Note

Transcriptional Regulation of 25-Hydroxyvitamin D-24-Hydroxylase (CYP24A1) by Calcemic Factors in Keratinocytes

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Summary

CYP24A1 regulates serum vitamin D (VD) levels by inactivating 25(OH)2D3, which is the precursor of the active form of VD [1α,25(OH)2D3], and CYP24A1 expression is controlled by multiple calcemic factors such as 1α,25(OH)2D3, calcium, and phosphate. A major phosphaturic factor, FGF23, has also been identified as a regulator of serum VD levels by affecting renal CYP24A1 gene expression; however, its effect on CYP24A1 in extrarenal cells remains largely unstudied. Therefore, the direct effect of FGF23 on CYP24A1 was examined in a human keratinocyte cell line (HaCaT). In this cell line, significant induction of CYP24A1 gene expression by 1α,25(OH)2D3 was seen within 4 h by qRT-PCR, and this was mediated by the VD receptor, as shown in a mutant cell line genetically deficient in this receptor. However, FGF23 treatment up to 12 h did not induce CYP24A1 expression, although the expected activation of the downstream MAPK signaling pathway was seen. High calcium and phosphate treatments were also ineffective in inducing CYP24A1 gene expression. Furthermore, a luciferase assay showed no activation of a VD-sensitive proximal CYP24A1 promoter in response to the calcium and phosphate treatments, suggesting that the effect of FGF23 on dermal CYP24A1 gene expression is indirect. From these findings, we speculate that CYP24A1 gene regulation by FGF23 occurs mainly in renal cells, but not in extrarenal cells, at least not in keratinocytes.

Key Words vitamin D, CYP24A1, FGF23, VDR, transcription

Vitamin D (VD) is a calcemic factor, and its serum level is tightly regulated through its biosynthesis in skin and dietary intake of VD precursors in response to the nutritional status of VD. The 25(OH)2D3 precursor is converted into the active form of VD, 1α,25(OH)2D3, by CYP27B1 (25(OH)D3 1α-hydroxylase) or, conversely, into inactive 24,25(OH)2D3 via 24-hydroxylation by CYP24A1 (1, 2). Calcium homeostasis regulated by VD is associated with the whole-body phosphate status, and the phosphaturic factor FGF23 and serum phosphate level modulate VD metabolism (3, 4). FGF23 acts to reduce the serum level of 1α,25(OH)2D3 by decreasing CYP27B1 expression and increasing CYP24A1 expression mainly in the kidney under physiological settings (3). However, under pathological settings such as chronic kidney disease–mineral and bone disorder, FGF23 induces CYP24A1 in extrarenal tissues (5, 6).

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VD regulates gene transcription via the nuclear VD receptor (VDR), which serves as a DNA-binding and VD-inducible transcription factor (1, 2). Upon VD binding, VDR binds to specific VD response elements (VDREs) within the promoters of VD target genes and activates their transcription. VDREs in the CYP24A1 promoter are critical for robust gene induction in response to VD stimulation in both humans and rats (7). Although the kidney is a pivotal organ in controlling the serum VD level (3, 4), extrarenal tissues such as the skin also express CYP24A1, presumably limiting local production of 1α,25(OH)2D3 by inactivation of its precursors. Calcemic factors such as FGF23, as well as minerals, have been shown to regulate CYP24A1 expression directly and indirectly in intact animals. Although CYP24A1 in the kidney and placenta (3, 4) appears to be regulated by FGF23 at the transcriptional level, FGF23 regulation of extrarenal CYP24A1 is unclear.

Given the physiological significance of dermal
Transcriptional Regulation of CYP24A1

CYP24A1 in the metabolism of VD and its associated minerals, here we evaluated the direct effect of FGF23 on CYP24A1 in a human keratinocyte cell line (HaCaT) after inhibiting VD signaling.

Materials and Methods

Cell culture and in vitro experiments. The human keratinocyte cell line HaCaT was cultured in DMEM (high glucose) (FUJIFILM Wako Pure Chemical Corporation, Saitama, Japan) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Beit HaEmek, Israel) an penicillin/streptomycin (FUJIFILM Wako Pure Chemical Corporation) (10). The human colorectal cancer cell line HCT116 was cultured in DMEM (low glucose) (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% FCS without antibiotics. VDR-knockout HaCaT cells were a kind gift from Setsuro Tech (Tokushima, Japan). All cells were cultured at 37˚C in 5% CO2. For quantitative real-time PCR (qRT-PCR), 1.0 × 10^6 cells were seeded in a 6-well plate, cultured in complete DMEM for 48 h, followed by incubation with DMEM without FBS for 24 h. Cells were stimulated with each reagent for the indicated time until harvesting for RNA extraction for qRT-PCR.

Generation of VDR-knockout HCT116 cells. VDR-knockout HCT116 cells were generated using the versatile non-homologous end joining-based knockin module for genome editing (VIKING) method (11). HCT116 cells were transfected with the VDR-pX330, VKG1-gRNA-pX330, and pVKG1-Puro vectors at a ratio of 17 : 2 : 1. At 24 h after transfection, the cells were treated with 3 μg/mL puromycin for 2 wk. Puromycin-selected colonies were cloned and subjected to PCR genotyping to confirm knockout of the VDR gene.

qRT-PCR. Total RNA was isolated from cells using TRIZOL reagent (Thermo Fisher, Waltham, MA, USA) following the manufacturer's instructions. cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). PCR was conducted on the CFX Connect Real-Time PCR System (Bio-Rad) (2, 3) using SYBR green master mix and the following primers: CYP24A1 forward, 5′-CAGCGAACT-GAAGCATAATGGTCG-3′, and reverse, 5′-GCTCGACTGGA-GTGACCACAT-3′; and β-actin forward, 5′-ATGGCAAT-GAGCGGTTC-3′, and reverse, 5′-CGTGGATGCCACAGTG-3′. RNA expression was calculated using the ΔΔCt method with normalization to β-actin expression. For verification of the PCR products, agarose gel electro-

Fig. 1. Transcriptional induction of human CYP24A1 by 1α,25(OH)2D3 requires VDR in human keratinocyte and colorectal cancer cell lines. (A) Time course of human CYP24A1 induction after 1α,25(OH)2D3 treatment. qRT-PCR of human CYP24A1 and human β-actin under the indicated treatments was performed. (B) PCR amplification of CYP24A1 and β-actin under the various treatments for 8 h and the expected transcript sizes. (C) Western blotting of VDR protein levels in HaCaT and HCT116 cell lines after VDR gene editing using the VIKING approach (11). (D) Abrogation of 1α,25(OH)2D3-mediated induction of CYP24A1 after VDR knockout in HaCaT and HCT116 cell lines. CYP24A1 induction after treatment with 1α,25(OH)2D3 for 4 h was assessed by qRT-PCR. Data are expressed as the mean±SE of two or three samples. 1α,25(OH)2D3: 1,25-dihydroxyvitamin D3. VDR KO: vitamin D receptor knockout. ACTB: β-actin. All data are representative of at least three independent experiments. Error bars in the figures indicate standard error. Comparison of mean values was done using Student’s t test, one-way analysis of variance (for non-repeated measures) and Bonferroni’s multiple comparison test. Significant differences between experimental groups are indicated with asterisks as follows: *p<0.05 and **p<0.01.
phoresis was performed using TBE buffer (89 mmol/L Tris-borate, 2 mmol/L EDTA, pH 8.3).

**Western blotting.** Cells were lysed with RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl at pH 7.5, 1% NP-40, 0.5% DOC, and 0.1% SDS) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). Total proteins (20 μg) in the cell lysates were resolved by SDS-PAGE. The following antibodies were used for Western blotting (13): anti-VDR (rabbit, 1:1,000; Cell Signaling Technology, Danvers, MA, USA), anti-α-tubulin (mouse, 1:1,000; Proteintech, Rosemont, IL, USA), anti-ERK (rabbit, 1:1,000; Santa Cruz Biotechnology, Dallas, TX, USA), and anti-phosphorylated-ERK (rabbit, 1:1,000 Santa Cruz Biotechnology).

**Luciferase assay.** HaCaT cells at 40–80% confluence were transfected with the indicated plasmids adjusted by the same total amounts with the empty vectors using the standard calcium phosphate method. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega). Renilla luciferase activity was also measured as a reference to normalize the transfection efficiencies in all experiments. All values are reported as means ±SE of at least three independent experiments. Error bars in the figures indicate standard error. Comparison of mean values was done using Student’s t test, one-way analysis of variance (for non-repeated measures) and Bonferroni’s multiple comparison test. Significant differences between experimental groups are indicated with asterisks as follows: *p < 0.05 and **p < 0.01.

**Results and Discussion**

The induction of CYP24A1 by 1α,25(OH)2D3 was assessed in HaCaT keratinocytes. The expression level of CYP24A1 in the absence of 1α,25(OH)2D3 was negligible, while 1α,25(OH)2D3 stimulation resulted in robust gene induction within 4 h (Fig. 1A). When VDR expression was inhibited by gene editing (Fig. 1C), the CYP24A1 gene induction by 1α,25(OH)2D3 was completely abrogated, but its expression level was slightly upregulated even in the absence of 1α,25(OH)2D3 (Fig. 1D). As the CYP24A1 gene locus is adjacent to a super enhancer (according to the dbSUPER database), its promoter activity is expected to be strong, considering from the 1α,25(OH)2D3-induced transcripts [presumably representing enhancer RNAs (2)] seen in the NET-
CAGE analysis of HaCaT cells (Fig. S1 (Supplemental Online Material)). However, in the absence to of 1α,25(OH)2D3, almost no CYP24A1 expression was observed (Fig. 1A). These observations suggest that VDR may act as a transcriptional repressor of the CYP24A1 promoter in skin cells. The indispensable role of VDR in CYP24A1 induction was further verified in the human colorectal cancer cell line HCT116. VDR expression was inhibited using the VIKING method, which has been applied in HaCaT cells previously (2) (Fig. 1C). As a result, the CYP24A1 induction by 1α,25(OH)2D3 was abolished. These findings suggest a pivotal role of VDR in the transcriptional regulation of CYP24A1 by VD.

Next, we evaluated whether FGF23 upregulates CYP24A1 in HaCaT cells, as previous reports demonstrated induction of CYP24A1 by FGF23 in the absence of 1α,25(OH)2D3 in HaCaT cells (12), as well as in renal tubular cells from intact animals and humans (3, 5). However, because the FGF23 treatment period was 24 h, and FGF23 induced cell proliferation, in HaCaT cells in the abovementioned study (12), it remains unclear whether FGF23 induces CYP24A1 at the transcriptional (direct) or post-transcriptional (indirect) level. To address this issue, a time-course experiment in HaCaT cells treated with FGF23 was performed to measure the expression level of CYP24A1. As shown in Fig. 2A, there was no increase in the CYP24A1 mRNA level after FGF23 treatment up to 12 h despite the presence of VDR, in contrast to the expected marked induction by VD at 4 h, peaking at 8 h. Together with the finding of almost no CYP24A1 expression in the absence of 1α,25(OH)2D3 (Fig. 1A), FGF23 does not appear to be capable of inducing CYP24A1 in HaCaT cells.

We also assessed the downstream signaling pathway activated by FGF23, as its receptor is expressed in HaCaT cells (12). In our previous work (4), we showed that FGF-induced gene expression was mediated by activation of the MAPK axis. As shown in Fig. 2B, FGF23 stimulation activated ERK, as detected by an increase in the level of phosphorylated ERK, consistent with a previous report (12). Both of αKloth and FGF receptor (FGFR) are indispensable for FGF23 signaling input in kidney and renal cells (3, 4, 14), and expectedly, expressions of αKloth and FGFR1–4 were seen in a renal cell line (HEK-293H cells) (Fig. S2 (Supplemental Online Material)), while in placenta, FGF23 action in CYP24A1 expression is inhibitory, even expressing αKloth and FGFRs (8, 9). Expressions of αKloth and FGFRs in HaCaT cells were documented in previous work (12), however, in our hand expression level of αKloth appeared marginal but with relevant expressions of FGFR2–4 (Fig. S2 (Supplemental Online Material)). The observed lack of FGF23 action in CYP24A1 induction in the present study may be owing to marginal expression level of αKloth sufficient to transmit FGF23 signal input. As CYP24A1 gene induction by VD via VDR was observed within 4 h, presumably at the transcriptional level, we speculate that regulation of CYP24A1 by FGF23 is indirect (post-transcriptional level). If this is the case, it might owe to cell fate, rather than gene regulation, in keratinocytes.

Subsequently, we examined whether phosphorus and calcium effectively modulate CYP24A1 gene expression, considering that these minerals mediate 1α,25(OH)2D3 and FGF23 gene regulation (4). As osmotic stimulation activates the MAPK signaling pathway, the total phosphorus and calcium concentrations in HaCaT cell culture medium were adjusted with Mg2+ for Ca2+ and PO4 3– for SO4 2–. However, no clear alteration in the CYP24A1 expression level was observed under the high mineral concentrations (Fig. 2C). To confirm this, CYP24A1 promoter activity was assessed by luciferase assay under the same conditions. No clear activation of CYP24A1 was seen (Fig. 2D), excluding the possibility that FGF23 indirectly regulates CYP24A1 gene expression via these minerals in this cell line. Although FGF23 regulates VD metabolism by regulating the expression of CYP24A1, the major site of this regulation appears to be renal tubular cells, but not extrarenal cells, at least not keratinocytes.

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Disclosure of state of COI
We have no conflicts of interest to declare.

Authorship
S.S., S.F., and S.K. conceived and designed the study. Y.K., A.H., T.S. and R.A. performed the experiments. T.K., Y.K., J.M. and A.H. analyzed the data. Y.K., A.H., and S.K. wrote the manuscript.

Supporting information
Supplemental Online Material is available on J-STAGE.

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