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A possible role of vitamin D receptors in regulating vitamin D activation in the kidney

(vitamin D metabolism/1α-hydroxylase/24-hydroxylase)

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ABSTRACT **The vitamin D endocrine system is regulated reciprocally by renal 25-hydroxyvitamin D_3 1α- and 24-hydroxylases. Previously, we reported that renal proximal convoluted tubules, the major site of 1α,25-dihydroxyvitamin D_3 production, have vitamin D receptors. In the presence of vitamin D receptors, renal proximal convoluted tubules cannot maintain the state of enhanced production of 1α,25-dihydroxyvitamin D_3. To clarify this discrepancy, we proposed a working hypothesis for the reciprocal control of renal 25-hydroxyvitamin D_3 1α- and 24-hydroxylase activities. In rat models of enhanced renal production of 1α,25-dihydroxyvitamin D_3, expression of vitamin D receptors and 25-hydroxyvitamin D_3 24-hydroxylase mRNAs was strikingly suppressed in renal proximal convoluted tubules but not in the cortical collecting ducts. In vitamin D-deficient rats with up-regulated renal 25-hydroxyvitamin D_3 1α-hydroxylase activity, expression of vitamin D receptor mRNA in renal proximal convoluted tubules was also down-regulated, indicating that the down-regulation of vitamin D receptor mRNA is not the result of the enhanced production of 1α,25-dihydroxyvitamin D_3. In Japanese quail models with up-regulated renal 25-hydroxyvitamin D_3 1α-hydroxylase activity by sex steroids, expression of vitamin D receptor mRNA was also down-regulated in the kidney but not in the duodenum. These results suggest that the down-regulation of vitamin D receptors plays a critical role in production of 1α,25-dihydroxyvitamin D_3 in renal proximal convoluted tubules.**

It is well established that vitamin D_3 is first metabolized in the liver to 25-hydroxyvitamin D_3 [25(OH)D_3] and then in the kidney to 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] (1). 1α,25$(OH)_2D_3$ synthesized by 25(OH)D_3 1α-hydroxylase (1α-OHase) is biologically the most active metabolite of the vitamin, and vitamin D receptors (VDRs) mediate its effects by controlling transcription of 1α,25$(OH)_2D_3$-responsive genes. 24,25-Dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] synthesized by 25(OH)D_3 24-hydroxylase (24-OHase) is thought to be an inactive metabolite. 1α,24,25-Trihydroxyvitamin D_3 is also a degradation product generated from 1α,25$(OH)_2D_3$ by 24-OHase. It is also known that renal 1α- and 24-OHase activities, which are located mainly in the mitochondria of proximal convoluted tubules (PCTs) (2, 3), are tightly and reciprocally regulated (4, 5). It was also reported that these two hydroxylases are distinct gene products (6). Previously, we reported that VDR exists in PCT, the major site for 1α,25$(OH)_2D_3$ production, in normal rats (7). If VDR exists in PCTs under conditions in which production of 1α,25$(OH)_2D_3$ is enhanced, the produced 1α,25$(OH)_2D_3$ would induce 24-OHase and suppress 1α-OHase (8–11) (Fig. 1*A*). However, this is unlikely, since PCTs have to continue to synthesize 1α,25$(OH)_2D_3$ when renal production of 1α,25$(OH)_2D_3$ is enhanced. So, we considered that VDRs in PCT cells must be down-regulated when renal production of 1α,25$(OH)_2D_3$ is stimulated, provided that there is no special mechanism that inhibits access of 1α,25$(OH)_2D_3$ synthesized in the mitochondria to the nucleus in the cell. We hypothesized that such a regulation of VDRs would allow reciprocal control of the 1α- and 24-OHase activities in PCTs (Fig. 1*B*). In support of this hypothesis, there have been several reports that demonstrate the decrease of renal VDR in rats fed a low calcium diet (12–15).

In the present study, we made several model animals with altered renal 25(OH)D_3 metabolism and investigated the expression of VDR mRNA in the endocrine and target tissues of 1α,25$(OH)_2D_3$ to test this working hypothesis.

MATERIALS AND METHODS

Animals. Male weanling rats (Sprague–Dawley strain), either intact or thyroparathyroidectomized (TPTX), were maintained on the diets (Teklad, Madison, WI) shown in Table 1 (16–20). Rats fed the vitamin D-deficient diet were maintained in a room with incandescent lighting, and all potential sources of ultraviolet light and vitamin D were excluded. After the indicated periods, all the animals were lightly anesthetized with diethyl ether and used for the experiments.

Male Japanese quail were fed a corn soy protein diet until they were 4–6 months old, when they were used for the experiments.

Hormones. Estradiol-17β valerate and testosterone enanthate were purchased from Sigma. Sex hormones were administered subcutaneously to Japanese quail as a single dose 24 hr before sacrifice (21, 22).

Measurement of Serum Levels of Calcium, Phosphorus, and Parathyroid Hormone (PTH) in Rats. Serum levels of calcium and phosphorus were measured with an autoanalyzer (model AU-550; Olympus, Tokyo). Serum levels of PTH were measured by radioimmunoassay with an intact N-terminal-specific PTH radioimmunoassay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA).

Measurement of the *in Vitro* Renal Cortical 1α- and 24-OHase Activities. *In vitro* renal cortical 1α- and 24-OHase activities of rats and quail were measured as described (14, 21). After blood was drawn from the aorta and perfusion was made with saline, the kidneys were removed. The perfused renal

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Abbreviations: VDR, vitamin D receptor; 25(OH)D_3, 25-hydroxyvitamin D_3; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 24,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 1α-OHase, 25(OH)D_3 1α-hydroxylase; 24-OHase, 25(OH)D_3 24-hydroxylase; PCT, proximal convoluted tubule; CCD, cortical collecting duct; TPTX, thyroparathyroidectomized; PTH, parathyroid hormone; RT, reverse transcription.

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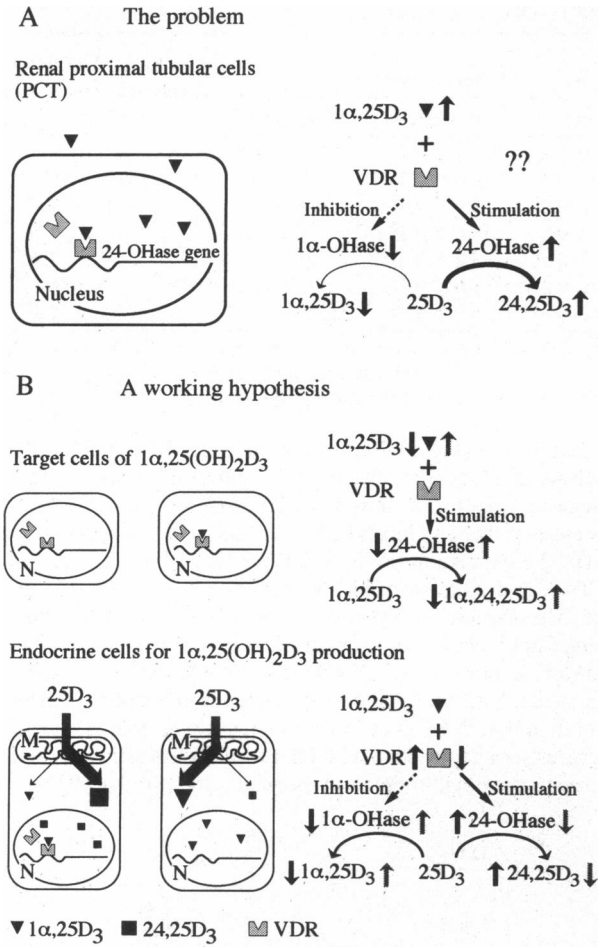

FIG. 1. The problem and a working hypothesis on the mode of regulation of vitamin D_3 metabolism. (*A*) The problem caused by the presence of VDRs in PCTs. Note that regulation of 1α- and 24-OHases, expected by the presence of VDRs, contradicts the enhanced production of $1\alpha,25(OH)_2D_3$ in the cell. (*B*) A working hypothesis for regulation of vitamin D_3 metabolism. (*Upper*) Target cells of $1\alpha,25(OH)_2D_3$. (*Lower*) Endocrine cells for $1\alpha,25(OH)_2D_3$ production (PCTs). (*Left*) Normal state. (*Middle*) State of enhanced production of $1\alpha,25(OH)_2D_3$ in the kidney. (*Right*) Possible mechanism of regulation of vitamin D_3 metabolism in the cell. Solid arrow, normal state; dashed arrow, enhanced production of $1\alpha,25(OH)_2D_3$ in the kidney. Upward and downward solid and dashed arrows, stimulation and inhibition, respectively. N, nucleus; M, mitochondria; $25D_3$, $25(OH)D_3$; $1\alpha,25D_3$, $1\alpha,25(OH)_2D_3$; $24,25D_3$, $24,25(OH)_2D_3$; $1\alpha,24,25D_3$; $1\alpha,24,25(OH)_3D_3$.

cortex was homogenized in 9 vol of a buffer containing 0.19 M sucrose, 15 mM Tris acetate (pH 7.5), 2 mM magnesium acetate, and 25 mM succinate. Each 3-ml portion of the 10% (rats) and 5% (quail) homogenates was incubated with 500 nM (66 GBq/pmol) and 1 μM (33 GBq/pmol) $[^3H]25(OH)D_3$, respectively, at 25°C for 20 min under oxygen gas with constant shaking. The incubation was terminated by adding 10 ml of methanol/$CHCl_3$ (2:1, vol/vol). Lipids were extracted by the method of Bligh and Dyer (23), and the chloroform phase was evaporated to dryness under a nitrogen stream. The residue was dissolved in a mixture of *n*-hexane/isopropanol/methanol (90:5:5, vol/vol) and subjected to a straight-phase HPLC, equipped with a Fine-Pak SIL column (4.6 mm × 25 cm; Jasco, Tokyo).

Preparation of Microdissected Rat Nephron Segments. Microdissection of the rat nephron segments was performed as described (7). PCTs and cortical collecting ducts (CCDs) (3 mm each) were microdissected. The length of the dissected tubules was measured with an eyepiece micrometer. Microdissected segments were transferred into fresh solution in another dish and rinsed carefully so that no cell debris or interstitial tissues were attached. The prepared segments were treated as described and the resultant pellets of nucleic acids were used for the following steps.

Reverse Transcription (RT). The RT reaction was performed as follows. The pellet in the ethanol precipitation tube was dissolved in 9.0 μl of RT solution and Vortexed mixed; 0.5 μl of RNasin (Promega) and 0.5 μl of Moloney murine leukemia virus H^- reverse transcriptase (Superscript; BRL) were added. The final composition of the RT reaction solution was 10 mM Tris·HCl, pH 8.3/50 mM KCl/50 mM $MgCl_2$/1 mM dithiothreitol/4 μM random hexamer (Takara Shuzo, Kyoto)/1.0 unit of RNasin per μl/5.0 units of reverse transcriptase per μl. RT tubes were incubated at 37°C for 60 min, and the reaction was terminated by heating the tubes at 95°C for 5 min. Twenty microliters of RT solution was added to the tube containing 10 μl of the RT reaction solution and it was divided into three, so that each 10 μl of aliquot corresponded to 1.0 mm of tubular segment, and they were used for PCR of β-actin, VDR, and 24-OHase.

PCR. PCR of β-actin, VDR, or 24-OHase was performed with the same primers as described (7). Detecting the expression of a housekeeping gene, β-actin, by PCR was used as a control for the quality of the sample RNA. The program of PCR was as follows: 30 cycles of 94°C for 40 s, 50°C for 1 min, 72°C for 1 min for VDR; 30 cycles of 94°C for 40 s, 60°C for 1 min, 72°C for 1 min 10 s for 24-OHase; and 30 cycles of 94°C for 40 s, 50°C for 1 min, 72°C for 30 s for β-actin.

Detection of PCR Products. For β-actin, 10 μl was taken from the tube containing the PCR product. After gel electrophoresis, gels were stained with ethidium bromide. For VDR and 24-OHase, 20 μl was taken from the tube containing RT-PCR product. After gel electrophoresis, samples were analyzed by Southern hybridization. Oligoprobes used for Southern hybridization were as described (7). These oligoprobes were labeled with $[\gamma\text{-}^{32}P]$ATP (Amersham) using T4 polynucleotide kinase (BRL). Hybridization was performed with a positively charged nylon membrane (Boehringer Mannheim).

Northern Blot Analysis of VDR and 24-OHase mRNAs. Total RNA was isolated by the method of Chomczynski and Sacchi. (24). Poly(A)$^+$ RNA was isolated by a batch method using Oligotex-dT30 (Takara Shuzo). Each 5 μg of poly(A)$^+$ RNA was electrophoresed and transferred to a Hybond-N membrane (Amersham) and applied for Northern hybridization of tubulin, VDR, and 24-OHase with $[\alpha\text{-}^{32}P]$dCTP-labeled cDNA probes as described (7). For Northern blotting of Japanese quail VDR mRNA, a cDNA probe corresponding to bases 160–961 of the avian VDR cDNA (25) was used for hybridization.

Statistics. The statistical significance of the differences between the experimental and the control groups was determined by Student's *t* test.

RESULTS

Serum Levels of Calcium, Phosphorus, and PTH and Renal Cortical 1α-OHase Activity in Model Rats. Serum levels of calcium, phosphorus, and PTH in model rats were similar to previous reports (Table 1) (16–20). Intact or TPTX rats fed the vitamin D-replete, low calcium diet or the low phosphorus diet were used as models with enhanced renal production of $1\alpha,25(OH)_2D_3$. Intact and TPTX rats fed the vitamin D-replete, normal diet were used as models with suppressed renal 1α-OHase activity. Intact rats fed the vitamin D-deficient, low calcium diet were used for the model of enhanced renal 1α-OHase activity without enhanced production of $1\alpha,25(OH)_2D_3$.

Expression of VDR and 24-OHase mRNAs in the Kidney in Vitamin D-Replete Rat Models. First, amplification products from several lengths of PCTs of intact rats fed the normal diet for VDR and 24-OHase were compared, assigning the 1-mm PCT sample

Table 1. Serum levels of calcium, phosphorus, PTH, and *in vitro* renal cortical 1α-OHase activities in model rats

Animal and diet	Diet: Ca, %	Diet: P, %	Diet: Vitamin D_3, units per g of diet	Feeding period, weeks	Serum level: Ca, mg/dl	Serum level: P, mg/dl	Serum level: PTH, pg/ml	Renal 1α-OHase activity, fmol per min per mg of protein
Intact; normal diet	0.6	0.6	2.0	2	10.1 ± 0.2	8.4 ± 0.2	22.3 ± 0.5	<10
Intact; low Ca diet	0.03	0.6	2.0	2	8.6 ± 0.2†	8.0 ± 0.2	56.2 ± 2.3†	101.5 ± 16.6†
Intact; low P diet	0.6	0.02	2.0	2	13.4 ± 0.3†	3.9 ± 0.4†	<10	31.3 ± 5.8*
TPTX; normal diet	0.6	0.6	2.0	2	5.1 ± 0.2†	11.6 ± 0.3†	<10	<10
TPTX; low P diet	0.6	0.02	2.0	2	13.6 ± 0.1†	3.4 ± 0.1†	<10	41.5 ± 3.9*
Intact; normal diet	0.6	0.6	2.0	4	9.9 ± 0.2	8.6 ± 0.2	21.5 ± 1.2	<10
Intact; low Ca diet	0.03	0.6	0	4	5.0 ± 0.3§	6.9 ± 0.3‡	355.0 ± 12.1§	494.5 ± 25.3§

Male weanling rats (SD strain), either intact or TPTX, were fed the diet indicated and all parameters were determined as described (14). Data were obtained from at least five rats. Values are expressed as means ± SE. *, $P < 0.01$; †, $P < 0.001$ (significantly different from intact rats fed the normal diet for 2 weeks). ‡, $P < 0.01$; §, $P < 0.001$ (significantly different from intact rats fed the normal diet for 4 weeks).

as an arbitrary unit of 100 (26). There was a high correlation between the length of PCTs of intact rats fed the normal diet (0.1–2.0 mm; $n = 12$) and the resulting PCR products ($r = 0.91$ for VDR; $r = 0.89$ for 24-OHase). Then, PCTs and CCDs were taken and used for RT-PCR—the former as an endocrine tissue for producing $1\alpha,25(OH)_2D_3$ and the latter as a target tissue of $1\alpha,25(OH)_2D_3$ (Fig. 2). Expression levels of VDR and 24-OHase mRNAs in PCTs were decreased significantly in the models of enhanced renal production of $1\alpha,25(OH)_2D_3$ (intact rats fed the low calcium diet and intact or TPTX rats fed the low phosphorus diet). The expression levels of VDR and 24-OHase mRNAs in PCTs decreased most strikingly in intact rats fed the low calcium diet. The expression levels of VDR mRNA in CCDs did not change appreciably between the intact rats fed either diet. In contrast, expression of 24-OHase mRNA in CCDs was up-regulated in intact rats fed the low calcium diet but at a much lower level than that in PCTs of intact rats fed the normal diet (Fig. 2). Previously, we reported that 24-OHase mRNA is expressed mainly in renal proximal tubules in normal rats (7). $1\alpha,25(OH)_2D_3$

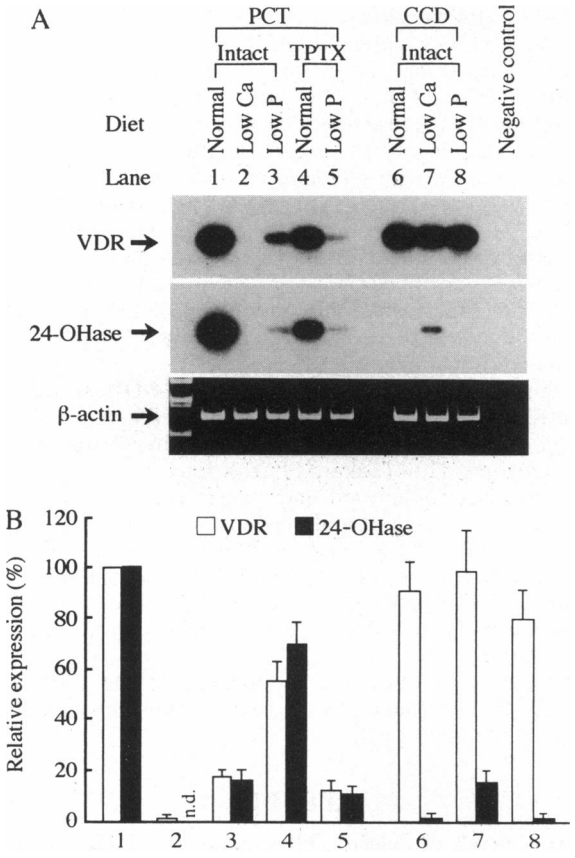

FIG. 2. Expression of VDR and 24-OHase mRNAs in the kidney in each rat model. (*A*) Southern blots of RT-PCR products of VDR and 24-OHase mRNAs in microdissected PCTs and CCDs. (*B*) Relative quantification of RT-PCR products of VDR and 24-OHase mRNAs in PCTs and CCDs in each rat model. Numbers correspond to lanes in *A*. Data are expressed as means ± SE of five rats. Open bar, VDR; solid bar, 24-OHase. Three-week-old rats, either intact or TPTX, were fed vitamin D-replete diets for 2 weeks. Microdissection and RT-PCR were performed as described (7) with slight modifications. Several PCT samples of intact rats fed the normal diet were applied to PCR without RT as negative controls. PCR products were detected with ^{32}P-labeled oligoprobes. Southern blots were quantified with a Fuji Bas 2000 image analyzer. For relative quantification, the value from PCTs of intact rats fed the normal diet was used as a standard (100%) (26). n.d., Not detected.

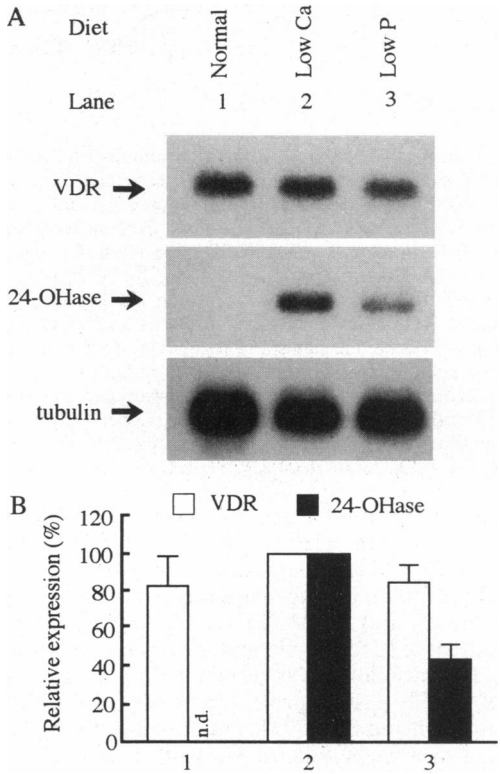

FIG. 3. Expression of VDR and 24-OHase mRNAs in the duodenum in each rat model. (*A*) Northern blots of VDR and 24-OHase mRNAs. (*B*) Relative expression of VDR and 24-OHase mRNAs. Expression of VDR and 24-OHase mRNAs was corrected by expression of tubulin mRNA. Numbers correspond to lanes in *A*. Data are expressed as means ± SE of five rats. Open bar, VDR; solid bar, 24-OHase. Northern blotting was performed as described (14). For relative quantification, the value from the duodenum of intact rats fed the low calcium diet was used as a standard (100%), and the percentage of each signal was calculated. n.d., Not detected.

produced by PCTs would have induced 24-OHase mRNA by an autocrine/paracrine mechanism in the same segments of intact rats fed the normal diet.

Expression of VDR and 24-OHase mRNAs in the Duodenum in Each Rat Model. Next we examined the expression of VDR and 24-OHase mRNAs in the duodenum, the classical target tissue of $1\alpha,25(OH)_2D_3$ (27) (Fig. 3). Expression of VDR mRNA in the duodenum did not change appreciably between the intact rats fed either diet. The expression of 24-OHase mRNA in the duodenum was greatly up-regulated in intact rats fed the low calcium or the low phosphorus diet.

Expression of VDR and 24-OHase mRNAs in the Kidney Between Intact Rats Fed the Vitamin D-Replete, Normal Diet or the Vitamin D-Deficient, Low Calcium Diet. As shown in Fig. 2, the expression of VDR mRNA in PCTs correlated well with the expression of 24-OHase mRNA and almost inversely with the activity of renal cortical 1α-OHase, which fits our working hypothesis. Furthermore, to exclude the possibility that the decrease in the level of VDR mRNA in PCTs is the result of enhanced production of $1\alpha,25(OH)_2D_3$, rats were fed a vitamin D-deficient, low calcium diet as a model of up-regulated renal 1α-OHase activity without enhanced production of $1\alpha,25(OH)_2D_3$. Compared to rats fed the vitamin D-replete, normal diet, expression of VDR mRNA in rats fed the vitamin D-deficient, low calcium diet was significantly suppressed in PCTs but not in CCDs (Fig. 4), as was also the case in rats fed the vitamin D-replete, low calcium diet (Fig. 2). This indicates that the decrease in the level of VDR mRNA in PCTs is not the result of enhanced production of $1\alpha,25(OH)_2D_3$.

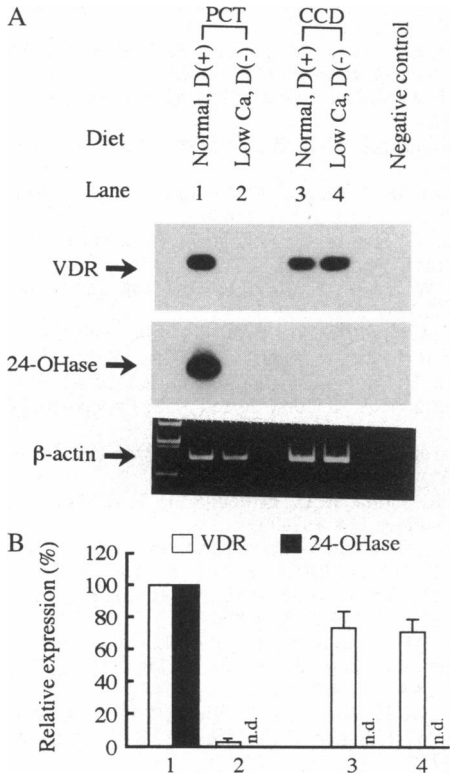

FIG. 4. Expression of VDR and 24-OHase mRNAs in the kidney between intact rats fed the vitamin D-replete [D(+)], normal diet or the vitamin D-deficient [D(−)], low calcium diet. (*A*) Southern blots of RT-PCR products of VDR and 24-OHase mRNAs in PCTs and CCDs in each rat model. (*B*) Relative quantification of RT-PCR products of VDR and 24-OHase mRNAs in PCTs and CCDs in each rat model. Numbers correspond to lanes in *A*. Data are expressed as means ± SE of five rats. Open bar, VDR; solid bar, 24-OHase. n.d., Not detected.

Effect of Sex Steroids on Renal Vitamin D Metabolism and VDR mRNA Expression in Japanese Quail. As another model of up-regulated renal 1α-OHase activity, expression of VDR mRNA was examined in Japanese quail treated with sex steroids (21, 28). Renal 1α- and 24-OHase activities were similar to previous reports (Fig. 5*A*). Treatment with sex steroids down-regulated the expression of VDR mRNA in the kidney but not in the duodenum as expected from our working hypothesis (Fig. 5 *B* and *C*).

DISCUSSION

The present study indicates that the model shown in Fig. 1*B* is a reasonable explanation for regulation of vitamin D metab-

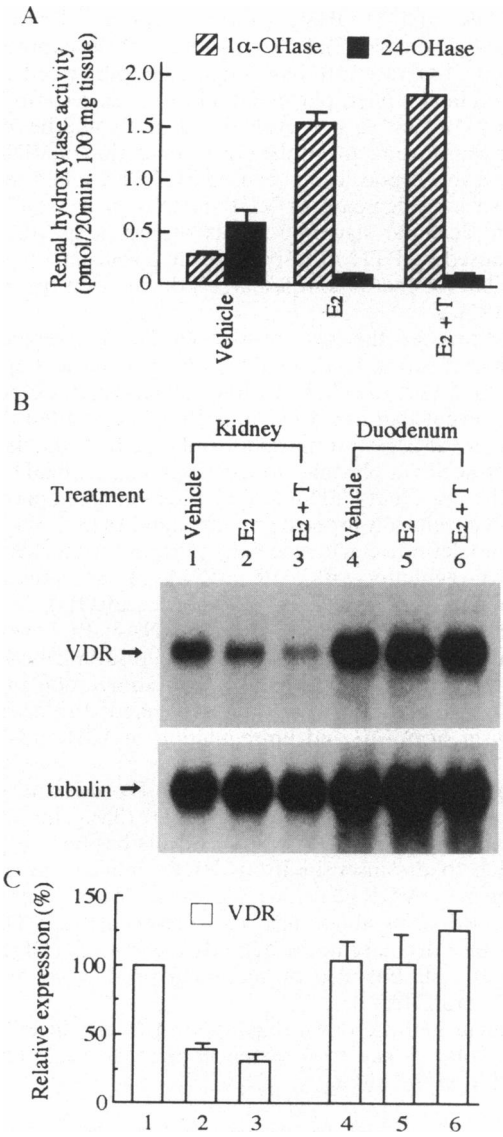

FIG. 5. Effect of sex steroids on renal vitamin D metabolism and VDR mRNA expression in Japanese quail. (*A*) *In vitro* renal cortical 1α- and 24-OHase activities. Mature male quail were given castor oil only (Vehicle), 5 mg of estradiol-17β valerate in castor oil (E2), or 5 mg of estradiol-17β valerate in castor oil and 10 mg of testosterone enanthate in sesame oil (E2 + T) 24 hr before sacrifice. Data are expressed as means ± SE of four quail. Hatched bar, 1α-OHase; solid bar, 24-OHase. (*B*) Northern blots of VDR mRNA in the kidney and duodenum. (*C*) Relative expression of VDR mRNA in the kidney and duodenum. Expression of VDR mRNA was corrected by expression of tubulin mRNA. Numbers correspond to lanes in *B*. Data are expressed as means ± SE of four quail. For relative quantification, the value from the kidney of the control quail was used as a standard (100%), and the percentage of each signal was calculated.

olism *in vivo*. In the target cells of $1\alpha,25(OH)_2D_3$, vitamin D metabolism is regulated preferentially by the $1\alpha,25(OH)_2D_3$ level produced by PCTs. However, in the endocrine cells for $1\alpha,25(OH)_2D_3$ production (PCTs), the content of VDRs is most likely the major regulator for $25(OH)D_3$ metabolism and therefore the production of $1\alpha,25(OH)_2D_3$. To further prove this model, detection of the changes in the quantity of VDRs at a protein level would be important, because the mRNA level of VDRs does not always correlate with the protein level (29).

The mechanism of the down-regulation of VDR mRNA in PCTs still remains unclear. PTH may play the most important role in the down-regulation of VDRs in PCTs of intact rats fed the low calcium diet (30, 31). However, continuous infusion of PTH into intact animals did not always cause decreases in renal VDRs and 24-OHase (30) or elevation of the serum $1\alpha,25(OH)_2D_3$ level (32). PTH did not affect the expression of VDR and 24-OHase mRNAs in opossum kidney cells, which retain sodium-coupled phosphate uptake and responsiveness to PTH (33). Low serum levels of calcium could be another possible causative factor of the down-regulation of VDRs (13, 15). However, hypocalcemia caused by TPTX failed to cause such a striking decrease of VDR mRNA in PCTs (Fig. 2). Some intracellular signaling events occurring specifically in PCTs caused by PTH in response to hypocalcemia would be responsible for the observed marked decrease in the level of VDR mRNA.

In the rats fed the low phosphorus diet, the direction of regulation of serum levels of PTH and calcium was opposite to that of intact rats fed the low calcium diet. A possible common event that can occur in both of the rats fed the low calcium diet and the low phosphorus diet may be the decrease of the intracellular phosphorus level in renal proximal tubules. It is well known that PTH inhibits sodium-coupled phosphate transport specifically in renal proximal tubules (34). But so far, there is no definitive evidence that indicates the mechanism of the down-regulation of VDR mRNA in the situation of up-regulated 1α-OHase in PCTs. When $1\alpha,25(OH)_2D_3$ acts in the target tissues satisfactorily, VDR mRNA in PCTs would be up-regulated by an unknown mechanism, resulting in suppression of $1\alpha,25(OH)_2D_3$ production and stimulation of 24,25 $(OH)_2D_3$ synthesis. Further studies are needed to clarify the mechanism of down- and up-regulation of VDR mRNA in PCTs.

Patients with hereditary $1\alpha,25(OH)_2D_3$-resistant rickets show high serum levels of $1\alpha,25(OH)_2D_3$ (35). The cause of hereditary $1\alpha,25(OH)_2D_3$-resistant rickets has been reported to be due to the insensitivity of VDRs induced by a point mutation in the VDR gene (36). We speculate that insensitivity of VDRs will bring about high serum levels of $1\alpha,25(OH)_2D_3$ not only by a decrease in the degradation of $1\alpha,25(OH)_2D_3$ in the target cells but also by increased production of $1\alpha,25$ $(OH)_2D_3$ in PCTs.

In conclusion, the down-regulation of VDRs in PCTs appears to play a key role in the control of $1\alpha,25(OH)_2D_3$ production in the kidney.

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