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INDUCTION OF VITAMIN D 24-HYDROXYLASE MESSENGER RNA AND ACTIVITY BY 22-OXACALCITRIOL IN MOUSE KIDNEY AND DUODENUM

POSSIBLE ROLE IN DECREASE OF PLASMA 1α,25-DIHYDROXYVITAMIN D_3

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Abstract—The synthetic analog of 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], 22-oxacalcitriol (OCT), retains most of the properties of 1,25$(OH)_2D_3$ but exhibits much less hypercalcemic action than the parent compound. The effects of OCT on plasma calcium, phosphorus, and 1α,25-dihydroxyvitamin D [1,25$(OH)_2D$] concentrations were examined in mice. Administration of a single dose (24 pmol/g body wt, i.p.) of OCT had no effect on plasma calcium for up to 48 hr, significantly increased plasma phosphorus at 4 and 8 hr and significantly reduced the concentration of 1,25$(OH)_2D$ in plasma between 4 and 48 hr. Both OCT and 1,25$(OH)_2D_3$ at 24 pmol/g body wt (i.p.) induced a single, 3.4-kb mRNA encoding vitamin D 24-hydroxylase (24-OHase), the cytochrome P450 enzyme responsible for 1,25$(OH)_2D_3$ degradation, in kidney and duodenum. The OCT-induced increase in 24-OHase mRNA and an increase in enzyme activity were marked at 2 and 4 hr in both tissues. In kidney, mRNA abundance had decreased by 8 hr but remained above basal values for up to 30 hr; activity remained relatively high for up to 48 hr. In duodenum, 24-OHase mRNA abundance returned virtually to control values by 8 hr after OCT treatment; activity remained at nearly maximal levels for up to 30 hr but was decreased at 48 hr. The effects of OCT and 1,25$(OH)_2D_3$ on 24-OHase mRNA abundance and enzyme activity were dose-dependent in kidney and duodenum. Whereas the dose–response relations for the effects of both compounds on 24-OHase mRNA were similar, OCT was slightly more potent than 1,25$(OH)_2D_3$ in stimulating 24-OHase activity in both tissues. These results suggest that the OCT-induced decrease in plasma 1,25$(OH)_2D_3$ is attributable, at least in part, to an increased degradation of 1,25$(OH)_2D_3$, which results from an increase in 24-OHase abundance and enzyme activity.

Key words: 22-oxacalcitriol; 1α,25-dihydroxyvitamin D_3; vitamin D 24-hydroxylase; gene expression; plasma calcium; cytochrome P450

The hormonally active form of vitamin D_3,1,25-$(OH)_2D_3$†, and related compounds, such as OCT, have been studied as potential therapeutic agents for the treatment of various diseases including osteoporosis, psoriasis, leukemia, and secondary hyperparathyroidism associated with chronic renal failure [1–5]. The recent development of vitamin D analogs that exhibit selective activities has also provided a new approach for studying the mechanism of vitamin D action in target tissues such as the duodenum and kidney. OCT mimics specific actions of 1,25$(OH)_2D_3$, including induction of differentiation of WEHI-3 myelomonocytic cells [6], inhibition of proliferation of psoriatic fibroblasts [7], and suppression of PTH secretion and gene expression [8, 9]. However, OCT exhibits little hypercalcemic activity, suggesting that bone and intestine are resistant to this analog [4]. Indeed, unlike 1,25$(OH)_2D_3$, OCT does not increase the intracellular calcium concentration in osteoblast-like osteosarcoma cells (ROS17/2.8) [10]. In contrast, OCT both binds to nuclear receptors and stimulates calcium absorption in the intestine [10, 11], although the stimulation is only transient because of the rapid clearance of the drug [11–13]. OCT has been shown to reduce the plasma concentration of 1,25$(OH)_2D$ independently of PTH, an important modulator of 1,25$(OH)_2D_3$ synthesis in the kidney; this effect of OCT may result from suppression of 1,25$(OH)_2D_3$ synthesis, increased hormone degradation, or both [12].

Further hydroxylation of 1,25$(OH)_2D_3$ is thought to result in catabolism to the end product of side-chain cleavage, calcitroic acid [14, 15]. The C-24 oxidation pathway is a major catabolic route for 1,25$(OH)_2D_3$ in target tissues, including intestine and kidney, and protects against the toxic effects of excess hormone [14–17]. The first enzyme in this degradative sequence, 24-OHase, catalyzes the

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† Abbreviations: 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1,25$(OH)_2D$, 1α,25-dihydroxyvitamin D; OCT, 22-oxacalcitriol; 24-OHase, vitamin D 24-hydroxylase; 25$(OH)D_3$, 25-hydroxyvitamin D_3; 1,24,25$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3; 24,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; and PTH, parathyroid hormone.

conversion of $1,25(OH)_2D_3$ to the biologically less active metabolite $1,24,25(OH)_3D_3$ and is identified as a mitochondrial cytochrome P450 [18]. The enzyme, which is expressed constitutively in the kidney, also converts $25(OH)D_3$ to $24,25(OH)_2D_3$ [19]. This catabolic pathway is also inducible in intestine and kidney with $1,25(OH)_2D_3$ treatment [20].

We have now examined the effect of OCT on 24-OHase activity and gene expression in mouse kidney and duodenum.

MATERIALS AND METHODS

Materials. $1,25(OH)_2[26,27\text{-}^3H]D_3$ (sp. act. 6.0 TBq/mmol) was obtained from DuPont NEN Research Products (Boston, MA, U.S.A.). Crystalline $25(OH)D_3$ was obtained from the Philips–Duphar Co. (Amsterdam, Netherlands). Crystalline $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ were donated by Dr. H. Yamato (Kureha Chemicals, Tokyo, Japan). OCT and $1,24,25(OH)_3D_3$ were gifts from Dr. H. Ohkawa (Chugai Pharmaceutical, Gotemba, Japan). $25(OH)[26,27\text{-}^3H]D_3$ (sp. act. 740 GBq/mmol), [α-^{32}P]dCTP (sp. act. 110 TBq/mmol), a Gigaprimer DNA labeling kit, nylon membrane (Hybond-N^+) and Hyperfilm were obtained from Amersham (Tokyo, Japan). A Micro-Fasttrack mRNA isolation kit [oligo(dT)-cellulose] was purchased from Invitrogen (San Diego, CA, U.S.A.). A Gene Amp RNA PCR kit was obtained from Perkin Elmer Cetus (Norwalk, CT, U.S.A.), and a pT7Blue T-vector kit was purchased from Novagen (Madison, WI, U.S.A.). Calcium C-Test was purchased from Wako Pure Chemical Industries (Osaka, Japan), and Pi Set was from Iatron (Tokyo, Japan). All other reagents and chemicals were of analytical grade.

Animals. Normal ddY male mice (SLC, Hamamatsu, Japan), weighing 35–45 g, were maintained on rodent chow (Oriental Yeast, Tokyo, Japan) containing vitamin D_3 (1.6 IU/g diet) for 1 week. OCT or $1,25(OH)_2D_3$ was administered i.p. to the mice in 100 μL of corn oil at doses of 0.24, 1.2, 3.6, or 24 pmol/g body wt. Control animals received vehicle alone. Mice were killed by cervical dislocation under anesthesia at the indicated time after the administration of vitamin D analogs. Plasma from five mice of the same group was pooled for the measurement of $1,25(OH)_2D$, as well as total calcium and phosphorus. Plasma $1,25(OH)_2D$ was measured by a radioreceptor assay with calf thymus receptor and C18/OH cartridge purification [21]. Total calcium and phosphorus in plasma were measured with the Calcium C-test and Pi Set, respectively. Kidneys were also removed, and the duodenum (5 cm from the pyloric sphincter) was scraped with a glass slide. Homogenates were prepared for the determination of 24-OHase activity. For the isolation of total and polyadenylated [poly(A)$^+$] RNA, kidney or whole duodenal tissue was quickly frozen with liquid N_2. Animal studies were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals [22].

Preparation and analysis of mRNA. For preparation of a probe for Northern and dot blot analysis, total RNA was isolated from the kidneys of normal ddY mice (40 g body wt) fed a normal diet and injected with $1,25(OH)_2D_3$ (24 pmol/g body wt i.p.) 4 hr previously [23]. Reverse transcription-polymerase chain reaction was performed using a Gene Amp RNA PCR kit. Oligo(dT)-primed single-strand cDNA was then generated by the reverse transcriptase reaction. Double-strand cDNA was synthesized with specific primers based on the rat cDNA sequence encoding $25(OH)D_3$-24-OHase [18] (5′ primer, nucleotides 844 to 865; 3′ primer, nucleotides 1867 to 1888) and the polymerase chain reaction (PCR). Thirty cycles of PCR were performed in the following sequence: denaturation at 93° for 1 min, annealing at 55° for 1 min, and extension at 72° for 2 min [24]. The PCR products were separated on a low melting temperature agarose gel and cloned into pT7Blue T-vector. The nucleotide sequence was determined by the dideoxynucleotide chain termination method [25]. A clone with high sequence homology to the confirmed sequence was used to prepare the probe.

Total RNA was isolated from kidney and duodenum by acid guanidium thiocyanate–phenol–chloroform extraction [23]. Poly(A)$^+$ RNA was isolated with oligo(dT)-cellulose (Micro-Fasttrack mRNA isolation kit). For Northern analysis, poly(A)$^+$ RNA or total RNA was fractionated on a 1.2% agarose gel containing formaldehyde and then transferred to a Hybond-N^+ membrane. Blots were hybridized with the cDNA probe, which had been labeled with [α-^{32}P]dCTP and the Gigaprimer DNA labeling system [26]. Northern analysis of poly(A)$^+$ RNA from kidney and duodenum was used to assess the integrity of the isolated RNA and the validity of the probe. Northern blots and dot blots of total RNA were used to quantify the amount of 24-OHase mRNA. Hybridization was performed in 50% formamide and 5 × SSPE (saline, sodium phosphate, EDTA) at 42°; blots were washed in 0.2 × SSPE at room temperature. These conditions allow hybridization between RNA species and labeled DNA probes that show >75% homology [27]. After hybridization, the membrane was exposed to Amersham Hyperfilm at −80° with intensifying screens. Cyclophilin mRNA, which is not affected by hormone administration, was used to verify the amount of mRNA on Northern blot analysis of poly(A)$^+$ RNA. RNA loading among lanes on Northern blotting of total RNA was compared by ethidium bromide staining.

Measurement of 24-OHase activity. 24-OHase activity in homogenates was assayed *in vitro*. The kidney cortex was minced and the duodenum was scraped, and both tissues were washed in ice-cold homogenization buffer containing 0.19 M sucrose, 25 mM sodium-succinate, 2 mM $MgCl_2$ and 20 mM Tris–HEPES (pH 7.4). Homogenates (5%, w/v) were prepared in homogenization buffer [20]. Substrate, dissolved in 10 μL of ethanol, was added to 1 mL of homogenate and incubated at 37° for 20 min. The substrates for renal and duodenal assays were [^{3}H]-$25(OH)D_3$(250 pmol/80,000 cpm) and [^{3}H]-$1,25(OH)_2D_3$ (50 pmol/80,000 cpm), respectively. The rate of conversion of the substrate into the product was nearly linear with time of incubation

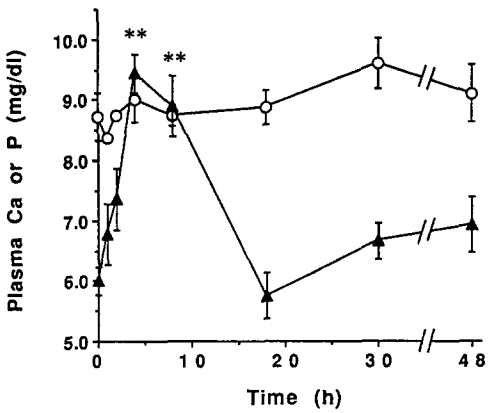

Fig. 1. Effects of OCT administration on plasma calcium and phosphorus. Mice were injected i.p. with OCT (24 pmol/g body wt), and plasma calcium (○) and phosphorus (▲) were measured at the indicated times. Values are the means ± SEM of 3–5 samples. Key: (**) $P < 0.01$ (vs time zero).

Table 1. Effects of OCT and $1,25(OH)_2D_3$ on plasma calcium concentrations

	Dose (pmol/g body wt)	Plasma calcium (mg/dL)
Control		9.5 ± 0.2
OCT	3.6	9.7 ± 0.4
	24.0	10.3 ± 0.3
$1,25(OH)_2D_3$	3.6	11.2 ± 0.4*
	24.0	12.1 ± 0.1*

Mice were injected i.p. with OCT or $1,25(OH)_2D_3$, and plasma calcium was measured at 18 hr. Values are the means ± SEM of 3–5 animals.
* $P < 0.01$ (vs control).

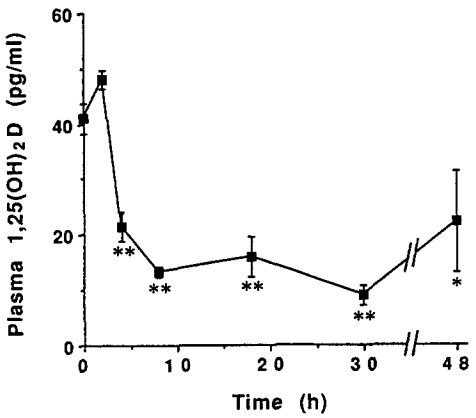

Fig. 2. Time course of plasma $1,25(OH)_2D$ in OCT-treated mice. Mice were injected i.p. with OCT (24 pmol/g body wt), and plasma $1,25(OH)_2D$ concentration was determined by radioreceptor assay at the indicated time. Results are expressed as the means ± SEM of 3–5 samples. Key: (*) $P < 0.05$, and (**) $P < 0.01$ (vs time zero).

up to 20 min and with the amount of protein up to 6% homogenate, corresponding to 12 mg protein in the kidney and 6 mg protein in the duodenum. [^{3}H]-$24,25(OH)_2D_3$ and [^{3}H]-$1,24,25(OH)_3D_3$ were separated by straight-phase HPLC on a Fine-Pak SIL column (25 cm × 3.9 mm i.d., JASCO, Tokyo, Japan) with *n*-hexane : isopropanol : methanol [90 : 5 : 5 (by vol.) for kidney; 88 : 6 : 6 for duodenum] as solvent at a flow rate of 1.5 mL/min. $24,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$ were identified as described previously [22]. To monitor the recovery, [^{3}H]-$1,25(OH)_2D_3$ (3000 cpm/sample) for the kidney homogenate or [^{3}H]-$25(OH)D_3$ (3000 cpm/sample) for the duodenal homogenate was added to each sample after the reaction was stopped. We corrected the amount of product and substrate by the radioactivity of recovery tracers on the HPLC. The recovery of each sample was 85–90%. Enzyme activity was expressed as pmol/mg tissue/20 min.

Statistical analysis. Results are expressed as means ± SEM and were analyzed by one-way ANOVA followed by a Fisher PLSD test with a computer program (Statview 512, Brain Power, Calabasas, CA, U.S.A.). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of OCT on plasma calcium, phosphorus and $1,25(OH)_2D$. Mice were injected i.p. with OCT (24 pmol/g body wt), and the plasma concentrations of calcium and phosphorus were measured at 1, 2, 4, 8, 18, 30, and 48 hr. OCT did not affect the plasma concentration of calcium but significantly increased plasma phosphorus at 4 and 8 hr (Fig. 1). The plasma phosphorus concentration had returned to basal values by 18 hr after OCT administration. To compare the hypercalcemic effect of OCT with that of $1,25(OH)_2D_3$, dose–response experiments on the plasma calcium were performed (Table 1). Mice were injected i.p. with graded doses of OCT or $1,25(OH)_2D_3$, and the plasma concentrations of calcium were determined at 18 hr. After administration of OCT at doses of 3.6 and 24 pmol/g body wt, there was no hypercalcemia, whereas $1,25(OH)_2D_3$ at the same doses led to hypercalcemia. We also examined the effect of OCT (24 pmol/g body wt, i.p.) on plasma $1,25(OH)_2D$ concentration (Fig. 2). Plasma $1,25(OH)_2D$ did not differ significantly from the control value at 2 hr, but was decreased significantly between 4 and 48 hr.

Evaluation of the specificity of the mouse 24-OHase cDNA probe. Renal and duodenal poly(A)$^+$ RNA from control and $1,25(OH)_2D_3$-treated mice was subjected to Northern blot analysis with a ^{32}P-labeled mouse 24-OHase cDNA probe (Fig. 3). The probe hybridized with a 3.4-kb mRNA from kidney and duodenum of mice treated with $1,25(OH)_2D_3$ (24 pmol/g body wt). This transcript was virtually identical in size to that of the rat 24-OHase mRNA described previously [18, 22]. The 3.4-kb mRNA

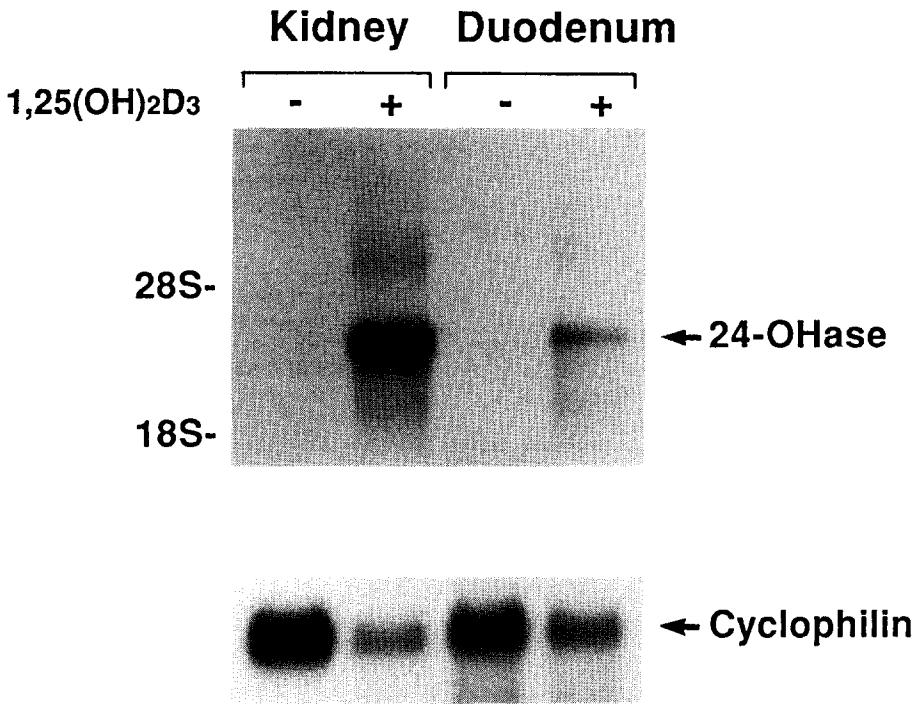

Fig. 3. Evaluation of the specificity of the mouse 24-OHase cDNA probe. Poly(A)$^+$ RNA was prepared from the kidneys and duodenum of mice that were killed 4 hr after the administration of $1,25(OH)_2D_3$ (24 pmol/g body wt) or vehicle. Poly(A)$^+$ RNA (5 μg) was fractionated by electrophoresis on a 1.2% agarose gel containing formaldehyde and then transferred to a nylon membrane. (Upper panel) The membrane was hybridized with the 1.0-kb ^{32}P-labeled mouse 24-OHase cDNA fragment and exposed to X-ray film for 24 hr at −80°. (Lower panel) The membrane was hybridized with the cyclophilin cDNA probe and exposed to X-ray film for 24 hr at −80°. The positions of 28S and 18S ribosomal RNA are indicated.

was not detected in the renal and duodenal poly(A)$^+$ RNA from control mice. The amount of 24-OHase mRNA relative to cyclophilin mRNA also increased in the kidney and duodenum of $1,25(OH)_2D_3$-treated mice. The mouse 24-OHase cDNA probe was thus shown to be suitable for the determination of 24-OHase mRNA abundance in the remaining studies.

Time course of the effects of OCT on 24-OHase mRNA and activity. Total RNA from kidneys and duodenum of mice that were killed at various times after administration of OCT (24 pmol/g body wt, i.p.) was subjected to northern blot analysis with the 24-OHase cDNA probe (Fig. 4). The time courses of the increase of 24-OHase mRNA were similar in both tissues; 24-OHase transcripts were observed at 1 hr and reached a maximum at 4 hr. However, the subsequent disappearance of 24-OHase mRNA was more rapid in the duodenum than in the kidney. We then directly compared the time courses of 24-OHase mRNA accumulation and enzyme activity in kidney and duodenum of mice treated with OCT (24 pmol/g body wt, i.p.) (Fig. 5). Both 24-OHase gene expression and enzyme activity were increased markedly in both tissues 2 and 4 hr after OCT administration. In kidney (Fig. 5A), mRNA abundance had decreased by 8 hr but remained above baseline until 30 hr. The activity remained relatively high even at 48 hr. In contrast, duodenal 24-OHase mRNA abundance returned to almost control values by 8 hr after OCT injection, whereas activity remained almost maximal at 30 hr and decreased at 48 hr (Fig. 5B).

Comparison of the effects of OCT and $1,25(OH)_2D_3$ on 24-OHase mRNA and activity. Northern analysis revealed that OCT and $1,25(OH)_2D_3$ each increased 24-OHase mRNA abundance in a dose-dependent manner in both kidney and duodenum 4 hr after administration (Fig. 6). The dose-dependent effects of OCT and $1,25(OH)_2D_3$ on relative 24-OHase mRNA abundance in kidney were quantified by dot blot analysis (Fig. 7A). The increase in 24-OHase mRNA in response to each analog was significant at a dose of 1.2 pmol/g body wt and reached a maximum between 3.6 and 24 pmol/g body wt. Both OCT and $1,25(OH)_2D_3$ also increased renal 24-OHase activity in a dose-dependent manner (Fig. 7B). Maximal stimulation was observed at 1.2 to 24 pmol/g body wt, with the effects of OCT being significantly greater than those of $1,25(OH)_2D_3$. The effects of OCT and $1,25(OH)_2D_3$ on duodenal 24-OHase mRNA relative abundance were significant at 1.2 pmol/g body wt (Fig. 8A). However, whereas maximal effects of OCT on 24-OHase mRNA were apparent at 3.6 and 24 pmol/g body wt, mRNA abundance continued to

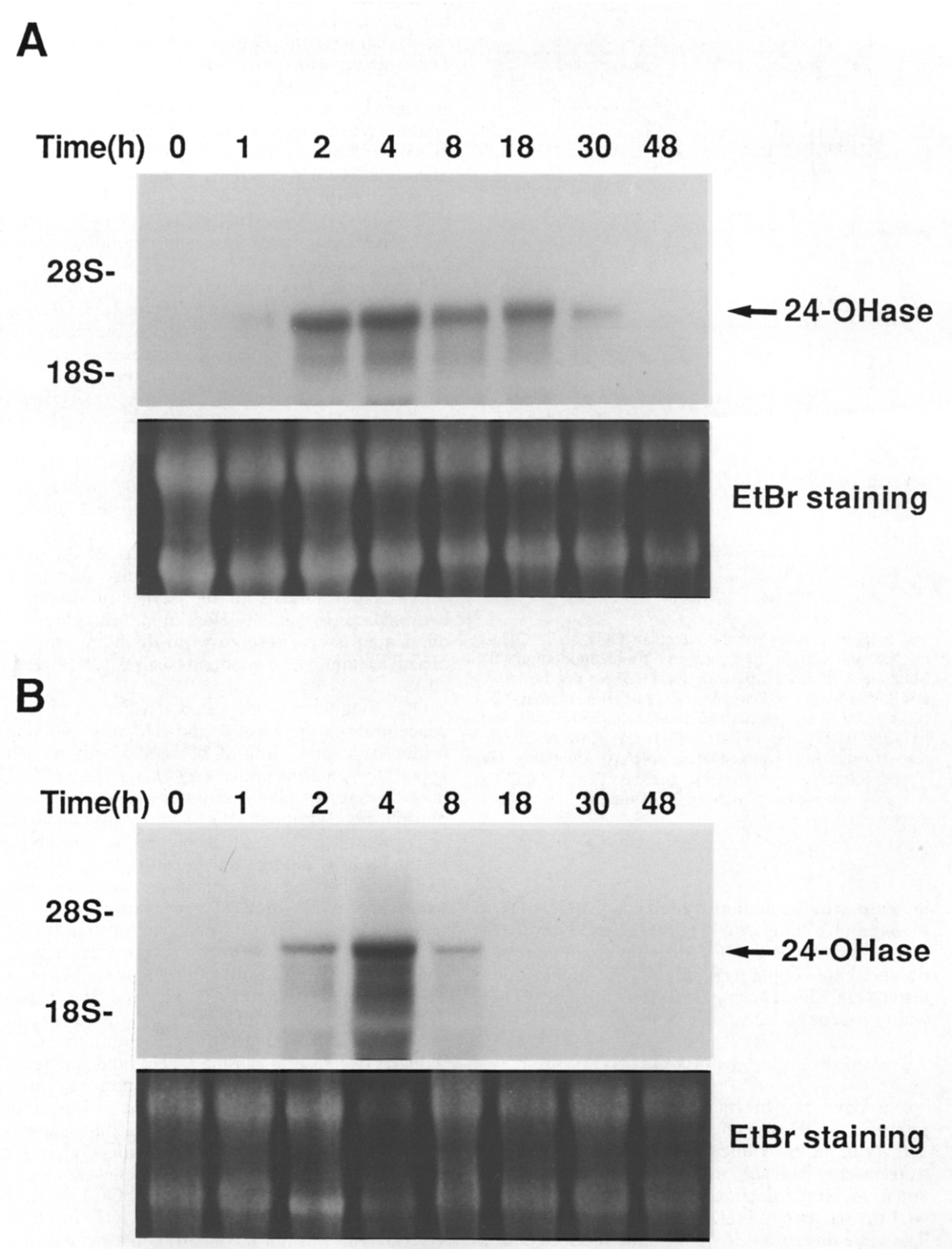

Fig. 4. Northern blot analysis of 24-OHase mRNA in kidneys and duodenum of mice treated with OCT. Total RNA (30 μg) from kidneys (A) and duodenum (B) of mice at various times after injection of OCT (24 pmol/g body wt) was subjected to Northern blot analysis. The bottom half of each panel shows gels stained with ethidium bromide (EtBr) before transfer of RNA to the nylon membrane.

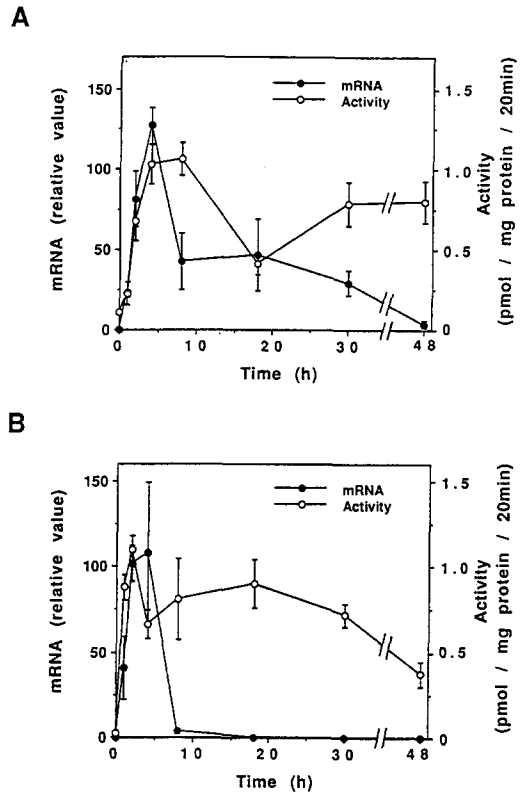

Fig. 5. Time courses for the effects of OCT on 24-OHase mRNA and activity in kidneys (A) and duodenum (B). Mice were injected i.p. with OCT (24 pmol/g body wt) and killed at the indicated times. 24-OHase activity (○) was measured using renal and duodenal homogenates with [^{3}H]-25(OH)D_3 and [^{3}H]-1,25(OH)$_2D_3$ as the substrate, respectively. Relative 24-OHase mRNA abundance (●) was determined by dot blot analysis. Values are the means ± SEM of 3–5 animals.

increase with the administration of 1,25(OH)$_2D_3$ up to 24 pmol/g body wt. Maximal stimulation of 24-OHase activity by OCT in the duodenum was observed at 0.24 pmol/g body wt, the smallest dose tested (Fig. 8B). The maximal effect of 1,25(OH)$_2D_3$ was apparent at 1.2 pmol/g body wt.

DISCUSSION

We have shown that OCT and 1,25(OH)$_2D_3$ increase 24-OHase gene expression and enzymatic activity in mouse kidney and duodenum. Although a transient increase in plasma phosphorus was apparent after injection of OCT (24 pmol/g body wt, i.p.), a hypercalcemic response was not observed. The same dose of OCT significantly reduced plasma 1,25(OH)$_2$D concentrations. These data suggest that the OCT-induced reduction in plasma 1,25(OH)$_2$D is attributable, at least in large part, to an increase in 1,25(OH)$_2$D degradation that results from OCT-induced 24-OHase gene expression in target tissues such as kidney and duodenum.

We used normal mice rather than rats for our studies. Although the 24-OHase activity assay with kidney homogenates prepared from rats that had been perfused extensively with saline was sensitive, reliable, and precise [22, 28], the assay did not show these characteristics with duodenal homogenates. The difficulty with activity measurement in rats may be due to the presence of proteins that inhibit the enzyme by reducing the substrate [29]. These inhibitors are much weaker in mice than in the plasma and tissues of rats. Thus, the measurement of mouse 24-OHase activity *in vitro* is possible by monitoring the conversion of [^{3}H]-25(OH)D_3 to [^{3}H]-24,25(OH)$_2D_3$ and of [^{3}H]-1,25(OH)$_2D_3$ to [^{3}H]-1,24,25(OH)$_3D_3$ in kidney and duodenal homogenates, respectively [20]. The activity of 24-hydroxylation was demonstrated *in vitro* with duodenal homogenates derived from 1,25(OH)$_2D_3$-treated mice. The same preparation converted 25(OH)D_3 to 24,25(OH)$_2D_3$, suggesting that duodenal 24-OHase can act on more than one vitamin D substrate. Furthermore, 1,25(OH)$_2D_3$ was converted to 1,24,25(OH)$_3D_3$, which was metabolized via pathways of C-24 oxidation and side-chain cleavage of the hormone [17]. In the intestine (duodenum), 1,25(OH)$_2D_3$ rather than 25(OH)D_3 is thought to be a physiological substrate for vitamin D 24-OHase [14, 15, 20]. We therefore used [^{3}H]-1,25(OH)$_2D_3$ for the assay of duodenal 24-OHase activity. Another advantage of mice as experimental animals for the studies of vitamin D hydroxylases is that 24-OHase in mouse kidney and duodenum is very responsive to the administration of active vitamin D_3 compounds such as 1,25(OH)$_2D_3$ [20].

OCT significantly increased plasma phosphorus concentrations between 4 and 8 hr after administration of a single dose of 24 pmol/g body wt. The hyperphosphatemia induced by a large dose of OCT may be due to increased intestinal absorption of phosphorus, although the analog is much less active than 1,25(OH)$_2D_3$ in increasing phosphorus metabolism in kidney and intestine [30]. Alternatively, this increase in plasma phosphorus may result from suppression of PTH gene expression [9, 31] and its secretion [8]. Our observation that OCT at the same dose had no effect on plasma calcium was consistent with the results of previous studies [4, 6]. The observation that OCT reduced the plasma 1,25(OH)$_2$D concentration in parathyroidectomized rats [12] demonstrates that the OCT-induced decrease in plasma PTH could not totally account for the significant reduction in plasma 1,25(OH)$_2$D concentration apparent in our study. Although increases in plasma calcium suppress 1,25(OH)$_2D_3$ synthesis in kidney independently of plasma PTH [32, 33], the fact that plasma calcium concentration was not affected by OCT administration suggests that the effect of OCT on plasma 1,25(OH)$_2$D was not mediated by plasma calcium.

The absence of a clear hypercalcemic action of OCT in the present study may be due to pharmacokinetic differences between the analog and 1,25(OH)$_2D_3$. OCT in rats disappeared from the circulation more rapidly than 1,25(OH)$_2D_3$ [11, 13]. OCT was quickly incorporated into the duodenum and formed an OCT–vitamin D receptor complex

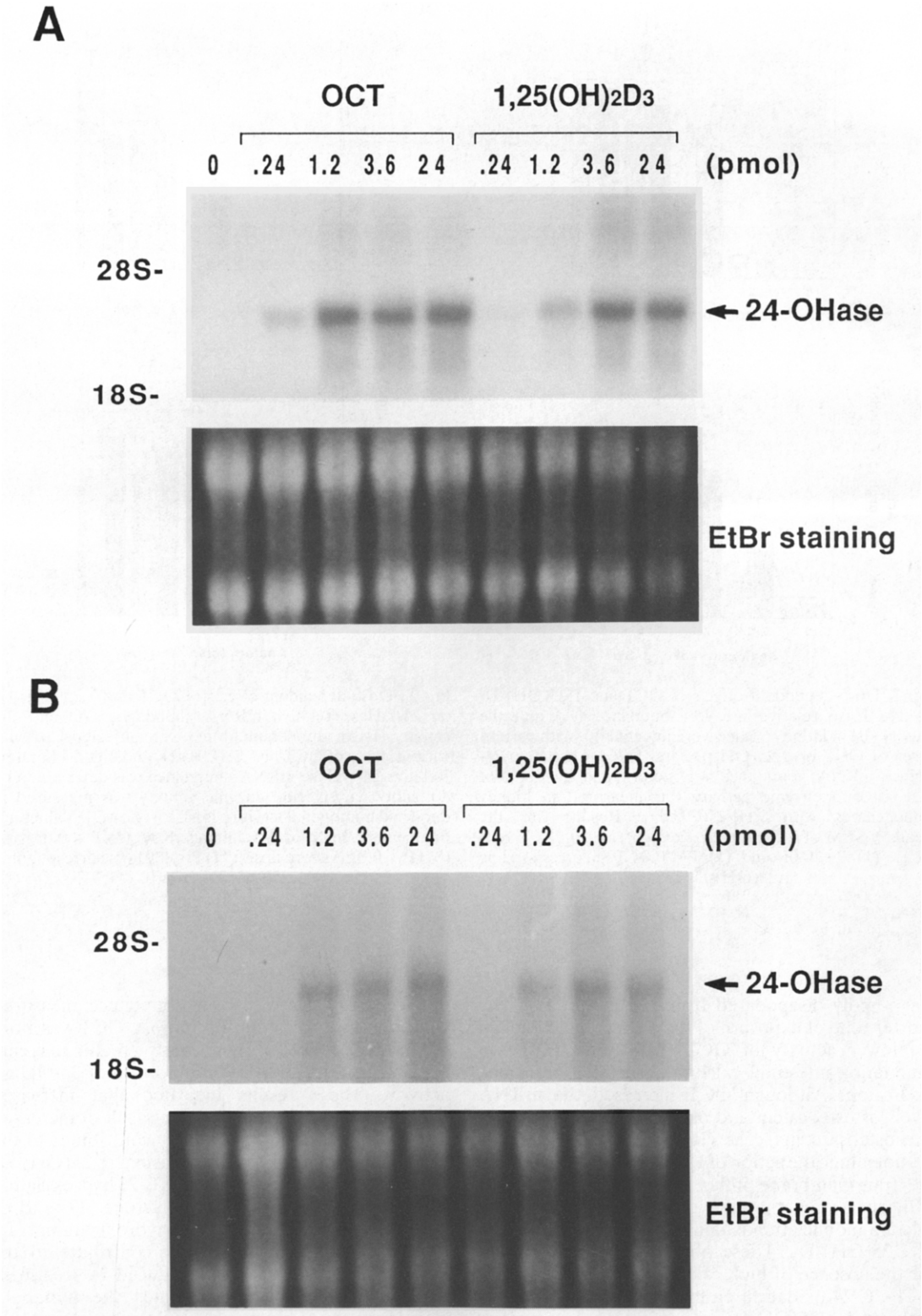

Fig. 6. Dose-dependent induction of renal (A) and duodenal (B) 24-OHase mRNA by $1,25(OH)_2D_3$ and OCT. Total RNA (20 μg) from kidneys and duodenum of mice killed 4 hr after an i.p. injection of various doses of OCT or $1,25(OH)_2D_3$ was subjected to northern blot analysis. The bottom half of each panel shows gels stained with ethidium bromide (EtBr) before transfer of RNA to the nylon membrane.

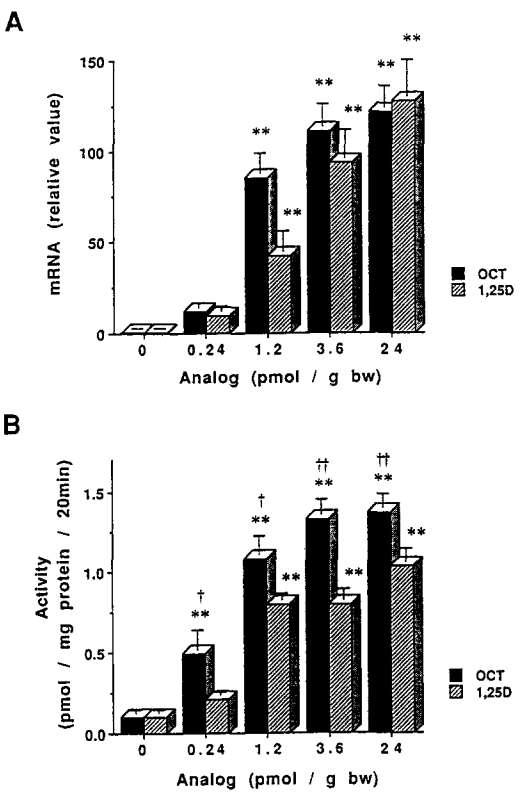

Fig. 7. Dose-dependent effects of OCT and $1,25(OH)_2D_3$ on 24-OHase relative mRNA abundance (A) and the activity (B) in kidneys. Mice were injected i.p. with various doses of OCT or $1,25(OH)_2D_3$ and killed 4 hr later. 24-OHase mRNA abundance was determined by dot blot analysis, and enzyme activity was measured in kidney homogenates with $[^3H]$-$25(OH)D_3$. Results are the means ± SEM of 3–5 animals. Key: (**) $P < 0.01$ (vs zero dose); (†) $P < 0.05$ and (‡) $P < 0.01$ (vs corresponding $1,25(OH)_2D_3$ group).

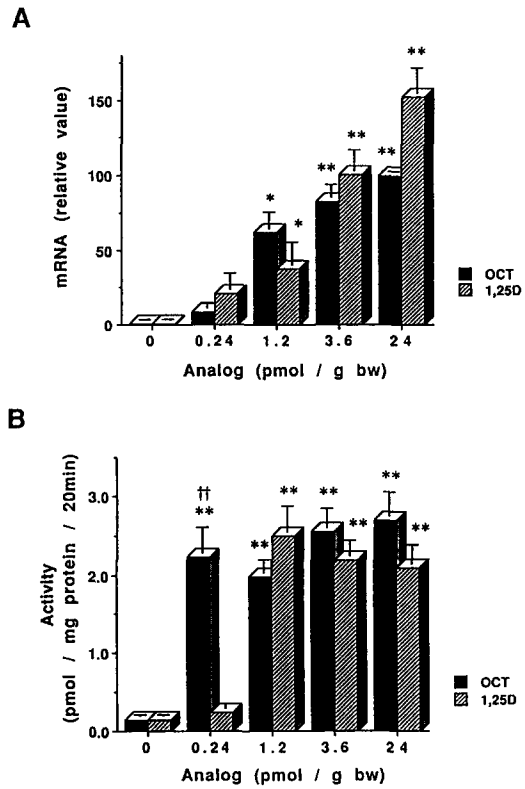

Fig. 8. Dose-dependent effects of OCT and $1,25(OH)_2D_3$ on 24-OHase relative mRNA abundance (A) and the activity (B) in duodenum. Mice were injected i.p. with various doses of OCT or $1,25(OH)_2D_3$ and killed 4 hr later. Relative 24-OHase mRNA abundance was determined by dot blot analysis, and enzyme activity was measured in duodenal homogenates with $[^3H]$-$1,25(OH)_2D_3$. Values are the means ± SEM of 3–5 animals. Key: (*) $P < 0.05$, and (**) $P < 0.01$ (vs zero dose); (‡) $P < 0.01$ (vs corresponding $1,25(OH)_2D_3$ group).

that rapidly disappeared from the tissue [11]. The shorter time of exposure of the analog could explain the lower activity of OCT than $1,25(OH)_2D_3$ in stimulating intestinal calcium absorption and bone resorption. Although OCT increased the mRNA levels of osteocalcin and osteopontin in osteoblast-like osteosarcoma cells (ROS 17/2.8), the analog did not mimic the action of $1,25(OH)_2D_3$ in increasing the transmembrane influx of calcium in the cells [10], suggesting that the effect of OCT on bone calcium mobilization is minimal compared with that of $1,25(OH)_2D_3$. These observations may account for the absence of high calcemic activity of OCT.

The C-24 oxidation pathway is a major catabolic pathway for $25(OH)D_3$ and $1,25(OH)_2D_3$ in many target tissues, including kidney and duodenum [15, 16]. Treatment with $1,25(OH)_2D_3$ induces 24-OHase expression in kidney and duodenum [14–16]. OCT also increased 24-OHase mRNA abundance and enzymic activity in a dose-dependent manner. OCT and $1,25(OH)_2D_3$ showed similar dose–response relations for their effects on renal and duodenal 24-OHase mRNA abundance measured 4 hr after administration. In contrast, OCT was more potent and showed a significantly greater maximal effect than $1,25(OH)_2D_3$ on induction of 24-OHase activity. These results indicated that increased degradation of $1,25(OH)_2D_3$ as a result of increased 24-OHase gene expression likely contributes to the OCT-induced decrease in plasma $1,25(OH)_2D$. Other catabolic pathways, such as C-23 hydroxylation to produce $1,25(OH)_2D_3$-26,23-lactone [17] and C-26 hydroxylation for 25,26-dihydroxyvitamin D_3 synthesis [14, 15, 34], also may contribute to the degradation of $1,25(OH)_2D_3$ induced by treatment with OCT. OCT also stimulates the metabolic clearance rate of plasma $1,25(OH)_2D$ in dogs [12].

The affinity of OCT for plasma vitamin D binding protein, a major recipient of vitamin D metabolites in the circulation, is extremely low [35], and the analog is mainly bound to lipoproteins, especially to chylomicrons and low density lipoprotein in the blood [13]. Thus, OCT was more accessible to target cells *in vivo* and formed larger amounts of a complex

with the vitamin D receptor in the intestine than $1,25(OH)_2D_3$, but the accumulation of the complex by OCT administration was transient [11]. These observations may help explain why OCT transiently induced 24-OHase mRNA in the duodenum. The observations that both compounds showed similar potencies with regard to their effect on 24-OHase mRNA but that OCT was slightly more potent than $1,25(OH)_2D_3$ in increasing the activity suggest that OCT also exerts a posttranslational action to prolong the half-life of the enzyme.

The effect of OCT on 24-OHase gene expression was rapid and transient in both kidney and duodenum, consistent with the results of Armbrecht and Boltz [36] showing that $1,25(OH)_2D_3$ induced a rapid, transient increase in 24-OHase mRNA in both of these tissues in young rats. In contrast, the effect of OCT on induction of 24-OHase activity was more prolonged than that on 24-OHase mRNA. Active forms of vitamin D_3, including OCT and $1,25(OH)_2D_3$, may inhibit degradation of the enzyme. Previous studies [19, 22] have suggested the presence of at least two forms (inducible and constitutive) of 24-OHase. In our mRNA determinations, only one inducible form of 24-OHase may have been detected, whereas the activity assay also detected an additional (not constitutive) form of the enzyme that could also be induced by OCT and remain during a long period in the tissues. Alternatively, it is likely that the methods used in the present study may not be sufficiently sensitive to detect such a small amount of mRNA.

In conclusion, our results indicate that OCT may prove effective in reducing plasma $1,25(OH)_2D$ concentration without inducing hypercalcemia in the treatment of various diseases, including sarcoidosis and adult T cell leukemia.

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REFERENCES

1. Gallagher JC, Jerpbak CM, Jee WSS, Johnson KA, DeLuca HF and Riggs BL, 1,25-Dihydroxyvitamin D_3: Short and long-term effects on bone and mineral metabolism in patients with postmenopausal osteoporosis. *Proc Natl Acad Sci USA* **79**: 3325–3329, 1982.
2. Slatopolsky E, Weerts C, Thielan J, Horst R, Harter H and Martin K, Marked suppression of secondary hyperparathyroidism by intravenous administration of 1,25-dihydroxycholecalciferol in uremic patients. *J Clin Invest* **74**: 2136–2143, 1984.
3. Holick MF, Smith E and Pincus E, Skin as the site of vitamin D synthesis and target tissue for 1,25-dihydroxyvitamin D_3. Use of calcitriol (1,25-dihydroxyvitamin D_3) for treatment of psoriasis. *Arch Dermatol* **123**: 164–183, 1987.
4. Abe J, Nakano T, Nishii Y, Matsumoto T, Ogata E and Ikeda K, A novel vitamin D_3 analog, 22-oxa-1,25-dihydroxyvitamin D_3, inhibits the growth of human breast cancer *in vitro* and *in vivo* without causing hypercalcemia. *Endocrinology* **129**: 832–837, 1991.
5. Inoue D, Matsumoto T, Ogata E and Ikeda K, 22-Oxacalcitriol, a noncalcemic analogue of calcitriol, suppresses both cell proliferation and parathyroid hormone-related peptide gene expression in human T cell lymphotrophic virus, type I-infected T cells. *J Biol Chem* **268**: 16730–16736, 1993.
6. Abe J, Morikawa M, Miyamoto K, Kaiho S, Fukushima M, Miyaura C, Abe E, Suda T and Nishii Y, Synthetic analogues of vitamin D_3 compounds with an oxygen atom in the side chain. *FEBS Lett* **226**: 58–62, 1987.
7. Morimoto S, Imanaka S, Koh E, Shiraishi T, Nabata T, Kitano S, Miyashita Y, Nishii Y and Ogihara T, Comparisons of the inhibition of proliferation of normal and psoriatic fibroblasts by 1,25-dihydroxyvitamin D_3 and synthetic analogues of vitamin D_3 with an oxygen atom in their side chain. *Biochem Int* **19**: 1143–1149, 1989.
8. Brown AJ, Ritter CS, Finch JL, Morrisey J, Martin K, Murayama E, Nishii Y and Slatopolsky E, The noncalcemic analogue of vitamin D, 22-oxacalcitriol, suppresses parathyroid hormone synthesis and secretion. *J Clin Invest* **84**: 728–732, 1989.
9. Naveh-Many T and Silver J, Effects of calcitriol, 22-oxacalcitriol, and calcipotriol on serum calcium and parathyroid hormone gene expression. *Endocrinology* **133**: 2724–2728, 1993.
10. Farach-Carson MC, Abe J, Nishii Y, Khoury R, Wright GC and Norman AW, 22-Oxacalcitriol: Dissection of $1,25(OH)_2D_3$ receptor-mediated and Ca^{2+} entry-stimulating pathways. *Am J Physiol* **265**: F705–F711, 1993.
11. Brown AJ, Finch J, Grieff M, Ritter C, Kubodera N, Nishii Y and Slatopolsky E, The mechanism for disparate actions of calcitriol and 22-oxacalcitriol in the intestine. *Endocrinology* **133**: 1158–1164, 1993.
12. Dusso AS, Negrea L, Finch J, Kamimura S, Lopez-Hilker S, Mori T, Nishii Y, Brown A and Slatopolsky E, The effect of 22-oxacalcitriol on serum calcitriol. *Endocrinology* **130**: 3129–3134, 1992.
13. Kobayashi T, Tsugawa N, Okano T, Masuda S, Takeuchi A, Kubodera N and Nishii Y, The binding properties, with blood proteins, and tissue distribution of 22-oxa-1α,25-dihydroxyvitamin D_3, a noncalcemic analogue of 1α,25-dihydroxyvitamin D_3, in rats. *J Biochem (Tokyo)* **115**: 373–380, 1994.
14. DeLuca HF, The vitamin D story: A collaborative effort of basic science and clinical medicine. *FASEB J* **2**: 224–236, 1988.
15. Kumar R, Metabolism of 1,25-dihydroxyvitamin D_3. *Physiol Rev* **64**: 478–504, 1984.
16. Jones G, Vriezen D, Lohnes D, Palda V and Edwards NS, Side-chain hydroxylation of vitamin D_3 and its physiological implications. *Steroids* **49**: 29–53, 1987.
17. Mayer E, Bishop JE, Chandraratna RAS, Okamura WH, Kruse JR, Popjak G, Ohnuma N and Norman AW, Isolation and identification of 1,25-dihydroxy-24-oxa-vitamin D_3 and 1,23,25-trihydroxy-24-oxa-vitamin D_3. New metabolites of vitamin D_3 produced by a C-24 oxidation pathway of metabolism for 1,25-dihydroxyvitamin D_3 present in intestine and kidney. *J Biol Chem* **258**: 13458–13465, 1983.
18. Ohyama Y, Noshiro M and Okuda K, Cloning and expression of cDNA encoding 25-hydroxyvitamin D_3 24-hydroxylase. *FEBS Lett* **278**: 195–198, 1991.
19. Mandla S, Boneh A and Tenenhouse HS, Evidence for protein kinase C involvement in the regulation of renal 25-hydroxyvitamin D_3-24-hydroxylase. *Endocrinology* **127**: 2639–2647, 1990.
20. Tomon M, Tenenhouse HS and Jones G, 1,25-Dihydroxyvitamin D_3-inducible catabolism of vitamin D metabolites in mouse intestine. *Am J Physiol* **258**: G557–G563, 1990.
21. Saikatsu S, Akeno N and Horiuchi N, Comparison of single cartridge (C18/OH) and high performance liquid

chromatography sample purification for the thymus radioreceptor assay of $1,25(OH)_2D$. *Klin Lab* **39**: 737–744, 1993.

22. Akeno N, Saikatsu S and Horiuchi N, Increase of renal 25-hydroxyvitamin D_3-24-hydroxylase activity and its messenger ribonucleic acid level in 1α-hydroxyvitamin D_3-administered rats: Possibility of the presence of two forms of 24-hydroxylase. *J Nutr Sci Vitaminol (Tokyo)* **39**: 89–100, 1993.
23. Puissant C and Houdebine L-M, An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Biotechniques* **8**: 148–149, 1990.
24. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB and Erlich HA, Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487–491, 1988.
25. Sanger F, Nicklen S and Coulsen AR, DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463–5467, 1977.
26. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
27. Anderson MLM and Young BD, Quantitative filter hybridization. In: *Nucleic Acid Hybridization: A Practical Approach* (Eds. Hames BD and Higgins SJ), pp. 73–111. IRL Press, Washington, 1985.
28. Horiuchi N and Rosenblatt M, Evaluation of a parathyroid hormone antagonist in an *in vivo* multiparameter bioassay. *Am J Physiol* **253**: E187–E192, 1987.
29. Botham KM, Ghazarian JG, Kream BE and DeLuca HF, Isolation of an inhibitor of 25-hydroxyvitamin D_3-1-hydroxylase from rat serum. *Biochemistry* **15**: 2130–2135, 1976.
30. Finch JL, Brown AJ, Kubodera N, Nishii Y and Slatopolsky E, Differential effects of $1,25(OH)_2D_3$ and 22-oxacalcitriol on phosphate and calcium metabolism. *Kidney Int* **43**: 561–566, 1993.
31. Fukagawa M, Kaname S, Igarashi T, Ogata E and Kurokawa K, Regulation of parathyroid hormone synthesis in chronic renal failure in rats. *Kidney Int* **39**: 874–881, 1991.
32. Hove K, Horst RL, Travis Littledike E and Beitz DC, Infusions of parathyroid hormone in ruminants: Hypercalcemia and reduced plasma 1,25-dihydroxyvitamin D concentrations. *Endocrinology* **114**: 897–903, 1984.
33. Bushinsky DA, Riera GS, Favus MJ and Coe FL, Evidence that blood ionized calcium can regulate serum $1,25(OH)_2D_3$ independently of parathyroid hormone and phosphorus in the rat. *J Clin Invest* **76**: 1599–1604, 1985.
34. Bergman T and Postlind H, Characterization of mitochondrial cytochromes P-450 from pig kidney and liver catalysing 26-hydroxylation of 25-hydroxyvitamin D_3 and C_{27} steroids. *Biochem J* **276**: 427–432, 1991.
35. Okano T, Tsugawa N, Masuda S, Takeuchi A, Kobayashi T and Nishii Y, Protein-binding properties of 22-oxa-1α,25-dihydroxyvitamin D_3, a synthetic analogue of 1α,25-dihydroxyvitamin D_3. *J Nutr Sci Vitaminol (Tokyo)* **35**: 529–533, 1989.
36. Armbrecht HJ and Boltz MA, Expression of 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 in kidney and intestine. Effect of 1,25-dihydroxyvitamin D and age. *FEBS Lett* **292**: 17–20, 1991.