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Nrf2 Activation Enhances Biliary Excretion of Sulfobromophthalein by Inducing Glutathione-*S*-Transferase Activity

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Sulfobromophthalein (BSP) is used to study hepatobiliary excretory function. BSP is conjugated with glutathione (GSH), whereas its dibrominated analog disulfobromophthalein (DBSP) is not conjugated with GSH prior to biliary excretion. In addition, both BSP and DBSP are transported into hepatocytes via organic anion–transporting polypeptides and excreted into bile via multidrug resistance–associated protein 2 (Mrp2). Nuclear factor erythroid 2–related factor 2 (Nrf2) is a transcription factor that under basal conditions is targeted for proteasomal degradation in the cytosol by kelch-like ECH–associated protein 1 (Keap1). Electrophilic and oxidative stress facilitate Nrf2 nuclear translocation and subsequent induction of cytoprotective genes, including GSH synthetic enzymes, GSH-*S*-transferases (Gsts), and Mrp transporters. The current study determined whether varying the amount of Nrf2 activation would effect the elimination of BSP and DBSP. Male wild-type (WT), Nrf2-null, and Keap1-knockdown (Keap1-kd) mice were administered BSP or DBSP. Within 30 min, Nrf2-null mice excreted 25%, WT mice 52%, and Keap1-kd mice 80% of the injected BSP. Liver GSH content was not altered by BSP. The biliary excretion of GSH and messenger RNA (mRNA) expression of major Gsts were directly proportional to the amount of Nrf2. Moreover, BSP-GSH conjugation activity in the liver of Nrf2-null and Keap1-kd mice was 42% and 237% of WT mice, respectively. In contrast to BSP, there were no differences in biliary excretion or plasma disappearance of DBSP among the three genotypes, suggesting that the modest differences in Mrp2 mRNA expression among genotypes do not affect BSP or DBSP biliary excretion. Collectively, these results indicate that increased biliary excretion of BSP, and possibly other compounds, is due to Nrf2-induced Gst mRNA expression and enzyme activity.

Key Words: **Nrf2; Gsts; BSP; biliary excretion.**

Sulfobromophthalein (BSP) is a prototypical compound used to assess hepatobiliary transport, biotransformation, and excretory mechanisms. BSP is transported into hepatocytes via the organic anion–transporting polypeptide (Oatp) family of uptake transporters (Cattori *et al*., 2001; Hagenbuch *et al*., 2000; Kanai *et al*., 1996; van Montfoort *et al*., 2002). BSP is conjugated with glutathione (GSH) in hepatocytes by glutathione-*S*-transferases (Gsts) (Alin *et al*., 1985; Brauer and Pessotti, 1949; Combes, 1965; Combes and Stakelum, 1960, 1961; Grodsky *et al*., 1959; Krebs and Brauer, 1958; Tahir *et al*., 1985; Yalcin *et al*., 1983) and then excreted from hepatocytes into the bile via the efflux transporter, multidrug resistance–associated protein 2 (Mrp2) (Cui *et al*., 2001; Tanaka *et al*., 2003). The dibrominated analog of BSP, disulfobromophthalein (DBSP), is also used to assess hepatobiliary function. Similar to BSP, DBSP is transported into the liver via Oatps and exported by Mrp2 (Johnson and Klaassen, 2002). In contrast to BSP, however, DBSP is not conjugated with GSH before biliary excretion and therefore is excreted into bile as the parent compound (Javitt, 1964).

The nuclear factor erythroid 2–related factor 2 (Nrf2)-kelch-like ECH–associated protein 1 (Keap1) pathway has been characterized over the past decade as an important endogenous cellular mechanism for coping with oxidative stress. Nrf2 is a transcription factor that promotes transcription of a battery of cytoprotective genes via antioxidant response elements (AREs) in promoter regions, thus restoring the intracellular balance between oxidants and antioxidants. Under conditions when oxidative stress is low, Keap1 sequesters Nrf2 in the cytosol by acting as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2 (Tong *et al*., 2006). Upon increased oxidative stress within the cell, Nrf2 circumvents Keap1-mediated proteasomal degradation and translocates into the nucleus (Bloom and Jaiswal, 2003). Once in the nucleus, Nrf2 heterodimerizes with a small musculo-aponeurotic fibrosarcoma protein and thereby promotes transcription of various cytoprotective genes through direct ARE binding (Itoh *et al*., 1997). Genes upregulated by Nrf2 include, but are not limited to, Mrp2, which effluxes BSP and DBSP into bile; glutamate-cysteine ligase catalytic and modifier subunits, which are responsible for the rate-limiting step in GSH synthesis and Gsts (Maher *et al*., 2007; Ramos-Gomez *et al*., 2001; Solis *et al*., 2002).

Because activation of the Nrf2-Keap1 pathway results in induction of Mrp2 as well as genes responsible for both the

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synthesis and conjugation of GSH, the present study investigated whether changes in the amount of Nrf2 *in vivo* (Nrf2-null, wild-type [WT], and Keap1-knockdown [Keap1-kd] mice) would alter the pharmacokinetics of BSP and DBSP. Nrf2-null mice, which have no functional Nrf2 protein, are highly susceptible to tissue injury due to a decreased capability to induce cytoprotective genes upon oxidative stress (Aoki *et al*., 2001; Enomoto *et al*., 2001; Kraft *et al*., 2004; Ramos-Gomez *et al*., 2001). Keap1-kd mice have decreased functional Keap1 protein and therefore, have increased Nrf2 in the nucleus, resulting in constitutively expressed, higher levels of cytoprotective genes (Okada *et al*., 2008; Reisman *et al*., 2009). The current study investigates the functional importance of Nrf2 on the pharmacokinetics of BSP and DBSP in WT, Nrf2-null, and Keap1-kd mice.

MATERIALS AND METHODS

Materials. BSP was purchased from Sigma-Aldrich (St. Louis, MO). DBSP was purchased from SERB Laboratories (Paris, France). All other chemicals were purchased from Sigma-Aldrich.

Animals and husbandry. Eight-week–old male C57BL/6 mice were purchased from Charles River Laboratories Inc. (Wilmington, MA). Nrf2-null mice were obtained from Dr Jefferson Chan (University of California, Irvine, CA) (Chan *et al*., 1996). Keap1-kd mice were graciously supplied by Dr Masayuki Yamamoto (Tohoku University, Aoba-ku, Sendai, Japan) (Okada *et al*., 2008).

Both Nrf2-null and Keap1-kd mice were backcrossed into C57BL/6 mice, and >99% congenicity was confirmed by the speed congenics group at Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature-, light-, and humidity-controlled environment and had access to Teklad Rodent Diet #8604 (Harlan Laboratories, Madison, WI) and water *ad libitum*. The housing facility is an American Animal Associations Laboratory Animal Care–accredited facility at the University of Kansas Medical Center, and all procedures were preapproved in accordance with Institutional Animal Care and Use Committee guidelines.

Determination of plasma concentration and biliary excretion of BSP and DBSP. WT, Nrf2-null, and Keap1-kd mice ($n = 5$, 8 weeks old) were anesthetized by injection of ketamine/midazolam (100 and 5 mg/kg, respectively, i.p.). Body temperature was maintained at 37°C by rectal probe–controlled heating pads. Subsequently, the right carotid artery was cannulated with PE-10 tubing and the common bile duct cannulated with the shaft of a 30-gauge needle attached to PE-10 tubing through a high abdominal incision. Depth of anesthesia was monitored by pinching the footpad before and throughout surgery, and if necessary, additional anesthetic drugs were administered during sample collection. Bile samples were collected in 15-min periods into preweighed 0.6-ml microcentrifuge tubes for five periods. The tubes into which bile was collected were immersed in ice. After the first bile collection, BSP (80 μmol/kg/10 ml) or DBSP (120 μmol/kg/10 ml) was injected via the carotid cannula. Thirty to 35 μl of blood were collected into heparinized tubes at −2, 7.5, 22.5, 37.5, and 52.5 min after BSP or DBSP injection. The volumes of bile samples were determined gravimetrically, taking 1.0 as specific gravity. Concentrations of BSP and DBSP in bile and plasma were quantified spectrophotometrically at 580 nm after an appropriate dilution of the samples with 0.1M sodium hydroxide.

Determination of hepatic GSH depletion by BSP. In WT mice, the peak excretion rate of BSP was within 20 min after injection. Therefore, WT mice were surgically prepared as described above, and after a 15-min bile collection, saline or BSP (80 μmol/kg/10 ml) was injected. During these experiments, bile and plasma samples were collected at the same time points described above. The livers were removed 20 min after saline or BSP administration, frozen in liquid nitrogen, and stored at −80°C.

Total GSH. Total GSH was quantified in bile and liver using a commercial GSH assay kit (Sigma). Bile was diluted 60× in 5% 5-sulfosalicylic acid (SSA) solution, whereas liver was homogenized (0.1 g/ml) in 5% SSA and further diluted 10× in 5% SSA. Samples were preincubated in assay buffer, GSH reductase, and 5,5′-dithiobis(2-nitrobenzoic acid) at room temperature prior to adding reduced nicotinamide adenine dinucleotide phosphate (NADPH). In the presence of NADPH, the GSH-dependent production of 5-thio-2-nitrobenzoic acid was quantified spectrophotometrically at 412 nm.

BSP-GSH conjugation. Optimal assay conditions were described previously (Goldstein and Combes, 1966; Klaassen and Plaa, 1967). The BSP-GSH solution was prepared with 0.1M (pH 8.4) sodium pyrophosphate buffer to achieve a final solution of 226μM BSP, 21.1mM GSH, at pH 8.0. All components were incubated at 37°C, and 546 μl of incubation medium (23 μl BSP + 68 μl GSH + 455 μl pyrophosphate buffer) was added to 1-ml cuvettes. Livers were homogenized (0.4 g/ml) in 250mM sucrose-10mM Tris buffer (pH 7.4) with protease inhibitors. The cytosolic fraction was separated after two consecutive centrifugations (1000 × g for 12 min; 100,000 g for 60 min at 4°C). Cytosols were diluted 50× in sodium pyrophosphate buffer, incubated at 37°C, and 454 μl of each sample was added to the BSP and GSH mixture in 1-ml cuvettes. GSH conjugation of BSP was determined spectrophotometrically at 330 nm and corrected for the amount of BSP per cuvette, cytosolic dilution factor, protein content of sample, time in minutes of conjugation reaction, and nonenzymatic conjugation.

Statistical analysis. All data were analyzed using one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$).

RESULTS

Bile Flow and GSH Excretion

For bile flow and biliary excretion, data were normalized to body weight, as performed previously (Gregus and Klaassen, 1982; Slitt *et al*., 2003). Mean body weight was not significantly different among 8-week–old WT (26 g), Nrf2-null (24 g), and Keap1-kd (26 g) mice. Bile flow tended to be lower in Nrf2-null mice, whereas it was higher (~30%) in Keap1-kd mice (Fig. 1A). Nrf2-null mice excreted GSH 30% slower, whereas Keap1-kd mice excreted GSH 30% faster than WT mice (Fig. 1B).

Plasma Concentration and Biliary Excretion of BSP

Nrf2-null mice had slower plasma disappearance of BSP than WT mice, whereas there was not a statistical difference in the plasma disappearance of BSP between Keap1-kd and WT mice (Fig. 2A). After BSP injection, Nrf2-null mice continued to have a slower bile flow, whereas Keap1-kd mice had a higher bile flow than WT mice (Fig. 2B). Nrf2-null mice had slower biliary excretion of BSP and lower cumulative biliary excretion of BSP than WT mice (Fig. 2C). In contrast, Keap1-kd mice had faster biliary excretion of BSP and higher cumulative biliary excretion of BSP than WT mice (Fig. 2D). Thirty minutes after BSP injection, Nrf2-null mice excreted 25%, WT mice 52%, and Keap1-kd mice 80% of the injected BSP.

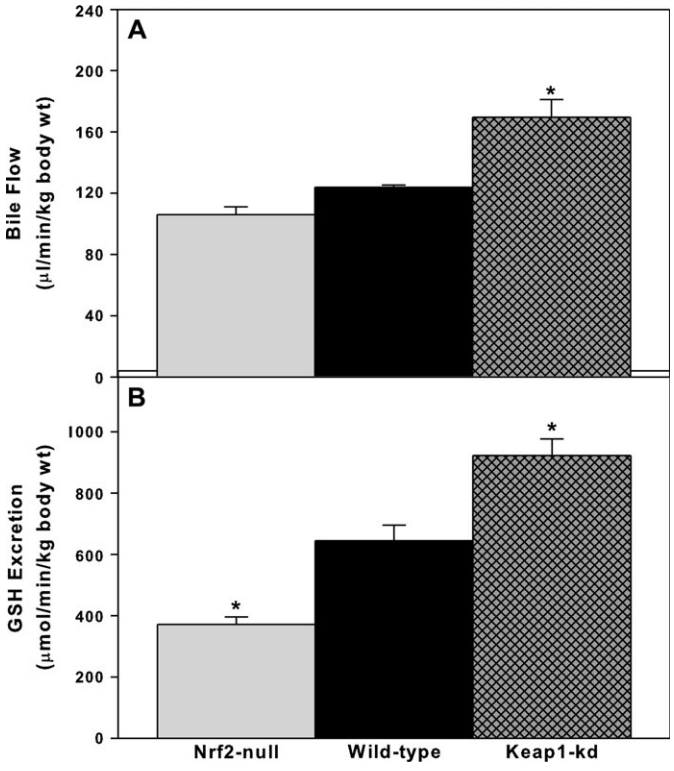

FIG. 1. Basal bile flow (A) and GSH excretion (B) in WT, Nrf2-null, and Keap1-kd mice ($n = 5$). Values are expressed as mean ± SEM. *indicate a statistically significant difference from WT mice ($p \leq 0.05$).

Plasma Concentration and Biliary Excretion of DBSP

There were no differences in plasma disappearance, biliary excretion, or cumulative biliary excretion of DBSP in WT, Nrf2-null, and Keap1-kd mice (Figs. 3A, C, and D). However, after DBSP administration, Keap1-kd mice maintained higher bile flow (Fig. 3B). It should be noted that Keap1-kd mice also had a higher bile flow rate than WT mice before injection of DBSP (Fig. 1A).

Effect of BSP Administration on Hepatic GSH Concentration

Twenty minutes after BSP (80 µmol/kg) administration, GSH concentrations were quantified in WT mice to determine whether GSH depletion after BSP administration might play a role in biliary excretion of BSP. GSH concentrations in liver were not decreased by BSP (Fig. 4).

BSP-GSH Conjugation Activity

BSP-GSH conjugation activity was determined in hepatic cytosol fractions. Consistent with the biliary excretion of BSP, conjugation of BSP with GSH is 42% less in Nrf2-null mice than in WT mice, whereas Keap1-kd mice have 137% higher conjugation activity than WT mice (Fig. 5).

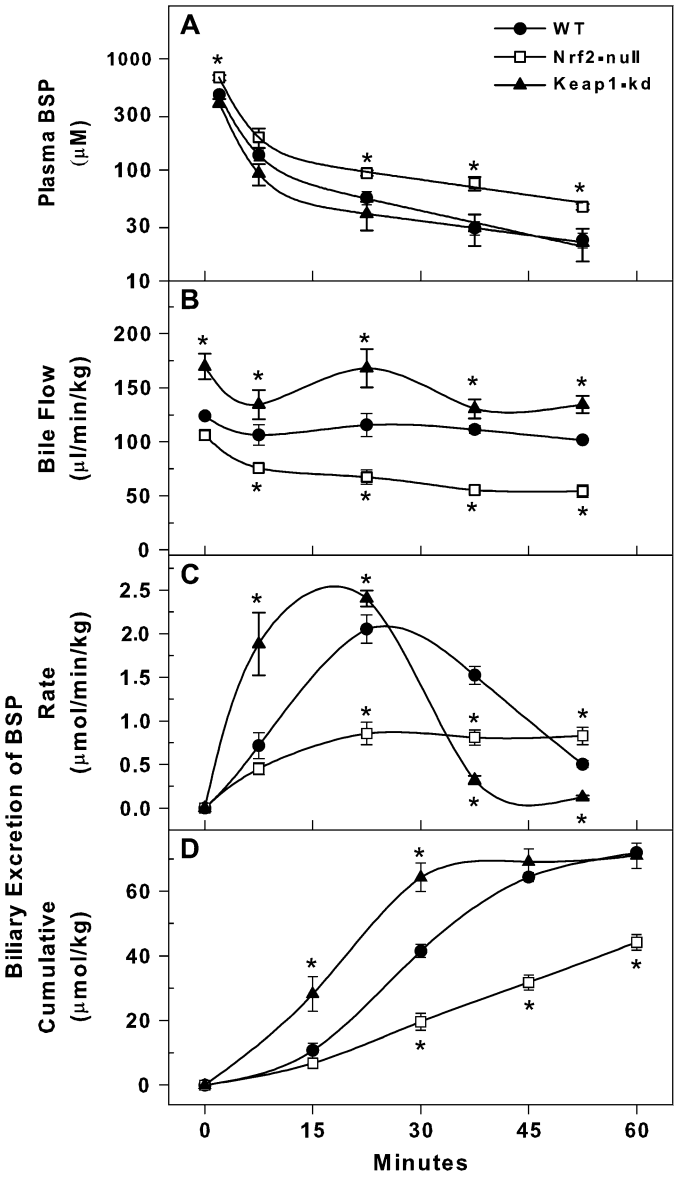

FIG. 2. Plasma disappearance (A), bile flow (B), biliary excretion (C), and cumulative biliary excretion (D) of BSP (80 µmol/kg) in WT, Nrf2-null, and Keap1-kd mice ($n = 5$). Values are expressed as mean ± SEM. *indicate a statistically significant difference from WT mice ($p \leq 0.05$).

DISCUSSION

BSP has been used for decades as a model compound to assess hepatobiliary function, both clinically and experimentally. In the present study, BSP was used to elucidate the role of Nrf2 in hepatobiliary function. Nrf2 is a transcription factor that upon activation induces a wide variety of cytoprotective genes, such as GSH synthesis enzymes, Gsts, and Mrps; all of which play a role in biliary excretion of xenobiotics.

Basal bile flow tends to be lower in Nrf2-null mice, whereas it is higher in Keap1-kd mice. These trends in bile flow

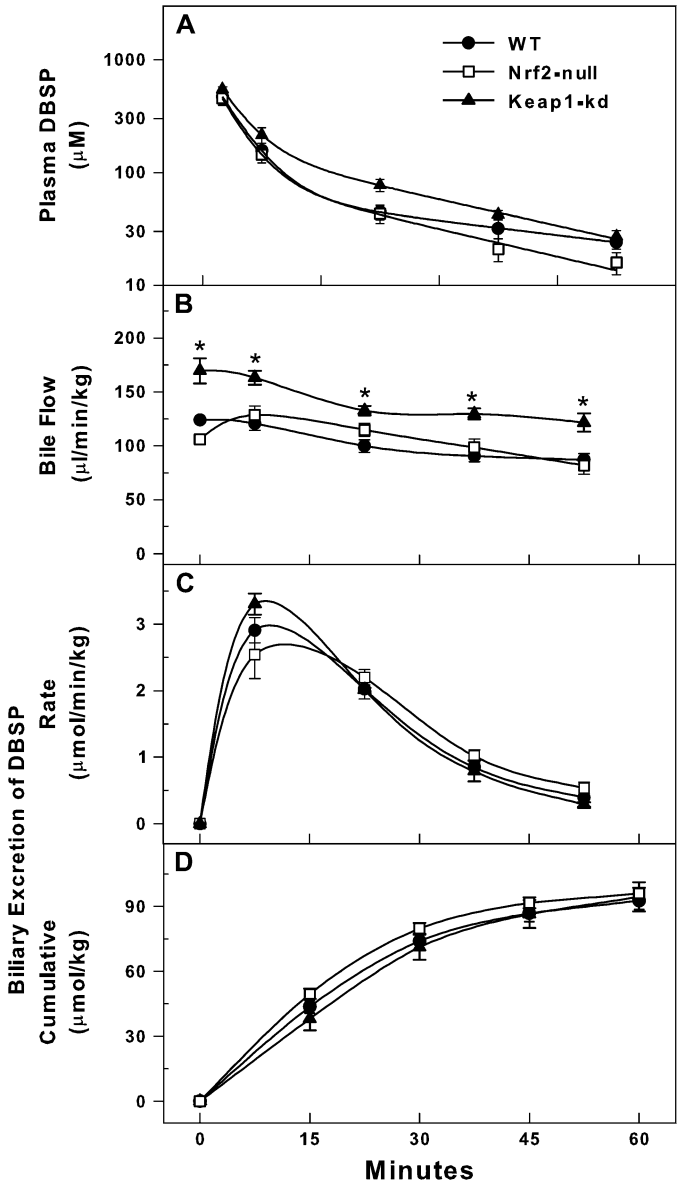

FIG. 3. Plasma disappearance (A), bile flow (B), biliary excretion (C), and cumulative biliary excretion (D) of DBSP (120 μmol/kg) in WT, Nrf2-null, and Keap1-kd mice ($n = 5$). Values are expressed as mean ± SEM. *indicate a statistically significant difference from WT mice ($p \leq 0.05$).

correlate with the decreased and increased biliary excretion of GSH in Nrf2-null and Keap1-kd mice, respectively (Fig. 1B). GSH provides the osmotic force that drives bile acid–independent bile flow (Ballatori and Truong, 1989, 1992). In addition, there were no differences in the biliary excretion of total bile acids among the three genotypes ($p > 0.05$, data not shown), as quantified colorimetrically (Bioquant, San Diego, CA).

Among WT, Nrf2-null, and Keap1-kd mice, Nrf2-null mice have the slowest disappearance of BSP from the plasma,

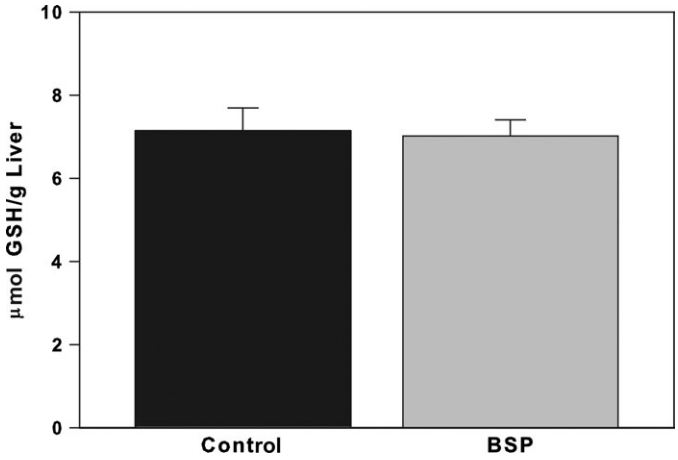

FIG. 4. Liver GSH concentration in WT mice 20 min after intravascular injection of saline or BSP (80 μmol/kg, $n = 5$). No differences were observed between treated and untreated groups.

slowest bile flow after BSP, slowest biliary excretion of BSP, and lowest cumulative excretion of BSP (Fig. 2). The opposite is true when Nrf2 is more highly activated (Keap1-kd mice), thus there is a direct correlation with the amount of activated Nrf2 and plasma disappearance of BSP, bile flow, and biliary excretion of BSP.

Clearly, Nrf2 influences bile flow and GSH biliary excretion; however, because Nrf2 affects multiple pathways that could affect BSP biliary excretion, additional experiments were performed to elucidate how Nrf2 alters BSP disposition. To investigate the possible role of transporters, the dibrominated analog of BSP, namely DBSP, was used in experiments similar to those performed with BSP. DBSP is transported into hepatocytes and effluxed into bile by the same transporters (Oatps and Mrp2) as BSP, but unlike BSP, DBSP is not

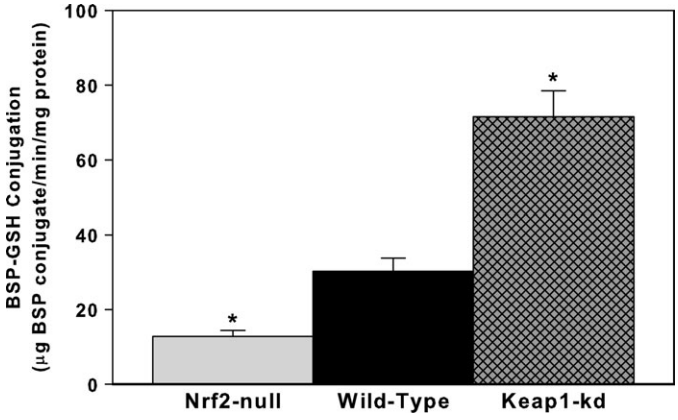

FIG. 5. BSP-GSH conjugation activity in WT, Nrf2-null, and Keap1-kd mice ($n = 5$). *indicate a statistically significant difference from WT mice ($p \leq 0.05$).

TABLE 1
Summary of Gst Liver mRNA Expression

Gene	Nrf2-null mice	Keap1-kd mice
Gsta1	↓	↑
Gsta4	↓	↑
Gstm1	↓	↑
Gstm2	↓	↑
Gstm3	↓	↑
Gstm4	↓	↑
Gstm6	↓	↔
Gstp2	↔	↑

The hepatic Gst mRNA expression from a previous report is summarized (Reisman *et al*., 2009). Down arrows (↓) represent a statistically significant decrease ($p < 0.05$) in mRNA expression, as compared to WT mice. Up arrows (↑) represent a statistically significant increase ($p < 0.05$) in mRNA expression, as compared to WT mice. Double-sided arrows (↔) represent no difference ($p > 0.05$) from WT mice.

conjugated with GSH. Therefore, if BSP biliary excretion differences are due to a transporter-mediated phenomenon rather than a biotransformation phenomenon, then there should be differences in the biliary excretion of DBSP among the three genotypes. However, no such differences in plasma disappearance and biliary excretion of DBSP were observed among the three genotypes, suggesting that transporter activity (at the dose of BSP administered) is not responsible for the changes observed in the biliary excretion of BSP (Fig. 3). Further evidence for a lack of transporter involvement in the differences observed in the biliary excretion of BSP among the three genotypes was reported previously, where only minor differences, if any, were observed in the messenger RNA (mRNA) expression of Oatps and Mrp2 among WT, Nrf2-null, and Keap1-kd mice (Reisman *et al*., 2009).

After parallel studies with DBSP failed to show differences in the biliary excretion among the genotypes, BSP metabolism was considered in more detail. Because BSP is conjugated with GSH before biliary excretion, and GSH excretion is a determinant of bile acid–independent flow, experiments were conducted to determine whether the differences in biliary excretion of BSP were due to depletion of GSH after BSP administration. The lack of decrease in GSH concentrations 20 min following BSP administration demonstrates that differences in biliary excretion of BSP are not due to depletion of the cosubstrate, GSH (Fig. 4).

Because the aforementioned studies suggested Nrf2-dependent differences in BSP biliary excretion might be due to GSH conjugation, Gst activity and mRNA expression were quantified. Interestingly, Nrf2-null mice have 42% less and Keap1-kd mice have 137% more enzyme activity to conjugate BSP with GSH, than do WT mice. Furthermore, the mRNA expression of eight Gst isoforms, namely Gsta1, a4, m1, m2, m3, m4, m6, and p2 were 40–85% lower in Nrf2-null mice and 20–58.5% higher in Keap1-kd mice, as summarized in Table 1 (Reisman *et al*., 2009). The differences in mRNA expression of Gsts correlates with the differences in the activity of liver to conjugate BSP with GSH and the biliary excretion of BSP in WT, Nrf2-null, and Keap1-kd mice. A proposed mechanism by which Nrf2 modulates BSP biliary excretion is summarized in Figure 6.

Numerous studies have shown that Nrf2 protects against toxicity, which is frequently attributed to decreased toxicodynamic effects, as exemplified by increased expression of cytoprotective genes, such as NAD(P)H:quinone oxidoreductase 1 (Nqo1), which contribute to protection against oxidative or electrophilic stress (Aleksunes and Manautou, 2007). The present study emphasizes the importance of the Nrf2-Keap1 pathway on the toxicokinetics of chemicals. Whereas activation of Nrf2 protects cells from electrophiles through induction of cytoprotective genes, Nrf2 also increases biliary excretion of potentially toxic xenobiotics, as exemplified by increased GSH conjugation and biliary excretion of BSP, which represents another important defense mechanism.

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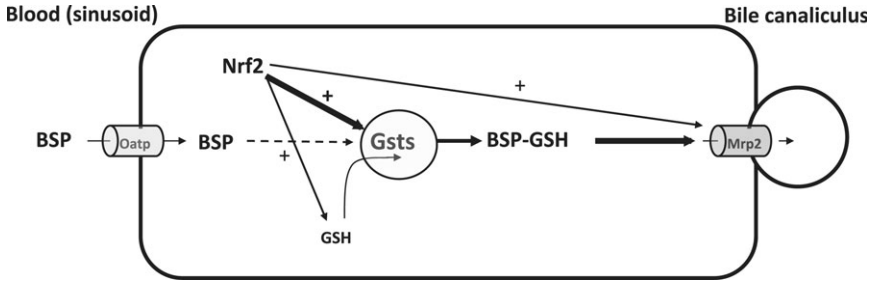

FIG. 6. Summary of Nrf2-dependent BSP excretion. Enhanced Nrf2 activation increases BSP biliary excretion by increasing the mRNA expression and activity of Gsts. BSP excretion is not affected by uptake transporters (Oatps), the efflux transporter Mrp2, or GSH content at the dose of BSP administered in this model.

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