Volume 282, number 2, 409–411 FEBS 09712 May 1991

ADONIS 001457939100422Y

Cultured human keratinocytes cannot metabolize vitamin D_3 to 25-hydroxyvitamin D_3

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Received 19 November 1990; revised version received 8 February 1991

The metabolism of [^{3}H]vitamin D_3 was studied in cultured human keratinocytes (CHK). Intact CHK were incubated for 1, 6, 12, 24 and 48 h with [^{3}H]vitamin D_3 and the lipid soluble fractions from the media and cells were extracted by high-performance liquid chromatography (HPLC). Vitamin D_3 and its metabolites, 25-OH-D_3, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ were added to the extracts, as markers, prior to HPLC. HPLC analysis of the lipid extracts did not reveal any monohydroxylated metabolites. CHK incubated for one hour with [^{3}H]25-OH-D_3 showed a 10±4% conversion to [^{3}H]1,25$(OH)_2D_3$ whereas no conversion to [^{3}H]1,25$(OH)_2D_3$ was observed in control CHKs that were boiled prior to incubation with [^{3}H]25-OH-D_3. These findings suggest that cultured neonatal keratinocytes are incapable of metabolizing vitamin D_3 to 25-OH-D_3.

Cultured human keratinocyte; Vitamin D_3; 25-Hydroxyvitamin D_3

1. INTRODUCTION

When human skin is exposed to sunlight, vitamin D_3 is produced [1]. Once formed, vitamin D_3 is transported via a vitamin D-binding protein to the liver where it is metabolized to 25-OH-D_3 which is further metabolized in the kidney to 1,25$(OH)_2D_3$, the most biologically active form of vitamin D_3 [2].

Receptors for 1,25$(OH)_2D_3$ have been demonstrated in cultured human and mouse keratinocytes [3, 4] and in cultured human dermal fibroblasts (CHDF) [3, 5, 6]. Incubation of both cultured human keratinocytes [CHK] and CHDF with 1,25$(OH)_2D_3$ caused a dose-dependent decrease in cell proliferation and in CHK, an increase in differentiation [7].

Cultured neonatal keratinocytes metabolize 25-OH-D_3 to 1,25$(OH)_2D_3$ [8]. Since the skin is the site for vitamin D_3 synthesis and a target organ for its 1,25-dihydroxy metabolite, it was of interest to determine whether CHK could also metabolize vitamin D_3 to 25-OH-D_3.

2. METHODS AND MATERIALS

2.1. *Cultured human keratinocytes*

Cultured human keratinocytes were grown in serum-free conditions as previously described [7]. Briefly, the medium consisted of Dulbecco's modified Eagle's medium (DMEM) with 0.15 mM calcium (M.A. Bioproducts, Walersville, MD, USA) containing 7 growth factors: transferrin (5 μg/ml), epidermal growth factor (25 ng/ml), hydrocortisone (203 mg/ml), insulin (5 μg/ml), prostaglandin E-1 (50 ng/ml), cholera toxin (0.1 μg/ml, Sigma, St. Louis, MO, USA), and selenous acid (2 ng/ml, Collaborative Research, Lexington, MA, USA). Penicillin G (75 U/ml) and streptomycin (50 ng/ml) were also added to the cultures. Keratinocytes were isolated from human neonatal foreskin according to a modification of the method of Rheinwald and Green [9]. For the experiments studied, fresh medium containing vehicle alone (0.01% ethanol) or one of the following compounds 1,25$(OH)_2D_3$, 24,25$(OH)_2D_3$ (gifts from Dr. M. Uskokovic of Hoffmann-La Roche, Nutley, New Yersey), 25-OH-D_3 (a gift from J. Babcock of Upjohn, Kalamazoo, Michigan) or vitamin D_3 (Sigma, St. Louis, MO) was added to each culture. Radioactive vitamin D_3 was from Amersham, TRK 346, Batch 33, [1α,2α-^{3}H]vitamin-D_3, 18.3 Ci/mmol.

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2.2. *Incubation of cultured human keratinocytes with [^{3}H]vitamin D_3*

Triplicate 35 mm cultures of CHK that were approximately 80% confluent were incubated with 100000 cpm of [^{3}H]vitamin D_3 for 1, 6, 12, 24, or 48 h. The medium was collected and the cells washed 2 times with medium which was combined with the first. Lipid extracts were obtained separately from the cells and the medium by adding 7 ml of methanol and 5 ml of H_2O and then transferring the mixture to a separating flask. Methylene chloride (4 ml) was added to the flask until a precipitate formed, and then an additional 16 ml was added. After mixing, the lipid-soluble (bottom) layer was removed and dried with nitrogen. To this, 20% IPA/hexane was added and the mixture was dried down with nitrogen. Cold standards were added to the extract (16 ng/ml of 1,25$(OH)_2D_3$, 35 ng/ml of 25-OH-D_3, 1 ng/ml of vitamin D_3 and 20 ng/ml 24,25$(OH)_2D_3$) prior to analysis by HPLC. Samples were eluted at a flow rate of 2.5 ml/min with a step-gradient of isopropanol in *n*-hexane on a 25×4.5 mm Zorbx-sil column as follows; 1.25% from 0 to 15 min; 2.5% from 16 to 35 min, and 5% from 36 to 70 min. The various standards were monitored by their UV absorbance at 254 nm. One ml fractions were collected and counted in a liquid scintillation counter. This system was used to separate the vitamin D_3, 25-OH-D_3, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$. For the study of the conversion of 25-OH-D_3 to 1,25$(OH)_2D_3$, the step-gradient of isopropanol in *n*-hexane was changed to 2.5% for 0 to 40 min and 5% for 41 to 70 min and the flow rate was 2.0 ml/min.

3. RESULTS

To be certain that [^{3}H]vitamin D_3 entered the keratinocytes, the cells were incubated with [^{3}H]vitamin D_3 and then at various times the cells and medium were harvested. At 1, 12 and 24 h after its addition, 28 ± 5%, 50 ± 9% and 61 ± 6%, respectively, of the [^{3}H]vitamin D_3 entered the cells and was available to intracellular enzymes. Accumulation of [^{3}H]vitamin D_3 peaked at 24 h with no significant difference from 24 (61 ± 6%) to 48 h (66 ± 8%), $P>0.05$. At all times tested, 88 + 6% of the radioactivity was recovered from both the cells and the medium as [^{3}H]vitamin D_3. The other 12 ± 4% was recovered in the water soluble phase of the extraction. A comparison of HPLC chromatograms of the standards (Fig. 1A) with the lipid extracts of cells (Fig. 1B, C) and medium (Fig. 1D, E) following incubation with [^{3}H]vitamin D_3 for 1 h (Fig. 1B, D) and 12 h (Fig. 1C, E) demonstrated that there was no metabolism of vitamin D_3 to known mono- and di-hydroxylated metabolites. Similar results were obtained from both cells and medium for the 15 min, 6 and 48 h incubations (data not shown). To be certain that the CHK retained the capacity to metabolize 25-OH-D_3, CHK were incubated, in triplicate, with [^{3}H]25-OH-D_3 for 1 h. As seen in Fig. 2A, 10% of the [^{3}H]25-OH-D_3 converted to [^{3}H]1,25(OH)$_2D_3$. The overall conversion for the triplicate samples was 10 ± 4%. The [^{3}H]1,25(OH)$_2D_3$ peak was collected and shown to migrate with 1,25(OH)$_2D_3$ standard (data not shown). CHKs that were boiled prior to incubation with [^{3}H]25-OH-D_3, demonstrated no conversion to [^{3}H]1,25(OH)$_2D_3$ (Fig. 2B).

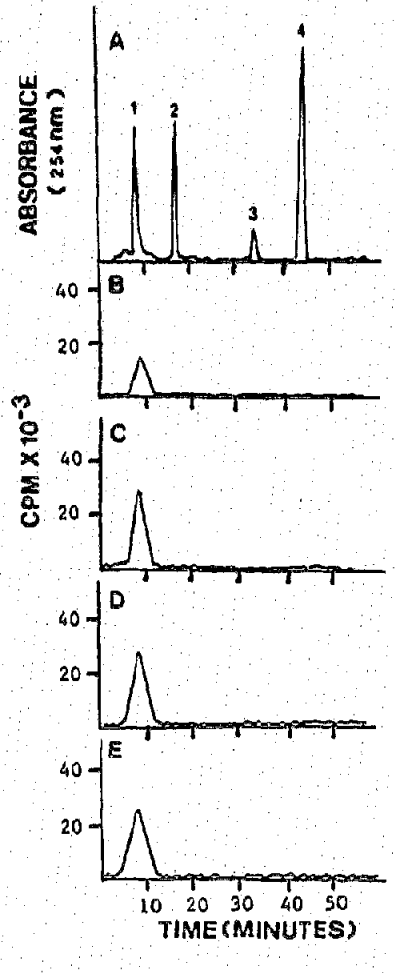

Fig. 1. Representative chromatograms from standards (A, 1, vitamin D_3; 2, 25-OH-D_3; 3, 24,25(OH)$_2D_3$; 4, 1,25(OH)$_2D_3$) and cultured human keratinocytes (B, C) and the incubation medium (D, E) after incubation with [^{3}H]vitamin D_3 (100 000 cpm) for 1 (B, D) and 12 h (C, E). The cells and the medium were kept separate and the lipid fractions were extracted and separated on HPLC (see section 2). 1 ml fractions were collected and counted on liquid scintillation counter.

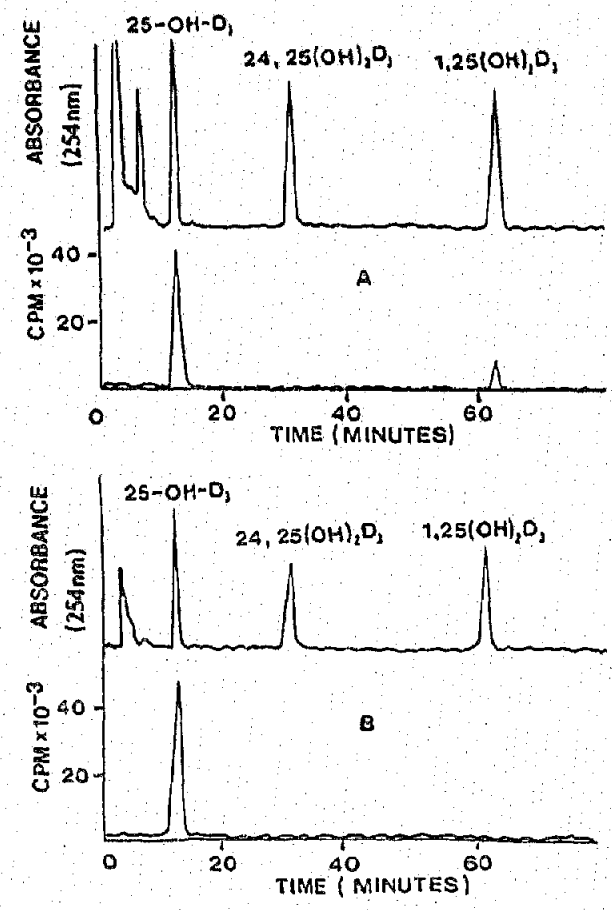

Fig. 2. Lipid extracts of cultured human keratinocytes (A) after 1 h incubation with [^{3}H]25-OH-D_3 at 37°C and analyzed by HPLC (see section 2). 1 ml fractions were collected and counted in a scintillation counter. The tritiated polar metabolite peak (rt = 61 min) co-eluted with standard 1,25(OH)$_2D_3$. Fig. 2B is an HPLC chromatogram of the lipid extract of keratinocytes that have been boiled prior to incubation with [^{3}H]25-OH-D_3. There was no conversion of the [^{3}H]25-OH-D_3 to [^{3}H]1,25(OH)$_2D_3$.

4. DISCUSSION

Investigators have shown that CHKs [3] possess nuclear receptors for $1,25(OH)_2D_3$ and that human skin may be a target tissue for $1,25(OH)_2D_3$ [6,7]. CHK metabolize 25-OH-D_3 to both $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ [7,8].

It has previously been shown [10] that hepatectomized, vitamin-D-deficient rats that received vitamin D_3, maintained low, but detectable levels of 25-OH-D_3. This suggests that the liver was not the sole site for the metabolism of vitamin D_3 to 25-OH-D_3. Our results demonstrate that CHK, which convert 25-OH-D_3 to $1,25(OH)_2D_3$, cannot metabolize vitamin D_3 to 25-OH-D_3. These results suggest that the human epidermis is unable to metabolize sunlight induced vitamin D_3 to $1,25(OH)_2D_3$.

Acknowledgement: This work was supported by NIH Grant AR36963.

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