Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 89–90 (2004) 39–42
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Cell specificity and properties of the C-3 epimerization of Vitamin D_3 metabolites☆

Maya Kamao[a], Syuichiro Tatematsu[a], Natsumi Sawada[b], Toshiyuki Sakaki[b], Susumi Hatakeyama[c], Noboru Kubodera[d], Toshio Okano[a,*]

[a] *Department of Hygienic Sciences, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan*
[b] *Laboratory of Enzyme Chemistry, Kyoto University, Kitasirakawa, Sakyo-ku, Kyoto 606-8502, Japan*
[c] *Faculty of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan*
[d] *Chugai Pharmaceutical Co. Ltd., Tokyo, 2-1-9 Kyobashi, Chuo-ku, Tokyo 104-0031, Japan*

Abstract

It is well documented that Vitamin D_3 metabolites and synthetic analogs are metabolized to their epimers of the hydroxyl group at C-3 of the A-ring. We investigated the C-3 epimerization of Vitamin D_3 metabolites in various cultured cells and basic properties of the enzyme responsible for the C-3 epimerization. 1α,25-Dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], 25-hydroxyvitamin D_3 [25$(OH)D_3$] and 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] were metabolized to the respective C-3 epimers in UMR-106 (rat osteosarcoma), MG-63 (human osteosarcoma), Caco-2 (human colon adenocarcinoma), LLC-PK_1 (porcine kidney) and HepG2 (human hepatoblastoma)] cells, although the differences existed in the amount of each C-3 epimer formed with different cell types. In terms of maximum velocity (V_{max}) and Michaelis constant (K_m) values for the C-3 epimerization in microsome fraction of UMR-106 cells, 25$(OH)D_3$ exhibited the highest specificity for the C-3 epimerization among 1α,25$(OH)_2D_3$, 25$(OH)D_3$ and 24,25$(OH)_2D_3$. C-3 epimerization activity was not inhibited by various cytochrome P450 inhibitors and antiserum against NADPH cytochrome P450 reductase. Neither CYP24, CYP27A1, CYP27B1 nor 3($\alpha \rightarrow \beta$)−hydroxysteroid epimerase (HSE) catalyzed the C-3 epimerization in vitro. Based on these results, the enzyme responsible for the C-3 epimerization of Vitamin D_3 are thought to be different from already-known cytochrome P450-related Vitamin D metabolic enzymes and HSE.

Keywords: C-3 Epimerization; Cell specificity; Enzymatic properties; Vitamin D metabolism; 1α,25-Dihydroxyvitamin D_3; 25-Hydroxyvitamin D_3; 24,25-Dihydroxyvitamin D_3

1. Introduction

25-Hydroxyvitamin D_3 [25$(OH)D_3$] and 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] are metabolized via C-24 or C-23/26 oxidation pathways by 25-hydroxyvitamin D_3 24-hydroxylase (24OHase, CYP24). Recently, a novel A-ring modification metabolic pathway of 1α,25$(OH)_2D_3$ was identified in a human colon adenocarcinoma-derived cell line [1] and other cultured cells [2], which is initiated by epimerization of the hydroxyl group at C-3 of the A-ring. 3-Epi-1α,25$(OH)_2D_3$ was also identified as a circulating metabolite of 1α,25$(OH)_2D_3$ in rats treated with pharmacological doses of 1α,25$(OH)_2D_3$ [3]. 3-Epi-1α,25$(OH)_2D_3$ was almost equipotent to 1α,25$(OH)_2D_3$ in suppressing parathyroid hormone secretion [4] and more potent than 1α,25$(OH)_2D_3$ in inducing HL-60 cell apoptosis [5] regardless of lower VDR binding affinity and weak regulatory activities of cell proliferation and differentiation. 25$(OH)D_3$, 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] [6,7] and the synthetic analog, 22-oxacalcitriol [22-oxa-1α,25$(OH)_2D_3$, OCT] [8] were also metabolized through the C-3 epimerization pathway. However, little is known about the biological significance of the C-3 epimerization pathway and its related enzyme(s). We performed experiments to characterize the C-3 epimerization of Vitamin D_3 metabolites in various cultured cells and basic enzymatic properties.

2. Materials and methods

2.1. Measurement of C-3 epimerization activity in cultured cells

UMR-106 (rat osteosarcoma), MG-63 (human osteosarcoma), Caco-2 (human colon adenocarcinoma), LLC-PK_1

☆ Presented at the 12 Workshop on Vitamin D (Maastricht, The Netherlands, 6–10 July 2003).

* Corresponding author. Tel.: +81-78-441-7563; fax: +81-78-441-7565.
E-mail address: t-okano@kobepharma-u.ac.jp (T. Okano).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.03.048

(porcine kidney) and HepG2 (human hepatoblastoma) cells were incubated with 10 μM of 1α,25(OH)$_2$D$_3$, 25(OH)D$_3$ or 24,25(OH)$_2$D$_3$ for 48 h at 37 °C. Lipid was extracted from cells and medium by methanol and dichloromethane. The lipid-soluble metabolites were purified by HPLC with photodiode array detector [Zorbax SIL, hexane:2-propanol:methanol (88:10:2), 1.0 ml/min]. The same metabolites were also separated by second HPLC system [Sumichiral OA-2000, 2-propanol:hexane (96.5:3.5 or 94.5:5.5), 1.0 ml/min].

2.2. Measurement of C-3 epimerization activity in microsome fraction of UMR-106 cells

The microsome fraction containing 4.0 mg of protein prepared from UMR-106 cells were incubated with 1α,25(OH)$_2$D$_3$, 25(OH)D$_3$ or 24,25(OH)$_2$D$_3$ in the presence of NADPH-generating reagents consisting of 10 μmol of NADP, 70 μmol of glucose 6-phosphate, 12 IU of glucose-6-phosphate dehydrogenase and 100 μmol of Mg^{2+} in 50 mM potassium phosphate buffer (pH 6.5) for 1 h at 37 °C. Generated amounts of C-3 epimers were measured by HPLC and V_{max} and K_m values were calculated from Lineweaver–Burk plot. The effect of various cytochrome P450 inhibitors (20 μM) or antiserum against NADPH cytochrome P450 reductase was also examined.

2.3. Measurement of C-3 epimerization activity in expression systems of human CYP24, CYP27A1, CYP27B1 and HSE

The expression plasmids for human CYP24 [9], CYP27A1 [10] or CYP27B1 [11] were constructed, transfected to *Escherichia coli* cells and the membrane fractions of recombinant *E. coli* cells were prepared. The C-3 epimerization activity for 25(OH)D$_3$ was measured in reconstituted system consisting of the membrane fractions and 0.5 mmol of NADPH in 100 mM Tris–HCl buffer (pH 7.4) for 1 h at 37 °C. Cos-7 (African green monkey kidney, SV40-transformed) cells were transfected with the expression vector pcDNA 3.1 (–)-3($\alpha \rightarrow \beta$)-hydroxysteroid epimerase (HSE) [12] and incubated with 25(OH)D$_3$, 3-epi-25(OH)D$_3$, androsterone or epi-androsterone for 48 h at 37 °C. The metabolites were separated by HPLC or TLC.

3. Results

3.1. Cell specificity of the C-3 epimerization of Vitamin D$_3$ metabolite in cultured cells

All the cells exhibited the C-3 epimerization of 1α,25(OH)$_2$D$_3$, 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$, although 25(OH)D$_3$ was also metabolized to 24,25(OH)$_2$D$_3$ beside 3-epi-25(OH)D$_3$ (Fig. 1). The C-3 epimerization activity for

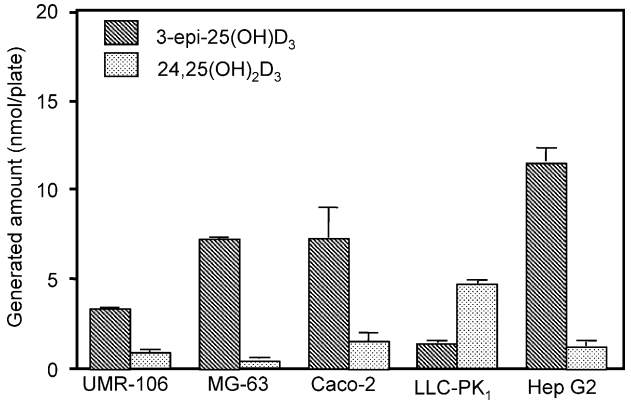

Fig. 1. Relative generated amounts of 25(OH)D$_3$ metabolites in UMR-106, MG-63, Caco-2, LLC-PK$_1$ and HepG2 cells. Each of the cells were incubated with 10 μM of 25(OH)D$_3$ for 48 h. The results are expressed as the total amount of product formed in μg/plate/48 h and represent the mean of three experiments (values in column).

24,25(OH)$_2$D$_3$ was commonly lower than that of 25(OH)D$_3$ and 1α,25(OH)$_2$D$_3$ in all the cells tested. In UMR-106, MG-63, Caco-2 and HepG2 cells, 3-epi-25(OH)D$_3$ was predominantly generated, whereas 24,25(OH)$_2$D$_3$ was the most major metabolite in LLC-PK$_1$ cells. HepG2 cells was the most active among the cells to metabolize 25(OH)D$_3$ to 3-epi-25(OH)D$_3$ and the generated amount of 3-epi-25(OH)D$_3$ was about eight-fold larger than that of 24,25(OH)$_2$D$_3$.

3.2. Basic properties of C-3 epimerization in microsome fraction of UMR-106 cells

In the microsome fraction of UMR-106 cells, V_{max} values for 1α,25(OH)$_2$D$_3$, 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$ were 1.52, 2.34 and 0.51 pmol/min/mg protein, respectively (Table 1). K_m values for 1α,25(OH)$_2$D$_3$, 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$ were 98.9, 73.7 and 200.1 μM, respectively. These results indicate that 25(OH)D$_3$ is the substrate with the highest specificity for the C-3 epimerization reaction among the three metabolites. C-3 epimerization activity for 25(OH)D$_3$ was not inhibited by commonly used cytochrome P450 inhibitors (ketoconazole, 1-aminobenzotriazol, benzilimidazole, methoxalen, metyrapon and SKF-525A), CYP2D6 specific inhibitor (qunidine), CYP3A4 specific inhibitor (troleandomycin) and antiserum against NADPH cytochrome P450 reductase.

Table 1
Kinetic parameters for C-3 epimerization

Substrate	V_{max} (pmol/min/mg protein)	K_m (μM)	V_{max}/K_m (pmol/min/mg protein/μM)
1α,25(OH)$_2$D$_3$	1.52	98.9	0.015
25(OH)D$_3$	2.34	73.7	0.032
24,25(OH)$_2$D$_3$	0.51	200.1	0.0025

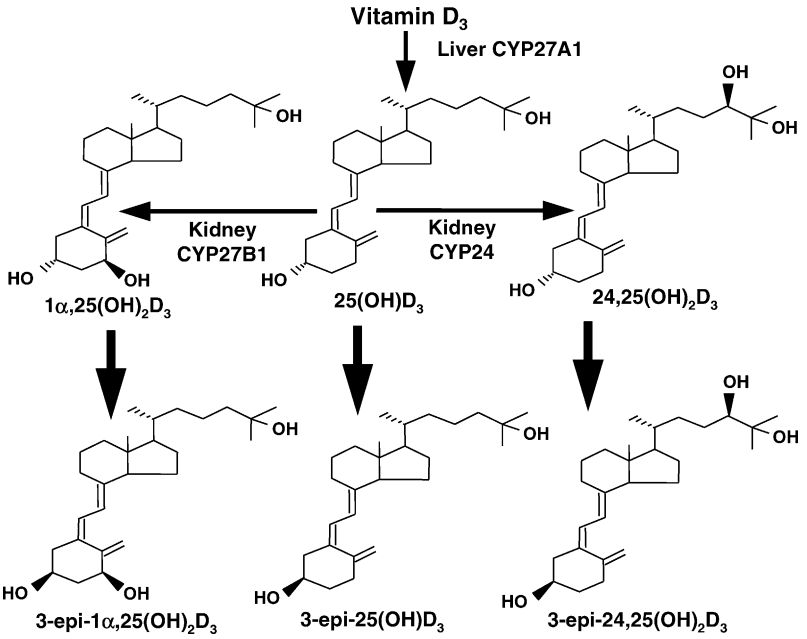

Fig. 2. C-3 epimerization pathway of major Vitamin D_3 metabolites.

3.3. C-3 epimerization activity in expression systems of human CYP24, CYP27A1, CYP27B1 and HSE

In the reconstituted system containing the membrane fractions prepared from the *E. coli* expressing CYP24, CYP27A1 or CYP27B1, 25(OH)D_3 was metabolized to 1α,25(OH)$_2D_3$ or 24,25(OH)$_2D_3$. However, the generation of 3-epi-25(OH)D_3 was not observed in all the systems tested. In Cos-7 cells transfected HSE expression vector, androsterone (3α) and epi-androsterone (3β) were converted to epi-androsterone (3β) and androsterone (3α), respectively. However, C-3 epimerization of 25(OH)D_3 was not changed in the HSE transfected Cos-7 cells when compared to the Cos-7 cells transfected with the expression vector only.

4. Discussion

The present study clearly indicate that UMR-106, MG-63, Caco-2, LLC-PK_1 and HepG2 cells possess the ability to metabolize 1α,25(OH)$_2D_3$, 25(OH)D_3 and 24,25(OH)$_2D_3$ to their C-3 epimers (Fig. 2). Thus, the C-3 epimerization appears to be a common metabolic pathway for the major Vitamin D_3 metabolites and may play an important role in the regulation of Vitamin D_3 metabolism. We also demonstrated that 25(OH)D_3 was better substrate for C-3 epimerization than 1α,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$. From this finding, it appears that the enzyme responsible for the C-3 epimerization can recognize the differences in the side chain and the A-ring structure of Vitamin D. In addition, since C-3 epimerization was not catalyzed by CYP24, CYP27A1, CYP27B1 and HSE, the C-3 epimerization enzyme for Vitamin D_3 metabolites are thought to be different from already-known cytochrome P450-related Vitamin D metabolic enzymes and HSE.

Acknowledgements

This work was supported in part by a grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, a Grant for Cooperative Research administered by the Japan Private School Promotion Foundation and a grant-in-aid from the Ministry of Health and Welfare of Japan.

References

[1] M.G. Bischof, M.–L. Siu-Caldera, A. Weiskopf, P. Vouros, H.S. Cross, M. Peterlok, G.S. Reddy, Differentiation-related pathways of 1α,25-dihydroxycholecalciferol metabolism in human colon adenocarcinoma-derived Caco-2 cells: production of 1α,25-dihydroxy-3-epi-cholecalciferol, Exp. Cell. Res. 241 (1998) 194–201.
[2] S. Masuda, M. Kamao, N.J. Schroeder, H.L.J. Makin, G. Jones, R. Kremer, J. Rhim, T. Okano, Characterization of 3-epi-1α,25-dihydroxyvitamin D_3 involved in 1α,25-dihydroxyvitamin D_3 metabolic pathway in cultured cell lines, Biol. Pharm. Bull. 23 (2000) 133–139.
[3] H. Sekimoto, M.-L. Siu-Caldera, A. Weiskopf, P. Vouros, K.R. Muralidharan, W.H. Okamura, M.R. Uskokovic, G.S. Reddy, 1α,25-Dihydroxy-3-epi-vitamin D_3: in vivo metabolite of 1α,25-dihydroxyvitamin D_3 in rats, FEBS Lett. 448 (1999) 278–282.
[4] A.J. Brown, C. Ritter, E. Slatopolsky, K.R. Muralidharan, W.H. Okamura, G.S. Reddy, 1α,25-dihydroxy-3-epi-vitamin D_3, a natural metabolite of 1α,25-dihydroxyvitamin D_3, is a potent suppressor of parathyroid hormone secretion, J. Cell Biochem. 73 (1999) 106–113.
[5] K. Nakagawa, Y. Sowa, M. Kurobe, K. Ozono, M.-L. Siu-Caldera, G.S. Reddy, M.R. Uskokovic, T. Okano, Differential activities of

1α,25-dihydroxy-16-ene-vitamin D_3 analogs and their 3-epimers on human promyelocytic leukemia (HL-60) cell differentiation and apoptosis, Steroids 66 (2001) 327–337.

[6] T. Higashi, R. Kikuchi, K. Miura, K. Shimada, H. Hiyamizu, H. Ooi, Y. Iwabuchi, S. Hatakeyama, N. Kubodera, New metabolic pathway of (24R)-24,25-dihydroxyvitamin D_3: epimerization of the 3-hydroxy group, Biol. Pharm. Bull. 22 (1999) 767–769.

[7] M. Kamao, S. Tatematsu, G.S. Reddy, S. Hatakeyama, M. Sugiura, N. Ohashi, N. Kubodera, T. Okano, Isolation, identification and biological activity of 24*R*,25-dihydroxy-3-epi-vitamin D_3: a novel metabolite of 24*R*,25-dihydroxyvitamin D_3 produced in rat osteosarcoma cells (UMR 106), J. Nutr. Sci. Vitaminol. 47 (2001) 108–115.

[8] M. Kamao, S. Tatematsu, S. Hatakeyama, K. Ozono, N. Kubodera, G.S. Reddy, T. Okano, Two novel metabolic pathways of 22-oxacalcitriol (OCT), J. Biol. Chem. 278 (2003) 1463–1471.

[9] T. Sakaki, N. Sawada, K. Komai, S. Shiozawa, S. Yamada, K. Yamamoto, Y. Ohyama, K. Inoue, Dual metabolic pathway of 25-hydroxyvitamin D_3 catalyzed by human CYP24, Eur. J. Biochem. 267 (2000) 6158–6165.

[10] N. Sawada, T. Sakaki, M. Ohta, K. Inoue, Metabolism of Vitamin D_3 by human CYP27A1, Biochem. Biophys. Res. Commun. 273 (2000) 977–984.

[11] N. Sawada, T. Sakaki, S. Kitanaka, K. Takeyama, S. Kato, K. Inoue, Enzymatic properties of human 25-hydroxyvitamin D_3 1α-hydroxylase, Eur. J. Biochem. 265 (1999) 950–956.

[12] X.-F. Huang, V. Luu-The, Molecular characterization of a first human 3($\alpha \rightarrow \beta$)-hydroxysteroid epimerase, J. Biol Chem. 275 (2000) 29452–29457.