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Effects of ablation and activation of Nrf2 on bile acid homeostasis in mice

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Abstract

Increasing evidence suggests that nuclear factor erythroid 2-related factor 2 (Nrf2) is a promising therapeutic target in cholestasis. However, the role of Nrf2 in bile acid (BA) homeostasis remains controversial. In this study, activation of Nrf2 was achieved either pharmacologically by CDDO-imidazolide (CDDO-Im) or genetically through a “gene dose-response” model consisting of Nrf2-null, wild-type (WT), Keap1-knockdown (Keap1-KD), and Keap1-hepatocyte knockout (Keap1-HKO) mice. In WT mice, CDDO-Im increased bile flow and decreased hepatic BAs, which was associated with a down-regulation of the canalicular BA efflux transporter Bsep and an increase in biliary BA excretion. In contrast, hepatic Bsep and biliary BA excretion were not altered in Keap1-KD or Keap1-HKO mice, suggesting that Nrf2 is not important for regulating Bsep or BA-dependent bile flow. In contrast, hepatic Mrp2 and Mrp3 were up-regulated by both pharmacological and genetic activations of Nrf2. Furthermore, ileal BA transporters (Asbt and Ostβ) and cholesterol transporters (Abcg5 and Abcg8) were down-regulated by both pharmacological and genetic activations of Nrf2, suggesting a role of Nrf2 in intestinal absorption of BAs and cholesterol. In Nrf2-null mice, CDDO-Im down-regulated hepatic BA uptake transporters (Ntcp, Oatp1a1, and Oatp1b2), leading to a 39-fold increase of serum BAs, suggesting a critical role of Nrf2 in protecting against CDDO-Im toxicities. To conclude, the present study demonstrates that activation of Nrf2 up-regulates Mrp2 and Mrp3 in the liver, down-regulates BA and cholesterol transporters in the intestine, as well as protects against the toxic effects of CDDO-Im in mice.

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#Both authors contributed equally to this work.

Conflict of Interest

All authors have no conflict of interest to declare.

Keywords

Nrf2; bile acid; CDDO-Im; bile acid signaling

Introduction

Bile acids (BAs) are well known for their role in assisting intestinal absorption of lipids and lipid-soluble vitamins. BAs are also signaling molecules for regulating the homeostasis of lipid and energy through BA receptors, including farnesoid X receptor (Fxr) and transmembrane G-protein-coupled receptor 5 (Tgr5) (Chiang, 2002). Generally, BAs are divided into primary BAs that are synthesized in the liver, and secondary BAs that are formed in the intestine. In humans, cholic acid (CA) and chenodeoxycholic acid (CDCA) are the two major primary BAs, whereas deoxycholic acid (DCA) and lithocholic acid (LCA) are the two major secondary BAs. In rodents, CDCA is further converted by Cyp2c70 in the liver to form α - and β -muricholic acid (α/β MCA) as well as ursodeoxycholic acid (UDCA) (Honda et al., 2020). Individual BAs differ in their physicochemical properties and activities in BA signaling. Abnormal alterations in BA profile and concentration have been associated with various liver and intestinal diseases, including cholestasis, steatosis, diabetes, and inflammatory bowel diseases (Hofmann, 2009a, b).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is considered a cellular sensor for oxidative/electrophilic stress and regulates a battery of cytoprotective genes. Under physiological conditions, Nrf2 is sequestered in the cytoplasm by binding to Kelch-like erythroid-cell-derived protein with CNC homology-associated protein 1 (Keap1). Upon oxidative/electrophilic stress, Nrf2 dissociates from Keap1 and translocates into the nucleus, where it heterodimerizes with small musculo-aponeurotic fibrosarcoma (Maf) proteins and binds to cis-acting antioxidant response elements (AREs) in the promoter regions of Nrf2 target genes (Kensler et al., 2007; Kobayashi et al., 2006). In our previous study, a “gene dose-response” Nrf2 model was established by using Nrf2-null, wild-type (WT), Keap1-knockdown (Keap1-KD), and Keap1-hepatocyte knockout (Keap1-HKO) mice. The order of hepatic Nrf2 activation is: Nrf2-null < WT < Keap1-KD < Keap1-HKO (Wu et al., 2012).

Increasing evidence suggests a crucial role of Nrf2 in cholestasis, a liver disease characterized by impaired bile flow. Toxic BAs, such as LCA, can activate Nrf2 and provoke adaptive antioxidative responses to BA toxicity in cholestasis (Tan et al., 2010). UDCA is one of the two drugs approved by the FDA to treat cholestasis. It stimulates efflux transport, detoxification, and antioxidative stress systems in mouse liver via Nrf2 activation (Okada et al., 2008). Nrf2 is also reported to be a positive regulator of human bile salt export pump (BSEP), which is the major transporter for biliary BA excretion (Weerachayaphorn et al., 2009). However, Nrf2-null mice were not more susceptible than WT mice to cholestatic liver injury induced by either α -naphthylisothiocyanate (ANIT) or bile duct ligation (BDL) (Tanaka et al., 2009; Weerachayaphorn et al., 2012). Thus, the role of Nrf2 in BA homeostasis remains controversial.

To date, numerous Nrf2 activators have been developed, and most of them are electrophilic compounds that covalently target the cysteine residues in Keap1 protein by oxidation or

alkylation (Robledinos-Anton et al., 2019). Synthetic triterpenoids (CDDO-Im, CDDO-Me, CDDO-EA, etc.) derived from the natural compound oleanolic acid are an important class of electrophilic Nrf2 activators. CDDO-Im is one of the most potent triterpenoid Nrf2 activators and has shown better activities of anti-inflammation and tumor suppression than other triterpenoids, including CDDO and CDDO-Me (Dinkova-Kostova et al., 2005; Yates et al., 2007). Studies in mice revealed that oleanolic acid alters BA metabolism (Liu et al., 2019). However, the effect of CDDO-Im on BA homeostasis remains unclear. The present study aimed to systematically investigate the effect of Nrf2 activation, by using either CDDO-Im or “gene dose-response” models, on the concentrations of individual BAs, bile flow, and mRNA expression of genes involved in BA homeostasis in male mice.

Materials and Methods

Chemicals.

CDDO-Im was provided by Dr. Michael Sporn (Dartmouth College, Hanover, New Hampshire). RNA Bee was purchased from TelTest Inc. (Friendswood, TX). All other chemicals including corn oil, unless indicated, were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Animals.

Eight-week-old male C57BL/6 mice were purchased from Charles River Laboratories Inc. (Wilmington, MA). Nrf2-null mice were provided by Dr. Jefferson Chan (University of California, Irvine, CA), and Keap1-KD mice were obtained from Dr. Masayuki Yamamoto (Tohoku University, Sendai, Japan). Keap1-HKO mice were generated as described in our previous study (Wu, et al., 2012). Nrf2-null, Keap1-KD, and Keap1-HKO mice showed > 99% congenicity with the C57BL/6 background, which was confirmed by Jackson Laboratories (Bar Harbor ME). All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. Mice were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), with a 12:12 hr light:dark cycle and provided chow (Teklad Rodent Diet #8604, Harlan Teklad, Madison, WI) and water *ad libitum*. Corn oil vehicle or CDDO-Im (1 mg/kg, i.p., in corn oil) was administered daily for 4 consecutive days to male wild-type (WT) and Nrf2-null mice (n=5-8 per group). The dosage was chosen according to a previous study (Reisman et al., 2009a). Keap1-KD and Keap1-HKO mice were administered corn oil vehicle. Thus, mice were divided into 6 groups: Nrf2-null mice treated with vehicle (Nrf2-null Veh), Nrf2-null mice treated with CDDO-Im (Nrf2-null CDDO-Im), WT mice treated with vehicle (WT Veh), WT mice treated with CDDO-Im (WT CDDO-Im), Keap1-KD mice treated with vehicle (Keap1-KD Veh), and Keap1-HKO mice treated with vehicle (Keap1-HKO Veh).

Sample collection.

At 24 hours after the fourth dose, one set of mice from the above mentioned six groups (n=6/group) were anesthetized using ketamine (100 mg/kg, i.p.)/midazolam (5 mg/kg, i.p.). The common bile duct of each mouse was cannulated with a 30-gauge needle attached to PE-10

tubing. Bile was collected from the cannula for 40 min in pre-weighed tubes on ice. The volume of bile sample was determined gravimetrically, using 1.0 for specific gravity.

Another set of mice (n=5-8/group) was anesthetized with 50 mg/kg pentobarbital at 24 hr after the last dose, blood was collected from the retro-orbital sinus, and serum was obtained by centrifuging blood at 6,000 *g* for 15 min. Livers, and ileum segments were harvested from the same animals, washed, frozen in liquid nitrogen, and stored at -80°C.

BA quantification.

Sample extraction and quantification of individual BAs were described previously (Alnouti et al., 2008; Zhang et al., 2010).

Multiplex mRNA quantification Array

Total RNA was isolated using RNA-Bee reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's protocol. The mRNAs were quantified using multiplex suspension arrays (Panomics/Affymetrix, Fremont, CA), as described previously (Lickteig et al., 2016; Zhang et al., 2017).

Statistical Analysis

Data were analyzed using GraphPad Prism software version 7.0. (GraphPad, San Diego, CA), and analyzed with one-way analysis of variance (ANOVA), followed by Duncan's post-hoc test. Statistical significance was set at $p < 0.05$. Data were expressed as means $\pm$ SEM.

Results

Effects of Nrf2 activation on liver weight and bile flow.

As shown in Figure 1, CDDO-Im did not alter the relative liver weights, but increased the bile flow calculated either per liver weight (22% $\uparrow$) or body weight (32% $\uparrow$) in WT mice. These effects of CDDO-Im were attenuated in Nrf2-null mice. Compared to WT mice, the relative weights were higher in Keap1-HKO mice (44% $\uparrow$), but not higher in Keap1-KD mice. The bile flow calculated per liver weight was higher in Keap1-KD mice, but not higher in Keap1-HKO mice. Additionally, the bile flow calculated per body weight of Keap1-KD and Keap1-HKO mice were not different from that of WT mice. These results suggest that Nrf2 is not an important mechanism for the enhanced bile flow in CDDO-Im-treated mice. It should be noted that both the relative liver weights (21% $\downarrow$) and bile flow calculated per body weight (39% $\downarrow$) were lower in Nrf2-null mice than WT mice.

Effects of Nrf2 activation on biliary excretion of BAs.

Biliary BA excretion is a major determinant of bile flow. As shown in Figure 2, CDDO-Im significantly increased the biliary excretion of total BAs (42% $\uparrow$) in WT mice. This was mainly due to the increased total conjugated (42% $\uparrow$) and total primary BAs (43% $\uparrow$), particularly TCA (54% $\uparrow$) and T β MCA (31% $\uparrow$). As a result, the biliary excretion of both 12 α -OH (54% $\uparrow$) and non-12 α -OH BAs (29% $\uparrow$) were significantly increased by CDDO-Im in WT mice. In contrast, CDDO-Im had no effect on the biliary BA excretion of Nrf2-null

mice. Compared to WT mice, the biliary excretion of total BAs was not significantly different in Nrf2-null, Keap1-KD or Keap1-HKO mice. These results indicate that Nrf2 activation is not the predominant mechanism for the enhanced biliary BA excretion observed in CDDO-Im-treated mice.

Further analysis revealed that the biliary excretion of non-12 α -OH BAs (33%↓) was lower in Keap1-KD mice than in WT mice, largely due to decreased T α MCA (34%↓) and T β MCA (42%↓). In contrast, the biliary excretion of non-12 α -OH BAs was higher in Nrf2-null mice (120%↑) than in WT mice, largely due to the enhanced excretion of TCDCA (300%↑), T α MCA (190%↑), TUDCA (170%↑), TLCA (310%↑), T ω MCA (59%↑), and α MCA (210%↑).

Effects of Nrf2 activation on BA concentrations in liver.

As shown in Figure 3, CDDO-Im significantly decreased hepatic total BAs (40%↓) in WT mice, mainly due to decreased conjugated (44%↓) and primary BAs (41%↓), including TCA (40%↓), TCDCA (70%↓), T α + β MCA (50%↓), and TUDCA (68%↓). CDDO-Im also decreased hepatic total secondary BAs (32%↓) in WT mice, largely due to decreased TLCA (55%↓) and T ω MCA (42%↓). As a result, CDDO-Im decreased both 12 α -OH (36%↓) and non-12 α -OH BAs (44%↓) in livers of WT mice. Interestingly, CDDO-Im increased hepatic total unconjugated BAs (87%↑) in WT mice, including both primary BAs (CA, 150%↑; CDCA, 170%↑; β MCA, 110%↑) and secondary BAs (DCA, 260%↑; ω MCA, 120%↑). In Nrf2-null mice, CDDO-Im did not significantly alter hepatic total or individual BA concentrations, except that it significantly increased total 12 α -OH BAs (32%↓). Compared to WT mice, Keap1-HKO mice had significantly lower hepatic total BAs (35%↓), conjugated BAs (35%↓), unconjugated BAs (70%↓), primary BAs (36%↓), and secondary BAs (31%↓). Individual BAs that were lower in Keap1-HKO mice than WT mice included TCA (22%↓), TCDCA (59%↓), T α + β MCA (57%↓), TUDCA (65%↓), TLCA (72%↓), T ω MCA (57%↓), CA (52%↓), CDCA (63%↓), α MCA (89%↓), β MCA (75%↓), UDCA (59%↓), and ω MCA (42%↓). As a result, both total 12 α -OH (23%↓) and non-12 α -OH BAs (56%↓) were lower in Keap1-HKO mice than WT mice. Notably, Keap1-KD mice also had lower non-12 α -OH BAs (32%↓), mainly due to decreased TCDCA (50%↓) and TUDCA (46%↓). These results suggest that hepatic Nrf2 activation either by CDDO-Im or liver-specific knockout of Keap1 decreases hepatic BAs in mice.

Compared to WT mice, Nrf2-null mice had lower hepatic total BAs (45%↓), due mainly to the lower conjugated (51%↓) and primary BAs (51%↓), particularly TCA (63%↓) and T α + β MCA (42%↓). Nrf2-null mice also had lower hepatic 12 α -OH BAs (60%↓) than WT mice. Thus, Nrf2 appears to play a role in maintaining the basal level of hepatic BAs.

Effects of Nrf2 activation on serum BA concentrations.

As shown in Figure 4, CDDO-Im tended to decrease serum total BAs in WT mice, but the difference was not statistically significant. Several BAs, including TDCA (77%↓), TUDCA (95%↓), CDCA (77%↓), UDCA (49%↓), and LCA (84%↓), were significantly decreased by CDDO-Im in WT mice. In contrast, CDDO-Im caused a 39-fold increase of total BAs in

serum of Nrf2-null mice, including all of the individual BAs. Thus, Nrf2 appears to protect against the toxicity of CDDO-Im in mice.

Further BA analysis revealed that both genetic ablation and activation of Nrf2 (Nrf2-null, Keap1-KD, and Keap1-HKO) had no effect on serum total BAs. Notably, both Keap1-KD and Keap1-HKO mice had 60% less serum secondary BAs than WT mice, which was mainly due to T ω MCA (Keap1-KD, 90% $\downarrow$; Keap1-HKO, 79% $\downarrow$) and LCA (Keap1-KD, 72% $\downarrow$; Keap1-HKO, 85% $\downarrow$).

Effects of Nrf2 activation on mRNA expression of hepatic BA transporters and BA synthetic enzymes.

In WT mice, CDDO-Im significantly increased Ntcp (32% $\uparrow$), Oatp1b2 (75% $\uparrow$), Bsep (35% $\uparrow$), Mrp2 (110% $\uparrow$), and Mrp3 (240% $\uparrow$). In Nrf2-null mice, CDDO-Im significantly decreased Ntcp (40% $\downarrow$), Oatp1a1 (53% $\downarrow$), and Oatp1b2 (52% $\downarrow$), but had no effect on Bsep, Mrp2 or Mrp3. Compared to WT mice, Nrf2-null mice had higher Ntcp (62% $\uparrow$) and Bsep (53% $\uparrow$), whereas Keap1-KD mice had higher Oatp1b2 (94% $\uparrow$). Notably, both Keap1-KD and Keap1-HKO mice had significantly higher Mrp2 (Keap1-KD, 130% $\uparrow$; Keap1-HKO, 210% $\downarrow$) and Mrp3 (Keap1-KD, 140% $\uparrow$; Keap1-HKO, 240% $\uparrow$) than WT mice. Therefore, both pharmacological and genetic activations of Nrf2 up-regulate hepatic Mrp2 and Mrp3 in mice.

The present study showed that CDDO-Im had little effect on BA synthetic enzymes in WT mice, except that it increased Cyp27a1 (34% $\uparrow$). In contrast, CDDO-Im decreased Cyp27a1 (21% $\downarrow$) and Bal (33% $\downarrow$) in Nrf2-null mice. Compared to WT mice, Nrf2-null mice had higher Bal (34% $\uparrow$), whereas Keap1-KD mice had higher Baat (42% $\uparrow$). Nonetheless, these findings indicate that Nrf2 is not a predominant mechanism for regulating hepatic BA synthesis.

Effects of Nrf2 activation on mRNA expression of ileal BA and cholesterol transporters.

As shown in Figure 6, CDDO-Im decreased both Asbt (56% $\downarrow$) and Ost β (39% $\downarrow$) in ilea of WT mice, but not in Nrf2-null mice (Figure 6). Additionally, CDDO-Im increased Mrp3 (170% $\uparrow$) in WT mice, but not in Nrf2-null mice. Compared to WT mice, Nrf2-null mice had lower Asbt (52% $\downarrow$) and Mrp2 (49% $\downarrow$), whereas both Keap1-KD and Keap1-HKO mice had lower Asbt (Keap1-KD, 24%; Keap1-HKO, 51% $\downarrow$) and Ost β (Keap1-KD, 27%; Keap1-HKO, 59% $\downarrow$). Additionally, Mrp2 (73% $\uparrow$) and Mrp3 (65% $\uparrow$) were higher in Keap1-KD mice than WT mice, but not altered in Keap1-HKO mice. Therefore, ileal Asbt and Ost β are down-regulated by both pharmacological (CDDO-Im) and genetic (Keap1-KD and Keap1-HKO) activation of Nrf2.

Npc111, Abca1, Abcg5 and Abcg8 are four transporters important for intestinal absorption of cholesterol. CDDO-Im decreased Npc111 (20% $\downarrow$), Abcg5 (38% $\downarrow$) and Abcg8 (26% $\downarrow$) in WT mice, but not in Nrf2-null mice (Figure 6). This suggests that CDDO-Im is able to down-regulate intestinal cholesterol transporters through a Nrf2-dependent manner. Compared to WT mice, Nrf2-null mice had lower Npc111 (27% $\downarrow$) and Abcg5 (23% $\downarrow$), whereas Keap1-HKO mice had lower Npc111 (31% $\downarrow$). Notably, both Keap1-KD and Keap1-HKO mice had lower Abcg5 (Keap1-KD, 22% $\downarrow$; Keap1-HKO, 49% $\downarrow$) and Abcg8 (Keap1-

KD, 22%; Keap1-HKO, 40%↓) than WT mice. Therefore, both pharmacological and genetic activations of Nrf2 down-regulate ileal Abcg5 and Abcg8.

Effects of Nrf2 activation on mRNA expression of major regulating factors of BA homeostasis.

In the liver, CDDO-Im significantly increased Shp (52%↑), Lrh-1 (74%↑) and Fgfr4 (53%↑) in WT mice (Figure 7). Compared to WT mice, Keap1-KD mice had higher Fxr (77%↑) and Lrh-1 (73%↑); Keap1-HKO mice had higher Lrh1 (46%↑); Nrf2-null mice had higher Fxr (58%↑), Shp (57%↑) and Lrh-1 (61%↑) in the liver than WT mice. These results suggest that Nrf2 is not important for regulating the hepatic Fxr-Shp signaling.

In the ileum, CDDO-Im did not altered Fxr or Fgf15, but decreased Shp (66%↓) and Lxra (28%↓) in WT mice. These changes were not observed in Nrf2-null mice. Generally, ileal Fxr and Fgf15 were not different in the four “gene dose-response” mouse models (Nrf2-null, WT, Keap1-KD, and Keap1-HKO), except that ileal Fxr was lower in Keap1-HKO mice (65) than WT mice. In contrast, Shp was lower in Nrf2-null (77%↓), Keap1-KD (74), and Keap1-HKO (66%↓) mice than WT mice. Compared to WT mice, Lxra was lower in Nrf2-null (34%↓) and Keap1-HKO (28%↓), but not lower in Keap1-KD mice. These results suggest that Nrf2 is not important for regulating the ileal Fxr-Fgf15 signaling.

Discussion

To date, many chemically diverse Nrf2 activators have been identified, and show promising actions in various diseases. Dimethyl fumarate (formerly known as BG-12 from Biogen Idec, Inc) is so far the most successful electrophilic Nrf2 activator, which has been approved in 2013 by the FDA for treating relapsing-remitting multiple sclerosis (Gold et al., 2012; Havrdova et al., 2013). The synthetic triterpenoid Nrf2 activator CDDO-Me (Bardoxolone methyl or RTA 402) has been investigated in clinical trials for the treatment of advanced chronic kidney disease (CKD) associated with type 2 diabetes mellitus (Pergola et al., 2011). However, the CDDO-Me trial was halted at Phase III due to cardiovascular safety issues (de Zeeuw et al., 2013). Furthermore, the CDDO-Me analog CDDO-EA (RTA 405) was reported to produce adverse effects, including bodyweight decline, liver injury, dyslipidemia, and increased blood pressure in rats with overt diabetes (Zoja et al., 2013). Oleanolic acid, a natural triterpenoid Nrf2 activator, was also reported to cause hepatotoxicity at high doses or after long-term use (Lu et al., 2013). These findings suggest potential hepatotoxicity of triterpenoid Nrf2 activators; however, the underlying mechanism remains unknown.

Increasing evidence demonstrates a critical role of BAs in regulating cardiovascular function, including heart rate and vascular tone (Khurana et al., 2011). Indeed, excess bile acids could decrease fatty acid oxidation in cardiomyocytes and induce cardiomyopathy (Desai et al., 2017). In the present study, one of the most striking findings was that CDDO-Im markedly increased serum BAs in Nrf2-null mice, which was associated with a down-regulation of hepatic BA uptake transporters (Ntcp and Oatp1b2). In contrast, CDDO-Im did not significantly alter serum BAs in WT mice. Therefore, the current findings suggest a critical role of Nrf2 in protecting against CDDO-Im toxicity. Future studies, such as dose-dependent toxicities of CDDO-Im in the four “gene dose-response” mouse modes, are

warranted to further elucidate the role of Nrf2 in the toxicities of synthetic triterpenoid Nrf2 activators.

Activation of Nrf2 is a potential therapeutic option for cholestasis, which is conventionally thought to be due to Nrf2-induced hepatic efflux transporters, such as hepatic Mrp2 (Maher et al., 2007; Reisman et al., 2009b). The present study showed that both pharmacological (CDDO-Im) and genetic (Keap1-KD and Keap1-HKO) activations of Nrf2 down-regulated ileal BA transporters (Asbt and Ost β), suggesting an important role of Nrf2 in intestinal BA absorption. This may also contribute to the therapeutic effect of Nrf2 activation in treating cholestasis. Human BSEP is reported to be regulated by Nrf2 (Weerachayaphorn, et al., 2009). In the present study, CDDO-Im increased hepatic Bsep in WT mice, which was associated with decreased hepatic BAs and increased biliary BA excretion. In contrast, genetic activations of Nrf2 (Keap1-KD and Keap1-HKO) had no effect on Bsep or biliary BA excretion. Furthermore, Nrf2-null mice had higher Bsep than WT mice. These results suggest that Nrf2 is not important for regulating mouse Bsep. Notably, both pharmacological (CDDO-Im) and genetic (Keap1-KD and Keap1-HKO) activations of Nrf2 markedly up-regulate hepatic Mrp2 and Mrp3, two Nrf2 target genes. Therefore, the present study suggests that Nrf2 is not important for regulating hepatic Bsep or biliary BA excretion in mice.

Previous studies showed that bile flow was lower in Nrf2-null mice when normalized to liver weight (Weerachayaphorn, et al., 2012), but higher in Keap1-KD mice when normalized to body weight (Reisman et al., 2009c). In contrast, the present study showed that Nrf2-null mice had lower bile flow when calculated per body weight, whereas Keap1-KD mice had higher bile flow when calculated per liver weight. This could be attributable to different experimental procedures and mouse status. For instance, different from the previous studies, mice in the present study were administered with vehicle control for 4 days and sacrificed without fasting overnight. Nonetheless, the present “gene dose-response” model indicates a role of Nrf2 in maintaining the basal bile flow in mice. It should be noted that the biliary excretion of BAs was not altered in all three genetic models. This suggests that Nrf2 is not important for regulating BA-dependent bile flow. Previous studies suggest that Nrf2 plays a critical role in biliary excretion of glutathione (Reisman, et al., 2009c; Weerachayaphorn, et al., 2012). These findings suggest that Nrf2 plays a role in maintaining the basal level of BA-independent bile flow in mice.

The present study suggests that Nrf2 is not important for regulating hepatic BA synthesis. Nrf2-null mice were previously reported to have lower Cyp7a1 in liver, with both hepatic Fxr-Shp and intestinal Fxr-Fgf15 signaling pathways significantly suppressed (Weerachayaphorn, et al., 2012). In contrast, the present study showed that Cyp7a1 was not altered in Nrf2-null mice. Although both Fxr and Shp were higher in livers of Nrf2-null mice than WT mice, they were not altered by either CDDO-Im or genetic activations of Nrf2 (Keap1-KD and Keap1-HKO). Furthermore, ileal Fxr and Fgf15 were not altered by CDDO-Im, and showed no difference in Nrf2-null and Keap1-KD mice when compared to WT mice. Taken together, Nrf2 is not important for regulating hepatic or ileal Fxr signaling pathway in mice.

In the present study, hepatic BAs were decreased in Nrf2-null and Keap1-HKO mice, but not in Keap1-KD mice. Notably, ileal BA transporters Asbt and Ost β were down-regulated by both genetic ablation (Nrf2-null) and genetic activations (Keap1-KD and Keap1-HKO) of Nrf2. The cholesterol uptake transporter Npc1l1 was decreased in Nrf2-null and Keap1-HKO mice, but not in Keap1-KD mice. This may explain why hepatic BAs are not altered in Keap1-KD mice. The reduced intestinal absorption of both BAs and cholesterol appears to contribute to the decrease of hepatic BAs in Nrf2-null and Keap1-HKO mice. Consistently, Nrf2-null mice were previously reported to have lower ileal Asbt and higher fecal BAs than WT mice (Weerachayaphorn, et al., 2012).

Lxra is important for cholesterol homeostasis by transcriptionally regulating genes involved in cholesterol metabolism. Kamisako et al. reported that high cholesterol diet induces hepatic Lxra target genes, including Abcg5 and Abcg8, in WT mice but not in Nrf2-null mice (Kamisako et al., 2014). The present study showed that hepatic Nrf2 activation by either CDDO-Im or liver-specific Keap1 knockout decreased ileal Lxra in mice. Furthermore, ileal Abcg5 and Abcg8 were down-regulated by both pharmacological (CDDO-Im) and genetic (Keap1-KD and Keap1-HKO) activations of Nrf2. These results further suggest a role of Nrf2 in cholesterol homeostasis by regulating Lxra and its target genes.

In conclusion, the present study provides a comprehensive analysis of the role of Nrf2 in regulating BA homeostasis in mice. Nrf2 is not important for regulating hepatic BA synthesis or biliary BA excretion, but plays a role in regulating intestinal absorption of BAs and cholesterol. Nrf2 regulates Mrp2 and Mrp3, but not Bsep, in livers of mice. Furthermore, Nrf2 is a critical mechanism for protecting against CDDO-Im toxicity.

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Abbreviations:

Abca1	ATP-binding cassette transporter a1
BA	bile acid
CA	cholic acid
Bsep	bile salt-export pump
CDCA	chenodeoxycholic acid
CDDO-Im	1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole
Cyp	cytochrome P450

DCA	deoxycholic acid
Fxr	farnesoid X receptor
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
Keap1	Kelch-like erythroid-cell-derived protein with CNC homology-associated protein 1
LCA	lithocholic acid
MCA	muricholic acid
MDCA	murideoxycholic acid
Mrp	multidrug resistance-associated protein
Nrf2	nuclear factor erythroid 2-related factor 2
Ntcp	sodium taurocholate cotransporting polypeptide
Oatp	organic anion transporting polypeptide
Ost	organic solute transporter
Shp	small heterodimer partner
TCA	taurocholic acid
UDCA	ursodeoxycholic acid
WT	wild-type

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Figure 1. Effects of Nrf2 activation on liver weight and bile flow.

Corn oil (Veh) or CDDO-Im (1 mg/kg) was administered daily (i.p.) for four consecutive days to male WT and Nrf2-null mice (n=5–8 per treatment group). Keap1-KD and Keap1-HKO mice were administered with corn oil vehicle. On day 5, mice were anesthetized and bile was collected for 40 minutes. Relative liver weight (LW) was expressed as a percent of bodyweight (BW). Bile flow rate was normalized to liver weight (LW) or body weight (BW). Data are expressed as means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT vehicle treatment group; # $P < 0.05$ versus Nrf2-null vehicle treatment group.

Figure 2. Effects of Nrf2 activation on biliary excretion of BAs.

BA concentrations in the bile were quantified by UPLC-MS/MS, and their excretion rates were normalized to body weights. The biliary excretion rates of total BAs (ΣBAs), total conjugated BAs (T-BAs), and total unconjugated BAs (U-BAs), total primary BAs (1°BAs), total secondary BAs (2°BAs), total 12α-hydroxylated BAs (12α-OHs), and total non-12α-hydroxylated BAs (non-12α-OHs) were calculated by summation of the corresponding BAs. Data are expressed as means ± S.E.M. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT vehicle treatment group; # $P < 0.05$ and ## $P < 0.01$ versus Nrf2-null vehicle treatment group.

Figure 3. Effect of Nrf2 activation on hepatic bile acids.

Concentrations of total BAs and individual BAs were quantified by UPLC-MS/MS. The total BAs (ΣBAs), total conjugated BAs (T-BAs), and total unconjugated BAs (U-BAs), total primary BAs (1°BAs), total secondary BAs (2°BAs), total 12α-hydroxylated BAs (12α-OHs), and total non-12α-hydroxylated BAs (non-12α-OHs) were calculated by summation of the corresponding BAs. Data are expressed as means ± S.E.M. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT vehicle treatment group.

Figure 4. Effects of Nrf2 activation on serum BA concentrations.

Concentrations of total BAs and individual BAs were quantified by UPLC-MS/MS. The total BAs (ΣBAs), total conjugated BAs (T-BAs), and total unconjugated BAs (U-BAs), total primary BAs (1°BAs), total secondary BAs (2°BAs), total 12α-hydroxylated BAs (12α-OHs), and total non-12α-hydroxylated BAs (non-12α-OHs) were calculated by summation of the corresponding BAs. Data are expressed as means ± S.E.M. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT vehicle treatment group; # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ versus Nrf2-null vehicle treatment group.

Figure 5. Effects of Nrf2 activation on mRNA expression of hepatic BA transporters and BA synthetic enzymes.

QuantiGene Plex 2.0 Assay was performed to quantify the mRNA levels of hepatic transporters (Ntcp, Oatp1a1, Oatp1b2, Bsep, Mdr2, Mrp2, and Mrp3) and BA-synthetic enzymes (Cyp7a1, Cyp8b1, Cyp27a1, Cyp7b1, Fatp5, and Baat). Relative mRNA levels were normalized to WT vehicle treatment group and expressed as fold change. Data represented means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT vehicle treatment group; # $P < 0.05$ and ## $P < 0.01$ versus Nrf2-null vehicle treatment group.

Figure 6. Effects of Nrf2 activation on mRNA expression of ileal BA and cholesterol transporters. QuantiGene Plex 2.0 Assay was performed to quantify the mRNA levels of BA transporters (Asbt, Ost β , Mrp2 and Mrp3) and cholesterol transporters (Npc111, Abca1, Abcg5, and Abcg8). Relative mRNA levels were normalized to WT vehicle treatment group and expressed as fold change. Data represented means $\pm$ S.E.M. * P < 0.05, ** P < 0.01, and *** P < 0.001 versus WT vehicle treatment group.

Figure 7. Effects of Nrf2 activation on mRNA expression of major regulating factors of BA homeostasis.

QuantiGene Plex 2.0 Assay was performed to quantify the mRNA levels of Fxr, Shp, Lrh-1 and Fgfr4 in the liver and Fxr, Shp, Fgf15, and Lxra in the ileum. Relative mRNA levels were normalized to WT vehicle treatment group and expressed as fold change. Data represented means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus WT vehicle treatment group.