Lithocholic acid derivatives act as selective vitamin D receptor modulators without inducing hypercalcemia

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Abstract 1α,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], a vitamin D receptor (VDR) ligand, regulates calcium homeostasis and also exhibits noncalcemic actions on immunity and cell differentiation. In addition to disorders of bone and calcium metabolism, VDR ligands are potential therapeutic agents in the treatment of immune disorders, microbial infections, and malignancies. Hypercalcemia, the major adverse effect of vitamin D_3 derivatives, limits their clinical application. The secondary bile acid lithocholic acid (LCA) is an additional physiological ligand for VDR, and its synthetic derivative, LCA acetate, is a potent VDR agonist. In this study, we found that an additional derivative, LCA propionate, is a more selective VDR activator than LCA acetate. LCA acetate and LCA propionate induced the expression of the calcium channel transient receptor potential vanilloid type 6 (TRPV6) as effectively as that of 1α,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24A1), whereas 1,25$(OH)_2D_3$ was more effective on TRPV6 than on CYP24A1 in intestinal cells. In vivo experiments showed that LCA acetate and LCA propionate effectively induced tissue VDR activation without causing hypercalcemia. These bile acid derivatives have the ability to function as selective VDR modulators.—Ishizawa, M., M. Matsunawa, R. Adachi, S. Uno, K. Ikeda, H. Masuno, M. Shimizu, K-i. Iwasaki, S. Yamada, and M. Makishima. **Lithocholic acid derivatives act as selective vitamin D receptor modulators without inducing hypercalcemia.** ***J. Lipid Res.*** **2008.** 49: **763–772.**

Supplementary key words nuclear receptor • intestine • leukemia • calcium

The vitamin D receptor (VDR; NR1I1), a member of the nuclear receptor superfamily, mediates the biological action of the active form of vitamin D, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], and regulates calcium and bone homeostasis, immunity, and cellular growth and differentiation (1–3). 1,25$(OH)_2D_3$ has been demonstrated to inhibit the proliferation and/or to induce the differentiation of various types of malignant cells, including breast, prostate, and colon cancers, as well as myeloid leukemia cells in vitro (1). The administration of 1,25$(OH)_2D_3$ and its analogs has therapeutic effects in mouse models of malignancies such as myeloid leukemia (4). 1,25$(OH)_2D_3$ was also demonstrated to exert immunomodulatory and antimicrobial functions (5). VDR activation by 1,25$(OH)_2D_3$ induces the cathelicidin antimicrobial peptide (CAMP) and kills *Mycobacterium tuberculosis* in monocytes (6). Although they have been used successfully in the treatment of bone and skin disorders, adverse effects, especially hypercalcemia, limit the clinical application of vitamin D and its synthetic analogs in the management of diseases other than bone and mineral disorders (5). Combined dosing of 1,25$(OH)_2D_3$ with other drugs is one approach to overcome its adverse effects (7, 8). The development of synthetic vitamin D analogs that retain VDR transactivation but have low calcemic activity provides another approach (9). With an improved understanding of the mechanisms of VDR signaling, the possibility of identifying VDR ligands with selective action is emerging (10).

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Abbreviations: AF2, activation function 2; CAMP, cathelicidin antimicrobial peptide; CYP24A1, 1α,25-dihydroxyvitamin D_3 24-hydroxylase; FXR, farnesoid X receptor; GPBAR1, G protein-coupled bile acid receptor 1; LCA, lithocholic acid; NBT, nitroblue tetrazolium; 1α$(OH)D_3$, 1α-hydroxyvitamin D_3; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PXR, pregnane X receptor; RXR, retinoid X receptor; TRPV6, transient receptor potential vanilloid type 6; VDR, vitamin D receptor.

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Nuclear receptors, including VDR, undergo a conformational change in the cofactor binding site and activation function 2 (AF2) domains upon ligand binding, a structural rearrangement that results in the dynamic exchange of cofactor complexes (11). In the absence of ligand, corepressors bind to the AF2 surface, composed of portions of helix 3, loop 3–4, helices 4/5, and helix 11. Ligand binding alters the AF2 surface by repositioning helix 12, reduces the affinity for corepressors, and increases the affinity for coactivator requirement, allowing nuclear receptors to induce the transcription of specific target genes. The secondary bile acid lithocholic acid (LCA) and its metabolite, 3-keto-cholanic acid, were recently identified as additional physiological VDR ligands (12). Our previous study showed that LCA derivatives modified at position 3, LCA formate and LCA acetate, activate VDR with 3 times and 30 times the potency of LCA, respectively (13). Structure-function analysis and docking models showed that LCA and LCA acetate interact with the VDR ligand binding pocket in a mode distinct from $1,25(OH)_2D_3$, particularly in interactions involving helix 3 and 4/5 residues (13), and these helices play an important role in the dynamic recruitment of cofactor proteins to the receptor (14, 15). These findings suggest that LCA derivatives may induce a VDR conformation distinct from vitamin D_3 and exhibit selective physiological functions.

In this study, we examined the effects of LCA derivatives, such as LCA acetate and LCA propionate, on VDR and other bile acid-responsive receptors and found that LCA propionate is a potent and more selective VDR agonist than LCA acetate. These LCA derivatives effectively induced the transcription of VDR target genes in various cells. Importantly, in vivo experiments showed that LCA acetate and LCA propionate can activate VDR in target organs without inducing hypercalcemia.

MATERIALS AND METHODS

Chemical compounds

LCA formate, LCA acetate, and LCA propionate (**Fig. 1**) were synthesized in our laboratory (H. Masuno, M. Shimizu, and S. Yamada, unpublished results). Proton NMR spectra (500 MHz) showed >99% purity of these compounds. LCA, chenodeoxycholic acid, and cholic acid were purchased from Nacalai (Kyoto, Japan), and LCA acetate methyl ester was from Steraloids (Newport, RI). $1,25(OH)_2D_3$ was obtained from Wako (Osaka, Japan). 1α-Hydroxyvitamin D_3 [$1\alpha(OH)D_3$] was kindly provided by Dr. Yoji Tachibana (Nisshin Flour Milling Co.).

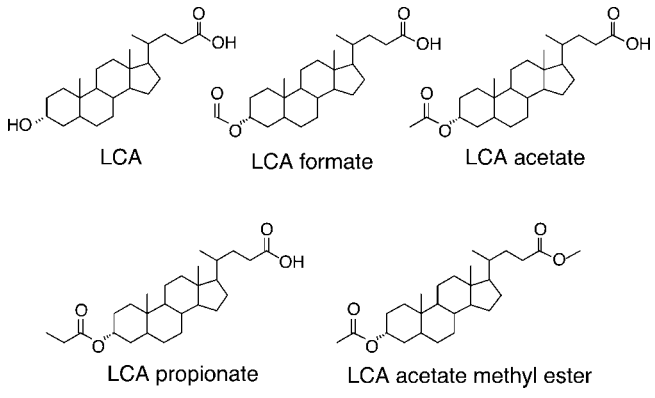

Fig. 1. Structures of lithocholic acid (LCA), LCA formate, LCA acetate, LCA propionate, and LCA acetate methyl ester.

Plasmids

The ligand binding domains of human VDR (GenBank accession number NM_000376) was inserted into the pCMX-GAL4 vector to make pCMX-GAL4-VDR (10). Fragments of human farnesoid X receptor (FXR; GenBank accession number NM_005123), pregnane X receptor (PXR; GenBank accession number NM_022002), and G protein-coupled bile acid receptor 1 (GPBAR1; GenBank accession number NM_170699) were inserted into the pCMX vector to make pCMX-FXR, pCMX-PXR, and pCMXGPBAR1, respectively (13, 16). IR1x3-tk-LUC, hCYP3A4-ER6x3-tk-LUC, Som-LUC, and GAL4-responsive MH100(UAS)x4-tk-LUC reporters were used to evaluate the activities of FXR, PXR, GPBAR1, and GAL4-VDR, respectively (13, 17). The ligand binding domain from VDR was inserted into pGEX vector (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) to generate pGEX-VDR (10). All plasmids were sequenced before use to verify DNA sequence fidelity.

Cell lines and cell culture

Human kidney HEK293 cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in DMEM containing 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Monkey kidney CV-1 (RIKEN Cell Bank), human colon carcinoma HCT116, SW480 (American Type Culture Collection, Rockville, MD), and immortalized keratinocyte HaCaT cells (kindly provided by Dr. Tadashi Terui, Department of Dermatology, Nihon University School of Medicine) were cultured in DMEM containing 10% FBS. Human colon carcinoma Caco-2, osteosarcoma MG63, and neuroblastoma SK-N-SH cells (RIKEN Cell Bank) were maintained in MEM containing 10% FBS, and myeloid leukemia THP-1, HL60, U937 (RIKEN Cell Bank), and breast carcinoma MCF-7 cells (American Type Culture Collection) were maintained in RPMI 1640 medium containing 10% FBS.

Transfection assay

Transfections in HEK293 cells were performed by the calcium phosphate coprecipitation assay as described previously (10). Eight hours after transfection, compounds were added. Cells were harvested after 16–18 h (for VDR and FXR) or 12 h (for GPBAR1) and were assayed for luciferase and β-galactosidase activities using a luminometer and a microplate reader (Molecular Devices, Sunnyvale, CA). Transfection experiments used 50 ng of reporter plasmid, 10 ng of pCMX-β-galactosidase, and 15 ng of each expression plasmid for each well of a 96-well plate. CV-1 cell transfection was performed with Fugene HD (Roche Diagnostics, Mannheim, Germany) using 100 ng of reporter plasmid, 50 ng of pCMX-β-galactosidase, and 50 ng of each expression plasmid for each well of a 96-well plate. Cells were harvested at 48 h after ligand addition. Luciferase data were normalized to the internal β-galactosidase control.

Competitive ligand binding assay

Glutathione *S*-transferase-VDR fusion protein was used for a competitive ligand binding assay (10). The proteins were dissolved in 0.05 M phosphate buffer (pH 7.5) containing 0.3 M KCl and 5 mM DTT and were incubated with [26,27-methyl-

^{3}H]1,25(OH)$_2$D$_3$ at 4°C in the presence or absence of nonradioactive competitor compounds. Bound and labeled 1,25(OH)$_2$D$_3$ was assessed using scintillation counting.

Quantitative real-time RT-PCR analysis

Total RNAs from samples were prepared by the acid guanidine thiocyanate-phenol/chloroform method (18). cDNAs were synthesized using the ImProm-II reverse transcription system (Promega, Madison, WI) (10). Real-time PCR was performed on the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences are listed in **Tables 1, 2**. The human and mouse RNA values were normalized to the level of β-actin and glyceraldehyde-3-phosphate dehydrogenase mRNA, respectively.

Nitroblue tetrazolium-reducing activity of myeloid leukemia cells

Nitroblue tetrazolium (NBT) reduction was assayed colorimetrically (7). Cells were incubated with 1 mg/ml NBT (Sigma-Aldrich, St. Louis, MO) and 100 ng/ml PMA (Sigma-Aldrich) in RPMI 1640 medium at 37°C for 30 min, and the reaction was stopped by adding HCl. Formazan deposits were solubilized in DMSO, and the absorption of the formazan solution at 570 nm per 10^6 cells was measured in a spectrophotometer (Molecular Devices).

Animal studies

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, Tokyo, Japan). Experiments were conducted with male mice between 8 and 9 weeks of age. Mice were injected intraperitoneally with test compounds diluted in PBS or treated orally with test compounds dissolved in corn oil (4, 12). Because LCA derivatives were dissolved incompletely in PBS, they were mixed vigorously before injection. Blood was collected from the tail or by heart puncture with a heparinized syringe and was immediately centrifuged to obtain plasma. Plasma total calcium was quantified by the *o*-cresolphthalein calcium method (Calcium C-Testwako; Wako). 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.

TABLE 1. Human primer sequences for quantitative real-time RT-PCR

Gene	GenBank Accession Number	Sequence (5′ to 3′)	Amplicon Size (bp)
CYP24A1	NM_000782	TGA ACG TTG GCT TCA GGA GAA (fw) AGG GTG CCT GAG TGT AGC ATC T (rev)	192
CD14	NM_000591	AAC TGA CGC TCG AGG ACC TAA A (fw) CGC AAG CTG GAA AGT GCA A (rev)	85
CD11b	NM_000632	CTG TCT GCC AGA GAA TCC AGT G (fw) GAG GTG GTT ATG CGA GGT CTT G (rev)	119
Cathelicidin antimicrobial peptide	NM_004345	GCT AAC CTC TAC CGC CTC CT (fw) GGT CAC TGT CCC CAT ACA CC (rev)	183
TRPV6	NM_018646	GCT ACT TCA GGA AGC CTA CAT G (fw) TGT CCA AAG AAG CGA GTG ACC (rev)	141
β-Actin	NM_001101	GAC AGG ATG CAG AAG GAG AT (fw) GAA GCA TTT GCG GTG GAC GAT (rev)	195

CYP24A1, 1α,25-dihydroxyvitamin D$_3$ 24-hydroxylase; fw, forward primer; rev, reverse primer; TRPV6, transient receptor potential vanilloid type 6.

TABLE 2. Mouse primer sequences for quantitative real-time RT-PCR

Gene	GenBank Accession Number	Sequence (5′ to 3′)	Amplicon Size (bp)
Cyp24a1	NM_009996	TGG AGA CGA CCG CAA ACA G (fw) AGG CAG CAC GCT CTG GAT T (rev)	101
Calbindin D$_{9k}$	NM_009789	GCT GCT GAT TCA GTC AGA GT (fw) CTT CGT AAC TAA CTT CTC CAT C (rev)	107
Trpv6	NM_022413	AGT TGC CCA TGA GCG AGA TG (fw) CGG TCG CCA AGA CCA TAC TC (rev)	132
Trpv5	NM_001007572	TGG AGA CCT ACT GCG CTT CTG (fw) GCA GGT CCG TCA ATG ATG GT (rev)	174
GAPDH	NM_008084	TGC ACC ACC AAC TGC TTA G (fw) GAT GCA GGG ATG ATG TTC (rev)	176

fw, forward primer; rev, reverse primer.

Statistics

Values are presented as means ± SD. Variables were compared using one-way ANOVA with compounds as factors, in conjunction with the Bonferroni post hoc test. The analysis was performed using SigmaStat (Systat Software, San Jose, CA).

RESULTS

Effects of LCA derivatives on bile acid receptors

Because there has been no reported physiological correlation between bile acid and intestinal calcium absorption, bile acid-derived ligands have the potential to selectively activate VDR without inducing hypercalcemia. We previously reported that modification of LCA at the 3α-hydroxyl group increases VDR transactivation, with LCA acetate being the most potent compound tested (13). We synthesized an additional LCA derivative, LCA propionate (Fig. 1), and compared its effect on VDR activation with those of other LCA derivatives. LCA weakly activated VDR, and LCA formate was more potent than LCA (**Fig. 2A**). As reported previously (13), LCA acetate induced VDR transactivation effectively, and methyl esterification of LCA acetate decreased the activity. LCA propionate was as

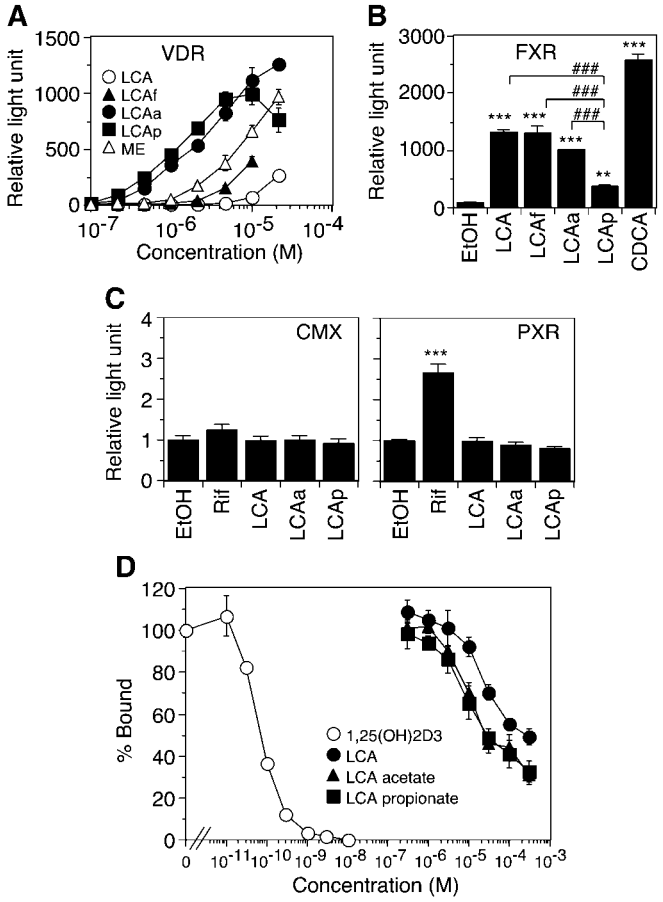

Fig. 2. Effects of LCA derivatives on vitamin D receptor (VDR), farnesoid X receptor (FXR), and pregnane X receptor (PXR). A: Transactivation of VDR by LCA derivatives. HEK293 cells were transfected with CMX-GAL4-VDR and MH100(UAS)x4-tk-LUC and then treated with several concentrations of LCA, LCA formate (LCAf), LCA acetate (LCAa), LCA propionate (LCAp), and LCA acetate methyl ester (ME). B: Transactivation of FXR by LCA derivatives. HEK293 cells were transfected with CMX-FXR and IR1x3-tk-LUC and treated with ethanol (EtOH), 18 μM LCA derivatives, or chenodeoxycholic acid (CDCA). ** $P < 0.01$, *** $P < 0.001$ compared with ethanol control; ### $P < 0.001$ compared with LCA propionate. C: Effects of LCA derivatives on PXR activation. CV-1 cells were transfected with CMX control or CMX-PXR in combination with hCYP3A4-ER6x3-tk-LUC and treated with ethanol, 30 μM rifampicin (Rif), LCA, LCA acetate, or LCA propionate. *** $P < 0.001$ compared with ethanol control. D: Direct binding of LCA derivatives to VDR. Glutathione *S*-transferase-VDR fusion proteins were incubated with [^{3}H]1α,25-dihydroxyvitamin D_3 [1,25(OH)$_2D_3$] in the presence of nonradioactive 1,25(OH)$_2D_3$, LCA, LCA acetate, or LCA propionate at a range of concentrations. All values represent means ± SD of triplicate assays.

potent as LCA acetate (Fig. 2A). Although VDR induces target genes that detoxify LCA and its derivatives, FXR (NR1H4) is activated by various bile acids, such as chenodeoxycholic acid and deoxycholic acid, and regulates the synthesis and enterohepatic circulation of bile acids (12, 19). Although chenodeoxycholic acid activated FXR effectively, LCA, LCA formate, and LCA acetate did so modestly, as reported previously (Fig. 2B) (13). The effect of LCA propionate on FXR transactivation was weaker than that of LCA acetate. PXR was reported to respond to high concentrations of LCA (20). Although rifampicin activated PXR, LCA, LCA acetate, and LCA propionate (30 μM) were not effective on PXR (Fig. 2C). LCA derivatives were not toxic to HEK293 and CV-1 cells at 30 μM in transfection assays but decreased the viability of these cells at concentrations >30 μM. VDR forms a heterodimer with retinoid X receptor (RXR) and binds to VDR response elements in the promoter region of target genes (1). Although the RXR ligand 9-*cis* retinoic acid induced the transactivation of RXRα (NR1B1), RXRβ (NR1B2), and RXRγ (NR1B3) very effectively, LCA, LCA acetate, and LCA propionate did not activate RXRs (data not shown). The VDR binding affinity of LCA and its derivatives was examined in a competitive binding assay. Isotopically labeled 1,25(OH)$_2D_3$ was incubated with glutathione *S*-transferase-VDR fusion proteins in the absence or presence of test compounds. While unlabeled 1,25(OH)$_2D_3$ competed the binding of [^{3}H]1,25(OH)$_2D_3$ to VDR with an IC_{50} of 0.08 nM, LCA, LCA acetate, and LCA propionate bound to VDR with IC_{50} of 300, 30, and 30 μM, respectively (Fig. 2D). Thus, LCA propionate is as potent a VDR ligand as LCA acetate.

Recently, the G protein-coupled receptor GPBAR1 was identified as a membrane receptor for bile acids (16, 21). In response to bile acid activation, GPBAR1 induces the production of cAMP and the subsequent activation of protein kinase A signaling pathways. To examine the effect of LCA derivatives on GPBAR1, we transfected a GPBAR1 expression vector and a luciferase reporter containing the cAMP-responsive element from the somatostatin promoter in HEK293 cells and treated cells with bile acids (10 μM) for 12 h (17). Although luciferase activity was only slightly induced by bile acids in the absence of GPBAR1 transfection, cholic acid, chenodeoxycholic acid, and LCA effectively induced GPBAR1-dependent luciferase reporter activation as reported (22). LCA formate had GPBAR1 agonist activity, although it was weaker than that of LCA. LCA acetate and LCA propionate, as well as LCA acetate methyl ester, did not have significant GPBAR1 activity (**Fig. 3**). Thus, LCA propionate is a bile acid that activates VDR at concentrations that are not effective on other bile acid receptors (FXR, PXR, and GPBAR1).

Effect of LCA derivatives on endogenous gene expression in cells

VDR is expressed in the vitamin D_3 target organs that mediate calcium homeostasis, such as intestine, bone, and kidney, and also in those that mediate noncalcemic actions of vitamin D_3 in other tissues, including blood cells and skin (23). 1,25(OH)$_2D_3$ induces its own metabolism through VDR-dependent activation of the enzyme 1α,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24A1) in many tissues (1). To examine the cell type-selective action of LCA derivatives, we treated intestinal mucosa-derived HCT116, SW480, Caco-2, myeloid-derived THP-1, U937, HL60, kidney epithelium-derived HEK293, osteoblast-derived MG63, mammary epithelium-derived MCF-7, skin

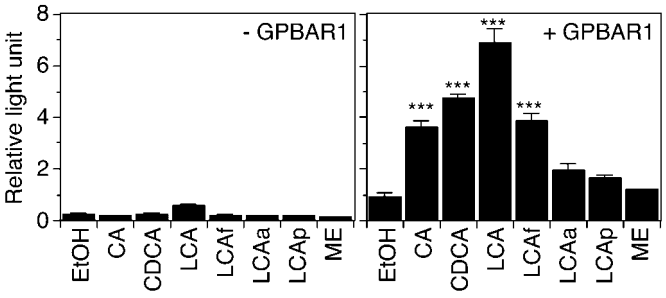

Fig. 3. Effects of bile acids and LCA derivatives on the plasma membrane G protein-coupled bile acid receptor 1 (GPBAR1). HEK293 cells were transfected with CMX control or CMX-GPBAR1 in combination with the cAMP-responsive Som-LUC reporter and treated with ethanol (EtOH), 10 μM cholic acid (CA), chenodeoxycholic acid (CDCA), LCA, LCA formate (LCAf), LCA acetate (LCAa), LCA propionate (LCAp), or LCA acetate methyl ester (ME). *** $P < 0.001$ compared with ethanol control. All values represent means ± SD of triplicate assays.

keratinocyte-derived HaCaT , and neuron-derived SK-N-SH cells with 100 nM $1,25(OH)_2D_3$ or 30 μM LCA derivatives and evaluated CYP24A1 mRNA expression by quantitative RT-PCR. LCA derivatives did not decrease the viability of these cells at 30 μM but induced toxic effects at 100 μM. $1,25(OH)_2D_3$ (100 nM) but not LCA (30 μM) induced CYP24A1 expression in all of the cell lines examined (**Fig. 4**). Activation of endogenous VDR by LCA required high ligand concentrations (~100 μM) (13). Although LCA formate induced CYP24A1 expression in U937 and MG63 cells, LCA acetate and LCA propionate induced its expression in HCT116, SW480, Caco-2, THP-1, U937, HL60, HEK293, MG63, and HaCaT cells (Fig. 4). LCA acetate was a weak activator in MCF-7 and SK-N-SH cells, and LCA propionate was less effective in these cells. These data indicate that LCA acetate and LCA propionate are potent VDR ligands in cells derived from the target organs of noncalcemic VDR action, such as blood cells and skin. LCA acetate methyl ester induced CYP24A1 expression in Caco-2 cells (Fig. 4). This suggests that LCA acetate methyl ester is activated by a cell-specific mechanism.

The human antimicrobial peptide CAMP gene is a VDR target that mediates innate immune function in bone marrow-derived cells and keratinocytes (24, 25). We examined the effects of LCA acetate and LCA propionate on CAMP mRNA expression in myeloid leukemia THP-1, U937, HL60, and immortalized keratinocyte HaCaT cells. $1,25(OH)_2D_3$ induced CAMP transcription effectively in these cells as reported (24, 25) (**Fig. 5**). LCA acetate and LCA propionate also increased CAMP expression in THP-1, U937, and HL60 cells. Thus, LCA derivatives may induce innate immunity in myeloid cells and keratinocytes.

$1,25(OH)_2D_3$ and LCA acetate are inducers of myeloid leukemia differentiation (8). $1,25(OH)_2D_3$ induced the expression of CD14 and CD11b genes in THP-1, U937, and HL60 cells (**Fig. 6A, B**). Increased expression of these genes may be attributable to the differentiation of these cells to monocytes. LCA acetate and LCA propionate at 10 or 30 μM concentration increased the expression of

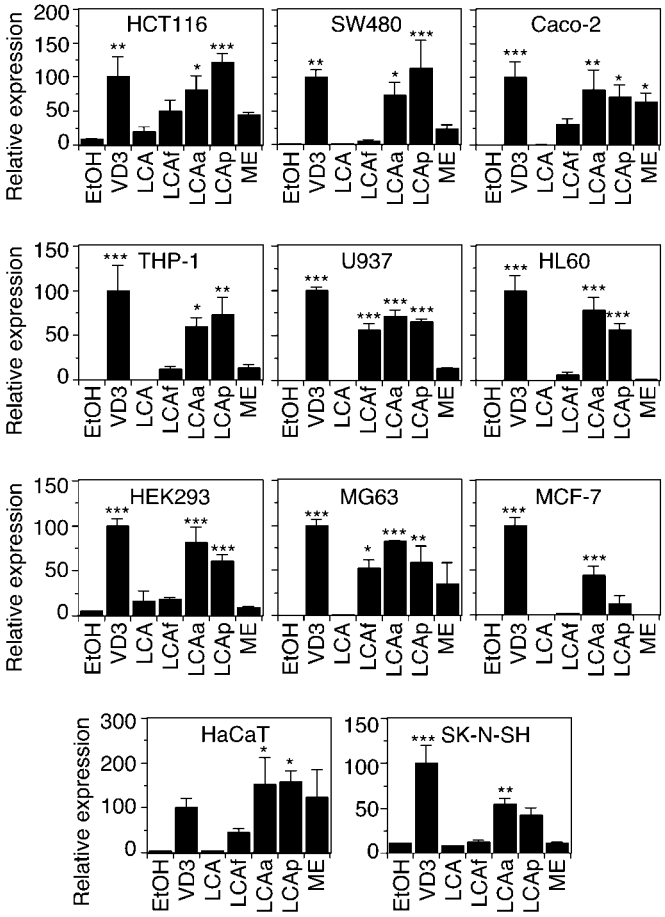

Fig. 4. Effects of LCA derivatives on the expression of the 1α,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24A1) gene in intestinal HCT116, SW480, Caco-2, myeloid THP-1, U937, HL60, kidney-derived HEK293, osteoblast MG63, mammary MCF-7, keratinocyte HaCaT, and neuronal SK-N-SH cells. Cells were treated with ethanol (EtOH), 100 nM $1,25(OH)_2D_3$ (VD3), 30 μM LCA, LCA formate (LCAf), LCA acetate (LCAa), LCA propionate (LCAp), or LCA acetate methyl ester (ME) for 24 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with ethanol control. All values represent means ± SD of triplicate assays.

CD14 and CD11b (Fig. 6A, B). LCA acetate, LCA propionate, and $1,25(OH)_2D_3$ induced NBT-reducing activity, a functional differentiation marker (Fig. 6C). Thus, LCA acetate and LCA propionate are inducers of myeloid leukemia cell differentiation.

Hypercalcemia is the major adverse effect of the therapeutic use of $1,25(OH)_2D_3$ and its derivatives (5). Administration of vitamin D_3 increases calcium absorption from the intestine by increasing calcium transport proteins, such as transient receptor potential vanilloid type 6 (TRPV6; also called calcium transport protein type 1). We treated intestinal mucosa-derived SW480 cells with $1,25(OH)_2D_3$ (1 μM), LCA acetate, or LCA propionate (30 μM) for 12, 24, and 48 h and examined the expression of CYP24A1 and TRPV6 genes. These compounds increased these expressions time-dependently (**Fig. 7A**). The concentration-dependent expression of CYP24A1 and TRPV6 was next examined in SW480 cells treated with

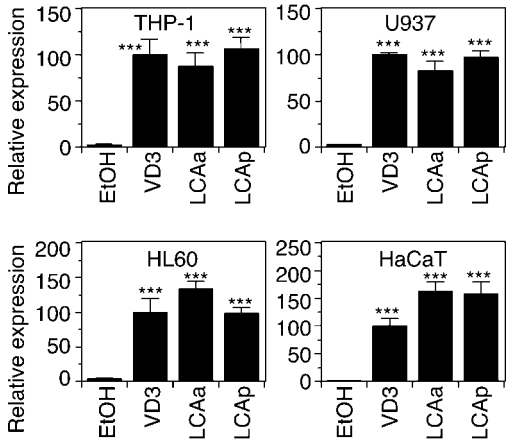

Fig. 5. Induction of cathelicidin antimicrobial peptide CAMP mRNA expression by LCA derivatives in myeloid THP-1, U937, HL60, and keratinocyte HaCaT cells. Cells were treated with ethanol (EtOH), 100 nM $1,25(OH)_2D_3$ (VD3), 30 μM LCA acetate (LCAa), or LCA propionate (LCAp) for 24 h. *** $P < 0.001$ compared with ethanol control. All values represent means ± SD of triplicate assays.

compounds for 12, 24, and 48 h. $1,25(OH)_2D_3$, LCA acetate, and LCA propionate induced the concentration-dependent expression of CYP24A1 and TRPV6 in intestinal mucosa-derived SW480 cells (Fig. 7B, C). The EC_{50} values of $1,25(OH)_2D_3$ on CYP24A1/TRPV6 induction at 12, 24, and 48 h were 30/8 nM, 40/6 nM, and 200/30 nM, respectively. $1,25(OH)_2D_3$ induced TRPV6 expression four to seven times more potently than CYP24A1 expression. The estimated EC_{50} values of LCA derivatives on CYP24A1/TPRV6 induction at 12, 24, and 48 h were 20/6 μM, 10/3 μM, and 2/1 μM, respectively. The potency of LCA derivatives on TRPV6 induction was two to three times greater than on CYP24A1 induction. These findings indicate that the TRPV6 gene is more sensitive to $1,25(OH)_2D_3$ than is the CYP24A1 gene.

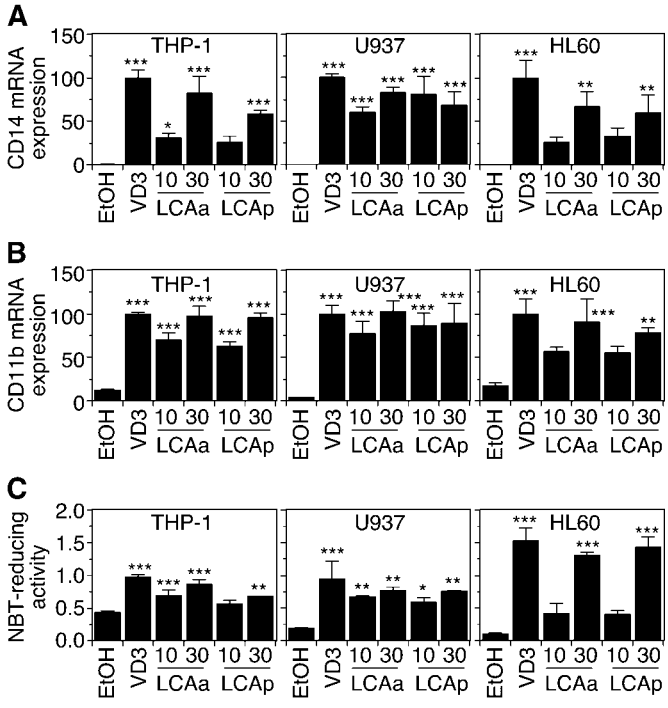

Fig. 6. Effects of LCA derivatives on the differentiation of myeloid leukemia THP-1, U937, and HL60 cells. A: Induction of CD14 mRNA expression. B: Induction of CD11b mRNA expression. Cells were treated with ethanol (EtOH), 100 nM $1,25(OH)_2D_3$ (VD3), or 10 or 30 μM LCA acetate (LCAa) or LCA propionate (LCAp) for 24 h. C: Induction of nitroblue tetrazolium (NBT)-reducing activity. Cells were treated with test compounds for 3 days. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with ethanol control. All values represent means ± SD of triplicate assays.

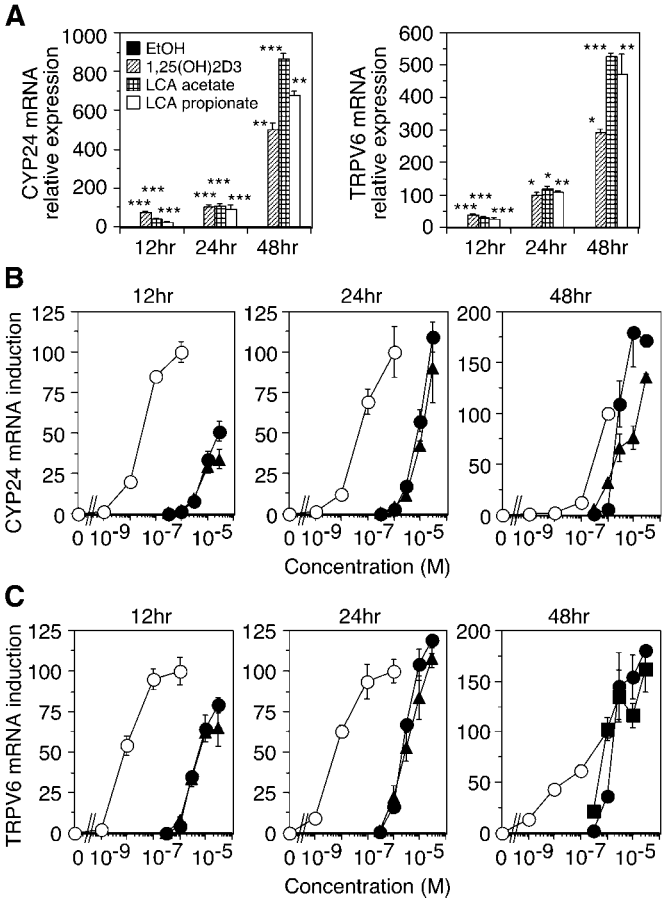

Fig. 7. Comparison of the effects of $1,25(OH)_2D_3$ and LCA derivatives on the induction of CYP24A1 and transient receptor potential vanilloid type 6 (TRPV6) mRNA expression in intestinal SW480 cells. A: Time course of induction of CYP24A1 and TRPV6 genes. Cells were treated with 1 μM $1,25(OH)_2D_3$, 30 μM LCA acetate, or 30 μM LCA propionate for 12, 24, and 48 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with ethanol control. B: Concentration-dependent effect of $1,25(OH)_2D_3$, LCA acetate, and LCA propionate on CYP24 mRNA expression. C: Concentration-dependent effects of $1,25(OH)_2D_3$, LCA acetate, and LCA propionate on TRPV6 mRNA expression. Cells were treated with $1,25(OH)_2D_3$ (open circles), LCA acetate (closed circles), or LCA propionate (closed triangles) at a range of concentrations for 12, 24, and 48 h. All values represent means ± SD of triplicate assays.

In vivo effects of LCA derivatives

To examine the in vivo effects of LCA acetate and LCA propionate, we treated mice with $1\alpha(OH)D_3$, LCA

acetate, or LCA propionate via intraperitoneal injection. $1\alpha(OH)D_3$ was rapidly converted to $1,25(OH)_2D_3$ after injection and was more effective than $1,25(OH)_2D_3$ at increasing the survival time of mice inoculated with leukemia cells (4). Intraperitoneal treatment of mice with $1\alpha(OH)D_3$ (12.5 nmol/kg) decreased body weight (**Fig. 8A**) and increased plasma calcium levels (Fig. 8B). $1\alpha(OH)D_3$ effectively induced expression of the kidney Cyp24a1, calbindin D_{9k}, Trpv6, and Trpv5 genes (Fig. 8C). It also induced intestinal Cyp24a1 expression (Fig. 8D). Treatment of mice with LCA acetate or LCA propionate (0.7 mmol/kg) did not decrease body weight (Fig. 8A). Importantly, LCA acetate and LCA propionate (0.7 mmol/kg) induced the expression of kidney Cyp24a1 as effectively as $1\alpha(OH)D_3$ (12.5 nmol/kg), but these LCA derivatives did not change the plasma calcium level or expression of calbindin D_{9k}, Trpv6, and Trpv5 (Fig. 8B, C). LCA acetate and LCA propionate were not effective at inducing intestinal target gene expression (Fig. 8D).

We next examined the in vivo effects of orally administered LCA derivatives. Oral $1\alpha(OH)D_3$ treatment decreased body weight (**Fig. 9A**) and increased the plasma calcium level, but LCA acetate and LCA propionate (1 mmol/kg) did not affect the body weight or plasma calcium (Fig. 9B). LCA acetate and LCA propionate (0.7 and 1 mmol/kg) induced kidney Cyp24a1 expression as

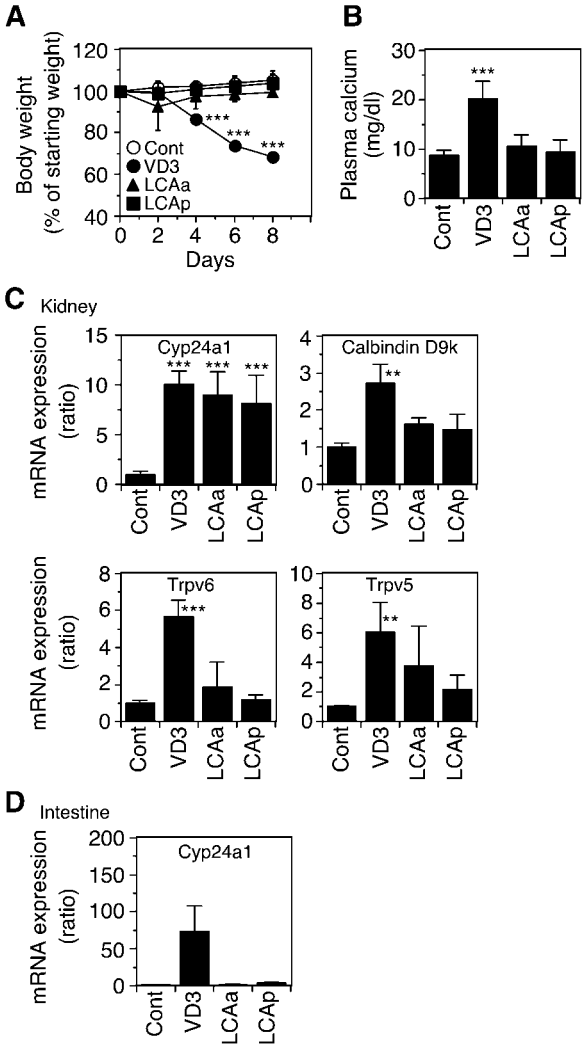

Fig. 8. Effects of intraperitoneal administration of LCA derivatives in mice. A: Body weight change. B: Plasma calcium level. C: mRNA expression of Cyp24a1, calbindin D_{9k}, Trpv6, and Trpv5 in kidney compared with vehicle control. D: mRNA expression of Cyp24a1 in intestine. $P = 0.190$ [vehicle control vs. $1\alpha(OH)D_3$]. Mice were administered vehicle control (Cont; n = 3), 12.5 nmol/kg $1\alpha(OH)D_3$ (VD3; n = 3), 0.7 mmol/kg LCA acetate (LCAa; n = 3), or 0.7 mmol /kg LCA propionate (LCAp; n = 3) via intraperitoneal injection on days 0, 2, 4, and 6. Blood was collected by heart puncture on day 8, and tissue mRNAs were examined on day 8. ** $P < 0.01$, *** $P < 0.001$ compared with vehicle control. All values represent means ± SD, and the experiments were repeated with similar results.

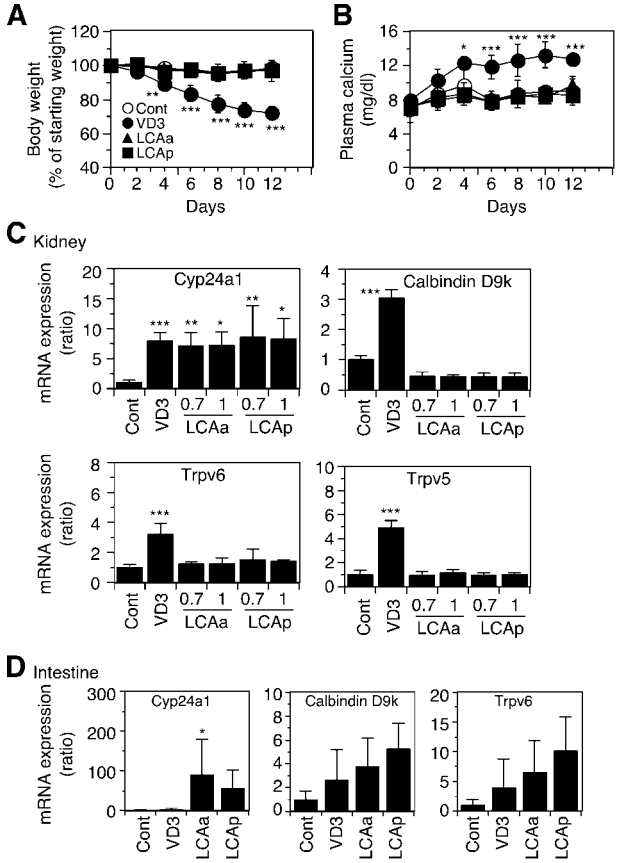

Fig. 9. Effects of oral administration of LCA derivatives in mice. A: Body weight change. B: Plasma calcium level. C: mRNA expression of Cyp24a1, calbindin D_{9k}, Trpv6, and Trpv5 in kidney. D: mRNA expression of Cyp24a1, calbindin D_{9k}, and Trpv6 in intestine. Mice were administered vehicle control (Cont; n = 3), 12.5 nmol/kg $1\alpha(OH)D_3$ (VD3; n = 3), 0.7 mmol/kg (n = 3) or 1 mmol/kg (n = 6) LCA acetate (LCAa), or 0.7 mmol/kg (n = 3) or 1 mmol/kg (n = 3) LCA propionate (LCAp) via gavage on days 0, 2, 4, 6, 8, and 10. Blood was collected from the tail on days 0, 2, 4, 6, 8, and 10 and by heart puncture on day 12. Tissue mRNAs were examined on day 12. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with vehicle control. All values represent means ± SD, and the experiments were repeated with similar results.

effectively as $1\alpha(OH)D_3$ (12.5 nmol/kg) (Fig. 9C). Unlike $1\alpha(OH)D_3$, the LCA derivatives did not increase kidney calbindin D_{9k}, Trpv6, and Trpv5 expression. The expression of intestinal Cyp24a1 was not increased after a 10 day oral administration of $1\alpha(OH)D_3$ (Fig. 9D). Because expression was observed after a 1 day single oral dose (data not shown), the lack of induction during a 10 day dose may be attributable to the adaptation mechanism(s) reported previously (26). The effect of LCA derivatives on intestinal Cyp24a1 expression was observed, but modestly. Therefore, LCA acetate and LCA propionate can activate VDR in vivo without the toxic effects of weight loss and hypercalcemia.

DISCUSSION

Bile acid-derived LCA acetate and LCA propionate act as VDR ligands. Bile acids are the major catabolic products of cholesterol and are essential detergents that are required for the ingestion and intestinal absorption of hydrophobic nutrients, such as cholesterol, fatty acids, and lipid-soluble vitamins, including vitamin D (27). Bile acid metabolism is regulated by nuclear receptors (19). FXR responds to primary and secondary bile acids in their free and conjugated forms, represses bile acid synthesis and hepatocellular import, stimulates bile acid export from cells, and protects hepatocytes from bile acid toxicity. VDR and PXR sense toxic secondary bile acids and induce their elimination through a xenobiotic metabolism pathway. Although the physiological role of VDR in bile acid metabolism is still under investigation, mounting evidence suggests that VDR acts as a bile acid sensor as well as an endocrine receptor for vitamin D signaling. Apart from direct effects on vitamin D absorption, a physiologic link between bile acids and calcium metabolism has not been demonstrated. According to these findings, we hypothesized that bile acid-derived VDR ligands may exhibit selective VDR activity without inducing hypercalcemia. Very recently, a potential in vivo role of LCA on VDR activation was investigated (28). Administration of high concentrations of LCA restored serum calcium level to the normal range in vitamin D-deficient rats by increasing VDR target gene expression and bone calcium mobilization. LCA administration was not effective in rats with normal vitamin D levels. These data indicate that LCA can substitute for vitamin D in calcium homeostasis only in vitamin D-deficient rats, and it is still unknown whether LCA or its derivatives can induce hypercalcemia. The secondary bile acid LCA is toxic to cells at concentrations that approximate those needed to activate VDR (13). In this study, we examined the effects of LCA acetate and a related compound, LCA propionate, on VDR activation in vivo and found that these derivatives can induce tissue VDR activation without inducing hypercalcemia.

There are several possible mechanisms by which LCA derivatives could selectively activate VDR. We recently reported that vitamin D_3 derivatives with adamantane or lactone ring side chain substituents are cell type-selective VDR modulators (10). Ma et al. (9) reported that nonsecosteroidal compounds act as noncalcemic and tissue-selective VDR ligands. These compounds are potent VDR agonists in keratinocytes, osteoblasts, and peripheral blood mononuclear cells but are less potent in intestinal cells. Distinct recruitment of cofactors may be responsible for the selective activity. We examined the effect of LCA acetate and LCA propionate on the induction of the VDR target gene CYP24A1 in several cell lines (Fig. 4). Except for mammary carcinoma MCF-7 cells and neuroblastoma SK-N-SH cells, LCA acetate and LCA propionate induced CYP24A1 mRNA expression in the cells examined, including intestinal cells. These findings suggest that noncalcemic VDR activation is not mediated by a cell type-specific mechanism.

TRPV6 is a key VDR target that mediates intestinal calcium absorption (29). We compared the effect of $1,25(OH)_2D_3$, LCA acetate, and LCA propionate on the induction of CYP24A1 and TRPV6 expression in intestinal SW480 cells (Fig. 7). Interestingly, the effect of $1,25(OH)_2D_3$ on TRPV6 induction was 4- to 7-fold greater than that on CYP24A1 induction, whereas the potency of LCA derivatives on TRPV6 induction was 2- to 3-fold greater than that on CYP24A1 induction (Fig. 7B, C), suggesting that the vitamin D signal is amplified for TRPV6 induction. This unique effect of $1,25(OH)_2D_3$ may cause the difficulty in developing vitamin D_3 derivatives without hypercalcemic action. Promoter-selective effects of VDR may be involved in the different potency of $1,25(OH)_2D_3$ on CYP24A1 and TRPV6 induction. Structure-function analysis and docking models show that LCA acetate interacts with the VDR ligand binding pocket in a mode distinct from $1,25(OH)_2D_3$, particularly in interactions involving helix 3 and 4/5 residues (13). These helices play an important role in the dynamic recruitment of cofactor proteins to the receptor (14, 15). The transcription of genes is regulated by multiple transcription factors, inducing nuclear receptors, and involves the dynamic recruitment of multisubunit cofactor complexes. Therefore, ligand-selective cofactor recruitment by promoter-specific transcription factors may lead to differential CYP24A1 and TRPV6 gene induction (Fig. 7), although the selective cofactors for bile acids and derivatives remain to be elucidated. In addition to the regulation of gene transcription, $1,25(OH)_2D_3$ elicits a variety of rapid nongenomic responses. $1,25(OH)_2D_3$-stimulated nongenomic responses may affect TRPV6 gene expression through the modification of transcription factor complexes. Mechanisms distinctly induced by $1,25(OH)_2D_3$ and LCA derivatives other than VDR transactivation may be related to their gene-selective actions.

Calbindin D_{9k} is an intracellular calcium transfer protein, and TRPV6 and TRPV5 are epithelial calcium channels (30). These target genes were induced by treatment with vitamin D_3 as reported (Figs. 8, 9) (31). TRPV6 is expressed in kidney and intestine, whereas TRPV5 (also called epithelial calcium channel or calcium transporter protein type 2) is restricted in the kidney (30). Mice lacking Trpv5 have diminished renal calcium reabsorption and severe hypercalciuria (32). Experiments using Trpv6-

deficient mice demonstrated that TRPV6 is necessary for intestinal calcium absorption and plays an important role in maintaining blood calcium levels (33). These findings suggest that renal and intestinal calcium absorption by TRPV5 and TRPV6 plays a role in vitamin D_3-induced hypercalcemia. In vivo experiments showed that treatment of mice with LCA acetate and LCA propionate induced kidney Cyp24a1 expression without inducing hypercalcemia (Figs. 8, 9). Administration of $1\alpha(OH)D_3$ at a dose that induces kidney Cyp24a1 to the same extent as LCA acetate and LCA propionate decreased body weight and increased plasma calcium concentrations. Administration of LCA acetate and LCA propionate did not induce the expression of kidney calbindin D_{9k}, Trpv6, or Trpv5 (Figs. 8, 9). Less efficient induction of these genes may be associated with the noncalcemic effect of LCA derivatives. Induction of these calcium metabolism-related genes by vitamin D_3 may require additional mechanisms, such as "vitamin D_3 signal amplification," as suggested from TRPV6 expression in SW480 cells (Fig. 7). LCA derivatives may not be effective on the vitamin D-specific mechanism(s), and VDR activation by LCA derivatives may not be sufficient for induction of the calcium metabolism-related genes. Figure 7 suggests that LCA derivatives are more stable than vitamin D_3 because they are not subject to vitamin D_3-metabolizing enzymes. However, the selective action of LCA derivatives without inducing hypercalcemia or the expression of calcium metabolism-related genes cannot be explained by their in vivo stability. Although the pharmacokinetics of LCA derivatives should be investigated, LCA and its derivatives, such as LCA propionate, may prove to be useful tools in the elucidation of the calcemic and noncalcemic actions of VDR.

Vitamin D receptor ligands with diminished calcium action have potential application in the treatment of immune disorders, malignancies, and infections (5, 23). $1,25(OH)_2D_3$ was initially found to induce the differentiation of mouse and human leukemia cells >25 years ago (8). LCA acetate and LCA propionate were shown to induce differentiation markers in myeloid leukemia cells (Fig. 6). Recently, vitamin D_3 was shown to play an important role in innate immune responses in monocytes and keratinocytes through the VDR-dependent induction of antimicrobial peptides such as CAMP (6, 25). LCA acetate and LCA propionate induced CAMP mRNA in myeloid cells and keratinocytes (Fig. 5). These data suggest that LCA derivatives can enhance the differentiation of myeloid leukemia cells and innate immunity in monocytes and keratinocytes.

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