Effect of Oestrogen and 1,25-Dihydroxycholecalciferol on 25-Hydroxycholecalciferol Metabolism in Primary Chick Kidney-Cell Cultures

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Primary cultures of chick kidney cells convert 25-hydroxycholecalciferol into more-polar metabolites. Cells from vitamin D-deficient chicks have high 25-hydroxycholecalciferol 1 α-hydroxylase (1 α-hydroxylase) activity, but no 25-hydroxycholecalciferol 24-hydroxylase (24-hydroxylase) activity. Physiological concentrations of 1,25-dihydroxycholeclaciferol suppress 1α-hydroxylase and induce 24-hydroxylase activity. The inhibition of 1α-hydroxylase preceded the induction of 24-hydroxylase. In contrast, oestradiol-17β had no effect on the activity of either hydroxylase under a variety of experimental conditions. These results clearly demonstrate that 1,25-dihydroxycholecalciferol, but not oestrogen, acts directly on the kidney cells to regulate the metabolism of 25-hydroxycholecalciferol.

Cholecalciferol (vitamin D_3) is converted into $25(OH)D_3$ in the liver (Horsting & DeLuca, 1969) and other tissues (Tucker et al., 1973). This metabolite is further hydroxylated in the kidney (Fraser & Kodicek, 1970), either by 1α-hydroxylase (25-hydroxycholecalciferol 1-hydroxylase) to $1,25(OH)_2D_3$, the most active form of the vitamin, or by 24-hydroxylase (25-hydroxycholecalciferol 24-hydroxylase) to $24,25(OH)_2D_3$, a metabolite of unknown physiological significance.

It is generally agreed that $1,25(OH)_2D_3$ is a Ca^{2+}-regulating hormone, with the kidney as the endocrine organ and the intestine, bone and possibly kidney as target tissues. The enzyme system, 1α-hydroxylase, responsible for the production of $1,25(OH)_2D_3$, has been shown to be modulated by ionic and hormonal factors such as Ca^{2+} (Boyle et al., 1971), phosphate (Tanaka & DeLuca, 1973), parathyrin (Garabedian et al., 1972), prolactin (Spanos et al., 1976a), growth hormone (MacIntyre et al., 1977), oestrogens (Tanaka et al., 1976) and $1,25(OH)_2D_3$ (MacIntyre et al., 1976).

Oestrogens and $1,25(OH)_2D_3$ have been reported to exert opposite effects on the activity of the $25(OH)D_3$ hydroxylases of the kidney. The suppression of 1α-hydroxylase and induction of 24-hydroxylase activities caused by administration of $1,25(OH)_2D_3$ in vivo led Colston et al. (1977) to suggest that $1,25(OH)_2D_3$ acts directly on the kidney cells to regulate its own production. The profound stimulation of 1α-hydroxylase and concomitant suppression of 24-hydroxylase observed when large

Abbreviations used: $25(OH)D_3$, 25-hydroxycholecalciferol; $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; $24,25(OH)_2D_3$, 24,25-dihydroxycholecalciferol.

doses of oestrogens are given to chicks are thought to be of physiological significance during the reproductive period in birds (Baksi & Kenny, 1977; Tanaka et al., 1976).

The objective of the present study was to investigate whether the well-documented effects of oestrogens and $1,25(OH)_2D_3$ in vivo could also be demonstrated in vitro. To do this we have developed a primary chick kidney-cell culture system that enabled us to study the long-term effects of these steroid hormones on the $25(OH)D_3$ hydroxylases of the kidney under controlled conditions in vitro.

Materials and Methods

Culture medium (Eagle's minimum essential medium with Earle's salts), foetal calf serum, trypsin, antibiotic (penicillin and streptomycin) solution and tryptose phosphate broth were purchased from Gibco–Biocult, Paisley, Renfrewshire, Scotland, U.K. Oestradiol-17β was from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Sephadex LH-20 was bought from Pharmacia (G.B.), London W.5, U.K. The Radiochemical Centre, Amersham, Bucks., U.K., supplied 25-hydroxy-[26,27-Me-^{3}H]cholecalciferol (initial sp. radioactivity 9Ci/mmol, but diluted to 160mCi/mmol with crystalline 25-hydroxycholecalciferol). Synthetic vitamin D_3 metabolites were a gift from Dr. N. T. Pollitt, Roche Products, P.O. Box 8, Welwyn Garden City, Herts., U.K.

Cell cultures

Cockerels (4 weeks old; Rhode Island Red × Light Sussex crossbreed; Orchard Farm, Great Missenden,

Bucks., U.K.), fed on a vitamin D-deficient diet (0.54% Ca^{2+}, 0.34% phosphate) from the day of hatching, were killed and washed with 70% (v/v) ethanol. The kidneys were removed aseptically and washed with phosphate-buffered saline [Dulbecco solution A, consisting of NaCl (8g/litre), KCl (0.2g/litre), Na_2HPO_4 (1.15g/litre) and KH_2PO_4 (0.2g/litre)], pH 7.2. The tissue was dissected into small fragments, washed twice with phosphate-buffered saline and incubated at 37°C in 40ml of 0.05% trypsin in phosphate-buffered saline with continuous stirring for 10min. This procedure was repeated three times. After each treatment the cell suspension was transferred to a centrifuge pot containing 10ml of foetal calf serum at 4°C. The pooled suspensions were centrifuged at 1000rev./min for 10min. The pellet was resuspended in Eagle's minimum essential medium supplemented with 8% (v/v) foetal calf serum, 10% (v/v) tryptose/phosphate broth, 60μg of penicillin/ml and 100μg of streptomycin/ml. Cells (5×10^5/ml) were seeded in 60ml flat glass medical flasks. Cell numbers were estimated by using a Neubauer haematocytometer. Cell viability was tested with Trypan Blue (Phillips, 1973) and averaged 85–90%. Unattached cells were discarded 24h after initial plating and the attached cells were washed with 2×5ml of phosphate-buffered saline. Fresh medium was added and cells reached confluency 48h later.

Enzyme assays

Confluent monolayers of chick kidney cells were incubated at 37°C for various time intervals, in 5ml of Eagle's minimum essential medium containing the substances to be tested. At the end of the incubation period, 100ng of ^{3}H-labelled 25(OH)D_3 was added to the medium. The reaction was terminated 1h later by the addition of 3vol. of methanol/chloroform (2:1, v/v) per volume of incubation medium. Lipids were extracted from the medium and cells by the method of Bligh & Dyer (1959). Extracts were chromatographed on Sephadex LH-20 columns (1.5cm × 18cm), eluted with 35% (v/v) hexane in chloroform at a constant flow rate of 0.6ml/min. The eluates were collected directly into plastic scintillation vials with an LKB 3403 fraction collector.

Enzyme activities were determined by the production of 1,25(OH)$_2$$D_3$ and 24,25(OH)$_2$$D_3$ from ^{3}H-labelled 25(OH)D_3. The amounts of 1,25(OH)$_2$$D_3$ and 24,25(OH)$_2$$D_3$ produced were calculated from the percentage of the total radioactivity in the respective peaks on the radiochromatogram.

Identification of vitamin D metabolites

The Sephadex LH-20 columns were calibrated with labelled 24,25(OH)$_2$$D_3$ and 1,25(OH)$_2$$D_3$ prepared biosynthetically from tritiated 25(OH)D_3 in homogenates from chick kidneys. Re-chromatography of

the peaks from Sephadex LH-20 corresponding to 24,25(OH)$_2$$D_3$ and 1,25(OH)$_2$$D_3$ on a Dupont 830 high-pressure liquid chromatograph showed that they were homogeneous and eluted in the same position as the synthetic metabolites.

Results

Effects of 1,25(OH)$_2$$D_3$ on the activities of 25(OH)D_3 hydroxylases of the kidney

Confluent monolayers of kidney cells from vitamin D-deficient chicks were incubated for 24h at 37°C in either the absence or the presence of synthetic 2.5nM-1,25(OH)$_2$$D_3$. Treatment with 1,25(OH)$_2$$D_3$ caused inhibition of 1α-hydroxylase activity and a marked induction of 24-hydroxylase activity (Fig. 1a). This
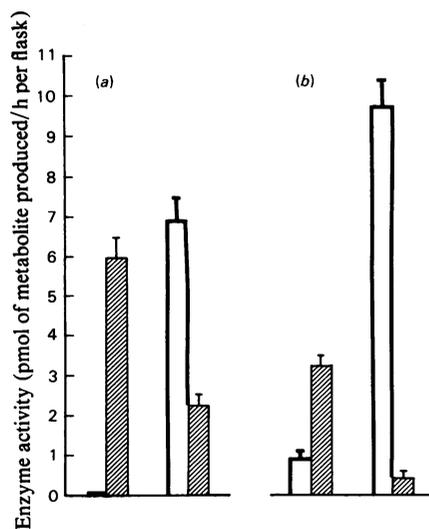

Fig. 1. *Effect of 1,25(OH)$_2$$D_3$ on the renal hydroxylases* (a) Confluent monolayers of kidney cells from vitamin D-deficient chicks were maintained in a serum-free medium and incubated for 24h at 37°C either with or without 2.5nM-1,25(OH)$_2$$D_3$. At the end of incubation 100ng of ^{3}H-labelled 25(OH)D_3 was added and enzyme reactions were allowed to proceed for 1h. Open bars represent 24-hydroxylase and hatched bars represent 1α-hydroxylase activity. Each bar represents the mean ± S.E.M. for ten determinations. Enzyme activity is expressed in pmol of metabolite produced/h per flask. (b) Confluent monolayers of kidney cells from vitamin D-deficient chicks were maintained in a serum-free medium containing 2.5nM-1,25(OH)$_2$$D_3$. Then 24h later the medium was discarded, and the cells were washed twice with phosphate-buffered saline and incubated for another 24h in fresh medium either with or without 2.5nM-1,25(OH)$_2$$D_3$. Open bars represent 24-hydroxylase activity and hatched bars represent 1α-hydroxylase activity. Each bar represents the mean ± S.E.M. for five determinations.
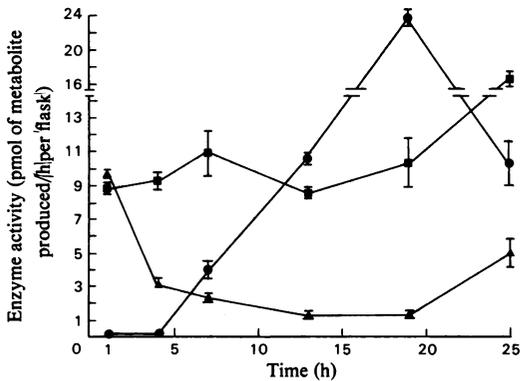
Fig. 2. *Time–response curve of the effects of* 1,25(OH)$_2D_3$
on the renal hydroxylases
Confluent monolayers of kidney cells from vitamin
D-deficient chicks were incubated at 37°C for dif-
ferent time intervals in 5 ml of a serum-free medium
with or without the presence of 25 nM-1,25(OH)$_2$D$_3$.
Symbols: ■, controls that had only 1α-hydroxylase
activity; ▲, 1α-hydroxylase activity; ●, 24-hydroxyl-
ase activity. Data are means±S.E.M. for five to six
determinations.

enzyme pattern was reversed when 1,25(OH)$_2$D$_3$ was
removed from the medium: 24-hydroxylase activity
declined and 1α-hydroxylase activity was stimulated
(Fig. 1b). The effects of 1,25(OH)$_2$D$_3$ studied at dif-
ferent time intervals are shown in Fig. 2. Control cells
had 1α-hydroxylase activity only, with higher values
observed during longer incubation periods. Treat-
ment for 4h with 1,25(OH)$_2$D$_3$ caused inhibition of
1α-hydroxylase, but had no effect on 24-hydroxylase.
After 7h treatment both inhibition of 1α-hydroxylase
and induction of 24-hydroxylase were observed.
Maximal effects on both enzymes were observed after
19h incubation with 1,25(OH)$_2$D$_3$.

A dose–response study revealed that the renal
hydroxylases responded to sub-physiological con-
centrations (25 pM) of 1,25(OH)$_2$D$_3$ in the medium
(Table 1). This concentration is one-third to one-
quarter the reported plasma value for this steroid
hormone in chicks (Spanos *et al.*, 1976b). The enzyme
responses were directly proportional to the concen-
trations of 1,25(OH)$_2$D$_3$ in the medium. However, a
very low 24-hydroxylase activity was observed when
1,25(OH)$_2$D$_3$ was present at a molar ratio of 5:1 with
the substrate (Table 1).

Effects of oestrogen

Oestradiol-17β had no effect on 1α-hydroxylase
activity of kidney cells from vitamin D-deficient
chicks (Table 2). However, it may be argued that
oestrogen could not stimulate 1α-hydroxylase because

Table 1. *Effect of various* 1,25(OH)$_2D_3$ *concentrations*
Kidney cells from 4-week-old vitamin D-deficient
chicks were grown to confluency in 8% (v/v) foetal
calf serum. After confluency was reached, the cells
were washed twice with phosphate-buffered saline
and placed in a serum-free medium containing
various concentrations of synthetic 1,25(OH)$_2$D$_3$.
After a 24h incubation at 37°C the medium was dis-
carded and the cells were washed with phosphate-
buffered saline. Incubation for a further 24h was
carried out in fresh medium containing the same
concentrations of synthetic 1,25(OH)$_2$D$_3$. Enzyme
assays were initiated by the addition of 100 ng of
^{3}H-labelled 25(OH)D$_3$ to the medium. The enzyme
reactions were terminated 1h later, by 3 vol. of
methanol/chloroform (2:1, v/v). Lipid extraction and
chromatography were then carried out as described
in the Materials and Methods section. Data
are means±S.E.M. for five to seven measurements.
Symbols indicate the following significance levels:
†$P<0.05$; *$P<0.01$; **$P<0.001$.

| Concentration of 1,25(OH)$_2$D$_3$ (nM) | Enzyme activity (pmol of metabolite produced/h per flask) | |
	24-Hydroxylase	1α-Hydroxylase
0	0	8.1±0.8
0.025	2.1±0.4	6.0±0.4†
0.25	4.0±0.5	5.8±0.3*
2.5	5.7±0.2	4.4±0.3**
25	17.4±0.7	1.2±0.07
250	0.8±0.3	0.8±0.07

Table 2. *Effect of oestradiol-17β in primary kidney-cell*
cultures from vitamin D-deficient chicks
Confluent monolayers of kidney cells from 4-week-
old vitamin D-deficient chicks were incubated at 37°C
for 24h in the presence of the indicated substances.
1,25(OH)$_2$D$_3$ treatment served as a positive control.
Data are means±S.E.M. for five determinations.

| Treatment | Enzyme activity (pmol of metabolite produced/h per flask) | |
	24-Hydroxylase	1α-Hydroxylase
None	0	23.7±2.1
Oestradiol-17β (1 μM)	0	25.8±2.5
1,25(OH)$_2$D$_3$ (25 nM)	5.7±0.4	9.9±0.9

its activity was already maximal owing to vitamin
D deficiency. Obviously we needed to study the effects
of oestrogens in cells with low 1α-hydroxylase
activity. To do this the cells were first treated for 24h
with 1,25(OH)$_2$D$_3$, then washed twice with phosphate-
buffered saline and incubated for a further 24h in the
presence of different oestradiol-17β concentrations.
Although this treatment was successful in lowering
1α-hydroxylase activity and inducing 24-hydroxylase

Table 3. *Effect of oestradiol-17β in 1,25$(OH)_2D_3$-treated cells*

Confluent monolayers of kidney cells from vitamin D-deficient chicks were first treated with 1,25$(OH)_2D_3$ (25 nM) for 24h. The medium was then discarded and the cells were washed twice with phosphate-buffered saline and incubated for another 24h in fresh medium containing various oestrogen concentrations, but not 1,25$(OH)_2D_3$. Data are means $\pm$ S.E.M. for five determinations.

Oestradiol-17β (μM)	Enzyme activity (pmol of metabolite produced/h per flask)	
	24-Hydroxylase	1α-Hydroxylase
0	3.8 ± 0.6	6.8 ± 0.5
0.1	3.3 ± 0.5	5.4 ± 0.1
1.0	2.8 ± 0.4	3.5 ± 0.3
10	2.6 ± 0.4	3.2 ± 0.1

Table 4. *Effect of oestradiol-17β in the presence of 1,25$(OH)_2D_3$ in the medium*

Confluent monolayers of kidney cells from vitamin D-deficient chicks were incubated in serum-free medium containing 1,25$(OH)_2D_3$ (25 nM). The medium was discarded 24h later and the cells were washed twice with phosphate-buffered saline and incubated for another 24h in fresh medium containing 1,25$(OH)_2D_3$ (25 nM) with or without oestradiol-17β. The values are means $\pm$ S.E.M. for four determinations.

Oestradiol-17β (μM)	Enzyme activity (pmol of metabolite produced/h per flask)	
	24-Hydroxylase	1α-Hydroxylase
0	8.6 ± 0.4	1.9 ± 0.3
1	8.8 ± 0.8	1.9 ± 0.5
10	9.8 ± 0.8	1.9 ± 0.2

activity, oestrogens again had no effect on these enzymes (Table 3).

The possibility that oestrogens may require the presence of 1,25$(OH)_2D_3$ in the medium to exert their effects was also investigated. As shown in Table 4, oestradiol-17β failed to stimulate an extremely low 1α-hydroxylase activity.

Discussion

It has been known for some time that the activity of the 25$(OH)D_3$ hydroxylases of the kidney is regulated by the vitamin D status of the animal. In vitamin D-deficient animals 1α-hydroxylase activity is very high, whereas 24-hydroxylase activity is undetectable. Vitamin D supplementation causes suppression of 1α-hydroxylase and induction of 24-hydroxylase

(Galante *et al.*, 1973; Henry *et al.*, 1974). This change-over in enzyme activity can be accelerated by administration of 1,25$(OH)_2D_3$ (Tanaka & DeLuca, 1974; MacIntyre *et al.*, 1976). Studies with the transcriptional inhibitors actinomycin D and α-amanitin indicate that the effects of 1,25$(OH)_2D_3$ on the renal 25$(OH)D_3$ hydroxylases are dependent on protein synthesis *de novo* (MacIntyre *et al.*, 1976; Omdahl, 1977). Consistent with this view are the results of Colston *et al.* (1977), who showed that 1,25$(OH)_2D_3$ given *in vivo* had a marked effect on gene transcription in the kidney cells. Two reports have appeared on the effects of 1,25$(OH)_2D_3$ in thyroid–parathyroidectomized rats. Omdahl (1977) found that the effects of 1,25$(OH)_2D_3$ on the renal 25$(OH)D_3$ hydroxylases are not affected by thyroid–parathyroidectomy. Suda *et al.* (1977), however, concluded that the feedback regulation of 1,25$(OH)_2D_3$ production by exogenous 1,25$(OH)_2D_3$ occurs by suppression of parathyrin secretion.

Attempts to study the regulation of 25$(OH)D_3$ metabolism *in vitro* have been hampered by the short life-span of the preparations used. The first to investigate the effects of 1,25$(OH)_2D_3$ *in vitro* were Larkins *et al.* (1974). They showed, in isolated renal tubules, an inhibitory effect of large doses of 1,25$(OH)_2D_3$ on 1α-hydroxylase, but they were unable to show an induction of 24-hydroxylase, possibly owing to the short life-span of their preparations.

To our knowledge the results reported in the present paper are the first demonstration that physiological concentrations of 1,25$(OH)_2D_3$ inhibit 1α-hydroxylase activity and induce 24-hydroxylase activity *in vitro*. Our results show clearly that the regulation of 25$(OH)D_3$ metabolism by 1,25$(OH)_2D_3$ is due to a direct action of this steroid hormone on the kidney cells. Thus the suggestion of Suda *et al.* (1977) that 1,25$(OH)_2D_3$ exerts its regulatory effects by acting on the parathyroids to inhibit parathyrin secretion seems most unlikely.

Henry (1977), with a similar system, reported that vitamin D-deficient cells treated with 1,25$(OH)_2D_3$ had 24-hydroxylase activity only. However, the concentration of 1,25$(OH)_2D_3$ used in her study was 500 times the physiological range. As shown in Table 1, physiological or sub-physiological concentrations of 1,25$(OH)_2D_3$ induced the activity of 24-hydroxylase, but only slightly suppressed 1α-hydroxylase activity. In accordance with the findings of Henry (1977), very high concentrations of 1,25$(OH)_2D_3$ caused a marked inhibition of 1α-hydroxylase. The activities of both hydroxylases were very low when 1,25$(OH)_2D_3$ was present at a molar ratio of 5:1 with the substrate. The very low activity of 24-hydroxylase under these conditions is consistent with the findings of Knutson & DeLuca (1974). These workers have shown, in isolated renal mitochondria, that 1,25$(OH)_2D_3$ when present

at a molar ratio of 2:1 to the substrate markedly inhibited 24-hydroxylase activity.

The time-course effects of $1,25(OH)_2D_3$ presented here are in very good agreement with our observations *in vivo* (Spanos *et al.*, 1977). The time difference between the suppressive and inductive effects of $1,25(OH)_2D_3$ may account for the failure of Larkins *et al.* (1974) to observe an induction of 24-hydroxylase in their short-lived isolated renal-tubule preparation.

The failure of oestrogens to stimulate 1α-hydroxylase activity *in vitro* is surprising in view of the well documented effects of these steroid hormones *in vivo* (Tanaka *et al.*, 1976; Baksi & Kenny, 1977). The high concentrations of oestrogen used in these experiments were chosen after measurements of plasma concentrations of oestrogen in chicks that had been given a single injection of 5 mg of oestradiol 24 h earlier. This dose of oestrogen in birds has been shown to stimulate 1α-hydroxylase activity markedly (Tanaka *et al.*, 1976) and increase circulating concentrations of $1,25(OH)_2D_3$ (J. W. Pike, E. Spanos, K. W. Colston, I. MacIntyre & M. R. Haussler, unpublished work). It has been suggested (Tanaka *et al.*, 1976) that oestrogen requires the presence of androgen to exert its effects. However, Baksi & Kenny (1977) found that oestrogen alone stimulated 1α-hydroxylase activity of immature male and female birds.

The inability of oestrogen to stimulate 1α-hydroxylase activity of kidney cells from vitamin D-deficient chicks may be due to the fact that the activity of 1α-hydroxylase in such cells is already maximal. It is interesting in this respect to note that oestrogen had no effect on 1α-hydroxylase when it was injected into vitamin D-deficient chicks (E. Spanos, K. W. Colston, D. I. Barrett & I. MacIntyre, unpublished work). Treatment of cells with $1,25(OH)_2D_3$ produced enzyme patterns very similar to those observed in vitamin D-supplemented chicks (Tables 3 and 4). Oestrogen, when tested in these cells, not only failed to stimulate 1α-hydroxylase activity but non-specifically inhibited both hydroxylases (Table 3). From our results it seems possible that oestrogen may stimulate 1α-hydroxylase activity not by acting directly on the kidney but by means of an indirect action. This possibility is supported by our observations *in vivo* (E. Spanos, K. W. Colston, D. I. Barrett & I. MacIntyre, unpublished work). We have found that intact liver function is required if oestrogen is to stimulate 1α-hydroxylase activity. No effect was observed in chicks after they had been treated with the hepatotoxic agent ethylene dibromide.

We have shown that oestrogen acts indirectly, whereas $1,25(OH)_2D_3$ acts directly on the kidney cells to regulate $25(OH)D_3$ metabolism. The physiological significance of our observations with $1,25(OH)_2D_3$ becomes clear when one takes into account the fact that known regulators of $25(OH)D_3$ metabolism, such

as Ca^{2+}, phosphate and parathyrin, require the presence of a form of vitamin D to exert their effects (Boyle *et al.*, 1971; Galante *et al.*, 1973; DeLuca & Schnoes, 1976). It is suggested that $1,25(OH)_2D_3$ by suppressing 1α-hydroxylase and inducing 24-hydroxylase not only regulates its own production but also provides the means for other regulators to exert their effects. The mechanism by which $1,25(OH)_2D_3$ sensitizes the kidney cells to respond to other regulators of $25(OH)D_3$ metabolism requires investigation.

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