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Regulation of hepatic bile acid transporters Ntcp and Bsep expression

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ABSTRACT

Sodium-taurocholate cotransporting polypeptide (Ntcp) and bile salt export pump (Bsep) are two key transporters for hepatic bile acid uptake and excretion. Alterations in Ntcp and Bsep expression have been reported in pathophysiological conditions. In the present study, the effects of age, gender, and various chemicals on the regulation of these two transporters were characterized in mice. Ntcp and Bsep mRNA levels in mouse liver were low in the fetus, but increased to its highest expression at parturition. After birth, mouse Ntcp and Bsep mRNA decreased by more than 50%, and then gradually increased to adult levels by day 30. Expression of mouse Ntcp mRNA and protein exhibit higher levels in female than male livers. No gender difference exists in BSEP/Bsep expression in human and mouse livers. Hormone replacements conducted in gonadectomized, hypophysectomized, and lit/lit mice indicate that female-predominant Ntcp expression in mouse liver is due to the inhibitory effect of male-pattern GH secretion, but not sex hormones. Ntcp and Bsep expression are in general resistant to induction by a large battery of microsomal enzyme inducers. Administration of cholestyramine increased Ntcp, whereas chenodeoxycholic acid (CDCA) increased Bsep mRNA expression. In conclusion, mouse Ntcp and Bsep are regulated by age, gender, cholestyramine, and bile acid, but resistant to induction by most microsomal enzyme inducers.

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1. Introduction

Bile acids secreted into bile undergo enterohepatic circulation (97%). Both hepatic uptake and biliary excretion of bile acids are driven by transport proteins. Bile acid uptake from blood into liver is mediated mainly by the Na⁺-taurocholate cotransporting polypeptide (Ntcp), and efflux from liver into bile by the bile salt export pump (Bsep).

Ntcp (gene symbol *Slc10a1*) has been cloned in various species [1–4], and localized to the basolateral membrane of

hepatocytes. Ntcp transports all physiological bile acids [5–7]. Bsep (gene symbol *Abcb11*) is responsible for the canalicular excretion of bile acids [8,9]. Targeted inactivation of the mouse Bsep gene results in mild, non-progressive, but persistent intrahepatic cholestasis [10]. In contrast, a mutation in human BSEP gene is responsible for progressive familial intrahepatic cholestasis subtype 2 (PFIC-2) [11,12].

Ntcp mRNA and protein expression is uniformly down-regulated in all experimental models of cholestasis and liver disease [13–17]. In contrast, Bsep expression is only modestly

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Abbreviations: bDNA, branched DNA signal amplification assay; Bsep, bile salt export pump; DHT, 5 α -dihydrotestosterone; E2, 17 β -estradiol; GHRH, growth hormone releasing hormone; GNX, gonadectomy; HX, hypophysectomy; Ntcp, sodium-taurocholate cotransporting polypeptide.

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impaired during cholestasis even with complete bile-duct obstruction [16,18].

Bile acids in general are thought to decrease Ntcp expression through farnesoid X receptor (FXR)-small heterodimer partner (Shp) pathway, and increase Bsep expression through direct FXR activation [19–21].

In rats, Ntcp mRNA expression is male-predominant, which is due to the inhibitory effect of female-pattern GH secretion [22]. However, it is not known whether mouse, rat, and human Ntcp/NTCP expression share similar gender predominance and underlying regulatory mechanisms. Except for the influence of bile acids, the regulation of Ntcp and Bsep is not complete. A few studies have been performed in rats, some in humans, but less in mice. Therefore, in the present study, we determined the effects of age, gender, and various chemicals, including microsomal enzyme inducers, cholestyramine, and chenodeoxycholic acid (CDCA) on the regulation of Ntcp and Bsep mRNA expression in mouse livers and evaluate their human relevance.

2. Materials and methods

2.1. Materials

Sodium chloride, HEPES sodium salt, HEPES free acid, lithium lauryl sulfate, EDTA, and D-(+)-glucose were purchased from Sigma–Aldrich (St. Louis, MO). Micro-O-protect was purchased from Roche Diagnostics (Indianapolis, IN). Formaldehyde, 3-(N-morpholino)propanesulfonic acid, sodium citrate, and NaHCO₃ were purchased from Fischer Chemicals (Fairlawn, NJ). Chloroform, agarose, and ethidium bromide were purchased from AMRESCO Inc. (Solon, OH). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was a gift from Dr. Karl Rozman (University of Kansas Medical Center, Kansas City, KS), and oltipraz was a gift from Dr. Steven Safe (Texas A&M University, TX). Polychlorinated biphenyl 126 (PCB 126) was obtained from AccuStandard (New Haven, CT). All other chemicals, unless otherwise indicated, were purchased from Sigma–Aldrich Co. All hormones and their respective vehicles used in the present study were purchased from Innovative Research of America (Sarasota, FL).

2.2. Animals and breeding

Eight-week-old adult male and female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine), and housed according to the American Animal Association Laboratory Animal Care guidance. For the ontogenic study, mice were bred in the animal facilities at the University of Kansas Medical Center. Livers from male and female C57BL/6 mice were collected at –2, 0, 5, 10, 15, 22, 30, 35, 40, 45, and 56 days of age ($n = 5/\text{gender}/\text{age}$). Livers were collected and snap-frozen in liquid nitrogen, and stored at -80°C . Breeding pairs of pregnane X receptor (PXR)-null mice in the C57BL/6 background were kindly provided by Dr. Frank J. Gonzalez [23].

For the animal experiments performed in the present study, unless otherwise indicated, mouse tissues were collected between 8:30 a.m. and 11:00 a.m.

2.3. Human liver tissues

One hundred individual human liver tissues (from 60 men and 40 women) were purchased from Xenotech (Lenexa, KS). Human livers were cut into pieces and preserved in the TRIzol[®] Reagent (Invitrogen, Carlsbad, CA).

2.4. Microsomal enzyme inducer treatments

Groups of five mice were administered one of the following chemicals once daily for 4 days: aryl hydrocarbon receptor (AhR) ligands: TCDD (40 $\mu\text{g}/\text{kg}$, i.p. in corn oil), β -naphthoflavone (BNF, 200 mg/kg, i.p. in corn oil), and PCB126 (300 $\mu\text{g}/\text{kg}$, p.o. in corn oil); constitutive androstane receptor (CAR) activators: phenobarbital (PB, 100 mg/kg, i.p. in saline), 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP, 3 mg/kg, i.p. in corn oil), and diallyl sulfide (DAS, 200 mg/kg, i.p. in corn oil); pregnane X receptor (PXR) ligands: pregnenolone-16 α -carbonitrile (PCN, 200 mg/kg, i.p. in corn oil), spironolactone (SPR, 200 mg/kg, i.p. in corn oil), and dexamethasone (DEX, 50 mg/kg, i.p. in corn oil); peroxisome proliferator-activated receptor alpha (PPAR α) ligands: clofibrate acid (CLFB, 500 mg/kg, i.p. in saline), ciprofibrate (CPFB, 40 mg/kg, i.p. in saline), and diethylhexylphthalate (DEHP, 1000 mg/kg, p.o. in corn oil); nuclear factor erythroid 2-related factor 2 (Nrf2) activators: butylated hydroxyanisole (BHA, 350 mg/kg, i.p. in corn oil), ethoxyquin (ETHOXYQ, 250 mg/kg, p.o. in corn oil), and oltipraz (OPZ, 150 mg/kg, p.o. in corn oil). Four different vehicle control groups (corn oil i.p., corn oil p.o., saline p.o., and saline i.p.) were used. All injections were administered in a volume of 10 ml/kg. Livers were removed on day 5, 24 h after the last treatment, snap-frozen in liquid nitrogen, and stored at -80°C .

Wild-type and PXR-null mice ($n = 5$) were treated once daily for 4 days with spironolactone (SPR, 200 mg/kg, i.p. in corn oil) and dexamethasone (DEX, 50 mg/kg, i.p. in corn oil). Control groups were treated with corn oil. On day 5, liver tissues were collected, snap-frozen in liquid nitrogen, and stored at -80°C .

2.5. Cholesterol/chenodeoxycholic acid/cholestyramine treatment

These treatments were reported previously [24]. Briefly, the 1% cholesterol diet was formulated from the control diet (Harlan Teklad Rodent Diet, type W) supplemented with 1% (w/w) cholesterol. After 1 week on experimental diets, mice ($n = 6/\text{treatment}$) were euthanized between the 5th and 7th hours of the light period. One percent chenodeoxycholic acid or 3% cholestyramine (both w/w) was added to powdered control diets (Harlan Teklad Rodent Diet, type W). After 10 days of feeding, mice ($n = 5\text{--}7/\text{treatment}$) were euthanized between the 5th and 7th hours of the dark period.

2.6. Sex hormone replacement in gonadectomized mice

Mice were castrated or ovariectomized at 37 days of age by Charles River Laboratories (Wilmington, MA). At 54 days of age, vehicle placebo, 5 α -dihydroxytestosterone (DHT, 5 mg), or

Table 1 – Oligonucleotide probes generated for analysis of mouse Ntcp and Bsep mRNA expression

Ntcp (MMU95131^a)	
CE ^b	gccttgatcttctgaactc- caTTTTTctcttggaagaaagt
CE	gaggatggccagagcct- caTTTTTctcttggaagaaagt
CE	ttgctgtagatgtataagagga- gaggTTTTTctcttggaagaaagt
CE	gccttttgacttcaggaa- gatcTTTTTctcttggaagaaagt
LE	tgtctgtggcccggtggTTTTTaggca- taggaccctgtgtct
LE	agaatgacgtgagcgcagTTTTTagg- cataggaccctgtgtct
LE	catgatgagcagcaacataactaccTTTT- Taggcataggaccctgtgtct
LE	tggtgcagccaagcgagagTTTTTagg- cataggaccctgtgtct
LE	gccactatggcgatgatcaccTTTTTagg- cataggaccctgtgtct
LE	caggagagcagccgagatTTTTTagg- cataggaccctgtgtct
LE	aagaggtagacaggttccccTTTT- Taggcataggaccctgtgtct
LE	cacaatgctgaggttcagtccTTTT- Taggcataggaccctgtgtct
LE	aagctggagcaggtgtcatTTTTTagg- cataggaccctgtgtct
LE	ttaagatctccgtcgtagattcctTTTT- Taggcataggaccctgtgtct
LE	ccctttagggcaccttgctcTTTTTagg- cataggaccctgtgtct
LE	cctatggcgcaaggaatgaTTTTTagg- cataggaccctgtgtct
BL	cccttgggcttcagaagtga
BL	ggggcatgataccgtactgg
BL	gccagaaggaagcactga
BL	atgctggtcagatgaagacctt
BL	cccttcattggccagggtg
BL	catcatgcccaggcagtg
BL	gaacctgacgagtgataacataatg
Bsep (NM_021022)	
CE	ggtcgagaaggatgttaaattta- caTTTTTctcttggaagaaagt
CE	atgtttgaacggaggaact- gaaTTTTTctcttggaagaaagt
CE	tttgcgcagcta- tagctctcTTTTTctcttggaagaaagt
CE	ccgcgagagagttga- gagccTTTTTctcttggaagaaagt
CE	ccctctctggcttgcga- gagTTTTTctcttggaagaaagt
LE	tgcccaggatccacagataaccTTTT- Taggcataggaccctgtgtct
LE	ccaacgaatgccagcgtcTTTTTaggca- taggaccctgtgtct
LE	gcttttaccacaccactgctcTTTTTagg- cataggaccctgtgtct
LE	tccaacagctggatgctggtTTTTTagg- cataggaccctgtgtct
LE	tgatcgggatcgtagaacctTTTTTagg- cataggaccctgtgtct
LE	ccatctatcatcaccgttcccTTTTTagg- cataggaccctgtgtct
LE	cattgactttttgtctgctgaTTTTTagg- cataggaccctgtgtct

Table 1 (Continued)

LE	gctcctgggagacaatcccaTTTTTagg- cataggaccctgtgtct
LE	cattatgtacagtgcaataa- cacggTTTTTaggcataggaccctgtgtct
LE	ttgtcccatacttgatgtgtcTTTTTagg- cataggaccctgtgtct
LE	agtcatgcagctgagcctgcTTTTTagg- cataggaccctgtgtct
LE	ctggatcccaacattagttcataTTTT- Taggcataggaccctgtgtct
LE	aatagcaatgcgtgtttctccTTTTTagg- cataggaccctgtgtct
LE	tcgtacaatggcccgagcTTTTTaggca- taggaccctgtgtct
LE	catccagtagcaagatttaggcatTTTT- Taggcataggaccctgtgtct
LE	tctgtgtctaaggcagatgtagctTTTT- Taggcataggaccctgtgtct
LE	caagctgcactgtcttttactTTTT- Taggcataggaccctgtgtct
LE	agcaatgacaatacaggtccgaTTTT- Taggcataggaccctgtgtct
LE	agttctggatagtagacaagcagtgTTTT- Taggcataggaccctgtgtct
LE	gacatgacggcgatgatctctTTTT- Taggcataggaccctgtgtct
BL	gacagtcattcagaactgtatatca
BL	tccacggagatctcttgggtg
BL	ttctctgggagtgacatgacga

^a GenBank accession numbers for each transcript are given in parenthesis after the gene name.

^b The type of function of each bDNA oligonucleotide probe. CE, capture extender; LE, label extender; BL, blocker.

17 β -estradiol (E2, 0.5 mg) in 21-day-release pellets (Innovative Research of America) was subcutaneously implanted in the gonadectomized male and female mice. The mice were separated into six treatment groups ($n = 5/\text{gender}/\text{treatment}$): (1) castrated male mice replaced with placebo, (2) castrated male mice replaced with DHT, (3) castrated male mice replaced with E2, (4) ovariectomized female mice replaced with placebo, (5) ovariectomized female mice replaced with DHT, and (6) ovariectomized female mice replaced with E2. Intact, untreated, age-matched mice were used as control. Livers were removed at 64 days of age from gonadectomized and age-matched intact control mice.

2.7. Growth hormone replacement in hypophysectomized mice

Mice were hypophysectomized at 30 days of age by Charles River Laboratories. Hypophysectomized mice received water with 5% glucose (w/v) ad libitum. Hypophysectomized mice that gained weight before the start of the study were excluded under the assumption that their surgery was incomplete. The mice were treated with placebo, rat GH in male-pattern (twice daily, intraperitoneal injection, dose at 2.5 mg/kg body weight), or rat GH in female-pattern (continuous infusion via subcutaneously implanted 21-day-release 1 mg rGH pellet). Intact, untreated, age-matched mice were used as controls. After 10 days of treatment, livers were removed for total RNA isolation.

2.8. Growth hormone replacement in lit/lit mice

Breeding pairs of GH-releasing hormone receptor mutant heterozygous mice (lit/+) were purchased from Jackson Laboratory (Bar Harbor, MA). After breeding in the University of Kansas Medical Center laboratory animal facilities, lit/lit (dwarf mice with an inactivating mutation of GHRH receptor) were used in this study. Their respective lit/+ and +/+ mice (characterized by normal body size) were used as controls. The mice were treated for 1 week with vehicle, rat GH in male-pattern (twice daily, intraperitoneal injection, dose of 2.5 mg/kg body weight), or rat GH in female-pattern (continuous infusion via subcutaneously implanted 21-day-release 1 mg rGH pellet). After the treatments, livers were removed for total RNA isolation.

2.9. Total RNA isolation

Total RNA was isolated from mouse 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. The integrity of each RNA sample was evaluated by formaldehyde–agarose gel electrophoresis before analysis.

Total RNA from human livers was isolated according to Invitrogen's protocol.

2.10. Branched DNA (bdNA) signal amplification assay

The mRNA for mouse Ntcp and Bsep was quantified using the QuantiGene[®] bdNA signal amplification kit (Panomics Inc., Fremont, CA). The gene sequences of mouse Ntcp and Bsep were accessed from GenBank (Table 1). The strategy of multiple oligonucleotide probe set design has been described previously [25]. All probes were synthesized by Operon Technologies (Palo Alto, CA). The luminescence for each gene is reported as relative light units (RLUs) per 8 µg of total RNA.

NTCP and BSEP mRNA expression in human livers were determined by the QuantiGene Plex assay (Panomics, Inc.). The data are reported as ratio of mRNA expression of NTCP (or BSEP) to GAPDH mRNA expression per 3 µg of total RNA.

2.11. Membrane protein preparation

Crude plasma membrane samples were prepared from mouse livers according to the method described previously [26]. Briefly, 0.2–0.3 g liver was minced in 10 ml ice-cold homogenizing buffer (0.25 M sucrose, 10 mM Tris-HCl [pH 7.5],

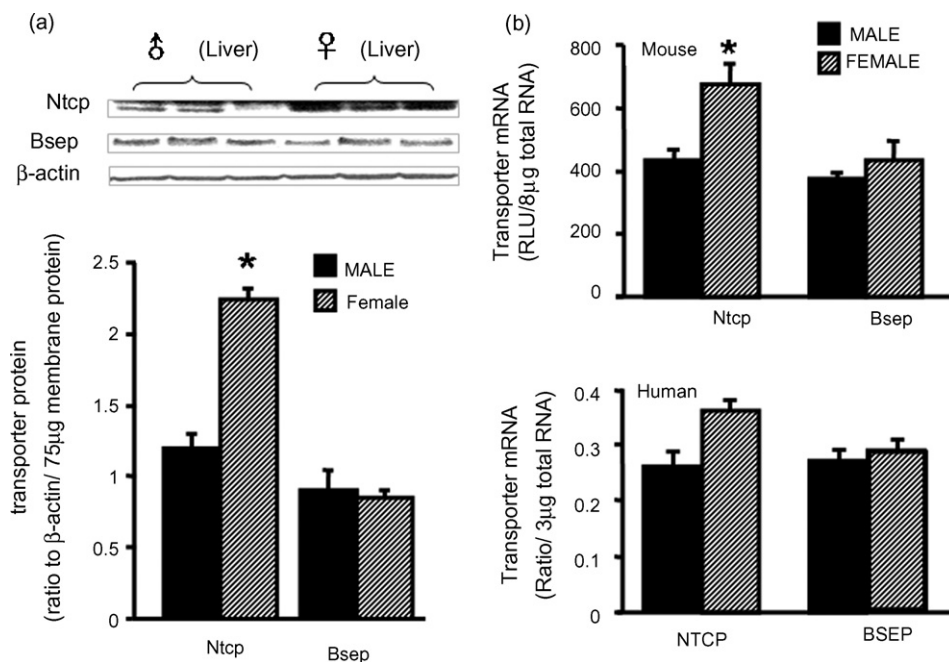


Fig. 1 – mRNA and protein expression of Ntcp/NTCP and Bsep/BSEP in mouse and human livers. (a) Protein levels of Ntcp and Bsep in male and female mouse livers. In the top panel, protein levels of Ntcp and Bsep in liver plasma membranes were analyzed by western blotting. In the bottom panel, protein levels of each transporter in male and female mouse livers are expressed as ratio of Ntcp (or Bsep) to β-actin protein levels per 75 µg total membrane protein. Data are presented as mean ± S.E.M. Asterisks indicate statistically significant differences between male and female mice ($p < 0.05$). (b) mRNA expression of Ntcp/NTCP and Bsep/BSEP in mouse and human livers. Total RNA from both male and female C57BL/6 mice ($n = 10$ /gender) and human livers (30 male Caucasians and 20 female Caucasians) was analyzed by the bdNA assay for mRNA expression of each transporter. Mouse data (on the top) are reported as relative light unit (RLU) per 10 µg of total RNA. Human data (on the bottom) are reported as ratio of NTCP (or BSEP) to GAPDH mRNA expression per 3 µg of total RNA. Both human and mouse data are presented as mean ± S.E.M. Asterisks indicate statistically significant differences between male and female mice ($p < 0.05$).

containing 25 $\mu\text{g/ml}$ leupeptin, 50 $\mu\text{g/ml}$ aprotinin, 40 $\mu\text{g/ml}$ PMSF, 0.5 $\mu\text{g/ml}$ pepstatin, and 50 $\mu\text{g/ml}$ antipain). The minced tissue was poured into a Dounce homogenizer (Kontes, Vineland, NJ) and homogenized on ice for 10 strokes. The homogenate was filtered through one layer of gauze sponges (Tyco Healthcare Group LP, Mansfield, MA), and then centrifuged at $100,000 \times g$ for 1 h at 4°C . The resulting pellet was dissolved in resuspension buffer (0.25 M sucrose, 10 mM HEPES [pH 7.5], and 40 $\mu\text{g/ml}$ PMSF). Protein concentration of each sample was determined with a Bradford protein assay kit from Sigma (St. Louis, MO).

2.12. Western blots

Membrane protein samples mixed with sample loading buffer (75 μg protein/lane) were loaded after heating onto a 10% SDS-polyacrylamide gel. Following electrophoresis, proteins in the gel were electrotransferred to nitrocellulose membrane for 4.5 h at 34 V at 4°C . Membranes were blocked for 2 h at room temperature with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Blots were then incubated overnight with polyclonal antibody of rat Ntcp and Bsep (kindly provided by Dr. Bruno Stieger, Department of Medicine, University Hospital, Zurich, Switzerland) at room temperature. β -actin antibody (Abcam Inc. Cambridge, MA) was used as a loading control. After thorough washing (three 20-min washes with excess TBS-T), blots were incubated with donkey anti-rabbit IgG horseradish peroxidase-linked secondary antibody (1:5000 dilution with 5% non-fat milk in TBS-T) for 1 h. Blots were washed again. Immunoreactive bands were detected with an enhanced chemical luminescence (ECL) kit (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). Ntcp and Bsep proteins were visualized by exposure to Fuji Medical X-Ray film. The protein band intensity on the films was quantified with Gel-Pro 3.1 image analysis software (Media Cybernetics, Silver Spring, MD).

2.13. In silico analysis of prospective DNA response elements in 6 KB of the promoter/enhancer region of mouse and human Ntcp/NTCP and Bsep/BSEP

Six KB of promoter/enhancer sequences of mouse and human Ntcp/NTCP and Bsep/BSEP were accessed from a genomic DNA database (www.ensembl.org). The DNA sequences were analyzed by Nubiscan (<http://www.nubiscan.unibas.ch/>) and Alibaba2.1 (<http://www.gene-regulation.com/pub/programs/alibaba2/>). Putative response element screening of signal transducer and activator of transcription (Stat)5b, a critical transcription factor for growth hormone signaling, was based on the Stat5b consensus DNA-binding sequence (TTCnnGGAA) [27,28].

2.14. Statistical analysis

Data are presented as mean \pm S.E.M. Data were analyzed by one-way ANOVA, followed by Duncan's post-hoc test. Statistical significance was set at $p < 0.05$. When the difference between genders in a specific tissue was determined, data were analyzed by Student's *T*-test, and statistical significance was considered at $p < 0.05$.

3. Results

3.1. Constitutive expression of Ntcp and Bsep in adult male and female mouse and human livers

Both Ntcp/NTCP and Bsep/BSEP have been shown to be highly expressed in mouse and human livers [1,18,29,30]. Ntcp protein expression is 60% higher in female than male mouse liver (Fig. 1a), and Ntcp mRNA is 70% higher in female than male mouse livers (Fig. 1b). In human livers, NTCP mRNA is 43% higher in women than men; however, it is not statistically different due to high variation in individual human NTCP expression. In contrast, no gender differences exist in mouse Bsep expression at either the protein or mRNA levels, or in human BSEP mRNA expression (Fig. 1a and b).

3.2. Ontogeny of Ntcp and Bsep in male and female mouse livers

The developmental patterns of Ntcp and Bsep mRNA expression in male and female mouse livers are shown in Fig. 2. Both Ntcp and Bsep mRNA expression are very low before birth. At birth, both Ntcp and Bsep mRNA increased to their highest expression at any age, about 50% higher than adult levels. Ntcp mRNA expression then rapidly returned to low levels after birth, and remained low until about 3 weeks of age, at which time the expression of both bile acid transporters reached adult levels. Gender-dimorphic Ntcp mRNA expression

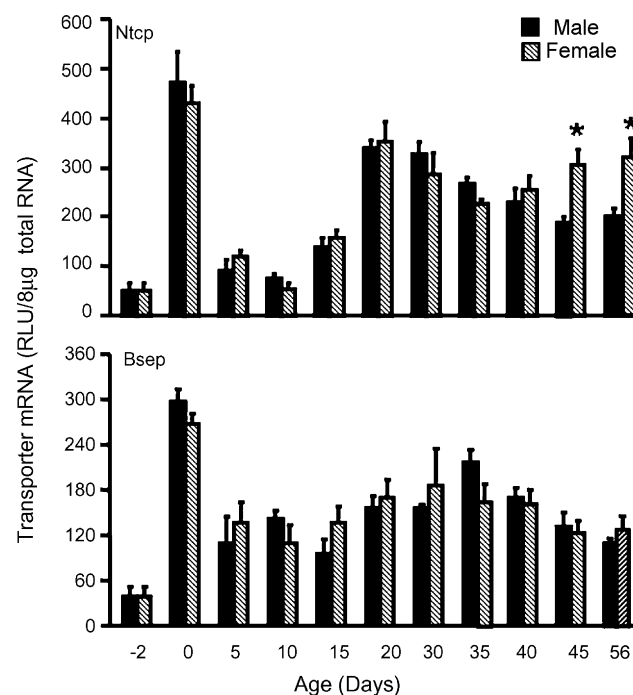


Fig. 2 – Ontogenic expression of mouse Ntcp and Bsep mRNA in mouse livers. Total RNA from C57BL/6 mice at each age ($n = 5/\text{gender}$) was analyzed by the bDNA assay for expression of Ntcp or Bsep mRNA. Data are presented as mean \pm S.E.M. Asterisks indicate statistically significant differences between male and female mice ($p < 0.05$).

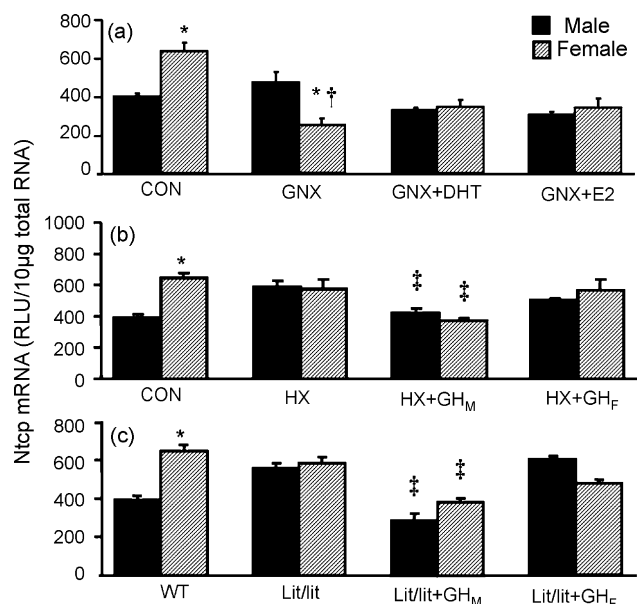


Fig. 3 – Regulatory mechanism characterization of female-predominant Ntcp expression in mouse liver. The solid, black bar represents Ntcp mRNA in males; the striated bar depicts Ntcp mRNA in females. Total liver RNA was isolated and analyzed by the bDNA signal amplification assay for Ntcp mRNA content. The data are presented as mean \pm S.E.M. (each group, $n = 5$ mice). (a) Effects of gonadectomy and sex hormone replacements on mouse Ntcp mRNA expression in livers from intact and gonadectomized male and female mice. GNX, placebo administered to gonadectomized mice; GNX + DHT, 5α-dihydroxytestosterone administered to gonadectomized mice; and GNX + E2, 17β-estradiol administered to gonadectomized mice. Asterisks (*) represent statistically significant differences ($p < 0.05$) between males and females in intact mice and the same treated gonadectomized mouse group; single dagger (†) represent statistically significant differences ($p < 0.05$) between intact mice and the same gender, untreated gonadectomized mice; and double dagger (‡) represents statistically significant differences ($p < 0.05$) between untreated gonadectomized mice and the same gender, gonadectomized mice administered 5α-dihydroxytestosterone, or 17β-estradiol. (b) Effects of hypophysectomy and growth hormone on mouse Ntcp mRNA expression in livers from intact and hypophysectomized male and female mice. HX, placebo administered to hypophysectomized mice; HX + GH_M, rat growth hormone twice daily administered by intraperitoneal injection to hypophysectomized mice mimicking male-pattern growth hormone secretion; and HX + GH_F, continuous infusion to hypophysectomized mice via subcutaneously implanted 21-day-release 1 mg rat GH pellet mimicking female-pattern growth hormone secretion. Asterisks (*) represents statistically significant differences ($p < 0.05$) between males and females in intact mice and the same treated hypophysectomized mouse group; single dagger (†) represent statistically significant differences ($p < 0.05$) between intact mice and the same

became apparent about 45 days of age, with higher expression in female than male mouse livers (Figs. 1 and 2).

3.3. Regulation of Ntcp mRNA by sex hormones and growth hormone

Figs. 1 and 2 illustrate that Ntcp mRNA expression is higher in adult female than male mouse livers. The importance of sex hormones and growth hormone (GH) in the Ntcp female-predominant expression was further determined.

Regulation of Ntcp expression by sex hormones was investigated in gonadectomized mice, as shown in Fig. 3a. Gonadectomy decreased Ntcp mRNA expression in female mouse livers, but not in males. Neither androgen (5α-dihydroxytestosterone, DHT) nor estrogen (17β-estradiol, E2) administration altered Ntcp mRNA expression in gonadectomized mice.

Two mouse models were used to determine the effects of GH on the regulation of Ntcp expression: hypophysectomized mice and lit/lit mice. In hypophysectomized mice, not only GH, but also other pituitary hormones, such as prolactin, luteinizing hormone, and follicle-stimulating hormone are depleted. In hypophysectomized mice, the gender difference in Ntcp mRNA expression disappeared due to an increase in Ntcp mRNA in the male mice (Fig. 3b). Male-pattern GH administration to hypophysectomized mice decreased Ntcp mRNA expression in both sexes; however female-pattern GH replacement did not alter Ntcp mRNA expression (Fig. 3b).

In lit/lit male mice, liver Ntcp mRNA expression was higher than that in wild-type male mice, and the gender difference in Ntcp was not observed in the lit/lit mice (Fig. 3c). Male-pattern GH administration to lit/lit mice decreased Ntcp mRNA expression. Female-pattern GH administration in lit/lit mice did not alter Ntcp mRNA expression (Fig. 3c).

3.4. Chemical regulation of mouse Ntcp and Bsep

Male C57BL/6 mice were administered five classes of prototypical drug-metabolizing enzyme inducers. For each class, three chemicals were used that are known to transcriptionally activate a specific signaling pathway. The doses were chosen according to what is commonly cited in the literature to activate specific transcription factors that target specific biotransformation enzymes: cytochrome P450 enzymes

gender, untreated hypophysectomized mice; and double dagger (‡) represents statistically significant differences ($p < 0.05$) between untreated hypophysectomized mice and the same gender, or hypophysectomized mice following hormone replacement treatments. (c) Effects of growth hormone on mouse Ntcp mRNA expression in livers from intact and lit/lit male and female mice. Lit/lit, placebo administered to Lit/lit mice; Lit/lit + GH_M, rat growth hormone twice daily administered by intraperitoneal injection to Lit/lit mice mimicking male-pattern growth hormone secretion; Lit/lit + GH_F, continuous infusion to Lit/lit mice via subcutaneously implanted 21-day-release 1 mg rat growth hormone pellet mimicking female-pattern growth hormone secretion.

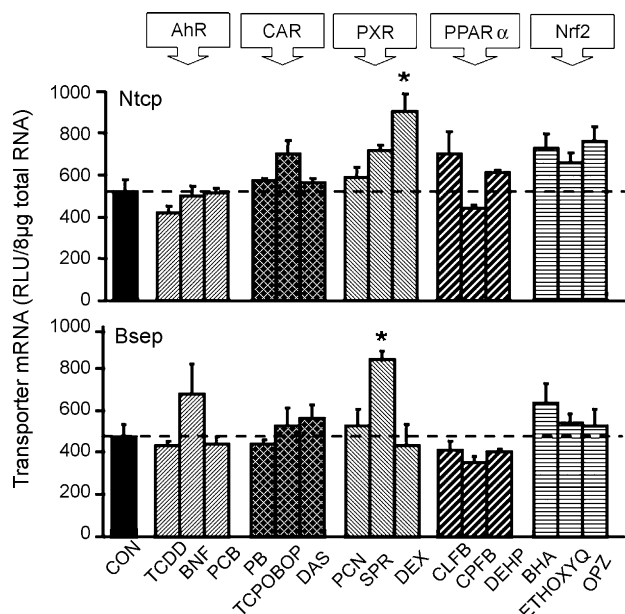


Fig. 4 – mRNA expression of *Ntcp* and *Bsep* in adult male C57BL/6 mouse liver after administration of prototypical phase-I and -II drug-metabolizing enzyme inducers. Mice were administered once daily for 4 days with each of microsomal enzyme inducers ($n = 5/\text{group}$) as detailed in Section 2. Total RNA from five chemically treated male livers was analyzed by the bDNA assay. All data were expressed as mean \pm S.E.M. for five mice in each group, except for control groups, which were combined from the four individual control groups after it was determined that they were not statistically different. Asterisk indicates statistically significant difference between treated and control mice ($p < 0.05$).

(Cyp1a1, Cyp2b10, Cyp3a11, and Cyp4A14) and NAD(P)H:quinone oxidoreductase (Nqo) 1, as reported previously [31]. The regulation of mouse *Ntcp* and *Bsep* by drug-metabolizing enzyme inducers is shown in Fig. 4. The AhR, CAR, PXR, PPAR α , and Nrf2 activators as a class did not alter *Ntcp* and *Bsep* mRNA expression. Individually, the PXR ligands dexamethasone and spironolactone increased mRNA expression of *Ntcp* and *Bsep*, respectively.

3.5. Regulation of *Ntcp* by dexamethasone and *Bsep* by spironolactone

Regulation of mouse *Ntcp* by DEX in PXR-null mice is depicted in Fig. 5a. In wild-type mice, DEX increased *Ntcp* mRNA expression (130%). Disruption of PXR function did not alter constitutive expression of *Ntcp* mRNA, however in PXR-null mice, DEX only increased *Ntcp* mRNA levels 34%, indicating an attenuation of the up-regulation of *Ntcp* by DEX compared with treatment of wild-type mice.

Regulation of *Bsep* by SPR in PXR-null mice is shown in Fig. 5b. In wild-type mice, SPR treatment increased *Bsep* mRNA expression 70%. Disruption of PXR protein, as demonstrated in PXR-null mice, decreased expression of *Bsep* mRNA approxi-

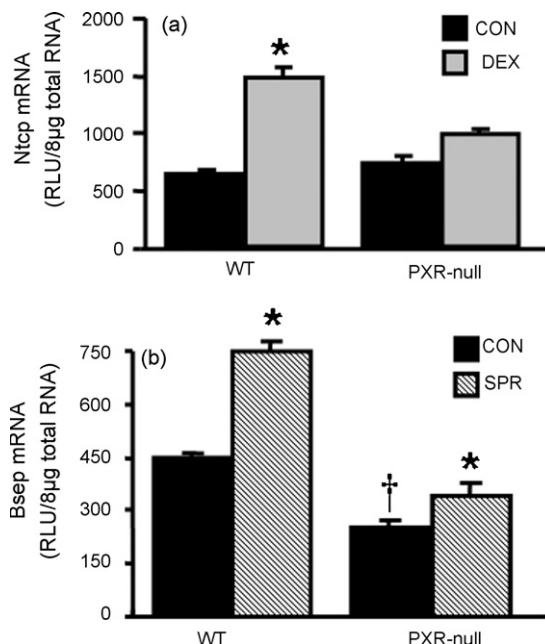


Fig. 5 – Regulation of mouse *Ntcp* by dexamethasone and *Bsep* by spironolactone. (a) Regulation of mouse *Ntcp* by dexamethasone in wild-type mice and PXR-null mice. Wild-type mice and PXR-null mice ($n = 4\text{--}6/\text{group}$) were treated once daily for 4 days with 50 mg/kg dexamethasone. Control group received corn oil. On day 5, liver tissues were removed and used for total RNA isolation. Individual total RNA sample ($n = 4\text{--}6$ mice) were analyzed by bDNA assay. The solid, black bar represents the data from mice after treatment with 50 mg/kg dexamethasone. (b) Regulation of mouse *Bsep* by spironolactone in wild-type mice and PXR-null mice. Wild-type and PXR-null mice ($n = 5/\text{group}$) were treated once daily for 4 days with corn oil (control) or spironolactone (200 mg/kg, i.p. in corn oil). On day 5, liver tissues were removed and used for total RNA isolation. Total RNA from five treated male livers was analyzed by the bDNA assay. All data were expressed as mean \pm S.E.M. for five mice in each group. Asterisk indicates statistically significant difference between treated and control mice ($p < 0.05$). Single dagger (†) indicates statistically significant difference between wild-type and PXR-null mice ($p < 0.05$).

mately 60%. In contrast, in PXR-null mice, SPR increased *Bsep* mRNA expression 35%.

3.6. Regulation of mouse *Ntcp* and *Bsep* by cholesterol, chenodeoxycholic acid, and cholestyramine

As depicted in Fig. 6, cholesterol administration did not alter either *Ntcp* or *Bsep* mRNA expression. Cholestyramine induced *Ntcp* mRNA expression about 50%, but had no effect on *Bsep*. Administration of CDCA induced *Bsep* mRNA expression about 160%, but it did not alter *Ntcp* mRNA expression.

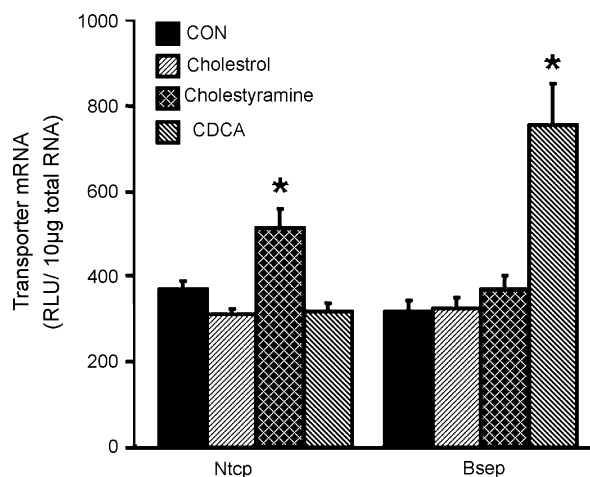


Fig. 6 – Regulation of mouse Ntcp and Bsep by feeding with cholesterol, chenodeoxycholic acid, or cholestyramine. Mice were fed diets containing 1% cholesterol for 7 days, or 1% chenodeoxycholic acid (CDCA) for 10 days, or 3% cholestyramine for 10 days as detailed in Section 2, while control mice received the standard diet. All data were expressed as mean \pm S.E.M. of 6–10 male mice for each group. Asterisk (*) denotes a statistically significant difference in mRNA levels in treated compared to control mice ($p < 0.05$).

3.7. Putative DNA response elements in 6 KB of the promoter/enhancer region of mouse and human Ntcp/NTCP and Bsep/BSEP

In the mouse Ntcp promoter, as shown in Fig. 7a, three putative glucocorticoid receptor (GR) response elements are

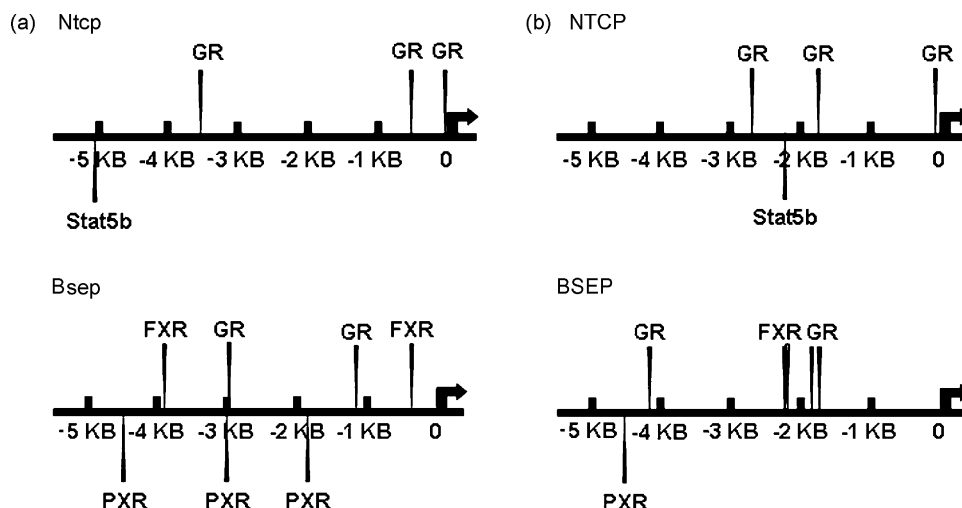


Fig. 7 – In silico analysis of prospective DNA response elements of Stat5b, and nuclear receptors including PXR, FXR, and GR in 6 KB of promoter/enhancer of mouse and human Ntcp/NTCP and Bsep/BSEP genes. Six KB of promoter/enhancer sequences of mouse and human Ntcp/NTCP and Bsep/BSEP are accessed from genomic DNA database (www.ensembl.org). The DNA sequences were analyzed by Nubiscan (<http://www.nubiscan.unibas.ch/>) and Alibaba2.1 (<http://www.gene-regulation.com/pub/programs/alibaba2/>). Response element search of Stat5b is based on their DNA-binding consensus sequence (TTcnnGGAA). (a) In silico analysis of promoter/enhancer sequences of mouse Ntcp and Bsep genes. (b) In silico analysis of promoter/enhancer sequences of human NTCP and BSEP genes.

found about 20, 500, and 3500 bp upstream from the transcription start site. One putative Stat5b binding site is shown in the Ntcp promoter (–5160 bp; CTCCAGTTCTTGGAA-TATTGT, consensus Stat5b response element underlined). In the promoter of mouse Bsep (Fig. 7a), there are two putative FXR-binding sites (–360 and –3930 bp), two putative GR-binding sites (–1110 and –3000 bp), and three putative PXR-binding sites (–1850, –3000, and –4450 bp).

In 6 KB of human NTCP promoter, one putative Stat5b binding site (–2270 bp; GTCAGGTTCTTGGAAAGAGATT, consensus Stat5b response element underlined), and three putative GR-response elements (–80, –1740, and –2760 bp) are localized (Fig. 7b). In contrast, putative response elements of PXR (one; at –4500 bp), GR (three; at –1730, –1880, and –4370 bp, respectively) and FXR (two; at –2200 and –2210 bp) exist in 6 KB of the human BSEP promoter/enhancer (Fig. 7b). There is no Stat5b response element observed in the promoter/enhancer of either mouse or human Bsep/BSEP.

4. Discussion

The goal of the present study was to obtain systematic information on the regulation of Ntcp and Bsep, two critical bile acid transporters in mouse and human livers. There are numerous reports on the regulation of Ntcp [19,32–34] and Bsep [20,32,34] by bile acids. The focus of this study was to characterize the ontogenic and gender-related constitutive expression of these two hepatic bile acid transporters, as well as examine the regulation of Ntcp and Bsep by microsomal enzyme inducers, chenodeoxycholic acid, and cholestyramine.

Human, rat, and mouse NTCP/Ntcp and BSEP/Bsep share similar postnatal developmental patterns, with lower levels in newborns and then gradually increasing to adult levels with

age. Both NTCP and BSEP mRNA expression in human livers is detectable in fetus at mid-gestational age (14–20 weeks) by real-time PCR. Human NTCP and BSEP mRNA levels are about 50-fold and 3-fold higher in adult than fetal livers, respectively [35]. Rat Ntcp mRNA has been detected at approximately 20 days of gestation, and gradually increases to adult levels after postnatal day 28 [36–38]. Hardikar et al. further showed that mRNA levels of rat Ntcp prior to birth were less than 20% of adult values, increased to 35% on the first postnatal day, and reached adult values by 1 week of age [39]. Rat Bsep was first detected at embryonic day 20 [37], and then gradually increases to adult levels. Bsep mRNA in adult rats is approximately twice that in newborn rats [37,40]. Hardikar et al. further showed that mRNA levels of rat Bsep were 12% of adult values prior to birth and showed a two-fold increase by the first day after birth, with adult levels being reached at 4 weeks of age [39]. Mouse Ntcp and Bsep mRNA expression in liver is minimal before birth, and then rapidly increases to the highest expression at birth (Fig. 2). After birth, mouse Ntcp mRNA expression decreases, and then gradually increases to adult levels at approximately 3 weeks of age (Fig. 2). In contrast, mouse Bsep mRNA decreased at 5 days after birth to half that at birth, and remained at this level thereafter (Fig. 2). Therefore, postnatal NTCP/Ntcp and BSEP/Bsep mRNA expression in humans, rats, and mice are low in newborns, and then increases to adult levels with age.

There is a species difference in the gender-divergent expression of Ntcp between rats, mice, and humans. In rats, Ntcp mRNA is male-predominant, with twice the amount in male as in female livers [22]. In contrast, mouse Ntcp mRNA expression is female-predominant with 70% higher expression in female than male livers. In addition, mouse Ntcp protein levels are 60% higher in female than male liver (Fig. 1a). NTCP mRNA expression in human livers was not statistically different between men and women, but mRNA levels were 43% higher in women than men (Fig. 1b). In rats, the gender differences in Ntcp mRNA levels were reported to be due to inhibitory effects of female-pattern GH secretion [22]. However, the present study indicates that in mice, female-predominant Ntcp mRNA expression is due to inhibitory effects of male-pattern GH secretion (Fig. 3b and c), but not sex hormones (Fig. 3a). In humans, GH gender-dimorphic secretion patterns are similar to that observed in rodents [41]. Therefore, mouse and human Ntcp/NTCP gender-dimorphic expression may share similar regulatory effects by GH. Recent microarray studies performed by Clodfelter et al. [42,43] showed that stat5a and stat5b play important roles in growth hormone-initiated gender-divergent liver gene expression. However, in both studies, regulation of mouse Ntcp by growth hormone was not included.

In silico analysis of 6 KB of the promoter/enhancer sequence of mouse Ntcp and Bsep showed that putative DNA binding sites of Stat5b exist in the promoter of mouse Ntcp, but not in mouse Bsep (Fig. 7). Stat5b is a down-stream target of the GH signaling pathway by which gender-specific liver gene expression occurs [27,42,44]. The existence of putative Stat5b response elements may explain why gender differences exist in the expression of mouse Ntcp, but not in mouse Bsep. In the human NTCP promoter, there is one putative DNA binding site of Stat5b (–2270 bp), but it does not

exist in the human BSEP promoter (Fig. 7b). Therefore, female-predominant human NTCP expression may be also due to gender-related GH secretion pattern.

Numerous chemicals, which are known to activate various transcription factors and induce phase-I and -II drug-metabolizing enzymes, regulate some hepatic transporters such as organic anion transporting polypeptides (Oatps) and multi-drug resistance-associated proteins (Mrps) [31,45,46]. It was therefore of interest to determine whether these chemicals might also alter Ntcp and/or Bsep expression. In human primary hepatocytes, TCDD (AhR ligand), rifampicin (a PXR activator), phenobarbital (a CAR activator), and oltipraz (a Nrf2 activator) treatment down-regulate Ntcp and Bsep mRNA expression [47]. In contrast, the present study indicates that mouse Ntcp or Bsep mRNA expression are relatively resistant to alteration by most microsomal enzyme inducers, but induced by a couple of these chemicals (Fig. 4). Variation in induction of Oatps, Mrps, xenobiotic enzymes, Ntcp, and Bsep by microsomal enzyme inducers is logical in relation to their functions. Both Oatps and Mrps are xenobiotic transporters, which are regulated by the challenge of drugs and other xenobiotics. Xenobiotics are detected by the nuclear receptors PXR and CAR [48–50] and consequently up-regulate various transporters and xenobiotic enzyme systems. However, Ntcp and Bsep are bile acid transporters, and not thought to be as important for xenobiotic transport, and as shown here, are not readily induced by xenobiotics [51].

Even through mouse Ntcp and Bsep mRNA expression are relatively resistant to alteration by most microsomal enzyme inducers, a couple of these chemicals alter their expression (Fig. 4). Specifically, dexamethasone induced Ntcp mRNA expression, whereas spironolactone induced Bsep mRNA expression.

Dexamethasone is a well-established inducer of CYP3A by activating the glucocorticoid receptor and PXR [52,53]. Rat Ntcp mRNA was shown to be up-regulated by dexamethasone in hypophysectomized rats and in isolated rat hepatocytes [22], but down-regulated in sandwich-cultured rat hepatocytes [54]. Human NTCP promoter can also be activated by dexamethasone via the GR [55]. In mouse liver, DEX increased Ntcp mRNA expression in wild-type mice, but not in PXR-null mice (Fig. 5a). However, there is no apparent PXR response element exists in the mouse Ntcp promoter (Fig. 7a). In addition, PCN, the prototypical PXR ligand did not increase mouse Ntcp (Fig. 4). Dexamethasone induced mouse Ntcp mRNA expression may be mediated by GR, which is suggested by the presence of GR-response elements in the mouse Ntcp promoter (Fig. 7).

Spironolactone increases bile flow in experimental animals [56,57]. Spironolactone is a ligand for PXR, GR, and the mineralocorticoid receptor. PXR was shown to be required for the up-regulation of mouse Bsep by PCN and RU486 during inflammation [58]. However, in the present study, PCN did not increase Bsep expression (Fig. 4). SPR increased Bsep mRNA expression 70% in wild-type mice (Figs. 4 and 5b), and 35% in PXR-null mice (Fig. 5b), suggesting partial involvement of PXR, which is supported by the presence of PXR response elements in the mouse Bsep promoter (Fig. 7a). However, PCN, the prototypical PXR agonist did not increase mouse Bsep (Fig. 7). Therefore, mechanisms other than PXR are involved in Bsep

induction by spironolactone. For example, Ghanem et al. [59] showed that in rats, spironolactone treatment increased P-glycoprotein expression and thus decreased intestinal absorption and liver content of digoxin. Therefore, spironolactone may be capable of altering disposition of some exogenous or endogenous compounds, such as bile acids, which might be responsible for regulation of Bsep.

Bile acids have been shown to either increase or decrease mouse Ntcp expression, but uniformly increase mouse Bsep expression. Ursodeoxycholic acid has no effect on mouse Ntcp expression, whereas it increased mouse Bsep mRNA and protein [32]. Taurocholate and/or cholic acid down-regulated mouse Ntcp via induction of the nuclear repressor short heterodimer partner (SHP) [33,34,60], whereas they up-regulated mouse Bsep expression [23,32,34]. Therefore, bile acids down-regulated Ntcp expression in some studies and had no effect in other studies, but consistently increased Bsep expression. In the present study, we further showed that mice treated with chenodeoxycholic acid increased Bsep mRNA expression, but did not alter Ntcp expression (Fig. 6).

Cholestyramine, which is a bile acid sequestrant in the intestine, has been shown to increase bile acid biosynthesis via Cyp7a1 induction in liver [61,62]. In the present study, cholestyramine treatment increased mouse Ntcp mRNA expression 40% (Fig. 6). In contrast, Wolters et al. [34] stated that cholestyramine did not alter mouse Ntcp expression; however, the figures in their publication depicted that cholestyramine slightly increased mouse Ntcp expression at both the protein and mRNA levels. The mechanism by which cholestyramine induces mouse Ntcp expression is not known. However, transcription factors, such as hepatic nuclear factor (Hnf) 4 α and Stat5b might be involved. Cholestyramine-induced human CYP7A1 expression is mediated by increased Hnf4 α expression [62,63]. In addition, Hnf4 α strongly inhibits Stat5b transcriptional activity via the inhibitory effects of Hnf4 α on JAK2 phosphorylation [44]. In Fig. 3, male-pattern GH secretion decreased hepatic Ntcp mRNA expression in hypophysectomized and lit/lit mice probably via activation of Stat5b [27,42]. Therefore, cholestyramine may increase Hnf4 α levels, which inhibits Stat5b transcriptional activity, and consequently increases Ntcp mRNA expression. Another potential mechanism is due to attenuation of the FXR-Shp pathway. By binding bile acids in the intestine, cholestyramine disrupts the enterohepatic circulation of bile acids. Less absorption of bile acids might lead to less activation of FXR, and as a consequence, attenuate the repressive effect of the FXR-Shp pathway on Ntcp expression. In rats, cholestyramine has been shown to decrease Shp mRNA levels [64]. However, a recent paper showed that cholestyramine administration to rats did not alter Shp expression [65]. The conflicting data on the regulation of Shp by cholestyramine, merits further studies.

Taken together, this study systemically characterized the regulation of Ntcp and Bsep mRNA expression in mouse livers. Liver at birth has the highest mRNA expression of both Ntcp and Bsep. Ntcp expression is female-predominant in mouse and human liver, which is due to the inhibitory effect of male-pattern GH secretion, and not sex hormones. Ntcp and Bsep expression in general are not regulated by microsomal enzyme inducers. Cholestyramine increased Ntcp and chenodeoxycholic acid increased Bsep mRNA expression in

mouse livers. In silico analysis indicates that human and mouse Ntcp/Ntcp and Bsep/Bsep genes may share similar regulatory mechanisms, and thus regulation of mouse Ntcp and Bsep may extrapolate to humans.

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