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Effects of patent ductus venosus on bile acid homeostasis in aryl hydrocarbon receptor (AhR)-null mice

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Abstract

The Aryl hydrocarbon receptor (AhR) is primarily known as one of the xenosensors and regulators of drug-metabolizing genes. Bile acids (BAs) are synthesized in the liver, and undergo several enterohepatic recirculations in which the liver removes BAs from the portal blood, minimizing the BAs that spill over into the systemic circulation. Previous studies revealed a lifelong patent ductus venosus (PDV) in AhR-null mice. Increased concentration of total BAs (Σ -BAs) in AhR-null mice is known; however, the impact of PDV on BA homeostasis in liver and bile remains unclear. This work investigated the consequences of PDV on BA homeostasis by comparing AhR-null and wild-type (WT) mice of both genders.

In serum, Σ -BAs were markedly higher (64–85-fold) in AhR-null mice than in WT mice, especially due to the increase of tri-OH primary BAs (86–142-fold). Despite the extremely high concentration of serum BAs, the concentration of BAs in livers of AhR-null mice remained similar to WT mice. AhR-null livers were protected against increased BA influx by downregulation of uptake transporters and BA synthetic enzymes in the alternative pathway. Although livers of AhR-null mice are 20–25% smaller than WT mice, biliary excretion of BAs was maintained in the AhR-null mice, and even tended to increase. Surprisingly, intestinal Fgf15 expression was not increased,

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even though there was a marked increase in serum BA concentrations. Although PDV resulted in extremely high BA concentrations in serum of AhR-null mice, they maintained a concentration of BAs in liver and biliary excretion of BAs similar to control mice.

Keywords

Aryl hydrocarbon receptor; bile acids; biliary excretion; ductus venosus

INTRODUCTION

Bile acids (BAs) are amphiphilic molecules participating in various physiological processes such as elimination of cholesterol, maintaining biliary excretion of endo- and xenobiotics, fat absorption, and metabolic/endocrine coordination of liver, intestine, and the intestinal microbiome. Primary BAs are synthesized by the classic and alternative pathways from cholesterol through several enzymatic steps. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the primary BAs in humans, whereas in rodents, additionally, two 6-OH BAs are formed, namely α - and β -muricholic acids (MCA). The synthesis and transporters of BA homeostasis are regulated by the intestinal Fxr (through Fgf15-Fgfr4) and the hepatic Fxr (through Shp) (Goodwin *et al.*, 2000; Inagaki *et al.*, 2005; Kong *et al.*, 2012). To decrease pKa and increase the water solubility, the primary BAs are amidated with either glycine or taurine and transported into the bile via the bile salt export protein (Bsep) (Wang *et al.*, 2001; Stieger *et al.*, 2007), and divalent sulfated-amidated BAs via multidrug resistance-associated protein 2 (Mrp2) (Keppler *et al.*, 1999; Akita *et al.*, 2001).

The intestinal microbiome metabolizes the primary BAs into secondary BAs such as deoxycholic acid (DCA) and lithocholic acid (LCA). In the terminal ileum, the apical sodium-dependent bile acid transporter (Asbt) reabsorbs the BAs (Dawson *et al.*, 2003), and the organic solute transporter (Ost) α + β effluxes the BAs into the portal circulation (Dawson *et al.*, 2005). In the liver, Ntcp and Oatp1b2 (OATP1B1/1B3) transporters are responsible for the uptake of conjugated and unconjugated BAs, minimizing BA spillover into the systemic circulation (Ananthanarayanan *et al.*, 1988; Cattori *et al.*, 2000; Csanaky *et al.*, 2011; Slijepcevic *et al.*, 2015).

Each BA molecule undergoes several enterohepatic recirculations, and only up to 5% of the excreted BAs pass each time with the feces. In the portal vein of humans, the concentration of BAs is 20–50 μ M, whereas in the systemic circulation in a fasting state, the BA concentration is less than 5 μ M (Hofmann, 1999). The BA extracting capacity of the liver depends on two factors: the hepatic blood flow from the intestine via the portal vein, and the transport maximum of the basolateral BA uptake transporters.

However, the blood supply to the fetal liver is different from the adult liver. A special fetal shunt, the ductus venosus, allows the highly oxygenated blood to bypass the liver from the umbilical vein to the inferior vena cava, and only a reduced portion of the oxygen and nutrient-rich blood goes to the liver. In adults, the portal blood flow is responsible for over 80% of the total hepatic blood flow. However, in the fetus, the ductus venosus diverts a significant portion (25–40%) of the portal blood flow (Kiserud, 1999; Kiserud, 2000). In

full-term neonates, the ductus venosus anatomically closes during the first week of life, but premature babies may have a patent ductus venosus (PDV) and its closure may take longer, causing an intrahepatic portosystemic shunt. The portosystemic venous shunt is characterized by prolonged blood coagulation time, hypergalactosaemia, hyperammonaemia, and elevated total serum BA concentrations (Murayama *et al.*, 2006). Still, the effects of patent ductus venosus and the high concentrations of serum BAs on the BA homeostasis, especially in liver and bile, have remained unclear.

The helix-loop-helix aryl hydrocarbon receptor (AhR) is primarily known as one of the xenosensors and regulators of drug metabolizing genes activated by numerous exogenous and endogenous compounds such as dioxin-like compounds, polycyclic aromatic hydrocarbons, plant flavonoids, polyphenols, indoles and tryptophan metabolites (Burbach *et al.*, 1992; Murray *et al.*, 2014). To better understand the physiological and pathological roles of AhR, AhR-null mouse models were developed, which happened to show various tissue and vascular abnormalities (Fernandez-Salguero *et al.*, 1996; Schmidt *et al.*, 1996; Mimura *et al.*, 1997). Dr. Bradfield's laboratory identified and characterized lifelong PDV, a developmental vascular abnormality, in AhR-null mice (Lahvis *et al.*, 2000; Harstad *et al.*, 2006). It has also been shown that the total BA concentrations are higher in both male and female AhR-null mice (Harrill *et al.*, 2013), similar to infants with patent ductus venosus (Murayama *et al.*, 2006). These similar changes in serum total BAs raise the question what is the impact of open ductus venosus on BA homeostasis, especially in the liver and bile. Recently, we have shown the effects of TCDD on BA homeostasis (Csanaky *et al.*, 2018), and in the present study we investigated the consequences of lack of AhR (with PDV) on BA homeostasis, comparing AhR-null and WT mice of both genders.

MATERIALS AND METHODS

Chemicals

Bile acid standards were purchased from Steraloids, Inc. (Newport, RI) and Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich unless otherwise noted.

Animals

Male and female C57BL/6 wild-type (WT) mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). AhR-null mice (>99% congenic for C57BL/6 background) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were characterized previously (Schmidt *et al.*, 1996). Mice were acclimated for at least one week in a standard temperature-, light-, and humidity-controlled facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Mice were provided Laboratory Rodent Chow 8604 (Harlan, Madison, WI) and drinking water *ad libitum*. Studies were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. To use approximately the same weight mice, male mice were used at 12–15 weeks of age, whereas females were 16–19 weeks of age.

Tissue collection

Corn oil (5 ml/kg) was administered intraperitoneally (i.p.) for four consecutive days to male and female mice (6–8 mice per group). At 24 hours after the last treatment, mice were anesthetized with 50 mg/kg pentobarbital (Nembutal, Lundbeck Inc, Deerfield, IL), blood was collected from the retro-orbital veins, and livers and ilea were harvested. Serum samples were separated using Microtainer separating tubes (BD Biosciences, San Jose, CA). Samples were frozen in liquid nitrogen and stored at –80°C until further analysis.

Bile collection

Separate groups of male and female mice (5–6 per group) were treated with corn oil as mentioned above. On day 5, mice were anesthetized with a ketamine/midazolam mixture (100 and 5 mg/kg, respectively, i.p.) and the common bile duct of each mouse was cannulated with the shaft of a 30-gauge needle attached to PE-10 tubing. Bile was collected for 40 min in pre-weighed 0.6-ml microcentrifuge tubes that were immersed in ice. The volume of bile samples was determined gravimetrically, taking 1.0 as specific gravity.

RNA extraction

The total RNA of livers and ilea was extracted using RNA-Bee reagent (Tel-Test, Inc., Friendswood, TX), according to the manufacturer's protocol. RNA concentrations were quantified using a NanoDrop1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at a wavelength of 260 nm. RNA integrity was confirmed by agarose gel electrophoresis and ethidium bromide staining of 5 µg of total RNA to visualize intact 18S and 28S bands.

Messenger RNA quantification

The majority of mRNA of genes in liver and ileum samples were quantified using QuantiGene Plex 2.0 Assay (Affymetrix/Panomics, Inc., Fremont, CA). Individual bead-based oligonucleotide probe sets, specific for each gene examined, were developed by Affymetrix/Panomics, Inc. Genes and reference sequence numbers are available at <https://www.thermofisher.com> (sets #21330 and #21383). Samples were analyzed using a Bio-Plex 200 System Array reader with Luminex 100 xMAP. Data were acquired using Bio-Plex Data Manager version 5.0 (Bio-Rad, Hercules, CA).

In addition to the bead array, for some mRNAs, reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used, namely for *Abca1*, *Abcg5*, *Abcg8*, *Atp8b1*, *Bcrp*, *β-klotho*, *Ent1*, *Ibapb*, *Mate1*, *Mdr1*, *Mrp1*, *Mrp4*, *Mrp6*, *Oatp1a4*, *Oatp2b1*, *Oat2*, *Oct1*, *Osta*, and *Ostβ* as described in detail (Lickteig *et al.*, 2016; Renaud *et al.*, 2016). Briefly, total RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA). Power SYBR Green Master Mix (Applied Biosystems) was used for qPCR analysis. Fluorescence was quantified with an Applied Biosystems 7300 Real Time PCR System. Differences in gene expression between groups were calculated using the comparative Ct method. All data were standardized to the internal control ribosomal protein L13A (liver) or glyceraldehyde 3-phosphate dehydrogenase (ileum). Relative mRNA levels were calculated with vehicle controls set as 100% for each gender.

Bile acid analysis in liver, bile, and serum

Sample extraction and quantification of individual BAs by UPLC-MS/MS were performed according to methods described previously (Alnouti *et al.*, 2008; Zhang and Klaassen, 2010).

Statistical analysis

All statistical analyses were performed with an IBM-SPSS 23.0 computer program (IBM, Armonk, NY). Individual values were log-transformed to obtain normal distribution. The differences between corresponding WT and AhR groups were determined by Student *t* test with significance set at $P < 0.05$. All data are presented as the mean $\pm$ S.E.M. Asterisks (*) denote differences between WT and AhR-null male or female mice.

RESULTS

Effects of AhR deletion on bile acid concentrations and composition in serum of mice

Concentrations of BAs in the serum of WT and AhR-null mice are shown in Fig.1. The total concentration of BAs in serum of male and female WT mice is fairly low (approximately 1 nmol/ml); it is composed of nearly equal concentrations of unconjugated- and T-conjugated BAs, with minuscule quantities of G-conjugated BAs (less than 1%, data not shown). The concentration of Σ -BAs was markedly higher in both male (63-fold) and female (83-fold) AhR-null mice compared to their corresponding male and female WT mice. The serum concentration of the 1°BAs was 86-fold higher in male and 124-fold higher in female AhR-null mice compared to WT mice, whereas the 2°BAs increased 20-fold in male and 21-fold in female AhR-null mice, compared to WT mice. This finding indicates that the accumulation of 1°BAs contributes the most to the extremely high concentration of Σ -BAs in AhR-null mice. The absence of AhR enhanced the serum concentration of T-BAs more in male (74-fold) than in female (59-fold) mice, whereas its absence increased the U-BAs more in female (125-fold) than in male mice (46-fold). The BAs that were elevated the most in AhR-null male mice were TCDCA (197-fold), T α + β MCA (155-fold), TCA (86-fold), α MCA (69-fold), CA (59-fold), and β MCA (41-fold). However, BAs that were elevated the least in AhR-null male mice were TDCA (2.3-fold) and LCA (4.5-fold). The more prominent increase in serum U-BAs and 1°BAs in female AhR-null mice was due to the elevated CA (1032-fold), β MCA (213-fold), α MCA (157-fold), and UDCA (46-fold). The concentrations of all other BAs in female AhR-null mice were higher than in WT mice, approximately to the same extent as in male AhR-null mice, compared to their corresponding WT mice.

Various changes in the concentration of individual BAs may result in significant alterations in the relative proportion of each BA. Changes in the relative proportion of BAs are important because the various BAs have different affinities for BA receptors (Fxr, Takeda-G-protein-coupled receptor 5 [Tgr5]) as well as to enzymes and transporters in BA metabolic pathways.

The relative proportion of each BA in the serum of WT and AhR-null mice is shown in Suppl. Fig. 1. The lack of AhR did not significantly change the proportions of individual T-BAs and U-BAs, although the percentage of U-BAs tended to decrease in males, but

increased in female AhR-null mice compared to their corresponding gender of WT mice. Interestingly, in AhR-null mice, the relative proportion of 1°BAs was higher (M: +26%, F: +29%), whereas the 2°BAs were lower than in WT mice. The proportion of 12-OH BAs in the serum was higher (M: +8.4%, F: +8.1%), whereas the non-6,12-OH BAs were lower (M: -2.9%, F: -7.3%) in AhR-null mice. The proportion of 6-OH BAs was similar in both male and female AhR-null and WT mice.

Surprisingly, the relative proportions of individual BAs had some similar and some opposite tendencies in male and female AhR-null compared to the corresponding genders of WT mice. The relative percentage of α + β MCA was higher in both male and female AhR-null mice compared to WT mice (M: +12%, F: +8.3%). The relative proportion of TCDCA was similar in both genders of AhR-null and WT mice. An interesting difference between the male and female AhR-null mice is that the proportion of TCA was higher in male (+11%), whereas it was lower in female (-13.4%) AhR-null mice compared to WT mice. Conversely, the relative contribution of unconjugated CA to the total serum BA composition in AhR-null females was 31.2% higher than in WT females, whereas in males, there was no significant difference in the relative ratio of CA between AhR-null and WT mice. It is important to note that the relative ratio of unconjugated α and β MCA had patterns similar to CA in AhR-null male and female mice. The relative ratio of α MCA (+1.5%) and β MCA (+5.3%) were higher in female AhR-null, but not in male AhR-null mice compared to WT mice. The ratio of all individual 2° BAs in the serum was lower in both male and female AhR-null than in WT mice.

Effects of AhR deletion on bile acid concentrations and composition in livers of mice

Fig. 2 demonstrates the concentrations of BAs in livers of AhR-null and WT mice. There were no significant differences in the major categories of BAs in the liver when comparing male AhR-null and WT mice. In the livers of female AhR-null mice, the 1°BAs were significantly higher (+41%) compared to WT mice, due to the higher 6-OH (+40%) and non-6,12-OH BAs (+54%). The TCDCA in liver was higher in both male (+39%) and female (+121%) AhR-null mice compared to WT mice. In addition, the concentration of α + β MCA (+58%) and TUDCA (+41%) were higher in livers of AhR-null female mice than in female WT mice. The concentration of CA was 1.2-fold higher, whereas the concentrations of the secondary BAs DCA (-64%) and LCA (-96%) were significantly lower in AhR-null female mice.

The relative proportions of the individual BAs in livers of male and female WT and AhR-null mice are presented in Suppl. Fig. 2. Over 93% of BAs in liver are T-conjugates, and the lack of AhR did not influence this proportion in either male or female mice. The proportion of 1° BAs was 84% in male and 80% in female WT mice. The fraction of 1°BAs was slightly higher in male (+3.4%) and female (+4.9%) in AhR-null mice than WT mice. The lack of AhR did not significantly alter the proportion of 12-OH, 6-OH, and non-6,12-OH BAs in livers of male mice, but it significantly decreased the proportion of 12-OH BAs (-4%) and increased the non-6,12-OH BAs (+1.2%) in female mice. The proportion of TCA (+5.5%) was higher in livers of AhR-null male mice, whereas the contribution of α + β MCA (+5%) and TCDCA (+1.7%) was higher in AhR-null female mice compared to WT

mice. In AhR-null mice, the relative proportion of secondary BAs such as TDCA (−2.4%) and T ω MCA (−1.8%) in livers of AhR-null mice, especially in female mice, was lower.

Effects of AhR deletion on liver weight and bile flow

The liver weight and bile flow in AhR-null and WT mice are shown in Fig. 3. The present data confirmed the earlier finding that the AhR-null mice have smaller livers (M: −26%, F: −20%) than the WT mice (Schmidt *et al.*, 1996). As a consequence of smaller livers, the bile flow per body weight was lower in both male (−17.3%) and female (−19.8%) AhR-null mice. However, there were no changes in bile flow per gram liver weight.

Effects of AhR deletion on biliary excretion of bile acids

The biliary excretion of BAs in WT and AhR-null mice is shown in Fig. 4. Contrary to the lower bile flow per body weight, there were no significant differences in the biliary excretion of Σ -BAs, T-BAs, and U-BAs between AhR-null and WT mice. The biliary excretion of Σ -BAs per g liver was significantly higher in AhR-null male (+133%) and female (+85%) mice than in WT mice (data not shown). These findings indicate that one gram of liver has to excrete more BAs in AhR-null mice than in WT mice to maintain the biliary excretion.

The relative contribution of excreted BAs is shown in Suppl. Fig. 3. In both male and female mice, the bile contains over 99% T-BAs in WT and AhR-null mice. Lack of AhR approximately halved the relative contribution of biliary excretion of 2°BAs in both male and female AhR-null mice. The lower contribution of 2°BAs in bile was due to less THDCA, TDCA, and DCA in both male and female AhR-null mice. In female AhR-null mice, the contribution of 6-OH BAs was higher than in WT mice, because the proportion of both T α - and T β MCA was higher. In contrast, the proportion of TCA was lower in AhR-null than WT mice.

Effects of AhR deletion on mRNA expression of major hepatic sinusoidal uptake, canalicular and basolateral efflux transporters

The top panel of Fig. 5 represents the relative gene expression of hepatic uptake transporters in AhR-null mice compared to WT mice. In both AhR-null male and female mice with the exception of Oatp1a1 and Ent1, all other uptake transporters tended to be lower (Ntcp in both male and female, Oct1 in male) or were significantly lower in AhR-null mice (Oatp1a1 M: −76%, F: −83%; Oatp1b2 M: −53%, F: −50%; Oatp2b1 M: −50.5%, F: −57.3; Oct1 F: −38.1%).

The middle panel of Fig. 5 depicts the relative gene expression of canalicular transporters in AhR-null mice compared to WT mice. With a few exceptions, absence of the AhR receptor did not alter the majority of canalicular transporters. Compared to WT mice, the mRNA expression of the cholesterol transporters Abcg5 (+181%) and Abcg8 (+239%) were markedly upregulated in male, whereas the Mdr1b (+74%) was higher in female AhR-null mice. The bottom panel shows the data for the basolateral efflux transporters. In the absence of AhR, the mRNA expression of Ost β were higher in both male (+170%) and female (+27%) mice. The expression of Mrp1 was lower in female (−37%) and tended to be lower

in male AhR-null mice. In male AhR-null mice, the expression of Mrp6 was also lower (−79%).

Effects of AhR deletion on mRNA expression of major BA synthesizing enzymes and major hepatic regulating factors of BA homeostasis

The mRNA expression of the major BA synthetic enzymes in WT and AhR-null mice are presented in the top panel of Fig. 6. The gene expression of enzymes in the alternative BA synthetic pathway is significantly lower in both male and female AhR-null mice compared to WT mice; the mRNA of Cyp27b1 was approximately one-third lower (M: −29%, F: −33%), whereas the Cyp7b1 expression was suppressed more in AhR-null mice (M: −77%, F: −61%). In contrast, Cyp7a1, the rate-limiting enzyme of the classic pathway, tended to be higher in both genders of AhR-null mice. The mRNA expression of Bal, which activates the BAs during conjugation, was similar in AhR-null and WT mice. However, the gene expression of bile acid conjugating enzyme Baat was lower in male (−17%) and tended to be lower in female AhR-null than in the corresponding WT mice.

In livers of AhR-null mice, the expression of the BA receptor Fxr was one-third lower (M: −29%, F: −31%) than in WT mice. In addition, the Fxr target gene Shp was also expressed 60% lower in female AhR-null mice. In contrast, Fgfr4 expression was 30% higher in male AhR-null mice than WT mice. Although in female AhR-null mice, the mRNA expression of Fgfr4 was not higher, and its co-receptor β -klotho tended to have a higher expression in female AhR-null mice compared to WT mice.

Effects of AhR deletion on mRNA expression of major ileal BA apical and basolateral transporters, and regulating factors of BA homeostasis

The top panel of Fig. 7 compares the mRNA expression of apical transporters in the ileum of WT and AhR-null mice. Expression of all the apical BA/cholesterol transporters were significantly lower in male AhR-null mice than in WT mice (Asbt −15%, Npc1l1 −32%, Abcg5 −19%, Abcg5 −21%). In contrast, no significant differences were detected in any of the apical BA/cholesterol transporters between female AhR-null and WT mice.

The mRNA expression of the regulators of BA homeostasis in the ileum is presented in the middle panel of Fig. 7. The mRNA expression of I-babp, facilitator of the intestinal uptake of bile acids, was 90% higher in male, but not in female AhR-null mice compared to the corresponding gender of WT mice. In contrast, the expression of Shp was higher (+243%) in female AhR-null mice, but not in males; moreover, Shp even tended to be lower in AhR-null than in WT mice. In male AhR-null mice, the gene expression of Fgf15 (−65%) and Lxra (−28%) was lower than WT mice, and a similar tendency was detected for Shp and Tgr5. It must be noted that the gene expression of Fxr was similar in both male and female mice of AhR-null mice compared to the respective gender of WT mice.

The bottom panel of Fig. 7 illustrates the basolateral transporters in the ilea of AhR-null and WT mice. The mRNA expression of Mrp2 (−39%) and Abca1 (−18%) were lower, whereas the expression of Mrp3 was (+68%) higher in male AhR-null than in WT mice. In contrast, the expression of Mrp3 was similar, but Mrp2 was (+42%) higher in AhR-null female mice than WT mice. In AhR-null female mice, Abca1 tended to be lower, like in the male null

mice. Osta was significantly higher in female (+38%), and it tended to be higher in male AhR-null mice. The expression of Ost β was similar in male and female mice of both genotypes.

DISCUSSION

The liver plays a central role in intermediary metabolism and chemical defense of the body. With synthesis and maintenance of continual BA flux, BAs play a pivotal role in orchestrating various functions of the liver, as well as communicating with other organs. Hepatic homeostasis is highly dependent on adequate perfusion and microcirculation in the hepatic sinusoids. It is well characterized that the ductus venosus stays open during the life of AhR-null mice (Lahvis *et al.*, 2000; Lahvis *et al.*, 2005; Harstad *et al.*, 2006), which anatomic and physiological condition predestined the AhR-null mice for altered BA homeostasis.

The present study (Fig1) confirmed the previous finding that the serum concentration of total BA is significantly higher in both male and female AhR-null than WT mice (Harrill *et al.*, 2013). The present study also shows that the increase in serum total BAs was even more marked in female AhR-null mice (85-fold) than in male AhR-null mice (64-fold). In contrast, in AhR-null rats, where the ductus venosus is closed, the total serum BA is elevated only in females (9-fold), but not in males, compared to the corresponding gender of WT rats (Harrill *et al.*, 2013). In AhR-null rats, the ductus venosus is not open throughout life like in the AhR-null mice. This species difference between AhR-null mice and rats points out that in AhR-null mice, the open ductus venosus is probably the main reason for the elevated serum BA concentration. However, in female AhR-null mice, some additional factors might also contribute to the more marked increase in serum BA concentrations, as seen in female AhR-null rats, where the ductus venosus is closed. One of these factors can be the differently regulated Asbt in male and female AhR-null mice. In male AhR-null mice, the mRNA expression of Asbt was 15% lower than in WT mice, whereas the Asbt expression in female AhR-null and WT mice were similar (Fig. 7). Therefore, in male AhR-null mice, the intestinal reuptake of BAs by Asbt may be lowered, whereas it is unchanged in female AhR-null compared to the corresponding gender of WT mice.

The present study provides further insight into the changes in individual BAs in Ahr-null mice. The accumulation of BAs in the serum of AhR-null mice is primarily due to the accumulation of primary BAs (Fig 1), significantly increasing their proportion in the serum of both genders (suppl. Fig 1) compared to WT mice (M: 62% to 88%; F: 60% to 89%). The 1° BAs that accumulated the most were the tri-OH primary bile acids (CA + TCA + α MCA + β MCA and their taurine conjugates) which increased in both male (87-fold) and female (142-fold) AhR-null mice and are also responsible for the higher increase of BAs in AhR-null female mice. The increase in major di-OH 1° BAs (CDCA+ UDCA and their taurine conjugates) were considerably lower than the tri-OH 1° BAs in the serum of both male (41-fold) and female (31-fold) AhR-null mice. Reichen and Paumgartner demonstrated that in perfused rat livers, di-OH BAs have a higher affinity for transport than tri-OH BAs (Reichen and Paumgartner, 1976). Similarly, our laboratory also showed in isolated rat hepatocytes that the initial rate of uptake of the di-OH BAs is about ten-fold higher than those of the tri-

OH BAs (Iga and Klaassen, 1982). Taken together, the difference in affinity between the di-OH and tri-OH BAs for the uptake transporters may contribute to the lower increase in the di-OH than tri-OH BAs in AhR-null mice. Although the absolute concentration of 2° BAs was higher in serum of both male and female AhR-null mice (Fig 1), the proportion of 2° BAs significantly decreased, due to the marked increase in primary BAs (Suppl. Fig 1). In female mice, surprisingly, the concentration of unconjugated BAs (especially CA, α MCA, β MCA) increased more in serum of AhR-null female mice (CA: 1033-fold, α MCA: 159-fold, β MCA: 214-fold) than in male mice (CA: 70-fold, α MCA: 69-fold, β MCA: 42-fold). It has also been shown that unconjugated CA has a lower affinity to the transport system than the taurine or glycine conjugated form of CA (Reichen and Paumgartner, 1976; Iga and Klaassen, 1982).

The liver is unique in that it has a double blood supply: the portal venous blood (75%) from the intestine and the oxygenized hepatic arterial blood (25%) from the heart (Rappaport, 1980). It has been shown that blood flow in the portal vein and the hepatic artery are inversely proportional; e.g., when portal blood flow decreases, the hepatic artery dilates and buffers up to 60% of the decreased portal flow. This mechanism, called hepatic arterial buffer response, minimizes the effect of portal venous flow changes on hepatic clearance and sustains sufficient oxygen supply to the liver (Lautt, 1983; Lautt *et al.*, 1990; Jakab *et al.*, 1995).

In AhR-null mice, the permanent ductus venosus diverts the blood from the portal vein, decreasing the first-pass hepatic extraction of the reabsorbed BAs, and setting a markedly higher steady-state serum concentration of BAs in the systemic circulation (Fig 1). Due to the diminished portal flow into the liver, the hepatic artery will deliver systemic blood with a higher BA concentration into the hepatic sinusoids, causing a constant high concentration of BAs (and other endogenous compounds). This situation may explain the downregulation of several basolateral uptake transporters, including bile acid transporters in both male and female AhR-null mice (Fig. 5, top panel). The downregulation of uptake transporters is crucial to protect the liver from the increased levels of BAs in the hepatic sinusoids.

Contrary to the extremely elevated serum BAs in the systemic circulation (Fig. 1), the concentrations of BAs in the liver were not significantly increased, - or were only minimally increased in females - in AhR-null mice (Fig. 2). The tendency of higher hepatic concentrations of BAs might be an artifact due to a small amount of residual blood in the liver after exsanguination. The minuscule amount of residual blood in livers of AhR-null mice can lead to a higher concentration of BAs in AhR-null mice than in WT mice. The hepatic concentration of total BAs in the liver of WT mice is about 100-fold higher in females and 116-fold higher in males than in the serum. In AhR-null mice, due to the extremely high concentration of BAs in the serum, the liver to serum ratio of total BA decreased to 2.2-fold in male and 1.5-fold in female AhR-null mice. Taken together, there is no meaningful difference in the hepatic concentrations of BAs between WT and AhR-null mice.

As reported earlier, the livers of both male and female AhR-null mice are smaller than the respective WT mice (Schmidt *et al.*, 1996; Harrill *et al.*, 2013). The smaller livers of AhR-

null mice yield lower bile flow calculated per kg bodyweight (Fig.3), but the bile flow calculated per g liver weight is similar in WT and AhR-null mice. This finding confirms that the lower bile flow is due to the smaller liver; even more, the smaller livers of males and females tend to excrete more BAs than the WT mice. This effective biliary process probably helps to maintain the optimal hepatic concentration of BAs in the AhR-null mice and the appropriate amount of BAs in the intestine for the absorption of lipids and lipid-soluble vitamins.

Although there is an enormous accumulation of BAs in the systemic circulation of AhR-null mice, resulting a significant increase in the BA pool, surprisingly, the Fxr-Fgf15-Fgfr4 pathway in the intestine is not activated to suppress the overall BA synthesis, but rather, tends to be downregulated (Fig 7), and Fxr transcription in the liver is significantly downregulated (Fig 6). These observations indicate that the intestinal Fxr does not play a role in the protection of liver of AhR-null mice against the high serum BAs concentration, but the hepatic Fxr may play a role.

In WT mice, the alternative pathway (Cyp27a1 and Cyp7b1) has been reported to contribute an average of 35% and 55% to the total bile acid synthesis in female and male mice, respectively (Schwarz *et al.*, 2001). The gene expression of Cyp27a1 and Cyp7b1 were significantly lower, whereas Cyp7a1 tended to be higher in AhR-null than WT mice (Fig. 6.). These findings suggest that the contribution of classic and alternative BA synthetic pathways shifts toward the classical pathway in AhR-null mice. It is important to mention that the lower expression of Cyp27a1 and Cyp7b1 is associated with the lower expression of the BA receptor Fxr in the liver and the basolateral uptake transporters in AhR-null mice, which yields coordinated protection of the liver against the enlarged and shifted BA pool in the systemic circulation.

In summary, due to open ductus venosus in AhR-null mice, there is a marked increase in the serum concentrations of all BAs, especially the tri-OH primary BAs (CA/TCA, MCAs, TMCAs). Although the BA concentrations of various BAs markedly increase as a result of the patent ductus venosus in AhR-null mice, the hepatic concentrations of BAs and biliary excretions of BAs are strictly maintained. While the liver is protected against increased BA influx, further studies are needed to understand the potential effects of the elevated level of systemic BAs in extrahepatic tissues of AhR-null mice. The finding of this study, similar to other preclinical models, emphasizes the importance of careful data interpretation of changes in serum BA. It is important to know the actual changes in liver or other compartments of BA homeostasis due to the complexity of the hepatic uptake and its consequence of spillover of BAs into the systemic circulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

1°BAs	Primary bile acids
2°BAs	Secondary bile acids
6-OH	6-hydroxylated
12-OH	12 α -hydroxylated
Abc	ATP-binding cassette
AhR	Aryl hydrocarbon receptor
Asbt	Apical sodium-dependent bile acid transporter
BA	Bile acid
Baat	Bile acid CoA:amino acid <i>N</i> -acyltransferase
Bal	Bile acid CoA ligase
Bcrp	Breast cancer resistance protein
Bsep	Bile salt export pump
CA	Cholic acid
CAR	Constitutive Androstane Receptor
CDCA	Chenodeoxycholic acid
Cyp	Cytochrome p450
DCA	Deoxycholic acid
di-OH	Dihydroxy
Ent	Equilibrative nucleoside transporter
F	Female
FXR	Farnesoid X Receptor
Fgfr4	Fibroblast growth factor receptor 4
Fgf15	Fibroblast growth factor 15
HDCA	Hyodeoxycholic acid
Hnf4α	Hepatocyte nuclear factor 4 α

i.p.	Intraperitoneal
LCA	Lithocholic acid
LRH1	Liver receptor homolog 1
Lxra	Liver x receptor α
M	Male
Mate	Multidrug and toxin extrusion transporter
MCA	Muricholic acid
Mdr	Multidrug resistance protein
Mrp	Multidrug resistance-associated protein
Ntcp	Na ⁽⁺⁾ -taurocholate cotransporting polypeptide
Npc1l1	Nieman-Pick c1-like 1
non-6,12-OH	Non-6,12-hydroxylated
Oatp	Organic anion transporting polypeptide
Oct	Organic cation transporter
Ost	Organic solute transporter
PDV	Patent ductus venosus
Σ-BAs	Sum (total) of bile acids
Shp	Small heterodimer partner
T-BAs	Taurine-conjugated bile acids
TCDD	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin
Tgr5	Takeda-G-protein-coupled receptor 5
tri-OH	Trihydroxy
U-BAs	Unconjugated bile acids
UDCA	Ursodeoxycholic acid
UPLC-MS/MS	Ultraperformance Liquid Chromatography–Tandem Mass Spectrometry

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HIGHLIGHTS

- Bile acids in serum of AhR-null mice were markedly higher than in wild type.
- Higher bile acid concentration in serum was largely due to the tri-OH bile acids.
- Bile acid concentrations in livers of AhR-null mice are similar to wild type.
- Biliary excretion of bile acids is maintained in AhR-null mice.
- Patent ductus venosus does not alter hepatic or biliary bile acid homeostasis.

Fig. 1.

Serum concentrations of total bile acids (top), individual T-conjugated bile acids (middle), and individual un-conjugated bile acids (bottom) in WT and AhR-null male (blue bars) and female (red bars) mice. Bars represent the mean $\pm$ SE of 5–6 mice per group. Asterisks indicate significant difference ($p < 0.05$) from the respective value of the WT. Primary bile acids (1° BAs), secondary bile acids (2° BAs), 6-hydroxylated bile acids (6-OH), 12 α -hydroxylated (12 α -OH) bile acids, aryl hydrocarbon receptor-null mice (AhR-null), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), females (F), hyodeoxycholic acid (HDCA), lithocholic acid (LCA), males (M), muricholic acid (MCA), Non 6-, non 12 α -hydroxylated bile acids (non 6,12-OH), total bile acids (Σ -BAs), T-conjugated bile acids (T-BAs), unconjugated bile acids (U-BAs), ursodeoxycholic acid (UDCA), wild-type mice (WT). Color image is available in the online version of the article.

Fig. 2.

Hepatic concentrations of total bile acids (top), individual T-conjugated bile acids (middle), and individual un-conjugated bile acids (bottom) in WT and AhR-null male (blue bars) and female (red bars) mice. Bars represent the mean $\pm$ SE of 5–6 mice per group. Asterisks indicate significant difference ($p < 0.05$) from the respective value of the WT. Primary bile acids (1° BAs), secondary bile acids (2°BAs), 6-hydroxylated bile acids (6-OH), 12 α -hydroxylated (12 α -OH) bile acids, aryl hydrocarbon receptor-null (AhR-null), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), females (F), lithocholic acid (LCA), males (M), muricholic acid (MCA), Non 6-, non 12 α -hydroxylated bile acids (non 6,12-OH), total bile acids (Σ -BAs), T-conjugated bile acids (T-BAs), unconjugated bile acids (U-BAs), ursodeoxycholic acid (UDCA), wild-type mice (WT). Color image is available in the online version of the article.

Fig 3. Relative liver weight and bile flow in male and female WT and AhR-null mice. Liver weight is expressed as a percent of bodyweight (BW) (top). Bile flow rates were normalized to BW (middle) and liver weight (bottom). Bars represent means $\pm$ SE of 5–6 mice per group. Asterisks indicate significant difference ($p < 0.05$) from the respective value of the WT. Females (F), males (M), Color image is available in the online version of the article.

Fig 4. Biliary excretion of total bile acids (top), individual T-conjugated bile acids (middle), and individual un-conjugated bile acids (bottom) in WT and AhR-null male (blue bars) and female (red bars) mice. Bars represent the mean $\pm$ SE of 5–6 mice per group. Asterisks indicate significant difference ($p < 0.05$) from the respective value of the WT. Primary bile acids (1° BAs), secondary bile acids (2°BAs), 6-hydroxylated bile acids (6-OH), 12 α -hydroxylated (12 α -OH) bile acids, aryl hydrocarbon receptor-null mice (AhR-null), Non 6-, non 12 α -hydroxylated bile acids (non 6,12-OH), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), females (F), hyocholic acid (HCA), hyodeoxycholic acid (HDCA), lithocholic acid (LCA), Males (M), muricholic acid (MCA), total bile acids (Σ -BAs), T-conjugated bile acids (T-BAs), unconjugated bile acids (U-BAs), ursodeoxycholic acid (UDCA), wild-type mice (WT). Color image is available in the online version of the article.

Fig 5. mRNA expression of basolateral uptake (top), canalicular (middle), and basolateral efflux (bottom) transporters in male and female WT and AhR-null mouse livers. Total RNA was analyzed by QuantiGene Plex 2.0 Assay, as well as by RT-qPCR. Bars represent the relative percentage mRNA expression $\pm$ SE of 5–6 mice per group. Asterisks indicate significant difference ($p < 0.05$) from the respective value of the WT. Aryl hydrocarbon receptor-null mice (AhR-null), Breast cancer resistance protein (Bcrp), Bile salt export pump (Bsep), Equilibrative nucleoside transporter (Ent), females (F), males (M), Multidrug and toxin extrusion transporter (Mate), Multidrug resistance protein (Mdr), Multidrug resistance-associated protein (Mrp), $\text{Na}^{(+)}$ -taurocholate cotransporting polypeptide (Ntcp), Organic anion transporting polypeptide (Oatp), Organic cation transporter (Oct), Organic solute transporter (Ost). Color image is available in the online version of the article.

Fig 6.

mRNA of BA synthesis (top) and regulation (bottom) in livers of male and female WT and AhR-null mice. Total RNA was analyzed by QuantiGene Plex 2.0 Assay, as well as by RT-qPCR. Relative mRNA levels were calculated with vehicle controls set as 100%. Bars represent the relative percentage mRNA expression $\pm$ SE of 5–6 mice per group. Asterisks indicate significant difference ($p < 0.05$) from the respective value of the WT. Aryl hydrocarbon receptor-null mice (AhR-null), Bile acid CoA:amino acid N-acyltransferase (Baat), Bile acid CoA ligase (Bal), Cytochrome p450 (Cyp), Farnesoid x receptor (Fxr), females (F), Fibroblast growth factor receptor (Fgfr4), Hepatocyte nuclear factor 4a (Hnf4a), Liver receptor homolog-1 (Lrh-1), males (M), Small heterodimer partner (Shp), wild-type mice (WT). Color image is available in the online version of the article.

Fig 7. mRNA BA regulators and transporters in ilea of male and female AT and AhR-null mice. Total RNA was analyzed by QuantiGene Plex 2.0 Assay, as well as by RT-qPCR. Relative mRNA levels were calculated with vehicle controls set as 100%. Bars represent the relative percentage mRNA expression $\pm$ SE of 5–6 mice per group. Asterisks indicate significant difference ($p < 0.05$) from the respective value of the WT. ATP-binding cassette (Abc), Apical sodium-dependent bile acid transporter (Asbt), Farnesoid X Receptor (Fxr), females (F), Fibroblast growth factor (Fgf), I-babp (ileal bile acid binding protein), Liver x receptor a (Lxra), males (M), Multidrug resistance-associated protein (Mrp), Nieman-Pick c1-like 1 (Npc1l1), Organic solute transporter (Ost), total bile acids (R-BAs), Small heterodimer partner (Shp), Transmembrane G protein-coupled receptor 5 (Tgr5). Color image is available in the online version of the article.