Note

Vitamin D Receptor Deletion Changes Bile Acid Composition in Mice Orally Administered Chenodeoxycholic Acid

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(Received December 29, 2019)

Summary The vitamin D receptor (VDR) is a nuclear receptor for the active form of vitamin D3 and also for the secondary bile acid lithocholic acid (LCA). The in vivo role of VDR in bile acid metabolism remains largely uncharacterized. We previously reported that pharmacological VDR activation enhances urinary bile acid excretion, particularly in mice fed chow supplemented with chenodeoxycholic acid (CDCA), which is metabolized to muricholic acid in mouse liver and is also converted to LCA by intestinal bacteria. In this study, we examined the effect of VDR deletion on bile acid composition utilizing VDR-knockout (VDR-KO) mice. VDR deletion did not change total bile acid levels in liver or feces of mice when fed standard chow supplemented with calcium, needed to prevent hypocalcemia in VDR-KO mice. Total bile acid levels in plasma and urine tended to be higher and lower, respectively, in VDR-KO mice. After feeding CDCA-supplemented chow, VDR-KO mice showed decreased hepatic, fecal and urinary total bile acid and CDCA levels compared to wild-type mice. Plasma total bile acids and LCA were relatively high in these mice. These results indicate that VDR deletion influences CDCA metabolism. VDR may play a role in the excretion of excess bile acids.

Key Words vitamin D, nuclear receptor, lithocholic acid, bile acid metabolism, bile acid excretion

Bile acids act as detergents necessary for the digestion and intestinal absorption of hydrophobic nutrients, such as triglycerides, fatty acids, cholesterol and lipid soluble vitamins including vitamin D (1). Primary bile acids, such as cholic acid (CA) and chenodeoxycholic acid (CDCA) (Fig. 1), are generated from cholesterol by the sequential action of liver enzymes and are secreted in bile as glycine and taurine conjugates in humans (2). The primary bile acids assist lipid digestion and absorption in the intestine, and most bile acids enter the enterohepatic circulation by intestinal reabsorption and portal circulation. Bile acids that escape reabsorption are converted to secondary bile acids, such as deoxycholic acid (DCA) and lithocholic acid (LCA) (Fig. 1), through deconjugation and dehydration by intestinal microflora (3). DCA and, to a much less extent, LCA are reabsorbed in the lower intestine. LCA can be detoxified into hyodeoxycholic acid (HDCA) by the xenobiotic metabolism enzyme cytochrome P450 (CYP) 3A4 (4). In mouse liver, LCA is also converted to ursodeoxycholic acid (UDCA) (5), and CDCA and UDCA are metabolized to α-muricholic acid (α-MCA) and β-MCA, respectively, by a newly identified enzyme, Cyp2c70 (6). UDCA and α-MCA are generated by intestinal bacteria in mice (3, 7), while UDCA is also present in germ-free mice (8). In comparison to primary bile acids, secondary bile acids, such as DCA, LCA and α-MCA, are more toxic and are thought to be involved in the pathogenesis of hepatic and colon cancers (3, 9, 10).

The vitamin D receptor (VDR) has been identified as a receptor for 1α,25-dihydroxyvitamin D3, the active form of vitamin D, and mediates vitamin D signaling in the regulation of calcium and bone metabolism, immunity, cardiovascular function, and cellular proliferation and differentiation (11, 12). VDR is also activated by the secondary bile acid LCA (13). Pharmacological VDR activation with 1α-hydroxyvitamin D3, which is rapidly converted to 1α,25-dihydroxyvitamin D3 and exhibits more stable VDR actions (14), reduces hepatic and plasma bile acids in mice fed chow supplemented with bile acids, such as CDCA and DCA (15). VDR activation increases urinary excretion of bile acids. In this study, we investigated the in vivo role of VDR in bile acid metabolism by utilizing VDR knockout (KO) mice.

Materials and Methods

Animal experiments. VDR-KO (Vdr(−/−)) mice and
wild-type (Vdr(+/+)) mice were generated by cross-breeding C57BL/6J Vdr(+/-) female mice and C57BL/6J Vdr(-/-) male mice (16, 17). During mating and weaning periods, parent mice were fed CLEA Rodent Diet CE-2 (CLEA Japan, Inc., Tokyo). After weaning, pups were raised on a powder rodent chow (Lab Animal Diet MF; Oriental Yeast Co., Ltd., Tokyo, Japan) supplemented with calcium (final 2%), phosphate (1.5%), and lactose (20%) (16, 17). Male mice were selected by genotyping and those between 7 and 10 wk of age were used for experiments. Individual mice were placed in metabolic cages for collection of feces and urine and were provided ad libitum water and chow with or without 1% CDCA for 7 d under controlled temperature (23 ± 1°C) and humidity (45–65%), and a standard 12-h light/dark cycle. The 24-h urine was collected in glass bottles in mouse metabolic cages. Bile samples were collected in a syringe by gallbladder puncture from over-night fasting mice, and biliary bile acids were analyzed (18). The experimental protocol adhered to the Guidelines for Animal Experiments of the Nihon University School of Medicine and was approved by the Ethics Review Committee for Animal Experimentation of the Nihon University School of Medicine (reference number AP10M036).

Analysis of bile acid contents. For gas chromatography–mass spectrometry analysis, bile acids were extracted from samples and were deconjugated as described previously (15, 18). Bile acids in urine (0.35–1.5 mL of urine) were prepared by solid phase extraction using C18 column. To obtain fecal bile acids, approximately 50 mg of feces were hydrolyzed by 2 mL of 1 mol/L NaOH containing 90% (v/v) ethanol at 70˚C for 60 min. Hydrolysates were washed with hexane three times, and acidified with HCl at around pH 1, then bile acids were separated as described (13). Total bile acids in feces of VDR-KO mice fed CDCA-supplemented diet were determined by gas chromatography after trimethylsilyl derivatives and gas-chromatographic separation was performed as described (15). Total bile acid concentrations were calculated from summation of trimethylsilyl derivatives and gas-chromatographic separation was performed as described (15).

Reverse transcription and quantitative real-time polymerase chain reaction. Total RNAs were prepared by the acid guanidium thiocyanate-pheno1/chloroform method, cDNAs were synthesized, and real-time polymerase chain reaction was performed as reported previously (15, 19).

Statistical analysis. Data are presented as means ± SE. We performed one-way ANOVA followed by Tukey’s multiple comparisons or unpaired two-group Student’s t test to assess significant differences.

Results
We previously reported that 1α-hydroxyvitamin D3 treatment reduces hepatic and plasma bile acids in mice fed CDCA- and DCA-supplemented chow while a diminished effect is seen in LCA or CA supplemented chow (15). Since CDCA is converted to the VDR ligand LCA by intestinal bacteria (3, 13), we examined the effect of VDR deletion on bile acid metabolism in mice fed chow supplemented with CDCA. To avoid hypocalcemia in VDR-KO mice, chow for both wild-type mice and VDR-KO mice was also supplemented with calcium, phosphate and lactose (16, 17). Total bile acid levels were not different in liver or feces between wild-type mice and VDR-KO mice fed control chow (Fig. 2). Total bile acid levels in plasma and urine were higher and lower, respectively, in VDR-KO mice, but these differences were not statistically significant. Renal mRNA expression of Abcc3 (the gene encoding multiple drug resistance-associated protein 3 (MRP3)) and Abcc4 (the gene encoding MRP4), but not Abcc2 (the gene encoding MRP2), was decreased in VDR-KO mice (Supplemental Online Material, Fig. S1).

CDCA feeding for 7 d elevated hepatic total bile acids, and this effect was weaker in VDR-KO mice than wild-type mice (Fig. 2A). Then, we further examined bile acid composition with gas chromatography–mass spectrometry analysis in mice fed CDCA-supplemented chow. CDCA and its metabolites (UDCA, α-MCA, β-MCA, and ω-MCA) were elevated in the liver after feeding a CDCA-supplemented diet. Hepatic levels of CDCA and α-MCA after CDCA feeding were lower in VDR-KO mice than wild-type mice (Fig. 2A). UDCA, β-MCA and ω-MCA levels were relatively low in VDR-KO mice. Thus, VDR deletion decreases hepatic levels of CDCA and its metabolites.

Plasma total bile acids were relatively high in VDR-KO mice fed a CDCA-supplemented diet (Fig. 2B). CDCA supplementation increased plasma total bile acid, CDCA and α-MCA levels in wild-type mice. While plasma CDCA levels were similar between wild-type mice and VDR-KO mice after CDCA feeding, LCA and UDCA levels were relatively high in VDR-KO mice. Thus, VDR deletion decreases hepatic levels of CDCA and its metabolites.

Total bile acids in feces of VDR-KO mice fed CDCA were lower compared to wild-type mice (Fig. 2C). Fecal UDCA and CDCA levels in these mice were decreased, and α-MCA and ω-MCA levels had a similar tendency. CDCA supplementation increased fecal LCA levels, but there was no difference in its levels between wild-type mice and VDR-KO mice.

Total bile acids in urine were relatively low in VDR-KO mice fed CDCA-supplemented chow compared to wild-type mice (Fig. 2D). While CDCA supplementation increased urine CDCA levels in wild-type mice, levels were
lower in VDR-KO mice fed chow with CDCA. α-MCA levels tended to be higher in the urine of VDR-KO mice fed CDCA.

Bile acid levels in bile were similar between wild-type mice and VDR-KO mice fed chow with CDCA (data not shown).

**Discussion**

CDCA supplementation increased total bile acid and CDCA levels in liver, plasma, faces, urine and bile samples in both wild-type mice and VDR-KO mice (Fig. 2). Interestingly, hepatic, fecal and urinary total bile acid levels were relatively high in VDR-KO mice compared to wild-type mice. A similar tendency was observed in urine and plasma in VDR-KO mice fed standard chow. These findings suggest that hepatic bile acids are decreased due to decreased import and/or increased efflux. Expression of Abcc3 and Abcc4, which encode the bile acid transporters MRP3 and MRP4, respectively (7), was decreased in the kidney of VDR-KO mice (Fig. S1). Decreased urinary excretion may induce accumulation of bile acids in the systemic circulation.

Bile acid overload activates farnesoid X receptor (FXR) (7). FXR is activated by CA, CDCA, DCA, LCA and their conjugates, but not by MCA (20–22), and FXR activation suppresses bile acid synthesis and import and induces bile acid export in the liver by regulating expression of genes involved in bile acid synthesis and enterohepatic circulation (7). Abnormal bile acid accumulation also activates pregnane X receptor (PXR), which is activated by the secondary bile acids, such as DCA and LCA (4, 23). PXR activation enhances excretion of bile acids from the systemic circulation into urine through the xenobiotic metabolism pathway (7). CDCA overload should inhibit bile acid synthesis and enterohepatic cir-
culation by FXR activation and enhance bile acid excretion by PXR activation. Plasma \( \alpha \)-MCA levels were decreased and urine \( \alpha \)-MCA levels were increased, suggesting that urinary excretion of \( \alpha \)-MCA is intact in VDR-KO mice. In contrast, excretion of other CDCA metabolites was disturbed in VDR-KO mice. These FXR- and PXR-mediated mechanisms may be enhanced in VDR-KO mice, but VDR deletion appears to disturb the excretion from the systemic circulation into urine. Intestinal epithelial VDR deletion changes bacterial profiles, such as increased \textit{Bacteroides} (24), which is involved in bile acid oxidation (25). In addition to changed expression of bile acid transporters, altered bile acid composition may influence the bile acid metabolism and transport in VDR-KO mice. Further studies are needed to elucidate the underlying mechanisms of bile acid transport.

In this study, we focused on the effect of VDR deletion on CDCA metabolism and utilized gas chromatography–mass spectrometry for analysis of bile acids. Since we derivatized samples before analysis, we did not discriminate between unconjugated and conjugated bile acids. More detailed analysis using liquid chromatography–mass spectrometry is needed for future more detailed studies. In order to examine the role of VDR in bile acid metabolism, we chose CDCA supplementation because treatment of mice with \( 1 \alpha \)-hydroxyvitamin \( D_3 \) reduces hepatic and plasma bile acids and increases urinary bile acid excretion in wild-type mice fed CDCA-supplemented chow (15). However, CDCA is not a main primary bile acid in mice, and the VDR ligand LCA is also not a principal secondary bile acid in mice. CDCA overload is not physiological, but LCA supplementation is toxic to mice. The species difference in bile acid composition is a disadvantage in the analysis of bile acids in mice. Mouse models for human bile acid metabolism, such as \textit{Cyp2c70}-KO mice (26), may be useful for future investigations of the role of VDR in bile acid metabolism.

Authorship
Research conception and design: SN and MM; experiments: SN and MI; statistical analysis of the data: SN and MM; interpretation of the data: SN, SK, and MM; writing of the manuscript: SN and MM.

Disclosure of state of COI
The authors declare no conflict of interest.

Acknowledgments
This word was supported by JSPS KAKENHI Grant Number JP 22590294, JP 25460394 and JP 16K08632 (to MM). The authors thank members of the Makishima laboratory for technical assistance and helpful comments, and Dr. Andrew I. Shulman for editorial assistance.

Supporting information
Supplemental online material is available on J-STAGE.

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