GF mice, respectively. Secondary BAs were almost absent in

Fig. 4. Biliary excretion of BAs in CV and GF mice. BAs in bile are expressed as nmol/kg body weight per min. (A) Concentrations of total BAs (Σ), total 1° (primary) and 2° (secondary) BAs, total 12 α -OH BAs [CA, TCA, DCA, and taurine-conjugated deoxycholic acid (TDCA)] and non-12 α -OH BAs (all other BAs), and total taurine-conjugated (T-Conj) and unconjugated (Unconj) BAs. (B) Concentrations of individual T-conj BAs. (C) Concentrations of individual Unconj BAs. Bile was collected for 40 minutes from each mouse. Data are presented as mean $\pm$ S.E.M., $n = 3-6$ mice/group. (D) Proportion of individual BAs in the bile of CV and GF mice. Asterisks (*) represent statistically significant differences between CV and GF mice ($P < 0.05$) by Student's t test. Dark blue and light blue bars represent CV and GF male mice, respectively, and red and pink bars represent CV and GF female mice, respectively. F, female; M, male.

the bile of GF mice. Total non-12 α -OH BAs increased 5-fold in male GF mice and 3-fold in female GF mice compared with CV mice. Taurine-conjugated BAs were increased 3- and 2-fold in the bile of male and female GF mice compared with their respective CV mice (Fig. 4A).

T α + β MCA increased 8-fold in male GF mice and 5-fold in female GF mice, although the other major primary BA, TCA, was

similar in the bile of GF and CV mice. The concentration of TUDCA increased 2-fold in the bile of male and female GF mice compared with CV mice (Fig. 4B). β MCA increased 9- and 2-fold in the bile of male and female GF mice, respectively, compared with their respective CV mice. CA was minimal in the bile of GF mice (Fig. 4C)

Fig. 5. Concentrations of BAs in the ileal tissue of CV and GF mice. (A) Concentrations of total BAs (Σ), total 1° (primary) and 2° (secondary) BAs, total 12 α -OH BAs [CA, TCA, DCA, and taurine-conjugated deoxycholic acid (TDCA)] and non-12 α -OH BAs (all other BAs), and total taurine-conjugated (T-Conj) and unconjugated (Unconj) BAs. (B) Concentrations of individual T-conj BAs. (C) Concentrations of individual Unconj BAs. Ileal tissue refers to the last one-third of the small intestine. Data are presented as mean $\pm$ S.E.M., $n = 7$ –10 mice/group. (D) Proportion of individual BAs in the ileal tissue of CV and GF mice. Asterisks (*) represent statistically significant differences between CV and GF mice ($P < 0.05$) by Student's t test. Dark blue and light blue bars represent CV and GF male mice, respectively, and red and pink bars represent CV and GF female mice, respectively. F, female; M, male.

T α + β MCA constituted 29% of total BAs in the bile of CV male mice but increased to 83% of total BAs in GF male mice (Fig. 4D). Similarly, in females, T α + β MCA constituted 29% of total BAs in CV mice but increased to 78% of total BAs in GF mice. In contrast, TCA was 47% of total BAs in the bile of male CV mice but decreased to 14% in male GF

mice. Similarly, in female mice, TCA was 44% of total BAs in the bile of CV mice but decreased to 18% in GF mice (Fig. 4D).

Ileal Tissue. Total BA concentrations in ileal tissue were 3-fold higher in male GF mice and 2-fold higher in female GF mice compared with their respective CV controls (Fig. 5A). This is probably due to

either increased uptake of BAs from the intestinal lumen or decreased efflux transport across the basolateral membrane. Primary BAs were increased 4- and 2-fold in the ileal tissue of male and female GF mice, respectively, compared with CV mice. Further, non-12 α -OH BAs were increased 6- and 4-fold in the ileal tissue of male and female GF mice,

respectively, compared with CV mice. Taurine-conjugated BAs were increased 5- and 3-fold in the ileal tissue of GF male and female mice compared with CV mice. Secondary and unconjugated BA concentrations were almost nonexistent in the ileal tissue of GF mice compared with CV mice (Fig. 5A).

Fig. 6. Concentrations of BAs in the feces of CV and GF mice. BAs are expressed as nmols/gram of feces. (A) Concentrations of total BAs (Σ), total 1° (primary) and 2° (secondary) BAs, total 12 α -OH BAs [CA, TCA, DCA, and taurine-conjugated deoxycholic acid (TDCA)] and non-12 α -OH BAs (all other BAs), and taurine-conjugated (total T-Conj) and unconjugated (Unconj) BAs. (B) Concentrations of individual T-conj BAs. (C) Concentrations of individual Unconj BAs. Mice were housed individually in metabolic chambers, and feces were collected over 24 hours. Data are presented as mean $\pm$ S.E.M., $n = 4$ mice/group. (D) Proportion of individual BAs in the feces of CV and GF mice. Asterisks (*) represent statistically significant differences between CV and GF mice ($P < 0.05$) by Student's t test. Dark blue and light blue bars represent CV and GF male mice. M, male.

Concentrations of $\text{T}\alpha\text{+}\beta\text{MCA}$ increased in the ileal tissue of male GF mice (10-fold) and female GF mice (5-fold) compared with CV mice. TCA concentration increased 3-fold in the ileal tissue of male GF mice and 1.5-fold in the ileal tissue of female GF mice compared with CV mice (Fig. 5B). TUDCA concentration in the ileal tissue was increased 6- and 3-fold in male and female GF mice, respectively, compared with their respective CV mice. In contrast, primary unconjugated BAs were almost absent in the ileal tissue of both male and female GF mice (Fig. 5C).

The proportions of individual BAs in the ileal tissues were altered in GF mice compared with CV mice (Fig. 5D), but were similar to changes in other tissues of GF mice. $\text{T}\alpha\text{+}\beta\text{MCA}$ constituted 28% of the total BAs in the ileal tissue of male CV mice but increased to 78% in male GF mice. Similarly, in females, $\text{T}\alpha\text{+}\beta\text{MCA}$ constituted 38% of the total BAs in the ileal tissue of CV mice but increased to 75% in GF mice.

Fecal Excretion of BAs in GF Mice

Male CV and GF mice were housed in individual metabolic cages, and feces were collected over a 24-hour period. Due to space restrictions inside the GF isolator, feces were collected only from male GF mice, and we expect the trend of changes in BAs to be similar in male and female GF mice. Generally, more than 90% of BAs in the intestinal lumen are reabsorbed and the remaining BAs are excreted in the feces.

There was a 63% decrease in the amount of total BAs in the feces of GF mice compared with CV mice, (Fig. 6A). This suggests an increased intestinal uptake of BAs in the absence of intestinal bacteria in GF mice. Total fecal excretion of $12\alpha\text{-OH}$ BAs in GF mice decreased 75% and non- $12\alpha\text{-OH}$ BAs decreased 57% compared with CV mice. Furthermore, the absence of intestinal bacteria that deconjugate BAs leads to a 4-fold increase in total conjugated BAs and a 99% decrease in total unconjugated BAs in the feces of GF mice (Fig. 6A).

Fecal excretion of primary taurine-conjugated BAs increased in GF mice compared with CV mice, more specifically, $\text{T}\alpha\text{+}\beta\text{MCA}$ increased 6-fold, TCA increased 4-fold, TCDCA increased 2-fold, and TUDCA increased 4-fold (Fig. 6B). Unconjugated BAs were essentially absent in the feces of GF mice compared with CV mice (Fig. 6C).

The proportions of the various BAs in the feces of GF mice were very different than those in CV mice. $\text{T}\alpha\text{+}\beta\text{MCA}$ were 5% of the total BAs in the feces of CV mice but increased to 74% of the total BAs in the feces of GF mice (Fig. 6D).

Gene Expression in the Liver

BA Enzymes. Major enzymes involved in BA biosynthesis include cytochrome P450 (Cyp) 7a1, Cyp7b1, Cyp8b1, Cyp27a1, and the BA conjugation enzymes bile acid-coenzyme A ligase (BAL) and bile acid-coenzyme A:amino acid N-acyltransferase (BAT). Surprisingly, the mRNA

Fig. 7. Gene expression in livers of CV and GF mice. (A) mRNA expression of genes involved in BA synthesis; (B) mRNA expression of genes involved in BA transport; and (C) mRNA expression of genes involved in FXR feedback regulation in the livers of male and female CV and GF mice. mRNA was quantified by beadplex assay; $n = 6$ per group. Data are presented as mean $\pm$ S.E.M. Asterisks (*) represent statistically significant differences between CV and GF mice ($P < 0.05$) by Student's t test. Dark blue and light blue bars represent CV and GF male mice, respectively. Red and pink bars represent CV and GF females, respectively. F, female; M, male.

of Cyp7a1, the rate-limiting enzyme in BA synthesis, was similar in the livers of CV and GF male mice (Fig. 7A). However, in female GF mice, Cyp7a1 mRNA decreased to about 50% of that in CV mice (Fig. 7A). The mRNA of Cyp27a1 was similar in the livers of male GF and CV mice, but decreased 15% in female GF mice (Fig. 7A). The mRNA of Cyp7b1 was decreased by 30% in the livers of male GF mice and 52% in the livers of female GF mice, respectively, compared with CV mice (Fig. 7A). This may be an attempt to decrease the alternate pathway of BA synthesis to ultimately decrease concentrations of BAs in the livers of GF mice.

Cyp8b1, the sterol 12 α -hydroxylase, is essential for the synthesis of CA. The mRNA of Cyp8b1 was decreased 32% in the livers of male GF mice and 73% in female GF mice compared with their CV controls (Fig. 7A). This correlates with the decrease in concentration of 12 α -OH BAs (TCA and CA) and the decrease in the proportion of TCA to T α + β MCA in the livers of GF mice (Fig. 3, A, B, and D).

Although taurine-conjugated BAs increased in the tissues of GF mice, the mRNA of enzymes involved in the conjugation of BAs, namely, BAL and BAT, were similar in the livers of male GF and CV mice. In the livers of female GF mice, BAL mRNA was decreased by 15% and the mRNA of BAT was decreased by 19% (Fig. 7A).

BA Transporters. The two BA uptake transporters in the livers are sodium taurocholate cotransporting polypeptide (Ntcp) and organic anion transporting polypeptide (Oatp) 1b2, where Ntcp transports conjugated BAs and Oatp1b2 transports unconjugated BAs. The GF condition increased the mRNA of Ntcp by 42% and Oatp1b2 by 48% in male mice compared with CV male mice (Fig. 7B). This increase in the mRNA of hepatic BA uptake transporters may enable the liver to remove more BAs from the circulation, given that total BAs are higher in the serum of both male and female GF mice. However, in female GF mice, the mRNA of Ntcp and Oatp1b2 is similar to that in female CV mice (Fig. 7B). The mRNA of the major hepatic BA efflux transporter bile salt export pump (Bsep) decreased somewhat (11%) in male GF mice but did not change in female GF mice compared with CV mice (Fig. 7B). The gene expression of multidrug resistance-associated protein (Mrp) 2 increased 25% in the livers of male GF mice but did not change in female GF mice compared with their respective controls (Fig. 7B).

BA Feedback Regulation. The mRNA of FXR and liver receptor homolog-1 increased minimally (16 and 13%, respectively) in the livers of male GF mice compared with their CV controls (Fig. 7C). However, these small increases may not be biologically significant as there was no subsequent increase in small heterodimer protein (SHP) mRNA or decrease in Cyp7a1 mRNA in the livers of male GF mice compared with CV mice (Fig. 7A). The mRNAs of other important genes involved in BA feedback regulation through FXR activation were similarly expressed in the livers of GF and CV mice (Fig. 7C).

Gene Expression in the Ileum

Transporters. Apical sodium dependent bile acid transporter (Asbt) is the only known apical BA uptake transporter in the ileum. Asbt mRNA increased 154% in the ileum of male GF mice compared with CV mice (Fig. 8A). This correlates with increased total BA concentrations in the ileal tissue and decreased total BA content in the feces observed in GF mice compared with CV mice. However, there was no difference in the expression of Asbt in female CV and GF mice (Fig. 8A). The mRNA of Ost α increased 46% in the ileal tissue of male GF mice compared with CV mice, but there was no change in Ost α mRNA in female GF mice compared with CV mice. The mRNA of Mrp3 was similar in the ileal tissue of male GF and CV mice but decreased minimally in the ileal tissue of female GF mice compared with CV mice. ATP-binding cassette (Abc) subfamily G member 5 mRNA remained

Fig. 8. Gene expression in ileum of CV and GF mice. (A) mRNA expression of BA transporters in the ileal tissue of male and female CV and GF mice. (B) mRNA expression of genes involved in BA signaling in the ileum of male and female CV and GF mice. mRNA was quantified by beadplex assay; $n = 6$ per group. Data are presented as mean $\pm$ S.E.M. Asterisks (*) represent statistically significant differences between CV and GF mice ($P < 0.05$) by Student's t test. Dark blue and light blue bars represent CV and GF male mice, respectively. Red and pink bars represent CV and GF females, respectively. F, female; M, male.

similar in male GF and CV mice, but decreased 35% in the ileum of female GF mice compared with female CV mice. However, the mRNA of Abc subfamily G member 8, which is the heterodimer partner of Abc subfamily G member 5, decreased 20% in male GF mice and 40% in female GF mice. The mRNA of the organic solute transporter (Ost) β and Mrp2 were similar in the ileal tissue of male and female GF mice compared with their respective controls (Fig. 8A).

Although the mRNAs of genes involved in BA-FXR signaling in the ileal tissue were similar in GF and CV mice (Fig. 8B), the mRNA of the cholesterol sensor Liver X receptor alpha (LXR α) was minimally induced in both male and female GF mice compared with CV mice (Fig. 8B).

Targets of TGR5 Signaling in CV and GF Mice

The major effects of TGR5 activation include increased bile flow and gallbladder size (Li et al., 2011) as well as increased GLP-1 secretion from the ileum (Katsuma et al., 2005). The gallbladders in GF mice were approximately 5-fold larger than those in CV mice (Fig. 9A). In addition, bile flow was 44% higher in GF male mice compared with their CV controls (Fig. 9B). GLP-1 was not detectable in the serum of CV mice, but was 78 and 62 μ M in male and female GF mice, respectively (Fig. 9C).

Gene Expression in Various Parts of the Intestine

Because there was a decrease in fecal excretion of BAs in the feces of GF mice but no consistent increase in the BA transporters in the ileum, the expression of BA transporters in various sections of the intestine of male CV and GF mice were examined by RNA-Seq. The mRNA of the BA uptake transporter Asbt decreased 93% in the jejunum, but increased 69% in the ileum and 96% in the colon of GF mice compared with CV mice (Fig. 10). The mRNA of the BA efflux transporters Ost α

Fig. 9. Targets of TGR5 signaling. (A) Gallbladder weights of CV and GF mice and (inset) representative pictures. (B) Bile flow in CV and GF mice. (C) Serum GLP-1 quantification by enzyme-linked immunosorbent assay. Data are presented as mean $\pm$ S.E.M.; $n = 3-6$ per group. Asterisks (*) represent statistically significant differences between CV and GF mice ($P < 0.05$) by Student's t test. Dark blue and light blue bars represent CV and GF male mice, respectively. Red and pink bars represent CV and GF females, respectively. F, female; M, male.

and *Ost β* increased 94 and 67% in the colon of GF mice compared with CV mice (Fig. 10). The mRNA of FXR increased in the large intestine of GF mice compared with CV mice (Supplemental Fig. 3).

UDCA Synthesis by Enzymes in Mouse Hepatic Microsomes

A striking finding of this study was that GF mice had elevated concentrations of TUDCA. TUDCA in serum was not detectable in male CV mice, but could be quantified in GF mice (Fig. 2B). Compared with CV mice, GF mice had a 3-fold increase in TUDCA in the liver, a 2-fold increase in bile (Fig. 3B; Fig. 4B), a 6-fold increase in ileal tissue, and a 4-fold increase in feces (Fig. 5B; Fig. 6B). Using a traditional

biotransformation assay, we found that incubating CDCA with hepatic microsomal protein from GF mice resulted in the appearance of a detectable peak, with the same retention time and mass as the UDCA standard (Supplemental Fig. 2). No UDCA peak was detected when the assay mixture did not contain CDCA or if CA was used instead of CDCA (data not shown), further suggesting that UDCA is a primary BA.

Discussion

The BA profiles of GF mice differ markedly from those of CV mice. In the absence of intestinal bacteria, GF mice have increased conjugated BAs and decreased secondary BAs. GF mice only have a few types of BAs (primary BAs and mainly taurine-conjugated BAs), which indicates that intestinal bacteria play a major role in the diversity of BAs. Somewhat surprisingly, there was an increase in UDCA.

UDCA is an oral drug used to dissolve cholesterol gallstones and treat liver diseases. In the present study, UDCA, which was thought to be a secondary BA made by intestinal bacteria, increased in the tissues of GF mice, similar to a recent report (Sayin et al., 2013). Results from previous studies in our laboratory demonstrate that feeding mice CDCA increases the hepatic concentration of UDCA and TUDCA, suggesting that UDCA is synthesized from CDCA (Zhang and Klaassen, 2010; Song et al., 2011). Indeed, *in vitro* experiments confirmed that UDCA can be synthesized from CDCA by enzymes in mouse liver microsomes (Supplemental Fig. 2).

The amount of BAs is much higher in GF mice than in CV mice. Total BAs increased 4-fold in the serum (Fig. 2A), 2-fold in the liver (Fig. 3A), 3-fold in the bile (Fig. 4A), and 3-fold in the ileum (Fig. 5A) but decreased 63% in the feces (Fig. 6A) of male GF mice compared with CV mice. These changes are likely due to an increase in the reabsorption of BAs. The ileal BA uptake transporter (*Asbt*) is thought to be responsible for the reabsorption of most of the BAs from the intestine (Dawson et al., 2003). It is known that antibiotics decrease the number of intestinal bacteria, increase intestinal BA absorption (Miyata et al., 2015), decrease fecal BA excretion, and increase hepatic BA concentrations (Hu et al., 2014; Zhang et al., 2014), with an increase in ileal *Asbt* expression. On the contrary, *Asbt*-null mice have limited enterohepatic circulation of BAs, increased fecal BA excretion, and decreased BA pool size (Dawson et al., 2003), which is just the opposite of what is observed in GF mice. GF mice also have decreased gut motility (Kashyap et al., 2013), and thus BAs remain in the intestine longer, providing more time for BAs to enter the enterohepatic circulation. This would also explain the increased half-life of CA in GF rats reported by others (Gustafsson et al., 1957). Although BAs are thought to be primarily reabsorbed by transporters in the ileum, surprisingly, data in the present manuscript (Fig. 10) indicate that the mRNA of the BA transporters *Asbt*, *Ost α* , and *Ost β* increase mainly in the large intestine of GF mice (Fig. 10). The mRNA of *Asbt* increases by 96%, *Ost α* increases by 94%, and *Ost β* increases by 67% compared with CV mice (Fig. 10). This suggests that in GF mice, BAs are taken up by transporters in the colon in addition to the small intestine to enter the enterohepatic circulation. Thus, the increase in BA transporters in the large intestine appears to be responsible for the increase in BAs in the tissues and the decrease of BAs in the feces.

The GF condition, as expected, led to an increase in taurine-conjugated BAs: 15-fold in the serum, 2-fold in the liver, 3-fold in the bile, 5-fold in the ileum, and 4-fold in the feces of male GF mice compared with CV mice. Taurine conjugation lowers the pK_a of BAs and increases their water solubility. In contrast, unconjugated BAs were decreased in all tissues, except in the liver, where they were 2-fold higher than in CV mice.

Fig. 10. Gene expression of BA-related genes across the intestinal sections. Total RNA was isolated from livers of adult male CV and GF C57BL/6 mice ($n = 3$ per group). The mRNA quantified by RNA-Seq as described in *Materials and Methods*. Asterisk (*) indicates differential expression determined using Cuffdiff (FDR-BH < 0.05). Dark blue and light blue bars represent CV and GF male mice, respectively. L.Int, large intestine (colon); M, male.

BA composition is similar in male and female GF mice, but differs markedly from their respective CV mice. For example, male GF mice have increased total non-12 α -OH BAs (MCAs, UDCA, and their taurine conjugates): 4-fold in the serum, 3-fold in the liver, 5-fold in bile, and 6-fold in the ileum. In contrast, there are similar concentrations of 12 α -OH BAs (CA, DCA, and their taurine conjugates) in each tissue, except the serum, where they were 3-fold higher in male GF mice than in CV mice.

T α + β MCA become the major BAs in GF mice as they increase 6-fold in the livers of male and female GF mice; 8-fold in the bile of male GF mice and 5-fold in the bile of female GF mice; 10-fold in the ileum of male GF mice and 5-fold in the ileum of female GF mice; and 6-fold in the feces of male GF mice compared with CV mice. Similarly, TUDCA increased 3-fold in the livers of male and female GF mice; 2-fold in the bile of male and female GF mice; 6-fold in the ileum of male GF mice and 3-fold in the ileum of female GF mice; and 4-fold in the feces of GF male mice compared with CV mice. Increased concentrations of MCA and UDCA make the GF BA pool more hydrophilic, which can protect against cell damage induced by hydrophobic bile acids (Heuman et al., 1991; Rodrigues et al., 1998).

Under normal conditions, FXR regulates hepatic BA synthesis through BA-mediated feedback mechanisms. Increased BAs in the liver activate FXR, which acts via SHP–liver receptor homolog-1 to decrease transcription of *Cyp7a1*. In addition, increased BAs in the ileum activate FXR, induce the secretion of Fgf15 into the portal circulation, which travels to the liver and via Fibroblast growth factor 4 (Fgf4)/ β -klotho signaling, and down-regulate the transcription of *Cyp7a1* (Chiang, 2004; Chiang et al., 2000; Inagaki et al., 2005; Kim et al., 2007). With the increase in total BAs in the liver and intestine of GF mice, one would expect an increase in FXR signaling in the liver and intestine and subsequent down-regulation of *Cyp7a1* in the liver. However, the mRNA of *Cyp7a1* was not lower in the livers of GF mice, and there was neither an increase in SHP or Fgf15 mRNA in the ileum of GF mice compared with CV mice. T β MCA is an FXR antagonist that decreases the feedback secretion of intestinal fibroblast growth factor 15 (Fgf15) and thus increases hepatic *Cyp7a1* mRNA (Li et al., 2013; Sayin et al., 2013; Hu et al., 2014). In the present study, there was an increase in T β MCA (FXR antagonist) but also an increase in other BAs, which are likely FXR agonists in vivo, and thus the mRNA of FXR-target genes in the intestine (Fgf15, SHP, or ileal bile acid-binding protein) and liver (SHP) are similar in GF and CV mice (Fig. 7, A and C; Fig. 8B). As a result, there is no alteration in *Cyp7a1*.

TGR5 activation by BAs in the intestine induces the secretion of GLP-1, which in turn stimulates insulin secretion and improves insulin sensitivity (Thomas et al., 2009). TGR5 receptor agonists and long-acting GLP-1 receptor agonists improve glucose tolerance and are promising drug candidates to treat metabolic disorders, such as type 2

diabetes and obesity (Nauck, 2011; Keitel and Haussinger, 2012). Although there are minimal changes in FXR signaling in either the liver or intestine of GF mice (Fig. 7, A and C; Fig. 8B), there was an increase in TGR5 signaling. GF mice displayed an increased gallbladder size and increased GLP-1 in the serum (Fig. 9), both of which are endpoints of TGR5 activation (Katsuma et al., 2005; Thomas et al., 2009; Li et al., 2011; Harach et al., 2012). It is interesting to note that the BA composition in TGR5-null mice is hydrophobic (Pean et al., 2013), which is in contrast to the phenotype of GF mice, in which TGR5 is activated.

Increasing GLP-1 is a therapeutic strategy for treating diabetes and obesity. Similar to the GF condition, reducing the number of bacteria in the intestine by antibiotics also alters the BA composition (Zhang et al., 2014) and increases GLP-1 (Hwang et al., 2015). Anionic resins that concentrate BAs in the intestinal lumen also increase GLP-1 secretion via TGR5 (Harach et al., 2012). Further, rectal administration of TCA in humans increases GLP-1 levels in plasma (Wu et al., 2013). The altered BA profile in GF mice might point to a BA that is a potent TGR5 agonist, and thus BAs could be used to increase TGR5 signaling and increase serum GLP-1 concentration. A recent study demonstrated that oral administration of the antioxidant tempol to mice decreases *Lactobacillus* species in the intestinal lumen, leading to accumulation of T β MCA, inhibition of FXR signaling, and resistance to obesity (Li et al., 2013). Although humans do not make T β MCA, appropriate studies might lead to the establishment of T β MCA as a drug.

Apart from BAs, other intestinal bacterial metabolites, such as short-chain fatty acids (SCFAs) also regulate GLP-1 secretion by activating the SCFA receptors, free fatty acid receptor 2 (FFAR 2) and FFAR3 (Tolhurst et al., 2012). Altering intestinal bacterial composition by dietary fiber administration also induces GLP-1 levels (Reimer and McBurney, 1996; Massimino et al., 1998; Tolhurst et al., 2012). Future experiments utilizing TGR5-null mice would help establish the relative importance of BAs (via TGR5) and SCFA (via FFAR2 or FFAR3) in regulating the secretion of GLP-1 from the intestine.

In conclusion, this study describes changes in BA homeostasis in male and female GF mice. The most notable changes in GF mice are 1) increases in total BAs in the serum, liver, bile, and ileal tissue; 2) a shift in BA composition toward an increase in T α + β MCA; and 3) a large increase in GLP-1. It appears that these effects are secondary to an increase in BA transporters in the colon.

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Authorship Contributions

Participated in research design: Selwyn, Csanaky, Zhang, Klaassen.

Conducted experiments: Selwyn, Csanaky, Zhang, Klaassen.

Performed data analysis: Selwyn, Csanaky, Zhang, Klaassen.

Wrote or contributed to the writing of the manuscript: Selwyn, Csanaky, Zhang, Klaassen.

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