Of mice and men: murine bile acids explain species differences in the regulation of bile acid and cholesterol metabolism

Sara Straniero, Amit Laskar, Christina Savva, Jennifer Härdfeldt, Bo Angelin, and Mats Rudling

Metabolism Unit, Endocrinology, Metabolism and Diabetes, and Integrated Cardio Metabolic Center (ICMC), Department of Medicine, Karolinska Institutet at Karolinska University Hospital, Huddinge, S-141 86 Stockholm, Sweden

ORCID IDs: 0000-0002-5918-3513 (S.S.); 0000-0002-1448-2368 (B.A.)

Abstract
Compared with humans, rodents have higher synthesis of cholesterol and bile acids (BAs) and faster clearance and lower levels of serum LDL-cholesterol. Paradoxically, they increase BA synthesis in response to bile duct ligation (BDL). Another difference is the production of hydrophilic 6-hydroxylated muricholic acids (MCAs), which may antagonize the activation of FXRs, in rodents versus humans. We hypothesized that the presence of MCAs is key for many of these metabolic differences between mice and humans. We thus studied the effects of genetic deletion of the Cyp2c70 gene, previously proposed to control MCA formation. Compared with WT animals, KO mice created using the CRISPR/Cas9 system completely lacked MCAs, and displayed >50% reductions in BA and cholesterol synthesis and hepatic LDL receptors, leading to a marked increase in serum LDL-cholesterol. The doubling of BA synthesis following BDL in WT animals was abolished in KO mice, despite extinguished intestinal fibroblast growth factor (Fgf15) expression in both groups. Accumulation of cholesterol-enriched particles (“Lp-X”) in serum was almost eliminated in KO mice. Livers of KO mice were increased 18% in weight, and serum markers of liver function indicated liver damage. The human-like phenotype of BA metabolism in KO mice could not be fully explained by the activation of FXR-mediated changes. In conclusion, the presence of MCAs is critical for many of the known metabolic differences between mice and humans. The Cyp2c70 KO mouse should be useful in studies exploring potential therapeutic targets for human disease.—Straniero, S., A. Laskar, C. Savva, J. Härdfeldt, B. Angelin, and M. Rudling. Of mice and men: murine bile acids explain species differences in the regulation of bile acid and cholesterol metabolism. J. Lipid Res. 2020. 61: 480–491.

Supplementary key words
cholesterol/metabolism • low density lipoprotein/metabolism • bile acids and salts/metabolism • Cyp2c70 • lipoproteins • cholestasis • CRISPR/Cas9 • liver

Bile acids (BAs) are amphiphilic molecules synthesized from cholesterol in the liver, stored in the gallbladder, and released into the gut after food intake. By solubilizing lipophilic molecules, they promote biliary lipid secretion and intestinal fat absorption. Through their subsequent active uptake in the distal ileum and reuptake by the liver, the pool size of BAs is conserved. The rate of formation of BAs from cholesterol in the liver is modulated by the flux of BAs through this enterohepatic circulation by regulation of the rate-limiting enzyme, cholesterol 7α-hydroxylase (CYP7A1). The recognition that BAs may also act as signaling molecules in overall regulation of body metabolism has enhanced the interest for how modulation of their metabolism may be used therapeutically for treatment of fatty liver disease, dyslipidemia, and type 2 diabetes (1–4).

A large part of our knowledge regarding cholesterol and BA metabolism emerges from studies in rats and mice. However, there are major known species differences limiting the translation of such data to human physiology. Compared with humans, the basal turnover of BAs and cholesterol, as well as that of circulating LDL, is higher and

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transferase; ASBT, apical sodium-dependent bile acid transporter/Scl10a2; AST, aspartate transaminase; BA, bile acid; BDL, bile duct ligation; C4, 7α-hydroxy-4-cholesten-3-one; CA, cholic acid; CDCA, Chenodeoxycholic acid; CTAC, Cyagen Transgenic Animal Center; CYP7A1, cholesterol 7α-hydroxylase; DCA, deoxycholic acid; FGF, fibroblast growth factor; MCA, muricholic acid; PCSK9, proprotein convertase subtilisin/kexin type 9; RT-qPCR, real-time quantitative PCR; UDCA, ursodeoxycholic acid.

1To whom correspondence should be addressed.

2Email: mats.rudling@ki.se.

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the concentration of LDL-cholesterol much lower in mice (5, 6). On the other hand, interruption of the enterohepatic circulation of BAs seems to elicit stronger effects on cholesterol metabolism and LDL levels in humans (7). Partly based on their different hydrophobicity, individual BAs have varying influences on biliary lipid secretion, intestinal fat absorption, and feedback inhibition of BA synthesis, the latter thought to occur predominantly through their interaction with the FXR in the distal ileum and in the liver (6). In contrast to humans, about half of the BA pool in mice consists of 6-hydroxylated muricholic acids (MCAs) (6). The physiological role of these hydrophilic BAs is still unclear, although their administration or overproduction reduces the intestinal absorption of dietary cholesterol and fat (1, 8–10). The presence of MCAs may also quench the responses elicited by hydrophobic BAs, such as cholic acid (CA), chenoecdexoycholic acid (CDCA), and deoxycholic acid (DCA) (6). The fact that taurine-conjugated MCAs display FXR-antagonistic properties (11) may indicate that their abundance explains the high basal synthesis of BAs and cholesterol in mice compared with humans (5, 6, 12, 13).

Another major difference between rodents and humans concerns the effect of cholestasis on BA production. Thus, bile duct ligation (BDL) increases BA synthesis by 2-fold in mice and rats (14–16), whereas cholestasis in humans is associated with suppressed BA synthesis (17–19). This response in rodents following BDL has been explained as a consequence of the deficient FXR-driven secretion of fibroblast growth factor (FGF)15 (human ortholog, FGF19) from the ileum in this situation (16), compatible with the concept of a gut-mediated negative feedback inhibition of BA synthesis regulated by transintestinal BA flux (20–22). In human cholestasis, the hepatic gene expression of Fgf19 is clearly induced, leading to higher circulating concentrations (17–19), whereas hepatic Fgf15 remains unexpressed in cholestatic mice (19). An alternative explanation that may be proposed for the paradoxical increase in BA production following BDL in rodents (14, 23) could be that it largely relates to the robust accumulation of hydrophilic MCAs known to occur in this situation (12, 13).

Recent observations in mice with deletion of the Cyp2c gene cluster indicated that the Cyp2c70 gene may be responsible for the 6-hydroxylation needed for MCA production in the mouse (24). Based on this finding, we constructed mice genetically deficient in this gene to test three initial hypotheses: i) that Cyp2c70 KO mice indeed lack MCAs as anticipated; ii) that BA and cholesterol synthesis, as well as hepatic LDL receptors, are reduced and serum LDL levels increased in such mice; and iii) that the paradoxical stimulation of BA synthesis following BDL is abolished in Cyp2c70 KO mice. Our results demonstrate that a broad set of differences in BA and cholesterol metabolism between mice and humans are strongly dependent on MCAs. The human-like metabolic phenotype of Cyp2c70 KO mice also indicates that this animal model may provide a useful tool in studies on lipid-modulating interventions for human disease.

MATERIALS AND METHODS

Mice and study design

Cyp2c70 KO mice were produced and supplied by Cyagen US Inc. (Santa Clara, CA). CRISPR/Cas-mediated genome editing was used to generate Cyp2c70 KO mice on a background strain of C57BL/6 mice. Exon 4 of the mouse Cyp2c70 gene, located on mouse chromosome 19 was selected as a target site. The targeting sequence of the Cyp2c70 sgRNA was CTCTCATCACGGCA-CAACTT. The sgRNA sequence was cloned into pBR[CRISPR] hCas9-U6 > 20nt CTCTCATCACGGCA-CAACTT (a cloning vector) to form a U6 promoter-mediated sgRNA expression vector. Cas9 mRNA and sgRNA were generated by in vitro transcription and microinjected into fertilized eggs. Thereafter, the injected embryos with normal morphology were transferred into oviducts.

Founder mice were genotyped by real-time quantitative RT-qPCR (RT-qPCR) and DNA sequencing. Primers used for genotyping were Cyp2c70 F (5'-GTGCCAGGTCTCATTCTGG-3') and Cyp2c70 R (5'-GCTTGTTAAAAATTTGAGCCCTCCTGATAC3'). Potential off-target fragments were amplified from the extracted DNA and identified by DNA sequencing, which confirmed absence of mutations. An identified positive founder was bred to the next generation (F1) and subsequently genotyped. Filial (F) breeding generation F3 and F4 mice were used for these experiments. Additional information is provided in supplemental Fig. S1.

First, the phenotype of Cyp2c70 KO mice was characterized using groups (n = 11–12) of 8-week-old Cyp2c70 KO mice and WT littermates matched for age and gender. Animals were housed in an exhaust-ventilated cage system in a pathogen-free and temperature-controlled (24 ± 2°C) room under a 12 h light-dark cycle with corn cob bedding and nestlet and paper cup enrichment. Presterilized standard chow diet was purchased to the standard laboratory Animals: Nutrients for Formula Feed). Animals were fasted for 4 h with free access to water before euthanization.

Second, groups (n = 8–10) of 12-week-old WT or Cyp2c70 KO mice matched for gender and age were subjected to BDL as described (25). Briefly, mice were anesthetized by isoflurane and BDL was performed after midline laparotomy. The common bile duct was ligated in two areas and was not transected between the ligations to reduce the risk of bile leakage. Sham operations were performed similarly, except for ligation of the bile duct. Animals were allowed to recover and moved to a cage with ad libitum access to water and food until 3 days after surgery and euthanized.

All animal experiments were performed at the Cyagen Transgenic Animal Center (CTAC), a specific-pathogen free barrier facility belonging to Cyagen’s Guangzhou branch, China. The CTAC is Association for Assessment and Accreditation of Laboratory Animal Care accredited and ISO 9001 certified. All animal experiments were approved by the Institutional Animal Care and Use Committee of CTAC (Oct 18, 2017), and meet Animal Research: Reporting of In Vivo Experiments standards. Frozen samples were transported from CTAC to Stockholm for analysis.

Methods

RT-qPCR. Total RNA was isolated using the RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany). Isolated RNA was reverse-transcribed with random-hexamer priming. Omniscript (Qiagen, Valencia, CA) and RNSatin (Promega, Madison, WI). RNA concentration was measured by spectrophotometry (NanoDrop; Thermo Scientific). RT-qPCR was performed by Quantstudio 5 real-time PCR system (Applied Biosystems, Foster City, CA) using primers for selected genes; their sequences are shown in the supplemental Materials and Methods.
Cyclophilin A and Gapdh were used as endogenous controls. The comparative Ct method was used to quantify the results presented as arbitrary units (a.u.).

Microsomal CYP7A1 enzymatic activity and cholesterol levels. Enzymatic activity of hepatic CYP7A1 was determined in liver microsomes by isotope dilution MS, as previously described (5), using D7-7a-hydroxycholesterol as internal standard.

Free and esterified cholesterol in microsomal membranes and liver homogenates. D7-cholesterol, water, NaCl, and Folch solutions were added to a 10% liver homogenate or microsomal fraction and samples centrifuged at 3,000 g for 5 min; the lower phase was then dried under nitrogen gas. Folch solution was added and the lower phase divided into two aliquots. One aliquot was dried under nitrogen for free cholesterol analysis. KOH (0.5 M) in ethanol was added in the other aliquot and the samples heated to 70°C in a water bath for 1.5 h, where water and hexane were added before centrifugation at 3,000 g for 5 min. The upper phase was dried under nitrogen for total cholesterol analysis. All samples were then silylated with 0.4 ml of pyridine/hexamethyl-disilizane/chlorotrimetylsilane (3:2:1, v/v/v) at room temperature overnight and thereafter dried under nitrogen at 60°C. Samples were dissolved in 100 μl of hexane and analyzed using GC/MS. Data were corrected to protein concentration, determined using Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA).

LDL receptor protein expression. Hepatic membrane proteins were prepared as described (26) from pooled liver tissue as detailed in the figure legends. Membranes were electrophoresed on 8% SDS-PAGE, electrotransferred to nitrocellulose, and probed against the LDL receptor (3839, rabbit polyclonal, diluted 1:1,000; GE Healthcare, Buckinghamshire, UK) and analyzed by CCD camera and quantified by a LAS 1000 Plus imager and Multi Gauge Software Science Lab 2005 version 3.1 (Fuji Photo). After stripping, β-actin was probed using the same protocol (A2228, diluted 1:10,000; Sigma-Aldrich, St. Louis, MO) followed by incubation with HRP-conjugated secondary antibody (A9044, diluted 1:20,000; Sigma-Aldrich) and analyzed as above.

Serum analyses. Lipoprotein profiles were obtained using fast-performance LC as described (27). Markers for BA production [7α-hydroxy-4-cholesten-3-one (C4)], cholesterol synthesis (lathosterol), and cholesterol absorption (stigasterol and campesterol) were analyzed as described (7, 28, 29). BAs in serum and bile were determined by LC-MS/MS using deuterium-labeled standards of unconjugated and taurine-conjugated BAs as described (7). Deuterium-labeled standards of unconjugated and glycine- and taurine-conjugated BAs were from Steraloids, Inc. (Newport, RI).

For liver function tests, serum activities of alanine transference (ALT), alkaline phosphatase (ALP), and aspartate transaminase (AST) were determined by colorimetric assays from Abbott Laboratories using an Abbott Architekt ci8200 (Abbott Park, IL).

Hepatic and intestinal BAs were analyzed by LC-MS/MS using deuterium-labeled standards of unconjugated and taurine-conjugated BAs as described above. One hundred microliters of a 10% homogenate in 1 M Tris-HCl (pH 7.5) were mixed with 400 μl of acetonitrile (VWR, Radnor, PA) and 5 μl of deuterium-labeled BA internal standards (10 ng d4-BA) and vortexed for 10 s. Supernatants were transferred to glass tubes and dried under a stream of nitrogen at 40°C following a 15 min centrifugation at 14,000 g. The residue was dissolved in 100 μl of methanol-water (1:1, v/v), and 5 μl of this solution were injected. Individual BAs were analyzed by LC-MS/MS as described (7).

All chemicals and solvents were of the highest purity available. Methanol, acetonitrile, and formic acid were obtained from VWR. Deuterium-labeled standards of unconjugated and glycine- and taurine-conjugated BAs were from Steraloids, Inc. (Newport, RI).

Statistics. The data are presented as mean ± SEM. The significance of difference was tested by unpaired two-tailed Student’s t-test after log transformation; P values <0.05 were considered significant. GraphPad Prism 7 version 7.03 was used.

RESULTS

An intact Cyp2c70 gene is crucial for MCA formation in vivo

We first evaluated to determine whether deletion of the Cyp2c70 gene eliminates MCAs by analyzing serum BAs by LC-MS/MS. Whereas MCAs comprised 69% of the serum BAs in WT mice, KO mice were devoid of MCAs, demonstrating a complete dependence on an intact CYP2C70 protein for their formation (Fig. 1A). Instead, 75% of the BA spectrum consisted of free and conjugated CDCA and CA. The MCA precursors, ursodeoxycholic acid (UDCA) and TUDCA, were increased by 6-fold compared with WT. Similar differences were observed for BAs in the liver and ileum, where conjugated BAs in general were enriched compared with serum (Fig. 1B, C). Thus, disruption of the Cyp2c70 gene resulted in elimination of the normally abundant MCAs, together with elevations of the two substrates for MCA synthesis, CDCA and CA, all in accordance with the proposal by Takahashi et al. (24) that the Cyp2c70 gene is crucial for MCA synthesis.

Loss of MCAs demonstrates their key role for the metabolic phenotype of WT mice

While body weight was similar in the two animal groups, liver weight was 18% higher in KO mice (Table 1). The enzymatic activity of the rate-limiting enzyme in BA synthesis, microsomal cholesterol 7α-hydroxylase, was reduced by 60% in KO mice (Fig. 2A). A corresponding reduction was found for hepatic Cyp7a1 mRNA (Fig. 2A), and a similar trend was observed for the serum marker of BA synthesis, C4 (Fig. 2A). The hepatic Cyp8b1 mRNA was reduced by 80% in KO mice (Fig. 2A). In the KO animals, the reduced conversion of cholesterol to BAs was associated with increased hepatic microsomal free and total cholesterol by 15% and 19%, respectively (Table 1). While the hepatic mRNA level of Srebp2 was not altered, transcripts of its target genes, Hmgc reductase (rate-limiting in cholesterol synthesis) and Ldlr, were reduced by 51% and 31%, respectively. There was also a trend for reduced expression of the proprotein convertase subtilisin/kexin type 9 (Pesk9) gene (Fig. 2B). Measurement of the LDL receptor protein showed a reduction by >50% in KO animals (Fig. 2C). In consonance with the downregulation of the LDLRs, serum total cholesterol was increased by 31% in KO mice (Table 1) due to a marked (141%) increase in LDL-cholesterol (Fig. 2D, Table 1). Total serum triglycerides were unchanged,
while LDL-triglycerides were also increased (Fig. 2D, Table 1). The serum marker for total body cholesterol synthesis (lathosterol) was unaltered in KO mice (Fig. 2E). Unexpectedly, two markers for cholesterol absorption, sitosterol and campesterol, were reduced by 50% in KO mice (Fig. 2F).

Whereas Cyp7a1 and Cyp8b1 mRNAs were lower in the livers of KO animals, the response pattern for other FXR-regulated genes was less clear (Fig. 3). Thus, there was no significant increase in Shp or Bsep and no decrease in Abcc4 (Mrp4) or Ntcp; the latter was actually increased. The expression of Fxr itself tended to be decreased. Of other transporter transcripts, Abcg5 and Abcg8, which are targets of Lxr, were not changed (supplemental Fig. S2B). Among potentially detoxifying genes, Cyp3a11 was reduced, and there was a similar trend for Sult1c2 (Fig. 3A, supplemental Fig. S2B).

In some contrast to what was seen in the liver, targets of Fxr, including Fgf15, Shp, and Ibabp, showed increased expression in the terminal ileum of KO animals; there was also a trend for enhanced levels of apical sodium-dependent BA transporter/Slc10a2 (Asbt), Ost alpha, and Ost beta mRNAs (Fig. 3B). Aбег5 and Aбег8 transcripts were unchanged in KO mice (Fig. 3B). Because the ileal expression of Fgf15 is considered to be regulated by the transintestinal flux of FXR-agonistic BAs, this is likely the consequence of the absence of MCAs in the enterohepatic circulation. The relative importance of increased ileal Fgf15 expression versus increased hepatic exposure to FXR-agonistic BAs for the reduction of hepatic BA synthesis seen in KO mice could not be evaluated from the present data. However, it appears clear that the suppressed BA synthesis in these animals leads to increased microsomal membrane cholesterol that, in turn, inactivates the Srebp2 pathway in the liver.

Loss of MCAs abolishes the paradoxical stimulation of BA synthesis following BDL in WT mice

To test our hypothesis that the accumulation of hydrophilic MCAs following BDL is involved in the paradoxical stimulation of BA synthesis (16), we compared BDL and sham-operated WT and KO mice 3 days after surgery. The differences observed between WT and KO mice in the previous experiment could generally be confirmed when comparing the two groups of sham-operated animals (supplemental Figs. S4, S5). While body weight decreased by ~3 g in both BDL groups, liver weight was unaltered in WT animals but was doubled in KO animals (Table 2). We measured serum markers of liver function (ALT, ALP, and AST) in this experiment, and could show that they were increased in sham-operated KO animals (Fig. 4A). After BDL, these

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**TABLE 1. BAs and lipids in serum and liver in WT and Cyp2c70-KO mice**

|                      | WT        | KO        | p*     |
|----------------------|-----------|-----------|--------|
| Body weight (g)      | 22.7 ± 0.92 | 23.5 ± 0.82 | NS     |
| Liver weight (g)     | 1.14 ± 0.06 | 1.34 ± 0.04 | 0.007  |
| Total serum cholesterol (mM) | 2.30 ± 0.10 | 3.05 ± 0.1 | <0.0001 |
| VLDL-cholesterol     | 0.08 ± 0.03 | 0.17 ± 0.03 | 0.005  |
| LDL-cholesterol      | 0.30 ± 0.05 | 0.73 ± 0.11 | 0.002  |
| HDL-cholesterol      | 1.99 ± 0.11 | 2.14 ± 0.14 | NS     |
| Total serum triglycerides (mM) | 1.31 ± 0.14 | 1.10 ± 0.07 | NS     |
| VLDL-triglycerides   | 0.16 ± 0.02 | 0.16 ± 0.03 | NS     |
| LDL-triglycerides    | 0.08 ± 0.01 | 0.15 ± 0.03 | 0.036  |
| HDL-triglycerides    | 0.05 ± 0.00 | 0.05 ± 0.01 | NS     |
| Glycerol             | 1.02 ± 0.13 | 0.75 ± 0.06 | NS     |
| Total serum BAs (nmol/ml) | 6.92 ± 1.39 | 3.76 ± 1.54 | NS     |
| Total liver BAs (nmol/g liver) | 191 ± 33.3 | 451 ± 127.1 | NS     |
| Total liver cholesterol homogenate (μg/mg protein) | 7.74 ± 0.70 | 8.06 ± 0.08 | NS     |
| F                    | 6.06 ± 0.38 | 6.71 ± 0.51 | NS     |
| Esterified           | 1.41 ± 0.45 | 1.35 ± 0.19 | NS     |
| Total liver cholesterol microsomes (μg/mg protein) | 36.8 ± 1.20 | 43.9 ± 1.41 | 0.001  |
| F                    | 35.1 ± 0.55 | 37.3 ± 1.04 | 0.002  |
| Esterified           | 3.75 ± 0.84 | 6.67 ± 1.06 | 0.045  |

*Data show mean ± SEM (n = 11–12).

*Values from unpaired two-tailed Student’s t-test.
Fig. 2. Hepatic CYP7A1 enzymatic activity. RT-qPCR analysis of liver Cyp7a1 and Cyp8b1 and serum marker C4 for BA synthesis in WT and KO mice (A). RT-qPCR analysis of liver Hmgcr, Ldlr, Psk9, and Srebp2 in WT and Cyp2c70-KO mice (B). LDLR expression by immunoblot of membranes from pooled liver (C). There were eight liver samples from each group (four males and four females) used for each pool. Serum lipoprotein profiles for cholesterol and triglycerides (D). Serum markers for cholesterol synthesis (lathosterol) in WT and Cyp2c70-KO mice (E). Serum markers for cholesterol absorption (sitosterol and campesterol) in WT and Cyp2c70-KO mice (F). Data show mean ± SEM (n = 7–12). Red circles represent females and black triangles represent males. P values from unpaired two-tailed Student’s t-test after log transformation. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
markers were significantly increased in both WT and KO mice, reaching the highest levels in the KO animals. Analysis of liver morphology was compatible with mild liver damage in the sham-operated KO animals and indicated more severe damage following BDL in this group (supplemental Fig. S6).

Following BDL, total serum BAs increased >3,800-fold in WT mice and ∼80-fold in KO mice (Fig. 4B, Table 2). The agonistic BAs (DCA, CDCA, TCDCA, and TCA), which comprised ∼10% of serum BAs in sham-operated WT mice, were eliminated following BDL, where MCAs comprised 78% and TCA the remainder (Fig. 4B). In agreement with the previous experiment, CA, CDCA, TCDCA, TDCA, and TCA comprised 74% of total BAs in sham-operated KO mice (Fig. 4B). Following BDL in KO mice, the relative contribution of UDCA was reduced while TCDCA did not change. TCA became the dominant BA, increasing from 20% to 52% (Fig. 4B). Similar results were obtained for BAs in the liver, ileum, and gallbladder bile (Fig. 4C–E). As anticipated (14–16), the enzymatic activity of cholesterol 7β-hydroxylase increased by 168% in WT BDL mice, accompanied by similar increases in mRNA and serum C4 (Fig. 5A), compatible with increased BA synthesis following BDL. These responses were

Fig. 3. RT-qPCR analysis of liver Fxr, Shp, Abcc3, Abcc4, Bsep, Ntcp, Abcb1a, and Cyp3a11 in WT and Cyp2c70KO mice (A). Intestinal gene expression of Fgf15, Asbt, Shp, Ostα, Ostβ, Abcg5, and Abcg8 (B). Data show mean ± SEM (n = 11–12). Red circles represent females and black triangles represent males. P values from unpaired two-tailed Student’s t test after log transformation. *P < 0.05, **P < 0.05 and ****P < 0.0001.
mRNA and circulating levels of FGF19 are increased and could not detect any gene expression of serum C4 reduced (17–19).

Strongly reduced in both groups following BDL (Fig. 5C). Thus, in mice following BDL required the presence of MCAs. The regulation of BA metabolism is a prototype for how the absence of Fgf15 may not be the major mechanism for the increased BA synthesis following BDL in WT mice.

The hepatic expression of Cyp8b1 mRNA was reduced following BDL in WT mice and remained very low in KO animals (Fig. 5B). The hepatic expression of Fxr mRNA was reduced following BDL in both WT and KO animals (Fig. 5B); reductions were also seen in both groups for Abcg5, Abcg8, and Sult1c transcripts (supplemental Fig. S3B), whereas no changes were observed for Shp, Bsep, or Cyp3a11 (Fig. 5B). In WT but not in KO animals, BDL resulted in increased mRNAs for Ntcp, Abcc4 (Mrp3), Abcc4 (Mrp4), and Abcb1a (Mdr3) (Fig. 5B).

In contrast, the intestinal mRNA level generally showed similar patterns following BDL in WT and KO animals. Thus, reflecting the absence of BAs, the FXR-regulated genes Shp and Bbap were markedly reduced (Fig. 5C), and the gene expression of Asbt was increased in WT mice, with a similar trend in the KO animals (Fig. 5C). While there were only trends toward a reduction in Ostα and Ostβ transcripts, the Lxr-regulated Abcg5 and Abcg8 mRNA levels were strongly reduced in both groups following BDL (Fig. 5C).

In agreement with previous work in mice (16, 19), we could not detect any gene expression of Fgf15 in the livers from any of the groups (data not shown). The metabolic response to BDL in WT mice is thus in clear contrast to that seen in humans with extrahepatic cholestasis, where hepatic mRNA and circulating levels of FGF19 are increased and serum C4 reduced (17–19).

### Table 2. BA and lipid profiles in serum and liver of sham operated or BDL WT and Cyp2c70 KO mice

|                        | WT Sham | WT BDL | KO Sham | KO BDL |
|------------------------|---------|--------|---------|--------|
| Body weight (g)        |         |        |         |        |
| Before operation       | 25.9 ± 1.4 | 25.9 ± 1.1 | 26.7 ± 1.3 | 30.6 ± 0.8 |
| After operation        | 25.5 ± 1.3 | 22.2 ± 1.1 | 27 ± 1.3 | 27.5 ± 0.7 |
| Liver weight (g)       | 1.3 ± 0.06 | 1.3 ± 0.06 | 1.7 ± 0.13 | 2.3 ± 0.17 |
| Total serum cholesterol (mM) | 2.4 ± 0.13 | 15 ± 1.60 | 2.6 ± 0.22 | 3.2 ± 0.19 |
| LDL-cholesterol        | 0.1 ± 0.03 | 12.7 ± 1.75 | 0.2 ± 0.02 | 1.2 ± 0.25 |
| HDL-cholesterol        | 0.4 ± 0.03 | 1.5 ± 0.21 | 0.8 ± 0.15 | 1.2 ± 0.13 |
| Total serum triglycerides (mM) | 0.9 ± 0.15 | 1.1 ± 0.08 | 0.8 ± 0.05 | 0.8 ± 0.08 |
| VLDL-triglycerides     | 0.3 ± 0.09 | 0.2 ± 0.02 | 0.1 ± 0.03 | 0.05 ± 0.02 |
| LDL-triglycerides      | 0.1 ± 0.03 | 0.08 ± 0.02 | 0.1 ± 0.01 | 0.2 ± 0.03 |
| HDL-triglycerides      | 0.03 ± 0.01 | 0.3 ± 0.05 | 0.04 ± 0.01 | 0.1 ± 0.02 |
| Glycolipids            | 0.5 ± 0.06 | 0.6 ± 0.03 | 0.5 ± 0.03 | 0.5 ± 0.05 |
| Total serum BAs (nmol/ml) | 6.5 ± 0.01 | 25,000 ± 0.05 | 21 ± 0.01 | 1680 ± 0.02 |
| Total liver BAs (nmol/g liver) | 273 ± 0.06 | 3,230 ± 0.03 | 354 ± 0.03 | 750 ± 0.05 |
| Total liver cholesterol homogenate (µg/mg protein) | 6.6 ± 0.93 | 8.9 ± 0.61 | 8.5 ± 0.92 | 10 ± 1.82 |
| Free                   | 5.5 ± 0.74 | 8.3 ± 0.55 | 6.8 ± 0.58 | 9.3 ± 1.55 |
| Esterified             | 1 ± 0.23 | 0.6 ± 0.08 | 1.8 ± 0.42 | 0.8 ± 0.28 |
| Total liver cholesterol micromoles (µg/mg protein) | 36 ± 2.36 | 52 ± 2.85 | 48 ± 2.94 | 54 ± 4.99 |
| Free                   | 32 ± 2.12 | 48 ± 3.04 | 43 ± 2.33 | 51 ± 4.76 |
| Esterified             | 3.8 ± 0.49 | 3.6 ± 0.74 | 4.5 ± 0.88 | 2.7 ± 0.61 |

Data show mean ± SEM (n = 8–10).

Possible role of MCAs for the response in hepatic cholesterol metabolism following BDL

In agreement with our previous results (Table 1), microsomal cholesterol in sham-operated KO mice was higher than in WT animals, and increased in both groups following BDL (Table 2). In accordance with the elevated BA synthesis, transcripts for Hmgcr reductase were increased in BDL WT mice, whereas no such changes occurred in BDL KO mice (Fig. 6A). The Ldlr mRNA was reduced following BDL in KO mice but not in the WT mice; no clear changes were observed for Pek9 or Sreb2 mRNAs in either group (Fig. 6A). The LDLR protein was reduced by >50% following BDL in both WT and KO mice (Fig. 6B). Compared to baseline, mRNA of Hmgcr reductase was increased following BDL in WT but remained low in KO animals. Serum lathosterol increased in both groups, remaining lower in the KO mice (Fig. 6C). The serum markers for cholesterol absorption, campsterol and sitosterol, were increased by 3- to 5-fold following BDL in WT mice, a response that was less pronounced in KO mice (Fig. 6D). Serum cholesterol increased 5-fold following BDL in WT mice, while the increase was only 22% in KO mice (Table 2). The cholesterol increase in WT mice was restricted to large lipoprotein particles poor in triglycerides, compatible with the formation of micellar lipoprotein-X complexes (30) (Fig. 6E); this response was markedly diminished in BDL KO animals. Of note, the absolute concentrations of micelle-promoting BAs were much higher in WT than in KO animals (supplemental Table S1).

**DISCUSSION**

The regulation of BA metabolism is a prototype for how molecules control their formation and distribution by modulation of rate-limiting steps of synthesis and transport,
Muricholic acids shape the metabolic phenotype in mice utilizing widespread sensing mechanisms throughout the enterohepatic circulation (31, 32). In addition to maintaining appropriate concentrations of BAs for micellar solubilization of lipids in the bile and in the upper small intestine, thereby promoting cholesterol and phospholipid excretion from the liver and absorption of fat and fat-soluble vitamins from the intestine, the degradation of cholesterol into BAs is a major regulator of body cholesterol homeostasis.

Fig. 4. Serum level of ALT, ALP, and AST activities in sham-operated or BDL WT and Cyp2c70-KO mice (A). Data represent mean ± SEM (n = 8–10). Red circles represent females and black triangles represent males. P values are from unpaired two-tailed Student’s t test after log transformation of sham versus BDL of each group. *P < 0.05, **P < 0.01, and ***P < 0.001. Total BA levels and spectrum in serum (B), liver (C), distal intestine (D), and gallbladder (E) in sham-operated or BDL WT and Cyp2c70-KO mice. Data represent mean ± SEM (n = 8–10). *In panel E, conjugated BAs could not be separated and presented as sum of Tα-, Tβ-, and Tω-MCA.
Although knowledge on BA and cholesterol turnover has partly been gained from human studies, our understanding of cholesterol metabolism is to a great extent based on animal models. It has long been established that there are major species differences in cholesterol, BA, and lipoprotein metabolism, which have hampered the straightforward translation of animal data to the human situation. Among such differences, the high capacity for BA and cholesterol synthesis and the rapid turnover and low concentration of LDL in mice and rats compared with humans have been of particular interest.

Our work presents strong evidence that abundant MCAs in normal mice play a key role for several major differences in their metabolic phenotype compared with humans. The complete elimination of MCAs in Cyp2c70-KO mice confirms the proposed critical role of this enzyme for their formation (24). When MCAs are absent, the synthesis of BAs and cholesterol as well as hepatic LDLR
numbers are all reduced by >50%, resulting in increased serum LDL-cholesterol levels. New set points of feedback control of BA synthesis are obviously reached when MCAs are absent, resulting in reduced synthesis as well as elimination of cholesterol from the body. The altered set points, in turn, cause adaptive modulations of hepatic cholesterol synthesis and lipoprotein clearance, leading to a more human-like metabolic phenotype, including relative hypercholesterolemia.

We have reason to believe that the total pool of BAs is reduced in the Cyp270KO mice. Three important observations support this. First, in the KO mice, the synthesis of BAs is reduced >50%. Second, the total BA content is strongly reduced in the intestine of KO mice. Third, BAs were reduced in bile from KO mice. On the other hand, to what extent the higher capacity for solubilization of biliary and intestinal cholesterol and other lipids of the BAs dominating the reduced pool of the KO animals, which was not

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**Fig. 6.** Hepatic mRNA expression of Hmgcr, Ldlr, Pcsk9, and Srebp2 (A). LDLR expression by immunoblot of membranes from pooled liver samples (B). There were eight liver samples from each group (four males and four females) except for the BDL Cyp270KO group where eight liver samples were from five males and three females. Serum markers for cholesterol synthesis (lathosterol) (C) and serum markers for cholesterol absorption (sitosterol and campesterol) (D) in sham-operated or BDL WT and Cyp270KO mice. Serum lipoprotein profiles for cholesterol and triglycerides (E) in sham-operated or BDL WT and Cyp270KO mice. Data represent mean ± SEM (n = 8–10). Red circles represent females and black triangles represent males. P values are from unpaired two-tailed Student’s t-test after log transformation of sham versus BDL of each group. *P < 0.05, **P < 0.01, and ****P < 0.0001.
directly assessed in the present work, should make the overall outcome more complex. An interesting observation was that the levels of serum plant sterol, which are generally considered to be markers of cholesterol absorption, were actually lower in the KO animals.

We had predicted that the change in BA pool size and composition following Cyp2c70 KO would result in an increased activation of FXR, which should be reflected in changes in mRNA levels of genes regulated by this transcription factor. However, our findings in two different experiments (comparing WT and KO animals under standard conditions or when sham-operated) showed a more complex picture, which may actually differ in the liver and in the intestine. Thus, although the downregulation of BA synthesis was not accompanied by an increase in hepatic Shp expression, mRNAs of Cyp7a1 and Cyp8b1, together with some other potentially FXR-regulated genes such as Cyp3a11 and Abcb1a, were changed in the liver. The expression of Ntcp was actually increased in the liver of KO animals. In the ileum, the pattern appeared more clear, with Shp, Fgf15, and Ibaip being increased in the KO animals, and Asbt and Ost α showing similar trends. During the revision of this article, two reports describing inactivation of the murine Cyp2c70 gene were published (33, 34). While the focus of their work was mostly different from ours, they describe some findings which confirm our results. Thus, comparing WT and KO animals, the differences that can be ascribed to hepatic FXR activation are not as clear as expected. Thus, the combined evidence indicates that there are probably additional mechanisms by which the differences in the composition of individual BAs result in species differences between mice and humans regarding BA and cholesterol metabolism. The possible role of gender differences for some of the regulatory mechanisms at work proposed by Honda et al. (34) was not consistently observed in our experiment (as indicated in our figures throughout), but further work will be needed to explore this important question.

In the KO animals, the absence of MCAs seemed to be associated with signs of liver damage, seen by increased liver weights, elevated serum liver enzymes, and histological changes (supplemental Fig. S6), also in accordance with the findings of Honda et al. (34). These findings may be related to the marked increase in “human” BAs in the KO mice. Thus, the sum of the concentrations of CA, TCA, CDCA, and TCDCA in the livers of WT animals in our study was ~75 nmol/g (supplemental Table S2), a value similar to that reported for human liver (35). The corresponding level in the KO livers was >400 nmol/g, being 5-fold higher that in normal humans, and actually similar to that found in children with biliary atresia (17). This occurs despite that the total pool size of BAs is probably reduced in the Cyp2c70-KO mice, again implying the importance of high levels of toxic BAs, such as CDCA.

In humans, BA synthesis is suppressed in extrahepatic cholestasis, while in rats and mice, cholestasis following BDL is known to result in a paradoxical stimulation of BA synthesis (14, 15). The latter response has been attributed to reduced FXR-mediated production of ileal Fgf15 signaling to the liver (16). Intriguingly, in spite of the fact that ileal Fgf15 mRNA was absent in all animals following BDL, the paradoxical stimulation of BA synthesis seen in WT animals was abolished or even reversed in KO mice. Although the response to BDL in the KO mice is more similar to that in humans, this observation, as well as the lack of induction of hepatic Fgf15 mRNA despite the very high hepatic levels of agonistic BAs reached following BDL, indicates that additional species differences may be involved in this situation. Because lipoprotein-X is considered to have a protective function in cholestatic liver (30), the stimulation of BA synthesis following BDL in mice may be seen as part of a “defense mechanism” in this species, in addition to the increased renal excretion of MCAs. Further studies using the Cyp2c70-KO mouse model should provide a useful approach to further explore mechanisms of liver damage in cholestatic conditions and, hopefully, novel therapeutic modalities in human disease.

Other important differences between mice and humans concern the diurnal rhythms of BA and cholesterol synthesis, where the production of cholesterol appears to be more synchronized with that of BAs in rodents (36). These may be relevant when trying to understand species differences, including those related to dietary challenges. The marked changes in cholesterol metabolism following modulation of BA pool composition in mice also highlight the possible importance of interindividual variation of BA synthesis in humans (37) in relation to the risk of developing disease. The more human-like metabolic phenotype of the Cyp2c70-KO mice should be useful for studies exploring the pathogenesis and treatment of human disease, including dyslipidemia, fatty liver disease, type 2 diabetes, and cholestasis (2, 3).

In conclusion, we have established that several of the major differences known to exist between humans and mice regarding cholesterol, BA, and lipoprotein metabolism can be explained to a large extent by the presence of species-specific hydrophilic MCAs in the latter. While differences between individual BAs related to activation of the BA-receptor FXR, particularly in the intestine, may partly explain this, it is clear that other mechanisms are also involved. We suggest that the Cyp2c70-KO mouse may be a useful tool in further studies of the pathogenic mechanisms of human disease and particularly for the exploration of compounds aimed at human therapy.

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