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Increased Bile Acids in Enterohepatic Circulation by Short-term Calorie Restriction in Male Mice

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Abstract

Previous studies showed glucose and insulin signaling can regulate bile acid (BA) metabolism during fasting or feeding. However, limited knowledge is available on the effect of calorie restriction (CR), a well-known anti-aging intervention, on BA homeostasis. To address this, the present study utilized a “dose-response” model of CR, where male C57BL/6 mice were fed 0, 15, 30, or 40% CR diets for one month, followed by BA profiling in various compartments of the enterohepatic circulation by UPLC-MS/MS technique. This study showed that 40% CR increased the BA pool size (162%) as well as total BAs in serum, gallbladder, and small intestinal contents. In addition, CR “dose-dependently” increased the concentrations of tauro-cholic acid (TCA) and many secondary BAs (produced by intestinal bacteria) in serum, such as tauro-deoxycholic acid (TDCA), DCA, lithocholic acid, ω-muricholic acid (ωMCA), and hyodeoxycholic acid. Notably, 40% CR increased TDCA over 1000% (serum, liver, and gallbladder). Interestingly, 40% CR increased the proportion of 12α-hydroxylated BAs (CA and DCA), which correlated with improved glucose tolerance and lipid parameters. The CR-induced increase in BAs correlated with increased expression of BA-synthetic (Cyp7a1) and conjugating enzymes (BAL), and the ileal BA-binding protein (Ibapb). These results suggest that CR increases BAs in male mice possibly through orchestrated increases in BA synthesis and conjugation in liver as well as intracellular transport in ileum.

Keywords

Calorie restriction; Bile acids; Enterohepatic circulation; 12α-hydroxylated BAs; BA profiling; UPLC-MS/MS

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Introduction

Calorie restriction (CR), which refers to decrease in calorie intake without causing malnutrition, is a well-known anti-aging intervention to decrease the incidence of age-related diseases. CR elicits multiple downstream signaling pathways that are orchestrated to achieve beneficial effects. CR alters many endogenous molecules, such as glucose, insulin, triglycerides, growth hormone, insulin-like growth factor 1, leptin, and dehydroepiandrosterone sulfate (Ramsey et al., 2000; Pasiakos et al., 2011). However, limited knowledge is available about the effect of CR on another endogenous molecule, bile acids (BAs).

BAs are end-products of cholesterol metabolism in liver, and well-known for their classic roles in facilitating cholesterol elimination in liver and dietary fat absorption in intestine as amphipathic molecules. There are two major BA receptors, namely the nuclear receptor farnesoid X receptor (FXR/Nr1h4) (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999) and the membrane-bound G-protein coupled receptor TGR5 (GPBAR1) (Kawamata et al., 2003). Upon BA activation, FXR translocates to the nucleus, and heterodimerizes with RXR, which binds to DNA and initiates target gene expression (Forman et al., 1995). FXR is crucial for regulating BA, glucose, and lipid metabolism (Caudel et al., 2005). TGR5 activation by BAs increases cAMP-deiodinase 2 and T3 signaling, which subsequently promotes energy expenditure in brown adipose tissue and muscle (Watanabe et al., 2006). Interacting with these receptors, BAs are versatile signaling molecules that regulate glucose, lipid, and energy metabolism (Lefebvre et al., 2009). Moreover, glucose and insulin are important regulators for BA homeostasis (Park and Pak, 2011; Li et al., 2012).

BA homeostasis is well maintained under normal conditions. Primary BAs are synthesized and conjugated in liver, from which secondary BAs are produced by bacterial enzymes mainly in the lumen of large intestine (LI) (Ridlon et al., 2006). BA *de-novo* synthesis involves many enzymes that are preferentially expressed in liver (Russell, 2003). The classic pathway starts with Cyp7a1, the rate-limiting enzyme (Chiang, 1998) that catalyzes cholesterol 7 α -hydroxylation. The alternative pathway starts with 27-hydroxylation of cholesterol by mitochondrial Cyp27a1, and involves 7 α -hydroxylases Cyp7b1 and Cyp39a1. Cyp8b1, a 12 α -hydroxylase, is required for cholic acid formation (Chiang, 2003). The expression of BA-synthetic enzymes (Cyp7a1 and Cyp8b1) is transcriptionally suppressed by BA-mediated feedback inhibition via two mechanisms. 1) FXR activation by BAs in liver induces small heterodimer partner (SHP), which suppresses the transactivation of BA-synthetic genes by liver receptor homolog-1 (LRH-1) (Goodwin et al., 2000). 2) FXR activation by BAs in intestine induces fibroblast growth factor 15 (Fgf15), an intestinal hormone that travels to the liver, where it interacts with its receptor Fgfr4 and inhibits BA synthesis (Inagaki et al., 2005).

BA transporters play crucial roles in facilitating the “enterohepatic circulation” (EHC) of BAs. In liver, conjugated BAs are taken up by the Na⁺/taurocholate cotransporting polypeptide (Ntcp) (Anwer, 2004), whereas unconjugated BAs by the organic anion transporting polypeptide 1b2 (Oatp1b2) (Csanaky et al., 2011), and BAs are effluxed into bile canaliculi by the bile salt export pump (Bsep) (Wang et al., 2001). At the end of small

intestine (SI), BAs are reabsorbed by the apical sodium-dependent bile acid transporter (Asbt) (Dawson et al., 2003), transferred from the apical to basolateral membrane by the ileal BA binding protein (Ibapb) (Grober et al., 1999), and pumped into portal blood by the organic solute transporter heterodimer Ost α and Ost β (Ballatori et al., 2008; Rao et al., 2008).

Previous reports about the effect of glucose and insulin signaling on BA metabolism are mainly on the regulation of the rate-limiting enzyme Cyp7a1 (Park and Pak, 2011; Li et al., 2012). Insulin suppresses the transcription of Cyp7a1, and therefore Cyp7a1 expression increases during fasting and decreases when fasted mice are re-fed (De Fabiani et al., 2003; Ponugoti et al., 2007). During CR, which resembles intermittent fasting and re-feeding cycles, the nutrient intake is restricted, and thus glucose and insulin levels are lower (Pasiakos et al., 2011). Therefore, it is hypothesized that CR can increase Cyp7a1 expression and thus likely BA concentrations. Increased bile flow was previously reported in CR rats (Tuchweber et al., 1987). However, how CR affects the concentrations of individual BAs as well as the BA composition is not known. Therefore, the present study aimed to systematically investigate the effect of CR on BA homeostasis using a “dose-response” model of CR (0, 15, 30, or 40%). BA profiling was performed in various compartments of the EHC following an improved UPLC-MS/MS method that was recently established in our laboratory. Furthermore, the expression of BA-related genes was determined to provide possible regulatory mechanisms for BA alterations by CR.

Materials and methods

Chemicals and Reagents

The sources of individual BA standards and internal standards were described previously (Zhang and Klaassen, 2010). All other chemicals and reagents, unless indicated, were purchased from Sigma-Aldrich (St. Louis, MO).

Animal Experiments and Study Design

Only male mice were used for this study. The majority of previous reports about CR have used male mice. Limited information is available on CR female mice, providing little evidence for design of percentages of CR that produce beneficial effects. Female mice have lower body weight compared to their age-matched male counterparts, and thus have varied ability to sustain CR. This leads to the technical difficulty of using female mice in a short-term CR study, which involves a rapid decrease in body weight within a relatively short period of time during preparation feeding. Furthermore, it is technically challenging to include mice of both genders in this “dose-response” study for “side-by-side” comparison, because of the extensive workload to perform CR on such large number of animals.

Mice were housed in an AAALAC-accredited facility at the University of Kansas Medical Center, with a 14-h light/10-h dark-cycle, temperature-, and humidity-controlled environment and *ad libitum* access to water. These studies were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

Previous studies on CR usually use one percentage of CR (15–60% CR) and various feeding regimes and diet formulae (Faulks et al., 2006; Yoshida et al., 2006; Rocha et al., 2007; Boily et al., 2008; Kim et al., 2008; Niemann et al., 2008; Yamamoto et al., 2009). Therefore, in order to determine which percentage of CR altered BA homeostasis, a “dose-response” CR model was utilized. The scheme of the current study design is illustrated in Fig. 1. Male C57BL/6 mice (3-month old) were purchased from Charles River Laboratories (Wilmington, MA), fed with purified AIN-93M diet (TD94048, Harlan Teklad, Madison, WI) for one week, and then randomly divided into four experimental groups, namely *ad libitum* (AL), 15% CR, 30% CR, and 40% CR (n=5). All mice were housed individually, and the AL control mice were given *ad libitum* access to TD94048 diet throughout this study. The diet consumption of the AL group was recorded daily, and subsequently 85%, 70%, and 60% of this amount were given to 15%, 30%, and 40% CR groups, respectively, for one month. Similar rationale of using short-term CR to recapitulate long-term effects (Cao et al., 2001; Estep et al., 2009) and designing enriched diets for CR animals (Lee et al., 2002; Barger et al., 2008; Nogueira et al., 2012) was published previously. Feed was provided between 17:00–18:00 daily. CR mice finished their daily feed within 2 hours after feeding. In order to prevent sudden weight loss, a gradual decrease in feed was provided to the mice one week before the start of CR feeding (Fig. 1B). In order to prevent malnutrition, which is a common side effect of CR studies (Cerqueira and Kowaltowski, 2010), custom enriched diets TD110466, TD110467, and TD110468 (Harlan Teklad, Madison, WI) were designed to feed 15%, 30%, and 40% CR groups respectively (Supplemental Table 1). Body weights were recorded weekly.

Glucose Tolerance Test

Glucose tolerance test (GTT) was conducted on Day 22 (after 21 days of CR feeding). Before the GTT, the AL mice were fasted for 6 h starting from 8:00, whereas the CR mice had finished their daily feed and literally started fasting around 19:00 the previous evening. A single dose of D-glucose (20% solution in water; 10 mL/kg) was injected i.p. Blood was taken from tails of mice at 0, 30, 60, 90, and 120 min thereafter, and glucose concentrations were determined using a ReliOn Ultima glucose monitor (Arkray USA, Inc., Minneapolis, MN).

Sample Collection

Fasting plasma was collected on Day 25. The AL mice were fasted for 4 h starting from 8:00, whereas the CR mice had finished their daily feed and literally started fasting around 19:00 the previous evening. All mice were anesthetized with pentobarbital (50 mg/kg), and then approximately 100 μ L of plasma was collected by retro-orbital bleeding using heparin-coated capillary tubes. Mice were returned to cages after blood was drawn and remained AL or CR feeding for the rest of the study.

At the end of the one-month CR feeding (Day 30), all mice were anesthetized with pentobarbital (50 mg/kg), and serum was collected by retro-orbital bleeding. After cervical dislocation, liver, gallbladder (GB), and ileum were removed, snap-frozen in liquid nitrogen, and stored at -80°C . SI and LI were flushed with 10 mL saline and the intestinal contents were collected separately on ice, and stored at -80°C . Tissue collections were between 9:00

and 12:00 in the morning to decrease the variations due to circadian rhythm of BAs (Zhang et al., 2011b).

Plasma Lipid Parameters

The triglycerides (TG; Pointe Scientific Inc, Canton, MI), total cholesterol (Wako Diagnostics, Richmond, VA), and non-esterified fatty acids (FAs; Wako Diagnostics, Richmond, VA) in fasting plasma were quantified using colorimetric assays according to the manufacturer's instructions.

Liver Lipid Parameters

Liver lipids were extracted as described (McGrath and Elliott, 1990), and the TG, total cholesterol, and FAs in liver were quantified with the same protocol as plasma lipids.

BA Extraction and Quantification

BAs were extracted from serum and livers (Zhang and Klaassen, 2010), GBs (tissue with bile inside) (Zhang et al., 2011a), as well as SI and LI contents (Zhang et al., 2012b). Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used to quantify 20 individual BAs, including primary and secondary BAs as well as their taurine conjugates (Zhang and Klaassen, 2010). Primary BAs include cholic acid (CA), chenodeoxycholic acid (CDCA), α -muricholic acid (α MCA), and β MCA. Secondary BAs converted from corresponding primary BAs include 1) deoxycholic acid (DCA) from CA, 2) lithocholic acid (LCA) and ursodeoxycholic acid (UDCA) and CDCA, and 3) murideoxycholic acid (MDCA), ω MCA, and hyodeoxycholic acid (HDCA) from α MCA and β MCA (Martin *et al.*, 2007; Hofmann, 2009; Zhang and Klaassen, 2010). The concentrations of individual BAs were summed to derive the concentrations of conjugated, unconjugated, and total BAs, as previously published (Fu et al., 2012).

Total RNA Isolation

Total RNA was isolated from liver and ileum tissues using RNA Bee reagent (Tel-Test Inc., Friendswood, TX) per the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm.

Multiplex Suspension Assay

The mRNAs of genes encoding BA synthetic enzymes (Cyp7a1, Cyp8b1, Cyp27a1, and Cyp7b1) and BA transporters in liver (Ntcp, Oatp1b2, and Bsep) were quantified by Panomics 2.0 QuantiGene Plex technology (Panomics/Affymetrix Inc., Fremont, CA). Individual gene information can be found on Panomics Web site (<http://www.panomics.com>) with Panel number 21150. Fluorescence was analyzed using a Bio-Plex 200 system array reader with Luminex 100 X-MAP technology, and data were acquired using Bio-Plex data manager software 5.0 (Bio-Rad, Hercules, CA). The mRNAs of target genes were normalized to Gapdh.

Reverse Transcription and Real-time PCR Analysis

Total RNA was transcribed to single-stranded cDNA using High Capacity cDNA Reverse Transcription Kit 1001073 (Applied Biosystems, Foster City, CA), and then the cDNA products were amplified by PCR, using Power SYBR Green PCR Master Mix in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The mRNAs of genes encoding BA conjugating enzymes (BAL and BAT), BA transporters in ileum (Asbt, Osta, and Ost β), ileal BA binding protein (Ibap), and proteins involved in Cyp7a1 regulation (FXR, SHP, Fgf15, and Fgfr4) were quantified, and normalized to β -actin. The sequences of real-time PCR primers (Integrated DNA Technologies, Coralville, IA) are listed in Supplemental Table 2.

Statistical Analysis

Data are presented as mean \pm SEM. To compare among AL and three CR groups, data were analyzed by one-way ANOVA, followed by Duncan's post-hoc test, differences being considered significant at $p < 0.05$. Due to space limitation on the figures, the authors decided not to show every significant difference between the various groups. Instead, the authors used asterisks (*) to indicate the differences between CR and AL groups. To compare the BA compositions between AL and 40% CR groups, data were analyzed by Student's *t*-test, differences being considered significant at $p < 0.05$.

Results

Body Weight, Glucose, and Lipid Parameters in CR Mice

In order to validate the graded CR model, body weight was recorded weekly. As expected, the body weights of CR mice were lower than AL mice, and they reached a "steady state" by the end of the study (Fig. 2A). Interestingly, the final body weights of 15%, 30%, and 40% CR mice were 15%, 28%, and 38% lower than AL mice, respectively. The health condition of the CR mice was monitored throughout the study, and no malnutrition was apparent. As shown in Fig. 2B, CR mice had better glucose tolerance, evidenced by lower AUCs in the glucose tolerance test (GTT). In addition, 30 and 40% CR mice had lower blood glucose than AL mice before glucose challenge as well, but they were not hypoglycemic. Lipid parameters in serum and liver of AL and CR mice were examined. TG levels in plasma were decreased by 40% CR, and interestingly, the levels in liver were decreased proportionally by CR. Moreover, cholesterol and FA concentrations were decreased by CR in plasma and liver (Fig. 2C).

Effect of Graded CR on Total BAs in Various Compartments of the Enterohepatic Circulation

Serum, liver, GB, SI, and LI are the major compartments of the EHC of BAs. BA pool size refers to the total amounts of BAs in the whole animal, and it is generally accepted to estimate BA pool size by adding the amounts of BAs in several BA-enriched compartments, including liver, GB, and intestine. Forty percent CR increased BA pool size (162%), due to the increased amount of total BAs in GB (133%) and SI (174%) (Fig. 3, top-left panel). However, CR did not alter the amount of total BAs in liver and LI.

In serum (Fig. 3, top-right panel), 40% CR increased total BA concentrations (231%). Specifically, 40% CR increased the concentrations of both conjugated (335%) and unconjugated BAs (178%). In addition, 40% CR increased similarly the concentrations of primary (224%) and secondary BAs (233%). In liver (Fig. 3), CR did not alter the amount of conjugated, unconjugated, primary, or secondary BAs. In GBs (Fig. 3), 40% CR increased the amount of conjugated (132%), primary (162%), and secondary BAs (77%), however, CR did not alter the amount of unconjugated BAs. In the SI contents (Fig. 3), 40% CR increased the amount of conjugated (179%), primary (219%), and secondary BAs (94%), however, CR did not alter the amount of unconjugated BAs. In the LI contents (Fig. 3), CR did not alter the amount of conjugated, unconjugated, primary, or secondary BAs.

Concentrations of Individual BAs in Serum of CR Mice

Four BA pathways are defined to aid in reading Fig. 4, each of which involves the primary BA and its secondary BA, as well as their taurine conjugates. For example, the CA pathway involves TCA, TDCA, CA, and DCA. In serum, 40% CR markedly increased the concentrations of TCA (872%), TDCA (over 1100%), and DCA (over 1000%) (Fig. 4). CA was increased by 30% CR (262%), but not by 40% CR. For the CDCA pathway, CR did not alter the concentrations of TCDCA, TLCA, TUDCA, or CDCA in serum, but LCA was increased by both 30% CR (262%) and 40% CR (408%), whereas, UDCA was increased by 30% CR (205%), but not 40% CR. For the α MCA pathway, 30% CR increased concentrations of T α MCA (558%) and α MCA (489%), whereas 40% CR did not alter either. The secondary BAs MDCA and TMDCA were not altered by CR. For the β MCA pathway, CR did not alter the concentrations of BA taurine conjugates (T β MCA, T ω MCA, or THDCA) or β MCA. The concentration of ω MCA was increased by both 30% CR (95.6%) and 40% CR (135%), as was HDCA (128% and 169%, respectively).

Amounts of Individual BAs in Livers and Gallbladders of CR Mice

In livers (Fig. 4), for the CA pathway, 30 and 40% CR tended to double the amount of TCA in livers (not statistically different). The amount of TDCA was increased by both 30% CR (222%) and 40% CR (484%). CR did not alter the amount of CA. For the CDCA pathway, 40% CR decreased the amount of TCDCA (45.9%), TUDCA (57.2%), but increased CDCA (146%). CR did not alter the amount of TLCA or UDCA in liver. For the α MCA pathway, CR did not alter the amount of BAs (T α MCA, TMDCA, α MCA, or MDCA) in liver. For the β MCA pathway, 40% CR decreased the amount of β MCA (86.6%) and HDCA (70.0%), but CR did not alter the amount of T β MCA, T ω MCA, THDCA, or ω MCA.

In gallbladders (GBs) (Fig. 4), for the CA pathway, 40% CR increased the amount of TCA (355%), TDCA (over 1000%), and DCA (over 1000%), and 15% CR increased the amount of CA (190%). CR did not alter the amount of BAs in the CDCA, α MCA, or β MCA pathways, except a modest increase in α MCA by 15% CR (180%).

Amounts of Individual BAs in Intestinal Contents of CR Mice

In the contents of the small intestines (SI) (Fig. 4), for the CA pathway, 40% CR increased the amount of TCA (506%) and TDCA (over 1800%), 30% CR increased the amount of CA (746%), whereas CR did not alter the amount of DCA. CR did not alter the amount of BAs

in the CDCA, α MCA, and β MCA pathways, except modest increases in α MCA (137%) and β MCA (210%) by 30% CR.

In the contents of the large intestines (LI) (Fig. 4), for the CA pathway, 40% CR increased the amount of TCA (694%), TDCA (639%), and DCA (253%), but CR did not alter the amount of CA. CR did not alter the amount of BAs in the CDCA pathway, except the increases in TCDCA (694%) and LCA (145%) by 15% CR. CR did not alter the amount of BAs in the α MCA or β MCA pathways, except decreases in MDCA (64.2%) and β MCA (73.6%) by 30% CR.

Altered Enterohepatic BA Composition by 40% CR

BA compositions in AL and 40% CR groups were analyzed by calculating the proportion of each BA and its taurine conjugates in total BA concentrations. In general, 40% CR increased the proportions of BAs in the CA pathway, but decreased the proportions of BAs in the CDCA, α MCA, and β MCA pathways (Fig. 5). Specifically, 40% CR doubled CA proportions in serum (8.4% to 17.2%), liver (20.5% to 41.1%), GB (26.4% to 53.5%), and SI (26.8% to 58.5%), and tended to increase CA proportion in LI (1.6% to 5.9%; not statistically different). Similarly, 40% CR increased DCA proportions in serum (9.3% to 29.3%), liver (2.7% to 12.1%), GB (2.5% to 7.8%), SI (1.1% to 7.9%), and LI (3.5% to 13.7%). In contrast, 40% CR decreased β MCA proportions in serum (10.9% to 2.9%), liver (26.5% to 13.2%), GB (20.7% to 11.0%), and SI (22.4% to 9.2%). In addition, 40% CR decreased ω MCA proportions in serum (44.1% to 31.9%), liver (22.3% to 14.9%), GB (20.5% to 11.5%), SI (22.6% to 10.0%), and LI (74.8% to 61.4%).

Expression of BA-Synthetic and Conjugating Enzymes in Liver of CR Mice

The expression of BA synthetic and conjugating enzymes in liver were quantified in the present study (Fig. 6). In the classic pathway, 40% CR increased the mRNA of the rate-limiting BA-synthetic enzyme Cyp7a1 (228%), but decreased the mRNA of Cyp8b1 (53.8%). In the alternative pathway, CR did not alter Cyp27a1 mRNA level. However, Cyp7b1 was decreased by 40% CR (76.2%), and Cyp39a1 was increased by 40% CR (440%). For BA conjugating enzymes, 40% CR increased BAL mRNA (123%), but CR had little or no effect on BAT mRNA level.

Expression of Regulatory Factors for Cyp7a1 Transcription in Liver and Ileum of CR Mice

The mRNAs of several key regulatory factors for Cyp7a1 transcription in the EHC of BAs were quantified (Fig. 7). Forty percent CR increased the mRNAs of FXR in liver (87.5%) and SHP in ileum (120%). In addition, 40% CR tended to increase the mRNA of Fgf15 in ileum, but was not statistically increased. CR did not alter the mRNA levels of other regulatory factors for Cyp7a1 transcription, such as SHP and Fgfr4 in liver, and FXR in ileum.

Expression of BA Transporters in the Enterohepatic Circulation in CR Mice

The expression of BA transporters in liver and ileum was quantified in the present study (Fig. 8). Forty percent CR increased the mRNAs of Ntcp (35%) in liver and Ibabp (206%) in

ileum. However, CR did not alter the mRNA levels of other BA transporters, including *Oatp1b2* and *Bsep* in liver, and *Asbt*, *Osta*, and *Ostβ* in ileum.

Discussion

The present study is the first to comprehensively characterize BA profiles in various compartments of the EHC using a “dose-response” CR model. In general, 15% CR is not sufficient to change most BAs, and 30% CR produces significant changes for some BAs. Obviously, 40% CR produces the most abundant and predominant changes.

Increased BA pool size by CR and increased expression of BA-related genes

The BA pool size usually maintains relatively constant. Interestingly, this study identifies an increase (162%) in BA pool size by 40% CR (Fig. 3). The increase in BA pool size by CR is mainly due to increase of BAs in the SI. It is noteworthy that the ratio of BAs in liver, GB, and intestine is approximately 1:2:20 under normal conditions. CR results in various changes in total BAs in these compartments. Specifically, 40% CR increases total BAs in GB and SI, but does not alter those in liver or LI. In addition, 30 and 40% CR increase total BA concentrations in serum, which might be associated with possible systemic effects (such as TGR5 activation in muscle).

CR-induced BA increase correlates with changes in the expression of BA-related genes. *Cyp7a1* plays the leading role in determining BA synthesis and BA pool size (Li et al., 2011). In this study, 40% CR increases *Cyp7a1* expression (Fig. 6), which correlates with the increase in BA pool size (Fig. 3). This finding indicates that the *Cyp7a1* increase probably contributes to the CR-induced increase in BA pool size. In addition, the increase in *BAL* expression by CR (Fig. 6), correlates with the increases in the proportions of taurine-conjugated BAs in serum, liver, and SI contents (Fig. 5). Furthermore, 40% CR increases the expression of *Ibabp* (Fig. 8), which is part of the regulatory machinery for BA reabsorption in the ileum. In summary, increased expression of *Cyp7a1*, *BAL*, and *Ibabp* by CR appears to be responsible for the increase in BA pool size during CR. One should be cautious when drawing conclusions from these mRNA data. Protein activity assays are needed to confirm whether CR increases the BA synthesis, conjugation, or reabsorption. For instance, an everted gut sac method may provide valuable information on possible changes of BA reabsorption in the ileum by CR.

Increased individual BAs by CR

In the “dose-response” model, CR increases several individual BAs in a “calorie-dependent” manner (Fig. 4). It appears that CA and its secondary BA (DCA) have the highest inverse correlation to calorie intake. In general, secondary BAs (such as DCA, LCA, ωMCA, and HDCA) increase more than primary BAs after CR. This suggests that the gut microbiome may play a role in determining the BA composition during CR, especially in the intestinal lumen. Currently, there is a lack of information on whether CR alters gut microflora, and which individual intestinal bacterial genus is responsible for the production of each secondary BAs. In future studies, it would be informative to characterize the changes of gut microflora by CR and correlate it to the changes in the BA profile.

BAs, as natural detergents, are considered cytotoxic at high concentrations (Hofmann, 1999). The CR-induced increase in serum total BAs (Fig. 3) is hundreds of magnitudes lower than that observed in the bile duct ligation-induced cholestatic mice (Zhang et al., 2012a). Low serum alanine aminotransferase activity (below 50 IU/L) in all groups of mice in the present study (data not shown) confirms that the CR-induced BA increases have not reached the concentration to cause hepatotoxicity.

Increased 12 α -hydroxylated BAs by CR correlated with improved glucose and lipid parameters

CR leads to interesting changes in BA composition in various compartments of the EHC (Fig. 5), namely increasing the proportions of BAs in the CA pathway (namely CA and DCA), and decreasing the proportions of BAs in MCA pathway (namely α -, β -, and ω MCAs). This is exactly opposite to the BA composition changes observed during aging, which is increased proportion of β MCA and decreased proportion of DCA as previously published by our laboratory (Fu et al., 2012). One major difference of BAs in the CA pathway compared to those in MCA pathway is that both CA and DCA have 12 α -hydroxyl groups, whereas the MCAs do not. Our finding on the correlation of increased proportions of 12 α -hydroxylated BAs and improved insulin sensitivity and decreased lipid parameters (Fig. 2B and 2C) in CR mice is consistent with the previous report that impaired generation of 12 α -hydroxylated BAs was associated with dyslipidemia and insulin resistance (Haeusler et al., 2012). Therefore, our study suggests the possible role of BA composition in regulating glucose and lipid metabolism.

CR-induced alterations in BA composition may lead to changes in the activation of the BA receptor TGR5. Individual BAs have various potencies for TGR5 activation: LCA > DCA > CDCA > CA (Chen et al., 2011). CR markedly increased DCA concentration and the proportion of DCA, which may lead to increased TGR5 activation. In future studies, it would be informative to quantify TGR5 targets (cAMP level and deiodinase 2) to determine changes of TGR5 activation during CR, as well as its relationship to energy expenditure in adipose and muscle tissue.

Feminized mRNA profiles of gender-divergently expressed BA-synthetic enzymes by CR

Some BA synthetic enzymes have gender-divergent expression in liver. Both Cyp8b1 and Cyp7b1 are expressed higher in male than female mice (Fu et al., 2012), which is opposite to Cyp39a1 (female > male) (Li-Hawkins et al., 2000a). Interestingly, the expression of Cyp8b1 and Cyp7b1 were decreased by CR, whereas Cyp39a1 was increased (Fig. 6). This appears to be attributable to feminization of the liver by CR. Previous reports have shown that CR can feminize genome-wide gene expression in male mice (Estep et al., 2009) and long-lived mice have lost the sex dimorphism of gene expression (Amador-Noguez et al., 2005). Therefore, the current finding of CR-induced feminization of some BA synthetic enzymes suggests that the connection between feminization and longevity is an interesting area of research.

FXR signaling in the regulation of BA synthetic enzymes during CR

Expression of BA-synthetic enzymes is feedback inhibited through two major mechanisms, namely the liver FXR-SHP pathway and the intestinal FXR-Fgf15 pathway. Recent data suggest that FXR-SHP plays a minor role in suppressing Cyp7a1, but is more important in Cyp8b1 suppression, whereas Fgf15 is important for both Cyp7a1 and 8b1 regulation (Kim et al., 2007; Kong et al., 2012). Expression of the FXR target gene SHP in liver is not altered by CR (Fig. 7), indicating that FXR activation by BAs in liver remains constant during CR. This correlates with the observation that CR does not alter the amounts of total BAs in liver (Fig. 3). In ileum, the expression of SHP is increased by CR, and another FXR target, Fgf15, has the tendency to increase although there is no statistical significance (Fig. 7). Moreover, the ileal bile acid-binding protein (Ibapb), a classic marker for FXR activation in ileum, also has increased expression during CR. Together, these findings indicate that FXR activation in ileum is likely increased by CR. This may be due to increased amounts of total BAs in the SI (Fig. 3). In summary, CR appears to increase FXR activation in intestine, and thus mediate feedback inhibition of Cyp8b1.

It is noteworthy that little or no feedback inhibition of Cyp7a1 is observed, indicating the possibility of an intrinsic induction of Cyp7a1 expression by CR, which may mask the FXR-mediated feedback inhibition of Cyp7a1, if any. The basal level of Cyp7a1 transcription is regulated by several factors, such as LXR, HNF4 α , PGC-1 α , and SREBP1c, which sense nutrient levels, such as glucose, oxysterols, cholesterol, as well as insulin. During fasting or high glucagon levels, the cAMP response element binding protein induces gluconeogenesis through PGC1 α (Herzig et al., 2001), meanwhile PGC-1 α binding to HNF4 increases, which leads to increased promoter-mediated Cyp7a1 transcription (De Fabiani et al., 2003; Shin et al., 2003). In contrast, during feeding or when there are high insulin levels, SREBP-1c, an insulin-induced lipogenic factor, competes with PGC-1 α for binding to HNF4, thus inhibiting the transcription of Cyp7a1 (Ponugoti et al., 2007). In the present study, 40% CR increases PGC1 α mRNA expression (157%) (data not shown), and whether this leads to promoter-mediated Cyp7a1 transcription increase by CR remains to be investigated.

Because of the difficulty in reasonable study design for percentage of CR in female mice as well as the extensive workload to perform the “dose-response” CR studies in both male and female mice for comparison, the current study has used only male mice. For future studies, it would be informative to perform the same studies in female mice to determine whether there is any gender-specificity in the regulation of BA homeostasis by CR in mice.

In conclusion, the present study demonstrates that short-term CR increases BAs in male mice, possibly through orchestrated increases in BA synthesis and conjugation in liver and intracellular transport in ileum (Fig. 9). This study also provides valuable information on the correlation of BA composition (for instance, increased ratio of 12 α - vs non-12 α -OH BAs) and improved glucose and lipid homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AL	<i>ad libitum</i>
Asbt	apical sodium-dependent bile acid transporter
BA	bile acid
BAL	bile acid-CoA ligase
BAT	bile acid-CoA: amino acid <i>N</i> -acyltransferase
Bsep	bile salt export pump
CA	cholic acid
CDCA	chenodeoxycholic acid
CR	calorie restriction
DCA	deoxycholic acid
EHC	enterohepatic circulation
Fgf	fibroblast growth factor
FXR	farnesoid X receptor
GB	gallbladder
HDCA	hyodeoxycholic acid
Ibabp	ileal bile acid binding protein
LCA	lithocholic acid
LI	large intestine
MCA	muricholic acid
MDCA	murideoxycholic acid
Ntcp	Na ⁺ /taurocholate cotransporting polypeptide
Oatp	organic anion transporting polypeptide
Ost	organic solute transporter
SHP	small heterodimer partner
SI	small intestine
T-BA	taurine-conjugated bile acid
U-BA	unconjugated bile acid
UDCA	ursodeoxycholic acid

UPLC-MS/MS ultra-performance liquid chromatography-tandem mass spectrometry

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Highlights

- Dose response effects of short-term CR on BA homeostasis in male mice.
- CR increased the BA pool size and many individual BAs.
- CR altered BA composition (increased proportion of 12 α -hydroxylated BAs).
- Increased mRNAs of BA enzymes in liver (Cyp7a1 and BAL) and ileal BA binding protein.

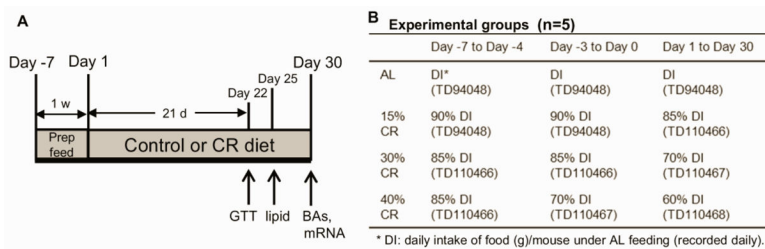


Fig. 1. Scheme of CR study design

(A) Mice (3-mon old) were randomly divided into four groups, namely AL, 15%, 30%, and 40% CR (n=5). Before the start of 1-mon CR feeding, mice were provided with gradual decrease amount of feed for 1 week. Glucose tolerance test (GTT) was performed on Day 22, and small amount of fasting plasma was drawn by retro-orbital bleeding for lipid quantification on Day 25. On Day 30, all mice were sacrificed and blood and tissues were collected for further quantification of BAAs and mRNA expression of BA-related genes. (B) CR mice were fed with decreased amount of feed compared to their AL controls. In addition, proportionally enriched diets were designed and provided to mice under a certain percentage of CR, respectively.

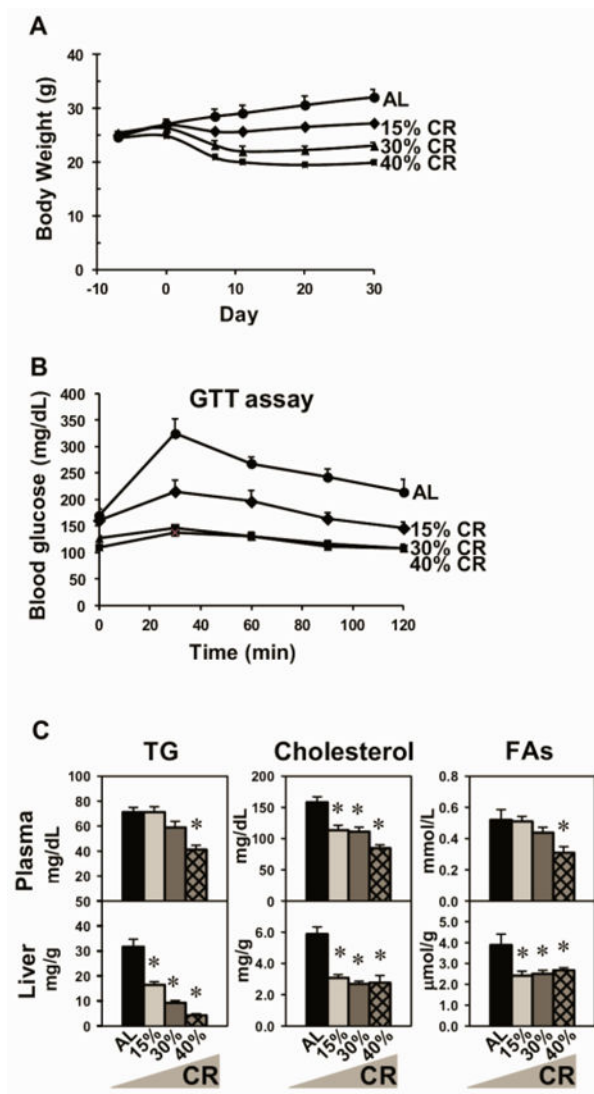


Fig. 2. Body weight, glucose, and lipid parameters of CR mice

(A) Body weight changes of male mice by graded CR (AL, 15%, 30%, or 40% CR) throughout the study. The body weights of CR mice started to be lower than AL mice after 1 week of CR feeding. (B) GTT. After 6 h of fasting, the basal blood glucose (time 0) was taken. Then a bolus of 20% D-glucose was given (i.p.) and serial glucose levels were taken at indicated time points thereafter. (C) Triglycerides (TG), cholesterol, and fatty acids (FAs) in plasma and liver. Data are presented as means \pm SEM of 5 mice. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test (not shown in A and B panels due to space limit)

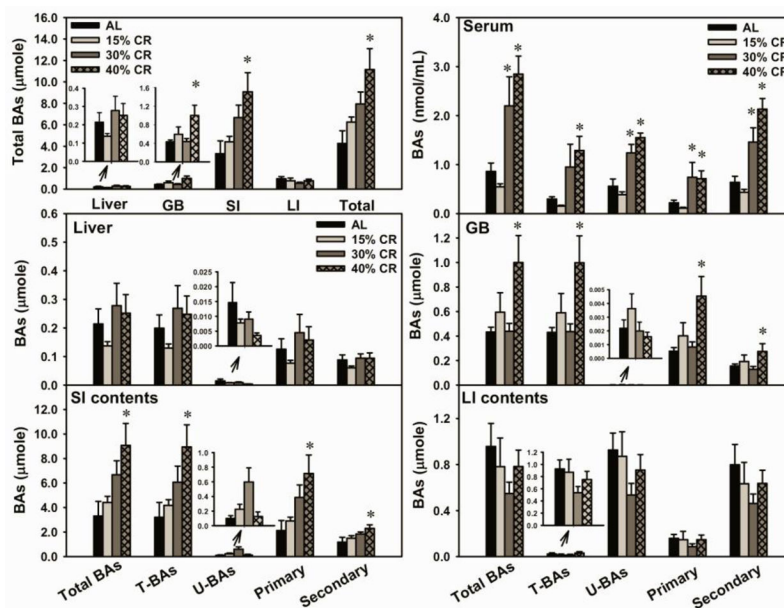


Fig. 3. Effect of graded CR on total BAs in various compartments of the enterohepatic circulation

(Top left) Total BA amounts in liver, gallbladder (GB), contents of small intestine (SI) and large intestine (LI), as well as total BA pool size in male mice. (Top right) Concentrations of total, taurine-conjugated (T-BAs), unconjugated (U-BAs), primary, and secondary BAs in serum. (Bottom) Amounts of total, conjugated, unconjugated, primary, and secondary BAs in liver, GB, SI, and LI. Data are presented as means \pm SEM of 5 mice. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test.

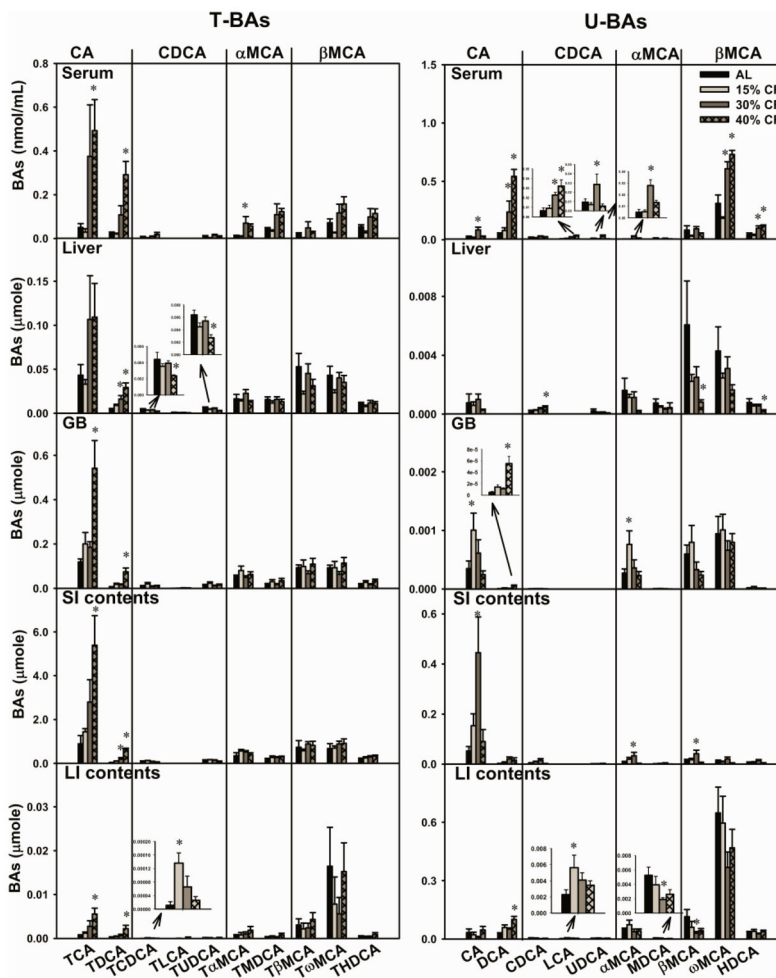


Fig. 4. Individual BAs in various compartments of the enterohepatic circulation in CR mice
 Four pathways are classified, and each (CA pathway for instance) involves primary BAs (CA), and secondary BAs (DCA), and their taurine conjugates (TCA and TDCA). CA, cholic acid; DCA, deoxycholic acid; CDCA: chenodeoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; MCA, muricholic acid; MDCA, murideoxycholic acid; HDCA, hyodeoxycholic acid. Data are presented as means \pm SEM of 5 mice. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test.

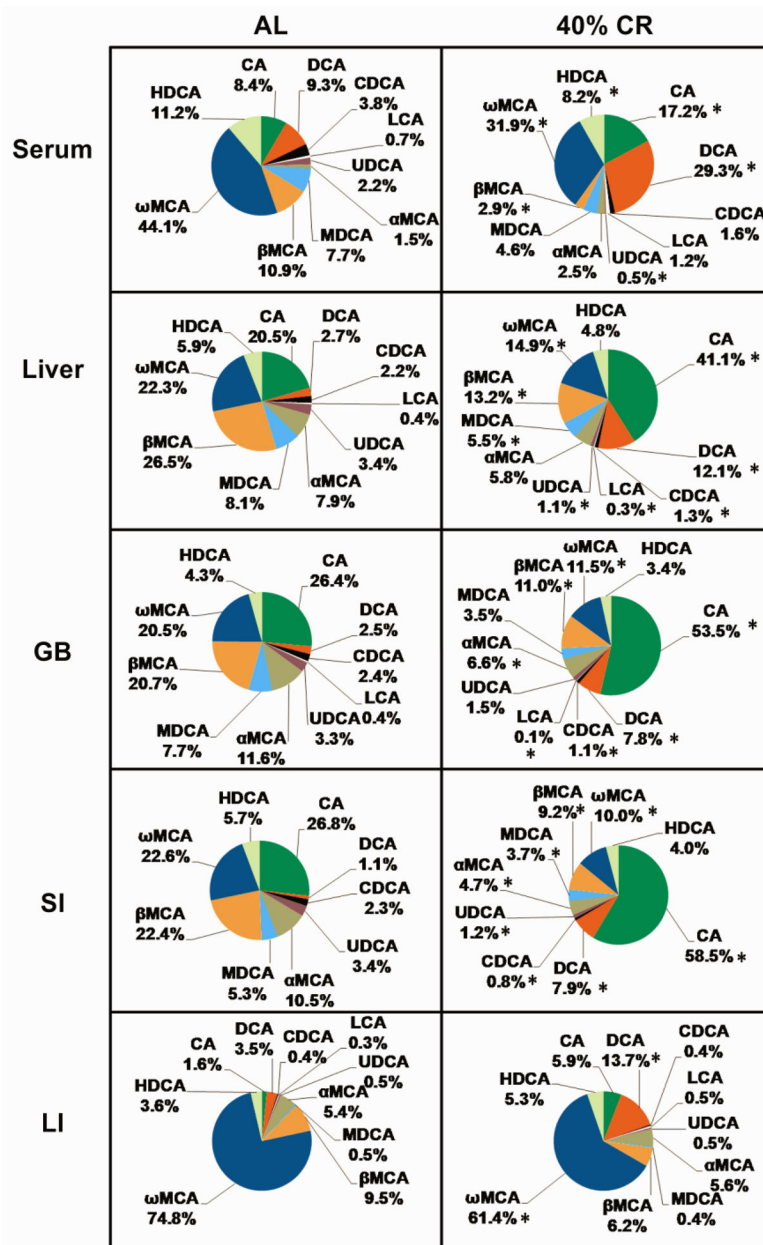


Fig. 5. Altered enterohepatic BA composition by 40% CR

For instance, the green portion of the pie chart represents the proportion of CA and TCA in total BA concentrations. Data are presented as means of 5 mice. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test.

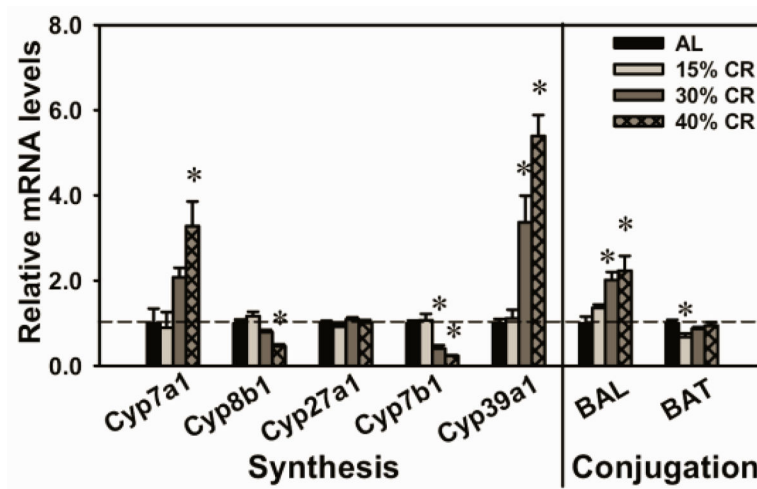


Fig. 6. Expression of BA-synthetic and conjugating enzymes in liver of CR mice

Cyp7a1 and Cyp8b1 are enzymes in the classic pathway for BA synthesis. Cyp7a1 is the rate-limiting enzyme for BA biosynthesis. Cyp27a1, Cyp7b1, and Cyp39a1 are enzymes in the alternative pathway for BA synthesis. BAL and BAT are BA conjugating enzymes. Data are presented as means \pm SEM of 5 mice. The mRNAs of each genes in AL mice were set as 1. * $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test.

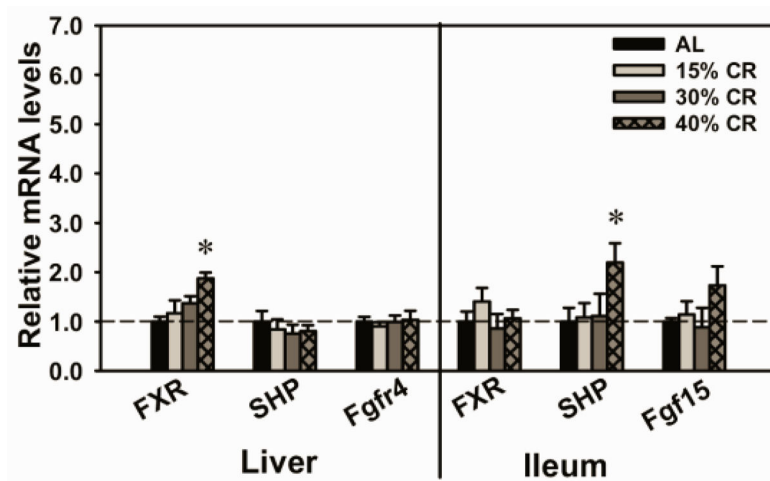


Fig. 7. Expression of regulatory factors for Cyp7a1 transcription in liver and ileum of CR mice
 The transcription of Cyp7a1 is regulated by BA-induced feedback inhibition. One proposed mechanism involves BA receptor FXR and its target gene nuclear receptor SHP in liver. The other mechanism involves “enterohepatic communication”, where BAs activate FXR in the intestine, inducing an intestinal hormone Fgf15, which travels through the circulation to the liver and through Fgfr4 down-regulates Cyp7a1 transcription. Data are presented as means \pm SEM of 5 mice. The mRNAs of each gene in AL mice were set as 1. * $p < 0.05$, by one-way ANOVA, followed by Duncan’s post-hoc test.

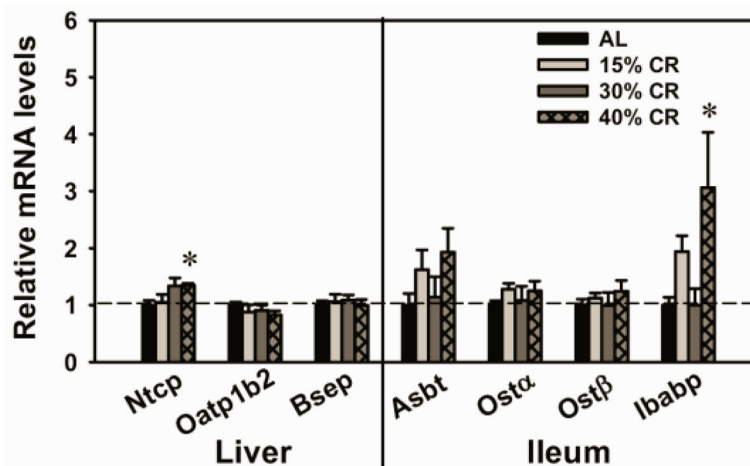


Fig. 8. Expression of BA transporters in the enterohepatic circulation in CR mice

In liver, BA transporters include uptake transporters Ntcp extracts conjugated BAs and Oatp1b2 extracts unconjugated BAs into hepatocytes, and the BA efflux transporter Bsep pumps out BAs into canalicular bile. In ileum, the BA uptake transporter Asbt mediates the reabsorption of BAs from the intestinal lumen into ileocytes, the BA binding protein Ibabp mediates the intracellular transfer of BAs from apical to basolateral side of ileocyte membrane, and the BA efflux transporter Osta/β pumps BAs out into portal blood. Data are presented as means ± SEM of 5 mice. The mRNAs of each gene in AL group were set as 1.

* $p < 0.05$, by one-way ANOVA, followed by Duncan's post-hoc test.

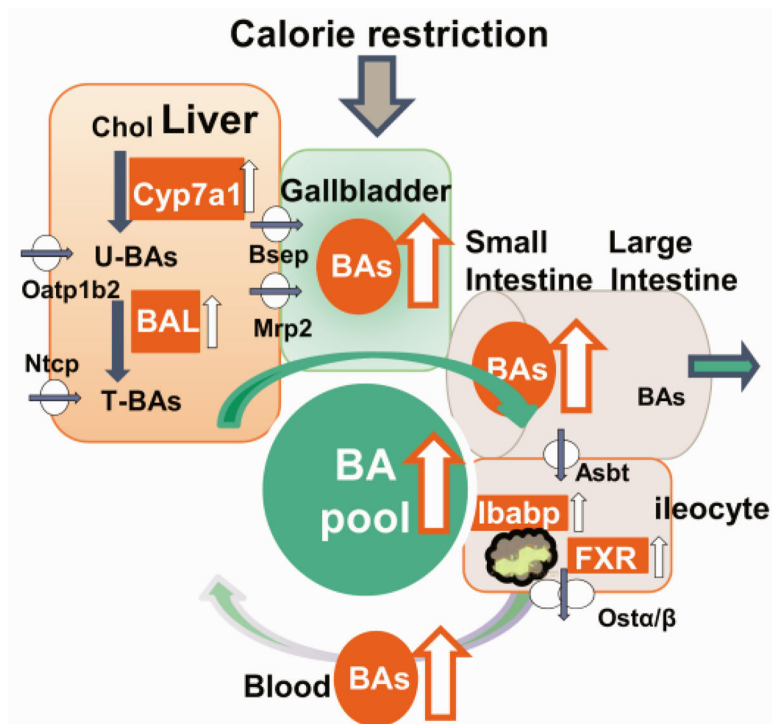


Fig. 9. Schematic view of the regulation of BA homeostasis by CR

CR increases BA pool size, which is likely due to increased BA synthesis (Cyp7a1) and conjugation (BAL) as well as intestinal BA reabsorption (Ibabp). Amounts of total BAs are increased by CR in gallbladder and small intestine, but remain relatively constant in liver.