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Lithocholic acid down-regulation of NF-κB activity through vitamin D receptor in colonic cancer cells

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Abstract

Lithocholic acid (LCA), a secondary bile acid, is a vitamin D receptor (VDR) ligand. 1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃), the hormonal form of vitamin D, is involved in the anti-inflammatory action through VDR. Therefore, we hypothesize that LCA acts like 1,25(OH)₂D₃ to drive anti-inflammatory signals. In present study, we used human colonic cancer cells to assess the role of LCA in regulation of the pro-inflammatory NF-κB pathway. We found that LCA treatment increased VDR levels, mimicking the effect of 1,25(OH)₂D₃. LCA pretreatment inhibited the IL-1β-induced IκBα degradation and decreased the NF-κB p65 phosphorylation. We also measured the production of IL-8, a well-known NF-κB target gene, as a read-out of the biological effect of LCA expression on NF-κB pathway. LCA significantly decreased IL-8 secretion induced by IL-1β. These LCA-induced effects were very similar to those of 1,25(OH)₂D₃. Thus, LCA recapitulated the effects of 1,25(OH)₂D₃ on IL-1β stimulated cells. Mouse embryonic fibroblast (MEF) cells lacking VDR have intrinsically high NF-κB activity. LCA pretreatment was not able to prevent TNFα-induced IκBα degradation in MEF VDR (−/−), whereas LCA stabilized IκBα in MEF VDR (+/−) cells. Collectively, our data indicated that LCA activated the VDR to block inflammatory signals in colon cells.

Keywords

Vitamin D receptor; IL-1β; NF-κB activity; Lithocholic acid; Intestine; Inflammation

1. Introduction

The primary bile acids in human bile are chenodeoxycholic acid and cholic acid. They are secreted into the intestine to aid the absorption of lipid-soluble nutrients. Within the intestines, the primary bile acids are converted to the secondary bile acids. Both primary and secondary bile acids are reabsorbed by the intestines and delivered back to the liver via the portal circulation. Bile acids regulate gene expression by acting as ligands for the nuclear receptor or by activating kinase signaling pathways [1-3].

Recently, lithocholic acid, a potent tumor-promoting secondary bile acid, was demonstrated to be a vitamin D receptor (VDR) ligand [4]. LCA binding to VDR induced the expression of

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colonic cytochrome P4503A (CYP3A) and dehydroepiandrosterone sulfotransferase, enzymes that detoxify LCA [4-7]. LCA activation of the VDR also induced the expression of the multi-drug resistance-associated protein 3 (MRP3) [8]. This basolateral membrane transporter exports LCA from the colonocyte to the blood for excretion by the liver or kidney. Thus, LCA activation of the VDR accelerates detoxification of the major secondary bile acids.

Moreover, recent study demonstrated that LCA can substitute for vitamin D *in vivo* [9]. LCA elevates the serum calcium in vitamin D-deficient rats. LCA in the diet also replaces vitamin D in the mobilization of calcium from bone. Further, LCA induces CYP24-hydroxylase mRNA gene expression in the kidney of vitamin D-deficient rats. It is clear, therefore, that LCA can be absorbed into the circulation to bind to the VDR [9].

1,25(OH)₂D₃, the hormonal form of vitamin D, is involved in the anti-inflammatory action through VDR [10,11]. Our previous study demonstrated that VDR negatively regulated the pro-inflammatory NF-κB pathway [12]. Mouse embryonic fibroblast (MEF) cells lacking VDR have intrinsically high NF-κB activity. VDR mutation led to a marked increase in nuclear p65 DNA binding and NF-κB transcriptional activity; consistently, induction of IL-6 by TNFα or IL-1β was much more robust in VDR (-/-) than in VDR (+/-) cells [12]. Collectively, cells lacking VDR are in a pro-inflammatory or pre-inflammatory state with high NF-κB activity.

LCA, like 1,25(OH)₂D₃, is expected to drive anti-inflammatory signals that could protect the colon against VDR-independent bile acid-induced pro-inflammatory signals. In present study, we use human colonic epithelial cells to assess the role of LCA in regulation of NF-κB pathway. We also use MEF lacking VDR to determine whether the LCA regulation of NF-κB is dependent on VDR. Our data indicate that LCA activates the VDR to block inflammatory signals in colon cells.

2. Materials and methods

2.1. Cell culture

Human colonic epithelial Caco-2 cells and HT29C19A (clone 19A) cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin–streptomycin and L-glutamine as previous described [13]. MEFs were isolated from E13.5 embryos generated from VDR (+/-) × VDR (+/-) mouse breeding [14]. The cells were cultured in DMEM containing 10% FBS. Cells from each embryo were genotyped by PCR. VDR (+/-) and VDR (-/-) MEFs were used in experiments after more than 15 passages when they had been immortalized as shown previously [15].

2.2. Immunoblotting

Cultured cells were rinsed twice in ice-cold HBSS, lysed in protein loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and sonicated. Equal amount of proteins or equal volumes of total cultured cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with primary antibodies (1:500 to 1000 dilution): anti-phospho-p65 on serine 536 (Cell Signaling Technology, Beverly, MA, USA), anti-IκBα, anti-VDR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or β-actin (Sigma–Aldrich, St. Louis, MO, USA) antibodies and visualized by ECL as previous described [13].

2.3. IL-8 secretion

Confluent Caco-2 cells were grown in 12-well plates. After treatment, the supernatant was collected and assayed for IL-8 using the R&D Systems human IL-8 ELISA kit (R&D, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

2.4. Statistical analysis

Data are expressed as mean $\pm$ S.D. Differences were analyzed by Student's *t*-test. *p*-Values <0.05 were considered significance.

3. Results

3.1. LCA treatment increases VDR expression

VDR is a nuclear transcription factor responsible for mediating the biological activities of 1,25(OH)₂D₃. As a ligand-dependent receptor, VDR expression is highly regulated by the vitamin D status [16,17]. Therefore, pretreatment of 1,25(OH)₂D₃ can increase the VDR protein expression. To confirm that LCA acts like 1,25(OH)₂D₃, we treated the human colonic epithelial Caco-2 cells with LCA and assessed VDR protein levels. Incubation of Caco-2 cells for 24 h with non-toxic concentrations of LCA (20 μ M) increased VDR levels, mimicking the effect of 1,25(OH)₂D₃ (Fig. 1A). In addition, the effect of LCA on VDR expression was also tested in the human colonic epithelial HT29C19A cells and mouse embryonic fibroblast cells. In HT29C19A and MEF cells, LCA treatment for 3 h up-regulated VDR expression (Fig. 1B and C), the similar trend as the Caco-2 cells did. It reveals that our results are not restricted to one cell line.

3.2. LCA pretreatment inhibits the IL-1 β -induced I κ B α degradation

To assess the effects of LCA on inflammatory signaling, we pretreated Caco-2 cells for 24 h with LCA, or 1,25(OH)₂D₃ as a positive control. Cells were then stimulated with IL-1 β . In unstimulated cells, NF- κ B is bound to the inhibitor I κ B α and restricted to the cytoplasm. With inflammatory stimuli, I κ B α is phosphorylated and subsequently degraded, releasing NF- κ B to traffic to the nucleus [18]. We examined the expression of I κ B α in the intestinal epithelial cells by immunoblotting. As expected, IL-1 β treatment reduced I κ B α level; LCA pretreatment was able to stabilize I κ B α as the positive control 1,25(OH)₂D₃ did (Fig. 2A I κ B α). We further test whether LCA has the similar effect in HT29C19A cells. Cells treated with IL-1 β or TNF α induced I κ B α degradation, whereas LCA pretreatment was able to stabilize I κ B α (Fig. 2B I κ B α).

3.3. LCA decreases the NF- κ B p65 phosphorylation

It should be noted that NF- κ B activity is regulated by phosphorylation. Increased level of phosphorylated-p65 (p-p65) indicates the high activity of NF- κ B pathway [19]. Interestingly, LCA treatment was able to inhibit the phosphorylation of the NF- κ B subunit, p65 (Fig. 2A p-p65). Without stimulation, the p-p65 level was very low in the control cells. With IL-1 β stimulation, the level of p-p65 increased dramatically. In contrast, the p-p65 remained low in the LCA pretreated cells after stimulation with IL-1 β . Collectively, LCA pretreatment decreased the NF- κ B activity by stabilizing I κ B α and decreasing p-p65.

3.4. IL-8 secretion regulated by LCA

Because NF- κ B is a key regulator involved in the synthesis of inflammatory cytokines, we also measured the production of IL-8, a well-known NF- κ B target gene, as a read-out of the biological effect of LCA expression on NF- κ B pathway [20]. As shown here, LCA significantly decreased IL-8 secretion induced by IL-1 β (Fig. 3, $p < 0.05$). These LCA-induced effects were very similar to those of 1,25(OH)₂D₃. Thus, LCA recapitulated the effects of 1,25(OH)₂D₃ on IL-1 β stimulated Caco-2 cells.

3.5. VDR knockout cells abolish the LCA effect

We further investigated the effect of VDR ablation on LCA regulation of NF- κ B using MEFs derived from VDR-null mice, because fibroblasts play an important role in inflammatory

reactions and have readily inducible NF- κ B activity. In the present study, we used VDR $^{+/-}$ cells, as we wanted to compare the difference between VDR-null and one allele of the Vdr gene. Similar results were seen when VDR $^{+/+}$ MEFs were used. As shown in Fig. 4, VDR expression was enhanced with LCA pretreatment in VDR $^{+/-}$ cells, whereas no VDR signal was detected in VDR $^{-/-}$ cells. In VDR $^{+/-}$, LCA pretreatment stabilized I κ B α level. And without LCA incubation in VDR $^{+/-}$, TNF α stimulation decreased I κ B α level. However, LCA cannot protect from TNF α -induced I κ B α degradation in VDR $^{-/-}$ (Fig. 4). Additionally, I κ B α baseline was lower in VDR $^{-/-}$ compared to that in the VDR $^{+/-}$, which is consistent with our previous results that VDR expression negatively regulates NF- κ B pathway and the baseline of I κ B α , the inhibitor of NF- κ B activity, in VDR $^{-/-}$ is lower than the wild-type cells (VDR $^{+/+}$) [12].

4. Discussion

Our studies demonstrated that the VDR-dependent effects of LCA on the pro-inflammatory NF- κ B signals in colon cancer cells. LCA is able to inhibit the inflammation by enhancing the VDR expression, stabilizing I κ B α , decreasing NF- κ B phosphorylation, and inhibiting IL-8 secretion. LCA pretreatment will not change the I κ B α level in VDR $^{-/-}$ cells without the receptor binding with LCA. These results indicate that LCA-VDR delivers an anti-inflammatory signal in colon cancer cells.

To our knowledge, it is the first report on the LCA activation of VDR, thus inhibiting TNF α - and IL-1 β -induced NF- κ B activation in colonic epithelial cells. 1,25(OH) $_2$ D $_3$ is involved in the anti-inflammatory action through VDR [10,11]. VDR expression is highly regulated by the vitamin D status [16,17]. Therefore, pretreatment of 1,25(OH) $_2$ D $_3$ can increase the VDR protein expression. LCA, like 1,25(OH) $_2$ D $_3$, also increases VDR expression and drives anti-inflammatory signals that could protect the colon against VDR-independent bile acid-induced pro-inflammatory signals. Based on our observation and previous studies [12], VDR expression negatively regulates NF- κ B pathway. VDR is enhanced with LCA pretreatment, thus decreasing the NF- κ B activity. In cells lacking VDR, the baseline of the NF- κ B activity is higher than the wild-type cells (VDR $^{+/+}$). LCA pretreatment will not change the NF- κ B activity in VDR $^{-/-}$ without the receptor binding with LCA. These data indicate that LCA regulation of NF- κ B activity is VDR dependent.

It should be noted that high fecal LCA concentrations in the micromolar range compensate for the lower affinity of LCA for the VDR compared to 1,25(OH) $_2$ D $_3$ [4]. Although LCA binds to VDR with low affinity, LCA can induce its own catabolism through the VDR. The molecular and functional comparison of 1,25(OH) $_2$ D $_3$ and lithocholic acid has been reported [7]. Recent *in vivo* study [9] demonstrated that LCA can substitute for vitamin D in the elevation of serum calcium in vitamin D-deficient rats. Therefore, it is clear that LCA can bind to VDR specifically and replace vitamin D. Thus, we believe LCA plays a quantitatively important role in VDR activation in the colon [21].

Epidemiological studies indicate that Western style high fat diets enhance colon cancer risk [22-24]. Since dietary factors are believed to contribute up to 70% of this tumor burden, increased understanding of the pathogenic mechanisms involving the diet could advance efforts to prevent this disease. Several mechanisms of inflammation and tumor promotion by dietary fat or bile acids have been identified in experimental models of colon cancer. In these models the effects of fat on tumorigenesis occur during the promotional phase. Bile acids can activate NF- κ B, AP-1 and β -catenin in colon cancer cells [25-27]. These transcription factors play key roles in controlling inflammation, proliferation and colonic tumorigenesis.

Studies of bile acid activated VDR pathways that can suppress inflammation will elucidate an unexplored area of diet-regulated tumor suppressor effects. In the future, studies in VDR $^{-/-}$

and VDR^{+/+} mice will help to understand whether LCA inhibits inflammation by a VDR-dependent mechanism. New understanding of pathways to block VDR-independent pro-inflammatory effects, while enhancing anti-inflammatory LCA–VDR signals will help develop potentially new chemopreventive strategies for colon malignancy.

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Fig. 1.

LCA induces VDR expression. (A) Caco-2 cells were incubated with 1,25(OH)₂D₃ (20 nM), LCA (20 μM) or ethanol (–) for 3 h. Cells were lysed and proteins (35 μg) probed for VDR expression. *Note:* VDR expression is increased by LCA and 1,25(OH)₂D₃. (B) LCA treatment increased VDR expression in HT29C19A cells. Cells were incubated with 1,25(OH)₂D₃ (20 nM), LCA (20 μM) or ethanol (–) for 3 h. Cells were lysed for VDR expression. (C) LCA treatment increased VDR expression in MEF cells. Cells were incubated with LCA (20 μM) or ethanol (–) for 3 h. Cells were lysed for VDR expression. Beta-actin is the loading control. Images shown are from a single experiment and are representative of three separate repeats.

(A) Caco-2 Cells

(B) HT29C19A

Fig. 2.

Effects of LCA and 1,25(OH)₂D₃ on IL-1β stimulated colon cancer cells. (A) Caco-2 cells were incubated for 24 h with 20 μM lithocholic acid (LCA, L) or 20 nM 1,25(OH)₂D₃ (D3). Cells were then treated with 10 ng/ml IL-1β (I) or vehicle (Ctl). After 30 min cells were lysed and whole cell lysates probed for IkBα and p-p65 expression. (B) HT29C19A cells were incubated for 24 h with 20 μM lithocholic acid (LCA, L). Cells were then treated with 10 ng/ml IL-1β(I), 10 ng/ml TNFα(T), or vehicle (Ctl). After 30 min cells were lysed and whole cell lysates probed for IkBα and β-actin expression. Images shown are from a single experiment and are representative of two to three separate repeats.

Fig. 3.

LCA inhibits IL-1 β stimulated IL-8 secretion in colon cancer cells. Caco-2 cells were incubated for 24 h with 20 μ M lithocholic acid (LCA, L) or 20 nM 1,25(OH)₂D₃ (D₃). Cells were then treated with 10 ng/ml IL-1 β (I) or vehicle (Ctl) for 24 h. The supernatant was collected and assayed for IL-8 using the R&D Systems human IL-8 ELISA kit (R&D, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. LCA and 1,25(OH)₂D₃ inhibit IL-1 β -induced IL-8 secretion in Caco-2 cells, * p < 0.05 compared with IL-1 β alone. Data are the mean $\pm$ S.D. of a single experiment assayed in triplicate and are representative of three separate experiments.

Fig. 4.

Effects of LCA is abolished in cells lacking VDR. MEF cells were incubated for 24 h with 20 μ M lithocholic acid (LCA, L). Cells were then treated with 10 ng/ml TNF α (T) or vehicle (Ctl). After 30 min cells were lysed and whole cell lysates were probed for VDR and I κ B α expression. Images shown are from a single experiment and are representative of two separate repeats.