SIGNALING CHARACTERIZATION OF UP-REGULATION OF MOUSE AND HUMAN BILE SALT EXPORT PUMP (BSEP) EXPRESSION BY BERBERINE

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ABSTRACT

SIGNALING CHARACTERIZATION OF UP-REGULATION OF MOUSE AND HUMAN BILE SALT EXPORT PUMP (BSEP) EXPRESSION BY BERBERINE

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Bile salt export pump (Bsep) is primarily responsible for biliary excretion of bile salts in the liver. Genetic mutation or drug-induced dysfunction of Bsep often leads to disruption of enterohepatic circulation of bile acids and consequently cholestatic liver injury. Berberine (BBR), a traditional herbal medicine, promotes bile flow and has been suggested to treat liver diseases, including cholestasis. We recently reported that BBR induces Bsep expression in mouse liver. However, the underlying mechanism by which BBR induces Bsep expression is unknown. My dissertation project showed that BBR induced mouse and human Bsep/BSEP mRNA and protein expression. In addition, BBR increased Bsep/BSEP transport activity, evidenced by increased cellular efflux of dichlorofluorescin diacetate, a selective Bsep substrate. BBR activated NRF2 signaling in human hepatoma cells, which contributed to BBR-induced human BSEP expression. However, activation of Nrf2 signaling was not essential for induction of mouse Bsep by BBR because BBR continued to increase Bsep expression in Nrf2-null mouse liver and Nrf2-silenced mouse hepatoma cells. In addition, BBR reversed LPS-decreased mouse Bsep expression in both mouse liver and cultured mouse hepatoma cells. Mechanistically, BBR attenuated LPS-activated TLR4-NF-κB signaling, which may contribute to BBR-induced mouse Bsep expression. In conclusion, BBR induced human BSEP expression

through NRF2 activation; whereas BBR induced mouse Bsep expression most likely through TLR4 inhibition.

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BBR, Berberine

Bsep, bile salt export pump

Cyp, cytochrome p450 enzymes

FXR, farnesoid X receptor

Ho-1, heme oxygenase-1

HCC, hepatocellular carcinoma

Keap1, Kelch like ECH associated protein 1

LPS, lipopolysaccharides

Mdr, Multidrug resistance transporter

Mrp, Multidrug resistance-associated protein

Nrf2, nuclear factor erythroid 2-related factor 2

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

NTCP, Na^+-taurocholate cotransporting polypeptide

Nqo-1, NAD(P)H quinone oxidoreductase-1

Oatp, organic anion transporting polypeptide

PFIC2, progressive familial intrahepatic cholestasis 2

qRT-PCR, quantitative real-time polymerase chain reaction

siRNA, small interfering RNA

TLR4, Toll-like receptor 4

CHAPTER 1 INTRODUCTION

1. Overview

Cholestasis is liver disease with impaired bile flow, primarily caused by impaired hepatic secretion or biliary obstruction (Reichen *et al.*, 1994). Occurrence of cholestasis is increasing in prevalence worldwide (Beste *et al.*, 2015; Asrani, *et al.*, 2013, 2019). Current research on cholestasis primarily focuses on the characterization of mechanisms by which cholestasis is produced, and the investigation of effective therapies to attenuate the progression of cholestasis. We have previously reported that berberine (BBR), a traditional herbal medicine historically used to treat bacterial infections, promote bile flow and increase expression and transport function of Na+-taurocholate cotransporting polypeptide (Ntcp), the major liver bile acid uptake transporter , by inhibiting Jak2-Stat5 signaling (Bu *et al.*, 2017). During this study, we also found that BBR induces mRNA expression of bile salt export pump (Bsep), the major liver bile acid efflux transporter, in mouse liver. The results of my dissertation project further demonstrated that BBR induces expression of mouse and human Bsep/BSEP transporter gene through different mechanisms.

In Chapter 1, the current understanding and ongoing research of traditional herbal medicine BBR, including its pharmaceutical applications, pharmacokinetics, and toxicological profiles are reviewed. In addition, the function and regulation of Bsep transporter is briefly discussed. In Chapter 2, methodology and experimental design is discussed. The results for the impact of Nrf2 activation on BBR-induced mouse and human Bsep/BSEP and the impact of TLR4 inhibition on mouse Bsep are determined in Chapter 3 and discussed in Chapter 4. In Chapter 5, the significance and overview of my

dissertation are summarized. In addition, the potential future directions of my dissertation are identified and discussed.

2. Traditional Herbal Medicines

Traditional herbal medicines are widely used in approximately 80 percent of developing countries because of their relatively less toxicity and cheap price (Chung *et al.*, 2012; Quartey *et al.*, 2012; Tillhon *et al.*, 2012).

Traditional herbal medicine generally consists of multiple active components, which work together to generate their therapeutic-effectiveness (Zhu and Woerdenbag, 1995). Recently, herbal medicine attracts more attention, especially its characteristic active components. For instance, berberine (BBR), a very old herbal medicine extracted from goldenseal and Hunaglian, has been extensively investigated because of its promising therapeutic potentials, including its anti-oxidative, anti-inflammatory, and anti-diabetic effects (Vickers *et al.*, 2001; Tillhon *et al.*, 2012). In addition, herbal medicine is very popular in the U.S. market, where several billions were spent on related products annually (Vickers *et al.*, 2001).

However, some drawbacks restrict the therapeutic applications of herbal medicine. For instance, herbal medicine normally contains a mixture of different substances. Thus, it is difficult to identify and purify the exact active component (Bandaranayake, 2006). In addition, exploring the mechanism of action of herbal medicines is also challenging because the synergistic or antagonistic interaction among different components (Bandaranayake, 2006). Moreover, herbal medicine generally contains extremely low concentrations of active components, leading to low clinical efficiency (Firenzuoli and

Gori, 2007). Furthermore, few data are available for toxicological profiles of herbal medicine (Bandaranayake, 2006).

With the rapid development and emergency of advanced separation methodology, especially HPLC-MS/MS, increased number of active components of herbal medicine have been separated and identified (Yang *et al.*, 2009). In addition, more elaborated animal studies have been conducted to investigate the mechanisms of action and the recommended dose of herbal medicine (Firenzuoli and Gori, 2007; Yi *et al.*, 2013). All of above advancements promote the applications of traditional herbal medicine in disease diagnosis, prevention and therapy.

In my dissertation, I report that BBR, a major active component in several herbal medicines, such as goldenseal, Oregon grape and barberry, can induce expression and transport function of Bsep, and thus can promote bile flow and relive endotoxemia-induced cholestasis.

3. Berberine (BBR)

Berberine (BBR), a cationic plant alkaloid, was originally isolated from *Xanthoxylon cava* by Cheballier and Pelletan in 1826 (Hahn and Ciak,1975). BBR can also be extracted from root, stem, bark, fruit and rhizome of other herbal medicine, such as *Hydrastic canadenis* (goldenseal) and *Berberis vulgaris* (barberry). In general, BBR is the major active component of above mentioned herbal medicine (Hahn and Ciak,1975; Harmon, 1990; Ivanovska and Philipov, 1996).

BBR has yellow fluorescence color and has been applied as a common fiber reactive dye in the textile industry and a special cell staining dye in the laboratory (Saxena and Raja, 2014; Weiß and Brandl, 2013). Herbal medicine containing BBR is extensively

used in India (*Berberis aristata Linn*), China (Huang Lian or *Copis chinesis*) and North America (*Hydrastis canadensis*) as the dietary supplement and folk remedies (Hahn and Ciak,1975; "Huang Lian", 1986; McKenna and Plotnikoff 2005; Komal *et al.*, 2011; Upton, 2011).

BBR shows broad pharmaceutical applications. Traditionally, BBR is used as an antibiotic (Hahn and Ciak,1975; Tillhon *et al.*, 2012). It has been thought that its isoquinoline nucleus with quaternary nitrogen and methylenedioxy structure on C-2 and C-3 is essential for its anti-microbial property (Fig. 1.3) (Qing *et al.*, 2017). In addition, positive charge of BBR can facilitate the interaction to negatively charged biological molecules, including bacteria DNA and RNA, and thus can inhibit bacteria growth (Davidson *et al.*, 1977; Creasey, 1979; Bhadra *et al.*, 2010). Furthermore, BBR has also been used to treat infections caused by fungi and protozoan (Amin *et al.*, 1969; Basha *et al.*, 2002).

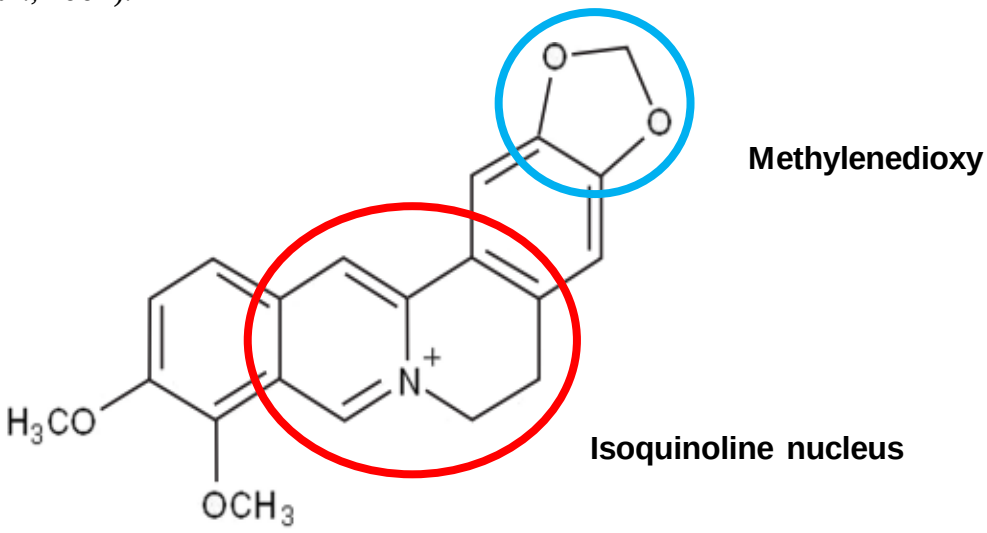

Figure 1.3 Chemical structure of berberine (BBR)

Isoquinoline nucleus and methylenedioxy structure of BBR as highlighted by red and blue circles, respectively.

Recently, BBR also shows potential to treat inflammatory diseases, type II diabetes and cancers. BBR suppresses inflammation most likely through blocking Toll-like receptor (TLR) 4-NF-κB inflammatory pathway, as well as inhibiting the synthesis of pro-inflammatory cytokines (Remppis, *et al.*, 2010; Lou *et al.*, 2011). BBR also suppresses the binding efficiency of lipopolysaccharides (LPS) to TLR4 (Kim *et al*, 2004; Chu *et al.*, 2014). In addition, BBR scavenges oxidative stress and modulates inflammation via activation of Nrf2 signaling, which play important anti-oxidative and anti-inflammatory roles (Mo *et al.*, 2014). The mechanisms by which BBR activates Nrf2 include the activation of PI3K/Akt-Nrf2 signaling and/or by following up-regulation of the expression of one LncRNA, *MARK052686* (Hsu *et al.*, 2012; Chen *et al.*, 2012; Chen *et al.*, 2014; Yuan *et al.*, 2015). Next, BBR has been applied to treat type II diabetes because it increases glucose metabolism and insulin sensitivity via AMPK activation (Creasey, 1979; Yin *et al.*, 2008; Brusq *et al.*, 2006; Lee *et al.*, 2006). BBR can also relieve diabetes-related complications, such as diabetic cardiovascular complications through preventing oxidative stress and reducing nitric oxide or cholesterol synthesis (Yin *et al.*, 2012). Moreover, BBR is a putative chemotherapeutic agent by inhibiting DNA topoisomerases, p53-depedent cell growth, Akt signaling and cell division progression (Kobayashi *et al.*, 1995; Serafim *et al.*, 2008; Choi *et al.*, 2009; Liu *et al.*, 2011; James *et al*, 2011; Kuo *et al.*, 2012).

More and more evidence indicates that BBR has hepatoprotective effects by preventing the progression of hepatocellular carcinomas, non-alcoholic fatty liver (NAFLD), cholestasis, liver fibrosis, and drug-induced liver injury (DILI) (Wei and Gilani, 2000; Zhang *et al.*, 2008; Sun *et al.*, 2009, 2017; Liu *et al.*, 2013). BBR promotes

autophagic cell death and mitochondrial apoptosis in hepatocellular carcinoma cells (Wang *et al.*, 2010). The mechanisms by which BBR ameliorates liver diseases involve anti-oxidative and anti-inflammatory effects, as well as improving lipid profile (Mo *et al.*, 2014; Fan *et al.*, 2015; Wei, *et al.*, 2016). For instance, BBR prevents chemical-induced hepatotoxicity and attenuates the progression of NAFLD in rats through Nrf2 activation (Deng *et al.*, 2019). Similar to animal study, BBR attenuates the progression of NAFLD in patients, indicated by down-regulation of serum triglycerides, low-density lipoprotein (LDL), alanine aminotransferase (ALT) and HemoglobinA1C (HbA1C) (Yan *et al.*, 2015). In addition, goldenseal, in which BBR is the major active component, promotes bile flow (Newall, *et al.*, 1996; Liu *et al.*, 2013; Guo *et al.*, 2016). We and others already reported that BBR induces the expression of Ntcp and Bsep, two bile acid transporters in mouse livers (Guo *et al.*, 2016; Bu *et al.*, 2017).

In addition to pharmaceutical effectiveness and safety, the pharmacokinetics of BBR has been well-documented. For example, oral administration of BBR shows extremely low bioavailability, which is less than 1% and 5% in human and rat, respectively (Chen *et al.*, 2011; Liu *et al.*, 2010; Liu *et al.*, 2016). The low bioavailability of BBR is due to poor absorption, intestinal first-pass elimination and/or transporter-mediated efflux (Chen *et al.*, 2011; Liu *et al.*, 2010; Liu *et al.*, 2016). After absorption, BBR primarily distributes to liver and can also be detected in kidney, muscle, lung, brain and heart in small amounts (Tan *et al.*, 2013). In the liver, BBR is uptaken and effluxed by organic cation transporter (Oct)-1 and multidrug-resistance protein (MDR)1, respectively (Nies *et al*, 2008). BBR can be metabolized into four metabolites, including berberrubine (M1), thalifendine (M2), demethyleneberberine (M3) and jatrorrhizine (M4) by cytochrome

p450 (CYP) enzymes, including CYP1A2, CYP2D6 and CYP3A4 (Tsai and Tsai, *et al.*, 2002; Nies *et al.*, 2008; Li *et al.*, 2011; Tan *et al.*, 2013). Finally, BBR is primarily eliminated through the urinary system (Chen and Chang, 1995; Tsai and Tsai, 2004). The half-life of BBR in rat serum and liver is 12.5±1.6 and 29.4±5.7 minutes, respectively (Tsai and Tsai, 2002). As a result, the effective doses of BBR in rodents are relatively higher because of poor absorption and short half-life.

Toxicological profile of BBR has also been reported. Oral LD50 of BBR is 1,000 mg/kg in rats and 329 mg/kg in mice (Haginiwa *et al.*,1962; Kheir *et al.*, 2010; Yi, *et al.*, 2013). To estimate the safe dose, mice were orally administrated with high doses of BBR for 6 hours. The limit of safety dose of BBR is 20.8 g/kg, which did not produce apparent mouse mortality (Kheir *et al.*, 2010). There is no clear LD50 value or dose-response relationship of BBR toxicity in humans (Mills *et al.*, 2004; Kheir *et al.*, 2010). According to the safety dose in mice, the predicted safety dose of BBR in patients is about 2.97 g/kg, approximate 1 x 10^3 times higher than the clinical dose (Kheir *et al.*, 2010). In general, BBR has been clinically used from 100 to 1,000 mg/kg/day without producing apparent toxicities (Vuddanda *et al.*, 2010; Yin *et al.*, 2008).

However, it has been reported that extremely high doses or chronic treatment of BBR could induce side effects and toxicities. For example, BBR can induce cardiotoxicity in dogs and immune toxicity in mice (Affuso *et al.*, 2010; Mahmoudi *et al.*, 2016). In addition, BBR may induce jaundice in pregnant women and kernicterus in the neonates by replacing bilirubin from its binding protein in rat serum and thus, resulting in an increase of serum level of bilirubin (Chan, 1993). Moreover, chronic treatment of BBR can induce gastrointestinal side effects, such as diarrhea and constipation in patients

(Kowalewski *et al.*, 1975; Bateman *et al.*, 1998; Imanshahidi and Hosseinzadeh, 2008; Affuso *et al.*, 2010)

4. The Major Bile Acid Transporters in the Liver

Bile acid transporters are located at both sinusoidal and canalicular membranes of hepatocytes, which transport either unconjugated or conjugated bile acids, or both (Roberts *et al.*, 2002; Klaassen and Aleksunes, 2010).

The primary function of liver bile acid transporters is to help to maintain bile acid homeostasis (Klaassen and Aleksunes, 2010). As shown in Figure 1.4, Na^+-taurocholate cotransporting polypeptide (Ntcp) is the major liver bile acids uptake transporter. Bile salt export pump (Bsep) is the major liver bile acids efflux transporter (Roberts *et al.*, 2002). Both Ntcp and Bsep can control bile flow in the liver. In addition, some other transporters can also contribute to hepatic transport of bile acids. For example, organic anion transporting polypeptides (Oatps) facilitate bile acids uptake. Multidrug resistance-associated proteins (Mrps) 2 and 3 are responsible for hepatic efflux of bile acids (Roberts *et al.*, 2002).

Disruption of bile flow or dysfunction of bile acid transporters often leads to cholestasis and even liver fibrosis and ultimately cirrhosis (Trauner *et al.*, 1998; Roberts *et al.*, 2002; Byrne *et al.*, 2002; Klaassen and Aleksunes, 2010;). For example, suppression or genetic knockout of Ntcp causes extrahepatic cholestasis in rats (Gartung *et al.*, 1996). Mutations of BSEP gene cause type 2 progressive familial intrahepatic cholestasis (PFIC2) in patients (Paulusma *et al.*, 1997; Strautnieks *et al.*, 1998; Anwer, 2004). In addition, patients with cholestatic liver injury often show disrupted or reduced

expression of hepatic bile acid transporters, including NTCP, MRPs and BSEP (Zollner *et al.*, 2001; Anwer, 2004).

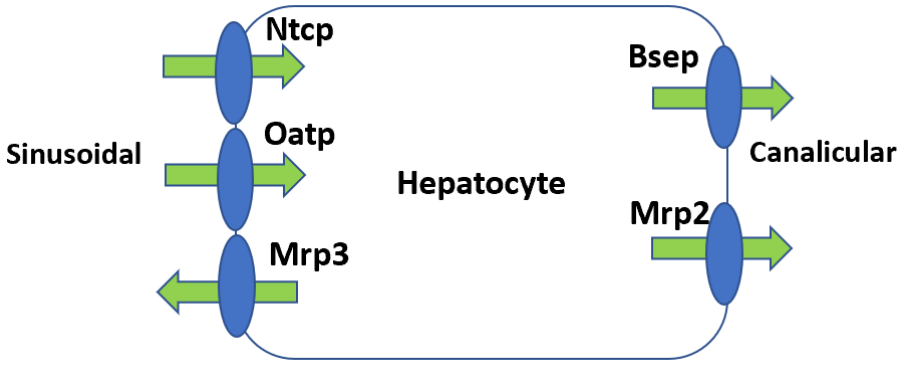

Figure 1.4 Major bile acid transporters in the hepatocyte

Ntcp and Oatp are responsible for hepatic uptake of bile acids whereas Bsep, Mrp2 and Mrp3 are responsible for cellular efflux of bile acids.

5. Bile Salt Export Pump (Bsep)

Bile salt export pump (Bsep), also named ATP-binding cassette (ABC) transporter family B member 11 (ABCB11), is predominantly expressed in the canalicular membrane of hepatocytes (Török *et al.* 1999; Meier and Stieger, 2002). In addition, Bsep is minimally detected in other tissues (Török *et al.* 1999).

Bsep has 12 membrane-spanning helices, which are functionally grouped into two transmembrane domains (Gerloff *et al.* 1998). Bsep utilizes the energy from ATP hydrolysis to facilitate the efflux of its substrates against a concentration gradient (Meier and Stieger, 2002). Bsep transports primarily monovalent bile acids, including taurine and glycine conjugated bile acids, and some secondary bile acids, such as deoxycholic acid, as well as certain drugs, cyclosporine and rifamycin (Byrne *et al.*, 2002; Stieger, 2011).

The regulation of Bsep expression has been extensively investigated. It is well-established that Bsep can be up-regulated following FXR activation, a master regulator that can sense the alteration of bile acid homeostasis (Parks *et al.*, 1999; Ananthanarayanan *et al.*, 2001; Gerloff *et al.*, 2002). FXR signaling can be activated by bile acids, such as chenodeoxycholic acid (CDCA) (Makishima *et al.*, 1999; Parks *et al.*, 1999). Activated FXR forms heterodimer with retinoid X receptor (RXR) alpha, and then binds to Inverted repeat-1 (IR-1) response elements in the promoter of its target genes to induce or suppress their expression (Makishima *et al.*, 1999; Parks *et al.*, 1999). In addition, Nrf2 signaling can up-regulate human BSEP expression in cultured human hepatoma cells (Weerachayaphorn *et al.*, 2009). Nrf2 activation is also essential for Bsep expression under ischemia condition in rat liver (Kim *et al.*, 2019). However, Nrf2 activation appears not to induce mouse Bsep expression (Klaassen and Reisman, 2010).

Bsep expression can be down-regulated by estrogens (Crocenzi *et al.*, 2003). For instance, estradiol-17β-D-glucuronide ($E_2$17G) inhibits Bsep transport function in rat liver (Crocenzi *et al.*, 2003). In addition, lipopolysaccharides (LPS), the endotoxins extracted from outer membrane of Gram-negative bacteria can activate Toll-like receptor (TLR) 4 and then inhibit Bsep expression through NF-κB, the downstream inflammatory pathway (Adachi *et al.*, 1998; Jiang *et al.*, 2000; Shimazu *et al.*, 1999; Sherry and Cerami, 1988; Raetz and Whitfield, 2002; Zollner *et al.*, 2001; Hartmann *et al.*, 2002). Furthermore, many drugs, like bosentan can inhibit Bsep efflux function in both *in vivo* and *in vitro* studies (Fattinger, *et al.*, 2001).

Genetic mutations or dysfunction of Bsep often lead to bile acid accumulation and consequently cholestasis in patients (Anwer, 2004). Mechanistically, genetic mutations of

Bsep often lead to disruption of mRNA expression stability, alterations of BSEP targeting, and even shorter half-life of Bsep expression and turnover time in the membrane (Trauner and Boyer, 2003; Kullak-Ublick *et al.*, 2004; Anwer, 2004). Therefore, mutations of Bsep gene cause defective bile secretion and severe cholestasis in the children, such as in the patients with progressive familial intrahepatic cholestasis (PFIC) 2 (Strautnieks, *et al.*, 1998; Jansen *et al.*, 1999). In addition, drug-induced cholestasis is often accompanied by reduced Bsep expression (Byrne *et al.*, 2002). For example, cyclosporin and rifampicin, two validated competitive Bsep inhibitors, cause drug-induced liver injury (DILI) in patients (Byrne *et al.*, 2002). Therefore, reversal of decreased Bsep expression can help to attenuate the progression of cholestasis.

6. Aims

We propose that the effects of BBR to increase bile flow is partly mediated by up-regulation of expression of major hepatic bile acid transporters, including Ntcp and Bsep. We have previously reported that BBR induced Ntcp expression via inhibition of Jak2-Stat5 signaling (Bu, *et al.*, 2017). The study objective of my dissertation focuses on characterization of regulation of Bsep expression by BBR. Specifically, I determined whether BBR induces Bsep expression and its transport function. In addition, the roles of Nrf2 and TLR4 signaling in BBR-induced Bsep/BSEP expression have been investigated. Specifically, in the first hypothesis, I determined the impact of Nrf2 activation on Bsep/BSEP induction by BRR. In the second hypothesis, I proposed that BBR induced mouse Bsep through TLR4 inactivation.

CHAPTER 2 METHOLOGY

1. Chemicals and Reagents

Berberine chloride was purchased from Alfa Aesar (cat. # AAJ6231106; Tewksbury, MA). Tak242 was purchased from Millipore (cat. # 50-833-600001; Burlington, MA). Lipopolysaccharides (LPS) was purchased from InvivoGen (cat. # NC0202558; San Diego, CA). Analysis kits of serum alanine aminotransferase (ALT) (cat. # A7526-150) and aspartate aminotransferase (AST) (cat. # A7561-150) were purchased from Pointe Scientific, Inc. (Ann Arbor, MI). Analysis kits of glucose (cat. # TR15221), triglycerides (cat. # TR22421) and cholesterol (cat. # TR13421), mem-PER Plus membrane protein extraction kit (cat. # 89842), NE-PER Nuclear and cytoplasmic extraction reagent kit (cat. # 78833), and Lipofectamine 2000 (cat. # 11668027) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Chemiluminescent HRP Substrate (cat. # WBKLS0100) for Western blotting was purchased from Millipore (Billerica, MA). Zymoclean Gel DNA Recovery kit (cat. # D4001), Zymo Plasmid Miniprep kit (cat. # D4036) and DNA Clean & Concentrator kit (cat. # D4013) were purchased from Zymo Research Corporation (Irvine, CA). All other reagents and chemicals, unless specifically indicated, were purchased from Thermo Fisher Scientific Inc. (Waltham, Massachusetts).

2. Animal and Treatment

Approximately 8 weeks of age of male C57BL/6 wild-type mice (cat. # 000664), TLR4-mutated mice, C3H/HeJ (cat. # 00659) and corresponding wild-type mice, C3H/OuJ (cat. # 00635) were purchased from the Jackson Laboratory (Bar Harbor, Maine). Nrf2-null mice, originally engineered in the Laboratory of Dr. Jefferson Chan at the University of California (Irvine, CA), were bred in the animal center at St. John's

University. All animals were maintained under a standard 12-hour light: dark cycle with free access to regular rodent chow and water ad libitum. To mimic the clinical use of BBR, oral administration is used in animal study. In the studies to test Hypothesis I, both wild-type and Nrf2-null mice were orally treated with BBR (30 or 100 mg/kg) once daily for 14 days (n=6/treatment). Control group received saline. In the studies of Hypothesis II, mice were randomly divided into four groups and treated with 1) BBR (100 mg/kg; p.o.) once daily for 7 days; 2) LPS (2 mg/kg; i.p.) for 12 hours; 3) BBR (100 mg/kg; p.o.) for 7 days following LPS (2 mg/kg; i.p.) for 12 hours. Control group received saline (p.o. or i.p.). According to previous reports, BBR or LPS doses used in this study will alter Bsep expression and will not produce apparent hepatotoxicity (Bu *et al.*, 2017; Li *et al.*, 2107).

Mouse condition was monitored daily. Either BBR or LPS treatment did not alter the body weight and animal behavior. After treatment, mouse livers and serum were collected and stored at -80°C for future analysis. One portion of freshly collected mouse liver was processed for histology study.

3. Histopathology

After LPS treatment with or without BBR pre-treatment, mouse livers were fixed in 10% formalin and then processed for Hematoxylin and Eosin (H&E) staining.

4. Serum chemical assay

The serum levels of ALT, glucose, triglycerides and cholesterol in wild-type C57/BL6 mice and serum levels of ALT and AST in wild-type C3H/OuJ mice were analyzed using commercially available kits according to their instructions.

5. Cell Culture

Mouse Hepa1c1c7 hepatoma cells (cat. # CRL-2026; ATCC®; Manassas, VA) were cultured in Corning® MEM (Minimum Essential Medium) Alpha Medium (cat. # MT15012CV; Corning, NY). Human SNU449 hepatoma cells (cat. # CRL-2234; ATCC®; Manassas, VA) were cultured in Gibco™ DMEM High Glucose medium (cat. # 11-995-065; Waltham, MA). The cell culture medium was supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals; Norcross, GA). Cells were cultured at 37°C with 5% CO_2 atmosphere. Both mouse and human hepatoma cells were treated by BBR as we previously described (Bu *et al.*, 2017). Briefly, mouse Hepa1c1c7 or human SNU449 hepatoma cells were seeded into 6-well plates (3 x10^5/well) and treated with BBR (5, 10 or 30 μM), in triplicate (Zhai *et al.*, 2008; Bu *et al.*, 2017). Mouse Hepa1c1c7 cells were also treated with LPS (10 ng/mL) in the presence or absence of BBR (10 μM) or Tak242 (0.3 mM), in triplicate. Control group received fresh medium. After 24 and 48 hours treatment, cells were collected and processed for mRNA and protein analysis, respectively.

6. Transfection of siRNA

The small interfering RNAs (siRNA) against mouse (cat. # L-003755-00-0005) or human Bsep/BSEP (cat. # L-04076655-00-0005), as well as nontargeting negative control siRNA (cat. #D0018101005) were purchased from Dharmacon™(Lafayette, CO). Mouse Hepa1c1c7 and human SNU449 hepatoma cells were transfected to test silence efficiency using Lipofectamine messengerMAX™ reagent per manufacturer's instructions (cat. # LMRNA003; Life Technologies; Carlsbad, CA). The successful gene silence should knockdown more than 75% of mRNA expression of target gene. After BBR treatment (10

μM) for 24 hours, cells were then collected and performed quantitative RT-PCR for mRNA analysis.

7. Quantitative Real time-PCR (qRT-PCR) assay

Total RNAs from mouse liver and cultured hepatoma cells were extracted using TRIzol™ RNA extraction reagent (cat. # 15596026; Life Technologies; Carlsbad, CA) per the manufacturer's instructions. RNA integrity was assessed by electrophoresis on a denatured agarose gel containing 3-(N-morpholino) propanesulfonic acid (MOPS) and formaldehyde. RNA concentrations were spectrophotometrically quantified at 260nm. Then, total RNAs were reversely transcribed into cDNA using SuperScript™ II reverse transcriptase (cat. # 18064014; Life Technologies; Carlsbad, CA). Quantitative RT-PCR was performed using SYBR™ Select Master Mix (cat. # 4472903; Life Technologies; Carlsbad, CA) in an AriaMx qRT-PCR system (Agilent Technologies; Santa Clara, CA). Data was calculated according to the comparative delta-delta CT method. The results were presented as relative fold of the control. Primers of mouse or human Bsep/BSEP, Nrf2/NRF2 and its target genes, as well as inflammation genes were listed in Table 2.1. All quantitative RT-PCR primers were designed using PubMed Primer-BLAST or Primer3 software (NIH) and synthesized by Integrated DNA Technologies (Coralville, IA) or Eurofins Genomics (Louisville, KY).

8. Immunocytochemistry (ICC) Staining

Mouse Hepa1c1c7 and human SNU449 hepatoma cells were cultured in chamber slides (cat. # 12-565-7; Thermo Fisher Scientific Inc.; Waltham, MA) and treated with or without BBR (5 or 10 μM) for 48 hours. Fixation and blocking were performed as previously described (Bu *et al.*, 2017). Then, cells were incubated with the antibody

(1:100) against mouse/human Bsep/BSEP protein (cat. # PAB4697; Abnova; Walnut, CA) or the antibody (1:100) against mouse/human Nrf2/NRF2 protein (cat. # 12721; Cell Signaling Technology; Danvers, MA) overnight at 4°C. Next day, chamber slides were incubated with 1:300 Pierce Goat Anti-Rabbit IgG (H+L) Biotin Conjugated secondary antibody (cat. # 31820; Thermo Fisher Scientific Inc.; Waltham, MA) for 4 hours at room temperature. Cells were then washed and incubated with 1:400 Pierce High Sensitivity Streptavidin-HRP (cat. # 21130; Thermo Fisher Scientific Inc.; Waltham, MA) or Alkaline phosphatase streptavidin (cat. # SA5100; Vector Laboratories Inc.; Burlingame, CA) for 1 hour at room temperature. After final wash, color development was achieved using BCIP/NBT Alkaline phosphatase substrate kit (cat. # SK5400) in Vector Laboratories Inc. (Burlingame, CA) according to the manufacturer's instructions.

9. Western Blotting

Membrane and nuclear proteins from mouse liver and hepatoma cells were extracted using the Mem-PER Plus membrane protein extraction kit and the NE-PER Nuclear and cytoplasmic extraction reagents kit, respectively. Protein concentrations were quantified at 280 nm using spectrophotometer. Protein samples (30 ng/lane) were mixed with SDS loading buffer and heated and separated by 12% or 15% SDS-PAGE gel. After electro-transfer blotting, PVDF membranes were blocked for 2 hours in 1xTBS supplemented with 5% non-fat milk or 5% BSA. PVDF membranes were then incubated with the antibody (1:1,000) against mouse/human Bsep/BSEP protein (cat. # PAB4697; Abnova; Walnut, CA), the antibody (1:1,000) against mouse/human Nrf2/NRF2 protein (cat. # 12721; Cell Signaling Technology; Danvers, MA) or the antibody (1:1,000) against mouse and human Bsep/BSEP (cat. # PAB4697; Abnova; Walnut, CA) or antibody

(1:1,000) against mouse and human p65-NF-κB (cat. # D14E12; Cell Signaling Technology; Danvers, MA) in 2.5% BSA at room temperature for 1 hour, followed by overnight incubation at 4°C. PVDF membranes were then washed and incubated with Pierce Goat Anti-Rabbit IgG (H+L) Biotin Conjugated secondary antibody (1:5,000; Thermo Fisher Scientific Inc.; Waltham, MA) in 1XTBS supplemented with 2.5% non-fat milk for 2 hours at room temperature. Last, the membranes were incubated with Pierce High Sensitivity Streptavidin-HRP (cat. # 21130; 1:5000; Thermo Fisher Scientific Inc.; Waltham, MA) in 1XTBS supplemented with 0.5% BSA for 30 minutes at room temperature. Beta-actin (cat. # MA5-15739; 1:2,000; Pierce biotechnology; Waltham, MA) and Histone-H3 (cat. # 4499; 1:1,000; Cell Signaling Technology; Danvers, MA) were used as loading controls for membrane and nuclear proteins, respectively. Immunoreactive protein bands were detected with Immobilon Western Chemiluminescent HRP Substrate (cat. # WBKL S00 50; EMD Millipore, Billerica, MA).

10. Analysis of Efflux Transport Function of Bsep/BSEP

The Bsep transport function was analyzed as previously reported (Li *et al.*, 2008). Specifically, mouse Hepa1c1c7 and human SNU449 hepatoma cells were treated with BBR (10 μM) and then harvested for 48 hours. Collected cells were washed with cold 1×PBS twice. The cells were aliquoted ($1x10^6$ per tube) into a 1.5 mL tube and incubated with dichlorofluorescin diacetate (cat. # D292648; Sigma-Aldrich; St. Louis, MO), a selective Bsep substrate for 40 minutes at 4°C. After incubation, cells were washed by cold 1XPBS twice and re-suspended with 1X PBS and then transferred into a new 1.5 mL tube. All tubes were incubated with a speed of 300 rpm at 37°C for 1 hour. Then, both

supernatant and cell pellet following centrifugation were collected. Cells were then lysated using 10% SDS solution. Dichlorofluorescin diacetate fluorescence was measured in both supernatant and cell lysate using Tecan Microplate Readers (Männedorf, Switzerland) at excitation 485 nm and emission 530 nm.

11. Cloning of Bsep/BSEP Gene Promoters of Different Lengths

BAC clones containing mouse Bsep (cat. # RP23-291P1) and human BSEP (cat. # RP11-527A7) genes were purchased from BACPAC resources (Children's Hospital Oakland; Oakland, CA). The 2.9-kb of mouse Bsep gene promoter or 3.6-kb of human BSEP gene promoter was cloned from BAC clones via PCR analysis, and then engineered into polycloning site of the pGL3-basic vector (cat. # E1751; Promega; Fitchburg, WI). For truncated promoter constructs, the locations and sequences of the forward primers, the shared reverse primer were listed in Table 2.2. The sequences of different lengths of Bsep/BSEP gene promoters that were inserted into pGL3 reporter construct were validated by DNA sequencing (Eurofins Genomics; Huntsville, AL).

12. Transfection and Dual-Luciferase® Reporter Assay

Recombinant pGL3 reporter constructs were transfected into mouse Hepa1c1c7 or human SNU449 cells, using Lipofectamine 2000 transfection reagent (cat. # 11668027; Life Technologies, Inc.; Carlsbad, CA) following the manufacturer's instructions. Briefly, the cells were seeded onto 96-well tissue culture plates 1 day prior to transfection. Plasmid DNA (180-200 ng) of the empty pGL3-basic vector (cat. # E1751; Promega, Fitchburg, WI) or Bsep/BSEP promoter-containing pGL3 reporter constructs, plus 10 ng of pRL Renilla luciferase reporter vector (cat. # E2261; Promega; Madison, Wisconsin) was mixed with Lipofectamine 2000 with 1:1 ratio and then dispensed to each well. After

24 hours of incubation, transfection medium was replaced with fresh mediums with or without BBR at indicated concentrations. Forty-eight hours later, the cells were lysed and processed for Dual-Luciferase® reporter assay per the manufacturer's instructions (cat. # E1910; Promega; Fitchburg, WI).

13. Statistical Analysis

Data were expressed as mean ± standard error. The data between control and BBR-treated group were analyzed by Student's t-test. Comparisons between more than two treatment groups were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's post-hoc tests (Sigmaplot; Systat Software, Inc.; San Jose, CA). The statistical significance was considered when $p < 0.05$.

Table 2.1: qPCR primers for analysis of mRNA expression of mouse and human 18s rDNA, Nrf2, Ho-1, Nqo-1, Bsep and inflammation genes by quantitative RT-PCR assays

Gene	Accession No.	Forward primer	Reverse primer
18s	NR_003278.3 (m, h)	GCAATTATTCCCCATGAACG	GGCCTCACTAAACCATCCAA
Nrf2	NM_007824.2 (m)	CGAGATATACGCAGGAGAGGTAAGA	GCTCGACAATGTTCTCCAGCTT
Ho-1	NM_010012.3 (m)	CCTCACTGGCAGGAAATCATC	CCTCGTGGAGACGCTTTACATA
Nqo-1	NM_024264.5 (m)	TATCCTTCCGAGTCATCTCTAGCA	TCTGCAGCTTCCAGCTTCTTG
Bsep	NM_031760.1 (m)	GGACAATGATGTGCTTGTGG	CACACAAAGCCCCTACCAGT
TNF-α	NM_001278601.1 (m)	GCCTCTTCTCATTCCTGCTTG	CTGATGAGAGGGAGGCCATT
IL-1β	NM_008361.4 (m)	AACCTGCTGGTGTGTGACGTTC	CAGCACGAGGCTTTTTTGTTGT
IL-6	NM_001314054.1 (m)	ACAACCACGGCCTTCCCTACTT	CACGATTTCCCAGAGAACATGTG
NRF2	NM_000780.3 (H)	CGGTATGCAACAGGACATTG	GTTTGGCTTCTGGACTTGGA
HO-1	NM_006446.4 (H)	GAAAAGCACATCCAGGCAAT	GCTGCCACATTAGGGTGTCT
NQO-1	NM_019844.3 (H)	GTTGCCTGAAAAATGGGAGA	AAAAACCACCAGTGCCAGTC
BSEP	NM_003742.4 (H)	TGTGGGAAGTTTGTGTGCAT	ACCCACAACGTGTTCCATTT

Table 2.2: Sequences of PCR cloning primers of mouse and human Bsep/BSEP promoter.

Bsep/BSEP promoter	**Promoter region**	**Forward primer sequence**
pGL3-Bsep-2928	-2,843 to +85	**P1:** AAGACTGACGCGTGTGAGCTCCTCT
pGL3-Bsep-2120	-2,035 to +85	**P2:** TGGACTTCACGCGTGGGTGAT
Shared reverse primers (m)		AGAGACTCGAGTGTGGAAAGTCA
pGL3-BSEP-3680	-3,602 to +78	**P1:** TTCTGCTAGCCTGGGTCTCT
pGL3-BSEP-377	-299 to +78	**P2:** GCAACACGCGTTTTCCAAGAG
pGL3-BSEP-178	-100 to +78	**P3:** TTCCAACGCGTGTTGACAC
Shared reverse primers (H)		CAACCTCGAGTTTCATCATTC

CHAPTER 3 RESULTS

1. Regulation of Mouse and Human Bsep/BSEP by BBR via Nrf2 Activation

1.1. BBR Decreased Serum Level of Cholesterol in Wild-type Mice

BBR did not increase serum level of ALT, a biomarker of liver injury in wild-type mice (Fig. 3.1A). BBR at 100 mg/kg tended to decrease serum levels of glucose (Fig. 3.1B) and triglycerides (Fig. 3.1C), and reduced serum levels of cholesterol (Fig. 3.1D).

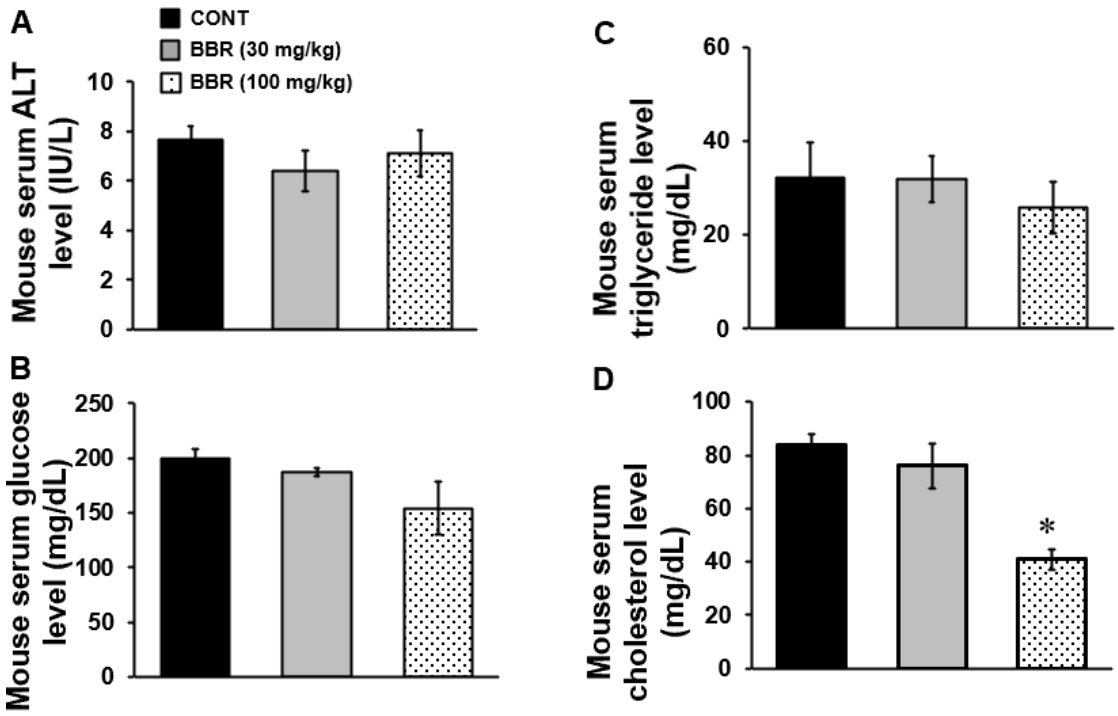

Figure 3.1 Effects of BBR on serum chemical levels in wild-type mice.

Wild-type C57BL/6 mouse sera were collected after BBR treatment (0, 30 or 100 mg/kg; p.o.) once daily for 14 days (n=6/treatment). Mice serum were collected and processed for serum chemical analysis of ALT (**A**), glucose (**B**), triglycerides (**C**) and cholesterol (**D**). Statistical differences (p <0.05) are indicated between BBR and control group (*).

1.2. BBR Increased Bsep mRNA and Protein Expression in Mouse Liver and Hepatoma Cells

BBR dose-dependently induced mRNA expression of Bsep in wild-type (Fig. 3.2A) but not in Nrf2-null mouse livers (Fig. 3.2B). In addition, BBR increased membrane protein expression of Bsep in both wild-type and Nrf2-null mouse livers (Fig. 3.3A and B).

BBR induced mRNA expression of Bsep/BSEP in both mouse Hepa1c1c7 (Fig. 3.4A) and human SNU449 (Fig. 3.4B) hepatoma cells. BBR dose-dependently increased membrane Bsep/BSEP protein expression in both mouse Hepa1c1c7 (Fig. 3.5A) and human SNU449 (Fig. 3.5B) hepatoma cells. In addition, immunocytochemistry (ICC) staining showed that BBR increased relative numbers of Bsep/BSEP protein-positive cells, which further demonstrated that BBR increased mouse and human Bsep/BSEP protein expression (Fig. 3.6A and B).

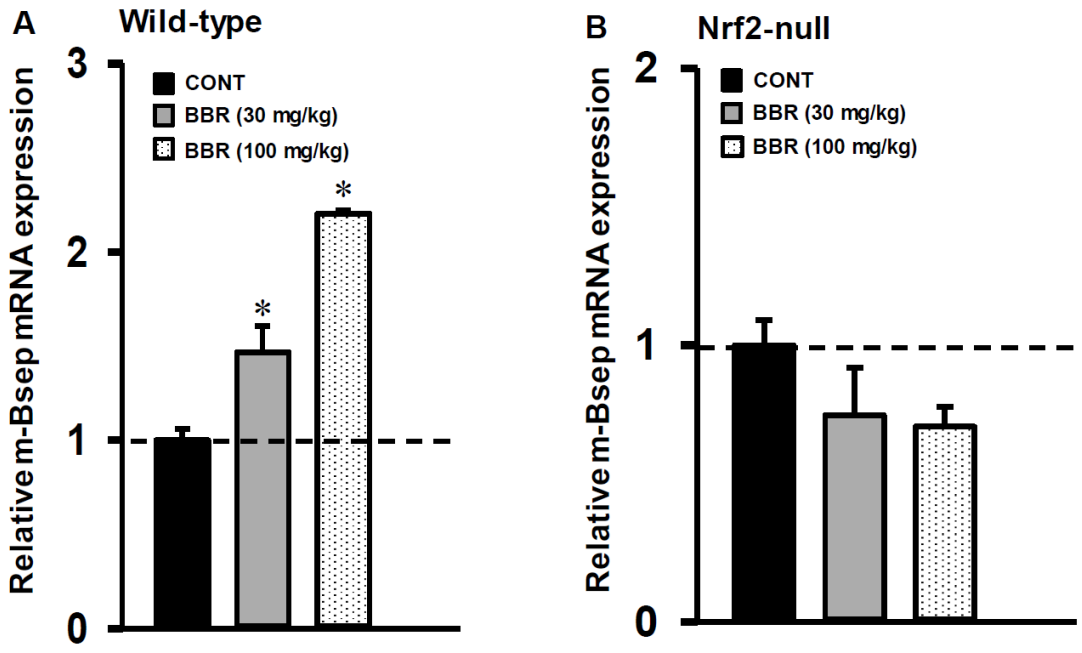

Figure 3.2 Regulation of Bsep mRNA expression by BBR in wild-type and Nrf2-null mouse livers.

Adult male C57BL/6 wild-type mice and age-matched male Nrf2-null mice were orally treated with BBR (0, 30 or 100 mg/kg) once daily for 14 days (n=6/treatment). Mouse livers were harvested 14 days later and processed for quantitative RT-PCR analysis of Bsep mRNA expression in wild-type (**A**) and Nrf2-null (**B**) mouse livers. Statistical differences (p <0.05) are indicated between BBR and control group (*).

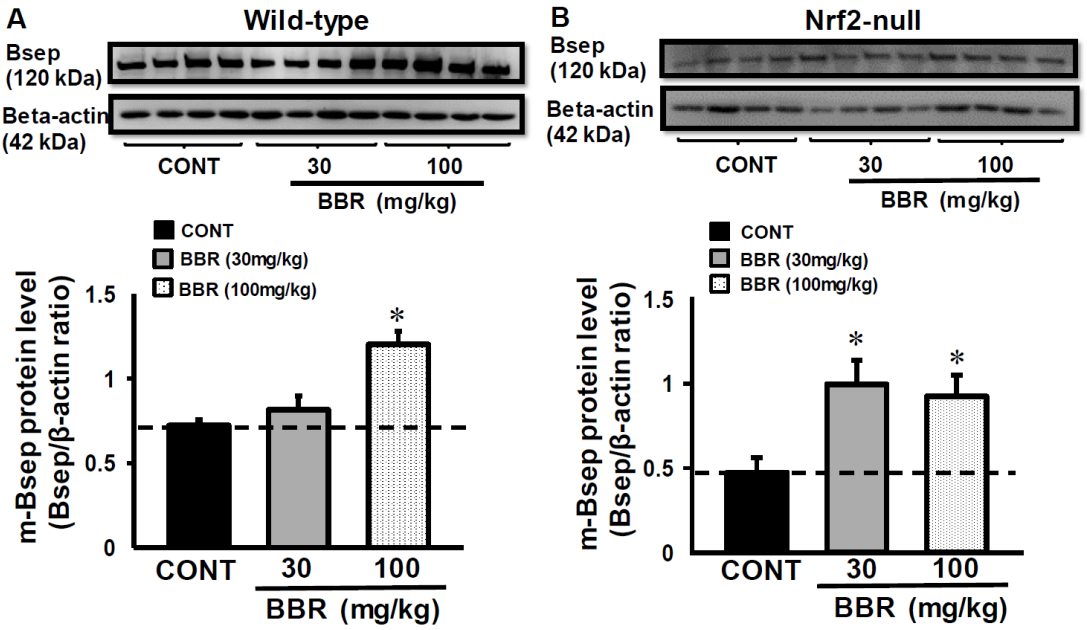

Figure 3.3 Regulation of Bsep protein expression by BBR in wild-type and Nrf2-null mouse livers.

Adult male C57BL/6 wild-type and age-matched male Nrf2-null mice were orally treated with BBR (0, 30 or 100 mg/kg) once daily for 14 days (n=6/treatment). Membrane proteins, extracted from wild-type (**A**) and Nrf2-null (**B**) mouse livers, were processed for analysis of Bsep protein expression by Western blotting. Statistical differences (p <0.05) are indicated between BBR and control group (*).

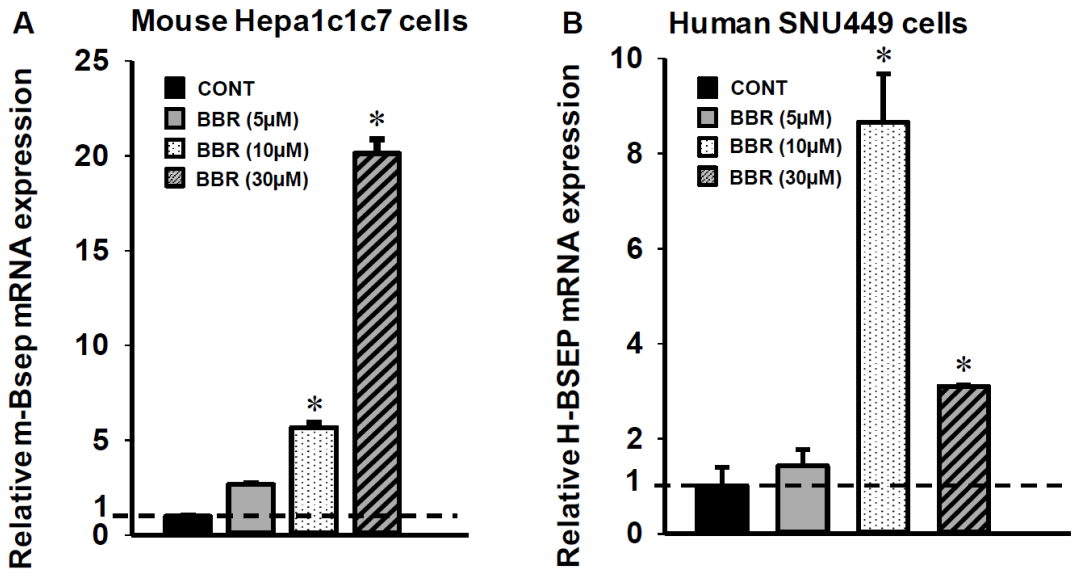

Figure 3.4 Regulation of Bsep/BSEP mRNA expression by BBR in cultured mouse and human hepatoma cells.

Quantitative RT-PCR was conducted to determine mRNA levels of Bsep/BSEP in cultured mouse Hepa1c1c7 (**A**) and human SNU449 (**B**) hepatoma cells following BBR treatment (0, 5, 10 or 30 μM) for 24 hours. Data are expressed as mean ± S.E., (n=3/treatment). Statistical differences (p <0.05) are indicated between BBR and control group (*).

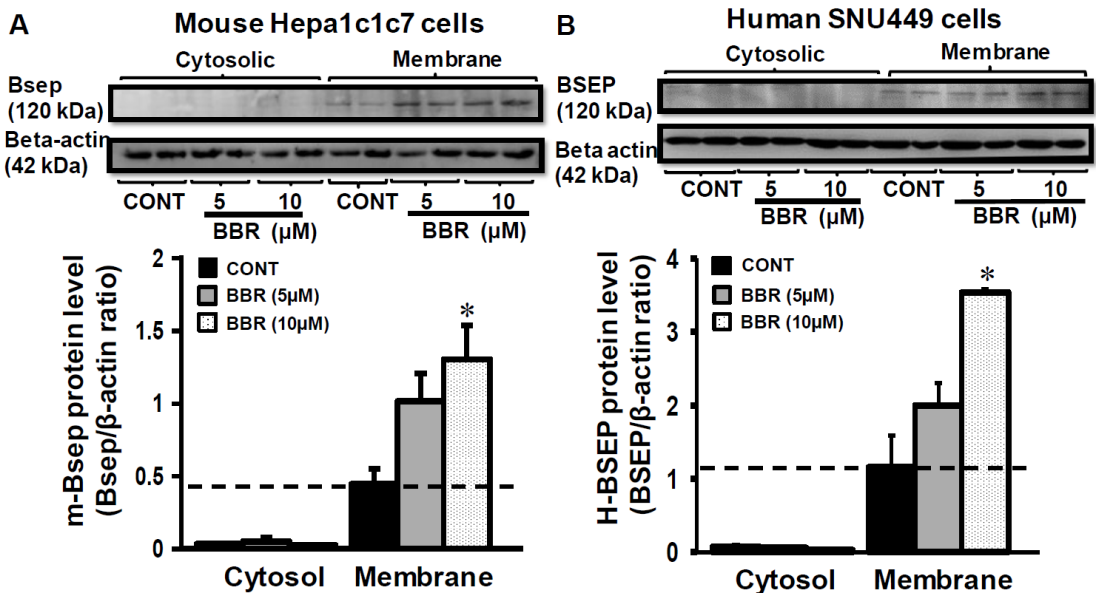

Figure 3.5 Regulation of Bsep/BSEP protein expression by BBR in cultured mouse and human hepatoma cells.

Mouse Hepa1c1c7 and human SNU449 hepatoma cells, in triplicate, were treated with BBR (0, 5, or 10 μM) for 48 hours. Cytosolic and membrane proteins, extracted from mouse (**A**) and human (**B**) hepatoma cells were analyzed for Western blotting of Bsep/BSEP protein expression. Data are expressed as mean ± S.E., (n=3/treatment). Statistical differences (p <0.05) are indicated between BBR and control group (*).

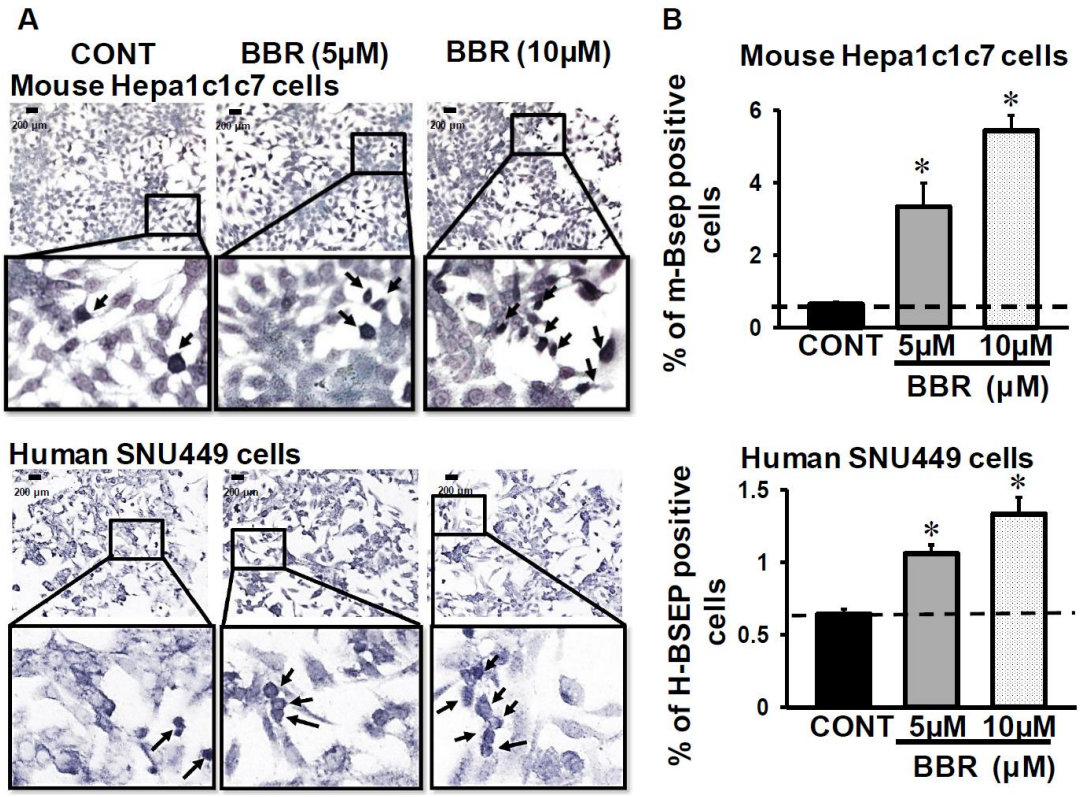

Figure 3.6 Immunocytochemistry staining of Bsep/BSEP protein in BBR-treated mouse and human hepatoma cells.

Immunocytochemistry staining of Bsep/BSEP protein in BBR treated mouse Hepa1c1c7 (**A**, top panel) and human SNU449 (**A**, bottom panel) hepatoma cells. 200× magnification, (Scale bar: 100 μm). Counting of Bsep/BSEP positive cells in a total of 1,000-1,500 cells (averaged on 20 random fields) per treatment in BBR-treated mouse (**B**, top panel) and human (**B**, bottom panel) hepatoma cells. Statistical differences (p <0.05) are indicated between BBR and control group (*).

1.3. BBR Increased Cellular Efflux of Bsep Substrate in Hepatoma Cells

Bsep/BSEP efflux function was assessed by monitoring cellular efflux of dichlorofluorescin diacetate, a selective Bsep substrate, into the culture medium. As shown in Fig. 3.7, BBR treatment leads to an increase of dichlorofluorescin diacetate concentrations in culture medium of both mouse Hepa1c1c7 and human SNU449 hepatoma cells. Accordingly, BBR treatment produces a decrease of dichlorofluorescin diacetate concentrations in the cell lysates of both mouse and human hepatoma cells (Fig. 3.7A and B).

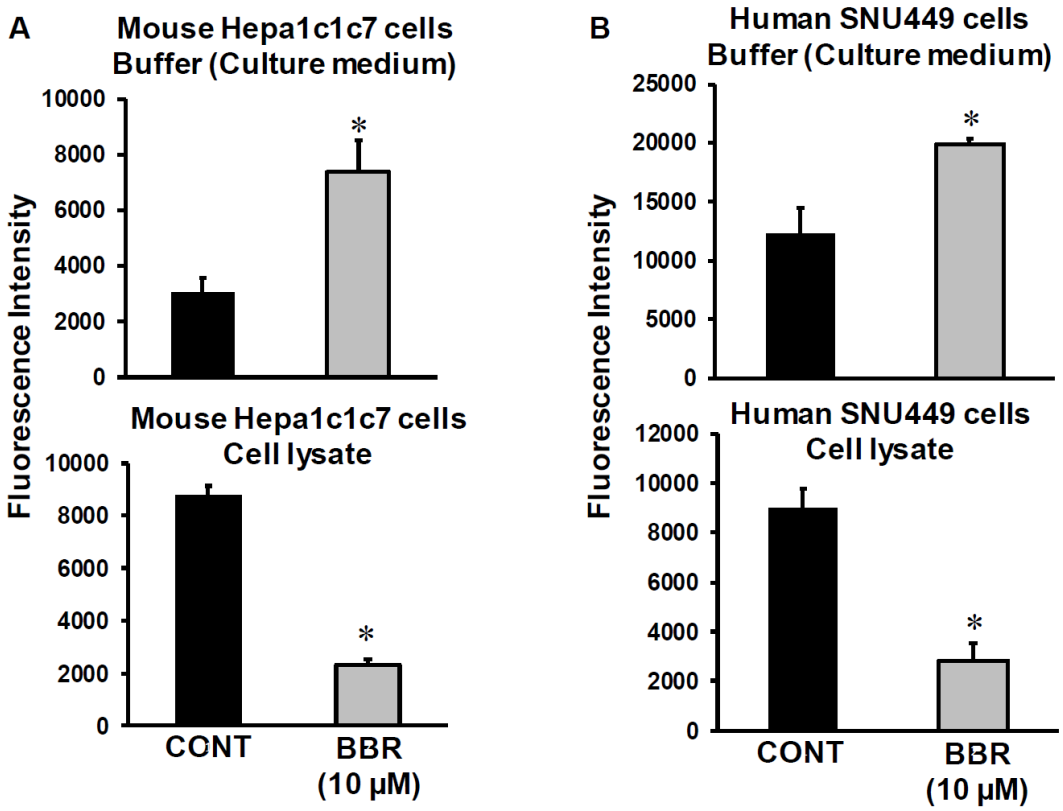

Figure 3.7 BBR enhances Bsep/BSEP efflux function in cultured mouse and human hepatoma cells.

Mouse Hepa1c1c7 and human SNU449 hepatoma cells, in triplicate, were treated with BBR (10 μM) for 48 hours. Efflux of dichlorofluorescin diacetate, a selective Bsep substrate was measured in mouse (**A**) and human (**B**) hepatoma cells. All data are expressed as mean ± S.E., (n=3/treatment). Statistical differences (p <0.05) are indicated between BBR and control group (*).

1.4. BBR Activates Nrf2 Signaling in Hepatoma Cells but not in Mouse Livers

BBR dose-dependently induced mRNA expression of Ho-1 and Nqo-1, two known Nrf2-target genes in wild-type (Fig. 3.8A) but not in Nrf2-null mouse livers (Fig. 3.8B). However, in wild-type mouse livers, BBR did not significantly increase nuclear Nrf2 protein level (Fig. 3.9).

In addition, BBR induced mRNA expression of Ho-1/HO-1 and Nqo-1/NQO-1 in both mouse Hepa1c1c7 (Fig. 3.10A) and human SNU449 (Fig. 3.10B) hepatoma cells. BBR also increased nuclear levels of Nrf2/NRF2 proteins (Fig. 3.11A and B), as well as the relative number of Nrf2/NRF2 protein-positive cells (Fig. 3.12A and B) in mouse and human hepatoma cells.

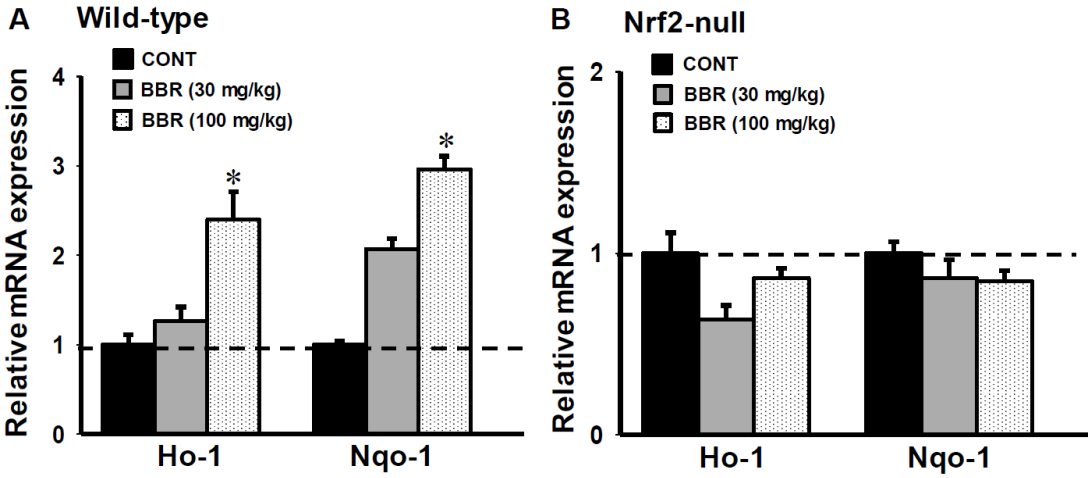

Figure 3.8 Regulation of mRNA expression of Ho-1 and Nqo-1 by BBR in wild-type and Nrf2-null mouse livers.

Adult male C57BL/6 wild-type and age-matched male Nrf2-null mice were orally treated with BBR (0, 30 or 100 mg/kg) once daily for 14 days (n=6/treatment). The mouse livers were collected 14 days later and then processed for quantitative RT-PCR analysis of mRNA expression of Ho-1 and Nqo-1 in wild-type (**A**) and Nrf2-null (**B**) mouse livers. Statistical differences (p <0.05) are indicated between BBR and control group (*).

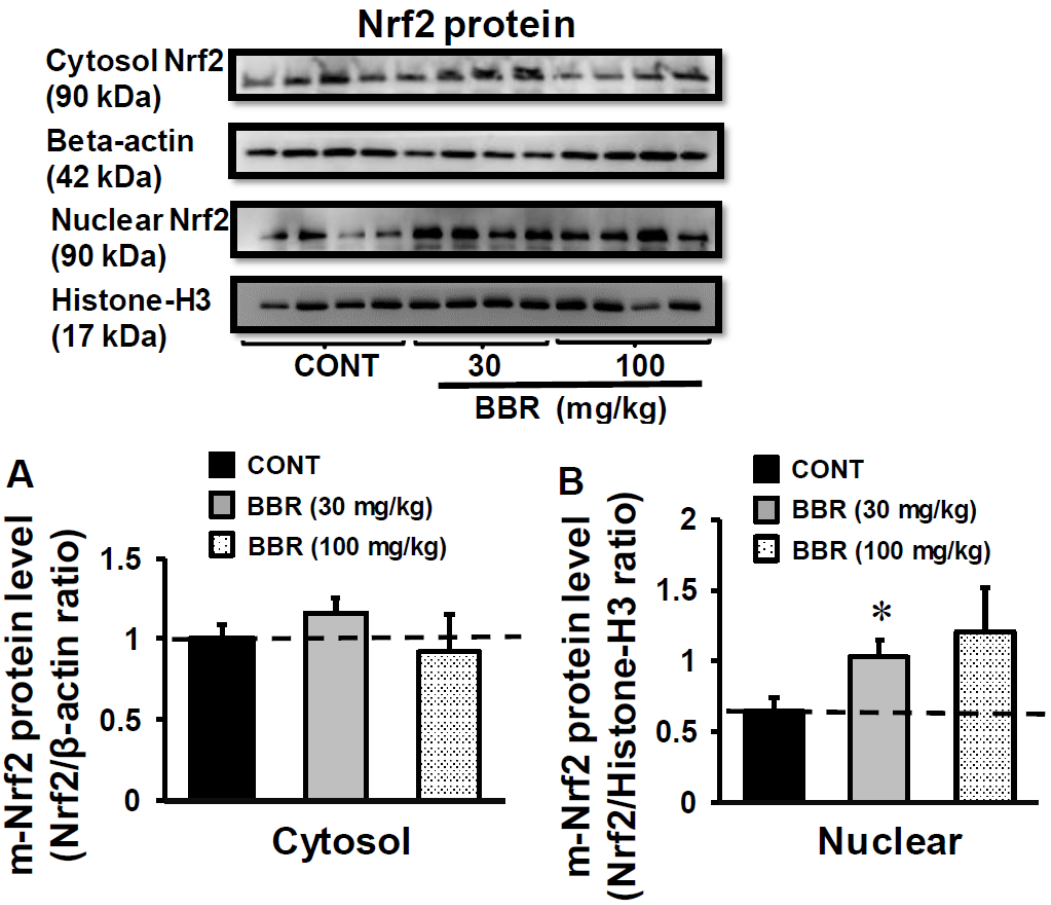

Figure 3.9 Regulation of cytosolic and nuclear Nrf2 protein levels by BBR in mouse livers.

Adult male C57BL/6 wild-type mice were orally treated with BBR (0, 30 or 100 mg/kg) once daily for 14 days (n=6/treatment). Cytosolic and nuclear proteins, extracted from wild-type mouse livers were processed for Western blotting of Nrf2 protein expression. Statistical differences (p <0.05) are indicated between BBR and control group (*).

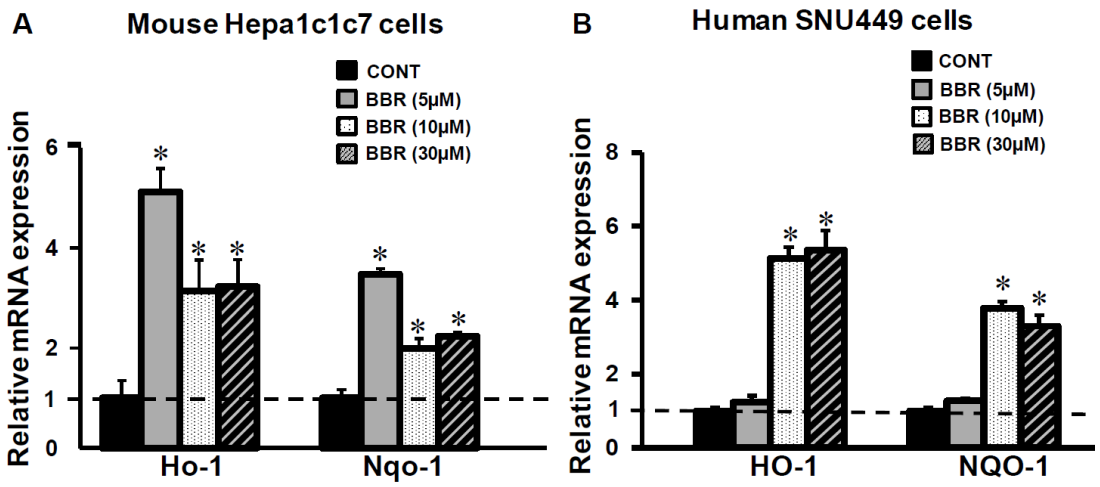

Figure 3.10 Regulation of mRNA expression of Ho-1/HO-1 and Nqo-1/NQO-1 in cultured mouse and human hepatoma cells.

Quantitative RT-PCR analysis of mRNA expression of Nrf2/NRF2-targte genes, Ho-1/NO-1 and Nqo-1/NQO-1 in cultured mouse Hepa1c1c7 (**A**) and human SNU449 (**B**) hepatoma cells following BBR treatment (0, 5, 10 or 30 μM). All data are expressed as mean ± S.E., (n=3/treatment). Statistical differences (p <0.05) are indicated between BBR and control group (*).

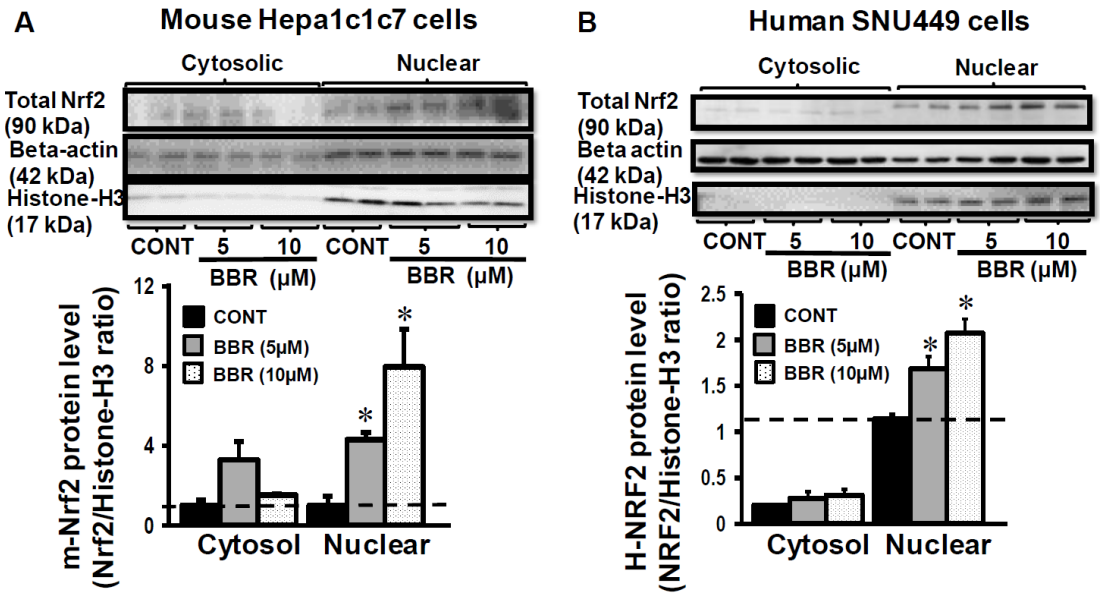

Figure 3.11 Regulation of nuclear Nrf2/NRF2 protein level in cultured mouse and human hepatoma cells.

Mouse Hepa1c1c7 and human SNU449 hepatoma cells were treated with BBR (0, 5, or 10 μM), in triplicate for 48 hours. Cytosolic and nuclear proteins, extracted from mouse (**A**) and human (**B**) hepatoma cells were analyzed for Western blotting of Nrf2/NRF2 protein expression. All data are expressed as mean ± S.E., (n=3/treatment). Statistical differences (p <0.05) are indicated between BBR and control group (*).

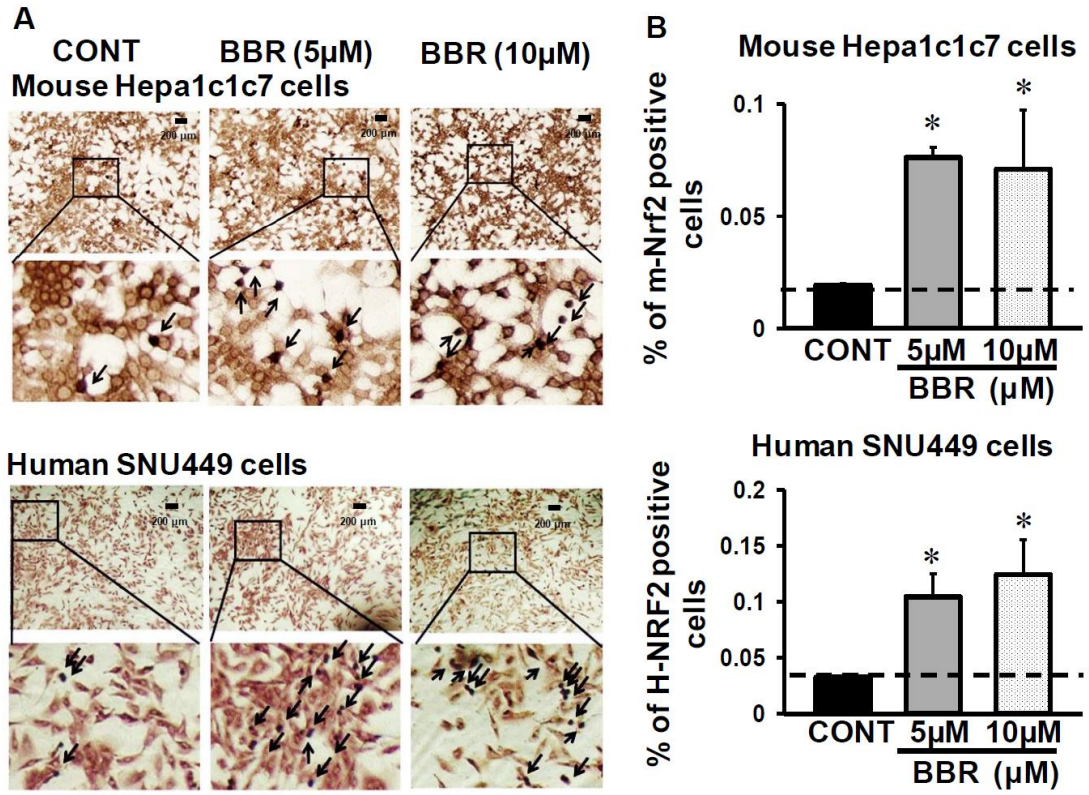

Figure 3.12 Immunocytochemistry staining of Nrf2/NRF2 protein in BBR-treated mouse and human hepatoma cells.

Immunocytochemistry staining of Nrf2/NRF2 protein in BBR treated mouse Hepa1c1c7 (**A**, top panel) and human SNU449 (**A**, bottom panel) hepatoma cells. 200× magnification, (Scale bar: 100 μm). Counting of Nrf2/NRF2 positive cells with a total of 1,000-1,500 cells (averaged on 20 random fields) in BBR or control treated mouse (**B**, top panel) and human (**B**, bottom panel) hepatoma cells. Statistical differences (p <0.05) are indicated between BBR and control group (*).

1.5. The Impact of Nrf2/NRF2 mRNA Silencing on BBR-induced Mouse and Human Bsep/BSEP Expression

In mouse hepatoma cells, Nrf2 siRNA treatment reduced Nrf2 mRNA levels about 75%, and largely repressed BBR-induced Bsep mRNA expression (Fig. 3.13). BBR still slightly increased Bsep mRNA expression after Nrf2 silence.

In human hepatoma cells, NRF2 siRNA treatment knocked down NRF2 mRNA levels more than 86%, and almost completely abolished BBR-induced BSEP mRNA expression (Fig. 3.14).

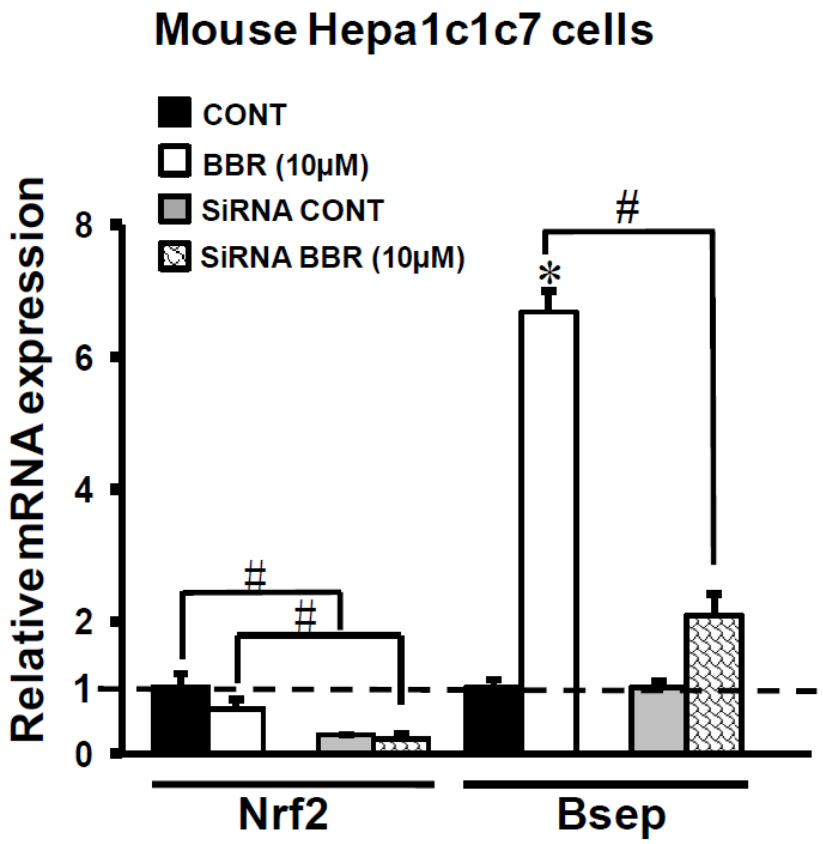

Figure 3.13 Nrf2 silence attenuated BBR-induced mouse Bsep expression.

Mouse Hepa1c1c7 hepatoma cells were treated with BBR (10 μM), in triplicate in the presence or absence of Nrf2 siRNA for 24 hours. Quantitative RT-PCR analysis for mRNA expression of Nrf2 (Left panel) and Bsep (Right panel) was determined in mouse hepatoma cells. All data are expressed as mean ± S.E., (n=3/treatment). Statistical differences (p <0.05) are indicated between BBR and control group (*), or between with Nrf2 siRNA treatment and without Nrf2 siRNA treatment (#).

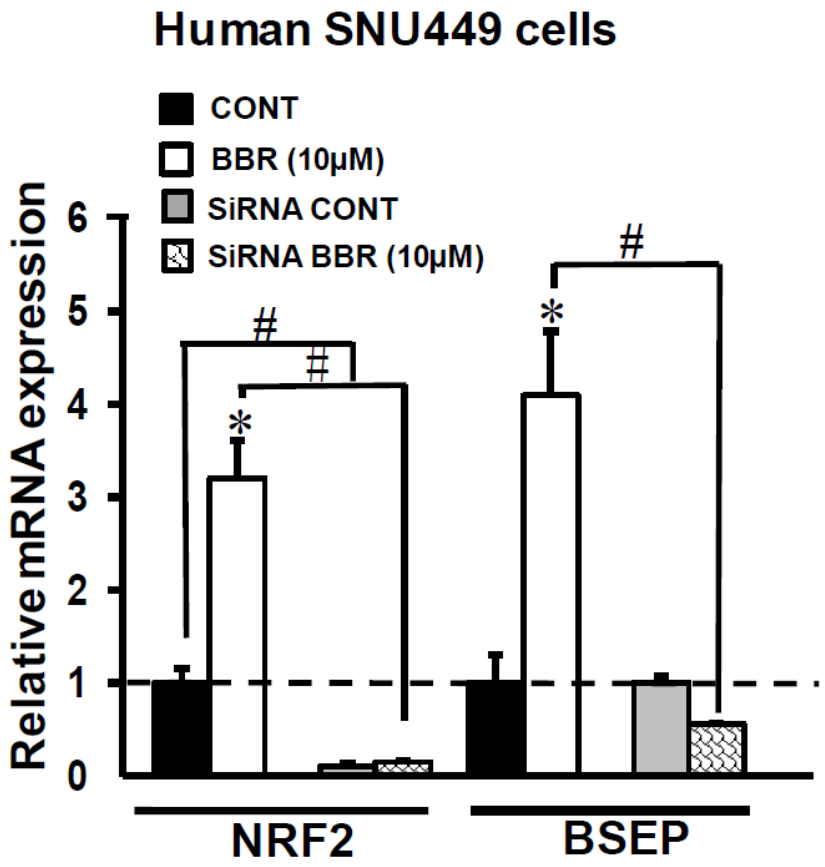

Figure 3.14 NRF2 silence abolished BBR-induced human BSEP expression.

Human SNU449 hepatoma cells were treated with BBR (10 μM), in triplicated in the presence or absence of NRF2 siRNA for 24 hours. Quantitative RT-PCR analysis for mRNA expression of NRF2 (Left panel) and BSEP (Right panel) was next determined in human hepatoma cells. All data are expressed as mean ± S.E., (n=3/treatment). Statistical differences (p <0.05) are indicated between BBR and control group (*), or between with NRF2 siRNA treatment and without NRF2 siRNA treatment (#).

1.6. Removal of Putative NRF2 ARE from Bsep/BSEP Promoter Results in Altered Promoter Activity

To determine the role of Nrf2 signaling in BBR-induced mouse and human Bsep/BSEP expression, we engineered a series of recombinant pGL3 reporter constructs containing different lengths of Bsep/BSEP promoters, as indicated in Figs. 3.15, 3.16 and 3.17. Luciferase activities that were driven by the 2.9-kb mouse and 3.7-kb human Bsep/BSEP gene promoters, were both concentration-dependently induced by BBR (Fig. 3.15A and B).

In mouse hepatoma cells, luciferase reporter activity driven by 2.9-kb mouse Bsep promoter (ranged from -2,843 to +85bp) with a putative antioxidative response element (ARE) located at -2,760 bp upstream of transcriptional start site (Fig. 3.16) is induced by BBR treatment in a concentration-dependent manner. In addition, we also engineered a truncated Bsep promoter that does not contain putative ARE (ranged from -2035 to +85bp) into pGL3 basic vector (Fig. 3.16). To our surprise, the luciferase activity of the truncated mouse Bsep promoter-driven pGL3 construct was even increased more by BBR treatment as compared to 2.9-kb mouse Bsep promoter-driven pGL3 construct, indicating that Nrf2 activation may suppress mouse Bsep expression.

In human hepatoma cells, the 3.7-kb human BSEP promoter (ranged from -3,602 to +78bp), which contains one putative ARE located at -100 to +78bp relative to transcriptional start site can be activated by BBR treatment (Fig. 3.17). We also engineered a shorter fragment of BSEP promoter with one putative ARE, as depicted in Fig. 2.16 to determine whether enhancer regulated human BSEP expression. The result showed that BBR still increased luciferase activity in these constructs with one putative

ARE. In contrast, the truncated human BSEP promoter that does not contain the putative ARE, as depicted in Fig. 3.17, cannot be activated by BBR treatment. This indicates that the putative ARE in human Bsep gene promoter is essential for BBR to induce human BSEP expression.

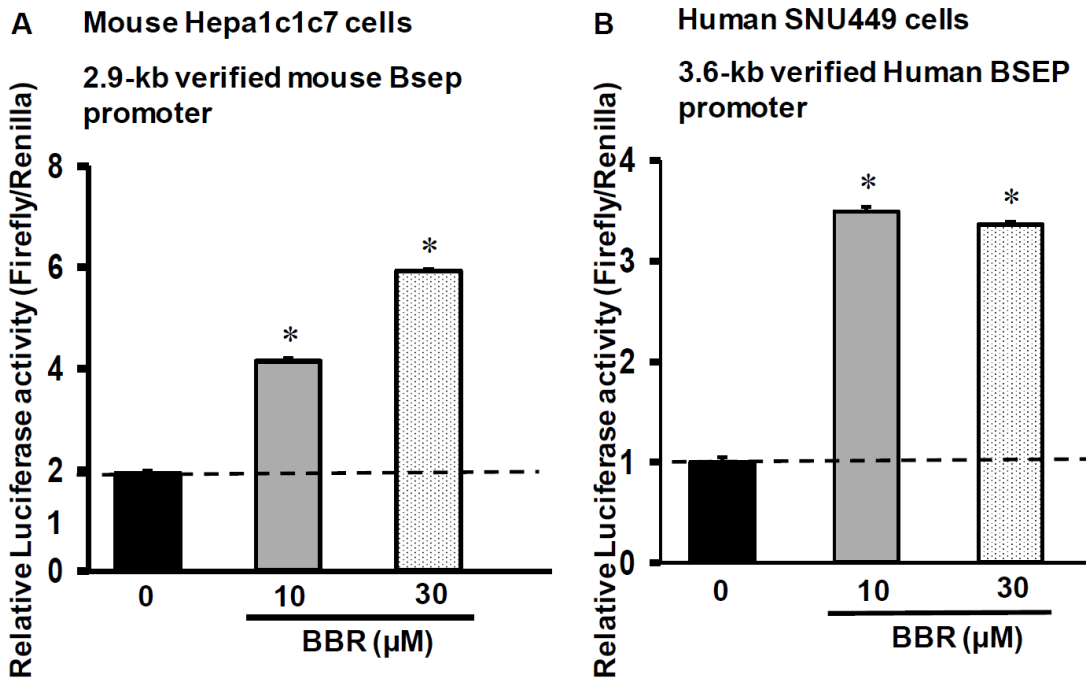

Figure 3.15 The impact of BBR on the activation of mouse and human Bsep/BSEP promoter determined by dual luciferase reporter assays in cultured mouse and human hepatoma cells.

Bsep/BSEP gene promoter-driven recombinant pGL3 reporter constructs were transfected into hepatoma cells. The regulation of luciferase activity, which is driven mouse or human Bsep/BSEP gene promoter was determined in mouse Hepa1c1c7 (**A**) and human SNU449 (**B**) hepatoma cells following BBR treatment (0, 10, or 30μM) for 48 hours. All data are expressed as mean ± S.E., (n=3/treatment). Statistical differences (p <0.05) are indicated between BBR and control group (*).

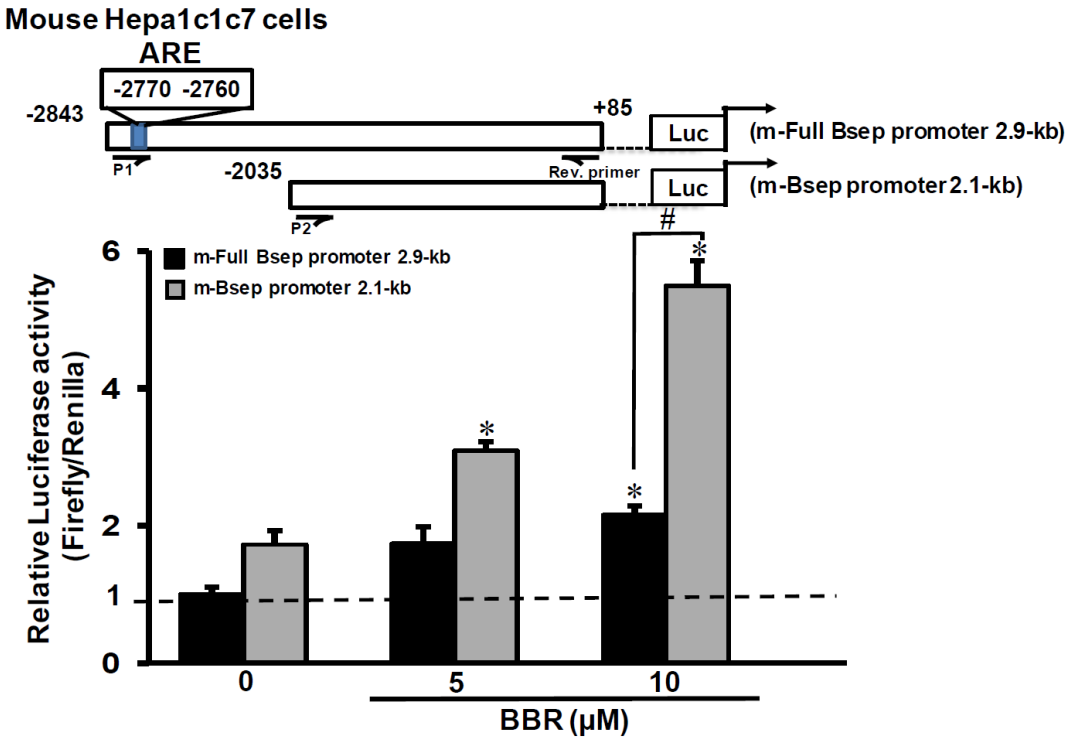

Figure 3.16 The impact of removal of putative ARE that is located in the Bsep gene promoter on the BBR-activated mouse Bsep promoter.

After deletion of putative ARE (-2,770 to -2,760) in the mouse Bsep promoter, BBR treatment (0, 5, or 10 μM) activated Bsep gene promoter even stronger in mouse hepatoma cells. All data are expressed as mean ± S.E., (n=3/treatment). Statistical differences (p <0.05) are indicated between BBR and control group (*), or between m-Bsep promoter groups (#).

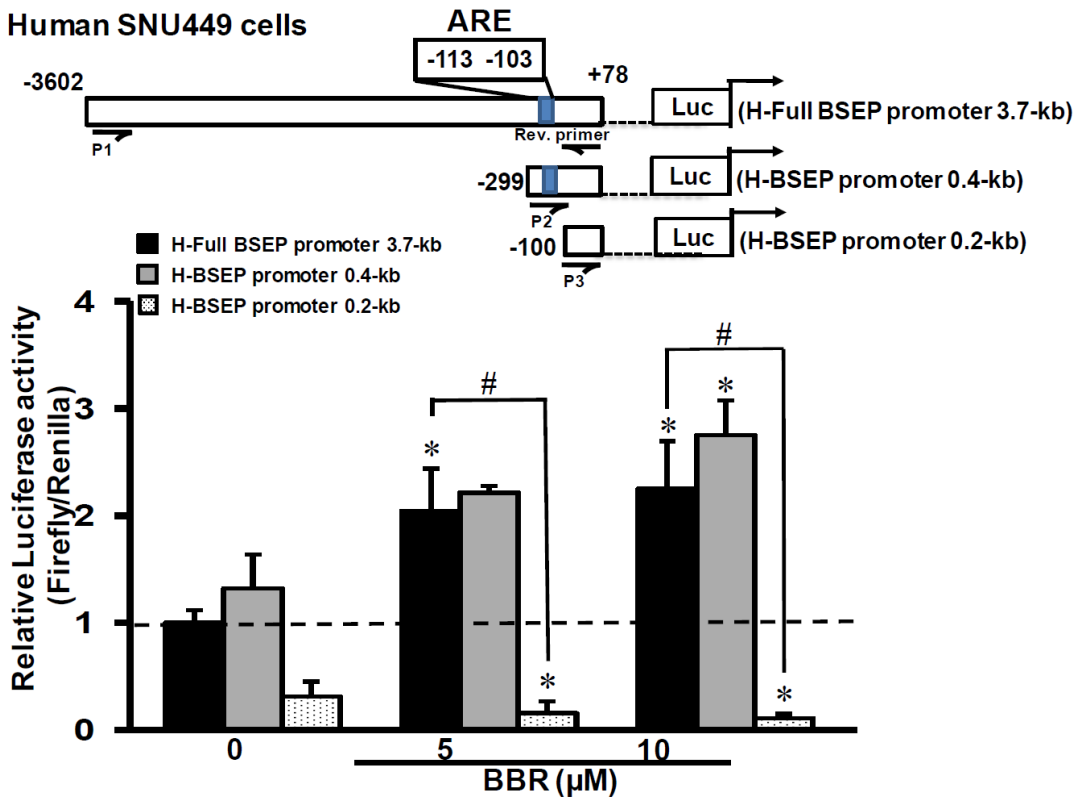

Fig. 3.17 The impact of removal of putative ARE that is located in human BSEP gene promoter on the BBR-activated human BSEP promoter.

Compared to 3.7-kb human BSEP promoter (-3,602 to +78) and truncated 0.4-kb human BSEP promoter (-299 to +78), deletion of putative ARE (-113 to -103) on human BSEP promoter led to no more activation of human BSEP gene promoter by any concentration of BBR treatment (0, 5, or 10 μM) in human hepatoma cells. All data are expressed as mean ± S.E., (n=3/treatment). Statistical differences (p <0.05) are indicated between BBR and control group (*), or between H-BSEP promoter groups (#).

2. Regulation of Mouse Bsep by BBR via TLR4 Inactivation

2.1. LPS Did Not Increase Serum Levels of Liver Injury Biomarkers in Wild-type C3H/OuJ Mice

LPS at 2 mg/kg did not increase serum levels of ALT or AST in wild-type C3H/OuJ mice (Fig. 3.18A and B). In addition, BBR or BBR pre-treatment did not alter serum levels of ALT or AST.

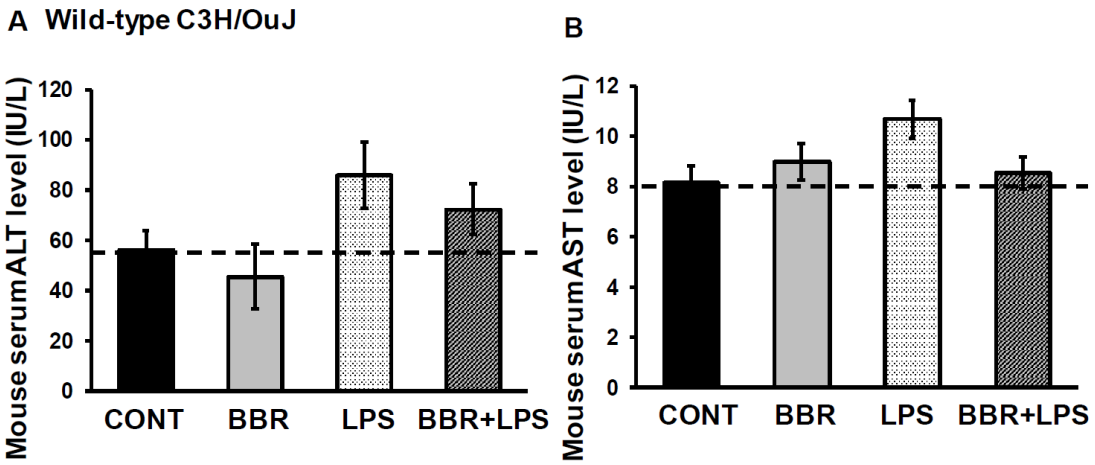

Figure 3.18 LPS did not increase serum levels of ALT or AST in wild-type mice

Wild-type C3H/OuJ mouse sera were collected after BBR treatment (100 mg/kg; p.o.) once daily for 7 days and/or LPS treatment (2 mg/kg; i.p.) once on day 7 for 12 hours (n=6/treatment). Serum levels of ALT (**A**) and AST (**B**) in mice were measured (n= 6/treatment). Statistical differences (p <0.05) are indicated between treatment and control group (*).

2.2. BBR Relieved LPS-Induced Liver Injury and Inflammation in Wild-type C3H/OuJ but not in TLR4-mutated C3H/HeJ Mouse Livers

BBR did not induce liver injury in wild-type C3H/OuJ mouse livers (Figs. 3.19). LPS induced apparent liver injury and inflammation, evidenced by cell swelling, diluted and congested sinusoidal space, and increased number of infiltrated immune cells (Figs. 3.19). Pre-treatment of BBR attenuated LPS-induced liver injury and inflammation, as indicated by recovered normal cell morphology and decreased number of infiltrated immune cells (Fig. 3.19). In contrast, in TL4-mutated C3H/HeJ mouse livers, LPS did not induce obvious liver injury and inflammation (Fig. 3.20).

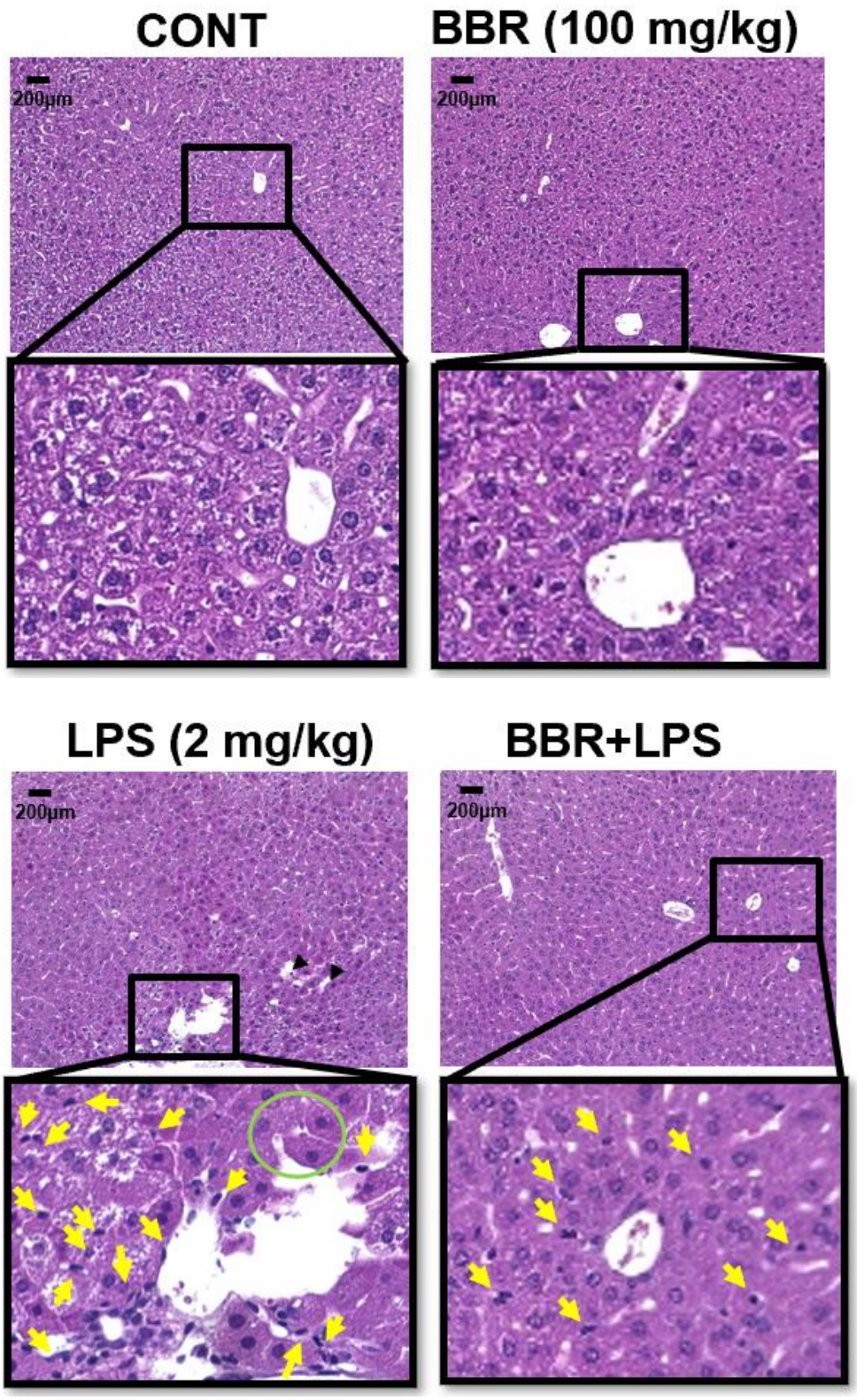
Wild-type C3H/OuJ
CONT
BBR (100 mg/kg)
LPS (2 mg/kg)
BBR+LPS
200μm
200μm
200μm
200μm

Figure 3.19 LPS-induced liver injury was attenuated by BBR in wild-type C3H/OuJ mouse livers.

Wild-type C3H/OuJ mouse livers were collected after BBR treatment (100 mg/kg; p.o.) once daily for 7 days and/or LPS treatment (2 mg/kg; i.p.) once on day 7 for 12 hours and processed for histology analysis (n=6/treatment). H & E staining of mouse livers following BBR and/or LPS treatment. 200× magnification, (Scale bar: 200 μm). Green cycle: cell damage; Black arrowhead, diluted sinusoidal space; Yellow shorter arrow: infiltrated immune cells.

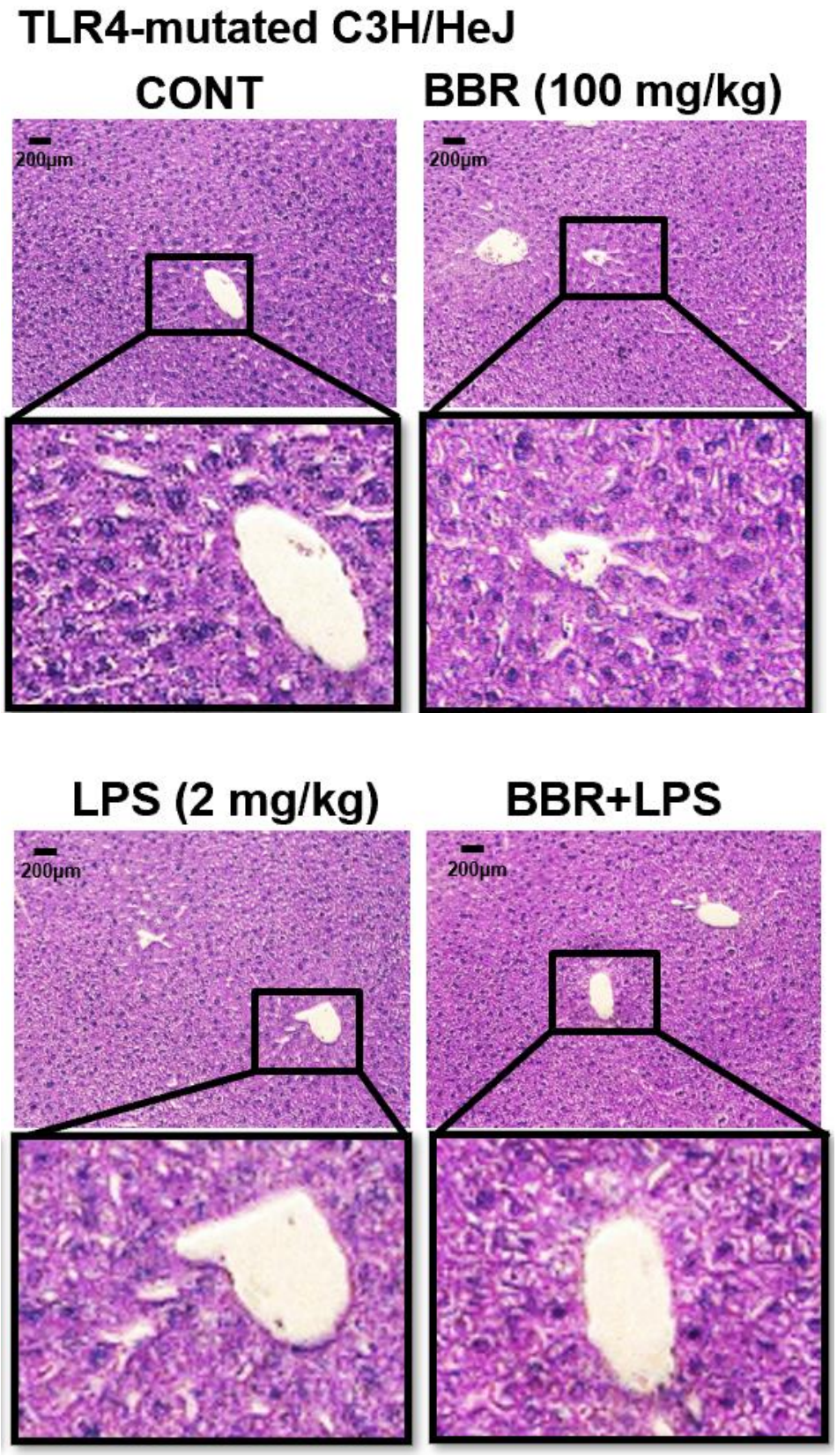
TLR4-mutated C3H/HeJ
CONT
BBR (100 mg/kg)
LPS (2 mg/kg)
BBR+LPS
200μm
200μm
200μm
200μm

Figure 3.20 LPS did not induce liver injury in TLR4-mutated C3H/HeJ mouse livers.

TLR4-mutated C3H/HeJ mouse livers were collected after BBR treatment (100 mg/kg; p.o.) once daily for 7 days and/or LPS treatment (2 mg/kg; i.p.) once on day 7 for 12 hours and processed for histology analysis (n=6/treatment). H & E staining of mouse livers following BBR and/or LPS treatment. 200× magnification, (Scale bar: 200 μm).

2.3. BBR Reversed LPS-Inhibited Bsep Expression Through Suppressing TLR4-NF-κB Signaling

In wild-type C3H/OuJ mouse livers, BBR induced Bsep mRNA expression but LPS decreased Bsep mRNA expression (Fig. 3.21A). Pre-treatment of BBR reversed LPS-inhibited Bsep expression. In contrast, in TLR4-mutated C3H/HeJ mouse livers, LPS did not decrease Bsep mRNA expression (Fig. 3.21B). In addition, pre-treatment of BBR did not alter Bsep expression as compared to LPS alone.

BBR also increased membrane Bsep protein expression but LPS decreased membrane Bsep protein expression in wild-type C3H/OuJ mouse livers (Fig. 3.22A). Pre-treatment of BBR reversed LPS-inhibited Bsep protein expression (Fig. 3.22A). In contrast, in TLR4-mutated C3H/HeJ mouse livers, LPS did not reduce Bsep protein expression (Fig. 3.22B). In addition, pre-treatment of BBR also tended to increase Bsep protein level in TLR4-mutated C3H/HeJ mouse livers (Fig. 3.22B).

We next determined the impact of BBR on NF-κB, the downstream molecules of TLR4 signaling. In wild-type C3H/OuJ mouse livers, LPS increased nuclear level of p65-NF-κB protein (Fig. 3.23A). Pre-treatment of BBR inhibited the nuclear translocation of p65-NF-κB protein. In contrast, in TLR4-mutated C3H/HeJ mouse livers, LPS did not increase nuclear level of p65-NF-κB protein (Fig. 3.23B).

In addition, LPS induced NF-κB downstream cytokine genes, such as TNF-α, IL-1β and IL-6 in wild-type C3H/OuJ mouse livers (Figs. 3.24A, B and C). Pre-treatment of BBR reversed LPS-induced mRNA expression of cytokine genes. In contrast, LPS did not induce mRNA expression of TNF-α in TLR4-mutated C3H/HeJ mouse livers (Fig.

3.24A). Compared to wild-type mouse livers, TLR4-mutated C3H/HeJ mouse livers also showed lower mRNA levels of IL-1β and IL-6 after LPS treatment (Figs. 3.24B and C).

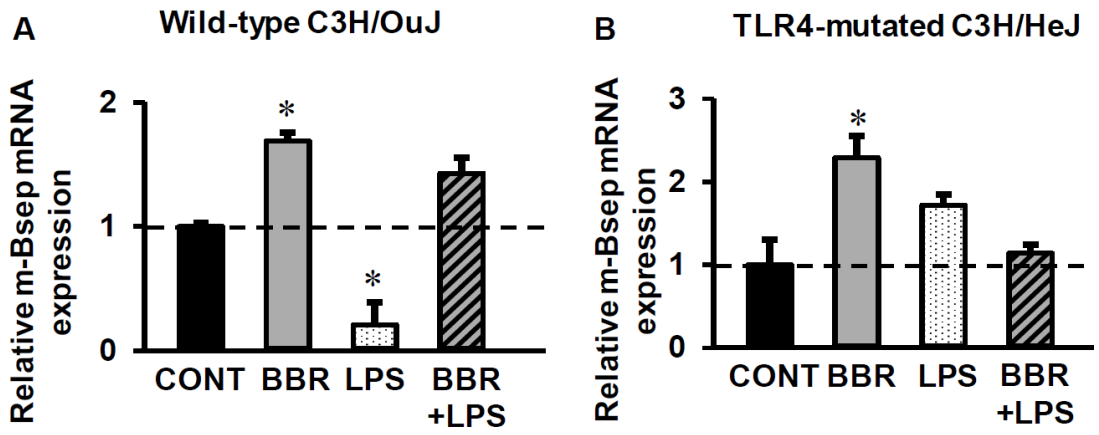

Figure 3.21 LPS-inhibited Bsep mRNA expression was reversed by BBR pre-treatment in mouse livers.

Wild-type C3H/OuJ and TLR4-mutated C3H/HeJ mice were treated with BBR (100 mg/kg; p.o.) once daily for 7 days or/and a single dose of LPS (2 mg/kg; i.p.) on day 7 for 12 hours (n=6/treatment). Quantitative RT-PCR analysis of mRNA expression of Bsep in wild-type (Fig. 3.21A) and TLR4-mutated (Fig. 3.21B) mouse livers. Statistical differences (p <0.05) are indicated between BBR and control group (*).

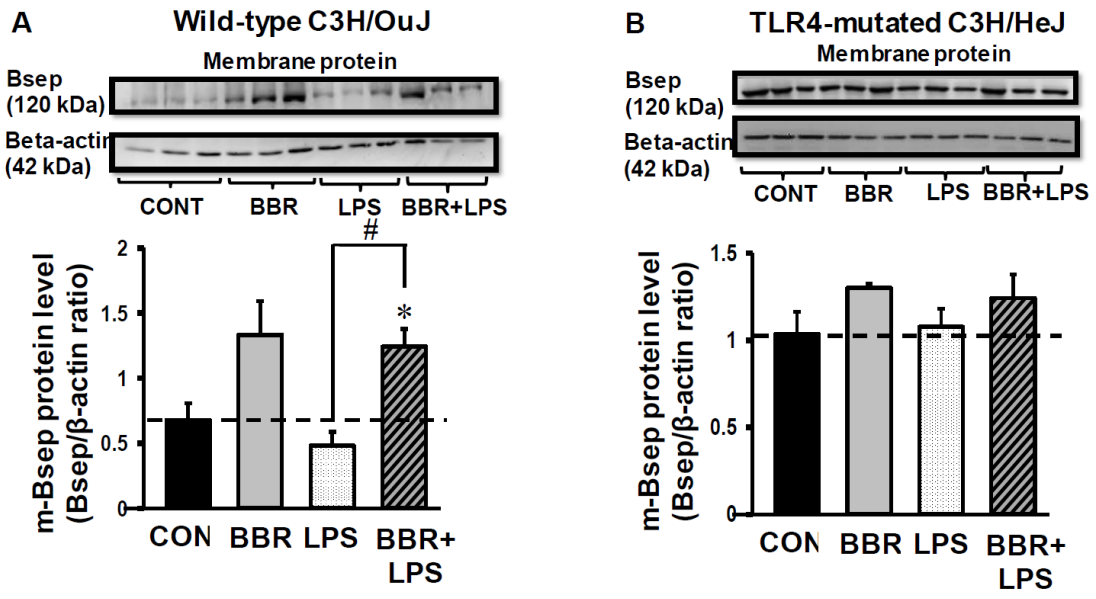

Figure 3.22 LPS-inhibited Bsep protein expression was reversed by BBR pre-treatment in mouse livers.

Wild-type C3H/OuJ and TLR4-mutated C3H/HeJ mice were treated with BBR (100 mg/kg; p.o.) once daily for 7 days or/and a single dose of LPS (2 mg/kg; i.p.) on day 7 for 12 hours. Membrane proteins extracted from wile-type (**A**) and TLR4-mutated (**B**) mouse livers were analyzed for Western blotting of Bsep protein expression. Statistical differences (p <0.05) are indicated between BBR and control group (*).

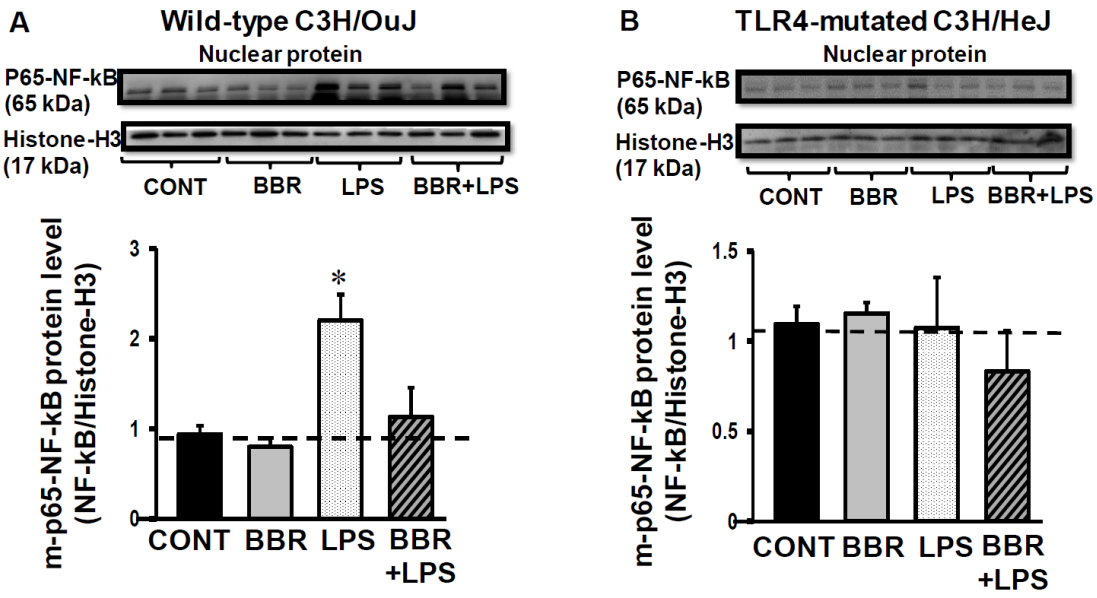

Figure 3.23 LPS-induced p65-NF-kB protein expression was attenuated by BBR pre-treatment in mouse livers.

Wild-type C3H/OuJ and TLR4-mutated C3H/HeJ mice were treated with BBR (100 mg/kg; p.o.) once daily for 7 days or/and a single dose of LPS (2 mg/kg; i.p.) on day 7 for 12 hours (n=6/treatment). Nuclear proteins extracted from wile-type (A) and TLR4-mutated (B) mouse livers were analyzed for Western blotting of p65-NF-kB protein expression. Statistical differences (p <0.05) are indicated between BBR and control group (*).

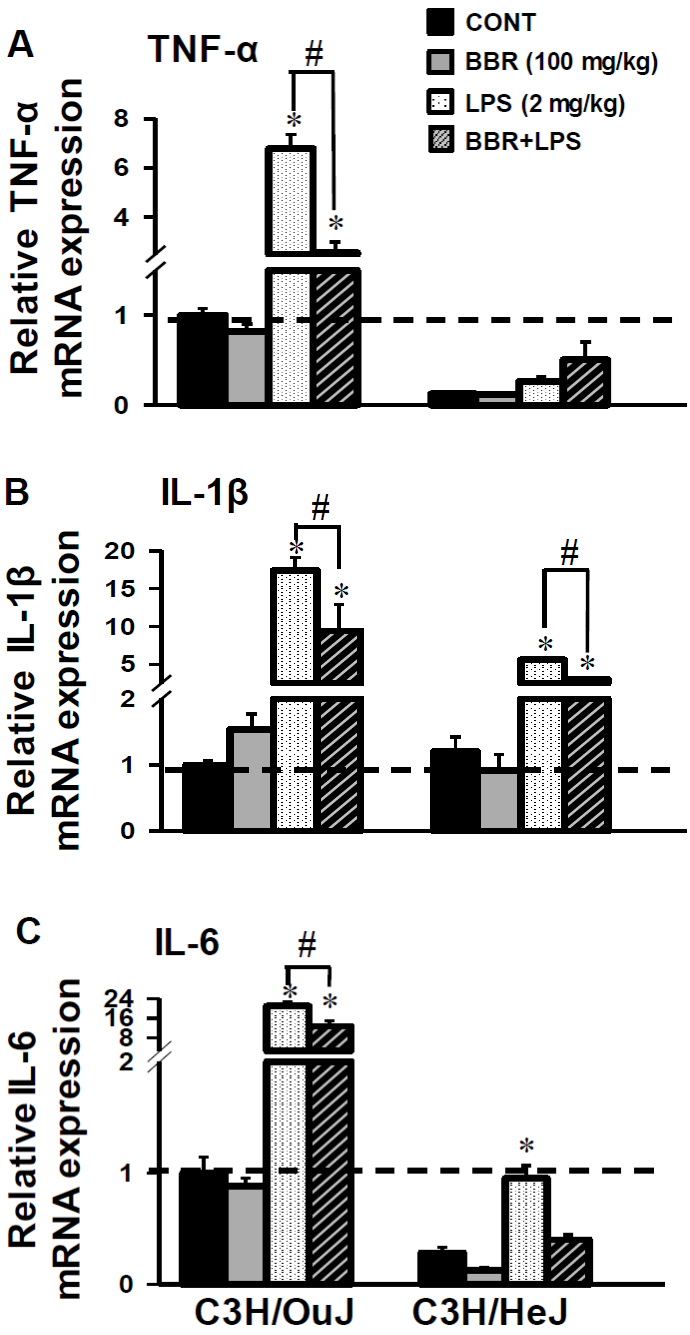

Figure 3.24 LPS-induced cytokine mRNA expression was attenuated by BBR pre-treatment in mouse livers.

Wild-type C3H/OuJ and TLR4-mutated C3H/HeJ mice were treated with BBR (100 mg/kg; p.o.) once daily for 7 days or/and a single dose of LPS (2 mg/kg; i.p.) on day 7 for 12 hours (n=6/treatment). Quantitative RT-PCR analysis of mRNA expression of TNF-α,

IL-1β and IL-6 in wild-type and TLR4-mutated mouse livers (Figs. 3.24**A**, **B** and **C**). Statistical differences (p <0.05) are indicated between BBR and control group (*).

2.4. BBR Reversed TLR4-Inhibited Bsep mRNA Expression in Mouse Hepatoma Cells

As shown in Fig. 3.25A, LPS reduced Bsep mRNA expression in mouse hepatoma cells. Pre-treatment of BBR reversed LPS-decreased Bsep mRNA expression (Fig. 3.25A). In addition, Tak242, the TLR4 inhibitor, also reversed LPS-suppressed Bsep mRNA expression (Fig. 3.25B).

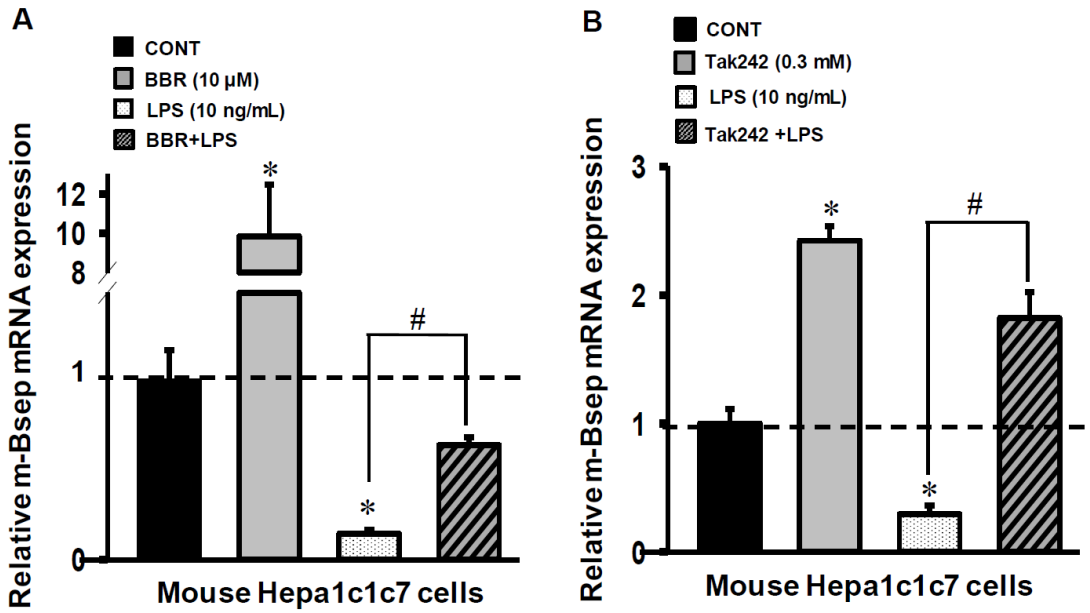

Figure 3.25 LPS-repressed Bsep mRNA expression was reversed by pre-treated with BBR in cultured mouse hepatoma cells.

Mouse Hepa1c1c7 hepatoma cells, in triplicate, were treated with LPS (10 ng/mL) in the presence or absence of BBR treatment (10 μM) or TLR4 inhibitor, TAK242 treatment (0.3mM) for 24 hours. Quantitative RT-PCR analysis of mRNA expression of Bsep in BBR (**A**) and TAK242 (**B**) treated mouse hepatoma cells. Data are expressed as mean ± S.E (n=3/treatment). Statistical differences (p <0.05) are indicated between treatment and control group (*).

CHAPTER 4 DISCUSSION

It has been previously reported that goldenseal, in which BBR is the major active component, promotes bile flow (Newall, *et al.*, 1996). We recently also reported that BBR promotes bile flow maybe via induction of cytochrome p450 (Cyp) 7a1, Ntcp and Bsep, several bile acid-related genes in the liver, and that BBR induces Ntcp expression via inhibiting Jak2-Stat5 signaling (Bu *et al.*, 2017). The studies proposed in my dissertation project characterized the underlying mechanisms by which BBR induces mouse and human Bsep/BSEP expression. Bsep expression is primarily induced by FXR signaling. However, the mechanisms other than FXR activation is responsible for BBR-induced Bsep expression because BBR below 300 mg/kg did not activate FXR signaling (Guo *et al.*, 2016). In addition, it is well-known that activation of FXR will down-regulate Ntcp and Cyp7a1 expression. However, as mentioned above, BBR actually induced the expression of Ntcp and Cyp7a1. In my dissertation, I reported that BBR induced Bsep/BSEP expression and transport function in mouse liver, as well as in mouse and human hepatoma cells. In addition, BBR induced human BSEP expression through NRF2 activation and induced mouse Bsep expression most likely through TLR4 inhibition.

First, similar as previously reported, BBR did not induce apparent liver injury, evidenced by no change in mouse behavior and body weight, as well as serum levels of ALT, a biomarker of liver injury (Fig. 3.1A). BBR also reduced serum levels of glucose and lipids in mice, indicating potential hepatoprotective roles (Yin *et al.*, 2008; Zhao *et al.*, 2019). It has been reported that BBR alone did not induce oxidative stress in both mouse liver and cultured hepatocytes (Dkhil *et al.*, 2014; Liu *et al.*, 2012). Pre-treatment

of BBR even inhibited tert-butyl hydroperoxide-induced ROS and lipid peroxidation in rat liver (Hwang *et al.*, 2002). Thus, BBR is a potential Nrf2 activator but not a pro-oxide.

In addition, BBR increased Bsep/BSEP mRNA and protein expression in mouse liver (Figs. 3.2 and 3.3), as well as in mouse and human hepatoma cells (Figs. 3.4, 3.5 and 3.6). Consistent to Bsep/BSEP gene regulation, BBR also enhanced Bsep/BSEP efflux transport function, evidenced by increased cellular efflux of dihydrofluorescein diacetate, a selective Bsep substrate in both mouse and human hepatoma cells (Wang *et al.*, 2003) (Fig. 3.7).

I next determined the impact of Nrf2 activation on Bsep/BSEP expression by BBR. Similar to previous reports, BBR activated Nrf2 signaling, evidenced by increased mRNA expression of Nrf2-target genes, including Ho-1/HO-1 and Nqo-1/NQO-1, in wild-type mouse livers (Fig. 3.8A) and mouse and human hepatoma cells (Fig. 3.10), but not in Nrf2-null mouse livers (Fig. 3.8B). To our surprise, BBR did not induce nuclear translocation of Nrf2 protein in mouse livers (Fig. 3.9), which was contradictory to previous reports (Deng *et al.*, 2019; Mo *et al.*, 2012; Hsu *et al.*, 2012). This discrepancy between our and other results could be interpreted by species and model difference, as well as different time period of BRR treatment. In contrast, as shown in Fig. 3.11 and 3.12, BBR induced nuclear translocation of Nrf2/NRF2 protein in both mouse and human hepatoma cells. This result was consistent to previous studies, BBR activated Nrf2 signaling in cultured neuron and human hepatoma cells (Hsu *et al.*, 2012 and 2013; Sun *et al.*, 2017). The differences regarding Nrf2 activation by BBR between mouse liver and mouse hepatoma cells is most likely due to loss of hepatocyte morphology and polarity in

cultured hepatoma cells, which leads to reduced expression or even complete loss of drug metabolized enzymes and transporters (Bachmann *et al.*, 2015). Consequently, without normal metabolism and efflux function, BBR can stay inside the hepatoma cells for longer time as compared in the liver.

Nrf2-null mouse model and siRNA knockdown were also applied to investigate the role of Nrf2/NRF2 activation in BBR-induced Bsep/BSEP expression. As shown in Fig. 3.3B, BBR increased Bsep protein expression in the membrane of both wild-type and Nrf2-null mouse livers, indicating that BBR-induced Bsep expression is independent of Nrf2 activation in mouse liver. This was consistent to several reports, which documented that mouse Bsep was not a downstream target of Nrf2 signaling (Klaassen and Reisman, 2010; Reisman *et al.*,2009; Cheng *et al.*, 2007). In addition, a potential Nrf2 activator, ursodeoxycholic acid did not increase Bsep expression in mouse liver (Okada *et al.*, 2008). Next, we used Nrf2/NRF2 siRNA to knockdown mRNA expression of Nrf2/NRF2 in hepatoma cells. In Nrf2-silenced mouse hepatoma cells, BBR still slightly increased Bsep mRNA expression (Fig. 3.13). This indicated that Nrf2 may be not solely responsible for BBR to induce mouse Bsep expression. In contrast, in NRF2-silenced human hepatoma cells, BBR-induced BSEP induction was blocked completely, indicating the necessity of NRF2 activation for BBR-induced human BSEP expression (Fig. 3.14).

To further determine the role of putative Nrf2 response element(s) in the BBR-induced Bsep/BSEP gene expression, luciferase reported assays were processed. Luciferase reported assays first showed that BBR activated mouse and human Bsep/BSEP promoter activity (Fig. 3.15). Then, a series of Bsep/BSEP gene promoters

with different lengths were cloned and then engineered cloned Bsep/BSEP gene promoters into pGL3-basic reporter construct as depicted in Figs. 3.16 and 3.17. In mouse hepatoma cells, BBR continued to increase the luciferase activity driven by mouse Bsep gene promoters in which putative Nrf2 binding site does not exist, indicating that Nrf2 activation was not required for BBR-induced mouse Bsep expression (Fig. 3.16). In contrast, in human hepatoma cells, BBR can no longer increase the luciferase activities that are driven by human BSEP gene promoters in which putative NRF2 binding site does not exist (Fig. 3.17). The difference between mouse and human hepatoma cells is due to species differences in the sequence of Bsep/BSEP gene promoter (Klaassen and Reisman, 2010).

Because BBR-induced mouse Bsep expression appeared not to be due to Nrf2 activation, we next determined whether other mechanisms are responsible for mouse Bsep induction by BBR. Several reports showed that BBR inhibited TLR4 activity and that TLR4-NF-kB inflammatory signaling repressed Bsep expression (Hsiang *et al.*, 2005; Enk *et al.*, 2007; Chu *et al.*, 2014). Therefore, we determined the impact of BBR-repressed TLR4 signaling on mouse Bsep expression.

Consistent to previous reports, pre-treatment of BBR inhibited LPS-induce liver injury and inflammation in mouse liver (Chu *et al.*, 2014; Lou *et al.*, 2010). In wild-type mice, LPS did not increase serum levels of ALT and AST (Fig. 3.18) but induced apparent liver injury and inflammation in mouse liver (Fig. 3.19). LPS-induced liver damage can be characterized by megatocytosis and dilated sinusoidal space (Liu *et al.*, 2018; Fang *et al.*, 2013; Greuter and Shah, 2016). LPS also stimulated acute inflammatory through activation of immune cells, such as macrophages/Kupffer cells and

neutrophils in mouse liver, which was evidenced by increased number of cell infiltration (Fig. 3.19) (Karlmark *et al.*, 2008). In addition, LPS induced TLR4 downstream molecules, NF-κB protein and its target cytokine expression (Figs. 3.23A and 3.24) (Chu *et al.*, 1999; Jiang *et al.*, 2000). In contrast, pre-treatment of BBR attenuated LPS-induced liver injury and inflammation in histology study (Fig. 3.19), and also modulated NF-κB signaling and its downstream gene expression (Figs. 3.23A and 3.24). The results were similar to previous reports, showing that BBR inhibited TLR4-mediated inflammation through inhibiting nuclear translocation of NF-κB protein and/or production of cytokines (Enk *et al.*, 2007; Chu *et al.*, 2014; Lou *et al.*, 2010).

Next, we determined the role of TLR4 signaling in BBR-induced mouse Bsep expression. In mouse livers, LPS inhibited Bsep mRNA and protein expression (Figs. 3.21 and 3.22A) (Hsiang *et al.*, 2005; Enk *et al.*, 2007). However, pre-treatment of BBR reversed LPS-decreased Bsep mRNA and protein expression (Figs. 3.21 and 3.22A). This indicated that the suppression of TLR4 by BBR can up-regulate mouse Bsep expression. To further investigate the role of TLR4 inhibition in mouse Bsep expression, we used TLR4-mutated C3H/HeJ mouse model. Since TLR4 is dysfunctional in C3H/HeJ mouse livers, LPS, as expected, did not induce liver injury and inflammation, and also did not inhibit Bsep expression (Figs. 3.20 and 3.23B and 3.24) (Paik *et al.*, 2003). This indicated that TLR4 inactivation is a putative strategy to reverse inflammation mediated Bsep inhibition. Furthermore, BBR and TAK242, a known TLR4 inhibitor, both reversed LPS-repressed Bsep expression in cultured mouse hepatoma cells (Fig. 3.25). Therefore, it is most likely that BBR inhibits TLR4 signaling and consequently induces mouse Bsep expression.

Taken together, our results demonstrated that BBR increased mouse and human Bsep/BSEP expression. In addition, NRF2 activation contributes to BBR-induced human BSEP expression. TLR4 inhibition most likely contributes to BBR-induced mouse Bsep expression.

CHAPTER 5 SUMMARY AND CONCLUSIONS

With extensive investigation, more and more therapeutic effects of BBR have been revealed, including anti-inflammatory, anti-diabetic, and anti-cancer effects (Puthdee *et al.*, 2017; Lee *et al.*, 2006; Diaz *et al.*, 2015; Doggrell, 2005). In addition, BBR has been recognized as a promising candidate to treat liver diseases, which is most likely due to its anti-oxidative and anti-inflammatory properties (Vuddanda *et al.*, 2010). We and others also demonstrated that BBR can promote bile flow (Newall, *et al.*, 1996; Bu *et al.*, 2017).

As an essential approach for absorption of lipid-soluble vitamins and elimination of xenobiotic, bile acid homeostasis is tightly regulated by multiple nuclear receptors and transporters (Anwer, 2004). For example, we previously reported that BBR induced mouse and human Ntcp/NTCP expression through down-regulation of Jak2-Stat5 signaling (Bu *et al.*, 2017). In this study, I also showed that BBR induced mouse and human Bsep/BSEP expression. However, the underlying mechanism is not known. The overall goal of my dissertation is to determine the underlying mechanisms by which BBR induces mouse and human Bsep/BSEP expression, especially the roles of Nrf2 activation and TLR4 inhibition.

In my dissertation, I first determined whether BBR-induced Bsep/BSEP expression can lead to enhanced transport function. As expected, BBR enhanced Bsep/BSEP efflux function, evidenced by increased efflux of dichlorofluorescin diacetate, the selective Bsep substrate, out of mouse and human hepatoma cells. Therefore, BBR-induced Bsep/BSEP expression consequently leads to increased cellular efflux activity of Bsep/BSEP.

Next, I determined the involvement of Nrf2 activation in BBR-induced Bsep/BSEP expression. The results first showed that BBR activated Nrf2 signaling, evidenced by

increased nuclear translocation of Nrf2 protein and increased expression of Nqo-1/NQO-1 and Ho-1/HO-1, two known Nrf2 target genes in mouse and human hepatoma cells. However, BBR apparently did not activate Nrf2 signaling in mouse liver. In addition, BBR increased mouse Bsep expression in both wild-type and Nrf2-null mouse livers, and even can activate mouse Bsep gene promoter in both presence and absence of putative ARE. These results indicated that Nrf2 is not essential for BBR to induce mouse Bsep expression. In contrast, after removal of putative ARE in human BSEP gene promoter, or in NRF2-silenced human hepatoma cells, BBR no longer induced human BSEP expression. Therefore, NRF2 activation is essential for BBR to induce human BSEP expression. Taken together, Nrf2/NRF2 plays important role in up-regulation of human BSEP, but not mouse Bsep by BBR.

I also investigated the impact of TLR4 inhibition on BBR-induced mouse Bsep expression by using TLR4-mutated C3H/HeJ mouse model. My findings demonstrated that pre-treatment of BBR suppressed TLR4-induced inflammation and then recovered Bsep expression in wild-type but not in TLR4-mutated mouse livers. In mouse hepatoma cells, BBR also reversed LPS-decreased Bsep expression. Because TLR4-NF-κB has been previously reported to suppress Bsep expression (Elferink *et al.*, 2004; Trauner *et al.*, 1998; Hartmann *et al.*, 2002), BBR-induced mouse Bsep expression maybe via TLR4 suppression.

My results further suggest that BBR is a promising therapeutic drug to manage cholestatic liver disease. However, to apply BBR for cholestasis therapy in patients, several limitations need to be taken into consideration. First, poor bioavailability of BBR is a big challenge. To solve this issue, silver nanoparticles containing BBR have been

designed to improve its bioavailability (Ling *et al.*, 2008). Second, it has been reported that BBR metabolites may have similar effects as BBR does. Therefore, it merits further investigation of Bsep regulation by BBR metabolites on. Third, due to the differences between *in vivo* and *in vitro* conditions, it is necessary to determine whether BBR similarly enhances Bsep efflux function in the whole animal. To perform this study, radiolabeled [^{18}F] LCATD (LithoCholic Acid Triazole Derivative), which is a Bsep substrate with radioactivity, may be used to evaluate the *in vivo* Bsep efflux function after BBR treatment (Testa *et al.*, 2018). Fourth, we may also consider using primary human hepatocytes and HepaRG cells. These cells have normal enzyme activity and transporter function, which are good models to investigate the effects of BBR in human livers.

In addition, my dissertation research also paves a way for future direction. In the studies proposed in Hypothesis I, the underlying mechanisms of how BBR activates human NRF2 signaling is not investigated. It has been suggested that PI3K/Akt signaling and epigenetic gene regulation can lead to human NRF2 activation (Ref). Therefore, these two signaling mechanisms can be further evaluated to determine whether they contribute to human NRF2 activation by BBR. In the studies proposed in Hypothesis II, I only reported that TLR4 repression may contribute to BBR-induced mouse Bsep expression. It merits further investigation, such as how TLR4 downstream signaling molecules, such as NF-κB or AP-1, contribute to BBR-induced mouse Bsep expression. Last, regulation of other bile flow-related transporters by BBR can also be investigated. For example, it has already been reported that Mdr2 can be induced by BBR and could be another potential target for cholestasis management (Guo *et al.*, 2016).

In conclusion, my dissertation demonstrates that BBR induced mouse and human Bsep/BSEP expression. In addition, BBR induced human BSEP expression via NRF2 activation, and induces mouse Bsep expression most likely via TLR4 suppression.

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