Gut Microbiota Regulates Bile Acid Metabolism by Reducing the Levels of Tauro-beta-muricholic Acid, a Naturally Occurring FXR Antagonist

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SUMMARY

Bile acids are synthesized from cholesterol in the liver and further metabolized by the gut microbiota into secondary bile acids. Bile acid synthesis is under negative feedback control through activation of the nuclear receptor farnesoid X receptor (FXR) in the ileum and liver. Here we profiled the bile acid composition throughout the enterohepatic system in germ-free (GF) and conventionally raised (CONV-R) mice. We confirmed a dramatic reduction in muricholic acid, but not cholic acid, levels in CONV-R mice. Re-derivation of Fxr-deficient mice as GF demonstrated that the gut microbiota regulated expression of fibroblast growth factor 15 in the ileum and cholesterol 7α-hydroxylase (CYP7A1) in the liver by FXR-dependent mechanisms. Importantly, we identified tauroconjugated beta- and alpha-muricholic acids as FXR antagonists. These studies suggest that the gut microbiota not only regulates secondary bile acid metabolism but also inhibits bile acid synthesis in the liver by alleviating FXR inhibition in the ileum.

INTRODUCTION

Bile acids are produced in hepatocytes, stored in the gallbladder, and released into the duodenum upon ingestion of a meal to facilitate absorption of triglycerides, cholesterol, and lipid-soluble vitamins (Li-Hawkins et al., 2002; Matakı et al., 2007). Bile acids are efficiently reabsorbed (>95%) from the intestine, mainly by active transport mediated by the ileal bile acid transporter (IBAT; also known as ASBT or SLC10A2) but also through passive diffusion in the upper small intestine and colon (Chiang, 2009). This efficient enterohepatic circulation of bile acids is maintained through a tight negative feedback control of their synthesis. Bile acids function as signaling molecules that not only regulate their own biosynthesis but also modulate key metabolic pathways involved in lipoprotein, glucose, drug, and energy metabolism by activation of nuclear receptors such as farnesoid X receptor (FXR) and the G protein-coupled receptor TGR5 (Hylemon et al., 2009; Thomas et al., 2008).

Bile acids are synthesized from cholesterol by a process that requires the concerted actions of at least 14 liver enzymes (Chiang, 2009; Russell, 2009). Humans synthesize cholic acid (CA) and chenodeoxycholic acid (CDCA), whereas mice synthesize CA and β-muricholic acid (MCA) (Russell, 2003). Bile acids are thereafter conjugated to glycine (predominant in humans) or taurine (predominant in mice) (Claus et al., 2011). The rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) initiates the classic pathway for bile acid synthesis, and CYP27A1 initiates the alternative pathway (Chiang, 2009). Sterol 12α-hydroxylase (CYP8B1) is required for CA synthesis (Li-Hawkins et al., 2002), whereas the enzyme(s) that catalyze 6- and 7-hydroxylation to produce MCA are not yet characterized. Hepatic expression of CYP7A1 and CYP8B1 is regulated by FXR, which is highly expressed in both the ileum and liver (Sinal et al., 2000). Although both CYP7A1 and CYP8B1 can be regulated by hepatic FXR activation through the nuclear receptors small heterodimer partner (SHP) and liver receptor homolog-1 (LRH1) (Chiang, 2009), recent data suggest that intestinal FXR regulates hepatic CYP7A1 through a fibroblast growth factor 15 (FGF15)-dependent mechanism (Inagaki et al., 2005; Kim et al., 2007; Zimmer et al., 2012).

It is well established that the gut microbiota has profound effects on bile acid metabolism by promoting deconjugation, dehydrogenation, and dehydroxylation of primary bile acids in the distal small intestine and colon, thus increasing the chemical diversity of bile acids (Midtvedt, 1974; Ridlon et al., 2006). Previous studies in rats have shown that the gut microbiota reduces the bile acid pool size with its greatest effect on βMCA rather than CA levels (Wostmann, 1973). However, the molecular mechanisms for how the gut microbiota suppresses bile acid synthesis are currently unknown. Here we comprehensively map the bile acid profiles of conventionally raised (CONV-R) and germ-free (GF) mice throughout the enterohepatic system.
and in serum to determine the role of the gut microbiota on bile acid metabolism. Rederivation of Fxr-deficient mice as GF showed that the gut microbiota regulates expression of Fgf15 and Cyp7a1 through this nuclear receptor. Importantly, we identified MCAs as potent FXR antagonists. We propose that the gut microbiota modulates bile acid synthesis by changing the bile acid pool composition and by alleviating FXR inhibition in the small intestine.

RESULTS

The Gut Microbiota Influences the Size and Composition of the Bile Acid Pool throughout the Enterohepatic System

Analysis of bile acids throughout the enterohepatic system of GF and CONV-R mice revealed that bile acid levels in the presence of a gut microbiota were reduced in the gallbladder and small intestine but increased in the cecum, colon, feces, and serum (Figure 1A). By combining the amounts measured in all of the tissues, we showed that the bile acid pool was reduced by 71% ± 2% in CONV-R mice (Figure 1B). Gallbladders from the GF mice had the highest bile acid levels of all the tissues analyzed (Figure 1A) and were significantly larger than gallbladders from CONV-R counterparts (Figure 1C). In contrast, the levels of cholesterol and phospholipids were reduced in GF gallbladders (Figures 1D and 1E). The larger size of the gallbladder and altered composition of bile in GF mice were not associated with inflammation of the gallbladder (Figures 1F and 1G).

To assess how the overall bile acid composition was affected by the gut microbiota, we performed principal component analysis (PCA) of the bile acid composition in each compartment of the enterohepatic system. The first principal component (PC) explained most of the variation in each tissue and reached >90% in the cecum and colon where clustering of samples was especially clear (Figure S1A available online). Furthermore, the second PC explained almost all of the remaining variation. The PCA explained somewhat less of the variation in the serum bile acids (PC1, 58%, and PC2, 26%; Figure S1A). Further network-based analysis using qp-graph (Castillo et al., 2011) revealed that all tissues were connected by edges in GF mice while the cecum and colon profiles formed their own cluster in CONV-R mice (Figure S1B), further emphasizing the markedly altered composition in these organs. Taken together, these results demonstrate that the gut microbiota affects the bile acid composition in all parts of the enterohepatic circulation and the greatest differences between GF and CONV-R mice are observed in the cecum and colon.

Analysis of individual bile acids revealed that taurine-conjugated CA and t-MCA (TCA and T-t-MCA, respectively) were the most common bile acids in the livers of both GF and CONV-R

Figure 1. The Gut Microbiota Alters the Size and Composition of the Total Bile Acid Pool
(A) Total amount of bile acids in individual organs/tissues throughout the enterohepatic circulation and in the feces and serum of GF and CONV-R Swiss Webster mice.
(B) Total amount of bile acids in the enterohepatic system obtained by combination of the values in (A).
(C) Gallbladder weights of GF and CONV-R mice. Representative gallbladders are shown (inset).
(D and E) Cholesterol (D) and phospholipids (E) in bile of GF and CONV-R mice.
(F and G) ALP levels in serum (F) and histology of the gallbladder (G) in GF and CONV-R mice.
Scale bars represent 20 μm. (GF mice weighed 30.2 ± 0.9 g and CONV-R mice 35.2 ± 0.8 g; p = 0.0007.) Mean values ± SEM are plotted; n = 14/group; *p < 0.05, **p < 0.01, ***p < 0.001 versus GF, Student’s t test.
mice (Figure 2). Livers from CONV-R mice contained reduced levels of TβMCA and increased levels of TCA and TαMCA compared with GF counterparts (Figure 2). TαMCA and TDCA were exclusively present in CONV-R livers (Figure 2). The bile acid profile of the gallbladder and the small intestine resembled that of the liver (Figure 2). Analyses of the individual intestinal segments revealed that the bile acid profile in CONV-R mice was dominated by TCA in the proximal intestine and CA in the distal small intestine (Figure S2), indicating efficient microbial deconjugation in the small intestine, which is essential for further microbial metabolism into secondary bile acids in the colon. In contrast, small intestines from GF mice almost exclusively contained TβMCA and TCA in addition to low levels of TUDCA (Figures 2 and S2) confirming that, in contrast to humans (Russell, 2003), UDCA is a primary bile acid in rodents.

Further microbial modifications of the bile acid pool were evident in the distal gut of CONV-R mice, which was dominated by DCA and ωMCA and almost depleted of taurine-conjugated bile acids (Figure 2). In contrast, the bile acid profile in the distal gut of GF mice was similar to that of the small intestine. As expected, the bile acid profile of feces in both groups reflected the profiles in the cecum and colon (Figure 2). The serum bile acid profile (Figure 2) most closely resembled the bile acids in the distal small intestine (Figure S2), which probably reflects the fact that most bile acids are reabsorbed from the ileum.

By combining the amounts measured in the entire enterohepatic system, we showed that the total content of conjugated and unconjugated metabolites of βMCA was reduced by 71% ± 3% in CONV-R mice whereas the total content of CA was unchanged (p = 0.75; Figure S3). Accordingly, the reduced MCA levels explain the lower bile acid pool size in CONV-R mice (Figure 1B).

The Gut Microbiota Alters the Expression Profile of Genes Involved in Bile Acid Synthesis, Conjugation, and Reabsorption

Next we determined whether the altered bile acid profile in CONV-R mice was associated with microbial regulation of enzymes in the bile acid synthesis pathway. Expression levels of most enzymes involved in bile acid synthesis were reduced in livers of CONV-R mice (Figure 3A). The activity of CYP7A1 (the rate-limiting enzyme in the bile acid synthetic pathway in the liver) was also reduced in microsomes isolated from livers of CONV-R mice (Figure 3B). However, the hepatic activity of CYP8B1 (which is essential for CA production) was not significantly affected by the presence of gut microbiota (Figure 3A,B).
The liver mainly in the distal ileum (Dawson et al., 2009). We determined the expression of genes involved in bile acid transport and taurine content. This indicates an attempt to compensate for the reduced hepatic homeostasis in the distal ileum and liver (Figures 3E and 3F). We found that expression of apical bile acid transporters was downregulated in the ileum of CONV-R mice, whereas basolateral transporters were upregulated (Figure 3E). The gut microbiota had less of an effect on expression of bile acid transporters in the liver (Figure 3F).

**Microbial-Induced Expression of Fgf15 in the Ileum Requires Functional FXR**

FXR is known to play a key role in the regulation of bile acid synthesis and homeostasis (Sinal et al., 2000), and we therefore investigated whether the effects of the gut microbiota on bile acid synthesis are mediated through FXR. We first showed that the presence of a gut microbiota upregulated expression of Fxr and its molecular targets Shp and Fgf15 in the distal ileum of CONV-R compared with GF mice (Figure S4A). In contrast, the gut microbiota had no effect on expression of Fxr or its downstream targets Shp and Lrh1 in the liver (Figure S4B), which suggests that the gut microbiota regulates the activity of FXR in the ileum but not in the liver.

To directly test the effect of the microbiota on targets downstream of FXR, we rederived Fxr–/– mice on a C57BL/6 background as GF. The microbiota-induced expression of Shp and Fgf15 in the ileum was completely abolished in Fxr-deficient mice (Figure 4A), demonstrating the requirement for FXR in mediating these transcriptional responses. It is noteworthy that the gut microbiota had a more pronounced effect on Fgf15 expression in C57BL/6 mice (Figure 4A) than in Swiss Webster mice (Figure S4A). Expression of the classical FXR target gene Ibabp was only slightly increased in the presence of gut microbiota, but the basal expression in GF mice was completely abolished in Fxr-deficient mice (Figure 4A). The expression of Ibat was reduced in colonized mice irrespective of genotype (Figure 4A).

Expression of Cyp7a1 in the liver was reduced in wild-type but not Fxr-deficient CONV-R mice (Figure 4B), consistent with the...
A novel concept that the microbiota- and FXR-dependent increases in Fgf15 in the ileum suppress Cyp7a1 expression in the liver. As previously described, Shp was suppressed by FXR in the liver (Sinal et al., 2000), but the regulation was microbiota independent (Figure 4B). We also showed that treatment of GF and CONV-R C57BL/6 mice with the FXR agonist INT-747 increased ileal Fgf15 expression and suppressed hepatic Cyp7a1 expression (Figure 4C), thus demonstrating that reduced FXR signaling in GF mice can be reversed by treatment with a specific FXR agonist.

In contrast to the clear effects of Fxr genotype on gene expression, bile acid levels in the gallbladder and small and large intestine appeared to be more dependent on colonization status (Figure S5). However, we found that the gut microbiota suppressed serum levels of TCA and TjIMCA in an Fxr-dependent fashion (Figure S5). We also noted increased levels of serum cholesterol in colonized Fxr-deficient mice (Figure S6A) and liver cholesterol in colonized mice (Figure S6B). In agreement, we also observed reductions in Hmgcr and Hmgcs1 expression (Figure S7).

**Antibiotic Treatment of CONV-R Mice Modifies Bile Acid Composition and Expression of Fgf15 and Cyp7a1**

To investigate whether microbiota-induced changes in bile acid composition and Fgf15 and Cyp7a1 expression are reversible in colonized mice, we treated CONV-R mice with an antibiotic cocktail consisting of bacitracin, neomycin, and streptomycin, which are nonabsorbable from the intestine. We showed that antibiotic treatment increased the levels of TCA and TjIMCA in the gallbladder of wild-type mice and reduced levels of secondary bile acids in serum (Figures 5A and 5B). These results are in agreement with a recent study showing increased levels of TCA and TjIMCA in the intestinal lumen of ampicillin-treated mice (Kuribayashi et al., 2012). Antibiotic treatment also promoted a dramatic suppression of Fgf15 expression and a corresponding increase of Cyp7a1 expression (Figure 5C). Thus, antibiotic treatment results in a phenotype similar to that observed in GF mice, namely modified bile acid composition together with reduced Fgf15 and increased Cyp7a1 expression.

**T-MCAs Are FXR Antagonists**

It is known that CAs but not MCAs are FXR agonists (Reschly et al., 2008), and thus the similar levels of CAs observed in small intestines of GF and CONV-R mice (Figures 2 and S2) are hard to reconcile with the higher expression of FXR-dependent genes in the ileum of CONV-R mice (Figures 4A and S4). However, MCA levels were significantly higher in the ileum of GF mice (Figure S2). MCAs are much less hydrophobic than primary human bile acids CDCA and CA, which have affinities to FXR directly related to their hydrophobicity (Reschly et al., 2008). Docking of TjIMCA into the ligand binding pocket of FXR with the FXR 6E-CDCA (OCA, obeticholic acid; INT-747) cocrystal structure suggested that, in contrast to the 6z ethyl group of 6E-CDCA, the 6β hydroxyl group of TjIMCA does not occupy the pocket near Tyr358, Phe363, and Tyr366 (Figure 6A). This pocket is known to be critical for the activation of FXR (Mi et al., 2003), and led us to hypothesize that TjIMCA may act as an FXR antagonist.

To test our hypothesis, we performed a coactivator recruitment assay and showed that both TzIMCA and TjIMCA were FXR antagonists with IC50 values of 28 μM and 40 μM, respectively (Figure 6B). Taurine conjugation was essential for...
antagonistic activity and, as expected, neither TαMCA nor TβMCA activated FXR in this assay (data not shown). To exclude the possibility that the observed antagonistic activity was due to nonspecific detergent effects at high bile acid concentrations, we showed that the concentration-response curve for the selective FXR agonist GW4064 was shifted to the right in the presence of 100 μM or 400 μM TβMCA (Figure 6C). We next investigated whether TβMCA could also function as an FXR antagonist in the small intestine, which has been shown to have the highest Fgf15 expression of all tissues (Larsson et al., 2012). We treated ileal explants from CONV-R mice with TCA to induce Fgf15 expression and found that TβMCA prevented this induction in a concentration-dependent fashion (Figure 6D). In addition, we induced ileal Fgf15 and Shp expression in GF mice in vivo by treatment with TCA and showed that simultaneous treatment with TβMCA significantly reduced this induction (Figures 6E and 6F). We could not repeat this experiment in CONV-R mice since the gut microbiota rapidly deconjugates TβMCA to βMCA. Taken together, these data indicate that TβMCA is a competitive and reversible antagonist for ligand-activated FXR.

DISCUSSION

Here we identified a profound role of the gut microbiota not only on secondary bile acid metabolism but also as regulator of hepatic bile acid synthesis. In the presence of a gut microbiota, mice had a smaller bile acid pool, with specific reductions in MCAs rather than CA. By comparing GF and CONV-R Fxr-deficient mice, we demonstrated that the gut microbiota regulates Fgf15 in the ileum through a FXR-dependent pathway. Finally, we identified TαMCA and TβMCA as FXR antagonists, and thus we propose that the higher levels of MCAs in GF mice at least partially account for the lower expression of FXR-dependent genes in the ileum of GF mice.

In the presence of a gut microbiota, bile acid levels were reduced in the gallbladder and small intestine, in agreement with earlier observations (Claus et al., 2008; Wostmann, 1973). By contrast, bile acid levels were higher in the cecum, colon, and feces of CONV-R mice compared with GF mice. Bile acids were more chemically diverse in CONV-R mice and the diversity was greatest in the cecum, colon, and feces of these animals. Thus, the most obvious microbial effects on the bile acid composition were in the compartments with the highest number of bacteria. No secondary bile acids were observed in GF mice. Our findings thus suggest that the reduced bile acid pool size in CONV-R mice may also reflect reduced bile acid reabsorption from the distal ileum and increased bile acid excretion in feces. In agreement, we showed that expression of Ibat and other transporters were reduced in CONV-R mice compared with their GF counterparts. Earlier studies have shown that TCA uptake is increased in ileal epithelium isolated from GF rats and that the half-life of 14C-labeled CA is four to five times longer in GF compared with CONV-R rats (Gustafsson et al., 1957; Riottot and Sacquet, 1985), supporting a role for increased reabsorption of bile acids that may further contribute to the larger bile acid pool in GF mice (Kellogg and Wostmann, 1969).

We observed a 71% reduction in MCA levels in CONV-R mice but with no effect on CA levels. CYP8B1 activity, which is required for CA formation (Li-Hawkins et al., 2002), was not altered in the presence of gut microbiota. By contrast, both gene expression and activity of CYP7A1 were reduced in CONV-R mice, supporting previous studies in rats (Gustafsson et al., 1975; Swann et al., 2011). CYP7A1 is required for CDCA formation, which is rapidly converted to MCAs through microsomal 6β hydroxylation in rodent livers (Gustafsson et al., 1975). We could not analyze the expression levels of the 6β
hydroxylase required for MCA synthesis as it has not yet been identified; however, 6β-hydroxylation is reduced in liver microsomes isolated from CONV-R mice and rats compared with GF counterparts (Claus et al., 2011; Gustafsson et al., 1975). Together, these changes in enzymatic activity are consistent with the reduced T-MCA/CA ratio observed in CONV-R mice.

FXR is highly expressed in liver and ileum, where it regulates distinct transcriptional networks (Thomas et al., 2010). It is generally recognized that FXR negatively regulates bile acid synthesis directly in the liver by inducing expression of SHP, which represses LRH-1, leading to reduced transcription of Cyp7a1 (Chiang, 2009). However, by comparing expression of direct FXR target genes in the liver (Shp and Lrh1) and ileum (Shp and Fgf15), we showed that the gut microbiota primarily affects FXR targets in the ileum and not the liver. Rederivation of Fxr−/− mice as GF confirmed that Fgf15 expression in the ileum required functional FXR signaling. The absence of established assays for FGF15, as noted in recent studies (Kir et al., 2011; Modica et al., 2012), prevented us from validating these findings at the protein level, but our data are in agreement with recent studies that showed an indirect effect of FXR signaling on bile acid synthesis by increasing expression of Fgf15 in the ileum (Inagaki et al., 2005; Zimmer et al., 2012). A recent study demonstrated that FGF19 administration suppressed bile acid synthesis in Fxr-deficient mice (Miyata et al., 2011), thus further implying that FGF15/19 are downstream of FXR and directly responsible for Cyp7a1 suppression. The precise mechanism remains to be elucidated, but it has been proposed that FGF15 is transported to the liver where it binds FGFR4 and acts to suppress Cyp7a1 (Inagaki et al., 2005; Zimmer et al., 2012). Tissue-specific knockouts have shown that Cyp7a1, but not Cyp8b1, is predominantly regulated by the FXR-FGF15 pathway in mice (Kim et al., 2007). Rederivation of tissue-specific Fxr-deficient mice as GF would be useful in future studies to assess the precise contribution of FXR signaling in the ileum and the liver to the microbiota-mediated regulation of the FGF15–CYP7A1 pathway. It is known that GF mice have altered gut physiology (Bäckhed et al., 2005), which may contribute to the observed changes in gene expression and bile acid composition. However, we showed that treatment of CONV-R mice with an antibiotic mixture resulted in modified bile acid composition, suppressed ileal Fgf15 expression and increased hepatic Cyp7a1 expression. Thus, the phenotype of antibiotic-treated mice was similar to that of GF mice, providing further evidence of an important role of the microbiota in the modulation of bile acid metabolism.

The presence of a gut microbiota increases the hydrophobicity of the bile acid pool and thus generates a bile acid profile that is a more potent activator of FXR. Importantly, we identified both TzMCA and TjMCA as FXR antagonists in vitro. TzMCA was not present in the distal small intestine of GF mice, but the combined TzMCA and TjMCA levels in the distal small intestine of CONV-R mice were 14-fold lower than the TjMCA levels in the same tissue from GF mice. The lower T-MCA/CA ratio in CONV-R mice will thus result in increased activity of FXR. Furthermore, we also showed that TjMCA significantly reduced the TCA-induced expression of Fgf15 in the ileum ex vivo as well as in

Figure 6. Taurine-Conjugated MCAs Are Competitive FXR Antagonists
(A) Docking of TzMCA (yellow) in the ligand-binding pocket of FXR using the FXR 6E-CDCA cocrystal structure (blue).
(B) Coactivator recruitment assay to assess whether TzMCA and TjMCA are functional antagonists to CDCA (80 μM)-mediated activation of FXR.
(C) Activation of FXR by GW4064 in the presence of 100 μM and 400 μM TjMCA to demonstrate reversibility of binding (assays were performed in triplicate and one out of two independent experiments is presented).
(D) Fgf15 expression in ileal explants treated with TCA in the presence of different amounts of TjMCA. The assay was performed in explants derived from five independent mice.
(E and F) Gene expression of Fgf15 (E) and Shp (F) in the ileum of GF C57BL/6 mice treated in vivo with TCA in the absence or presence of TjMCA (n = 6–7 mice/group).
Data were analyzed with one-way ANOVA followed by Bonferroni post hoc test. Mean values ± SEM are plotted; *p < 0.05, **p < 0.01, ***p < 0.001 versus control.
in vivo. Our data thus indicate that the gut microbiota may suppress Cyp7a1 and bile acid synthesis by reducing the levels of T-MCAs and promoting FXR-dependent activation of Fgf15 in the ileum (Figure 7). Furthermore, it predominantly suggests that the gut microbiota modulates FXR signaling in the gut.

In summary, we have demonstrated that the gut microbiota has a profound systemic effect on bile acid metabolism. Not only does the gut microbiota exert its effects within the gut, but also in other parts of the enterohepatic system, such as regulating bile acid synthesis in the liver. We demonstrate that the microbial suppression of biosynthetic genes in the liver is consistent with increased FXR-dependent activation of Fgf15 in the ileum due to reduced levels of TjmCA.

**EXPERIMENTAL PROCEDURES**

**Mice**

GF male Swiss Webster and C57BL/6 mice were maintained under a strict 12 hr light cycle in flexible plastic film isolators. Fxr−/− mice backcrossed four generations on to a C57BL/6 background were obtained from the Jackson Laboratory through a MTA with Frank Gonzalez and backcrossed four more generations on to a C57BL/6 background were obtained from the Jackson Laboratory.

**Analysis of Bile Acids**

**Reagents**

LCA was obtained from Calbiochem (Gibbstown, NJ); UDCA from Fluka (Buchs, Switzerland); CA, DCA, TCA, CDCA, DCA-d4, and DCA-d3 were obtained from Sigma-Aldrich; and 7α,12α-MCA, TCDCA-d4, and TCA-d4 were obtained from Steraloids (Newport, RI). The internal standards CA-d4, DCA-d4, UDCA-d4, CDCA-d4, and DCA-d4 were obtained from Qmx Laboratories (Essex, UK) and TCDCA-d4, TLCA-d4, TDCA-d4, TUDCA-d4, and TCA-d4 were synthesized at VTT (Tseng et al., 1977).

**Sample Preparation for Serum and Tissues**

Internal standards (10 μl 100 ppm) and water (240 μl) were added to 40 μl serum sample and vortexed. Acetonitrile (300 μl) was added to precipitate proteins. The centrifuged sample was filtrated with Sirocco Protein Precipitation Plate (Waters, Milford, MA). DCA-d4 was added to 40 μl serum sample and then precipitated with 0.5 M NaOH (10 μl) and purified with Oasis HLB LP 96-well plate...
60 mg (Waters). Cartridges were conditioned with 1 ml methanol and 2 ml water; the sample was loaded and washed twice with 300 µl water. Samples were eluted with 300 µl 90% acetonitrile, frozen, evaporated and reconstituted with 30% methanol.

Tissues were weighed and internal standards were added before extraction with 0.2 M NaOH (0.5–6 ml) at 80°C for 20 min. After cooling, 1.5–6 ml of water was added, and the samples were purified from lipids using liquid-liquid extraction with 1.5–6 ml of hexane. The extraction step was repeated three times and water phases were combined and the samples were further purified with Oasis HLB 3 cc 60 mg (Waters). The cartridges were conditioned with 1 ml methanol and 3 ml water; 4 ml sample was loaded and washed twice with 500 µl water. Samples were eluted with 1 ml acetonitrile:methanol:water (4:4:2), frozen, evaporated, and reconstituted with 50% methanol. For tauin analysis, 20 µl of the aqueous phase was aspirated and 80 µl boric acid buffer, and 20 µl AccQ-Fluor reagent was added to the extract. The sample mixture was instantly vortexed for 60 s before analysis. Norvaline was used as an internal standard for tauin analysis.

**UPLC-MS Analysis of Bile Acids**

Bile acids were analyzed on an Acquity UPLC system coupled to a Waters Xevo TQ-S MS (Waters, Manchester, UK) with an Acquity HSS T3 (2.1 x 100 mm, 1.7 µm) column (Waters) and gradient elution with 10 mM formic acid in water and 10 mM formic acid in acetonitrile:methanol (35:65) as mobile phases. Cone voltage was 70 V and collision energy 2 eV for unconjugated bile acids and 90 V and 65 eV for tauin conjugates. Anayses were detected by selected ion monitoring and quantified by internal standard methods. The desolvation temperature was 650°C, and the source temperature 150°C. Selected reaction monitoring (SRM) was used with dwell times of 32–247 ms. Analytes were quantified with deuterized internal standards for all analytes except for MCA and 7-oxo-MCA, for which CA-d4 was used. TCA-d4 was used as an internal standard for all tauin conjugates. Results were calculated as response (area analyte/area internal std). Retention times of analytes are presented in Table S2.

**UPLC-UV Analysis of Taurine**

Taurine was analyzed on an Acquity UPLC system (Waters, Milford, MA) with a diode array detector, and chromatography was performed with an Acquity Mass Trak (2.1 x 150 mm, 1.7 µm) column (Waters) kept at 43°C. Separation of 1 µl sample was performed with gradient elution with 10% (v/v) Amino Acid Analysis Concentrate A in water (A) and Amino Acid Analysis Eluent B (B) at a flow rate of 0.4 ml/min. Signal was detected at 280 nm (2.4 nm resolution, 20 points/s).

**Quantitative Real-Time PCR**

Liver or distal ileum (30 mg) was homogenized with TissueLyzer (QIAGEN), and total RNA was isolated with the RNeasy kit (QIAGEN). The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize 20 µl cDNA templates from 500 ng purified RNA with random hexamer primers, and the products were diluted 7x before use in subsequent reactions. SYBR Green Master Mix buffer (1x; Thermo Scientific) was used for quantitative real-time PCR at final reaction volumes of 25 µl. Gene-specific primers (900 nM) were used in each reaction and all results were normalized to the ribosomal protein L32 mRNA (primer sequences can be found in Table S3). PCr product specificity was verified by performance of a melting curve for each primer set. Assays were performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems) or CFX96 Real-Time System (Bio-Rad Laboratories). The reactions were analyzed with the ΔΔCt analysis method.

**Enzyme Activity of CYP7A1 and CYP8B1**

Microsomes were prepared from each mouse liver by differential ultracentrifugation of liver homogenates as described previously (Einarsson et al., 1986). The activity of CYP7A1 was determined as the formation of 7α-hydroxycholesterol (pmol/mg protein/min) from endogenous microsomal cholesterol at pH 7.4 via isotope dilution mass spectrometry in duplicate samples from each mouse (Einarsson et al., 1986). CYP8B1 activity was determined as the formation of 7a, 12α-dihydroxy-4-cholesten-3-one (pmol/mg/min) from added 7α-hydroxy-4-cholesten-3-one at pH 7.0 with isotope dilution mass spectrometry in duplicate samples from each mouse (Andersson et al., 1999).

**FXR Coactivator Recruitment Assay**

sMCA and jMCA and their respective tauin conjugates were tested for direct FXR activity in a coactivator recruitment assay as previously described (Solaas et al., 2004). The ligand binding domain of human FXR (amino acids 222–472) was expressed in Escherichia coli as an N-terminal His-tagged protein with the PET28a vector (Novagen) and the protein was purified by affinity chromatography. The activity of recombinant human FXR was determined in a FXR coactivator recruitment assay in white 384-well plates (Greiner). Eu3+-coupled anti-His antibody (anti-His-Eu3+) and aliphoyccyacin-co coupled streptavidin were obtained from PerkinElmer Life Sciences. An N-terminally biotinylated peptide (NH2–HSSLTRKHILRHLQEGSSP–COOH) (Innovagen, Sweden) derived from steroid receptor coactivator 1 was used as coactivator. A 20 µl reaction volume contained 20 mM Tris (pH 7.5), 0.125% CHAPS, 2 mM dithiotreitol, 0.05% bovine serum albumin, 0.14 µg/ml anti-His-Eu3+ and 2.9 µg/ml aliphoyccyacin-co coupled streptavidin, 75 mM human FXR-ligand binding domain, 150 mM biotinylated steroid receptor coactivator 1 peptide, and the appropriate ligand. Antagonist activity was measured in the presence of 80 µM CDCA in the reaction mixture. After addition of all reagents, plates were incubated for 1 hr at room temperature and time-resolved fluorescence was measured in a Pherasar platereader (BMG Labtech). Excitation was at 340 nm, and fluorescence was measured at 615 nm and 665 nm. Specific signals were calculated by dividing the 665 nm signal by the 615 nm signal and multiplying the fraction by 10,000. TsMCA and TjIMCA were purchased from Steraloids. GW4064 was obtained from AstraZeneca R&D Möndal and was used at concentrations ranging from 5.1 nM to 100 µM. XLFit (JDBusiness Solutions) was used to fit the experimental data points to curves and to calculate IC50 values.

**Ex Vivo Experiments**

CONV-R mice were killed by cervical dislocation, and approximately 1 cm section of distal ileum was collected. Intestinal contents from the section were removed through flushing with cold PBS, and the section was divided into four equal longitudinal parts. Each part was placed in cell culture plates containing growth medium (Dulbecco’s modified Eagle’s medium with glutamine and pyruvate, 4.5 g/l glucose, 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin) with different concentrations of the bile acids TCA and TjMCA. The plates were then incubated overnight at 37°C in 5% CO2. The mixture containing the tissue was transferred to Eppendorf tubes and centrifuged at 14,000 rpm for 5 min. The tissue was collected after removal of the supernatant and used for analysis of mRNA expression.

**Statistical Analysis**

Values are expressed as mean ± SEM. Significant differences between two groups were evaluated with a two-tailed, unpaired Student’s t test or Mann-Whitney U test for samples that were not normally distributed. Multiple groups were analyzed by one-way or two-way ANOVA followed by Bonferroni or Dunnnett’s multiple comparison test. PCA was performed with the Guineau software for metabolomics data analysis, version 0.8 (Castelo and Roverato, 2008). Construction of the association network of tissues based on bile acid profiles was performed with undirected Gaussian graphical Markov networks that represent q-order partial correlations between variables, implemented in an R package “qgraph” (Castillo et al., 2011). In these networks, missing edges denote zero partial correlations between pairs of variables and thus imply the conditional independence relationships in the Gaussian case. The network was visualized with Cytoscape (Cline et al., 2007).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.01.003.

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