Berberine-induced Inactivation of Signal Transducer and Activator of Transcription 5 Signaling Promotes Male-specific Expression of a Bile Acid Uptake Transporter*

Received for publication, September 7, 2016, and in revised form, January 29, 2017

Published, JBC Papers in Press, February 1, 2017, DOI 10.1074/jbc.M116.757567

Pengli Bu1†1, Yuan Le†1, Yue Zhang1, Youcai Zhang6, and Xingguo Cheng5,2

From the Departments of 1Pharmaceutical Sciences and 2Biological Sciences, St. John’s University, Queens, New York 11439 and the §School of Pharmaceutical Science and Technology, Tianjin University, Tianjin 300072, China

Edited by Jeffrey E. Pessin

Sodium-taurocholate co-transporting polypeptide (Ntcp/NTCP) is the major uptake transporter of bile salts in mouse and human livers. In certain diseases, including endotoxemia, cholestasis, diabetes, and hepatocarcinoma, Ntcp/NTCP expression is markedly reduced, which interferes with enterohepatic circulation of bile salts, impairing the absorption of lipophilic compounds. Therefore, normal Ntcp/NTCP expression in the liver is physiologically important. Berberine is an herbal medicine used historically to improve liver function and has recently been shown to repress STAT signaling. However, berberine effects on Ntcp/NTCP expression are unknown, prompting use to investigate this possible connection. Our results showed that berberine dose-dependently increased Ntcp expression in male mouse liver and decreased taurocholic acid levels in serum but increased them in the liver. In mouse and human hepatoma cells, berberine induced Ntcp/NTCP mRNA and protein expression and increased cellular uptake of [3H]taurocholate. Mechanistically, berberine decreased nuclear protein levels of phospho-JAK2 and phospho-STAT5, thus disrupting the JAK2-STAT5 signaling. Moreover, berberine stimulated luciferase reporter expression from the mouse Ntcp promoter when one putative STAT5 response element (RE) (−1137 bp) was deleted and from the human NTCP promoter when three putative STAT5REs (−2898, −2164, and −691 bp) were deleted. Chromatin immunoprecipitation demonstrated that berberine decreased binding of phospho-STAT5 protein to the −2164 and −691 bp STAT5REs in the human NTCP promoter. In summary, berberine-disrupted STAT5 signaling promoted mouse and human Ntcp/NTCP expression, resulting in enhanced bile acid uptake. Therefore, berberine may be a therapeutic candidate compound for maintaining bile acid homeostasis.

Berberine-induced Inactivation of Signal Transducer and Activator of Transcription 5 Signaling Promotes Male-specific Expression of a Bile Acid Uptake Transporter*

*This work is supported in part by internal research funds including the new faculty start fund and a seed grant (to X. C.) from St. John’s University. The authors declare that they have no conflicts of interest with the contents of this article.

†These authors contributed equally to this work.

2To whom correspondence should be addressed: Dept. of Pharmaceutical Sciences, St. John’s University, Queens, NY 11439. Tel.: 718-990-7985; Fax: 718-990-1877; E-mail: chengx@stjohns.edu.

The abbreviations used are: Ntcp, sodium-taurocholate co-transporting polypeptide; ALT, alanine aminotransferase; BBR, berberine; Bsep, bile salt export pump; JAK, Janus kinase; Oatp, organic anion transporting polypeptide; phospho-STAT, phosphorylated STAT; Slc, solute carrier transporter; qPCR, quantitative PCR; RE, response element; tauro-CA, taurocholic acid; HBV, hepatitis B virus; UPLC, ultra performance liquid chromatography-tandem mass spectrometer.

4602 JOURNAL OF BIOLOGICAL CHEMISTRY

© 2017 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 292, NO. 11, pp. 4602–4613, MARCH 17, 2017

© 2017 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
Results

**BBR Did Not Cause Apparent Liver Injury**—Hematoxylin and eosin staining showed that BBR treatment, at all doses tested, did not change liver histology (Fig. 1A). In addition, BBR administration once daily for 2 weeks did not markedly alter the liver to body weight ratios. Only 300 mg/kg BBR slightly decreased liver to body weight ratio (Fig. 1B). Moreover, BBR treatment did not increase serum levels of alanine aminotransferase (ALT), one of the common biomarkers of liver injury (Fig. 1C).

**BBR Enlarged Gallbladder**—BBR treatment caused marked enlargement of the gall bladder at doses higher than 10 mg/kg body weight. A representative image of a gall bladder from a BBR-treated (100 mg/kg body weight) mouse is shown in Fig. 2A.

**BBR Increased Expression of Major Genes Involved in Bile acid Biosynthesis and Transport in Male Mouse Liver**—We next assessed the expression of major genes involved in bile acid biosynthesis and transport in male mouse liver in response to BBR treatment. The enzyme that catalyzes the first and rate-limiting step in the classic pathway, cholesterol 7α-hydroxylase (Cyp7a1), was transcriptionally induced by BBR treatment at higher doses (30, 100, and 300 mg/kg body weight) (Fig. 2B). In addition, the transporters that play critical roles in transporting bile acids were also induced by BBR treatment (Fig. 2B). Ntcp and Bsep were both induced at doses equal and higher than 30 mg/kg body weight.

**BBR Increased Hepatic Extraction of Taurocholate**—Ntcp primarily transports taurine-conjugated bile acids, such as taurocholic acid (tauro-CA), into hepatocytes. As shown in Fig. 2C, BBR treatment decreased serum tauro-CA levels, and in the meantime increased liver tauro-CA levels, suggesting a stimulated tauro-CA extraction from mouse serum by the liver. Taken together, increased *de novo* production of bile acids and enhanced bile acid transport are responsible, at least partially, for the stimulated bile formation in BBR-treated mice (Fig. 2A).

**BBR Induced Ntcp/NTCP Expression and Increased[^3H]Taurocholate Uptake in Mouse and Human Hepatoma Cells**—In cultured mouse Hepa1c1c7 cells, BBR concentration-dependently induced Ntcp and Bsep mRNA levels at 12 (Fig. 3A) and 24 h (Fig. 3B). In contrast, BBR did not markedly induce Cyp7a1 mRNA expression (Fig. 3, A and B). Ntcp protein levels were elevated in Hepa1c1c7 cells exposed to BBR for 48 h, as revealed

---

**FIGURE 1.** BBR did not cause apparent liver injury in mice. Adult male C57BL/6 mice were treated with BBR (3, 10, 30, 100, and 300 mg/kg) or saline via gavage once daily for 2 weeks (*n* = 5/treatment). After treatment, the mouse serum and liver were collected. A, images of mouse liver after hematoxylin and eosin staining. B, the ratio of liver to body weight. C, serum ALT levels. The data are expressed as means ± S.E. (*n* = 5 mice/group). Asterisks (*) represent a statistical difference (p < 0.05). CONT, control.
Inactivation of STAT5-induced Ntcp/NTCP Expression

**FIGURE 2.** BBR increased the expression of bile acid biosynthesis and transport genes in male mouse liver, as well as hepatic extraction of taurocholate. A, representative image of gallbladders from BBR-treated adult male mice (100 mg/kg) and from mice receiving saline. B, qRT-PCR analysis of mRNA levels of Cyp7a1, Ntcp, and Bsep. C, concentrations of taurocholate in serum and liver from control and BBR-treated mice were quantified by established UPLC-MS/MS methods described under “Materials and Methods.” The data are expressed as means ± S.E. (n = 5 mice/group). Asterisks (*) represent a statistical difference (p < 0.05). CONT, control.

by immunocytochemistry (Fig. 3, C and D) and immunoblotting (Fig. 3, E and F). Specifically, consistent with its transporter function, the induced Ntcp protein appeared to be enriched in the membrane preparation (Fig. 3E). As expected, the [3H] taurocholate uptake activity of Ntcp protein was also increased ~2.5-fold in the BBR-treated Hepa1c1c7 cells compared with control (Fig. 3G). In cultured human Hep3B cells, NTCP mRNA was induced by BBR at 12 h (Fig. 4A) and 24 h (Fig. 4B). BBR did not increase CYP7A1 expression in Hep3B cells either (Fig. 4, A and B). NTCP protein levels were also increased after a 48-h treatment, as evidenced by immunocytochemistry (Fig. 4, C and D) and immunoblotting (Fig. 4, E and F). Uptake of [3H] taurocholate by human NTCP protein was increased ~3.0-fold in the BBR-treated Hep3B cells (Fig. 4G). Together, these results indicated that the induction of Ntcp/NTCP expression by BBR may be a conserved hepatic response existing in both human and mouse.

**BBR Induced NTCP Expression in Additional Human Hepatoma Cell Lines**—To ascertain the inductive effect of BBR on NTCP transcripts, we tested additional human hepatoma cell lines and identified three responsive lines, namely SK-Hep-1, SNU449, and SNU 475 (Fig. 5), in addition to Hep3B cells. Similar to the response seen in Hep3B cells, BBR concentration-dependently induced NTCP transcript levels after 24 h, with a more prominent effect at higher concentrations (10 and 30 μM).

**BBR Stimulated Ntcp/NTCP Promoter Activity in Cultured Hepatoma Cells**—Because BBR increased Ntcp/NTCP transcription in both mouse liver (Fig. 1B) and cultured hepatoma cells (Figs. 3 and 4), the influence of BBR on the promoter activity of mouse and human Ntcp/NTCP gene was investigated. As shown in Fig. 5, BBR treatment concentration-dependently increased the luciferase reporter activity, which was driven by the mouse Ntcp gene promoter (Fig. 6A) or the human NTCP gene promoter (Fig. 6B).

**BBR Decreased Nuclear Phospho-STAT5 and Phospho-JAK2 Levels Prior to Ntcp Induction**—Phospho-STAT5 has been implicated in prolactin-dependent Ntcp induction in rat (13). The impact of BBR treatment on protein levels of phospho-STAT5 was determined in cultured mouse and human hepatoma cells. Prior to the induction of Ntcp/NTCP protein (which was prominently detected after 48 h of BBR treatment), a significant reduction in phospho-STAT5 levels was observed in BBR-treated mouse and human hepatoma cells. As early as 12 h after BBR treatment, a concentration-dependent reduction in phospho-STAT5-positive cells was observed in mouse Hepa1c1c7 cells (Fig. 7, A and B) and in human Hep3B cells (Fig. 7, C and D). Furthermore, immunoblotting results confirmed that the nuclear phospho-STAT5 protein levels were decreased following BBR treatment (Fig. 7E). We next investigated what may cause the reduction in phosphorylation of STAT5. The Janus kinase (JAK) is a known upstream activator of STAT5. Because JAK is activated by phosphorylation, we determined the levels of phospho-JAK2 and -JAK3 by immunoblotting. We found decreased phospho-JAK2 but not JAK3 in the nucleus of BBR-treated Hepa1c1c7 cells, which occurred after a 12-h treatment (Fig. 7, G and H).

**Deletion of STAT5 Binding Sites from the Ntcp Promoter Resulted in Augmented Promoter Activity**—We performed *in silico* analysis of the mouse and human Ntcp/NTCP gene promoter sequences (3.5 kb upstream from transcription start site) and identified two and three consensus STAT5 binding sites in mouse and human Ntcp/NTCP gene promoter, respectively. We next investigated whether the identified STAT5 binding sites in the Ntcp promoter are responsible for BBR-induced Ntcp/NTCP expression by performing promoter partial deletion assay. In this study, we first engineered a series of truncated promoter constructs lacking one or more of these STAT5 binding sites. The schematic of truncated Ntcp promoter constructs...
is shown in Fig. 8A (mouse) and Fig. 8C (human). The full-length mouse Ntcp promoter construct (spanning from \(-3462\) to \(+125\) bp and containing two putative STAT5 sites; Fig. 8A) exhibited an inducible activity upon BBR treatment (Fig. 8B, black bars), whereas the truncated Ntcp promoter construct spanning from \(-790\) to \(+125\) bp lacking the more distal STAT5 site \((-1137\) to \(-1129\) bp) (Fig. 8A) showed constitutive activity without BBR treatment and a further elevated activity upon BBR treatment (Fig. 8B, gray bars). This result supports the idea that this distal STAT5 site has a negative influence on the Ntcp promoter activity, and its absence increased the basal and inducible promoter activity. For the truncated promoter excluding both STAT5 sites (spanning from \(-671\) to \(+125\) bp; Fig. 8A), its activity was restored to control level (empty vector), with or without BBR treatment (Fig. 8B, striped bars). It is possible that this proximal STAT5 site \((-679\) bp to \(-671\) bp) is part of the core promoter required for the assembly of the transcription initiation complex, and deleting this site may impair the transcription initiation even when cells were stimulated. With respect to human NTCP promoter, three putative STAT5 sites were identified (Fig. 7C). It seemed the effect was modest when the distal one or two sites were deleted; however, when
the third site (also most proximal to the transcription start site) was deleted, the greatest induction upon BBR treatment was observed (Fig. 8D, dotted bars). These results indicate that in the human NTCP promoter, the third and most proximal (−691 bp) STAT5 binding site was most likely to contribute to transcription repression by mediating p-STAT5 binding events.

**BBR Decreased the Binding of Phospho-STAT5 to the Human NTCP Promoter**—To investigate whether phospho-STAT5 actually binds to these putative binding sites (STAT5Res), we performed ChIP-qPCR analysis with human Hep3B cells. As shown in Fig. 9B, phospho-STAT5 protein was significantly enriched at −2164- and −691-bp STAT5Res, but not at the distal −2898-bp STAT5RE. BBR treatment significantly decreased the binding of phospho-STAT5 protein to both −2164- and −691-bp binding sites. This further demonstrated that the −2164- and −691-bp STAT5 binding sites are cis-regulatory elements mediating phospho-STAT5-dependent transcriptional repression on NTCP expression.

**Discussion**

In the present study, we reported that BBR reduced nuclear levels of phospho-STAT5 and phospho-JAK2 and decreased
the binding of phospho-STAT5 protein to the mouse and human Ntcp/NTCP gene promoter, thus relieving the transcriptional suppression by STAT5 and increasing Ntcp/NTCP gene expression. As a result of elevated Ntcp/NTCP levels, hepatic extraction of taurocholic acid, a typical substrate of Ntcp/NTCP transporter, was enhanced.

More than two decades ago, it was observed that a herbal plant goldenseal (Hydrastis canadensis) increased bile flow in humans (17). The major active component in the goldenseal is BBR; however, the underlying mechanism is not known. Our findings provide a mechanistic explanation for the above observation. Bile flow is influenced by enterohepatic circulation and biosynthesis of bile acids. In the present study, we showed that BBR increased expression of Ntcp and Bsep, two key bile acid transporters in the liver, as well as Cyp7a1, the rate-limiting enzyme in the classical bile acid biosynthesis pathway. All of these actions of BBR lead to an enhanced bile flow. In addition to goldenseal, BBR is also found in many other medicinally important plants such as Berberis aquifolium (Oregon grape) and Berberis vulgaris (barberry) (18). These plants have been used for over 3000 years in human history to treat various diseases (19). Therefore, it is of clinical importance to investigate the effect of BBR on transporters and its potential role in influencing drug-drug interactions where these herbal medicines are applied.

The relatively low bioavailability of BBR was suggested to be due, at least in part, to its poor intestinal absorption (20). BBR was identified as a substrate for P-glycoprotein, and the action of P-glycoprotein played an important role in the absorption of BBR (21). To accurately mimic the pharmacokinetics of BBR in vivo, we chose oral gavage as the administration route for BBR in the mouse study. By using a range of BBR doses (3–300 mg/kg body weight), we demonstrated that BBR affected bile acid homeostasis in mice, particularly at 100 mg/kg, without apparent liver toxicity (Figs. 1 and 2). The positive in vivo results thus served as a proof of concept of the novel influence of BBR on the regulation of bile acid transporters, and we proceeded to investigate the underlying molecular mechanisms using mouse and human hepatoma cells.

A previous report showed that activation of STAT5 signaling by prolactin induced rat Ntcp mRNA and transporter activity (12, 13, 22, 23). In the current study, however, a decrease in nuclear phospho-STAT5 protein level and a reduced binding of STAT5 to Ntcp gene promoter are found to precede BBR-induced Ntcp/NTCP expression. Regarding the apparent discrepancy between the previous and the current studies, the answer lies in a species-specific context. Whether the STAT5 signaling has a positive or negative impact on Ntcp expression depends on the species employed in the study. For example, it has been demonstrated that STAT5 signaling influences gender-dimorphic expression of Ntcp in the liver in a species-specific manner. STAT5 signaling is stimulated by male pattern growth hormone secretion. Humans, mice, and rats all exhibit similar gender-divergent growth hormone secretion patterns (24). However, liver Ntcp/NTCP expression is higher in females.
than in males in mice and humans (25) but higher in males than in females in rats (26). It was discovered that in mice and humans, increased STAT5 signaling by male pattern growth hormone repressed Ntcp/NTCP expression, which lead to a female-predominant Ntcp/NTCP mRNA expression pattern (25). In contrast, increased STAT5 signaling by growth hormone replacement mimicking male pattern secretion increased Ntcp expression in primary rat hepatocytes (27). Therefore, the same STAT5 signaling stimulated by the same growth hormone resulted in opposite impacts on Ntcp expression in different species. In addition, activation of estrogen receptor decreased nuclear levels of phospho-STAT5 in female mice (28), which correlated with higher Ntcp expression in female mice (25). Together, activation of STAT5 signaling increased Ntcp expression in rats but decreased its expression in mice and humans. On the other hand, inactivation of STAT5 signaling, by BBR or activation of estrogen receptor, increased Ntcp/NTCP expression in mice and humans.

In addition, because STAT5 signaling pathway similarly regulated mouse and human Ntcp/NTCP expression, the mouse
may serve as a better animal model compared with the rat for modeling human NTCP expression and regulation. Caution should be exercised when extrapolating data of Ntcp regulation between species.

In the present study, we showed that BBR decreased nuclear level of phospho-STAT5 protein. Furthermore, we demonstrated that a decrease in nuclear phosphorylated/active JAK2 was concomitant with the reduced phospho-STAT5 (Fig. 7, G and H). Cellular signals relayed by JAK family members often lead to phosphorylation and activation of STATs (29, 30). Recently, BBR chloride has been identified as a novel inhibitor for JAK3 with specificity and potency in a myeloid cell line (31). However, in hepatocytes we identified JAK2 not JAK3, whose activation (as reflected by phosphorylated protein level) was decreased upon BBR treatment. Therefore, the reduced phosphorylated JAK2 consequently contributed, at least in part, to the decreased phosphorylation of STAT5.

In mice receiving BBR via gavage for 2 weeks, we observed markedly enlarged gallbladder volume on the day of sacrifice (between 9:00 and 9:30 a.m.). This observation indicates elevated gallbladder filling activities in BBR-treated mice. It has been reported that FGF15 (FGF19 in human) derived from ileum and G protein-coupled bile acid receptor (TGR5) expressed in gallbladder play important roles in gallbladder filling via independent mechanisms (32, 33). The expression of ileal FGF15 was not affected by BBR treatment (data not shown), and thus it is of interest to investigate the involvement of TGR5 signaling between the IgG and corresponding phospho-STAT5 group (p < 0.05). Asterisks (*) represent a statistical difference of phospho-STAT5 binding between the IgG and corresponding phospho-STAT5 group (p < 0.05). #, indicating statistical differences of phospho-STAT5 binding between the control and BBR treatment group (p < 0.05).
expression in hepatocytes, and consequently, its usage may increase the risk of hepatitis virus infection. In this regard, our study calls for caution when employing BBR-containing herbal medicines as antibiotics against bacterial infection and recommends risk assessment for potential hepatitis virus infection.

Materials and Methods

Chemicals—Berberine chloride was purchased from Alfa Aesar (catalog no. 50330221; Ward Hill, MA). Formaldehyde was purchased from TCI America (Cambridge, MA). Paraformaldehyde was purchased from Acros (catalog no. AC4167850003; Belgium, Brussels). The ChIP assay kit (EZ-ChIP, catalog no. 17-391) was purchased from Millipore (Billerica, MA). [3H]Taurocholic acid (1–5 Ci/mmol) was purchased from PerkinElmer Life Sciences. The Dual-Luciferase reporter assay system (catalog no. E1910) was purchased from Promega (Madison, WI). Accutase cell detachment solution (catalog no. 083300; Sigma) was acquired from EMD Millipore (Billerica, MA). [3H]Taurocholic acid in mouse serum and liver (38, 39).

Liver—Animals—Eight-week-old adult male C57BL/6 mice were purchased from the Jackson Laboratory, maintained in an environmentally controlled facility at the University of Kansas Medical Center with lights on from 6:00 a.m. to 6:00 p.m., and allowed free access to water and standard rodent chow (Teklad: Harlan, Indianapolis, IN). At approximately 2 months of age, the mice were treated with either BBR or saline (vehicle) via gavage twice daily for 2 weeks. Upon completion of treatment, gallbladder, blood, and liver from control and treated mice were collected between 8:30 and 10:00 a.m. and treated mice were collected between 8:30 and 10:00 a.m. The University of Kansas Medical Center Animal Care and Use Committee approved the protocols for the care and use of the animals.

Quantification of Taurocholic Acid in Mouse Serum and Liver—The blood was allowed to coagulate and centrifuged at 10,000 × g for 15 min. The resultant supernatant (serum) was collected for analysis. Total bile acids were extracted from mouse serum and liver as previously described (38, 39). UPLC-MS/MS analysis was performed to determine the levels of taurocholic acid in mouse serum and liver (38, 39).

Quantification of ALT Levels in Mouse Serum—Freshly collected mouse serum samples were analyzed by enzymatic colorimetric assays using ALT assay kit in accordance with manufacturer’s protocols (Pointe Scientific).

Histology—Fresh liver samples were embedded in 10% zinc formalin (Fisher Scientific). Liver sections (4 µm in thickness) were stained with hematoxylin and eosin for the evaluation of liver histology and hepatic injury.

Cell Culture and Treatment—Mouse hepatoma cells Hepa1c1c7 (ATCC, catalog no. CRL-2026) and human hepatoma cells Hep3B (ATCC, catalog no. HB-8064) were maintained in minimum essential medium α MEM (Mediatech), respectively, supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO2. Human hepatoma cells SK-HEP-1, SNU449, and SNU475 were cultured in either DMEM (high glucose with pyruvate; Gibco, catalog no. 11995081) or PRMI-1640 medium (Gibco, catalog no. A1049101) as previously described (40). The cells were seeded into 6-well plates (3 × 10^5/well) or chamber slides (1.0 × 10^5/chamber) and allowed to adhere for 16–24 h before BBR treatment. The cells were then incubated with or without BBR at the specified concentrations for indicated duration. The medium was refreshed every 24 h if the treatment was longer than 24 h.

RNA Extraction and Quantification—Total RNA was extracted using RNA Bee reagent (Fisher Scientific, Inc.) or TRIzol RNA extraction reagent (Life Technologies, Inc.) as per the manufacturer’s instructions. RNA concentrations were quantified at 260 nm with a spectrophotometer (Eppendorf Biocounter, Hauppauge, NY). RNA samples with an A 260/A 280 ratio between 1.8 and 2.0 were used for further analysis.

RT-qPCR Assay—Reverse transcription and qPCR were performed as previously described (41). Briefly, total RNA was first reverse-transcribed into cDNA using SuperScript II reverse transcriptase (Life Technologies, Inc.) following the manufacturer’s instructions. Quantitative PCR was performed using SYBR Select Master Mix (Life Technologies, Inc.) in a StepOne Plus (Applied Biosystems, Foster City, CA) or AriaMx (Agilent Technologies, Santa Clara, CA) real time qPCR system. The data were calculated according to the comparative ΔΔCT method and presented as relative fold of the control. Primers used in qRT-PCR were designed with Primer3 software (version 4), were synthesized by Integrated DNA Technologies (Coralville, IA) or Eurofins Genomics (Huntsville, AL), and are listed in Table 1. All primers were validated to confirm optimal amplification efficiency prior to qPCR analysis.

Immunocytochemistry—The cells were cultured in chamber slides and treated with or without BBR. Following fixation (4% freshly prepared formaldehyde) and permeation (0.1% Triton X-100 in PBS), the cells were incubated with BLOXALL Solution (Vector Laboratories) to eliminate endogenous peroxidase activity. Upon blocking in 5% goat serum in PBS, the cells were incubated with anti-Ntcp/NTCP antibody (catalog no. ab131084; Abcam, Cambridge, MA; 1:200) or anti-phosphoSTAT5 (Tyre99) (catalog no. 4322; Cell Signaling Technology, Danvers, MA; 1:100) in 2.5% goat serum in PBS at 4 °C overnight followed by incubation with Pierce goat anti-rabbit IgG (H + L) biotin-conjugated secondary antibody (Thermo Fisher Scientific) at room temperature for 2–4 h. Cells were then
washed and incubated with Pierce high sensitivity streptavidin-HRP (Thermo Fisher Scientific) for 1 h at room temperature. Color development was achieved using ImmPACT NovaRed peroxidase (HRP) substrate (Vector Laboratories) according to the manufacturer’s instructions.

Protein Extraction and Western Blots—Upon treatment completion, the cells were harvested and membrane and cytoplasmic portions were separated and extracted using Mem-PER Plus membrane protein extraction kit (catalog no. 89842, Thermo Fisher Scientific). The nuclear and cytoplasmic proteins were separated and extracted using a NE-PER nuclear and cytoplasmic extraction kit (catalog no. 78835, Thermo Fisher Scientific). Protein concentrations were quantified with a spectrophotometer and separated by a SDS-PAGE (12% gel for Ntcp/NTCP protein; 10% gel for phospho-STAT5 and phospho-JAK2; 15% gel for histone H3). After blotting, PVDF membranes were blocked for 2 h in 5% nonfat milk (for Ntcp/NTCP) or 5% BSA (for phospho-STAT5 and phospho-JAK2) in TBS. The membranes were washed in TBS and incubated with anti-Ntcp/NTCP antibody (catalog no. ab31084, Abcam; 1:1000 for Hep3B cells and 1:600 for Hepa1c1c7 cells) in 2.5% nonfat milk, anti-phospho-STAT5 (Tyr694) (catalog no. 4322, 1:1000; Cell Signaling Technology), or phospho-JAK2 (Tyr1007/1008) (catalog no. 81E2, 1:1000; Cell Signaling Technology) followed by 30 min of incubation with Pierce high sensitivity streptavidin-HRP (1:5000, Thermo Fisher Scientific) in TBS containing 0.1 mg/ml BSA and 10 mM HEPES at room temperature. Membranes were washed again, followed by 30 min of incubation with Pierce high sensitivity streptavidin-HRP (1:5000, Thermo Fisher Scientific) in TBS containing 0.1 mg/ml BSA and 10 mM HEPES at room temperature. β-Actin and histone H3 were used as loading controls. Immunoreactive protein bands were detected with Immobilon Western chemiluminescent HRP substrate (catalog no. WBKL S00 50, EMD Millipore, Billerica, MA).

[^3]H\text{Taurocholic Acid Uptake Assay}—The cells were treated with or without BBR for 48 h in T-75 flasks and detached with the BD Accutase cell detachment solution and counted immediately before the uptake assay. The uptake assay was performed following protocols previously described (2, 3, 42). Briefly, aliquots of cells (1.5 × 10⁶/aliquot) were incubated at 37 °C with uptake buffer containing [3H]taurocholic acid (3–10 μCi) for 6 h with gentle shaking every 20 min. The cells were then washed with ice-cold PBS, lysed, and placed in 5 ml of scintillation fluid. Radioactivity was measured in a Packard TRI-CARB 1900CA liquid scintillation analyzer (Packard Instrument, Downers Grove, IL).

Cloning of Full-length and Truncated Ntcp/NTCP Gene Promoter—BAC clones containing either mouse Ntcp or human NTCP promoters were purchased from BACPAC resources (Children’s Hospital Oakland, Oakland, CA). The proximal portion of the Ntcp/NTCP gene promoter (3.5 kb upstream of the transcription start site) was defined as the full-length promoter and cloned from BAC clones into the pGL3-basic vector (Promega) with KpnI and XhoI restriction sites and primer pairs specific to mouse Ntcp promoter or human NTCP promoter. For truncated promoter constructs, the locations of the forward primers and the shared reverse primer are depicted in Fig. 8A (mouse) and 8C (human). Primer sequences and restriction sites used are listed in Table 2. The accuracy of all promoter constructs was confirmed by DNA sequencing (Eurofins Genomics, Huntsville, AL).

Transfection and Dual-Luciferase® Reporter Assay—Corresponding promoter constructs were transfected into mouse Hepa1c1c7 or human Hep3B cells, using Lipofectamine 2000 transfection reagent (Life Technologies, Inc.) following the manufacturer’s instructions. Briefly, the cells were seeded onto 24-well tissue culture plates 1 day before transfection. Plasmid DNA (0.8 μg) of the pGL3 basic vector (catalog no. E1751, Promega) or promoter-containing pGL3 basic vector, plus 10 ng of pRL Renilla luciferase reporter vector (catalog no. E2261, Promega) was mixed with Lipofectamine 2000 at 1:3 ratio (2.4 μl) and dispensed to each well. After 24 h of incubation, transfection medium was replaced with fresh growth medium containing BBR at indicated concentrations. After a 48-h treatment, the cells were harvested and processed for Dual-Luciferase® reporter assays per the manufacturer’s instructions (catalog no. E1910, Promega).

ChIP-qPCR Assay—The putative STAT5 binding sites present in the 3.5-kb promoter regions of the mouse or human Ntcp/NTCP gene promoters were identified using on-line

### Table 1

| Gene          | Accession no.   | Forward primer | Reverse primer | Amplicon |
|---------------|-----------------|----------------|----------------|----------|
| Gapdh         | NM_000804.3 (m) | TGTGAGACCTAAGGTCAGG | ACTGCTCCGGTTGACCTCCA | 123 bp   |
| Cyp7a1        | NM_007824.2 (m) | GCGAGCGGTCTGAGATTATTG | ACACTCTAGACCCAGGCTCTT | 159 bp   |
| Cyp8b1        | NM_010012.3 (m) | AGTGTGACCTGCCCCTCCAAT | CTGTGCTGCAAACTGAGTCT | 169 bp   |
| Cyp27a1       | NM_28264.5 (m)  | GAGAGGCTCCACGGGTCACCA | TCACCGTTGAGGGGCTTACG | 106 bp   |
| Cyp7b1        | NM_007825.4 (m) | ATCCCGCTGCTGACTGGAAA | GAAGCAGTGTGGCTGGAACA | 122 bp   |
| Oatp1a1       | NM_013797.5 (m) | GAGGACGTTGCGCTACAT | GCGGTGTTGCTTGCTTGT | 127 bp   |
| Oatp1a4       | NM_030687.1 (m) | GAGAGGACATTGCACTGACAT | CTGTGGCTGCTGACTGGAG | 147 bp   |
| Oatp1b2       | NM_020495.1 (m) | GAACATCGACATCAGCAAGA | GCGGCAGAAATATCTTGCA | 87 bp    |
| Ntcp          | NM_011387.2 (m) | GTCGCTCTACAAAGGATATT | ACAACACAGGATGGGGAAGA | 104 bp   |
| Bsep          | NM_021022.3 (m) | GACACAGATGATCCTGATTG | CACACAAGCCGCTTACAT | 147 bp   |
| GAPDH         | NM_001256799.2 (h) | GAGAGGCTGTCGGCCTATTT | AGTAGGATGTTAGCAGTG | 231 bp   |
| Cyp7a1        | NM_000780.3 (h) | GAGAGGCGAACAGGCTGAC | ATCGGTTCAATGCTTGTG | 276 bp   |
| Oatp1b1       | NM_000466.4 (h) | ACTGTCTTCTGCTGCTGG | TATTTSGATTTGCGACAG | 106 bp   |
| OATP1B3       | NM_019844.3 (h) | AAGACGCCTCAGAATGGGTTT | ATGCGGGGCTGCTGACTGG | 117 bp   |
| NTCP          | NM_003049.3 (h) | CTGGACTAGAGAGGAGGCT | GTGCGAGGCTGAATTGGA | 107 bp   |
| BSEP          | NM_003742.2 (h) | TGTGGAGGTTTCTGCTGAC | ACCACACACTGGCTATTC | 203 bp   |
transcription factor binding site searching program Alibaba 2.1. The binding of p-STAT5 to these putative STAT5 binding sites was analyzed by ChIP-qPCR assays in human hepatoma Hep3B cells using an EZ-ChIP assay kit as per the manufacturer’s instructions. Hep3B cells cultured in T-75 flask were treated with water (vehicle) or BBR (5 and 10 μM) for 12 h followed by cross-linking with formaldehyde at a final concentration of 1% for 10 min at room temperature. Sonication of cell genomic DNA was achieved by using Sonic Dismembrator ultrasonic processor (Fisher Scientific) at the following specific settings: amplitude 30; 10 s on and 30 s off, 26 cycles. Confirmed by agarose-gel electrophoresis, the resultant DNA fragments predominantly ranged in length from 200 to 1,000 bp. Sonicated samples were proceeded with chromatin immunoprecipitation using an IgG control antibody or anti-p-STAT5 antibody (catalog no. 9351, phospho-STAT5 (Tyr694), Cell Signaling Technology). The precipitated DNA fragments were then purified and analyzed by qPCR. The primers flanking the identified STAT5 binding sites present in the human NTCP gene promoter are depicted in Fig. 9A, and their sequences are listed in Table 3.

**Statistical Analysis**—The data are expressed as means ± S.E. The data from the two-sample comparison were analyzed by Student’s t test. The data of three or more sample comparison were analyzed by one-way analysis of variance, followed by Duncan’s post-hoc test using statistics software (StatSoft Inc., Tulsa, OK). Statistical significance was set at p < 0.05.

**Author Contributions**—P. B., Y. L., and X. C. participated in research design, and P. B., Y. L., Yu. Z., Yo. Z., and X. C. conducted experiments, performed data analysis, and wrote or contributed to the writing of the manuscript.

**Acknowledgments**—We thank Dr. Amaia Lujambio (Mount Sinai) for generously providing us human hepatocellular carcinoma cells and Dr. Zhe-Sheng Chen (St. John’s University) for the assistance with [3H] taurocholic acid uptake assay. We are also grateful for the insightful comments from Dr. Curtis D. Klaassen.

### References

1. Hagenbuch, B., and Dawson, P. (2004) The sodium bile salt cotransporter family SLC10. *Pflugers Arch.* **447**, 566–570
2. Hagenbuch, B., and Meier, P. J. (1994) Molecular cloning, chromosomal localization, and functional characterization of a human liver Na+/bile acid cotransporter. *J. Clin. Invest.* **93**, 1326–1331
3. Hagenbuch, B., Stieger, B., Fuguet, M., Lilbret, H., and Meier, P. J. (1991) Expression cloning function and characterization of the hepatocyte Na+/bile acid cotransport system. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10629–10633
4. Chiang, J. Y. (2009) Bile acids: regulation of synthesis. *J. Lipid Res.* **50**, 1952–1966
5. You, G., and Morris, M. E. (2014) Drug Transporters: Molecular Characterization and Role in Drug Disposition, 2nd Ed., John Wiley & Sons, Inc., New York
6. Ayrton, A., and Morgan, P. (2001) Role of transport proteins in drug absorption, distribution and excretion. *Xenobiotica* **31**, 469–497
7. You, G. (2004) The role of organic ion transporters in drug disposition: an update. *Curr. Drug Metab.* **5**, 55–62
8. Green, R. M., Beier, D., and Gollan, J. I. (1996) Regulation of hepatocyte bile salt transporters by endotoxin and inflammatory cytokines in rodents. *Gastroenterology* **111**, 193–198
9. Gartung, C., Ananthanarayanan, M., Rahman, M. A., Schuele, S., Nundy, S., Soroka, C. I., Stolz, A., Suchy, F. J., and Boyer, J. L. (1996) Down-regulation of expression and function of the rat liver Na+/bile acid cotransporter in extrahepatic cholestasis. *Gastroenterology* **110**, 199–209
10. Kang, I., Wang, J., Cheng, J., Cao, Z., Chen, R., Li, H., Liu, S., Chen, X., Sui, J., and Lu, F. (2016) Down-regulation of NTCP expression by cyclin D1 in hepatitis B virus-related hepatocellular carcinoma has clinical significance. *Oncotarget* 10:18362/oncotarget.10241
11. Zollner, G., Wagner, M., Fickert, P., Silbert, D., Fuchsbiicher, A., Zatloukal, K., Denk, H., and Trauner, M. (2005) Hepatobiliary transporter expression in human hepatocellular carcinoma. *Liver Int.* **25**, 357–379
12. Ganguly, T. C., Liu, Y., Hyde, J. F., Hagenbuch, B., Meier, P. J., and Vore, M. (1994) Prolactin increases hepatic Na+/taurocholate co-transport activity and messenger RNA post partum. *Biochem. J.* **303**, 33–36
13. Ganguly, T. C., O’Brien, M. L., Karpen, S. J., Hyde, J. F., Suchy, F. J., and Vore, M. (1997) Regulation of the rat liver sodium-dependent bile acid cotransporter gene by prolactin: mediation of transcriptional activation by Stat5. *J. Clin. Invest.* **99**, 2906–2914
14. Liu, X., Zhang, X., Ye, L., and Yuan, H. (2016) Protective mechanisms of berberine against experimental autoimmune myocarditis in a rat model. *Biomed. Pharmacoch.* **79**, 222–230

### Table 2

| NTCP/NTCP promoter | Promoter region | Restriction site | Forward primer sequence |
|--------------------|-----------------|-----------------|-------------------------|
| pGL3-NTCP-3462     | −3462 to +125   | KpnI            | P1: CAGGCTACGTTAGACACAGCACATCTGGTTCATAGT |
| pGL3-NTCP-790      | −790 to +125    | MluI            | P2: GACCAAGTCCTACGGCGTTCGTGGTT |
| pGL3-NTCP-671      | −671 to +125    | HindIII         | P3: CTTCCCTGCTACGGCTCAGTATAG |
| pGL3-NTCP-3403     | −3403 to +75    | XhoI            | CAGCTGAGACCTACACCCCTCTCTCTT |
| pGL3-NTCP-2398     | −2398 to +75    | KpnI            | P1: CAGGCTACGTTAGACACAGCACATCTGGTTCATAGT |
| pGL3-NTCP-1021     | −1021 to +75    | KpnI            | P2: CAGGCTACGTTAGACACAGCACATCTGGTTCATAGT |
| pGL3-NTCP-596      | −596 to +75     | KpnI            | P3: CAGGCTACGTTAGACACAGCACATCTGGTTCATAGT |
| Shared reverse primers (m) |                  |                 |                         |
| P1: CAGGCTACGTTAGACACAGCACATCTGGTTCATAGT |
| P2: GACCAAGTCCTACGGCGTTCGTGGTT |
| P3: CTTCCCTGCTACGGCTCAGTATAG |

### Table 3

| Promoter/target site | Target site location | Forward primer sequence |
|----------------------|----------------------|-------------------------|
| Human NTCP promoter/p-STAT5 | −2898 to −2890 bp | AGGGAACACCTTATACGTCTGGTTC |
|                      | −2164 to −2156 bp   | ATCCATACACAGCATGCTGCTCAA |
|                      | −691 to −683 bp     | ACAGGACATCTCCACAGCAGG |

| Amplicon | bp |
|----------|----|
| P1        | 187|
| P2        | 198|
| P3        | 137|

| Promoter/target site | Target site location | Reverse primer sequence |
|----------------------|----------------------|-------------------------|
| Human NTCP promoter/p-STAT5 | −2898 to −2890 bp | AGATGAACACGACATGCTGCTGATG |
|                      | −2164 to −2156 bp   | TCTCCATACACGGCTGCTGCTCAA |
|                      | −691 to −683 bp     | GGCTGCTCCACTCTTGGCAG |

| Amplicon | bp |
|----------|----|
| P1        | 187|
| P2        | 198|
| P3        | 137|

### Author Contributions

P. B., Y. L., and X. C. participated in research design, and P. B., Y. L., Yu. Z., Yo. Z., and X. C. conducted experiments, performed data analysis, and wrote or contributed to the writing of the manuscript.
Inactivation of STAT5-induced Ntcp/NTCP Expression

15. Li, Y. H., Xiao, H. T., Hu, D. D., Fatima, S., Lin, C. Y., Mu, H. X., Lee, N. P., and Bian, Z. X. (2016) Berberine ameliorates chronic relapsing dextran sulfate sodium-induced colitis in C57BL/6 mice by suppressing Th17 responses. *Pharmacol. Res.* **110**, 227–239

16. Zhu, T., Li, L. L., Xiao, G. F., Luo, Q. Z., Liu, Q. Z., Yao, K. T., and Xiao, G. H. (2015) Berberine increases doxorubicin sensitivity by suppressing STAT3 in lung cancer. *Am J. Chin. Med.* **43**, 1487–1502

17. Newall, C. A., Anderson, L. A., and Phillipson, J. D. (1996) *Herbal Medicines: A Guide for Health-Care Professionals*, 2nd Ed., The Pharmaceutical Press, London, UK

18. Kumar, A., Ekavali, Chopra, K., Mukherjee, M., Pottabathini, R., and Dhull, D. K. (2015) Current knowledge and pharmacological profile of berberine: an update. *Eur J. Pharmacol.* **761**, 288–297

19. Abd El-Wahab, A. E., Ghareeb, D. A., Sarhan, E. E., Abu-Serie, M. M., and El Demellawy, M. A. (2013) *In vitro* biological assessment of *Berberis vulgaris* and its active constituent, berberine: antioxidants, anti-acyethylcholinesterase, anti-diabetic and anticancer effects. *BMC Complement Altern. Med.* **13**, 218

20. Chen, W., Miao, Y. Q., Fan, D. J., Yang, S. S., Lin, X., Meng, L. K., and Tang, X. (2011) Bioavailability study of berberine and the enhancing effects of TPGS on intestinal absorption in rats. *AAPS PharmSciTech.* **12**, 705–711

21. Pan, G. Y., Wang, G. J., Liu, X. D., Fawcett, J. P., and Xie, Y. Y. (2002) The involvement of P-glycoprotein in berberine absorption. *Pharmacol. Toxicol.* **91**, 193–197

22. Ganguly, T., Hyde, J. F., and Vore, M. (1993) Prolactin increases Na⁺/taurocholate cotransport in isolated hepatocytes from postpartum rats and ovariectomized rats. *J. Pharmacol. Exp. Ther.* **267**, 82–87

23. Liu, Y., Ganguly, T., Hyde, J. F., and Vore, M. (1995) Prolactin increases mRNA encoding Na⁺-TC cotransport polypeptide and hepatic Na⁺-TC cotransport. *Am J. Physiol.* **268**, G11–G17

24. Jaffe, C. A., Ocampo-Lim, B., Guo, W., Krueger, K., Sugahara, I., DeMott-Friberg, R., Berman, M., and Barkan, A. L. (1998) Regulatory mechanisms of growth hormone secretion are sexually dimorphic. *J. Clin. Invest.* **102**, 153–164

25. Cheng, X., Buckley, D., and Klaassen, C. D. (2007) Regulation of hepatic bile acid transporters Ntcp and Bsep expression. *Biochem. Pharmacol.* **74**, 1665–1676

26. Simon, F. R., Fortune, J., Iwahashi, M., Qadri, I., and Sutherland, E. (2004) Multihormonal regulation of hepatic sinusoidal Ntcp gene expression. *Am. J. Physiol. Gastrointest. Liver Physiol.* **287**, G782–G794

27. Cao, J., Gowri, P. M., Ganguly, T. C., Wood, M., Hyde, J. F., Talamantes, F., and Vore, M. (2001) PRL, placental lactogen, and GH induce Na⁺/taurocholate-cotransporting polypeptide gene expression by activating signal transducer and activator of transcription-5 in liver cells. *Endocrinology* **142**, 4212–4222

28. Wyzsomierski, S. L., Yeh, J., and Rosen, J. M. (1999) Glucocorticoid receptor/signal transducer and activator of transcription 5 (STAT5) interactions enhance STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation. *Mol. Endocrinol.* **13**, 330–343

29. Gao, B., Wang, H., Laďfil, D., and Feng, D. (2012) STAT proteins: key regulators of anti-viral responses, inflammation, and tumorigenesis in the liver. *J. Hepatol.* **57**, 430–441

30. Heim, M. H. (1999) The Jak-STAT pathway: cytokine signalling from the receptor to the nucleus. *J. Recept. Signal Transduct. Res.* **19**, 75–120

31. Kim, B. H., Kim, M., Yin, C. H., Jee, J. G., Sandoval, C., Lee, H., Bach, E. A., Hahn, D. H., and Baeg, G. H. (2011) Inhibition of the signalling kinase JAK3 alleviates inflammation in monoarthritic rats. *Br. J. Pharmacol.* **164**, 106–118

32. Choi, M., Moschetta, A., Bookout, A. L., Peng, L., Umetani, M., Holmstrom, S. R., Suino-Powell, K., Xu, H. E., Richardson, J. A., Gerard, R. D., Mangelsdorf, D. J., and Kliewer, S. A. (2006) Identification of a hormonal basis for gallbladder filling. *Nat. Med.* **12**, 1253–1255

33. Li, T., Holmstrom, S. R., Kir, S., Umetani, M., Schmidt, D. R., Kliewer, S. A., and Mangelsdorf, D. J. (2011) The G protein-coupled bile acid receptor, TGR5, stimulates gallbladder filling. *Mol. Endocrinol.* **25**, 1066–1071

34. Watashi, K., Urban, S., Li, W., and Wakita, T. (2014) NTCP and beyond: opening the door to unveil hepatitis B virus entry. **Int. J. Mol. Sci.** **15**, 2892–2905

35. Yan, H., Peng, B., Liu, Y., Xu, G., He, W., Ren, B., Jing, Z., Sui, J., and Li, W. (2014) Viral entry of hepatitis B and D viruses and bile salts transportation share common molecular determinants on sodium taurocholate cotransporting polypeptide. *J. Virol.* **88**, 3273–3284

36. Ni, Y., Lempp, F. A., Mehrle, S., Nkongolo, S., Kaufman, C., Falth, M., Stindt, J., König, C., Nassal, M., Kubitz, R., Sültmann, H., and Urban, S. (2014) Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. *Gastroenterology* **146**, 1070–1083

37. Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y., Peng, B., Wang, H., Fu, L., Song, M., Chen, P., Gao, W., Ren, B., et al. (2011) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* **1**, e00049

38. Alnouti, Y., Csanaky, I. L., and Klaassen, C. D. (2008) Quantitative-profiling of bile acids and their conjugates in mouse liver, bile, plasma, and urine using LC-MS/MS. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **873**, 209–217

39. Zhang, Y., and Klaassen, C. D. (2010) Effects of feeding bile acids and a bile acid sequestrant on hepatic bile acid composition in mice. *J. Lipid Res.* **51**, 3230–3242

40. Bollard, J., Miguela, V., Ruiz de Galarreta, M., Venkatesh, A., Bian, C. B., Roberto, M. P., Tovar, V., Sia, D., Molina-Sanchez, P., Nguyen, C. B., Nakagawa, S., Llovet, J. M., Hoshida, Y., and Lujambio, A. (2016) Palbociclib (PD-0332991), a selective CDK4/6 inhibitor, restricts tumour growth in preclinical models of hepatocellular carcinoma. *Gut* **10.1136/gutjnl-2016-312686

41. Liu, J., Lu, H., Li, Y. F., Lei, X., Cui, J. Y., Ellis, E., Strom, S. C., and Klaassen, C. D. (2014) Potency of individual bile acids to regulate bile acid synthesis and transport genes in primary human hepatocyte cultures. *Toxicol. Sci.* **141**, 538–546

42. Hagenbuch, B., Lüübert, B., Stieger, B., and Meier, P. J. (1990) Expression of the hepatocyte Na⁺/bile acid cotransporter in Xenopus laevis oocytes. *J. Biol. Chem.* **265**, 5357–5360