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Review

Bridging cell surface receptor with nuclear receptors in control of bile acid homeostasis

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Bile acids (BAs) are traditionally considered as "physiological detergents" for emulsifying hydrophobic lipids and vitamins due to their amphipathic nature. But accumulating clinical and experimental evidence shows an association between disrupted BA homeostasis and various liver disease conditions including hepatitis infection, diabetes and cancer. Consequently, BA homeostasis regulation has become a field of heavy interest and investigation. After identification of the Farnesoid X Receptor (FXR) as an endogenous receptor for BAs, several nuclear receptors (SHP, HNF4α, and LRH-1) were also found to be important in regulation of BA homeostasis. Some post-translational modifications of these nuclear receptors have been demonstrated, but their physiological significance is still elusive. Gut secrets FGF15/19 that can activate hepatic FGFR4 and its downstream signaling cascade, leading to repressed hepatic BA biosynthesis. However, the link between the activated kinases and these nuclear receptors is not fully elucidated. Here, we review the recent literature on signal crosstalk in BA homeostasis.

Keywords: bile acids; FXR; HNF4α; LXR; FGFR4; FGF15/19; Shp2; phosphorylation

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Introduction

Cholesterol is directly converted by a series of chemical reactions into more soluble amphipathic primary bile acids (BAs) in hepatocytes. Therefore, the BA biosynthesis pathway is the primary route of cholesterol disposal from the body[1]. Through specialized hepatic transporters, hepatic BAs are then transported into bile canaliculi, which carry them into the gallbladder, where bile is concentrated. After each meal, bile is emptied from the gallbladder and, due to its amphipathic nature, emulsifies lipids and hydrophobic vitamins to facilitate their intestinal absorption. Excess BAs are reabsorbed by the ileum, which is the distal part of the intestine, and travel back to hepatocytes through the portal vein. Up to 95% of BAs will be reabsorbed in this process, which is fittingly termed the "enterohepatic recycle of BAs."

A number of diseases cause cholestasis, a condition in which bile fails to flow out of the liver. Cholestasis usually induces chronic or acute liver toxicity and damage[2]. Furthermore, BA dysregulation has been associated with a variety of liver and metabolic diseases, other than those directly affected by blocked BA transport. Hepatitis B viruses (HBVs) enter hepatocytes through the transporter for BA uptake, namely the Na^+-taurocholate cotransporting polypeptide (NTCP)[3]. HBV infection causes increased BA biosynthesis[4]. When added to the existing type 2 diabetes treatment regimens, BA sequestrants, which bind BAs to prevent their ileal reabsorption, exhibited beneficial effects for these patients[5]. BA alteration is also associated with obesity, and increased circulating BAs were observed in patients who had undergone bariatric surgeries[6, 7]. Elucidating the regulatory mechanisms behind BA homeostasis could allow for the identification of new therapeutic targets useful for improved treatment of relevant diseases.

In BA biosynthesis, p450 family member cholesterol 7α-hydroxylase (Cyp7a1) is the rate-limiting enzyme for the conversion of cholesterol to BAs[8, 9]. Further demonstration of the functional significance of Cyp7a1 comes from the loss-of-function mutation in humans, which results in a hypercholesterolemic phenotype[10]. With the cloning of Cyp7a1, the feedback regulation of Cyp7a1 transcription was also observed as excess BAs inhibited Cyp7a1 transcription[11]. Given the functional importance of Cyp7a1 in both cholesterol and BA homeostasis, the transcriptional regulation of Cyp7a1 has been the attention of research in the field for over 20 years[1] and a number of regulatory factors have been identified. Specifically, identification of the Farnesoid X Receptor (FXR, also known as NR1H4) as the endogenous receptor for BAs[12, 13] has ushered our understanding of BA biosynthesis, at both molecular and physiological levels, into a new era.

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Before the identification of FXR, BAs were never considered anything more than "physiological detergents." However, accumulating data has led to the designation of BAs as important "signaling hormones"[14]. Inside the intestine, BAs are further modified into structurally diverse secondary BA species by gut microbiota[15]. More intriguingly, different primary and secondary BA species exhibit different capacities to bind and activate FXR, making BA composition a crucial factor for determining FXR activity in various tissues[15, 16]. Within hepatocytes, BAs bind FXR, and together they transactivate the small heterodimer partner (SHP, also known as NR0B2). SHP represses Cyp7a1 transcription by binding with two other nuclear receptors, including liver receptor homolog-1 (LRH-1, also known as NR5A2) and hepatocyte nuclear factor 4α (HNF4α, also known as NR2A1), both of which are required for the basal transactivation of *Cyp7a1*[17–20]. The inhibition of *Cyp7a1* by the BA-FXR-SHP axis resonates with the feedback regulation of *Cyp7a1* transcription by excess BAs, which provided the molecular basis for the intrahepatic feedback regulation of BA biosynthesis. Despite the complex phenotypes, including hypertriglyceridemia, the analysis of FXR knockout (KO) mice further supported this model, as FXR KO mice displayed elevated BA levels, decreased *SHP* expression, and increased *Cyp7a1* expression[21]. However, SHP KO mice only exhibited a mild increase in BA and were still responsive to feedback regulation of BA biosynthesis[22]. Moreover, BA synthesis and *Cyp7a1* expression are dramatically higher in mice with double knockout (DKO) of FXR and SHP than with either gene alone[23]. Although these observations were still considered consistent with the intrahepatic feedback model, they also allow for additional pathway or factor involvement in the tight control of BA biosynthesis.

The ileum has long been recognized as the site for BA absorption[24, 25], which is physiologically indispensable for the intrahepatic feedback model. It was a surprising discovery that the ileum acts as more than simply a canal for BA recycling[26]. In ileocytes, BAs bind to FXR and transactivate *fibroblast growth factor 19* (*FGF19, FGF15* as the mouse ortholog)[26]. Then, FGF15/19, specially expressed in the ileum[27], binds to FGF receptor 4 (FGFR4), which activates a cascade of mitogen-activated protein (MAPK) kinase signaling[26, 28, 29]. Accordingly, *FGF15* or *FGFR4* KO mice exhibited increased BA levels[26, 30]. Regulators of this pathway were also proven to be essential players in BA biosynthesis. For example, FGF15-interacting protein Diet, which is also specifically expressed in intestine, regulates *FGF15* expression. Therefore, Diet regulates hepatic *Cyp7a1* expression and lipid homeostasis[31]. Shp2, which is a cytoplasmic tyrosine phosphatase with two SH2 domains, positively regulates FGFR4 and was proven to be indispensable for the regulation of hepatic BA biosynthesis and *Cyp7a1* expression[32]. Undoubtedly, the FGF19/FGFR4 pathway constitutes another feedback loop for repression of BA biosynthesis.

The ileum is responsible for eliciting two feedback signals: BAs and FGF19/15. BAs, together with FXR, provide intrahepatic feedback regulation of *Cyp7a1* expression. Meanwhile, FGF19/15, the ligand for FGFR4, is secreted from the ileum, further providing an extrahepatic feedback signal for *Cyp7a1* repression. Overall, the research on these two feedback branches remains two parallel paths. However, several studies implied the intertwining of these two pathways. In *FXR* or *SHP* KO mice, at the very least, impaired *Cyp7a1* expression followed FGF19/15 injection[26, 33]. In turn, in *FGFR4* or *FGF15* KO mice, FXR agonists failed to repress *Cyp7a1* expression[26, 33]. Also, in the mice with Shp2 deleted in hepatocytes, several lines of evidence suggested the cross-regulation of these two pathways[32]. Despite increased BAs and FGF15, the activation of both pathways was impaired in this mouse line. Furthermore, failed activation of FXR by a synthetic FXR agonist was also detected in the same animals with uncompromised expression and subcellular localization of FXR[32]. All of these studies, albeit not directly, point to the possible regulation of bile acid synthesis beyond transcription and translation levels.

The post-translational regulation of nuclear receptors (FXR, SHP, HNF4α, and LRH-1) has been highlighted in the literature, but the physiological significance of this regulation remains inconclusive[34]. Due to metabolic roles of these nuclear receptors in BA homeostasis, these receptors are well suited for therapeutic targeting[16, 35]. In this review, we summarize the functional phosphorylation sites of these nuclear receptors, sites that alter the transactivity of these receptors *in vitro*, and discuss the possible physiological implications of these receptors in BA biosynthesis (Figure 1).

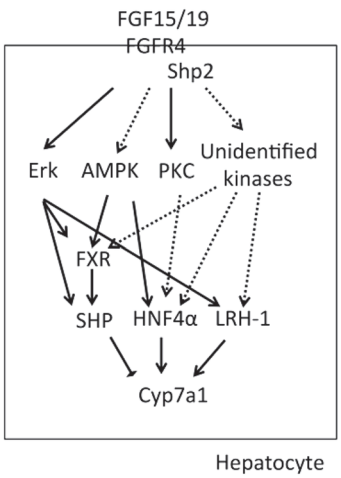

Figure 1. Summary of possible kinases in the regulation of nuclear receptors in bile acid homeostasis. The confirmed substrates for specific kinases (at least by *in vitro* assays) are indicated with solid arrows, whereas proteins merely implicated to be the substrates of specific kinases are shown with dashed arrows.

FXR

In human hepatocytes, activating FXR via BAs induces *FGF19* expression[36, 37]. Therefore, FGFR4 downstream kinase Erk1/2 is activated by BA treatments[37]. Inhibition of FGF19 by neu-

tralizing antibodies or FGFR4 activity by siRNA knockdown abrogates inhibition of *Cyp7A1* by FXR agonist GW4064 in human hepatocytes, further supporting FGFR4-dependent FXR activity[37].

FXR was first shown to be phosphorylated at serine 135 and 154 by protein kinase C (PKC) α and βI[38]. Mutation of both serines led to reduced FXR transcription activity without impairment of its DNA-binding capacity[38]. BAs are able to activate several isoforms of PKC in cultured cell lines and *in vitro* kinase assays[39–41]. In another study, a distinct PKC isoform, PKCζ, could phosphorylate FXR at threonine 456 to prevent cholestasis[42]. Despite the completely different PKC isoforms and phosphorylation sites, both studies indicated a possible role of PKC activation in the feedback regulation of BA biosynthesis mediated by BAs. More recently, PKCζ was shown to be activated by FGF19 in both cultured hepatocytes and in mouse livers[43]. Although FGF19 failed to induce PKCζ in another study, specific phosphorylation of other PKC isoforms was detected[32].

In vitro data showed that activated PKC could phosphorylate FXR and enhance its transcription activity[38]. However, careful studies are still needed to establish PKC's role in BA homeostasis. First, FXR mutations at phosphorylation sites have to exhibit defective activity *in vivo* either in individuals carrying such mutations or knockin models. Second, the isoform(s) of PKC need(s) to be characterized in experimental models. Third, if PKC isoform(s) are involved in BA homeostasis, how PKC isoform(s) are activated under physiological or pathological conditions still requires further investigation.

Another possible kinase for regulation of BA biosynthesis is the AMP-activated protein kinase (AMPK). AMPK can be activated by BAs for establishing hepatocyte polarization[44]. Moreover, metformin, activator of AMPK, protects BA-induced hepatocyte apoptosis[45]. Although both studies investigated other important aspects of BA regulatory functions, they highlighted the possible role of AMPK in BA biosynthesis. A recent study reported that AMPK could phosphorylate FXR *in vivo* at serine 250[46]. More importantly, in both *in vitro* and *in vivo* models, AMPK activators can antagonize BA- or FXR agonist-induced SHP expression[46]. Given the important roles of AMPK in lipid and glucose metabolism, AMPK shows functional relevance with both BA/FXR and FGF19/FGFR4 signaling. Elucidation of AMPK's roles in FXR regulation could shed new light on the fine-tuning of this important signaling pathway.

SHP

The transcription of SHP is dynamic, possibly because BA biosynthesis is regulated by nutrient supply and the circadian clock[47, 48]. Given that the transcription of SHP is regulated by the circadian clock[47], the stability of its protein is low[49]. Erk can phosphorylate SHP in a BA- and FGF19-dependent manner[49]. This is the first time that FGF19/FGFR4 and BA/FXR signaling converge in the regulation of a specific Cyp7a1 regulator. FGF19/15 could mildly induce SHP expression[26, 32] in some studies and failed to induce any SHP expression in other reports. This could be due to subtle experimental design differences such as sample collection timing. However, they further argued for additional factor(s) in the regulation of BA biosynthesis, especially because SHP knockout mice showed only mild defects in BA biosynthesis regulation[22].

More recently, the same group that reported SHP phosphorylation identified another phosphorylation site (threonine 55) on SHP[43]. This phosphorylation site was required for SHP binding with HNF4α and LRH-1, resulting in the failure to repress Cyp7a1 by BA and FGF19 signals. Of note, this site was phosphorylated by PKCζ, the activation of which by BA and FGF19 signals still needs further independent confirmation. Still, a better approach for evaluating these sites is to establish knockin mouse lines, especially when the SHP knockout mice exhibited only mild defects of BA biosynthesis regulations.

HNF4α

HNF4α is a definite regulator of Cyp7a1 and binds to the Cyp7a1 promoter for its basal activation. HNF4α is also required for the repression of Cyp7a1 through its association with SHP. However, its binding to the Cyp7a1 promoter does not change with FGFR activation by FGF19. Mass spectrometry studies have shown HNF4α can be phosphorylated at several serine/threonine sites[50, 51], suggesting that phosphorylation of HNF4α is an important route for regulating its activity.

Glucagon and cAMP were able to repress *Cyp7a1* expression[52]. Increased HNF4α phosphorylation by protein kinase A (PKA) consequently reduced HNF4α binding to the *Cyp7a1* promoter[52]. Although only PKA was the focus in this study, the possible roles of AMPK in the regulation of HNF4α during *Cyp7a1* transcriptional regulation cannot be ruled out. Furthermore, not only can HNF4α be phosphorylated by AMPK but its dimerization and stability can also be reduced by the phosphorylation[53]. It should be noted that both FXR and HNF4α could be the targeted BA biosynthesis regulators by AMPK.

Due to the pleiotropic functions of HNF4α in various metabolic processes, multiple kinases have been shown to phosphorylate HNF4α under different conditions, which include mitogen-activated protein kinase p38[54], Erk[55], Src tyrosine kinase[56], JNK[57], and PKC[58]. Erk activation downstream of FGFR4 was proven to be indispensable for the repression of *Cyp7a1*[26, 32, 33, 48]. Among these kinases, Erk is the only one that is undoubtedly activated by FGF15/19. It would be interesting to examine the possible phosphorylation of HNF4α by both FGF19 and BA signals to determine whether the phosphorylation is involved in the direct switch of the downstream target in the feedback regulation of BA biosynthesis.

LRH-1

LRH-1 is another definite regulator of Cyp7a1 transcription, which binds with HNF4α and SHP at the Cyp7a1 promoter. Hepatocyte-specific knockout of LRH-1 showed that regula-

tion of BA homeostasis by LRH-1 is not essential for feedback regulation of BA synthesis[19]. Erk can phosphorylate LRH-1 *in vitro* at serines 238 and 243 in the hinge domain, which increases the transcription activity of LRH-1[59].

Shp2 and regulation of BA-related kinases

As discussed above, FGF15/19/FGFR4 represents an extracellular mechanism for the repression of BA biosynthesis and BA/FXR signaling is the main player in the nuclei. The post-translational modification is an appealing mechanism to bridge these two spatially restricted pathways. Recent work on the cytoplasmic protein tyrosine phosphatase has shed new light on the intersection of these two pathways. In mice with Shp2 deleted in hepatocytes ($Shp2^{hep-/-}$), BA biosynthesis is uncontrollably high with both FGF15/FGFR4 and FXR signaling exhibited defective activation, despite the high expression of the respective ligands[32].

Detailed BA composition analysis revealed that contrary to the dramatic increase in the FXR agonist species, the FXR antagonist species remained unchanged in $Shp2^{hep-/-}$ mice. Without changes in expression, the impaired FXR activation could be due to post-translational changes in the protein or changes in co-activators. This observation was confirmed when synthetic FXR agonist treatment failed to activate FXR in $Shp2^{hep-/-}$ liver.

In the same mice, increased FGF15 expression also failed to activate FGFR4, as evidenced by lower Erk phosphorylation in the $Shp2^{hep-/-}$ liver. Shp2 binds with the direct target of FGFR4, FRS2α and is required for activation of the receptor. Defective activation of FGFR4 resulted in not only impaired Erk activation but also defective activation of multiple kinases, including p90RSK and PKC isoforms.

The results from $Shp2^{hep-/-}$ mice further proved that both the extrahepatic FGF15/19 signal and nuclear BA/FXR signal are orchestrated in hepatocytes by a cascade of signaling events. Furthermore, this model provided experimental evidence of crosstalk between these two pathways.

Perspectives

BAs have penetrated all areas of liver diseases as well as some gastrointestinal diseases. Given the toxicity of excess BAs, complicated regulatory circuits for BA homeostasis maintenance are necessary. Recently, two main repression signals for BA biosynthesis, namely BAs themselves and FGF15/19, have gained full recognition. However, the means by which these two signals are integrated inside hepatocytes still need further research. Post-translational modifications for nuclear receptors could provide the link for a fully integrated view of this important biological process. A more thorough understanding of the functional diversity of these nuclear receptors and their post-translational modifications is required to target them for future therapeutics.

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The authors have extensively referenced the resources on phosphosite.org, which curates both published and unpublished datasets.

Due to the abundance of review and research papers on BA homeostasis, we regret having to exclude some excellent relevant references due to space limitation.

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