Regulation and Its Refractoriness of 25-Hydroxyvitamin D₃ Metabolism in Vitamin D Deficiency

Naoyuki TAKAHASHI,¹ Chisato MIYAURA,¹ Noboru HORIUCHI,¹
and Tatsuo SUDA^{1,2}

*Department of Biochemistry, School of Dentistry, Showa University,
1–5–8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan*

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Summary The time course of change in plasma calcium levels and renal metabolism of 25-hydroxyvitamin D₃ [25(OH)D₃] was investigated in chicks maintained on a vitamin D-deficient diet for 4 weeks. Plasma calcium concentrations dropped sharply between the 7th and 14th day of the feeding period. Renal 25(OH)D₃-1 α -hydroxylase activity was reciprocally enhanced concurrently with the decrease in plasma calcium levels. The elevated activity of 1 α -hydroxylase had declined significantly by the 21st and 28th days in spite of the more severe hypocalcemia. When graded amounts of vitamin D₃ were administered to the chicks maintained on this diet for 14 or 28 days, there were considerable differences in the change of plasma calcium levels and 25(OH)D₃ metabolism induced by vitamin D₃ administration between the 14-day and 28-day birds. The minimal dose levels of vitamin D₃ to completely suppress renal 1 α -hydroxylase activity were 25 μ g in the 14-day, and 2.5 mg in the 28-day birds. These differences were not observed between the 14-day and 28-day birds when 1 α -hydroxyvitamin D₃ [1 α (OH)D₃] was administered. Renal adenylate cyclase activity induced by parathyroid hormone (PTH) was much lower in the 28-day chicks than that in 1-day-old and the 14-day birds. These results are explained by the hypersecretion of PTH and the subsequent refractoriness of the target organs in severe vitamin D deficiency. Plasma calcium levels *per se* did not appear to be a major factor in the regulation of 25(OH)D₃ metabolism.

Key Words vitamin D deficiency, plasma calcium, secondary hyperparathyroidism, PTH-sensitive adenylate cyclase, refractoriness of the control of 25(OH)D₃ metabolism, renal 1 α -hydroxylase, end-organ refractoriness to PTH

¹ 高橋直之, 宮浦千里, 堀内 登, 須田立雄

² To whom all correspondence should be addressed.

Abbreviations: 25(OH)D₃, 25-hydroxyvitamin D₃; 1 α ,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; 24,25(OH)₂D₃, 24,25-dihydroxyvitamin D₃; 1 α (OH)D₃, 1 α -hydroxyvitamin D₃; TPTX, thyroparathyroidectomy or thyroparathyroidectomized; PTH, parathyroid hormone.

It is known that vitamin D₃ is first hydroxylated to 25-hydroxyvitamin D₃ [25(OH)D₃] in the liver and subsequently to 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃] or 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃] in the kidney (1). The 1 α ,25(OH)₂D₃, presently known as the most potent and the most rapidly acting metabolite of vitamin D₃, is regarded as its hormonal form (1). In the pathway of vitamin D₃ metabolism, renal 1 α -hydroxylation of 25(OH)D₃ is the rate-limiting step in the production of 1 α ,25(OH)₂D₃ and is strictly regulated by plasma calcium (2-4), plasma phosphorus (3-5), parathyroid hormone (PTH) (3, 6, 7) and vitamin D status in animals (8, 9).

It has been demonstrated that hypocalcemia stimulates PTH secretion which in turn increases 1 α ,25(OH)₂D₃ production in the kidney (3, 6, 7). The stimulatory effect of PTH on renal 1 α ,25(OH)₂D₃ synthesis is mediated by cyclic AMP (10). Using single nephron preparations from rabbits, Chabardès *et al.* (11) showed that PTH-dependent adenylate cyclase activity is located in both proximal and distal tubules. It has also been reported that renal 1 α -hydroxylase activity is located exclusively in the proximal tubules in vitamin D-deficient chicks (12) and rats (13) and in fetuses from normal rabbits (14). Although the role of PTH-dependent adenylate cyclase activity in the distal tubules is not known, these findings strongly support the concept that cyclic AMP acts as a second messenger in stimulating the PTH-induced production of 1 α ,25(OH)₂D₃.

Very recently, Trechsel *et al.* (15) reported that in pair-fed thyroparathyroidectomized (TPTX) and sham-operated rats plasma levels of 1 α ,25(OH)₂D₃ are influenced not only by PTH but also by plasma calcium levels *per se*, suggesting the existence of a calcium-dependent, PTH-independent regulatory mechanism in the renal biosynthesis of 1 α ,25(OH)₂D₃.

In this paper, we show that renal 25(OH)D₃-1 α -hydroxylase activity is suppressed significantly in the course of vitamin D deficiency, suggesting that this enzyme activity is regulated mainly by PTH rather than plasma calcium levels *per se*.

MATERIALS AND METHODS

Animals. One-day-old White Leghorn cockerels were maintained on a vitamin D-deficient diet (16) containing 1.0% calcium and 0.45% phosphorus for 28 days. On the 4th, 7th, 14th, 21st or 28th days of the feeding period, the birds were sacrificed. Graded amounts of either vitamin D₃ (0.25-2,500 μ g) or 1 α -hydroxyvitamin D₃ [1 α (OH)D₃] (0.074-6.0 μ g) in 0.1 ml of cotton-seed oil were orally administered either 48 or 24 hr prior to sacrifice. The control birds received vehicle only.

Materials. Crystalline D₃ was purchased from Wako Pure Chemical Industry, Osaka, Japan, and 25(OH)D₃ from Philips-Duphar Co., Amsterdam, Netherlands. Crystalline 24R,25(OH)₂D₃ and 1 α ,25(OH)₂D₃ were kindly donated by Dr. M. R. Uskokovic, Hoffmann-LaRoche, Inc., Nutley NJ. The 1 α (OH)D₃ was a gift from

Dr. I. Matsunaga, Chugai Pharmaceutical Co., Tokyo, Japan. The 25(OH)[26,27-³H]D₃ (9.0 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, U. K. The parathyroid hormone (bovine TCA powder, 218 USP units/mg) was obtained from the Wilson Laboratories, Chicago, IL. All other reagents and chemicals used in this study were of analytical grade.

Analysis of renal 25(OH)D₃-1 α - and 24-hydroxylase activities. The chicks were killed by decapitation and blood was collected to determine plasma concentrations of calcium. Kidneys were quickly removed, rinsed and homogenized in 4 volumes of 0.2 M sucrose containing 2 mM MgCl₂, 5 mM succinate and 15 mM Tris-HCl (pH 7.4). Each aliquot (1.5 ml) of the homogenates was incubated with 712 ng (0.15 μ Ci) of 25(OH)[26,27-³H]D₃ at 37°C for 20 min under 100% oxygen. The reaction was terminated by the addition of 9 ml of methanol-chloroform (2:1, v/v) and extracted by the method of Bligh and Dyer (17). For analysis of 25(OH)[³H]D₃ metabolites, extracts were applied to Sephadex LH-20 columns (1.2 $\times$ 22 cm) which were eluted with a mixture of chloroform and *n*-hexane (65:35, v/v). Four-ml fractions were collected and the radioactivity in each fraction was counted with a Packard Tri-Carb liquid scintillation counter, Model 3385. Suspected 1 α ,25(OH)₂[³H]D₃ and 24,25(OH)₂[³H]D₃ obtained from the Sephadex LH-20 columns were identified by co-chromatography with authentic 1 α ,25(OH)₂D₃ and 24R,25(OH)₂D₃ on a high-pressure liquid chromatograph (Waters, Model 204), equipped with a Zorbax-Sil column (4.6 $\times$ 150 mm, DuPont) as previously described (18). Enzyme activities were expressed as pg/min/mg of kidney tissue. Over 79% of the radioactivity suspected to be 1 α ,25(OH)₂[³H]D₃ in the Sephadex LH-20 column migrated to exactly the same position as authentic 1 α ,25(OH)₂D₃ on HPLC. When the suspected 24,25(OH)₂D₃ fraction in the Sephadex column was applied to HPLC, at least 71% of the radioactivity co-migrated with authentic 24R,25(OH)₂D₃.

Assay of PTH-dependent adenylate cyclase activity. The chicks were sacrificed, and kidneys were removed, rinsed with 0.25 M sucrose containing 1 mM EDTA and 10 mM Tris-HCl (pH 7.4) and homogenized in 9 volumes of the same buffer. The homogenate was centrifuged at 150 g for 5 min. The supernatant was subsequently centrifuged at 2,200 g for 15 min. The resulting crude membrane pellet was washed twice and suspended in 10 volumes (of the initial tissue weight) of 50 mM Tris-HCl (pH 7.4). An aliquot was used for the assay of PTH-dependent adenylate cyclase activity. The reaction mixture consisted of 1 mM ATP, 2 mM MgCl₂, 0.2 mM 3-isobutyl methylxanthine, 0.05 mM GTP, 0.4 mg/ml of BSA, 25 mM Tris-HCl (pH 7.4), 10 U/ml of PTH (or vehicle), and 30 μ l of the prepared enzyme solutions in a total of 0.2 ml. The reaction was started by the addition of the enzyme solution. After incubation at 37°C for 10 min, the reaction was terminated by the addition of 0.2 ml of 10% TCA and the mixture was centrifuged at 3,000 rpm for 10 min. TCA in the supernatant was removed by washing three times with water-saturated ether. The amount of cyclic AMP in the supernatant was estimated using a Yamasa cyclic AMP assay kit. Protein was determined by the method of Lowry *et al.* (19) with bovine serum albumin as the standard.

Determination of plasma calcium and phosphorus. Plasma calcium was measured with an atomic absorption spectrometer (Hitachi, Model 170-50A). Plasma phosphorus was determined by the method of Fiske-Subbarow (26).

RESULTS

The sequence of changes in plasma calcium levels and renal 25(OH)D₃-1 α - and 24-hydroxylase activities is shown in Fig. 1. The plasma calcium levels dropped sharply from 9.2 to 7.6 mg/100 ml between the 7th and 14th day of the feeding period and then declined gradually thereafter. One-day-old chicks showed activities of both the renal 25(OH)D₃-1 α - and 24-hydroxylases. The former activity was lower than the latter in 1-day-old chicks. Feeding of the vitamin D-deficient diet caused an increase in the 1 α -hydroxylase, and disappearance of the 24-hydroxylase activity. The 1 α -hydroxylase activity was markedly enhanced coincidentally with the sharp decrease in plasma calcium levels. The activity attained a maximum on the 14th day and declined thereafter in spite of the more severe hypocalcemia. Plasma concentrations of phosphorus in chicks maintained for 14 days were 5.4 ± 0.5 mg/100 ml and those for 28 days were 5.2 ± 0.5 mg/100 ml.

Figure 2 shows the comparative effectiveness of graded amounts of vitamin D₃

Fig. 1. Time course of change in plasma calcium levels and renal 25(OH)D₃-1 α - and 24-hydroxylase activities in chicks fed a vitamin D-deficient diet for 28 days. (A) change in plasma calcium ($\circ$), (B) change in renal activities of 25(OH)D₃-1 α -hydroxylase ($\square$) and 24-hydroxylase ($\blacksquare$). Each point indicates the mean $\pm$ SEM of 4–12 chicks.

Fig. 2. Effect of vitamin D₃ on plasma calcium and renal 25(OH)D₃-1 α - and 24-hydroxylase activities in chicks fed a vitamin D-deficient diet for 14 (A) or 28 (B) days. Chicks were maintained on this diet for 12 or 26 days, then given orally 0.25–2,500 μ g of vitamin D₃, and killed 48 hr later. Plasma calcium (○) and renal activities of 1 α -hydroxylase (□) and 24-hydroxylase (▨) were determined as described in MATERIALS AND METHODS. Data are expressed as the means $\pm$ SEM of 4–6 chicks.

on plasma calcium levels and renal 25(OH)D₃-1 α - and 24-hydroxylase activities in chicks fed the vitamin D-deficient diet for 14 or 28 days (hereafter called 14-day and 28-day chicks). Administration of vitamin D₃ to the 14-day chicks led to a dose-dependent increase in plasma calcium levels. Vitamin D₃ at 2.5 mg resulted in an apparent hypercalcemia (plasma calcium: 14.2 mg/100 ml). In the 28-day chicks, however, the same dose level of vitamin D₃ was found to induce much less hypercalcemic action (plasma calcium: from 7.0 to 10.6 mg/100 ml).

Similarly, there was a considerable difference in the change of 25(OH)D₃ metabolism induced by vitamin D₃ administration between the 14-day and 28-day chicks (Fig. 2). The minimal dose level of vitamin D₃ to completely suppress renal 1 α -hydroxylase activity in the 14-day chicks was 25 μ g. On the other hand, as much as 2.5 mg of vitamin D₃ were required to completely suppress 1 α -hydroxylase activity in the 28-day birds. The 24-hydroxylase activity was first induced by 0.25 μ g of vitamin D₃ in the 14-day chicks and by 25 μ g of vitamin D₃ in the 28-day birds.

These differences, however, were not observed when 1 α (OH)D₃ was administered to the chicks fed this diet for either 14 or 28 days (Fig. 3). The hypercalcemic action of 1 α (OH)D₃ was almost identical in both groups of chicks. Further, in both the 14- and 28-day birds the 1 α -hydroxylase activity disappeared with the administration of 2.0 μ g of 1 α (OH)D₃, and similarly in both groups the 24-hydroxylase

Fig. 3. Effect of $1\alpha(\text{OH})\text{D}_3$ on plasma calcium and renal $25(\text{OH})\text{D}_3$ - 1α - and 24 -hydroxylase activities in chicks fed a vitamin D-deficient diet for 14 (A) or 28 (B) days. Chicks were maintained on this diet for 13 or 27 days, then given orally 0.074 – $6.0 \mu\text{g}$ of $1\alpha(\text{OH})\text{D}_3$, and killed 24 hr later. Plasma calcium ($\circ$) and renal activities of 1α -hydroxylase ($\square$) and 24 -hydroxylase ($\blacksquare$) were determined as described in MATERIALS AND METHODS. Data are expressed as the means $\pm$ SEM of 4–5 chicks.

Table 1. PTH-dependent renal adenylate cyclase activity in 1-day-old chicks and in 14- and 28-day chicks.

Feeding period (days)	Number of chicks	Adenylate cyclase activity		
		Control (pmol/min/mg of protein)	PTH	PTH/Control (%)
0	4	5.3 ± 0.3	$10.3 \pm 0.7^*$	193 ± 5
14	4	4.0 ± 0.2	$8.2 \pm 0.7^*$	206 ± 22
28	4	5.2 ± 0.3	6.2 ± 0.5	$118 \pm 3^{**}$

Data are expressed as the means $\pm$ SEM. * Significantly different from each control, $p < 0.01$. ** Significantly different from 1-day-old chicks and those fed this diet for 14 days, $p < 0.01$.

activity appeared with $0.22 \mu\text{g}$ of $1\alpha(\text{OH})\text{D}_3$. Plasma levels of phosphorus in each group of chicks were not changed by treatment with vitamin D_3 or $1\alpha(\text{OH})\text{D}_3$.

Table 1 shows the PTH-dependent adenylate cyclase activity of renal membrane fractions in 1-day-old chicks and in the 14- or 28-day birds. The basal adenylate cyclase activity was almost identical in each of the groups, but the PTH-dependent adenylate cyclase activity in the 28-day birds was significantly lower than

that from either the 1-day-old chicks or the 14-day ones.

DISCUSSION

In 1971, Boyle *et al.* (2) reported that a hypocalcemic state enhances renal conversion of 25(OH)D₃ to 1 α ,25(OH)₂D₃. Subsequent investigations proved that hypocalcemia stimulates the secretion of PTH which then enhances renal biosynthesis of 1 α ,25(OH)₂D₃ as a trophic hormone (6, 7). Thyroparathyroidectomy (TPTX) almost completely abolishes the production of 1 α ,25(OH)₂D₃, while administration of either PTH or cyclic AMP to TPTX animals restores the synthesis of 1 α ,25(OH)₂D₃ to the sham-operated control levels (3, 6, 7, 10). These results suggest that the 25(OH)D₃-1 α -hydroxylase activity is regulated by circulating levels of PTH rather than calcium. However, very recently Trechsel *et al.* (15) have demonstrated by their experiments in pair-fed TPTX and sham-operated rats that a change in plasma PTH levels is not essential for elevating the plasma 1 α ,25(OH)₂D₃ levels and that hypocalcemia *per se* stimulates renal production of 1 α ,25(OH)₂D₃.

In the present study, we found that renal 1 α -hydroxylase activity does not necessarily change in response to plasma calcium levels in the course of vitamin D deficiency. First, the renal 1 α -hydroxylase activity in the 28-day chicks declined to about half that in the 14-day chicks, though the former birds showed apparently more severe hypocalcemia than the latter ones. Second, much larger amounts of vitamin D₃ were required to suppress 1 α -hydroxylase activity as well as to induce 24-hydroxylase activity in the 28-day chicks than in the 14-day birds. 1 α (OH)D₃, however, did not exhibit such different effects between the 14- and 28-day chicks. In addition, administration of vitamin D₃ exhibited only weak hypercalcemic action in the 28-day chicks, while that of 1 α (OH)D₃ induced marked hypercalcemia in those birds. Third, the adenylate cyclase activity of the renal membrane fraction induced by PTH was also impaired in the 28-day chicks.

These results suggest that severe vitamin D deficiency induces a state of end-organ refractoriness to PTH. In the course of vitamin D deficiency, the decreased formation of cyclic AMP stimulated by PTH in the kidney leads to a reduction in 1 α -hydroxylase activity. Vitamin D₃ administered to these animals is not sufficiently converted to 1 α ,25(OH)₂D₃ in the kidney. Thus, the plasma calcium levels and renal metabolism of 25(OH)D₃ do not respond to vitamin D₃ administration. On the other hand, administration of 1 α (OH)D₃ which is rapidly converted to 1 α ,25(OH)₂D₃ in the liver without any control mechanism (20, 21) is effective even in severe vitamin D deficiency.

Forte *et al.* (22) reported that the renal cortical adenylate cyclase activity induced by PTH was markedly reduced in vitamin D-deficient rats. Kakuta *et al.* (23) and Carnes *et al.* (24) reported independently that the blunted renal cyclic AMP response to PTH in vitamin D-deficient rats is due to hypocalcemia or associated secondary hyperparathyroidism, but not to vitamin D deficiency *per se*. It has also been reported that plasma levels of 1 α ,25(OH)₂D₃ in rats maintained on

a low calcium diet increase rapidly, attain a maximal level at the third week of the feeding period, and decline thereafter (25). These findings are consistent with our results.

In conclusion, vitamin D deficiency leads to the decrease in plasma calcium levels. Trechsel *et al.* (15) reported that a change in plasma calcium *per se* is important for the regulation of renal biosynthesis of $1\alpha,25(\text{OH})_2\text{D}_3$. However, hypocalcemia *per se* does not appear to be a major factor in the stimulation of $1\alpha,25(\text{OH})_2\text{D}_3$ production, since the elevated activity of 1α -hydroxylase had declined significantly by the 21st and 28th days in spite of the more severe hypocalcemia. Hypocalcemia stimulates the secretion of PTH which then enhances renal biosynthesis of $1\alpha,25(\text{OH})_2\text{D}_3$ as a trophic hormone. In the course of vitamin D deficiency, the secretion of PTH proceeds increasingly and kidney tissues become insensitive to PTH. The end-organ refractoriness in a state of severe vitamin D deficiency is due at least in part to the impairment of renal adenylate cyclase activity induced by PTH.

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