Vitamin D₃ Modulates the Expression of Bile Acid Regulatory Genes and Represses Inflammation in Bile Duct-Ligated Mice

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ABSTRACT

Vitamin D receptor (VDR), a nuclear receptor that regulates calcium homeostasis, has been found to function as a receptor for secondary bile acids. Because the in vivo role of VDR in bile acid metabolism remains unknown, we investigated the effect of VDR activation in a mouse model of cholestasis. We treated mice with 1α-hydroxyvitamin D₃ [1α(OH)D₃] after bile duct ligation (BDL) and examined mRNA expression and cytokine levels. 1α(OH)D₃ treatment altered the expression of genes involved in bile acid synthesis and transport in the liver, kidney, and intestine but did not decrease bile acid levels in the plasma and liver of BDL mice. 1α(OH)D₃ treatment suppressed mRNA expression of proinflammatory cytokines in the liver and strongly decreased the plasma levels of proinflammatory cytokines in BDL mice. These findings indicate that 1α(OH)D₃ regulates a network of bile acid metabolic genes and represses proinflammatory cytokine expression in BDL mice. VDR ligands have the potential to prevent the cholestasis-induced inflammatory response.

Bile acids are the major metabolic products of cholesterol and are essential detergents that are required for the digestion and intestinal absorption of hydrophobic nutrients, such as cholesterol, fatty acids, and lipid-soluble vitamins, including vitamin D (Hofmann, 1999). Primary bile acids, such as cholic acid and chenodeoxycholic acid, are generated from cholesterol by the sequential actions of liver enzymes and are secreted in bile as glycine or taurine conjugates (Russell, 2003). After assisting in lipid digestion and absorption, bile acids are reabsorbed in the intestine and recirculate to the liver through a mechanism called the enterohepatic circulation. Bile acids that escape reabsorption are converted to secondary bile acids, such as deoxycholic acid and lithocholic acid, by intestinal microflora (Ridlon et al., 2006). The nuclear receptor superfamily of ligand-dependent transcription factors regulates bile acid metabolism (Makishima, 2005).

The farnesoid X receptor (FXR; NR1H4) binds to primary and secondary bile acids, represses bile acid synthesis and hepatocellular import, stimulates bile acid export from cells, and protects hepatocytes from bile acid toxicity. The pregnane X receptor (PXR; NR1I2) senses toxic secondary bile acids and induces their elimination through a xenobiotic metabolism pathway. The constitutive androstane receptor (CAR; NR1I3) has also been reported to be involved in bile acid detoxification, although the potential bile acid-sensing role of CAR remains unclear (Stedman et al., 2005). The vitamin D receptor (VDR; NR1I1), a receptor for 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃, calcitriol], also acts as a bile acid receptor with specificity for the secondary bile acid lithocholic acid and its derivatives (Makishima et al., 2002). Although the roles of VDR in calcium and bone homeostasis have been investigated for decades, understanding of the biology of VDR regulation of bile acid metabolism is newly emerging.

Cholestasis is associated with hepatic and systemic accumulation of toxic biliary compounds, such as bile acids and bilirubin and subsequent liver damage and jaundice (Zollner et al., 2006). Decreased secretion of bile acids into intestine induces proliferation and translocation of intestinal bacteria,
which can result in endotoxemia and sepsis (Chand and Sanyal, 2007). Endotoxin and proinflammatory cytokines, such as tumor necrosis factor (TNF) α, are implicated in endotoxin-induced cholestasis. The bile acid receptors FXR and PXR have been investigated in a bile duct ligation (BDL) model of cholestasis. FXR activation by a synthetic ligand protects against cholestatic liver damage by decreasing expression of bile acid biosynthetic genes, such as sterol 12α-hydroxylase (Cyp8b1), and by increased expression of genes involved in bile acid transport, such as the bile salt export pump (Bsep; Abcb11) (Liu et al., 2003). FXR also plays a role in protection of intestine from bacterial invasion (Inagaki et al., 2006). It is interesting that Stedman et al. (2006) reported that FXR-null mice are protected from obstructive cholestasis, which seems to contradict the prior finding. PXR agonists enhance bile acid detoxification by inducing an import transporter, organic anion transporting polypeptide (Oatp) 1a4 (Scl01a1, Oatp2), the detoxifying enzyme Cyp3a11, and a basolateral export transporter, multidrug resistance-associated protein (Mrp) 3 (Abcc3), resulting in decreased serum bile acids and increased urinary bile acid excretion (Wagner et al., 2005). In this study, we investigated the in vivo role of VDR in cholestasis using BDL in mice and found that vitamin D₃-induced VDR activation modulates the expression of genes involved in bile acid transport and represses the expression of proinflammatory cytokines.

Materials and Methods

Compounds. 1α-Hydroxyvitamin D₃ [1α(OH)D₃, alfalcacidol] was kindly provided by Dr. Yoji Tachibana (Nisshin Flour Milling Inc., Tokyo, Japan).

Animals and BDL. C57BL/6J mice were obtained from Charles River Laboratories Japan (Yokohama, Japan) and were maintained under controlled temperature (23 ± 1°C) and humidity (45–65%) with free access to water and chow (Lab. Animal Diet MF; Oriental Yeast Co., Ltd., Tokyo, Japan). Experiments were conducted with female mice between 7 and 8 weeks of age. After mice were anesthetized with diethyl ether, and the common bile duct was ligated. Sham surgery was performed under the same conditions with the exception of BDL. 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.

Administration of 1α(OH)D₃. After surgery, mice were injected intraperitoneally with 1α(OH)D₃ (31 nmol/kg) or vehicle control (phosphate-buffered saline) four times, every other day. Plasma, liver, kidney, and small intestine were collected 7 days after surgery. Tissues were snap-frozen in liquid nitrogen or dry ice and were stored until analysis. Plasma total calcium, bilirubin, alanine aminotransferase, and total bile acids were quantified with Calcium C-Testwako (Wako Pure Chemicals, Osaka, Japan), Bilirubin BII-Testwako (Wako Pure Chemicals), Transaminase CII-Testwako, and Total bile acid-Testwako (Wako Pure Chemicals), respectively. Total bile acid contents in the liver extract were also measured with Total bile acid-Testwako (Wako Pure Chemicals). Plasma cytokine levels were determined with enzyme-linked immunosorbent assay kits (BioSource International, Camarillo, CA).

Real-Time Quantitative Polymerase Chain Reaction. Total RNAs from samples were prepared by the acid guanidine thiocyanate-phenol/chloroform method (Tavangar et al., 1990). cDNAs were synthesized using the ImProm-II Reverse Transcription system (Promega, Madison, WI) (Nishigori et al., 2007). Real-time polymerase chain reaction was performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems). Some primer sequences have been reported previously (Ishizawa et al., 2008), and others are listed in Table 1. The mRNA values were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Results

Effect of BDL and Vitamin D₃ on Expression of Genes Involved in Vitamin D₃ and Calcium Metabolism. To examine the effect of BDL on VDR-regulated gene expression, we treated sham-operated mice and BDL mice with 1α(OH)D₃ via intraperitoneal injection to avoid the effect of bile deficiency on intestinal absorption of the lipid-soluble vitamin. 1α(OH)D₃ is rapidly converted to 1,25(OH)₂D₃ after injection and is more effective than 1,25(OH)₂D₃ in prolonging survival time of mice inoculated with leukemia cells (Honma et al., 1983). As reported previously (Ishizawa et al., 2008), 1α(OH)D₃ treatment increased renal mRNA expression of 25-hydroxyvitamin D 24-hydroxylase (Cyp24a1), an enzyme that inactivates vitamin D₃ in the intracellular calcium binding protein calbindin D9k, and the membrane calcium channels transient receptor potential vanilloid (Trpv) types 5 and 6 (Fig. 1A). The expression of 25-hydroxyvitamin D 1α-hydroxylase (Cyp27b1), an enzyme that produces 1,25(OH)₂D₃, was decreased. It is interesting that BDL increased the expression of Cyp24a1, calbindin D9k, Trpv5, and Trpv6, but not Cyp27b1 or calbindin D9k. The BDL-dependent regulation of these genes may not be mediated by VDR. 1α(OH)D₃ treatment in BDL mice increased the expression of Cyp24a1, calbindin D9k, Trpv5, and Trpv6 and decreased Cyp27b1 expression as effectively as in sham-operated mice. 1α(OH)D₃ treatment induced intestinal VDR target gene expression in both sham-operated mice and BDL mice (data not shown). 1α(OH)D₃-induced VDR target gene expression was associated with increased plasma calcium levels (Table 2). These findings indicate that BDL does not affect VDR target gene expression induced by intraperitoneal 1α(OH)D₃ treatment.

Effect of BDL and Vitamin D₃ on the Expression of Genes Involved in Bile Acid Metabolism. BDL results in changes in the expression of genes involved in bile acid metabolism (Zollner et al., 2006). We next examined the effect of BDL and 1α(OH)D₃ on bile acid metabolism. Cholesterol 7α-hydroxylase (Cyp7a1) catalyzes the rate-limiting step of the classical bile acid synthesis pathway and is negatively regulated by FXR through induction of an FXR target gene, short heterodimer partner (Shp; Nr0b2) (Makishima, 2005). Cyp8b1 is also involved in bile acid synthesis and is suppressed by the FXR-SHP cascade. BDL decreased the expression of Cyp8b1 and did not change the expression of Cyp7a1 and Shp in the liver (Fig. 2A), as shown previously (Inagaki et al., 2005; Stedman et al., 2005). 1α(OH)D₃ treatment increased Cyp7a1 expression and decreased Shp expression. Sodium taurocholate-cotransporting polypeptide (Ntcp; Slc10a1) and Oatps are involved in bile acid uptake at the basolateral membrane of hepatocytes (Zollner et al., 2006). As reported previously (Alrefai and Gill, 2007; Geier et al., 2007), BDL repressed hepatic expression of Ntcp, Oatp1a1, Oatp1a4, and Oatp1b2 (Fig. 2A). 1α(OH)D₃ treatment increased Ntcp expression, an effect not observed in BDL mice. 1α(OH)D₃ strongly suppressed the expression of Oatp1a1, but not Oatp1a4 or Oatp1b2. Bile acids are excreted by the canalicular export pumps Bsep and Mrp2 (Abcc2) (Zollner et al., 2006). At the hepatocyte basolateral membrane, Mrp3,
Mrp4 (Abcc4), and the heterodimeric transporter organic solute transporter (Ost) α/β play a role in the alternative excretion of bile acids into the systemic circulation. As reported previously (Liu et al., 2003; Stedman et al., 2005; Boyer et al., 2006), BDL did not affect the expression of Bsep and Mrp2 but strongly increased Ostβ expression (Fig. 2A). 1α(OH)D3 treatment decreased Bsep expression and increased Mrp2 expression in the liver of both sham-operated mice and BDL mice and did not change Ostβ expression. Expression of Cyp24a1, Cyp2a11, Mrp3, Mrp4, and Ostα was not changed by BDL or 1α(OH)D3 treatment (data not shown).

The bile acid transporters Mrp2, Mrp4, and Ostα/β are expressed in renal tubular cells and are thought to be involved in renal bile acid transport (Zollner et al., 2006). Under cholestatic conditions, renal excretion of bile acids through these transporters is thought to be a major alternative elimination route. BDL increased the renal expression of Mrp2, Mrp4, Ostα, and Ostβ (Fig. 2B). 1α(OH)D3 treatment induced renal Mrp2 and Mrp4 expression to similar levels in both sham-operated mice and BDL mice. In contrast, 1α(OH)D3 decreased the Ostα expression induced by BDL, whereas it did not change its expression in sham-operated mice. Apical sodium-dependent bile acid transporter (Slc10a2) is responsible for intestinal bile acid absorption and has been reported to be induced by VDR activation in the rat intestine (Chen et al., 2006). 1α(OH)D3 treatment increased intestinal apical sodium-dependent bile acid transporter expression in BDL mice but not in sham-operated mice (Fig. 2C).

Plasma and hepatic bile acid levels were increased by BDL and were unaffected by 1α(OH)D3 treatment (Table 2). Although VDR activation modified the mRNA expression of bile acid transporters in liver and kidney, such as Ntcp, Bsep, Mrp2, and Ostα (Fig. 2), the effect of 1α(OH)D3 on accumulated bile acids in BDL mice was not significant. We determined the types of bile acids present in the BDL liver with gas chromatography-mass spectrometry. The primary bile acids cholic acid (41%), β-muricholic acid (28%), and α-muricholic acid (28%) were accumulated, and 1α(OH)D3 treatment did not induce a significant change in the composition of bile acids (data not shown). 1α(OH)D3 did not change plasma bilirubin and alanine aminotransferase (ALT) levels increased by BDL (Table 2).

**Effect of Vitamin D3 on BDL-Induced Cytokine Expression.** Cholestasis induces the release of proinflammatory cytokines, and the expression of bile acid transporters is regulated by both bile acid-sensing receptors and cytokine signaling (Alrefai and Gill, 2007). As reported previously (Zollner et al., 2005), BDL increased the mRNA expression of

<table>
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fw, forward primer; rev, reverse primer; bp, base pair.
that BDL mice (Fig. 3). VDR activation has been reported to
suppress inflammation and to inhibit the expression of IL-1β,
TNFα, and IFNγ in a mouse model of inflammatory bowel
disease (Froicu et al., 2006). VDR ligands are also candidates
for treatment for other inflammatory and autoimmune dis-
eeases, such as multiple sclerosis and rheumatoid arthritis
(Nagpal et al., 2005). VDR is expressed in immune cells such as
antigen-presenting cells, T cells, B cells, and natural killer
cells. Maturation of dendritic cells and alloreactive T cell
activation are inhibited by treatment with 1,25(OH)2D3, and
VDR-null mice have hypertrophy of subcutaneous lymph
nodes and an increase in mature dendritic cells in lymph
nodes (Griffin et al., 2001). 1,25(OH)2D3 has a direct effect on
naïve CD4+ T cells to enhance Th2 cell development (Boon-
stra et al., 2001). These findings indicate that the immuno-
modulatory effects of 1,25(OH)2D3 are mediated through the
action on dendritic cells and T cells, although the underlying
molecular mechanism remains to be elucidated. Cholestasis-
induced inflammation potentiates liver injury and sup-
presses immune function, which can further enhance bacte-
rial translocation (Chand and Sanyal, 2007) (Fig. 4). Kupffer
cells, which are tissue macrophages residing within the lu-
men of the liver sinusoids, are considered to play a role in
modulating the inflammation (Chand and Sanyal, 2007).
Kupffer cells take up bacteria and endotoxins and are stimu-
lated to release a wide range of products implicated in liver
injury, such as proinflammatory cytokines including TNFα
and IL-1, superoxides, and lysosomal enzymes. Kupffer cells
also produce an anti-inflammatory cytokine IL-10 (Abe et al.,
2004). Although hepatocellular dysfunction is associated
with the release of TNFα, IL-1, and IL-10 (Sewnath et al.,
2002; Abe et al., 2004), Kupffer cell-derived IL-6 abrogates
cholestatic liver injury (Gehring et al., 2006). Although
Kupffer cells play a role in immunity in the liver, dysregu-
lated release of cytokines from Kupffer cells may enhance
liver injury. Because VDR is expressed and functions in
Kupffer cells (Gascon-Barré et al., 2003), the effect of
1α(OH)D3 treatment on suppression of cytokine expression
in BDL mice may be mediated through the action on Kupffer
cells. Accumulation of toxic biliary compounds, endotoxemia,
and proinflammatory cytokines released from Kupffer cells
in cholestasis can induce systemic inflammatory response.
The immunomodulatory effect of 1α(OH)D3 may be also me-
diated through the action on extrahepatic lymphoid tissues.
1,25(OH)2D3 does not induce transcription of a target gene.

proinflammatory cytokines in the liver, including interleukin
(IL)-1β and IL-6 (Fig. 3A). BDL also induced the expression
of nitric oxide synthase 2. 1α(OH)D3 treatment repressed the
expression of IL-1β and nitric oxide synthase 2 induced by
BDL. In addition, 1α(OH)D3 inhibited the expression of inter-
feron (IFN) γ in sham-operated mice. Although transcrip-
tional induction of IL-10, TNFα, and IFNγ was not signifi-
cant, BDL increased the plasma levels of these cytokines and
those of IL-1β and IL-6 (Fig. 3B). 1α(OH)D3 treatment signi-
nificantly decreased the plasma levels of IL-1β, IL-6, IL-10,
TNFα, and IFNγ induced by BDL. These findings indicate
that 1α(OH)D3 treatment is effective in inhibiting the pro-
inflammatory response secondary to cholestasis.

Discussion

In this study, we examined the in vivo effect of 1α(OH)D3
in a mouse model of cholestasis. 1α(OH)D3 treatment effect-
tively suppressed proinflammatory cytokine expression
in BDL mice (Fig. 3). VDR activation has been reported to

TABLE 2

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<tr>
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<td>+VD3</td>
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<tr>
<td><strong>Plasma</strong></td>
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<td>Calcium (mg/dl)</td>
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<td>15.09 ± 2.08</td>
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<td>Bilirubin (mg/dl)</td>
<td>0.64 ± 0.68</td>
<td>0.15 ± 0.19</td>
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<td>ALT (IU/l)</td>
<td>44.4 ± 18.7</td>
<td>39.0 ± 12.8</td>
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<td>Bile acids (µM)</td>
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<td>91 ± 152</td>
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<td><strong>Liver</strong></td>
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<td>Bile acids (nmol/mg)</td>
<td>0.57 ± 0.17</td>
<td>0.52 ± 0.16</td>
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* Comparison with sham operation without 1α(OH)D3 treatment.

** Comparison with BDL without 1α(OH)D3 treatment.
in hepatocytes because of low expression of VDR mRNA and protein (Gascon-Barre et al., 2003). 1α(OH)D₃ treatment changed expression of several genes involved in bile acid synthesis and transport in the liver, such as Ntcp, Oatp1a1, and Mrp2 (Fig. 2A). Because hepatic expression of the VDR target genes Cyp24a1 and Cyp3a11 was not induced in BDL mice, the 1α(OH)D₃-induced transcriptional regulation may be through indirect mechanisms. Increased plasma calcium levels by vitamin D3 may influence liver gene expression, although calcium-dependent regulation of bile acid metabolism-related genes has not been described. Proinflammatory cytokines have been demonstrated to down-regulate the expression of liver transporter genes, including Oatp1a1 and Bsep (Hartmann et al., 2002). Although 1α(OH)D₃ treatment effectively decreased proinflammatory cytokine levels, it did not restore BDL-suppressed Oatp1a1 expression. 1α(OH)D₃ further decreased Bsep expression in BDL mice. These findings suggest that 1α(OH)D₃-dependent suppression of proinflammatory cytokine expression does not influence the expression of these transporters.

Fig. 2. Effects of 1α(OH)D₃ and BDL on mRNA expression of genes involved in bile acid metabolism in liver (A), kidney (B), and intestine (C). Total RNA was prepared from the liver, kidney, and small intestine of mice, and expression of the indicated genes was measured as in Fig. 1. The values represent the means ± S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Fig. 3. 1α(OH)D₃ treatment suppresses the release of proinflammatory cytokines in BDL mice. A, mRNA expression of liver inflammation-related genes. B, plasma levels of proinflammatory cytokines. Total RNA was prepared from the liver of mice, and expression of the indicated genes was measured as in Fig. 1. Plasma samples were also prepared, and concentrations of the indicated cytokines were measured with enzyme-linked immunosorbent assay. The values represent the means ± S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
1α(OH)D₃ treatment increased expression of genes involved in bile acid transport, such as renal Mrp2 and Mrp4, but did not decrease the elevated plasma bile acid levels in BDL mice. Insufficient or aberrant expression of bile acid transporters may disturb the efficient excretion of bile acids in cholestasis. Cholic acid and β-muricholic acid accumulate in the serum of BDL mice (Wagner et al., 2003), but VDR is not activated by these bile acids (Makishima et al., 2002). VDR is effectively activated by secondary bile acids, such as lithocholic acid, which are produced by intestinal microflora. In cholestasis, secondary bile acid production is decreased because of deficient bile secretion into intestine, suggesting that VDR plays a limited role in bile acid metabolism in cholestasis (Fig. 4). We used female mice in this study. Sex-specific cholestatic sensitivity has been reported in mouse models of cholestasis (Uppal et al., 2005, 2007). Sex hormones affect expression of genes involved in bile acid detoxification and transport. Primary biliary cirrhosis, which is a slowly progressive autoimmune disease of the liver, primarily affects women (Kaplan and Gershwin, 2005), and intrahepatic cholestasis of pregnancy complicates approximately 1 of 100 pregnancies (Ropponen et al., 2006). The molecular mechanism for the sex specificity remains unknown, and further studies are required to elucidate whether the effects of vitamin D₃ on cholestasis are sex selective. FXR inhibition has been reported to change bile acid composition and to protect against liver injury (Stedman et al., 2006), and agonists for FXR and CAR stimulate hepatocellular bile acid detoxification (Wagner et al., 2005). VDR agonists may be effective in enhancing the elimination of bile acids in combination with an FXR modulator or PXR and CAR agonists.

We demonstrate in this study that VDR ligands are effective in suppressing inflammatory cytokine expression in a mouse model of cholestasis (Fig. 4). Vitamin D₃ has been shown to enhance innate immunity and to play a role in protection against bacterial infections, such as tuberculosis (Li et al., 2006). These findings indicate that vitamin D₃ treatment has two potential benefits in cholestasis treatment, suppression of inflammation and stimulation of antimicrobial immunity. Although further investigation is required to elucidate the role of VDR in bile acid metabolism and immunity, VDR should be a promising molecular target in the treatment of cholestatic diseases.

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References


Makishima M (2005) Nuclear receptors as targets for drug development: regulation


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