Feedback Regulation of Vitamin D Metabolism by 1,25-Dihydroxycholecalciferol

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Many factors influence the production of 1,25(OH)₂D₃ (1,25-dihydroxycholecalciferol) by the kidney. One important factor seems to be feedback regulation by 1,25(OH)₂D₃ itself. Administration of 1,25(OH)₂D₃ to vitamin D-deficient chicks abolishes renal 25(OH)D₃ (25-hydroxycholecalciferol) 1-hydroxylase activity and induces the appearance of 25(OH)D₃ 24-hydroxylase activity. It is likely that these effects are mediated via a nuclear effect, as they are prevented by pretreatment with actinomycin D and α -amanitin. Further, 1,25(OH)₂D₃ has a marked effect on gene transcription in the kidney cell, as assessed by measurement of RNA polymerase activities. RNA polymerase I and II activities are 80–90% inhibited by 12.5 nmol of 1,25(OH)₂D₃ within 30 min of subcutaneous administration, indicating an immediate and massive decrease in total gene transcription. By 4 h RNA polymerase II activity has returned to control values, but RNA polymerase I activity is markedly enhanced. These results are consistent with the view that regulation of cholecalciferol metabolism in the kidney is associated with an effect of the active metabolite on the kidney nucleus.

Vitamin D₃ (cholecalciferol) is now considered to be a prohormone which must be converted into hormonally active forms before it can exert its physiological effects on calcium metabolism. The major circulating form of the vitamin is 25(OH)D₃ (25-hydroxycholecalciferol) which is formed in the liver (Blunt *et al.*, 1968) and other tissues (Tucker *et al.*, 1973). 25(OH)D₃ itself is further hydroxylated by a mitochondrial enzyme system located only in the kidney (Fraser & Kodicek, 1970) to 1,25(OH)₂D₃ (1,25-dihydroxycholecalciferol), the most active form of the vitamin yet known (DeLuca, 1974). An alternative renal hydroxylation converts 25(OH)D₃ into 24,25(OH)₂D₃ (24,25-dihydroxycholecalciferol) (Holick *et al.*, 1972), a less active metabolite whose physiological significance is not yet understood.

As with other endocrine systems, it is known that the kidney alters its output of the hormonally active metabolite in an appropriate manner, depending on vitamin D intake and the prevailing electrolyte status. However, the mechanisms by which the activity of the renal 25(OH)D₃ 1-hydroxylase and 25(OH)D₃ 24-hydroxylase enzymes are regulated have not been fully elucidated. Many factors have been suggested as regulators of 1,25(OH)₂D₃ production, among them serum calcium (Boyle *et al.*, 1971), renal phosphate concentration (Tanaka & DeLuca, 1973), parathyroid hormone (DeLuca, 1972; Fraser & Kodicek, 1973), calcitonin (Galante *et al.*, 1972a) and intra-

cellular calcium concentration (Colston *et al.*, 1973; Galante *et al.*, 1972b). It may be that some or all of these factors act in an interrelated manner to influence the rate of production of 1,25(OH)₂D₃ *in vivo*.

In the present paper, experimental evidence is put forward to indicate that one further important control mechanism is feedback regulation of vitamin D metabolism by cholecalciferol itself or, more probably, by the hormonally active metabolite. Moreover, it appears that this process is not one of simple product inhibition but involves an action of 1,25(OH)₂D₃ on the nucleus.

Materials and Methods

Animals and treatment

One-day-old chicks (Rhode Island Red $\times$ Light Sussex crossbreed; Orchard Farm, Great Missenden, Bucks., U.K.) were fed on a vitamin D-deficient diet (Ca 0.34%, P 0.54%) for 8–14 days before the start of each experiment. Chicks were maintained on the vitamin D-deficient diet from the day of hatching. The birds were treated with vitamin D (Sigma, Kingston-upon-Thames, Surrey, U.K.) or 1,25(OH)₂D₃ (Hoffmann-La Roche, Nutley, NJ, U.S.A.) subcutaneously in 0.1 ml of ethanol at doses and times as stated in the legends. Control birds received ethanol alone. In some instances, the animals received actinomycin D (Dactinomycin; Merck,

Sharpe and Dohme International, Rahway, NJ, U.S.A.) or α -amanitin (Boehringer, Ingelheim am Rhein, Germany), each in 0.1 ml of 0.9% NaCl.

Analysis of renal 25(OH)D₃ 1-hydroxylase and 24-hydroxylase activities

The chicks were killed by decapitation at various times after treatment as stated in the legends. Kidney homogenates were assayed for 1-hydroxylase and 24-hydroxylase activity as previously described (Galante *et al.*, 1973; Colston *et al.*, 1973). The concentration of substrate (25-hydroxy[26(27)-Me-³H]-cholecalciferol; The Radiochemical Centre, Amersham, Bucks., U.K.) was 25 pmol/3 ml of incubation mixture of the kidney homogenate.

RNA polymerase assays

In some experiments, one kidney from each chick was removed for hydroxylase assays and the other was removed for measurement of RNA polymerase activity. The excised kidneys for the latter were snap-frozen in liquid N₂ until all groups could be assayed together. The isolation of the nuclear fraction and assay for endogenous RNA polymerase I (nucleolar) and II (nucleoplasmic) activities, which assess synthesis of RNA and DNA-like RNA respectively, have been described elsewhere (Glasser *et al.*, 1972). Freezing the whole tissue has previously been shown

to have only mild effects on RNA polymerase activities in the isolated nuclei of chick oviducts and liver (Knowler *et al.*, 1973).

Fig. 1. *Effect of dietary vitamin D₃ restriction on renal 1-hydroxylase and 24-hydroxylase activities*

Chicks were maintained on a vitamin D-deficient diet (0.34% Ca, 0.54% PO₄) from the day of hatching. Renal 25(OH)D₃ 1- and 24-hydroxylase activities were assayed at intervals from 1 to 16 days after restriction of dietary vitamin D intake. Enzyme activity is expressed as fmol of dihydroxy metabolite produced/min per mg of protein, $\pm$ s.e.m., with three birds per time and two duplicate measurements of (□) 1-hydroxylase and (■) 24-hydroxylase activity per bird.

Fig. 2. *Effect of synthetic 1,25(OH)₂D₃ on renal 1- and 24-hydroxylase activities in chick kidney homogenates*

The results were from two separate experiments. Chicks were kept on the vitamin D-deficient diet for 16 days. Renal enzyme activities were assayed 9 h after a single injection of 1,25(OH)₂D₃ given in the doses indicated. Open bars represent 1-hydroxylase and solid bars 24-hydroxylase activity expressed as fmol of dihydroxy metabolite produced/min per mg of protein. The mean ($\pm$ s.e.m.) of two measurements from each of three birds per treatment group is shown.

Chemical assays

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and RNA by the method of Burton (1956). Plasma calcium was measured by flame spectrophotometry (MacIntyre, 1961).

Results

Effect of vitamin D deficiency on renal hydroxylase activities

Before vitamin D restriction, 1-day-old chicks have comparable activities of renal 1-hydroxylase and 24-hydroxylase. Vitamin D deficiency caused a marked increase in the activity of the renal 25(OH) $_2$ D $_3$ 1-hydroxylase enzyme such that this activity increased 4-fold 4 days after restriction of vitamin D

intake. This increase in enzyme activity was maintained over the following 2 weeks. Conversely, 25(OH) $_2$ D $_3$ 24-hydroxylase activity decreased rapidly during vitamin D restriction (Fig. 1).

Effect of cholecalciferol and 1,25-dihydroxycholecalciferol on renal hydroxylase activities

Administration of either cholecalciferol or 1,25-(OH) $_2$ D $_3$ to vitamin D-deficient chicks leads to a decrease in 1-hydroxylase activity and an appearance of 24-hydroxylase activity. We have previously shown that a physiological dose (6.25 nmol) of cholecalciferol induces the appearance of 24-hydroxylase activity and a significant decrease of 1-hydroxylase activity at 38 h (Galante *et al.*, 1973). As might be expected, 1,25(OH) $_2$ D $_3$, the most active metabolite of cholecalciferol, produced the same effects, but at a much earlier time. This dose (6.25

Fig. 3. *Effect of actinomycin D on the induction of 24-hydroxylase activity by 1,25(OH) $_2$ D $_3$*

Two groups of birds (three per group) received 125 nmol of 1,25(OH) $_2$ D $_3$ and one group in addition received actinomycin D to a total dose of 400 nmol (500 μ g) per bird, given in five equal doses of 80 nmol at 2 h intervals. The first injection of actinomycin was given 30 min before administration of 1,25(OH) $_2$ D $_3$. A third group received actinomycin D alone and a fourth group served as controls. Renal enzyme activities were assayed 9 h after 1,25(OH) $_2$ D $_3$ treatment. Solid bars represent 24-hydroxylase activity and open bars 1-hydroxylase activity, expressed as fmol of dihydroxy metabolite produced/min per mg of protein, mean ($\pm$ S.E.M.).

Fig. 4. Effect of α -amanitin on the induction of 24-hydroxylase activity by $1,25(\text{OH})_2\text{D}_3$

Two groups of birds received $1,25(\text{OH})_2\text{D}_3$ (125 nmol per bird) and one group in addition received a single dose of α -amanitin (100 nmol; 100 μg per bird), 30 min before $1,25(\text{OH})_2\text{D}_3$ treatment. A third group received α -amanitin alone and a fourth group served as control. Renal 1- and 24-hydroxylase activities were assayed 9 h after $1,25(\text{OH})_2\text{D}_3$ treatment. Solid bars represent 24-hydroxylase activity and open bars represent 1-hydroxylase activity expressed as fmol of dihydroxy metabolite produced/min per mg of protein, mean ($\pm$ S.E.M.).

Table 1. Effect of the selected dose of α -amanitin and $1,25(\text{OH})_2\text{D}_3$ on RNA polymerase activities in chick kidney nuclei

The chicks were treated as described in the legend to Fig. 4 and below in the Table. The kidneys from each of three chicks per group were combined, the nuclear fraction was isolated and the RNA polymerase I and II activities were assayed as described in the Materials and Methods section. Activity is expressed as pmol of [^3H]UMP incorporated/mg of nuclear DNA. Values are means $\pm$ S.E.M. The RNA polymerase I assays did not include the additional α -amanitin.

Treatment	RNA polymerase activity	
	I	II
(a) 30 min after injection		
Control	26.84 $\pm$ 2.54	139.13 $\pm$ 14.59
+ 100 nmol of α -amanitin	24.04 $\pm$ 1.90	4.05 $\pm$ 1.39
(b) 9 h after injection		
Control	36.42 $\pm$ 2.43	153.83 $\pm$ 6.20
+ 12.5 nmol of $1,25(\text{OH})_2\text{D}_3$	46.78 $\pm$ 2.68	179.25 $\pm$ 15.50
+ 125 nmol of $1,25(\text{OH})_2\text{D}_3$	42.21 $\pm$ 0.00	145.82 $\pm$ 4.22
+ 100 nmol of α -amanitin	25.73 $\pm$ 0.80	6.56 $\pm$ 1.04
+ 125 nmol of $1,25(\text{OH})_2\text{D}_3$ + 100 nmol of α -amanitin	21.8 $\pm$ 0.9	6.74 $\pm$ 0.26

nmol) of synthetic $1,25(\text{OH})_2\text{D}_3$ produced changes in the activities of the renal enzymes at 24 h, but even more rapid effects were required to allow analysis of these changes by using inhibitors of transcription. Fig. 2 shows that 12.5 nmol of $1,25(\text{OH})_2\text{D}_3$ was sufficient to abolish the 1-hydroxylase activity and to induce the appearance of the 24-hydroxylase within 9 h. Administration of 62.5 nmol and 125 nmol of

$1,25(\text{OH})_2\text{D}_3$ yielded approximately the same results. Consequently, doses within this range were selected for subsequent experiments to ensure a full effect of the metabolite on the renal hydroxylases.

Effect of transcriptional inhibitors

The relatively slow changeover from 1-hydroxylase to 24-hydroxylase indicates that this effect is not

simply one of product inhibition. A change in the number or activity of enzyme molecules dependent on new protein synthesis seems more likely. Further, it has previously been shown that these doses of $1,25(\text{OH})_2\text{D}_3$ do not inhibit the conversion of $[\text{H}]25(\text{OH})\text{D}_3$ into $1,25(\text{OH})_2\text{D}_3$ *in vitro* (MacIntyre *et al.*, 1974). Thus inhibition of transcription, and hence of protein synthesis, would be expected to inhibit the change in renal enzyme activities in response to $1,25(\text{OH})_2\text{D}_3$. Actinomycin D abolished both the appearance of 24-hydroxylase activity and the disappearance of 1-hydroxylase activity induced by $1,25(\text{OH})_2\text{D}_3$ (Fig. 3). Interestingly, actinomycin D

itself had little or no effect on either enzyme activity. Similarly, pretreatment with α -amanitin also abolished the effects of $1,25(\text{OH})_2\text{D}_3$ on both renal hydroxylases (Fig. 4). Further, this dose of α -amanitin (100 nmol) was shown to inhibit RNA polymerase II activity 30 min after administration, and the activity of this nuclear enzyme remained low for the duration of the experiment (Table 1). Although α -amanitin alone lowers 1-hydroxylase activity, $1,25(\text{OH})_2\text{D}_3$ has a greater effect which is largely blocked by the combined treatment with α -amanitin + $1,25(\text{OH})_2\text{D}_3$ (Fig. 4).

Thus blocking transcription via the DNA template (actinomycin D) or the RNA polymerase II enzyme (α -amanitin) negates the effects of $1,25(\text{OH})_2\text{D}_3$ or the renal hydroxylase activities. These results suggest that the observed effects of $1,25(\text{OH})_2\text{D}_3$ are dependent on changes in gene transcription and protein synthesis.

Effect of $1,25(\text{OH})_2\text{D}_3$ on RNA polymerase activities

The effects of two doses of $1,25(\text{OH})_2\text{D}_3$ on the activities of RNA polymerase I (synthesizing RNA) and polymerase II (synthesizing DNA-like RNA) were assessed (Fig. 5).

RNA polymerase I activity was inhibited up to 2 h after treatment with both doses of $1,25(\text{OH})_2\text{D}_3$. However, by 4 h an enhancement of this enzyme activity occurred. Both doses of $1,25(\text{OH})_2\text{D}_3$ also inhibited RNA polymerase II activity up to 1 h after injection. This inhibition of enzyme activity persisted up to 4 h with the higher dose, but was minimal by 2 h with the lower dose. With the higher dose, RNA polymerase II activity approached control values by 9 h.

Discussion

These results strongly suggest that $1,25(\text{OH})_2\text{D}_3$ plays an important physiological role in regulating the metabolism of $25(\text{OH})\text{D}_3$ by the kidney, although caution is necessary in interpretation because of the large doses used. In states of vitamin D deficiency, renal 1-hydroxylase activity is markedly increased whereas 24-hydroxylase activity is markedly decreased.

Administration of a physiological dose of cholecalciferol (6.25 nmol) decreases 1-hydroxylase activity and induces the appearance of 24-hydroxylase activity at 38 h. This dose (6.25 nmol) of $1,25(\text{OH})_2\text{D}_3$, the most active metabolite of cholecalciferol, has the same effect on the activities of the renal enzymes, but at an earlier time. Larger doses of $1,25(\text{OH})_2\text{D}_3$ act even more rapidly, such that at 9 h 1-hydroxylase activity is abolished and there is high 24-hydroxylase activity. Both the disappearance of 1-hydroxylase

Fig. 5. Effects of $1,25(\text{OH})_2\text{D}_3$ on RNA polymerase I (a) and II (b) activities in the chick kidney

Chicks were maintained on the vitamin D-deficient diet for 10 days and were then injected (subcutaneously) with (■) 12.5 nmol (5 μg) or with (□) 125 nmol (50 μg) of $1,25(\text{OH})_2\text{D}_3$. The chicks were killed at the designated times (three chicks per time from each treatment group). Control chicks (receiving the ethanol vehicle alone) were killed at 4 h. Both kidneys from each chick from each group were combined and snap-frozen in liquid N_2 and stored at -20°C until assayed for RNA polymerase activity as described in the Materials and Methods section. The average and range of two replicate analyses for each time interval are presented.

activity and the appearance of 24-hydroxylase activity seem to depend on transcription and therefore new protein synthesis. Treatment with actinomycin D, which inhibits transcription by blocking the DNA template, abolished both the appearance of 24-hydroxylase activity and the disappearance of 1-hydroxylase activity seen in response to $1,25(\text{OH})_2\text{D}_3$. Similarly α -amanitin, which inhibits RNA synthesis by binding to RNA polymerase II, also prevented the change in renal enzyme activities 9h after administration of $1,25(\text{OH})_2\text{D}_3$.

The most natural interpretation of our results would be that they are consequent on an effect of $1,25(\text{OH})_2\text{D}_3$ on calcium metabolism which would influence the enzyme activities in the kidney. In fact, there is evidence that calcium may have an important regulatory influence on the 1-hydroxylase enzyme (Boyle *et al.*, 1971; Colston *et al.*, 1973). However, an effect via serum calcium is unlikely to be an essential part of the observed action of $1,25(\text{OH})_2\text{D}_3$ on the kidney enzyme activities, since the synthetic analogue $1\alpha(\text{OH})\text{D}_3$ (1α -hydroxycholecalciferol) produced the same effects at 9h without changes in calcium concentration. Thus 9h after a 625nmol dose of $1\alpha(\text{OH})\text{D}_3$ there was no change in the plasma calcium concentration. Mean plasma calcium concentrations were: controls 1.72mM; $1\alpha(\text{OH})\text{D}_3$ -treated 1.90mM; $1\alpha(\text{OH})\text{D}_3$ +actinomycin D-treated, 1.84mM.

Although the control of $25(\text{OH})\text{D}_3$ metabolism was first thought to be exerted via an effect on calcium metabolism, there are now several situations known where this cannot be the case. Thus prolactin (Spanos *et al.*, 1976) and oestrogen (Tanaka *et al.*, 1976), both of which increase serum calcium, stimulate 1α -hydroxylase.

Our hypothesis is that the observed effect of $1,25(\text{OH})_2\text{D}_3$ is an acceleration of a slower physiological regulation of major importance. The most likely mechanism of this effect is, in our view, a direct nuclear action on the kidney cell of a similar nature to that established for other steroid hormones. Further, there is proof of such a direct effect of $1,25(\text{OH})_2\text{D}_3$ in the studies of Larkins *et al.* (1974) on isolated renal tubules.

Our studies on the activities of RNA polymerases I and II seem to confirm that $1,25(\text{OH})_2\text{D}_3$ exerts an immediate effect on transcription, thereby regulating the activity of the renal hydroxylase enzymes. Even the 12.5nmol dose causes a marked decrease in both polymerase enzyme activities within 30min. The rapid inhibition of RNA polymerase II activity by $1,25(\text{OH})_2\text{D}_3$ is similar to the effects seen with other steroid hormones, notably the action of progesterone on the chick oviduct (T. C. Spelsberg & R. F. Cox, personal communication). The overall decrease in RNA polymerase II activity in response to $1,25(\text{OH})_2\text{D}_3$ does not necessarily imply that new mRNA

species and thus new proteins are not being synthesized, but merely indicates that total gene transcription in the cell is being decreased by this cholecalciferol metabolite. The transcription of new genes would be masked by the massive decrease in the transcription of genes that were being transcribed before $1,25(\text{OH})_2\text{D}_3$ administration.

It should be emphasized that the action of actinomycin D and α -amanitin in preventing the effects of $1,25(\text{OH})_2\text{D}_3$ on the renal hydroxylase enzymes and the rapid effects of this steroid on RNA polymerase activities suggest, but do not prove, that the induced changes in the hydroxylases originate at the transcription level. Alternative possibilities are via actions on post-transcriptional processes such as RNA processing and transport, translation and enzyme activation by such mechanisms as phosphorylation. Our results provide strong experimental evidence that one important control of vitamin D metabolism is feedback regulation at the kidney level by $1,25(\text{OH})_2\text{D}_3$.

These experimental results are consistent with the view that vitamin D regulates its own metabolism in a feedback manner and that it exerts its effects, as do other steroid hormones, by a nuclear effect involving changes in gene transcription. Such a nuclear-feedback control would explain how the kidney responds to the prevailing vitamin D status of the animal. Thus variation in the amount and type of renal enzyme, dependent on the intake of vitamin D, may prove to be an important factor in the physiological regulation of cholecalciferol metabolism. To confirm that this feedback inhibition involves changes in the synthesis of the renal hydroxylase enzymes, the best approach in future may be the preparation of antisera against both (purified) hydroxylases and the titration of their appearance or disappearance in response to $1,25(\text{OH})_2\text{D}_3$.

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