METABOLISM OF 25-HYDROXY-VITAMIN D_3 BY PRIMARY CULTURES OF CHICK KIDNEY CELLS

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Received November 29,1976

Summary

The primary culture of kidney cells from vitamin D deficient chicks is described. After four days in culture the cells reach confluency and retain their ability to metabolize 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3. Addition of one unit of bovine parathyroid hormone to the culture medium for 48 hours prior to assay had no effect on the cells' ability to produce 1,25-dihydroxy vitamin D_3, whereas after 24 hours in the presence of 5×10^{-8} M 1,25-dihydroxyvitamin D_3 the cells produced not this metabolite, but 24,25-dihydroxyvitamin D_3. This cell culture system will allow the investigation of the regulation of renal 25-hydroxyvitamin D_3 metabolism under controlled in vitro conditions.

The role of the kidney in the conversion of 25-hydroxyvitamin D_3 to $1,25\text{-}(OH)_2D_3$[1] (1-3) and $24,25\text{-}(OH)_2D_3$ (4) is now well established. Also well recognized is that the renal enzyme, 25-OH-D_3-1-hydroxylase, is subject to strong regulation such that under conditions of ample calcium availability the enzyme is present at very low activity, while under conditions of restricted availability of calcium, kidney levels of 25-OH-D_3-1-hydroxylase activity increases. The benefit to the organism of this adaptive response is clear in that in the latter situation, more $1,25\text{-}(OH)_2D_3$, the biologically active form of the steroid, is made available to the intestine where the absorption of dietary calcium can be increased.

What is not clear, in spite of intensive study by several investigators, are which factors mediate these fluctuations in the 25-OH-D_3-1-hydroxylase and the mechanisms by which they operate. Among those factors which have been suggested as regulators of the 25-OH-D_3-1-hydroxylase are serum calcium

[1] Abbreviations used are: 25-hydroxyvitamin D_3 (25-OH-D_3); 1,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2D_3$); 24,25-dihydroxyvitamin D_3 ($24,25\text{-}(OH)_2D_3$); parathyroid hormone (PTH).

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levels (5), serum or renal tissue phosphorous concentrations (6), parathyroid hormone (7-9) calcitonin (10) and vitamin D or its metabolites (9-12). Much of the experimental evidence for these suggestions has been obtained *in vivo* and it has proved difficult to thoroughly substantiate any of them *in vitro*. A whole cell preparation of renal tubule fragments has been described (13) which has been the subject of several studies of the 25-OH-D_3-1-hydroxylase (14-17). These studies have yielded interesting but conflicting results. A major disadvantage of this system is the short time scale (3-6 hours) over which the cells are viable and experiments can be carried out and the fact that during at least part of the experimental period, one may be dealing with a dying population of cells.

For these reasons, the development of the culture kidney cells which could be studied in a steady state is an attractive approach to the investigation of the renal metabolism of 25-OH-D_3. The present report describes such a system with the important attribute that the 25-OH-D_3-1-hydroxylase remains activ e after several days in culture and appears to be subject to regulation by at least one of the factors known to affect it *in vivo*, 1,25-$(OH)_2D_3$.

Methods

Three week old chicks, fed a rachitogenic diet (18) from the day of hatching, were sacrificed and thoroughly washed with 70% ethanol. The kidneys were perfused with 0.9% NaCl and removed, under sterile conditions, to a petri dish containing a buffer of the following composition: 137 mM NaCl; 4 mM KCl; 0.4 mM NaH_2PO_4; 0.2 mM KH_2PO_4; 9.4 mM $NaHCO_3$; 11 mM glucose. Gentle teasing with forceps at reduced the tissue to pieces approximately 1 mm which were incubated at 37°C with 60 mg collagenase (Sigma, Type I) and 40 mg hyaluronidase (Sigma, Type I) for 15 minutes. Gentle pipetting (no more than 3-4 passes) aided in tissue dispersion. At this time, 2.5% trypsin (GIBCO) was added to a final concentration of 0.25% and the incubation continued for five minutes more. Cells and tubule fragments were removed by centrifugation, resuspended in and washed several times in GIBCO medium 199 before the final suspension in the medium was obtained. Large fragments were removed by allowing them to settle to the bottom of the tube between centrifugations. Cell number in the final suspension was determined with a hemacytometer and viability was estimated by the ability of the cells to exclude trypan blue dye.

Falcon flasks (75 cm^2) were inoculated with 15 ml medium 199 containing 2-4x10^5 cells/ml and 15% fetal calf serum (GIBCO). After 24 hours incubation at 37°C in an atmosphere of 5% CO_2/95% air, the medium, containing unattached and dead cells was replaced with fresh medium. Medium was changed again at 72 hours following the initiation of the cultures.

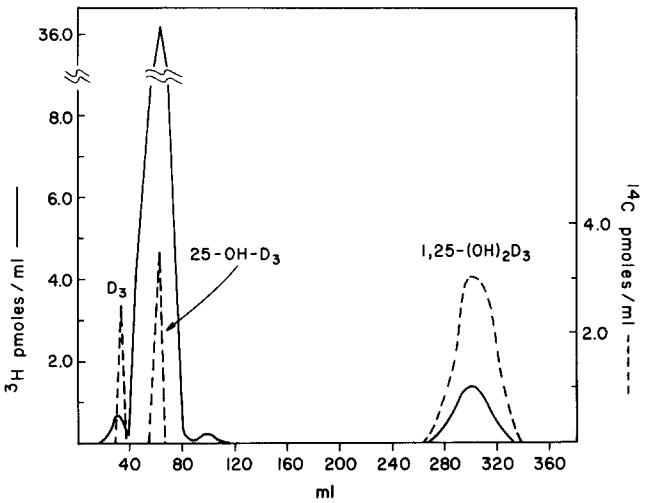

Figure 1. Production of 1,25-(OH)$_2$-[^{3}H]-D$_3$ by freshly isolated chick kidney cells. Cells were incubated with 25-OH-[^{3}H]-D$_3$ for one hour and the lipid extract was chromatographed on Sephadex LH-20 as described in Methods. The solid line represents the elution pattern of tritium while the dashed line indicates the elution position of the co-chromatographed ^{14}C-labeled D$_3$, 25-OH-D$_3$ and 1,25-(OH)$_2$D$_3$.

The ability of the cultures to metabolize 25-OH-D$_3$ was determined by replacing the growth medium with 10 ml of medium 199 and adding, in 0.05 ml ethanol, 1 nmole 25-hydroxy-[26,27-^{3}H]-vitamin D$_3$ (Amersham-Searle; 70 mCi/mmole). After a one hour incubation at 37°C, lipids were extracted from the medium and cells according to the method of Bligh and Dyer (19). Extracts were chromatographed on 1x80 cm Sephadex LH-20 columns in 35% hexane in CHCl$_3$. The elution positions of 25-OH-[^{3}H]-D$_3$ and 1,25-(OH)$_2$-[^{3}H]-D$_3$ were determined by co-chromatography with ^{14}C-labeled vitamin D metabolites prepared as described previously (20). The amount of radioactivity in each sample was determined in a Beckman LS-200 liquid scintillation counter with the use of an external standard measure efficiency.

Crystalline, chemically synthesized 1,25-(OH)$_2$D$_3$ was a generous gift of M. Uskokovic, Hoffman-LaRoche. Partially purified bovine parathyroid hormone (580 U/mg) was purchased from Wilson Laboratories.

Results and Discussion

In five preparations, the yield of kidney cells from the isolation procedure described above was 5-9 x 10^7 cells per gram of kidney tissue with 80-90% viability as judged by the ability of the cells to exclude trypan blue dye. The majority of cells were present as single cells and although some occurred as pairs and triplets, there were no tubule fragments or clumps of more than 4-5 cells.

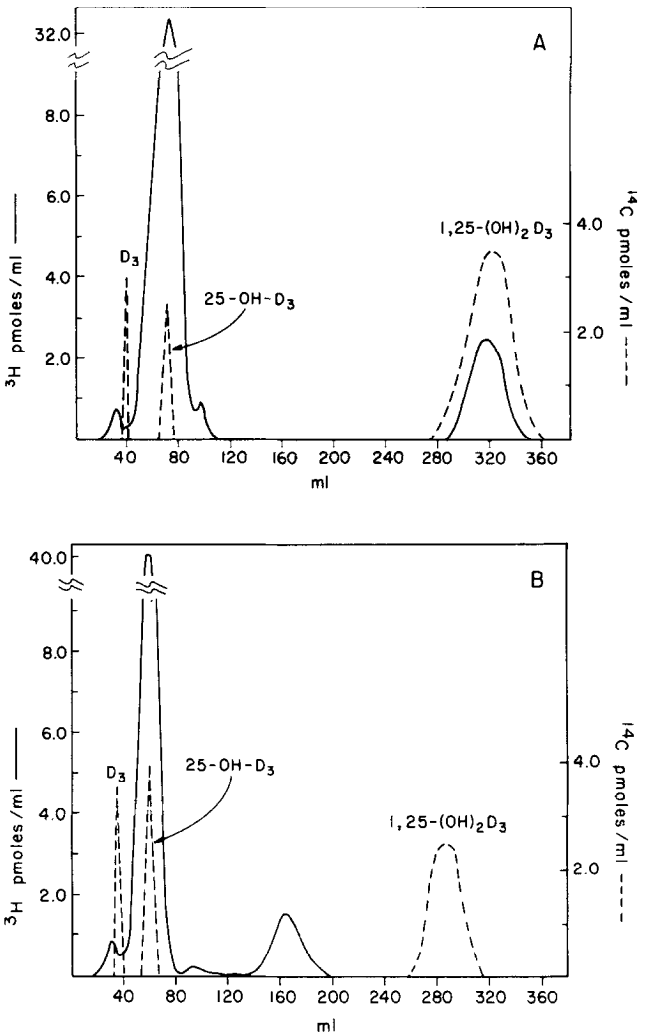

Figure 2. Metabolism of 25-OH-[^{3}H]-D$_3$ by chick kidney cells after four days in culture in the absence (A; top) and presence (B; bottom) of 5×10^{-8} M 1,25-(OH)$_2$-D$_3$ for 24 hours prior to assay. As in Figure 1, the solid line is the metabolite pattern produced by the cells and the dashed line is the elution profile of co-chromatographed ^{14}C-labeled D$_3$, 25-OH-D$_3$, and 1,25-(OH)$_2$D$_3$.

The ability of the final cell suspension to metabolize 25-OH-[^{3}H]-D$_3$ was tested by incubating 5×10^6 cells in 5 ml of medium 199 at a substrate concentration of 10^{-7} M. Lipids were extracted and chromatographed as described in Methods, and a typical result is shown in Figure 1. Under these conditions, the percentage of radioactivity occurring in the 1,25-(OH)$_2$-[^{3}H]-D$_3$

peak varied from 4-8%. No other metabolites were detected and recovery of radioactivity from the column was 95-105%.

Culture medium was replaced at 24 and 72 hours often initiation of cultures and confluency was always reached within 96 hours. As shown in Figure 2A, the ability of the cells to metabolize 25-OH-[^{3}H]-D_3 remained after 4 days in culture. As in the case of cells assayed prior to culture, no metabolites other than 1,25-$(OH)_2D_3$ were observed.

As shown in Figure 2B when 5 x 10^{-8} M 1,25-$(OH)_2D_3$ was added to the culture for 24 hours prior to assay (i.e ,from 72 to 96 hours of culture) the cells produced upon incubation with 25-OH-[^{3}H]-D_3, no 1,25-$(OH)_2$-[^{3}H]-D_3, but a substantial amount (7.1% of the total radioactivity) of a metabolite migrating in the position of 24,25-$(OH)_2D_3$ (4). The results shown in Figure 2A and 2B indicate that not only is the 25-OH-D_3-1-hydroxylase activity retained by cultured chick kidney cells but that it remains subject to modulation by at least one of the factors known to affect it *in vivo*, 1,25-$(OH)_2D_3$ (9,11,12).

In view of the importance of parathyroid hormone (PTH) in the regulation of calcium metabolism and the possibility that this peptide hormone may be important in the regulation of the 25-OH-D_3-1-hydroxylase, it was of interest to determine whether an effect of PTH on 1-hydroxylation of 25-OH-D_3 could be demonstrated in the cultured cells. A partially purified preparation of bovine PTH was added at a concentration of 1 unit/ml (approximately 6x10^{-8} M) at 48 and 72 hours after the initiation of the cultures and the cells were assayed as described above at 96 hours. Vehicle was added to control cultures. No effect of PTH was observed on either the growth rate or morphology of the cells during the 48 hours of its presence in the cultures. The chromatograms obtained after incubation of the cells with 25-OH-[^{3}H]-D_3 were similar to those observed in Figures 1 and 2A and the results are summarized in Table 1. No metabolites other than 1,25-$(OH)_2$-[^{3}H]-D_3 were observed and, as seen in Table 1, in three separate experiments PTH did not influence the ability of the cells to produce 1,25-$(OH)_2D_3$.

Table I. Production of 1,25-Dihydroxyvitamin D_3 by Cultured Chick Kidney Cells in the Presence and Absence of Parathyroid Hormone

	% of Radioactivity as $1,25\text{-}(OH)_2\text{-}[^3H]\text{-}D_3$		
	Exp. 1	Exp. 2	Exp. 3
+PTH	4.0	4.0	3.5
-PTH	3.4	4.0	3.7
+PTH/-PTH x 100	118	100	95

Bovine parathyroid hormone (1 unit/ml) was added to the cell cultures 48 hours prior to the assay for 25-OH-D_3-1-hydroxylation. Incubation of the cells and chromatography was carried out as described in Methods. (See Figures 1 and 2A.

The effect of PTH on the 1-hydroxylation of 25-OH-D_3 is generally held to be a positive one (7-9) and the failure to demonstrate this effect under the present conditions could be the result of a number of factors. For example, perhaps a higher concentration of the hormone is required, although the concentration of PTH chosen is similar to those used to elicit resorption of bone in organ culture (21,22). Alternatively, these kidney cells, isolated from vitamin D deficient and presumably hyperparathyroid chicks, may already be expressing their maximum capacity for 1-hydroxylation which cannot, therefore, be increased by an additional stimulus. Experiments are currently underway to test these possibilities.

In summary, a chick renal cell culture system has been described which shows great potential for the investigation of the metabolism of 25-OH-D_3 to $1,25\text{-}(OH)_2D_3$ and its regulation. The importance of this metabolic step to the maintenance of calcium homeostasis makes the elucidation of the mechanisms of its control a critical area of investigation. The ability to carry out this investigation under controlled conditions *in vitro* will clearly contribute to our understanding of calcium metabolism in the whole organism.

Acknowledgements

This work was supported in part by USPHS grant AM-09012 and AM-14,750. The author thanks Ms. June Bishop for excellent technical assistance and Professor A. Karu for invaluble advice and is especially grateful to Professor A. W. Norman for support and advice throughout the investigation.

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