Hairless Modulates Ligand-Dependent Activation of the Vitamin D Receptor-Retinoid X Receptor Heterodimer

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The active form of vitamin D, 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3], binds to the vitamin D receptor (VDR) and regulates various physiological and pharmacological processes. Secondary bile acids, such as lithocholic acid (LCA), also act as endogenous VDR ligands. The molecular basis of ligand-selective VDR action remains largely unknown. Hairless (HR) acts as a coregulator of VDR through a direct interaction. HR mutations confer an alopecia phenotype similar to VDR mutations in mice and humans, but the underlying molecular mechanisms have not been elucidated. We examined the effect of HR on VDR activation induced by 1,25(OH)2D3 and LCA. HR repressed VDR transactivation induced by both 1,25(OH)2D3 and LCA. HR also repressed transactivation of VDR E269A and R391A mutants, but less effectively than that of wild-type VDR. These residues are involved in retinoid X receptor (RXR) heterodimer allosteric communication, through which information from ligands is transmitted to dimer and coactivator interfaces. In the presence of HR cotransfection, LCA activated these VDR mutants more effectively than wild-type VDR. In mammalian two-hybrid assays, HR enhanced the association of VDR with a corepressor, nuclear receptor corepressor. These findings indicate that HR affects VDR-RXR heterodimer allosteric communication and corepressor complex formation. Interestingly, HR knockdown in keratinocyte-derived HaCaT cells increased ligand-induced cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24A1) expression but suppressed expression of cathelicidin antimicrobial peptide, indicating that HR acts not only as a corepressor but also as a coactivator. HR may be a VDR modulator that affects the RXR allosteric communication network in order to regulate transcription in a gene-selective manner.

Key words vitamin D receptor; hairless; gene expression; cofactor; cathelicidin antimicrobial peptide

Vitamin D receptor (VDR; NR1I1) is a nuclear receptor that mediates the biological action of the active form of vitamin D, 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3], in numerous physiological and pharmacological processes, including bone and calcium metabolism, cellular growth and differentiation, immunity, and cardiovascular function.1) VDR acts as an allosteric transcription factor that undergoes a ligand-dependent conformational change in the cofactor binding site and activation function 2 (AF2) domain, structural rearrangements that result in dynamic interaction with the heterodimer partner retinoid X receptor (RXR; NR2B) and exchange of cofactor complexes.2,3) While corepressors bind to the VDR-RXR heterodimer in the absence of ligand, the ligand binding reduces the affinity of corepressors and increases the affinity for coactivators, a structural transition that induces transcription of specific genes. The VDR-RXR heterodimer binds preferentially to a DNA response element that consists of a two hexanucleotide (AGGTCA or a related sequence) direct repeat motif separated by three nucleotides (DR3).4) The DR3 VDR-binding element has been identified in the regulatory regions of many target genes, including cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24A1), cathelicidin antimicrobial peptide (CAMP), and osteopontin.2,4) An everted repeat of the hexanucleotide motif separated by six nucleotides (ER6) is another VDR-binding element that regulates expression of the human CYP3A4 gene.5) Dynamic and coordinated interactions of VDR with RXR and cofactor complexes are required for the efficient regulation of transcription.1,2,3) Lithocholic acid (LCA), a secondary bile acid, acts as an additional physiological VDR ligand and interacts with the VDR ligand-binding pocket in a mode distinct from 1,25(OH)2D3, and alternate contacts are seen particularly with helices 3 and 4/5, components of the AF2 surface.6–9) A statistical coupling analysis has demonstrated that RXR heterodimer allosteric communication is required for effective activation of VDR by LCA but not by 1,25(OH)2D3.9) Indeed, LCA induces the interaction of VDR with RXR, steroid receptor coactivator 1 (SRC-1), and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) in a manner distinct from 1,25(OH)2D3 in cells.3) Although the role of VDR in mediating the biological effects of 1,25(OH)2D3 has been investigated for decades, an understanding of the biology of VDR regulation by LCA is only now emerging.

Hairless (HR) is a nuclear protein that functions as a co-repressor of nuclear receptors, including thyroid hormone receptor, retinoic acid receptor-related orphan receptors and VDR.10–12) HR interacts with VDR via multiple protein–protein interfaces in the absence of ligand and represses VDR transactivation by 1,25(OH)2D3 and LCA.12,13) Interestingly, LCA but not 1,25(OH)2D3 enhances the interaction,12) suggesting that LCA induces a conformation of the VDR-RXR heterodimer with enhanced affinity for HR binding. In both mice and humans, mutation of HR results in hair-loss phenotypes similar to VDR mutation, suggesting a functional interaction of HR and VDR in vivo.14) In contrast to the in vitro interaction, 1,25(OH)2D3 treatment decreases HR binding to VDR in keratinocytes.15) Although the in vivo role of HR in VDR function remains unclear, HR may be a ligand-selective
VDR regulator. In this study, we examined the effects of HR on VDR activation by 1,25(OH)₂D₃ and LCA and found that HR affects VDR-RXR heterodimer allosteric communication and nuclear receptor corepressor (N-CoR) association. In addition, HR knockdown experiments showed that HR can act as a coactivator in a gene-selective manner.

MATERIALS AND METHODS

Chemicals 1,25(OH)₂D₃ and trichostatin A were purchased from Wako Pure Chemical Industries (Osaka, Japan) and LCA was from Nacalai Tesque (Kyoto, Japan).

Plasmids A mouse HR expression plasmid, pcDNA-HR, was kindly provided by Dr. Yuko Oda (University of California, San Francisco and Veterans Affairs Medical Center San Francisco, San Francisco, CA, U.S.A.), pCMX-FLAG-VDR, pCMX-VP16-VDR, pCMX-GAL4-SRC-1, pCMX-GAL4-N-CoR, pCMX-GAL4-SMRT, Spp×3-tk-LUC, hCYP3A4-ER6×3-tk-LUC, and MHI100 (UAS)×4-tk-LUC were previously reported. Mutations were introduced into pCMX-FLAG-VDR using a QuickChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, U.S.A.) and pCMX-FLAG-VDR mutants were sequenced before use to verify DNA sequence fidelity.

Cell Culture and Transfection Assay Human embryonic kidney (HEK) 293 cells (RIKEN Cell Bank, Tsukuba, Japan) and human immortalized keratinocyte HaCaT cells (kindly provided by Dr. Tadashi Terui, Department of Dermatology, Nihon University School of Medicine) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Transfections in HEK293 cells were performed by the calcium phosphate coprecipitation method. Transfection experiments used 150 ng of each expression plasmid in combination with 50 ng of reporter plasmid and 10 ng of pCMX-β-galactosidase in each well of a 96-well plate. Eight hours after transfection, compounds were added. Cells were harvested after 16–20 h and were assayed for luciferase and β-galactosidase activities using a luminometer and a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). Luciferase data were normalized to the internal β-galactosidase control.

Western Immunoblotting Analysis Total cell lysates (30 μg for each lane) from HEK293 cells were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transferred to a membrane for immunoblotting. Western blot analysis was performed using antibodies to VDR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) and FLAG (Sigma-Aldrich, St. Louis, MO, U.S.A.), visualized with an alkaline phosphatase conjugate substrate system.

RNA Interference, and Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction Small interfering RNAs (siRNAs) directed against human HR (HR-siRNA) and control siRNA were purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A.). siRNA oligonucleotides were transfected into HaCaT cells using a DharmaFECT 1 Transfection Reagent (Thermo Fisher Scientific). Twenty-four hours after transfection, compounds were added. Cells were harvested after 24 h and total RNAs were prepared by the acid guanidine thiocyanate-phenol/chloroform method. CDNA were synthesized using an ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI, U.S.A.). Quantitative real-time polymerase chain reaction was performed on an ABI PRISM 7000 Sequence Detection System (Life Technologies Corporation, Rockville, MD, U.S.A.) with Power SYBR Green PCR Master Mix (Life Technologies Corporation). Primers for HR were 5'-ACC GGG CAC AGA AAG ACT TC-3' and 5'-AGC CCT GCA TCC AGG TAG CA-3'. Primers for CYP24A1, CAMP and glyceroldehyde-3-phosphate dehydrogenase were reported previously. RNA values were normalized to the level of glyceroldehyde-3-phosphate dehydrogenase mRNA.

RESULTS AND DISCUSSION

Suppression of Ligand-Dependent Transactivation of VDR Mutants by HR We examined the effects of HR on ligand-dependent VDR transactivation in HEK293 cells. Both 1,25(OH)₂D₃ and LCA induced transactivation of human VDR on luciferase reporters containing a DR3 element from the mouse osteopontin gene (Spp×3-tk-LUC) (Fig. 1) and an ER6 element from the human CYP3A4 gene (hCYP3A4-ER6×3-tk-LUC) (Figs. 2C, D, left and D, left). As reported in COS-1 cells, HR cotransfection suppressed VDR transactivation induced by 1,25(OH)₂D₃ and LCA in HEK293 cells (Fig. 1). For example, 1,25(OH)₂D₃ (1 nM) induced luciferase activity to 2826 units in HEK293 cells transfected with VDR, and HR cotransfection decreased the activity to one-seventh (406 units). HR decreased luciferase activity induced by VDR with 30 μM LCA from 1326 units to 89 units. We prepared alanine point mutants of the VDR ligand binding domain residues listed in Figure 2A. Protein expression of all VDR mutants was detected in HEK293 cells with immunoblotting using anti-VDR and anti-FLAG antibodies (Fig. 2B). As expected, VDR activation by 1,25(OH)₂D₃ and LCA was abolished by mutation of residues necessary for ligand binding (R274A, H305A and H397A) (Figs. 2C, D). The charge clamp residues, K246 and E420, are essential for VDR-coactivator interaction. Consistent with previous reports, 1,25(OH)₂D₃ and LCA could not activate the K246A and E420A mutants (Figs. 2 C, D). The charge clamp residues, K246 and E420, are essential for VDR-coactivator interaction. As expected, 1,25(OH)₂D₃ but not LCA induced activation of the S278A mutant (Figs. 2C, D).
HR cotransfection suppressed 1,25(OH)\textsubscript{2}D\textsubscript{3}-induced transactivation of the S278A mutant as effectively as that of wild-type VDR. The E269 and R391 residues of VDR are involved in allosteric communication with RXR across the heterodimer interface.\textsuperscript{9) 1,25(OH)\textsubscript{2}D\textsubscript{3} and LCA effectively induced activation of the E269A and R391A mutants in HEK293 cells without RXR cotransfection (Figs. 2C, D). Compared to previous reports using cells cotransfected with both VDR and RXR.
and RXR expression plasmids,7,9 response of these mutants to 1,25(OH)2D3 and LCA was still observed in the absence of RXR cotransfection. The concentration of RXR available for VDR-RXR heterodimerization may influence the difference and further investigation is needed to elucidate the mechanism responsible. Interestingly, HR repressed 1,25(OH)2D3-induced transactivation of the E269A and R391A mutants less effectively than that of wild-type VDR (Fig. 2C). For example, HR cotransfection decreased wild-type VDR, VDR-E269A, and VDR-R391A induced by 1,25(OH)2D3 (1 nM) from 1716 units, 1192 units, and 947 units to 149 units (9%), 261 units (22%), and 238 units (25%), respectively. LCA-induced transactivation of E269A and R391A was more refractory to HR repression (Fig. 2D). For example, LCA (30 µM) induced luciferase values mediated by wild-type VDR, VDR-E269A, and VDR-R391A to 1013 units, 823 units, and 693 units, respectively. Although HR repressed these VDR activities, luciferase values induced by LCA (30 µM) in the presence of HR cotransfection were 28 units, 137 units, and 99 units for wild-type VDR, VDR-E269A, and VDR-R391A, respectively. LCA also activated these VDR mutants more effectively than wild-type VDR in cells cotransfected with HR and Spp×3-tk-LUC (data not shown). This finding is consistent with previous reports demonstrating that LCA but not 1,25(OH)2D3 increases the interaction of VDR and HR25 and that the VDR-RXR allosteric network is more important for the response to LCA than for 1,25(OH)2D3.12 A surface interaction of VDR including residues involved in the RXR heterodimer allosteric network may target a site of HR. Previous biochemical studies show that three regions of VDR (residues 109–111, 134–01 and 202–303) are necessary for association with HR24 and that the 202–303 domain is a major interface for HR interaction.12 VDR E269-L390-R391-D342 residues in the major HR interaction site form an allosteric network that transmits the energy of ligand binding to the dimer interface.9,9 Since an LCA response requires allosteric contribution from RXR, VDR transactivation by LCA may be more susceptible to HR inhibition than for 1,25(OH)2D3. The finding that the VDR mutation R391C causes severe rickets and alopecia25 also supports a significant role for the heterodimer allosteric network in HR interaction.

**HR Enhances Association of N-CoR with VDR** Upon ligand binding, nuclear receptors undergo a conformational change that results in dissociation of repressors such as N-CoR and SMRT and their associated histone deacetylases (HDACs), and recruitment of coactivators such as SRC-1, resulting in complex formation with histone acetyltransferases and other complexes necessary for transcription.24 HR interacts with HDACs such as HDAC1.10,25 We examined the effects of HR on the interaction of VDR with cofactors in a mammalian two-hybrid assay as reported previously.26 1,25(OH)2D3 and, to a lesser extent, LCA increased the association of VDR with SRC-1 (Fig. 3A), consistent with our previous reports.23 Ligand-induced interaction of VDR and SRC-1 was not altered by HR cotransfection (Fig. 3A). VDR interacted with the corepressors N-CoR and SMRT in the absence of ligand. Although luciferase values were small compared to those in Figs. 1, 2 and 3A, we have confirmed interaction of N-CoR and SMRT fragments with VDR in previous studies.5,3 1,25(OH)2D3 and LCA induced the dissociation of N-CoR and SMRT from VDR in a concentration-dependent manner (Figs. 3B,C). Interestingly, the presence of HR increased the interaction of VDR and N-CoR and suppressed ligand-induced dissociation of N-CoR (Fig. 3B). A recent study shows that HR associates and colocalizes with N-CoR in nucleus.27 HR did not affect interaction of VDR and SMRT in the absence or presence of ligand (Fig. 3C). These findings indicate that HR modulates the interaction of VDR with a selective cofactor. A HDAC inhibitor, trichostatin A, reverses the repression of thyroid hormone receptor activity by HR.28 Treatment with trichostatin A reversed ligand-dependent VDR activation inhibited by HR, and the effect of trichostatin A was less effective on LCA-induced VDR activity (Fig. 3D). HR possesses a Jumonji C domain, which is a common feature of histone demethylases, although the function of this domain in HR is unclear.29 Repressive activity of HR may be mediated not only by corepressors like N-CoR and HDACs but also by other mechanisms such as histone demethylation.

**Repressive and Inducible Effects of HR on VDR Target Gene Expression** As we examined in Fig. 1, repressive activity of HR on VDR transactivation has been demonstrated in transient transfection assays using VDR and/or HR expres-
HR has also been reported to inhibit endogenous CYP24A1 expression induced by 1,25(OH)2D3 in HaCaT cells transfected with HR and/or VDR plasmids.30) We examined the effects of endogenous HR on CYP24A1 gene expression in HaCaT cells using HR mRNA interference knockdown (Fig. 4A). As expected from the previous finding that HR overexpression inhibits 1,25(OH)2D3-induced CYP24A1 expression,30) HR knockdown increased CYP24A1 expression induced by 1,25(OH)2D3 and LCA (Fig. 4B). Surprisingly, ligand-dependent expression of CAMP was repressed in HR knocked-down HaCaT cells (Fig. 4C). These results suggest that HR acts as a coactivator for CAMP,32) and CAMP is unlikely to be involved in regulation of the hair cycle by HR. The hedgehog and Wnt pathways are affected by both HR mutation and VDR knockout in mice.33) HR may be involved in transcriptional induction of unknown VDR target genes, for example, in the hedgehog and Wnt pathways, as in the case of CAMP. CAMP is an antimicrobial peptide, which acts as an effector of vitamin D-dependent innate immunity in humans.34) The roles of HR in immunity remain to be elucidated.

Keratinocyte-specific expression of a VDR transgene with a mutation abolishing ligand binding or with a mutation in the AF2 domain restores hair cycling in VDR null mice, suggesting that VDR effects on the hair follicle are ligand independent.35) In contrast, a rickets patient with the VDR mutation R391C has alopecia,23) a finding that supports the importance of a ligand-induced VDR-RXR allostERIC network. RXR disruption results in defective hair cycling.36) Since LCA is a secondary bile acid produced by intestinal microflora, it is not likely to be involved in hair cycle regulation in the skin. Unknown endogenous VDR ligand(s) may be present in the skin and play a role in hair cycle regulation by activating VDR in a manner dependent on the RXR allostERIC network and HR association.

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