Orally administered berberine modulates hepatic lipid metabolism by altering microbial bile acid metabolism and the intestinal FXR signaling pathway

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Abbreviations: BBR, Berberine; BSH, Bile salt hydrolase; CYP7A1, Cholesterol 7α-hydroxylase; FXR, Farnesoid X receptor; SHP, Small heterodimer partner; FGF15/19, Fibroblast growth factor 15/19; OGTT, Oral glucose tolerance test; O.C.T., optimal cutting temperature compound; PY medium, peptone-yeast extract medium; BA, Bile acid; CA, Cholic acid; CDCA, Chenodeoxycholic acid; DCA, Deoxycholic acid; UDCA, Ursodeoxycholic acid; αMCA, α-muricholic acid; βMCA, β-muricholic acid; ωMCA, ω-muricholic acid; HDCA, Hyodeoxycholic acid; LCA, Lithocholic acid; GCA,
Glycocholic acid; TCA, Taurocholic acid; TCDCA, Taurochenodeoxycholic acid; TβMCA, Tauro-β-muricholic acid; TDCA, Tauro-deoxycholic acid; TLCA, Tauro-lithocholic acid; TUDCA, Tauro-ursodeoxycholic acid; THDCA, Tauro-hyodeoxycholic acid; d4-CA, Cholic-2,2,4,4-d4 Acid; d4-TCA, Taurocholic-2,2,4,4-D4 acid; d4-GCA, Glycocholic-2,2,4,4-D4 acid.
Abstract

Previous studies suggest that the lipid-lowering effect of BBR involves actions on the LDL receptor and the AMPK signaling pathways. However, the implication of these mechanisms is unclear because of the low bioavailability of BBR. Because the main action site of BBR is the gut and intestinal FXR plays a pivotal role in the regulation of lipid metabolism, we hypothesized that the effects of BBR on intestinal FXR signaling pathway might account for its pharmacological effectiveness. Using wild type (WT) and intestine-specific FXR knockout (FXR<sup>int−/−</sup>) mice, we found that BBR prevented the development of high-fat-diet induced obesity and ameliorated triglyceride accumulation in livers of WT, but not FXR<sup>int−/−</sup> mice. BBR increased conjugated bile acids in serum and their excretion in feces. Furthermore, BBR inhibited bile salt hydrolase (BSH) activity in gut microbiota, significantly increased the levels of tauro-conjugated bile acids, especially taurocholic acid, in the intestine. Both BBR and TCA treatment activated the intestinal FXR pathway and reduced the expression of fatty acid translocase CD36 in the liver. These results indicate that the lipid lowering effect of BBR maybe primarily exert in the gut by modulating the turnover of bile acids and subsequently the ileal FXR signaling pathway. In summary, for the first time, we provide evidence to suggest a new mechanism of BBR action in the intestine that involves, sequentially, inhibiting BSH, elevating TCA, and activating FXR, which leads to the suppression of hepatic expression of CD36 to result in reduced uptake of long-chain fatty acids in the liver.
Introduction

Obesity with excess fat accumulation and extensively distorted metabolic regulation is a major risk factor for cardiovascular disease (CVD) (Hubert et al., 1983; Lavie et al., 2009), type 2 diabetes (Mokdad et al., 2003), a variety types of cancer (Carroll, 1998; Vaughan et al., 1995) and non-alcoholic fatty liver disease (NAFLD) (Wanless and Lentz, 1990). The prevalence of NAFLD is high in developed countries (up to 30%) (Angulo, 2007; Williams, 2006) and is increasing in developing countries (nearly 10%) (Fan and Farrell, 2009). Non-alcoholic steatohepatitis (NASH) is a severe form of NAFLD that includes steatosis, inflammation and fibrosis in the liver. NASH is becoming a major cause of hepatic cirrhosis and hepatocellular carcinoma (HCC). According to the “two-hit” model of NASH, two sequential injuries, lipid accumulation followed by a second insult, lead to the development of NASH. Thus, preventing the accumulation of lipids in the liver may be extremely important in the prevention of NASH (James and Day, 1998; Polyzos et al., 2009).

Berberine (BBR), which is extracted from the roots of *Rhizoma Coptidis*, has been traditionally used to treat diarrhea. Interestingly, BBR decreases serum lipids in humans, hamsters, mice and rats (Chang et al., 2010; Kong et al., 2004; Wang et al., 2010; Wang et al., 2014). BBR was also reported to be effective in the prevention and treatment of NAFLD (Chang et al., 2010; Guo et al., 2016; Yuan et al., 2015). In a previous study using hamsters, we found that BBR was poorly absorbed into the systemic circulation but significantly accumulated in the intestine (Gu et al., 2015). Furthermore, some studies have revealed that BBR treatment could change the composition of gut microbiota (Xie et al., 2011; Zhang et al., 2015; Zhang et al., 2012). Therefore we hypothesized that multiple mechanisms in the
intestine might be responsible for the lipid-lowering effects of BBR.

Farnesoid X receptor (FXR, NRIH4) is a nuclear receptor that is mainly expressed in the liver, intestine, kidney and adrenals (Lee et al., 2006a; Lee et al., 2006b). FXR is essential in maintaining bile acid homeostasis, and is important in the regulation of cholesterol metabolism (Sinal et al., 2000). Bile acids are endogenous ligands of FXR. Intestinal, followed by hepatic, FXR signaling pathways are important for suppressing bile acid synthesis. This suppression is achieved by regulation of the expression of the *Cyp7a1/CYP7A1* gene, which encodes cholesterol 7 alpha-hydroxylase, the rate-limiting enzyme in the conversion of cholesterol into bile acids (Kim et al., 2007). FXR also regulates the expression of genes encoding various bile acid transporters, including NTCP, BSEP, MRP2, and OSTα/β in the liver and ASBT and OSTα/β in the intestine (Jung et al., 2007; Laffitte et al., 2000). FXR has also been found to modulate triglyceride and glucose homeostasis (Potthoff et al., 2011; Trauner et al., 2010; Watanabe et al., 2004), reduce energy expenditure (Watanabe et al., 2011) and exert anti-inflammatory and anti-fibrotic effects (Zhang et al., 2009). However, to the best of our knowledge, the effects of BBR on intestinal FXR function have not been evaluated.

Gut microbiota, or gut flora, are bacteria that have colonized within the intestines. Interactions between these microbiota and the human host play an important role in metabolic homeostasis and human health (Nicholson et al., 2012; Serino et al., 2009). Bile acid metabolism is closely related to gut microbiota composition. It is well established that secondary bile acids are derived from the primary bile acids by the action of bacteria present in the intestine (Chiang, 2009). Bile salt hydrolase (BSH), an enzyme synthesized by gut
microbiota, acts on carbon-nitrogen bonds to hydrolyze taurine- or glycine-conjugated bile acids for de-conjugation (Begley et al., 2006). BSH activity can be modulated by various antibiotics and probiotics, and this modulation may affect lipid metabolism and host health (Li et al., 2013; Smet et al., 1995; Smet et al., 1998).

As shown in our previous study, BBR enhanced the production of bile acids in hamsters. Moreover, CYP7A1 and CYP27A1 mRNA and protein expression was induced by BBR in a concentration-dependent manner (Gu et al., 2015). In the current study, we have used wild type (WT) and intestine-specific FXR knockout (FXR int−/−) mice to evaluate the regulatory effects of BBR on BSH activity and bile acid turnover, with the purpose of gaining insight into the anti-obesity and lipid-lowering mechanisms of BBR.
Materials and Methods

Chemicals and reagents

BBR (≥98%) was purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). CDCA, UDCA and HDCA were purchased from the National Institutes for Food and Drug Control (Beijing, China). CA, DCA, TCA, LCA, GCDCA, GCA, TCDCA, TDCA, d4-CA, Triton X-114 and tert-Butanol were purchased from Sigma-Aldrich (St Louis, MO, USA), d4-TCA and d4-GCA were purchased from TLC PharmaChem (Concord, ON, Canada). αMCA, βMCA, TβMCA, GLCA, GUDCA, GDCA, TUDCA, THDCA and TLCA were purchased from Steraloids Inc. (Newport, RI). Bio-Bag and AnaeroPack (2.5 L) were purchased from Mitsubishi Gas Chemical Company, Inc. (Kyoto, Japan).

Animal treatment

WT and FXR\textsuperscript{int/-} mice with a C57BL/6J background (6 to 8 weeks old, male) were bred and maintained at Rutgers, the State University of New Jersey (Kong et al., 2012). All animals were housed under specific-pathogen-free conditions, fed \textit{ad libitum} and maintained under a 12-hour light-dark cycle. All animal experiments were performed with approval from the Institutional Animal Care and Use Committee (IACUC) of Rutgers University and from the Animal Ethics Committee of China Pharmaceutical University.

To determine the effects of BBR on the hepatic accumulation of triglycerides and its anti-obesity activity, four groups of WT mice were used. Two groups of mice (n=6) were fed with control diet (AIN-93M, Research Diets, New Brunswick, NJ, USA) and treated with CMC-Na (0.5%) or BBR (150 mg/Kg). Other two groups were fed with high-fat diet (HFD, 60% calories from fat and 1% cholesterol, Research Diets, New Brunswick, NJ, USA), the
HFD vehicle group (n=6) was administered with CMC-Na, and the treatment group (n=6) was administered with BBR (150 mg/Kg). For the FXR\textsuperscript{int-/-} study, four groups of FXR\textsuperscript{int-/-} mice were fed with the AIN-93M or HFD. Groups were paired and intragastrically administered either CMC-Na as a vehicle control (n=4) or BBR (150 mg/Kg, n=4) for 8 weeks. BBR was grounded into fine particles and passed through a sieve of 120 meshes. The powder was then stirred and suspended with 0.5% CMC-Na aqueous solution to achieve a final concentration of 15 mg/mL. This suspension was given to mice by oral gavage at a dose of 10 mL/Kg body weight. The body weights of the mice were recorded every week. The animals were fasted overnight before euthanasia. At the time of euthanasia, the mice were anaesthetized by xylazine-ketamine and avertin, and samples of blood, liver, gallbladder, small intestine and cecal content were collected and stored at -80°C for further analysis.

For the taurocholic acid (TCA) treatment, WT mice (6 weeks old, male) were provided by Yangzhou University, China. Two groups (n=6) of mice were intragastrically administered with either vehicle (saline) or 100 mg/Kg TCA for 7 days. Tissue collection was performed as described above.

**Oral glucose tolerance test (OGTT)**

7 weeks after treatment with BBR, OGTT was performed, mice were fasted for 6 hrs (9:00 a.m. to 3:00 p.m.) and oral administrated with 1 g/Kg glucose. Blood was drawn at 0, 15, 30, 60, 90 and 120 mins after administration and blood glucose was measured by Contour\textsuperscript{TS} blood glucose meter (Bayer HealthCare LLC, Mishawaka, IN). Area under the curve (AUC) was calculated.

**Measurement of hepatic total triglycerides**
To further evaluate the lipid-lowering effect of BBR, hepatic total triglycerides were measured. Briefly, a 100-mg sample of liver was homogenized with 1 mL of Buffer I (18 mM Tris HCl pH=7.5, 300 mM Mannitol, 50 mM EGTA and 0.1 mM PMSF), and 400 μL of homogenate was extracted with 2 mL of chloroform: methanol mixture (2:1). After overnight mixing, 1 mL of water was added to each tube and mixed, followed by centrifugation at 3000 g for 5 min, afterwards the lower lipid phase was collected and concentrated using a Speed-Vac. The lipid pellets were re-suspended with Buffer II (60 μL of tert-Butanol and 40 μL of Triton X-114: Methanol (2:1) in 100 μL). Triglycerides were measured using an enzymatic-colorimetric assay kit purchased from Pointe Scientific (Canton, MI, USA) according to the protocol provided.

**Oil-Red-O staining**

Livers were frozen in optimal cutting temperature compound (O.C.T.) during tissue collection, sectioned into 8-μM-thick slices and stained with Oil-Red-O using a previously described protocol (Kong et al., 2009).

**qRT-PCR analysis**

Total RNA was isolated using TRI Reagent (Sigma). The mRNA concentration was quantified using a Nano Drop ND-1000 spectrophotometer (Thermo Scientific). The diluted mRNA (0.5 μg/μL) was reverse-transcribed according to the manufacture’s protocol, and the gene expression levels were determined by SYBR-green-based real-time-PCR technique (ABI ViiA 7 Real-time PCR system, Applied Biosystems, USA). The mRNA levels of β-actin were used for an internal normalization. The sequences of primers used for qRT-PCR are listed in Table 1.
Western blot

Total protein in the liver was extracted and western blot analysis was performed as previously described (Cao et al., 2013; Kong et al., 2012). Antibodies against p-ERK1/2 and T-ERK1/2 were purchased from Cell Signaling Technology, Inc. (Beverly, MA, US), antibodies against CD36 and GAPDH (the loading control) were purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). The intensity of bands on the Western blots was quantified by Image Lab statistical software (Bio-Rad Laboratories, Hercules, CA, US).

Determination of bile acid composition in serum, intestine and feces

Bile acids in serum, liver, intestine and feces were analyzed by LC-MS/MS as previously reported with some revisions (Zhou et al., 2014). Briefly, bile acids were extracted with 70% ethanol at 55°C for 4 hrs, and then subjected to solid-phase extraction using solid-phase extraction technique (UCT-CLEAN UP C18, Oasis-HLB and Oasis-MAX cartridges). The resultant residue was re-dissolved in 100 μL of methanol and centrifuged at 18,000 rpm for 10 mins. Chromatography was performed on a Shimadzu HPLC system (Kyoto, Japan) coupled to an AB SCIEX 4000 mass spectrometer (AB Sciex). A Waters Atlantis T3 column (2.1×100 mm, 3 μm) protected by a Security Guard (Phenomenex Inc., CA, USA) was used for chromatographic separation. The mobile phase consisted of 0.1% formic acid in water and methanol. Cholic-2,2,4,4-D4 acid (d4-CA) and glycocholic-2,2,4,4-D4 (d4-GCA) acid were used as internal standards.

Measurement of BSH activity

After 8 weeks of treatment, the cecal contents of mice were recovered and pooled in sterile tubes. The gut contents were boiled with an aqueous solution containing 20% glycerol and 1.8%
sodium chloride at a 1:3 ratio (g: mL). The samples were stored at -80°C for further analysis. All experimental procedures were performed in a sterile environment.

Gut flora were diluted 10-fold in a peptone-yeast extract (PY) medium prepared as previously described (Zhao et al., 2013) and then cultured at 37°C and 200 rpm for 12 hrs to allow anaerobic bacteria to proliferate. For analysis, 10 μL of PY medium (containing 50 μg/mL of d4-TCA) and 90 μL of the fermented gut content were mixed in 1.5 mL Eppendorf tubes and placed into a 2.5 L seal can with a bio-bag. The seal can was then placed into an incubator and shaken at 200 rpm at 37°C for 20 mins. After incubation, 300 μL of methanol (containing 100 ng/mL of IS, d4-GCA) was added to each tube, and the tubes were vigorously vortexed for 5 mins. After centrifugation at 12,000 × g for 10 mins, 200 μL of the supernatant was transferred into a LC vial for LC-MS/MS analysis of d4-CA.

To analyze the gut contents of the mice fed with the control diet or the HFD, BBR was added into PY medium at concentrations of 0 and 100 μg/mL. Following this, the gut flora were diluted in PY medium by 10-fold and then cultured at 37°C and 200 rpm for 12 hrs to allow anaerobic bacteria to proliferate. The following procedure was the same as previously described, and the production of d4-CA was determined by LC-MS/MS.

**Statistical Analysis**

All data were expressed as mean ± SD. Differences among groups were tested by one-way ANOVA and/or two-tailed Student’s t tests. Heatmap was constructed using the R package pheatmap (http://cran.r-project.org/web/packages/pheatmap/index.html).
Results

BBR prevented diet-induced obesity and triglyceride accumulation in the liver

To investigate the anti-obesity and lipid-lowering effects of BBR, the body weight of mice were recorded weekly. After 8 weeks, the mice fed the HFD showed a significant increase in body weight (31.03±1.82 g vs. 27.57±0.9 g, \( P<0.001 \)), while treatment with BBR reduced this effect (25.33±1.76 g vs. 31.03±1.82 g, \( P<0.001 \)) (Fig. 1A). To explore the effect of BBR on glucose homoeostasis, OGTT was performed and the results revealed that after 7 weeks of HFD, compared with vehicle groups, mice treated with BBR displayed significantly reduced blood glucose levels after glucose loading, suggesting improved insulin sensitivity. For mice fed with AIN-93M, there was no change (Fig. 1B and 1C). Moreover, fasting serum glucose levels from BBR-treated groups decreased significantly (Fig. 1D). Triglyceride levels in serum and livers of mice fed with HFD were significantly increased by 1.2 fold and 4.4 fold, respectively. After BBR treatment, there was a significant decrease in serum and hepatic triglycerides by 19% and 47%, respectively (Fig. 1E and 1F). Lipid droplets in the liver also appeared to be reduced in size revealed by Oil-Red-O staining (Fig. 1G).

BBR activated the intestinal FXR pathway and reduced CD36 gene expression in liver

For genes involved in fatty acid metabolism and transportation, the expression of Fas was decreased by BBR treatment, and the expression of Cyp4a10 was upregulated significantly. The expression of Mtp and ApoB were not changed (Fig. 2A and 2B). The intestinal FXR pathway plays a key role in the regulation of cholesterol metabolism and bile acid homeostasis. The 8-week treatment with BBR may have activated the intestinal, but not hepatic FXR pathway, as revealed by increased ERK1/2 phosphorylation in the liver and the
up-regulation of Fgf15 and Ibabp mRNA levels in the distal ileum (Fig. 2C, 2D, 2E and 2F). Furthermore, oral administration of BBR reduced Cyp7a1 mRNA levels in mice (Fig. 2D), which is consistent with the up-regulation of intestinal Fgf15. Because CD36 is critical to the uptake of long-chain fatty acids into the liver and has been shown to be reduced by FXR activation, we also examined its mRNA expression and protein level. We found that treatment with BBR markedly down-regulated the mRNA expression of CD36 in the liver by approximately 48% (P=0.045, Fig. 2B). The protein level of CD36 was also reduced by BBR treatment (Fig. 2E and 2F).

BBR treatment caused modifications in bile acid composition

Bile acid composition is an indicator of the homeostasis and turnover of cholesterol as well as of bile acid metabolism activity. There is species difference in bile acid composition and muricholic acid (MCA) only exists in rodent. In mice, with respect to individual bile acid, TCA and TβMCA were agonist and antagonist of FXR, respectively (Sayin et al., 2013). Therefore, we measured bile acid profiles in serum, liver, intestine and feces using LC-MS/MS. The composition of bile acids was shown in Fig. 3A, 4A, 5A and 6A.

In serum, HFD increased the levels of GCA, TCA and TUDCA, whereas decreased the levels of other bile acids. Treatment of BBR increased the levels of TCA and LCA, but decreased the levels of other bile acids (Fig. 3A). Specifically, for TβMCA and TCA, we found that the consumption of the HFD did not change TCA levels, whereas TβMCA levels increased in serum. Treatment of BBR reduced TβMCA in the serum of mice fed with both AIN-93M and HFD. For TCA, treatment of BBR had no influence on mice fed with AIN-93M, but significantly increased TCA level in serum of mice fed with HFD (Fig. 3B and 3C).
For bile acids in the liver, the consumption of the HFD did not change the levels of bile acids significantly. Treatment of BBR increased the level of TUDCA, TDCA, LCA and TCA, but not the levels of GCA and CA. The rest of bile acids trended towards reduction (Fig. 4A). Specifically, for TβMCA and TCA, treatment with BBR decreased TβMCA levels in mice fed with both diets, and BBR also decreased TCA levels in mice fed with AIN-93M, whereas there was no obvious change for TCA in mice fed with the HFD (Fig. 4B and 4C).

In the intestine, HFD rarely changed the levels of individual bile acids. BBR also had no effect on THDCA, LCA and GCA; however, the levels of UDCA, αMCA, βMCA, ωMCA, CA, CDCA, HDCA and DCA were decreased. The levels of TDCA, TCDCA and TUDCA showed a trend of increase (Fig. 5A). Specifically, for TβMCA and TCA, BBR showed rare regulation effect on TβMCA. TCA levels were both elevated after treated with BBR (Fig. 5B and 5C).

With respect to bile acid composition in the feces, the consumption of a HFD increased TDCA, αMCA, ωMCA, βMCA, TCDCA, TLCA, CDCA, DCA, LCA, UDCA and HDCA levels (Fig. 6A). Specifically, both TβMCA (Fig. 6B) and TCA (Fig. 6C) levels were increased after the consumption of the HFD and were even further elevated after treatment with BBR.

**BBR inhibited BSH activity**

BSH activity is critically involved in the capacity of gut microbiota to hydrolyze conjugated bile acids. A previously established *in vitro* metabolism system was used to evaluate the BSH activity of gut microbiota (Zhao et al., 2013). Overall, a decrease in d4-TCA level was found, and d4-CA production is a measure of BSH activity (Fig. 7A). Relative to the gut microbiota
of the mice that were fed the control diet, d4-TCA was hydrolyzed at a slower rate and there was less of an increase of d4-CA in the mice fed the HFD. Moreover, treatment with BBR reduced the hydrolyzation of d4-TCA and reduced d4-CA to undetectable levels, indicating a marked decrease in the production of d4-CA. Accordantly, co-incubation of BBR (100 μg/mL) in vitro also showed an inhibition of BSH activity for gut microbiota from mice fed with both control diet and HFD (Fig. 7B and 7C).

**TCA activated the intestinal FXR pathway and reduced Cd36 expression in the liver**

BBR treatment decreased BSH activity and increased the levels of TCA in the small intestine (Fig. 5E). Therefore, we determined the effects of a 7-day TCA treatment on FXR signaling pathway. After TCA treatment, hepatic FXR target gene, Shp, and intestinal FXR target genes, Fgf15 and Ibabp mRNA levels were up-regulated (Fig. 8A and 8B). The expression of Fas was decreased and the expression of Cyp4a10 was increased significantly (Fig. 8C). Consistent with the effects of BBR treatment, in the liver, ERK1/2 phosphorylation was activated and the Cyp7a1 mRNA levels were significantly reduced, the mRNA expression and protein level of CD36 were both reduced (Fig. 8A, 8D, 8E and 8F).

**Anti-obesity and lipid-lowering effects of BBR was diminished in FXRint-/- mice**

To confirm the role of the intestinal FXR signaling pathway in the lipid-lowering effect of BBR, FXRint-/- mice were used. The data showed that BBR treatment did not change either the body weights or the serum triglyceride levels of FXRint-/- mice that were fed with HFD (Fig. 9A and 9B). Measurement of lipids and Oil-Red-O staining showed that triglycerides in the livers were not affected by BBR treatment as well (Fig. 9C and 9D). The expression of the genes involved in fatty acid metabolism and transportation, including Srebp-1c, Ppara, Acc,
Fas, Mtp and ApoB, was not changed significantly by BBR treatment, Cyp4a10 was induced ($P=0.049$) (Fig. 10A and 10B). In contrast to an increasing trend of intestinal mRNA levels of Fgf15 that were observed, the mRNA levels of Ibabp and Ostβ were significantly reduced, which is an opposite trend from that observed in WT mice (Fig. 10C). Moreover, the expression of the genes encoding Cyp7a1 ($P=0.005$) and Cd36 (not significantly) was increased after BBR treatment (Fig. 10B and 10D). These results indicate that the lipid-lowering effect of BBR relies on an intact intestinal FXR signaling pathway, and regulation of Cd36 expression by increased Fgf15 signaling in the liver might be the key mechanism.

Discussion

Anti-obesity and lipid-lowering effects of orally administered BBR

BBR is reported to possess anti-obesity and lipid-lowering effects in different species, and previous studies suggest that the lipid-lowering mechanisms exerted by BBR include the up-regulation of LDLR and the activation of the AMPK pathway (Brusq et al., 2006; Kong et al., 2004). Other studies have reported that BBR can ameliorate NAFLD by modulating hepatic mRNA and lncRNA expression profiles (Yuan et al., 2015). However, the activation of AMPK was not observed in an in vivo study (Guo et al., 2016). In addition, the in vitro concentrations of BBR used in the referenced studies were two orders of magnitude higher than the blood concentrations achieved following the oral administration, and when BBR was present in the liver at low concentrations, it failed to produce pharmacological effects (Kong et al., 2004; Liu et al., 2010). In contrast, high levels of BBR accumulate in the
gastrointestinal tract after the oral administration. Therefore, we hypothesized that the pharmacological effects of orally administered BBR exert through BBR-mediated modulation of intestinal microbiota. We first tested this hypothesis in the WT mice. We found that BBR indeed exerted an anti-obesity effect, improved insulin resistance and ameliorated the accumulation of triglycerides in the liver (Fig. 1). Moreover, we hypothesized the intestinal, but not hepatic FXR signaling pathway was involved in these beneficial effects (Fig. 2C and 2D).

**Intestinal FXR plays a pivotal role in the pharmacological effects of BBR**

The intestinal FXR signaling pathway plays an important role in the development of NAFLD. It has been documented that FXR agonists inhibit inflammation, repress macrophage activation, reduce adipose differentiation-related protein levels, increase cholesteryl ester transfer protein expression, reduce \(Cd36\) gene expression and promote the conversion of adipose tissue from white adipose tissue (WAT) to brown adipose tissue (BAT). These actions reduce obesity and improve insulin resistance (Fang et al., 2015; Ma et al., 2013; Yao et al., 2014; Zhang et al., 2009). As the intestinal FXR signaling pathway appeared to be activated in WT mice following treatment with BBR (Fig. 2C), FXR\(^{int/-}\) mice were used to further test the role of intestinal FXR in the pharmacological effects of BBR. We found that BBR did not produce anti-obesity and lipid-lowering effects in FXR\(^{int/-}\) mice (Fig. 9), which further suggests that intestinal FXR may be the critical mediator of the pharmacological effects of BBR.

According to previous tissue distribution studies that in the liver and small intestine the concentration of BBR is approximately 100 ng/g tissue (Hu et al., 2014; Tan et al., 2013). Due
to the low bioavailability of BBR, the pharmacological effects of BBR may be a secondary
effect following its modulation of gut microbiota and, subsequently, bile acid metabolism in
the gut.

**BBR modulated bile acid composition and BSH activity**

It is well known that different bile acids differentially regulate the activity of FXR, and the
modulation of FXR activity by endogenous bile acids creates complicated and comprehensive
effects. CDCA, CA and TCA are endogenous agonists of FXR (Makishima et al., 1999; Parks
et al., 1999), whereas ursodeoxycholic acid (UDCA) (Mueller et al., 2015) and TβMCA (Li et
al., 2013; Sayin et al., 2013) are endogenous antagonists of FXR. We measured the
compositions of bile acids in the sera and feces of mice treated with BBR. In the sera of the
mice that were treated with BBR, the majority of conjugated bile acids, especially TCA, were
present at elevated levels, whereas the level of TβMCA was unchanged, and the majority of
unconjugated bile acids were present at reduced levels. A similar phenomenon was also
observed in the feces, in which tauro-conjugated bile acid levels increased following BBR
treatment. Bile acid metabolism is closely related with gut microbiota composition; therefore,
the modulation of gut microbiota should be considered in the context of BBR treatment.

Both obesity and NAFLD are considered to be closely related to changes in gut
microbiota composition, indicating the importance in modulation of gut microbiota
(Abu-Shanab and Quigley, 2010; Compare et al., 2012; Musso et al., 2011; Nicholson et al.,
2012; Tremaroli and Bäckhed, 2012). BSH, an enzyme produced by gut microbiota, serves as
one of the mechanisms by which microbes and hosts interact, and such interactions regulate
lipid and cholesterol metabolism. Previous reports have shown that the expression of
exogenous BSH in mouse intestines or treatment with probiotics can significantly reduce weight gain and plasma and liver lipids, as well as improve insulin resistance (Degirolamo et al., 2014; Joyce et al., 2014). In the current study, we found that BBR significantly inhibited BSH activity both \textit{in vivo} and \textit{in vitro}, and the levels of tauro-conjugated bile acids, especially TCA, became substantially elevated following the inhibition of BSH activity. However, BSH has been found to be expressed by all major bacterial divisions and archaeal species in the gut based on metagenomic analyses (Jones et al., 2008), and BBR was found to reduce the proportions of both fecal \textit{Firmicutes} and fecal \textit{Bacteroidetes} in HFD-fed mice (Xie et al., 2011). Based on these findings, the modulation of BSH activity by BBR may be due to an indiscriminant inhibition of gut microbiota.

\textbf{Reduction of Cd36 expression ameliorated hepatic lipid accumulation}

The activation of the intestinal FXR signaling pathway induces the expression of Fgf15 (human homologue, Fgf19), which acts on the liver and inhibits the expression of Cd36. CD36 (cluster of differentiation 36), also known as FAT (fatty acid translocase), belongs to the class B scavenger receptor family, which includes receptors for selective cholesteryl ester uptake and is important for lipid transportation and metabolism (Bieghs et al., 2010; Febbraio et al., 2001). The FXR agonist GW4064 showed an inhibitory effect on Cd36 expression and reduced the accumulation of triglycerides in the liver without affecting expression of genes that are directly involved in lipogenesis (Ma et al., 2013). Furthermore, the treatment of FXR knockout mice with FGF19 protein reduced liver expression of Cd36, reduced the transportation of free fatty acids and inhibited the synthesis of triglycerides (Miyata et al., 2011).
As TCA was elevated in both serum and feces after BBR treatment, we treated a group of mice with TCA (100 mg/Kg) for a week. We found that the TCA treatment produced consistent results to those achieved following BBR treatment: activated intestinal FXR signaling pathway and reduced expression of Cd36. Thus, the anti-obesity and lipid-lowering effects of BBR are mainly due to the activation of intestinal FXR and a reduction in Cd36 expression. In FXR<sup>int/-</sup> mice treated with BBR, the expression of FXR target genes in the intestine was inhibited, and Cd36 expression was not changed obviously. Furthermore, the pharmacological effects of BBR were no longer evident, which further validates our hypothesis.

In summary, in mice fed with HFD, BBR shows anti-obesity and lipid-lowering effects in WT mice, but not in FXR<sup>int/-</sup> mice. BBR inhibits the BSH activity of gut microbiota, leading to increased levels of conjugated bile acids. Our data suggest, for the first time, that the lipid-lowering effect of BBR is achieved via sequential events involving the inhibition of BSH, elevation of TCA, activation of the intestinal FXR signaling pathway, and reduction of Cd36 expression in the liver, which results in the reduced hepatic uptake of long-chain fatty acids. This underlying mechanism helps explain the observed discrepancy between the low oral bioavailability of BBR and its effective lipid-lowering and anti-obesity effects.
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Conducted experiments: Runbin Sun, Na Yang, Bo Kong, Bei Cao, Dong Feng, Xiaoyi Yu, Chun Ge, Jingqiu Huang, Jianliang Shen, Pei Wang, Siqi Feng, Fei Fei, Jiahua Guo, Jun He, Nan Aa, Qiang Chen, Yang Pan

Performed data analysis: Runbin Sun, Na Yang, Bo Kong, Grace L. Guo, and Jiye Aa

Wrote or contributed to the writing of the manuscript: Runbin Sun, Na Yang, Bo Kong, Bei Cao, Justin D. Schumacher, Chung S. Yang, Grace L. Guo, Jiye Aa, and Guangji Wang
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**Hepatology** 56(3): 1034-1043.


Footnotes

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Legends for figures

Figure 1. BBR prevented HFD-induced weight gain and ameliorated hepatic triglyceride accumulation in WT mice.

Male WT mice were treated with BBR (150 mg/Kg, i.g.) or vehicle (CMC-Na) for 8 weeks. A. Weekly body weight changes. B. Oral glucose tolerance test. C. AUC of blood glucose. D. Blood glucose after fasted for 6 hrs. E. Serum triglycerides at the end of the treatment. F. Hepatic triglycerides at the end of the experiment. G. Oil-Red-O staining (20×). WT AIN-93M Vehicle, mice fed with control diet and treated with CMC-Na; WT AIN-93M BBR, mice fed with control diet and treated with BBR; WT HFD Vehicle, mice fed with HFD and treated with CMC-Na; WT HFD BBR, mice fed with control diet and treated with BBR. Error bars show SD of replicates (n=6). *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\), compared with normal control group; #, \(P < 0.05\); ##, \(P < 0.01\), compared with high fat control group.

Figure 2. BBR activated intestinal but not hepatic FXR signaling pathway and reduced Cd36 expression in WT mice.

Total RNA were isolated form the liver and intestine from male WT mice after treatment of BBR (150 mg/Kg, i.g.) or vehicle (CMC-Na) for 8 weeks and qRT-PCR was performed to measure the expression of genes. A. Relative mRNA levels of \(Ppara\), \(Srebp-1c\), \(Acc\) and \(Fas\) in liver. B. Relative mRNA levels of \(Cyp4a10\), \(Mtp\), \(ApoB\) and \(Cd36\) gene in the liver. C. Relative mRNA levels of \(Fxr\), \(Fgf15\), \(Shp\) and \(Ibabp\) gene in the distal ileum. D. Relative mRNA levels of \(Fxr\), \(Shp\) and \(Cyp7a1\) gene in the liver. E. Semi-quantitive analysis of the protein levels in liver. F. Protein level of CD36, p-ERK1/2 and T-ERK1/2 in liver. Error bars show SD of replicates (n=6). *, \(P < 0.05\); **, \(P < 0.01\), compared with normal control.
group; #, $P < 0.05$; ##, $P < 0.01$, compared with high fat control group.

**Figure 3. Changes in bile acid composition in the serum after BBR treatment.**

Bile acids in serum of male WT mice after treatment of BBR (150 mg/Kg, i.g.) or vehicle (CMC-Na) for 8 weeks were measured using LC-MS/MS. A. Heatmap detailing the bile acid composition of serum from WT mice. The color key represents the calibrated contents of bile acids, and the dendrogram on the left showed the clustering of bile acids with the similar changes. B. TβMCA levels in the serum. C. TCA levels in the serum. Error bars show SD of replicates (n=6). *, $P < 0.05$, compared with normal control group; #, $P < 0.05$; ##, $P < 0.01$, compared with high fat control group.

**Figure 4. Changes in bile acid composition in the liver after BBR treatment.**

Bile acids in liver of male WT mice after treatment of BBR (150 mg/Kg, i.g.) or vehicle (CMC-Na) for 8 weeks were measured using LC-MS/MS. A. Heatmap detailing the bile acid composition of liver. The color key represents the calibrated contents of bile acids, and the dendrogram on the left showed the clustering of bile acids with the similar changes. B. TβMCA levels in the liver. C. TCA levels in the liver. Error bars show SD of replicates (n=6). *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, compared with normal control group; #, $P < 0.05$, compared with high fat control group.

**Figure 5. Changes in bile acid composition in the intestine after BBR treatment.**

Bile acids in the intestine of male WT mice after treatment of BBR (150 mg/Kg, i.g.) or vehicle (CMC-Na) for 8 weeks were measured. A. Heatmap detailing the bile acid composition of intestine. The color key represents the calibrated contents of bile acids, and the dendrogram on the left showed the clustering of bile acids with the similar changes. B.
TβMCA levels in the intestine. C. TCA levels in the intestine. Error bars show SD of replicates (n=6). **, $P < 0.01$, compared with normal control group; #, $P < 0.05$, compared with high fat control group.

**Figure 6. Changes in bile acid composition in feces after BBR treatment.**

Bile acids were extracted from feces of male WT mice after treatment of BBR (150 mg/Kg, i.g.) or vehicle (CMC-Na) for 8 weeks and measured. A. Heatmap detailing the bile acid composition of feces. The color key represents the calibrated contents of bile acids, and the dendrogram on the left showed the clustering of bile acids with the similar changes. B. TβMCA levels increased in feces after BBR treatment. C. TCA levels increased in feces after BBR treatment. Error bars show SD of replicates (n=6). ***, $P < 0.001$, compared with normal control group; ###, $P < 0.001$, compared with high fat control group.

**Figure 7. BBR suppressed the activity of BSH in gut microbiota.**

BSH activity was evaluated in gut microbiota. A. In gut microbiota, BSH hydrolyzed d4-TCA to d4-CA and taurine. B. The consumption of HFD increased the reduction of d4-TCA, and BBR aggravated this function. C. The consumption of HFD also reduced the production of d4-CA, and BBR aggravated this function. Error bars show SD of replicates (n=6). *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$, compared with control group.

**Figure 8. TCA treatment activated the intestinal FXR pathway and reduced Cdx36 expression.**

After TCA treatment for 7 days, livers and intestines of male WT C57BL/6J mice were collected. Protein levels and gene expressions at mRNA level were measured. A. Relative mRNA levels of Fxr, Shp and Cyp7a1 gene in the liver. B. Relative mRNA levels of Fxr,
Fgf15, Shp and Ibabp gene in the distal ileum. C. Relative mRNA levels of Srebp-1c, Ppara, Acc, Fas and Cyp4a10 gene in the liver. D. Relative mRNA levels of Mtp, ApoB and Cd36 gene in the liver. E. Semi-quantitive analysis of the protein levels in the liver. F. Protein level of CD36, p-ERK1/2 and T-ERK1/2 in the liver. Error bars show SD of replicates (n=6). *, P < 0.05; **, P < 0.01, *** P < 0.001, compared with control group.

**Figure 9.** BBR treatment did not change body weight or hepatic triglyceride in FXR$^{\text{int-/-}}$ mice.

To further evaluate the role of the intestinal FXR pathway in the pharmacological effects of BBR, intestinal-specific FXR knockout (FXR$^{\text{int-/-}}$) mice were fed a HFD and treated with either BBR (150 mg/Kg/d, i.g.) or vehicle (CMC-Na) for 8 weeks. Livers and intestines were collected, and mRNA was extracted and measured by qRT-PCR. A. The ratio change of mouse body weight at the time of euthanasia to the body weight at the time of the beginning of the experiment revealed that BBR treatment only slightly reduced the body weight gain of FXR$^{\text{int-/-}}$ mice. B. Serum triglycerides at the end of the treatment. C. Hepatic triglycerides at the end of the experiment. D. Oil-Red-O staining (20×). FXR$^{\text{int-/-}}$ AIN-93M Vehicle, mice fed the control diet and treated with CMC-Na; FXR$^{\text{int-/-}}$ AIN-93M BBR, mice fed the control diet and treated with BBR; FXR$^{\text{int-/-}}$ HFD Vehicle, mice fed the HFD and treated with CMC-Na; FXR$^{\text{int-/-}}$ HFD BBR, mice fed the HFD and treated with BBR. Error bars show SD of replicates (n=4).

**Figure 10.** BBR inhibited intestinal FXR signaling pathway but did not change the expression of Cd36 in FXR$^{\text{int-/-}}$ mice.

Total RNA were isolated form the liver and intestine from male FXR$^{\text{int-/-}}$ mice after treatment
of BBR (150 mg/Kg, i.g.) or vehicle (CMC-Na) for 8 weeks and qRT-PCR was performed to measure the expression of genes. A. Relative mRNA levels of Ppara, Srebp-1c, Acc and Fas gene in the liver. B. Relative mRNA levels of Cyp4a10, Mtp, ApoB and Cd36 gene in the liver. C. Relative mRNA levels of Fgf15, Ibabp and OSTβ gene in the distal ileum. D. Relative mRNA levels of Fxr, Shp and Cyp7a1 gene in the liver. Error bars show SD of replicates (n=4). *, P < 0.05; **, P < 0.01, compared with normal control group; #, P < 0.05; ###, P < 0.01, compared with high fat control group.
Table 1. Primers used for gene expression analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Primer Sequence (5' - 3')</th>
<th>Reverse Primer Sequence (5' - 3')</th>
</tr>
</thead>
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<tr>
<td>β-actin</td>
<td>GCGTGACATCAAAGAGAAGC</td>
<td>CTCGTTGCCAATAGTGATGAC</td>
</tr>
<tr>
<td>Cd36</td>
<td>GATGACGTGGCAAAGAAAGC</td>
<td>TCCTCGGGGTCTCTGAGTTAT</td>
</tr>
<tr>
<td>Cyp7a1</td>
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<td>GTGTAGAGTGAAGTCCTCCTTAGC</td>
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<td>Fgf15</td>
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<td>Fxr</td>
<td>TCCGGACATTCAACCATCAC</td>
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<td>TGGGATTTTCCATCTGCCCCTCGGAG</td>
<td>GTACAGATCCATCAGAGGAATG</td>
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<tr>
<td>Mtp</td>
<td>GACCACCTGGATCTCCATA</td>
<td>AGCGTGGTAGGAAAGGGCTTAT</td>
</tr>
</tbody>
</table>
Figure 6

Table A

|      | C1  | C2  | C3  | C4  | C5  | C6  | H1  | H2  | H3  | H4  | H5  | H6  | CB1 | CB2 | CB3 | CB4 | CB5 | CB6 | HB1 | HB2 | HB3 | HB4 | HB5 | HB6 |
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| αMCA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| CA   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| TUDCA|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| GCA  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| TCA  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| TβMCA|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| THDCA|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| TCDCA|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| TLCA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| TLCA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| βMCA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| UDCA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| UDCA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| HDCA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| HDCA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| LCA  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| LCA  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| DCA  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| DCA  |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| TDCDA|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| TDCA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| wMCA |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

Figure B

B) Fecal Tβ MCA

C) Fecal TCA

Graphs showing the comparison between Vehicle and BBR, with statistical significance indicated by asterisks.
Figure 7

A

\[ \text{d4-TCA} \xrightarrow{\text{BSH, Gut microbiota}} \text{d4-CA} + \text{Taurine} \]

B

\begin{align*}
\text{d4-TCA} & \quad \text{ng/ml} \\
\text{AIN-93M} & \quad \text{Vehicle} \\
\text{HFD} & \quad \text{BBR (100 µg/ml, in vitro)} \\
& \quad \text{BBR (150 mg/Kg, in vivo)}
\end{align*}

C

\begin{align*}
\text{d4-CA} & \quad \text{ng/ml} \\
\text{AIN-93M} & \quad \text{Vehicle} \\
\text{HFD} & \quad \text{BBR (100 µg/ml, in vitro)} \\
& \quad \text{BBR (150 mg/Kg, in vivo)}
\end{align*}
A  
**Hepatic FXR pathway**

- **Vehicle**
- **TCA**

B  
**Intestinal FXR pathway**

C  
**TG metabolism**

D  
**TG transporter**

E  
**Fold change**

F  
**Western blots**

- **CD36**
- **p-ERK**
- **T-ERK**
- **GAPDH**