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Metabolism of ^{3}H-1α,25-Dihydroxyvitamin D_3 in Cultured Human Keratinocytes

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Abstract In the present investigation we studied the metabolism of 1α,25-dihydroxy-[1β-^{3}H]vitamin D_3 (^{3}H-1,25$(OH)_2D_3$) in culture-grown human keratinocytes (CHK). Our results showed that the cellular uptake of ^{3}H-1,25$(OH)_2D_3$, upon incubation with CHK, occurred very rapidly; and it paralleled a decrease in the concentration of ^{3}H-1,25$(OH)_2D_3$ in the medium. The amount of ^{3}H-calcitroic acid, on the other hand, increased slowly in the medium, while the concentration of ^{3}H-calcitroic acid in the cell remained undetectable during the whole period of incubation. When the cells were preincubated with 1,25$(OH)_2D_3$ (10^{-8}M), conversion of ^{3}H-1,25$(OH)_2D_3$ to ^{3}H-calcitroic acid increased almost twofold, indicating that 1,25$(OH)_2D_3$ catalyzed its own catabolism.

Key words: cultured human keratinocytes, CHK, ^{3}H-1α,25-dihydroxyvitamin D_3, ^{3}H-calcitroic acid, cellular uptake, metabolism

Multiple physiologic roles of the calciotropic hormone 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] are well-recognized [Holick, 1989]. These properties are dependent on its concentration in circulation which is under strict physiologic control. The circulating concentration of 1,25$(OH)_2D_3$, is also dependent on its catabolic degradation ultimately to calcitroic acid, a C_{23}-acid with four fewer carbon atoms in the side chain than the parent compound, i.e., 1,25$(OH)_2D_3$ [Esvelt et al., 1979].

While the in vivo production of 1,25$(OH)_2D_3$ is known to take place almost exclusively in the kidney [Fraser and Kodicek, 1970], the in vitro production of 1,25$(OH)_2D_3$ has been reported in culture-grown cells from various organs and tissues such as sarcoid tissue [Barbour et al., 1981; Mason et al., 1984], bone cells [Howard et al., 1981], placenta [Whisett et al., 1981], and skin [Bikle et al., 1986; Holick et al., 1987]. The metabolic activation/degradation of 1,25$(OH)_2D_3$ is well-known to be a stepwise process involving numerous metabolic intermediates [Holick, 1989; Esvelt et al., 1979; Mayer et al., 1983]. Structural identities of many of these metabolites have been unequivocally determined, and as a result, biosynthetic pathway from 1,25$(OH)_2D_3$ to calcitroic acid has been established in rat kidney perfusates [Reddy and Tserng, 1989] and in bone cells [Makin et al., 1989].

As a part of our ongoing interest in studying the effects of 1,25$(OH)_2D_3$ on human skin [Smith et al., 1986] we examined the metabolism of ^{3}H-1,25$(OH)_2D_3$ in human skin cells (keratinocytes) grown in culture. Results of these studies are described herein.

MATERIALS AND METHODS

1α,25-Dihydroxy-[1β-^{3}H]vitamin D_3 (specific activity 15 Ci/mmol) was synthesized according to a method developed in our laboratory [Holick et al., 1980]. HPLC solvents were obtained from E. Merck and Co. (Jamestown, NJ). HPLC analyses were carried out on a Waters HPLC system (Millipore Co., Milford, MA) connected directly to a Radiomatic Flo-One Beta On-line radioactivity detector (Radiomatic Instruments Inc., Tampa, FL). 1,25$(OH)_2D_3$ and its metabolites were generous gifts from Drs. Milan Uskokovic (Hoffman La Roche Inc., Nutley, NJ), G. Satya Reddy (Womens and Infants Hospital, Providence, RI), and Glenville Jones (Queen's University, Ontario, Canada). These standard samples

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were used to identify various radioactive elution peaks in the HPLC chromatograms of the experimental samples.

Cell Culture and Incubation Methods

Keratinocytes were grown in culture using a modification of the method by Rheinwald and Green [Rheinwald and Green, 1975]. 3T3 cells were plated at 10^4 cells per 35 mm tissue-culture dish, and were irradiated lethally after 2 days with a ^{60}Co source (5,000 rads). Keratinocytes were obtained from neonatal foreskin after overnight trypsinization at 4°C and treatment with 0.2% EDTA. The cells, in 1 ml of serum-free medium, were plated on the lethally irradiated 3T3 cells. Each experiment was performed on primary or secondary keratinocyte cultures obtained from different skin samples. The serum-free medium consisted of MCDB 153 medium (Sigma Chemical Co., St. Louis, MO) with additives and calcium (0.15 mM). The cells were grown to 50–60% confluence, when medium was removed and replaced with 1 ml of fresh medium containing either ethanol (0.1% v/v) or $1,25(OH)_2D_3$ (10^{-8}M in ethanol). The cells were incubated at 37°C for 4 h followed by the removal of the media. One milliliter of fresh medium containing an ethanolic solution of ^3H-$1,25(OH)_2D_3$ (0.2 μCi) was added to each of all the plates which were further incubated for designated periods. After the specified time either medium was removed and methanol was added to terminate the reactions, or methanol was added to the cells in the medium. This procedure was applied to cells in four dishes for each time point. Control experiments were carried out by boiling a set of cells in a microwave oven for 2 minutes followed by the addition of ^3H-$1,25(OH)_2D_3$ (0.2 μCi) and incubation for the maximum time period.

Procedures for the Lipid Extraction of Cells and HPLC Analysis of the Extract

After the designated time of incubation the medium was removed from four plates and the cells were lysed by the addition of methanol (one ml). The lysed cells were extracted with methanol-chloroform. The chloroform-methanol extracts of the cells were dried over anhydrous magnesium sulfate and evaporated to dryness with nitrogen. The medium from each sample was extracted in the same fashion as with the cell samples. In some cases medium was not removed prior to the addition of methanol, and the whole mixture was extracted with chloroform-methanol. For each experiment, aliquots from aqueous layers from cell and medium samples, after methanol-chloroform extraction, were mixed with scintillation cocktail and counted for radioactivity in a β-liquid scintillation counter. The amount of radioactivity present in the aqueous solution was insignificant for incubations up to 2 h. With increasing time of incubation the amount of radioactivity in the aqueous solutions increased slowly to a maximum of 10% of the total. This was probably due to the presence of highly polar and water-soluble metabolites of ^3H-$1,25(OH)_2D_3$ in the incubates. Although the majority of radioactivity was found in the organic extracts of the cells and their medium, the total amount varied from sample to sample. It is likely that the total number of cells in each dish differed accounting for the variability.

HPLC analysis of the extracts were carried out using ECONOSPHERE C_{18} cartridge (Altech Associates, State College, PA) and a binary solvent system consisting of acetonitrile/water/glacial acetic acid (40:60:1) for 20/25 min followed by acetonitrile/water/glacial acetic acid (90:10:1) for an additional 20 min with a constant flow rate of 2 ml/min. In the case of radioactive samples, effluents from the HPLC were directly fed into the on-line radioactivity detector. Standard samples (nonradioactive) of $1,25(OH)_2D_3$ and its metabolites were used in the HPLC analyses to identify various radioactive peaks which were integrated by a data analyzer. Integration for each peak was taken as the relative amount of the metabolite present in the experimental mixture. For each time point, HPLC analyses were carried out on organic-extracts from four samples in each group. Although time-course profiles for all the samples were strikingly similar, the absolute amount of any particular metabolite present in a sample often varied significantly from another, which precluded us from taking averages of several samples. This was probably due to the presence of varying number of cells in different dishes as discussed earlier. Hence the time-course profiles included in this communication are representative of the four individual HPLC analyses for each group. Control experiments, which involved killing the cells by heating followed by incubation with ^3H-$1,25(OH)_2D_3$, did not show any appreciable degradation/metabolism as determined by HPLC analysis.

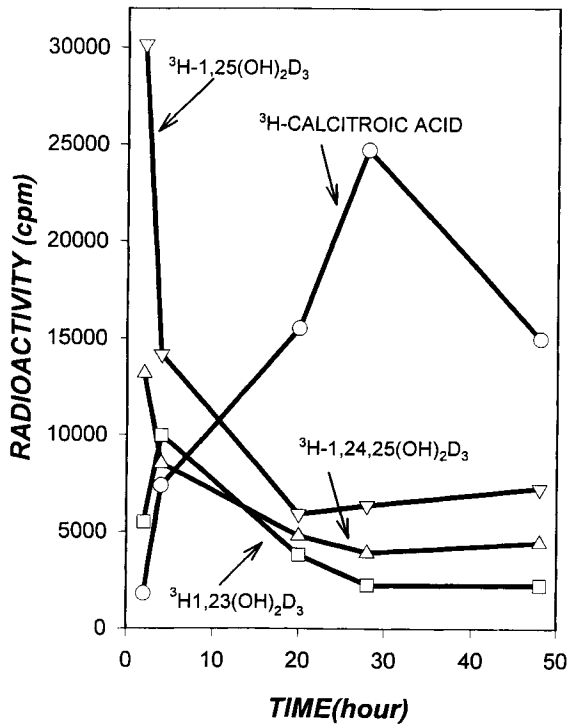

Fig. 1. Time-course for the metabolic degradation of ^{3}H-1,25-dihydroxyvitamin D$_3$ in cutured human keratinocytes (CHK). CHK samples, incubated with ^{3}H-1,25-dihydroxyvitamin D$_3$, were lipid-extracted and analyzed by reverse phase HPLC with a C$_{18}$-column and an acetonitrile-water-acetic acid binary solvent system. Radioactivity in the samples was monitored by on-line radioactivity detection. Peaks corresponding to standard metabolite samples were integrated and their concentrations (radioactivity) were plotted against the duration of incubation.

RESULTS AND DISCUSSION

Biosynthesis of calcitroic acid is a stepwise process which is initiated by the introduction of a hydroxyl group at the C-24 position of 1,25(OH)$_2$D$_3$ molecule leading to the formation of 1,24,25-trihydroxyvitamin D$_3$ [1,24,25(OH)$_3$D$_3$], the latter is sequentially metabolized to 1,25-dihydroxy-24-oxo-vitamin D$_3$, 1,23,25-trihydroxy-24-oxo-vitamin D$_3$, 24,25,26,27-tetranor-1,23-dihydroxyvitamin D$_3$ [1,23(OH)$_2$D$_3$] and finally to calcitroic acid [Reddy and Tserng, 1989; Makin et al., 1989]. Thus, concentrations of some or all of these key intermediates in a mixture at various time points could be used as a guide to determine the rate of metabolic transformations of 1,25(OH)$_2$D$_3$. We used standard samples of 1,24,25(OH)$_3$D$_3$, 1,23(OH)$_2$D$_3$ and calcitroic acid, which were available to us, to chart a time-course for the degradation of ^{3}H-1,25(OH)$_2$D$_3$ in cultured human keratinocytes (CHK).

We incubated CHK with ^{3}H-1,25(OH)$_2$D$_3$ for various time periods ranging from 2 to 48 h in order to determine a time course at which various metabolites of ^{3}H-1,25(OH)$_2$D$_3$ were formed/degraded. At the end of each incubation each sample of cells from a group of four was lysed and the mixture (cell and medium) was extracted with chloroform/methanol. The lipid-extract from each experiment was analyzed by HPLC. As shown in Figure 1, where concentrations of various metabolites are plotted against time, there is a sharp decline in the concentration of ^{3}H-1,25(OH)$_2$D$_3$ within the first four hours of incubation. This decline correlated well with the appearance and then the decline of ^{3}H-1,24,25(OH)$_3$D$_3$ and ^{3}H-1,23(OH)$_2$D$_3$. ^{3}H-calcitroic acid appeared in the reaction mixture

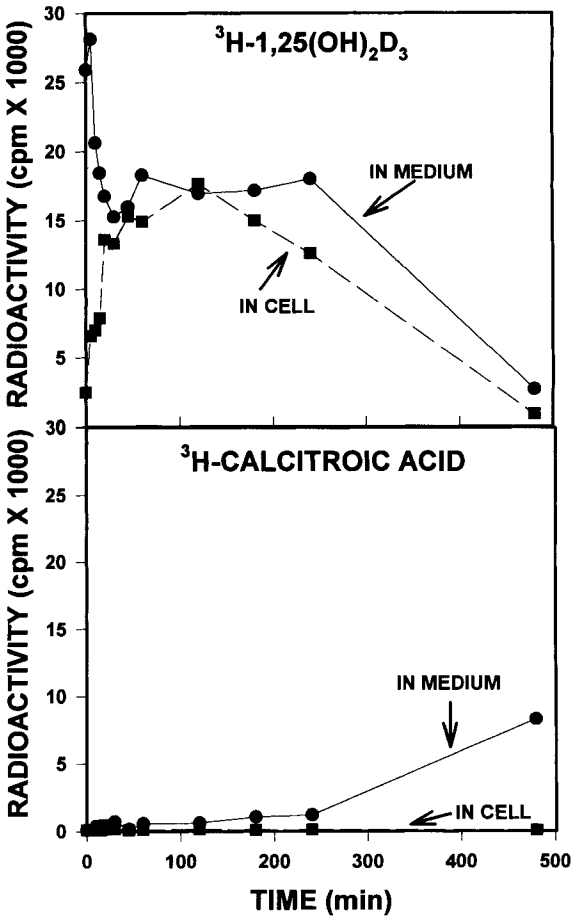

Fig. 2. Time-course for the conversion of ^{3}H-1,25(OH)$_2$D$_3$ to ^{3}H-calcitroic acid in cellular and medium fractions of keratinocytes incubated with ^{3}H-1,25(OH)$_2$D$_3$ from 0 through 500 min followed by lipid-extraction and HPLC-analysis/on-line radioactivity detection. **Top panel:** Concentration of ^{3}H-1,25(OH)$_2$D$_3$ in cell and medium samples. **Bottom panel:** Concentration of ^{3}H-calcitroic acid in cell and medium samples.

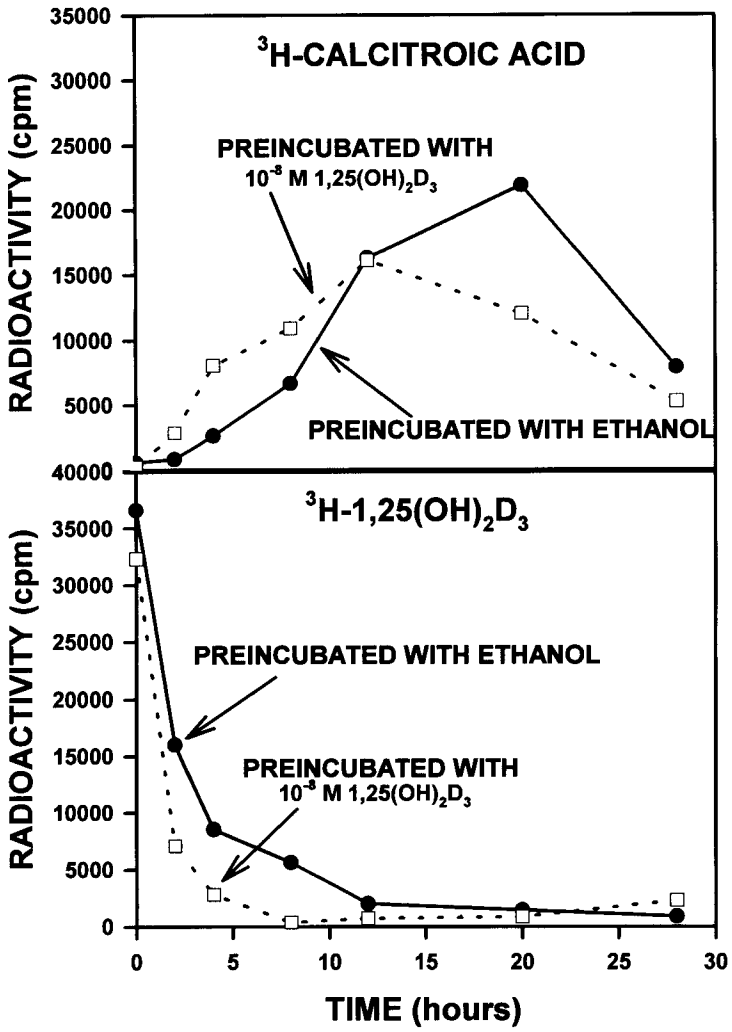

Fig. 3. Time-course for the conversion of ^{3}H-1,25(OH)$_2$D$_3$ to ^{3}H-calcitroic acid in keratinocytes preincubated with 10^{-8} M of 1,25(OH)$_2$D$_3$ or ethanol. Following the incubation for specified times, the samples (cell and medium together) were extracted with chloroform/methanol and analysed by HPLC/on-line radioactivity detection. **Top panel:** Concentration of ^{3}H-calcitroic acid in cells preincubated with 10^{-8} M of 1,25(OH)$_2$D$_3$ or ethanol. **Bottom panel:** Concentration of ^{3}H-1,25(OH)$_2$D$_3$ in in cells preincubated with 10^{-8} M of 1,25(OH)$_2$D$_3$ or ethanol.

within two hours and reached a peak at approximately 28 h.

It is clear from Figure 1 that most ^{3}H-1,25(OH)$_2$D$_3$ was metabolized within the first 2–10 h of incubation. Hence we carried out another set of experiments where incubation-time was considerably decreased (5 min to 8 h). Furthermore, we separated each incubate into cell and medium fractions following the incubation, and extracted them separately for HPLC analysis. Results of these experiments are shown in Figure 2. The amount of ^{3}H-1,25(OH)$_2$D$_3$ increased rapidly inside the cells during the first thirty minutes representing the cellular uptake, and it paralleled a decrease in the concentration of ^{3}H-1,25(OH)$_2$D$_3$ in the medium during the same period (Fig. 2, top panel). After the initial 30 min, the amount of ^{3}H-1,25(OH)$_2$D$_3$ did not change substantially for the ensuing 2 and 3 h in the cell and medium respectively. After this time concentration of ^{3}H-1,25(OH)$_2$D$_3$ decreased rapidly in both fractions, and at the end of 8 h, concentrations of ^{3}H-1,25(OH)$_2$D$_3$ in cell and medium fractions were extremely low.

The concentration of ^{3}H-calcitroic acid in the medium, on the other hand, slowly increased during the ensuing 8 h of incubation. The concentration of ^{3}H-calcitroic acid in the cells was

very low throughout the incubation period, and remained low even after 48 h of incubation (Fig. 2, bottom panel).

We were also interested in determining whether preincubation of the cells with 1,25(OH)$_2$D$_3$ had any effect on the catbolism of ^{3}H-1,25(OH)$_2$D$_3$. One set of cells were incubated with 1,25(OH)$_2$D$_3$ (10^{-8} M) for 4 h followed by the addition of ^{3}H-1,25(OH)$_2$D$_3$, while the other set was incubated with ethanol (vehicle) for the same period of time prior to the addition and incubation with ^{3}H-1,25(OH)$_2$D$_3$. After the designated time of incubation, methanol was added to the samples; and they were extracted with chloroform/methanol as described earlier. As shown in Figure 3 (top panel), preincubation of the cells with 1,25(OH)$_2$D$_3$ (10^{-8}M) significantly enhanced the degradation of ^{3}H-1,25(OH)$_2$D$_3$ so that the concentration of ^{3}H-calcitroic acid increased rapidly and reached its maximum within approximately 12 h. The same was reached for ethanol-treated cells at approximately 20 h (Fig. 3, top panel). On the other hand, concentration of ^{3}H-1,25(OH)$_2$D$_3$ declined sharply and reached a minimum at approximately 8 h for the 1,25(OH)$_2$D$_3$-treated cells. For the ethanol-treated cells, the initial sharp decline (in the concentration of ^{3}H-1,25(OH)$_2$D$_3$) was followed by a shallow gradient, and the minimum was reached between 12 through 17 h. These results demonstrate that preincubation of the cells with 1,25(OH)$_2$D$_3$ significantly enhanced the catabolism of ^{3}H-1,25(OH)$_2$D$_3$. It is plausible that preincubation of the cells with 1,25(OH)$_2$D$_3$ accelerated the induction of the oxidative enzymes responsible for the degradation of ^{3}H-1,25(OH)$_2$D$_3$. It is noteworthy that addition of ^{3}H-1,25(OH)$_2$D$_3$ to 1,25(OH)$_2$D$_3$-treated cells did not significantly add to the mass of 1,25(OH)$_2$D$_3$ that was presented to the cells. This was due to the relatively high specific activity of ^{3}H-1,25(OH)$_2$D$_3$ (15 Ci/mmol), which minimized the kinetic isotope effect, and rendered the kinetic data obtained by us more realistic.

For the past several years our laboratory has been conducting biochemical and clinical studies to determine the effects of 1,25(OH)$_2$D$_3$ on human skin, as well as developing 1,25(OH)$_2$D$_3$ as a therapeutic agent for treating hyperproliferative skin disorders such as psoraisis [Holick et al., 1987; Smith et al., 1988]. These studies often involved supraphysiological doses of 1,25(OH)$_2$D$_3$ with potential side effects. However, to date we have failed to notice any untoward side effects in patients treated with topical doses of 1,25(OH)$_2$D$_3$ [Smith et al., 1988]. The topical application of 1,25(OH)$_2$D$_3$ possibly triggers a rapid degradation of 1,25(OH)$_2$D$_3$ so that the potential toxic side effects including hypercalciurea and hypercalcemia are not observed.

The results included in this communication clearly demonstrate that culture-grown skin cells, obtained from normal human subjects, are capable of metabolizing ^{3}H-1,25(OH)$_2$D$_3$ to calcitroic acid in a time-dependent manner.

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