— Minireview —

Novel Metabolic Pathways of Vitamin D_3 — Identification of C-3 Epimerization and C-25 Dehydration Pathways and Biological Activity of Novel Metabolites

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The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], functions to maintain calcium and phosphorus homeostasis and plays an important role in cell proliferation and differentiation. Since the discovery of non-classical functions of 1α,25$(OH)_2D_3$, many 1α,25$(OH)_2D_3$ analogs have been synthesized to separate calcemic properties from the antiproliferative cell-differentiating properties. 1α,25$(OH)_2D_3$ and its precursor, 25-hydroxyvitamin D_3 [25$(OH)D_3$], are metabolized *via* C-24 and C-23/26 oxidation pathways. Recently, a novel A-ring modification metabolic pathway of 1α,25$(OH)_2D_3$, the C-3 epimerization pathway, was identified. In our laboratory, C-3 epimerized metabolites of major natural vitamin D_3 metabolites, 1α,25$(OH)_2D_3$, 25$(OH)D_3$ and 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$], and a synthetic analog, 22-oxacalcitriol [22-oxa-1α,25$(OH)_2D_3$, OCT], were identified. In addition, other novel metabolites of OCT were assigned to two kinds of C-25 dehydrates, 25-dehydroxy-25-ene-22-oxa-1α-hydroxyvitamin D_3 [25-ene-22-oxa-1α(OH)D_3] and 25-dehydroxy-24-ene-22-oxa-1α-hydroxyvitamin D_3 [24-ene-22-oxa-1α(OH)D_3]. In this mini-review, the identification of C-3 epimers of vitamin D_3 compounds and C-25 dehydrates of OCT using ^{1}H-NMR and LC-MS techniques is described. Furthermore, the cell-specific generation and biological activity of these novel metabolites are reviewed.

Key words ——— vitamin D_3, C-3 epimerization, C-25 dehydration, metabolism, biological activity

INTRODUCTION

Vitamin D_3 is obtained from the diet and synthesized in the skin from the precursor 7-dehydrocholesterol by exposure to sunlight. Vitamin D_3 is metabolized to 25-hydroxyvitamin D_3 [25$(OH)D_3$] in the liver and subsequently to the active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], or the inactive form of vitamin D_3, 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$], in the kidney. 1α,25$(OH)_2D_3$ plays an essential role in calcium homeostasis,[1] cell proliferation and differentiation.[2–4] 1α,25$(OH)_2D_3$ exerts its functions through the steroid hormone receptor family.[5] It is evident that a variety of cells and tissues not primarily related to calcium or bone metabolism also contain the vitamin D receptor (VDR).[6] The widespread distribution of the VDR in target cells indicates that 1α,25$(OH)_2D_3$ is an important modulator of proliferation and differentiation in normal and malignant cells. 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ are further metabolized *via* C-24 or C-23/26 oxidation pathways by 25-hydroxyvitamin D_3 24-hydroxylase (24OHase, CYP24).[7,8] The C-24 oxidation pathway, initiated by C-24 hydroxylation, yields C-24 oxo compounds and ultimately results in the formation of side-chain cleavage products.[9,10] The C-23/26 oxidation pathway, initiated by C-23 or C-26 hydroxylation, results in the production of metabolites with a lactone-ring in the side-chain.[11,12] Recently, a novel A-ring modification metabolic pathway of 1α,25$(OH)_2D_3$ was identified in human colon

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adenocartinoma cells,[13] rat osteosarcoma[14] and various cell lines,[15] which is initiated by epimerization of the hydroxyl group at C-3, the C-3 epimerization pathway. The C-3 epimerization results from a change in the orientation of the hydroxyl group at the C-3 position from the β to the α. 3-Epi-1α,25(OH)$_2$D$_3$ was also identified as a circulating metabolite of 1α,25(OH)$_2$D$_3$ in rats treated with pharmacological doses of 1α,25(OH)$_2$D$_3$.[16] It was indicated that the C-3 epimerization pathway is target cell-specific[14,15] and cell differentiation-related.[13] However, little is known about the biological functions of this metabolic pathway. We performed experiments to characterize the C-3 epimerization of natural vitamin D$_3$ metabolites, 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$,[17] as well as 1α,25(OH)$_2$D$_3$. We also examined the C-3 epimerization of a 1α,25(OH)$_2$D$_3$ analog, 22-oxacalcitriol [22-oxa-1α,25(OH)$_2$D$_3$, OCT], containing an oxygen atom at position 22 and identified another novel metabolic pathway, the C-25 dehydration pathway.[18] Furthermore, the production of the C-3 epimer and C-25 dehydrates in various cell lines and the biological activities of the novel metabolites were tested.

Identification of Novel Metabolites of Vitamin D$_3$ Compounds

After incubation of 10 μM of 1α,25(OH)$_2$D$_3$ with rat osteosarcoma-derived UMR-106 cells for 48 hr, lipid extracts from the media along with the cells were applied to a HPLC system using a silica gel column. The metabolite corresponding to an authentic standard of 3-epi-1α,25(OH)$_2$D$_3$ was observed as well as metabolites from the C-24 oxidation pathway and assigned to 3-epi-1α,25(OH)$_2$D$_3$ by ^{1}H-NMR and GC-MS analyses.[15] In addition, lipid extracts from the media and cells incubated with 10 μM of 25(OH)D$_3$ for 48 hr were applied to two kinds of HPLC systems using a silica gel column or a chiral column for the separation of 3-epi-25(OH)D$_3$ from 25(OH)D$_3$. The metabolite corresponding to an authentic standard of 3-epi-25(OH)D$_3$ was purified for structure assignments by ^{1}H-NMR spectroscopy and LC-MS analysis. The chemical shift of H-3 of the 25(OH)D$_3$ metabolite (3.86 ppm) was observed in the upfield when compared to 25(OH)D$_3$ (3.93 ppm) as well as 3-epi-25(OH)D$_3$ (3.86 ppm). Such an upfield shift appears to be responsible for the C-3 epimerization of vitamin D compounds. In the LC-MS spectra of authentic 25(OH)D$_3$ and 3-epi-25(OH)D$_3$, $[M+H]^+$ and $[M+NH_4]^+$ were observed at *m/z* 401.8 and 418.8, respectively. In the spectrum of the 25(OH)D$_3$ metabolite, $[M+H]^+$ and $[M+NH_4]^+$ were also observed at *m/z* 401.8 and 418.8, respectively. From the results of HPLC, ^{1}H-NMR and LC-MS analyses, the 25(OH)D$_3$ metabolite could be assigned to 3-epi-25(OH)D$_3$, a diastereomer of a hydroxyl group at C-3 of the A-ring of 25(OH)D$_3$. C-3 epimerization of 24,25(OH)$_2$D$_3$ was likewise observed in UMR-106 cells.[17] Higashi *et al*. also identified 3-epi-24,25(OH)$_2$D$_3$[19] and 3-epi-24,25(OH)$_2$D$_3$-24-glucuronide[20] in the plasma and bile of rats administered pharmacological doses of 24,25(OH)$_2$D$_3$. The amount of 3-epi-25(OH)D$_3$ from 25(OH)D$_3$ was similar to that of 3-epi-1α,25(OH)$_2$D$_3$ from 1α,25(OH)$_2$D$_3$ in UMR-106 cells. On the other hand, the amount of 3-epi-24,25(OH)$_2$D$_3$ was smaller than that of 3-epi-25(OH)D$_3$ and 3-epi-1α,25(OH)$_2$D$_3$ in all the cells tested.

We also examined the metabolism of a 1α,25(OH)$_2$D$_3$ analog, OCT. OCT has received government approval for use as an agent for the treatment of secondary hyperparathyroidism and psoriasis in Japan. Incubation of 10 μM of OCT with UMR-106 cells for 48 hr resulted in the formation of five metabolites. Two of the five were polar metabolites of OCT and identical to the previously identified 1α,20-dihydroxyvitamin D$_3$ [1α,20(OH)$_2$D$_3$] and 24-hydroxy-OCT [24(OH)OCT]. The other three metabolites were less polar than substrate OCT and have not been identified to date. The retention times of HPLC, ^{1}H-NMR and LC-MS spectra of one of three less polar metabolites were completely congruent with those of authentic 3-epi-OCT and this metabolite was identified as 3-epi-OCT.[18] Reddy *et al*.[21] also demonstrated that synthetic vitamin D analogs, 1α,25(OH)$_2$-16-ene-23-yne-vitamin D$_3$ and 1α,25(OH)$_2$-16-ene-23-yne-20-epi-vitamin D$_3$, are metabolized to their respective C-3 epimers in UMR-106 cells. Two other less polar metabolites of OCT were predicted to have OCT-like structure with an additional exo-methylene group at the end of the side-chain and an olefin group introduced between C-24 and C-25 as a result of dehydration at the C-25 hydroxyl group of OCT from the findings of ^{1}H-NMR.[18] In the LC-MS spectra of these less polar metabolites, $[M+NH_4]^+$ was observed at *m/z* 418.6 and indicated a reduction of 18 mass units from OCT. Thus, the two other less polar metabolites were identified as 25-dehydroxy-25-ene-22-oxa-1α-hydroxyvitamin D$_3$ [25-ene-22-oxa-1α(OH)D$_3$] and

25-dehydroxy-24-ene-22-oxa-1α-hydroxyvitamin D_3 [24-ene-22-oxa-1α(OH)D_3]. We also observed the two C-3 epimers of the C-25 dehydrates, 25-ene-3-epi-22-oxa-1α(OH)D_3 and 24-ene-3-epi-22-oxa-1α(OH)D_3, as metabolites of 3-epi-OCT. These results suggest that C-3 epimerization of the A-ring is a common metabolic pathway for the major metabolites of vitamin D_3 and synthetic analogs (Fig. 1). In addition, we demonstrated for the first time that OCT was metabolized *via* the C-25 dehydration pathway. The less polar metabolites of 1α,25$(OH)_2D_3$ corresponding to dehydrates of 1α,25$(OH)_2D_3$ were observed in UMR-106 and ROS 17/2.8 cells. However, definite structure assignments of the dehydrated metabolites of 1α,25$(OH)_2D_3$ have not been completed.

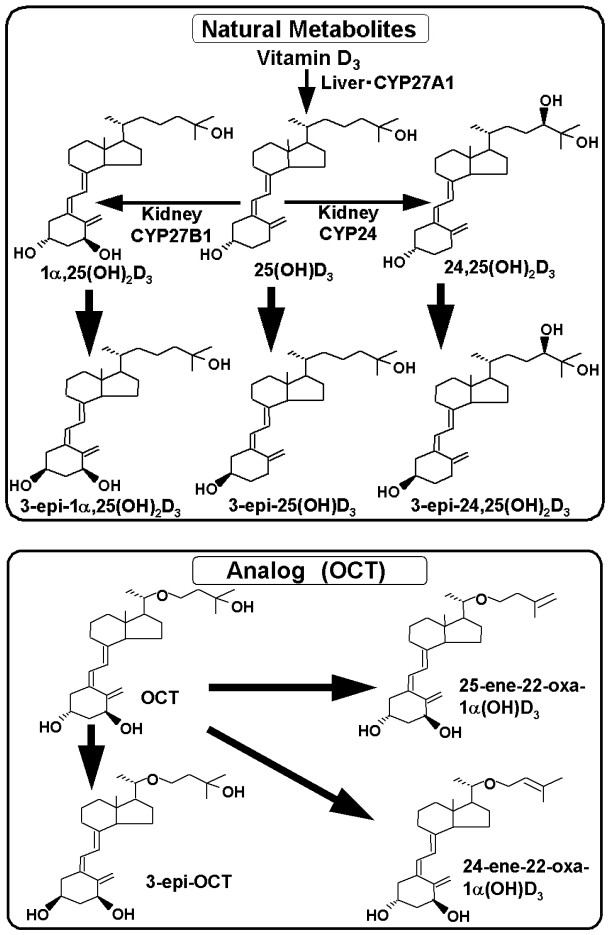

Fig. 1. Novel Metabolic Pathway of Vitamin D_3 and Synthetic Analog, OCT

Cell-Specific Generation of Novel Metabolites

When metabolism of 25$(OH)D_3$ in five kinds of cell culture systems was examined, 3-epi-25$(OH)D_3$ and 24,25$(OH)_2D_3$ were formed in all cell lines (Fig. 2). In cell cultures of UMR-106 (rat, osteosarcoma), MG-63 (human, osteosarcoma), Caco-2 (human, colon adenocarcinoma) and Hep G2 (human, hepatoblastoma), 3-epi-25$(OH)D_3$ was predominantly generated, whereas 24,25$(OH)_2D_3$ was the major metabolite in LLC-PK_1 cells (porcine, kidney). In Hep G2 cells, which generated 3-epi-25$(OH)D_3$ most abundantly among the cells, the amount of 3-epi-25$(OH)D_3$ was approximately 8-fold grater than that of 24,25$(OH)_2D_3$. This finding suggests that vitamin D_3 is metabolized to 25$(OH)D_3$ and subsequently metabolized to 3-epi-25$(OH)D_3$ in the liver. In contrast, the kidney may work in the metabolism of vitamin D compounds *via* the C-24 oxidation pathway rather than the C-3 epimerization pathway.

The production rates of OCT metabolites in UMR-106, Caco-2 and LLC-PK_1 cells were also examined. 25-Ene-22-oxa-1α(OH)D_3, 24-ene-22-oxa-1α(OH)D_3 and 3-epi-OCT were generated in all cell lines tested, although there were differences in the amounts of products formed among the cell types (Fig. 3).[18] The major metabolite found in the cell cultures of Caco-2 and LLC-PK_1 was 24(OH)OCT, whereas 25-ene-22-oxa-1α(OH)D_3 appeared to be more prevalent in UMR-106 cells. Interestingly, in UMR-106 cells the production ratio of 25-ene-22-oxa-1α(OH)D_3 to 24-ene-22-oxa-1α(OH)D_3 was 2 : 1, however in Caco-2 and LLC-PK_1 cells, the production ratios of 25-ene-22-oxa-1α(OH)D_3 to 24-ene-22-oxa-1α(OH)D_3 were 1 : 4 and 1 : 11, respectively. Therefore, the C-25 dehydration process of OCT appears to be under strict cell-specific control, or further metabolism of the dehydrates may differ with cell line.

Biological Activity of the Novel Metabolites

VDR and vitamin D binding protein (DBP) binding affinity, transcriptional activity and anti-proliferative and differentiation-inducing activity of 1α,25$(OH)_2D_3$, 25$(OH)D_3$, 24,25$(OH)_2D_3$ and their C-3 epimers are shown in Table 1.[15,17] The results indicate that the VDR binding affinities of C-3 epimers are lower than those of 1α,25$(OH)_2D_3$, 25$(OH)D_3$ and 24,25$(OH)_2D_3$, while 3-epi-25$(OH)D_3$ and 3-epi-24,25$(OH)_2D_3$ have high binding affinity to DBP and likewise for 25$(OH)D_3$ and 24,25$(OH)_2D_3$. The relative transcriptional activity on a human osteocalcin gene promoter containing vitamin D response element (VDRE) in transfected MG-63 cells was about 12% of 1α,25$(OH)_2D_3$ for 3-

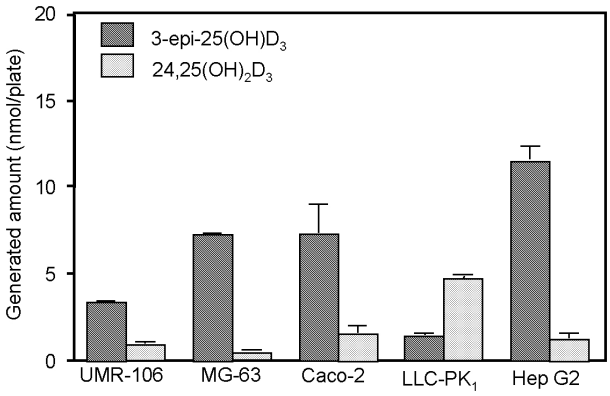

Fig. 2. Relative Amounts of Generated 25(OH)D_3 Metabolites in UMR-106, MG-63, Caco-2, LLC-PK_1 and Hep G2 Cells

Each of the cells were incubated with 10 μM of 25(OH)D_3 for 48 hr. The results are expressed as the total amount of product formed in nmol/plate/48 hr and represent the mean of three experiments (values in column).

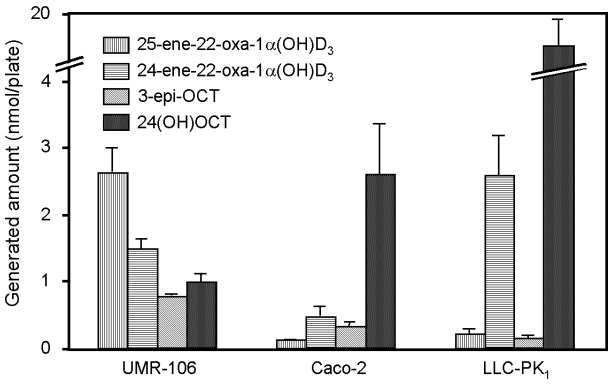

Fig. 3. Relative Amounts of Generated OCT Metabolites in UMR-106, Caco-2 and LLC-PK_1 Cells

Each cell line was incubated with 10 μM OCT for 48 hr. The results are expressed as the total amount of product formed in nmol/plate/48 hr, and represent the mean of three experiments (values in column).

epi-1α,25$(OH)_2D_3$. 25(OH)D_3 and 24,25$(OH)_2D_3$ and their C-3 epimers exhibited potencies at 10^{-6} M. The rat 24OHase gene transactivation results for the metabolites were very consistent with those for human osteocalcin gene transactivation. Relative anti-proliferative and differentiation-inducing activities were about 29 and 9% of 1α,25$(OH)_2D_3$ for 3-epi-1α,25$(OH)_2D_3$, respectively. 25(OH)D_3 and 24,25$(OH)_2D_3$ and their C-3 epimers had quite low activities in arresting the cell cycle at the G0-G1 phase and inducing cell differentiation. However, it was reported that 3-epi-1α,25$(OH)_2D_3$ was almost equipotent to 1α,25$(OH)_2D_3$ in suppressing parathyroid hormone secretion in bovine parathyroid cells [22] and in inhibiting keratinocyte proliferation,[23] and more potent than 1α,25$(OH)_2D_3$ in inducing HL-60 cell apoptosis.[24–26] In addition, a high metabolic stability of 3-epi-1α,25$(OH)_2D_3$ in target cells has been proposed by Reddy *et al.*[27] Thus, the C-3 epimerization pathway appears to play an important role not only in the regulation of intracellular concentrations of 1α,25$(OH)_2D_3$ and its analogs, but also in the formation of metabolite(s) with a different biological activity profile.

The biological activities of OCT and its less polar metabolites are shown in Table 2.[18] All of the metabolites tested had a lower binding affinity for VDR than OCT. The relative binding affinities were only 0.13, 0.30 and 0.47% of OCT for 25-ene-22-oxa-1α(OH)D_3, 24-ene-22-oxa-1α(OH)D_3 and 3-epi-OCT, respectively. All of the metabolites tested had an extremely low binding affinity for DBP, like OCT. At 10^{-8} M, the transcription-inducing activities on a human osteocalcin gene promoter in transfected MG-63 cells were about 7, 24 and 32% of OCT for 25-ene-22-oxa-1α(OH)D_3, 24-ene-22-oxa-1α(OH)D_3 and 3-epi-OCT, respectively. Similarly, at 10^{-8} M,

Table 1. Biological Activity of C-3 Epimers of Vitamin D_3 Metabolites

	VDR binding affinity[a]	DBP binding affinity[a]	Transcriptional activity: human osteocalcin gene[b]	Transcriptional activity: rat 24OHase gene[b]	Anti-proliferative activity[b]	Differentiation-inducing activity[b]
1α,25$(OH)_2D_3$	100	100	100	100	100	100
3-epi-1α,25$(OH)_2D_3$	2.1	46	12.1	13.4	29	9.4
25(OH)D_3	0.1	9091	1.4	2.6	2.2	1.1
3-epi-25(OH)D_3	0.003	3273	< 0.5	< 0.8	< 1.5	< 0.3
24,25$(OH)_2D_3$	0.03	7182	0.9	2.3	3.9	0.9
3-epi-24,25$(OH)_2D_3$	0.0005	4364	< 0.5	< 0.8	< 1.5	< 0.3

a)The values represent the relative binding affinity to 1α,25$(OH)_2D_3$ (expressed as 100), calculated from the concentration of each compound needed to achieve 50% displacement of [^{3}H]-1α,25$(OH)_2D_3$ from VDR or DBP. *b*) The values are expressed as percentage activity (at 50% of the dose–response) in comparison with 1α,25$(OH)_2D_3$ (expressed as 100% activity).

Table 2. Biological Activity of Novel OCT Metabolites

	VDR	Transcriptional activity		Differentiation-inducing activity[b]
	binding affinity[a]	human osteocalcin gene[b]	rat 24OHase gene[b]	
OCT	100	100	100	100
25-ene-22-oxa-1α(OH)D_3	0.13	7.3	2.4	13.2
24-ene-22-oxa-1α(OH)D_3	0.30	23.8	10.6	12.4
3-epi-OCT	0.47	32.0	14.9	19.0

a) The values represent the relative binding affinity to OCT (expressed as 100), calculated from the concentration of each compound needed to achieve 50% displacement of [^{3}H]-1α,25$(OH)_2D_3$ from VDR or DBP. *b*) The values are expressed as percentage activity at 10^{-8} M in comparison with OCT (expressed as 100% activity).

the transcription-inducing activities on a rat 24OHase gene promoter in transfected MG-63 cells were about 2, 11 and 15% of OCT for 25-ene-22-oxa-1α(OH)D_3, 24-ene-22-oxa-1α(OH)D_3 and 3-epi-OCT, respectively. Thus, 24-ene-22-oxa-1α(OH)D_3 and 3-epi-OCT were found to be less active than OCT, with potencies between 1/3 and 1/10 in terms of the activation of vitamin D-target genes. At 10^{-8} M, no metabolites of OCT showed significant activity in arresting the cell cycle at the G0-G1 phase compared to 1α,25$(OH)_2D_3$ and OCT. Three OCT metabolites had little inducing effect on cell surface CD11b antigen expression in a human promyelocytic leukemia cell line, HL-60. The findings of the biological studies here with two dehydrates of OCT demonstrated that their biological activities are considerably lower than OCT. Thus, it appears that like the C-23/C-24 hydroxylation pathways, the C-25 dehydration pathway contributes to reducing the high potency of OCT.

In summary, we demonstrated that both natural vitamin D_3 metabolites [25$(OH)D_3$, 1α,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$] and a synthetic 1α,25$(OH)_2D_3$ analog (OCT) were metabolized *via* the C-3 epimerization pathway. Furthermore, we also demonstrated that OCT is metabolized to two kinds of dehydrates *via* the C-25 dehydration pathway. The interplay of these metabolic pathways may be important in the regulation of vitamin D metabolism and its biological activity. To understand the biological function of these novel metabolic pathways of vitamin D, identification of the enzymes responsible for C-3 epimerization and C-25 dehydration will be required.

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