Improved Cholecalciferol Nutrition in Rats Is Noncalcemic, Suppresses Parathyroid Hormone and Increases Responsiveness to 1,25-Dihydroxycholecalciferol[1]

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ABSTRACT We examined how cholecalciferol (vitamin D) nutrition affected serum 25-hydroxycholecalciferol (25(OH)D) and 1,25-dihydroxycholecalciferol ($1,25(OH)_2D$). Rats were fed conventional diet (vitamin D, 4.5 IU/g, or 7 nmol/d) or the same diet plus 18 nmol/d of extra vitamin D for 3 wk. The extra vitamin D resulted in greater serum 25(OH)D (51 ± 3, vs. control of 21 ± 2 nmol/L), and kidney mRNA for vitamin D receptor [VDR mRNA] ($P = 0.026$) and lower serum $1,25(OH)_2D$ (72 ± 16 vs. control of 161 ± 10 pmol/L, $P = 0.001$), and parathyroid hormone (PTH) (89 ± 4 vs. control of 160 ± 15 ng/L, $P = 0.001$). Kidney VDR mRNA relative to GAPDH mRNA correlated inversely with serum $1,25(OH)_2D$ ($r = -0.714$, $P = 0.006$). There were no differences in serum calcium, phosphate, alkaline phosphatase, or weight gain. Experiment 2 compared groups supplemented with 0.2, 2 or 20 nmol/d of vitamin D orally, or 20 nmol/d dermally to see how vitamin D nutrition influenced the response of $1,25(OH)_2D$ to changes in diet calcium. Vitamin D did not affect urinary calcium or pyridinoline excretion, serum calcium, phosphate, vitamin D binding protein or alkaline phosphatase. In groups given 20 nmol/d of vitamin D, renal mitochondrial 25(OH)D-1α-hydroxylase was lower ($P < 0.01$) and 25(OH)D-24-hydroxylase was higher ($P < 0.05$). Higher 25(OH)D concentration was related to proportionally lower $1,25(OH)_2D$ at every calcium intake, indicating greater tissue sensitivity to $1,25(OH)_2D$. We conclude suppression of $1,25(OH)_2D$ and PTH, and higher renal VDR mRNA and 24-hydroxylase did not involve higher free $1,25(OH)_2D$ concentration or a first pass effect at the gut. Thus, 25(OH)D or a metabolite other than $1,25(OH)_2D$ is a physiological, transcriptionally and biochemically active, noncalcemic vitamin D metabolite. J. Nutr. 130: 578–584, 2000.

KEY WORDS: • *vitamin D* • *calcium* • *adaptation* • *parathyroid hormone* • *rats* • *transcription*

Cholecalciferol (vitamin D_3)[3] is the raw material from which the calcium-regulating hormone, 1,25-dihydroxycholecalciferol ($1,25(OH)_2D$) is generated. The intermediate compound, 25-hydroxycholecalciferol [25(OH)D], is usually thought of as an inactive metabolite whose concentration reflects calciferol nutrition. In humans, 25(OH)D may play a direct role, meaning that it can increase calcium absorption in the absence of corresponding changes to serum $1,25(OH)_2D$ (Colodro et al. 1978, Heaney et al. 1997, Reasner et al. 1990). In humans, intestinal calcium absorption agrees more closely with serum 25(OH)D than with $1,25(OH)_2D$ levels (Bell et al. 1988, Francis et al. 1983, Reasner et al. 1990). Furthermore, an inverse relationship between circulating 25(OH)D levels and parathyroid hormone (PTH) has been recognized for years in humans. As a result, the clinical definition of hypovitaminosis D has changed dramatically, to the point where serum 25(OH)D levels in excess of 100 nmol/L are seen as desirable (McKenna and Freaney 1998).

For rats, Brommage and DeLuca (1985) argued that there is no functional role for any vitamin D metabolite, other than $1,25(OH)_2D$. Later, Sandgren and DeLuca (1990) showed that when normocalcemic, vitamin D-deprived rats are given replacement amounts of ergocalciferol, $1,25(OH)_2D$ receptor (VDR) levels in kidney double, based on an immunoassay for VDR. The issue of which vitamin D metabolite may have caused the increase in VDR was not addressed, but other work shows that if $1,25(OH)_2D$ is upregulated physiologically due to dietary calcium restriction, it does not increase VDR (Goff et al. 1990). There has been no follow-up to investigate the in vivo implications of how serum levels of vitamin D metabolites, PTH, or indices of calcium metabolism might have responded to the increase in VDR that Sandgren and DeLuca (1990) demonstrated at the molecular level.

Hormone receptor levels generally correlate with tissue responsiveness to hormone (Bamberger et al. 1996, Meyer and Schmidt 1997). In ovariectomized rats, estrogen raises VDR levels in the intestine, and the intestinal responsiveness to $1,25(OH)_2D$ increases (Liel et al. 1999). In rats fed conventional diet, serum $1,25(OH)_2D$ levels correlate inversely with 25(OH)D levels (Vieth et al. 1995). This reflects a suppression

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[3] Abbreviations used: $1,25(OH)_2D$, 1,25-dihydroxy-cholecalciferol; 25(OH)D, 25-hydroxycholecalciferol; cholecalciferol, vitamin D_3; DBP, vitamin D-binding protein; PTH, parathyroid hormone; VDR, vitamin D receptor, intracellular receptor for $1,25(OH)_2D$.

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of $1,25(OH)_2D$ that may be explainable by higher tissue VDR levels. Here we examined the effects of nutritionally moderate differences in vitamin D supply, to verify the results of Sandgren and DeLuca (1990) at the level of VDR mRNA, and to characterize the in vivo implications of VDR upregulation on how rats adapt to changes in dietary calcium.

We also addressed the question of whether it makes any difference from which route the calciferol is acquired (through the skin or the gut) by administering calciferol by either gastric or dermal routes. This was done because results obtained with orally administered vitamin D may reflect an unnatural situation. For most mammals, including humans and rats, the skin is a physiological route of entry of vitamin D. Haddad et al. (1993) showed that orally acquired vitamin D is absorbed with chylomicrons and taken up quickly by liver metabolism, while dermally acquired vitamin D is bound to vitamin D binding protein, and metabolized gradually (Haddad et al. 1993). We asked whether the unexpected findings of VDR upregulation (Sandgren and DeLuca 1990), and suppression of $1,25(OH)_2D$ likely associated with it (Vieth et al. 1995), were attributable to the nonphysiological way the vitamin D was administered in those studies. Artifacts with oral treatments could have been due to chemical or enzymatic modification of the vitamin D in the gut, or due to first-pass action at the target organ, the intestine.

METHODS

Animals. An animal-care committee at the University of Toronto had approved the protocols and procedures used. For the first experiment, 14 male Wistar rats (Canadian Breeding Laboratories, Montreal, Canada) initially weighing 214 (±5) g wt were fed conventional diet, (Rodent Laboratory Chow, Ralston Purina Co., St. Louis, MO), which contained 1 g/100 g of elemental calcium, and vitamin D3, 4.5 IU/g (0.3 nmol/g). During the 3 wk they were maintained, they consumed an average of 25 g diet/d, (7 nmol/d vitamin D from diet). Half of the rats were supplemented with another 18 nmol/d vitamin D in ethanol.

For expt. 2, male Wistar rats, initially 120–130 g, were fed a 0.01% calcium, 0.7% phosphate diet that was vitamin D-deficient (Teklad Test Diets, Madison, WI) (**Table 1**) for 1 wk to deplete vitamin D levels (Vieth et al. 1987). For the next 3 wk the rats were fed the vitamin D-deficient diet, supplemented to 0.5% elemental calcium (as calcium carbonate powder, USP). The rats were divided into four vitamin D-treatment groups: *i*) supplemented once weekly with 1.5 nmol of calciferol (0.2 nmol/d); *ii*) once every 2nd d with calciferol to provide 2 nmol/d; and *iii*) every 2nd d with calciferol to provide 20 nmol/d. The preceding groups all received the calciferol through an infant feeding tube directly into the gut. One group, *iv*), received its calciferol in ethanol, applied onto a shaved patch of skin on its back, this was done every 2nd d to provide calciferol at 20 nmol/d (40 nmol calciferol per 80 μL ethanol application). The volume of each dose of calciferol given was adjusted so that the stated doses are per 250 g body weight. After the 3-wk preparation period, the rats ranged in weight from 350 to 424 g, with no weight differences among the groups. Over the next 3 wk while maintaining the described calciferol dosing regimens, diet calcium content was changed weekly between 0.01 and 1.5 g/100 g diet. In each week, blood was obtained at d 0 (the day of, but just prior to, the change in diet calcium), and d 1, 3 and 7 after the change in calcium intake. During wk 1, 0.5 mL of blood was taken on the first two of these days and anticoagulated with heparin, the cells were isolated and resuspended in normal rat plasma and reinjected into the femoral vein (Fox 1992). This procedure resulted in an unacceptable mortality rate over the following day that was probably due to emboli in the reinfused blood. For the subsequent samplings, only 0.2–0.3 mL of blood was taken by bleeding from the tail into precalibrated microcentrifuge tubes. In this way, a total of <2 mL of blood was taken that we did not replace, that is, less than or equal to 10% of the blood volume. Samples of daytime urine were collected into microfuge tubes that were fastened to stainless steel metabolic collection funnels designed to hang under the wire cages. The cages and funnels were rinsed with 0.1 mol/L of hydrochloric acid prior to each collection to remove calcium. To eliminate contamination of the urine with diet, stool or water, the rats were placed into these separate cages only during the urine-collection period, until about 1 mL of urine was present in the microfuge tube; this was typically within 5 min to 1 h.

TABLE 1

Composition of custom powdered diet fed rats in expt. 2. Vitamin D-deficient diet (calcium 0.01 g/100 g, phosphate 0.7 g/100 g diet)

	g/kg diet
Casein, "vitamin-free" Test	200
DL-Methionine	3
Sucrose	340
Corn starch	300
Corn oil	60
Cellulose	50
Sodium chloride (NaCl)	2.58
Potassium citrate, monohydrate	7.68
Potassium sulfate (K_2SO_4)	1.82
Magnesium oxide (MgO)	0.838
Manganous carbonate	0.122
Ferric citrate	0.210
Zinc carbonate	0.056
Cupric carbonate	0.010
Potassium iodate KIO_3	0.00035
Sodium selenite Na_2SeO_3 $5H_2O$	0.00035
Chromium potassium sulfate $CrK(SO_4)_2$ $12H_2O$	0.0192
Sodium phosphate, monobasic (NaH_2PO_4 H_2O)	12.3
Potassium phosphate, monobasic (KH_2PO_4)	12.13
Ethoxyquin (antioxidant)	0.012
Biotin	0.0004
Calcium pantothenate	0.066
Folic acid	0.002
Inositol	0.110
Menadione	0.050
Niacin	0.099
p-Aminobenzoic acid	0.110
Pyridoxine HCl	0.022
Riboflavin	0.022
Thiamin HC	0.022
Vitamin B-12 (0.1% in mannitol)	0.030
Ascorbic acid, coated (97.5%)	1.02
Corn starch	3.45
Choline bitartrate	3.50
α Tocopheryl acetate (preparation, 500 U/g)	0.24
Dry retinol palmitate (500000 U/g)	0.04

[1] Approximately 0.005–0.01% of inherent background Ca.
[2] Diet and composition supplied Harlan Teklad (Madison, WI), diet number TD 97140.

Biochemical measurements. $1,25(OH)_2D$ in serum was measured by calf-thymus receptor-binding assay after initial purification of $1,25(OH)_2D$ with C18-OH cartridges (Hollis and Kilbo 1988). For the serum volumes that were minimal, serum was measured out in 50-μL increments, and the final result was adjusted for the volume of sample analyzed. To measure 1α-hydroxylase in renal mitochondria, the incubation was as described previously, except the substrate was 20 μmol/L of nonradioactive 25(OH)D. The assay was stopped by addition of 2.5 mL of methanol. To monitor recovery of $1,25(OH)_2D$, 33 Bq/mL of [^{3}H]-$1,25(OH)_2D$ (Amersham, Oakville, Ontario, Canada) were added in 50 μL of ethanol prior to further extraction with two additions of 1.25 mL of methylene chloride. The methylene chloride layer was taken and evaporated to dryness, redissolved in hexane/isopropanol/methanol (90:9:1) for purification by HPLC, Zorbax-sil column. The material eluting as $1,25(OH)_2D$ was evaporated and redissolved in acetonitrile/water for further purification with C18-OH cartridges and quantitation by thymus receptor assay,

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TABLE 2

Serum biochemical and renal transcription effects of extra vitamin D in rats fed conventional diet[1]

Cholecalciferol intake, *nmol/d*	7	25
Serum levels		
25(OH)D, *nmol/L*	21 ± 2	51 ± 3[2]
$1,25(OH)_2D$, *pmol/L*	161 ± 10	72 ± 16[2]
PTH, *ng/L*	160 ± 15	89 ± 4[2]
Calcium, *mmol/L*	2.87 ± 0.04	2.84 ± 0.04
Phosphate, *mmol/L*	2.96 ± 0.06	2.98 ± 0.06
Magnesium, *mmol/L*	0.92 ± 0.03	0.96 ± 0.03
Alkaline phosphatase, *U/L*	216 ± 23	242 ± 15
Renal tissue mRNA		
VDR/GAPDH,[4] *relative units*	0.62 ± 0.05	0.80 ± 0.04[3]

[1] Rats fed conventional rat diet consumed the equivalent of 7 nmol of calciferol/d from the diet, which for one group was supplemented with another 18 nmol. Values in table are means ± SEM, $n = 7$ per group.
[2] Means are significantly different, two-tailed t-test, $P < 0.001$.
[3] Means are significantly different, two-tailed t-test, $P = 0.026$.
[4] Ratio of vitamin D receptor mRNA band intensity vs. glyceraldehyde phosphate dehydrogenase mRNA intensity, by Northern blot.

as described for serum. Renal 24-hydroxylase was measured by incubating mitochondria with [^{3}H]-25(OH)D as substrate (Vieth and Fraser 1979). Vitamin D-binding capacity was measured, based on the specific uptake of [^{3}H]-25(OH)D3 by rat serum, involving the removal of nonspecifically bound [^{3}H]-25(OH)D with charcoal (Vieth 1994). To measure 25(OH)D, 0.2 mL of serum was spiked with 33 kBq/L with 25(OH),26,26[^{3}H]-vitamin D (0.74 TBq/mmol; Amersham) and treated with 1 mL of acetone. Precipitated proteins were removed by centrifugation, and the supernatant was removed and evaporated to dryness under nitrogen. The calciferol metabolites were redissolved with hexane and extracted with cartridges (Vieth et al. 1995). In short, material eluting at the positions of authentic 25(OH)D was measured with competitive protein-binding assay against standards of crystalline 25(OH)D that had been purified by HPLC and quantified, based on spectral absorbance at 265 nm, assuming 18,300 AU/(mol/L). Urinary pyridinoline bone collagen crosslinks were measured by HPLC following hydrolysis in hydrochloric acid (Eyre et al. 1984) except that an internal recovery marker (Metra Labs, Mountain View, CA) was incorporated (Pratt et al. 1992) prior to initial extraction with cellulose cartridge in preparation for HPLC. Other biochemical measures were made using routine methods established for the Kodak Ektachem 700, dry-chemistry slide system (Rochester, NY). PTH was measured with the rat PTH (IRMA) kit from Nichols Institute Diagnostics (San Juan Capistrano, CA), which has a within-run CV of 5%.

Northern blot analysis of rat kidneys. Total RNA was isolated from kidneys of control and treated rats using TriZol (Gibco BRL, Burlington, Ontario, Canada) according to the protocol provided by the manufacturer. This was electrophoresed on formaldehyde agarose gels and capillary-transferred to a positively charged nylon membrane (Boehringer Mannheim, Laval, Quebec, Canada) using 20XSSC (Sambrook et al. 1989). RNA was then crosslinked to the membrane by exposing it to ultraviolet light in a Stratalinker (Strategene, La Jolla, CA). Rat VDR cDNA (2.1 kbp EcoR1 fragment in plasmid pSG5) was kindly provided by Dr. Geoff Hendy (Royal Victoria Hospital, Montreal, Canada). Rat VDR and GAPDH cDNA were labeled with the digoxygenin-dUTP using the DIG-high prime DNA labeling kit (Boehringer Mannheim). Hybridization, washing and detection were performed using a DIG Easy Hyb and Detection Starter Kit with the conditions provided by the manufacturer (Boehringer Mannheim). Intensities of the VDR mRNA and GAPDH mRNA signals were read with a gel scanner, and VDR mRNA signal normalized to the GAPDH mRNA.

Statistical analyses and calculations. ANOVA was used to identify significant differences among the means of the four groups, and if there was evidence of differences, the Tukey-Kramer multiple-comparison test was carried out (This was with the SPSS 8.0, SPSS Chicago, IL). Furthermore, repeated measures ANOVA was used for each group of rats to compare $1,25(OH)_2D$ levels across the days of a given diet treatment, to determine whether a plateau level had been attained. This was done by carrying out ANOVA sequentially, starting from the last day of a period of diet treatment, and working toward the earlier days until ANOVA indicated statistical variability. All statistical P-values are two-tailed unless one-tailed based on a priori expectation, as indicated.

RESULTS

Compared to the group fed conventional, nonpurified diet, the group given additional vitamin D had lower serum $1,25(OH)_2D$, lower PTH ($P < 0.001$) and higher kidney tissue vitamin D receptor mRNA ($P < 0.04$) (**Table 2**). The level of VDR mRNA (VDR mRNA relative to GAPDH mRNA) in kidney correlated inversely with serum $1,25(OH)_2D$ (**Fig. 1**, $r = -0.714$, $P = 0.006$). This was a stronger relationship than that between VDR mRNA and 25(OH)D ($r = 0.527$, $P = 0.032$, in the expected one-tail direction).

In expt. 2, the supply of vitamin D influenced serum 25(OH)D in a manner appropriate for the doses used (**Table 3**). Application of vitamin D to shaved rat skin proved to be an effective way to provide vitamin D. All groups differed from one another in serum 25(OH)D. Renal 1α-hydroxylase was suppressed and renal 24-hydroxylase was increased by vitamin D nutrition. 1α-Hydroxylase in the group with the lowest 25(OH)D concentration was significantly higher than in either of the two groups with the highest 25(OH)D. 24-Hydroxylase differed significantly between the groups with the lowest and highest 25(OH)D. There were no significant differences among any of the groups in serum calcium, inorganic phosphate, alkaline phosphatase or vitamin D binding protein, or in weight gain.

Expt. 2 was designed to examine the dynamics of bone metabolism and serum $1,25(OH)_2D$ change in response to changes in diet calcium (as opposed to the steady state). Diet calcium was adjusted weekly as shown in **Figures 2** and **3**. In all groups, urinary pyridinoline fluctuated, but its changes did not coincide with changes in dietary calcium. At no time were

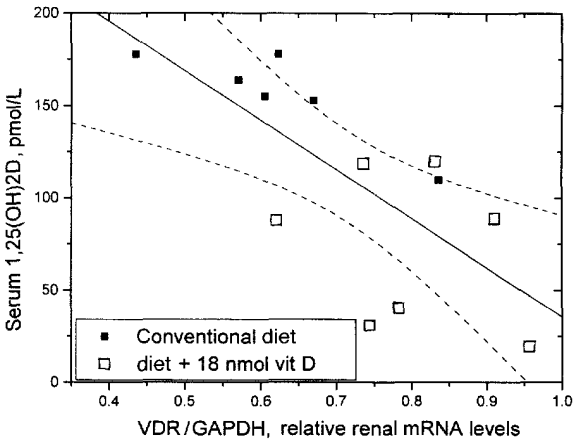

FIGURE 1 Relationship between kidney tissue ratio of vitamin D receptor (VDR) mRNA/GAPDH mRNA, and serum 1,25-dihydroxycholecalciferol [$1,25(OH)_2D$] in rats fed Purina nonpurified diet without or with additional vitamin D. The regression line is shown, along with its 95% confidence limits. The data show a significant negative relationship ($r = -0.714$, $P = 0.006$), with rats given a moderately higher vitamin D intake having lower $1,25(OH)_2D$ and higher renal VDR mRNA by Northern blot, relative to expression of the GAPDH housekeeping gene.

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TABLE 3

Biochemical comparisons in rats whose vitamin D intake ranged over 100-fold, both orally and dermally[1]

Vitamin D intake and route, nmol/d	0.2 diet	2 GI	20 GI	20 dermal
n per group	7	7	5	9
Serum				
25(OH)D, *nmol/L*	3.3 ± .6[d]	29 ± 3[c]	148 ± 10[b]	127 ± 4[a]
$1,25(OH)_2D$ (initial, +Ca diet), *pmol/L*	769 ± 168[a]	593 ± 173[ab]	262 ± 62[ab]	213 ± 58[b]
$1,25(OH)_2D$ (final, −Ca diet), *pmol/L*	1233 ± 209[a]	1227 ± 174[a]	566 ± 120[b]	957 ± 240[ab]
Calcium,[2] *mmol/L*	2.38 ± .06	2.41 ± .12	2.39 ± .12	2.58 ± .07
Inorganic phosphate,[2] *mmol/L*	2.41 ± .13	2.50 ± .15	2.19 ± .10	2.54 ± .13
Alkaline phosphatase,[2] *U/L*	119 ± 9	151 ± 23	147 ± 12	152 ± 18
Binding capacity for 25(OH)D,[2] *μmol/L*	7.1 ± .3	6.93 ± .3	7.13 ± .4	7.96 ± .2
Renal mitochondria				
1α-hydroxylase, *fmol/(mg × min)*	1052 ± 69[a]	993 ± 62[a]	539 ± 35[b]	630 ± 79[b]
24-hydroxylase, *pmol/(mg × min)*	1.3 ± 0.9[b]	3.67 ± 1.2[ab]	8.88 ± 2.3[a]	6.26 ± 1.9[a]
Final wt,[2] *g*	385 ± 9	374 ± 6	374 ± 13	367 ± 12

[1] Results for groups of rats, differing according to vitamin D intake, as indicated at the top of the table (GI indicates vitamin D by gastric tube; dermal indicates vitamin D applied to shaved skin). Except for the initial $1,25(OH)_2D$ value on day 0, obtained while rats were fed 0.5 g calcium/100 g diet, all results are for the end of the 21-d experimental protocol, during the consumption of calcium-deficient diet. Values are mean ± SEM.

[a–d] Means that do not share common letters are different by two-tailed comparisons ($P < 0.05$).

[2] ANOVA indicated no significant differences for the variable among groups.

there differences in pyridinoline excretion due to the vitamin D treatment (Fig. 2). Deoxypyridinoline was also measured, but the results are not presented since they were similar to those for pyridinoline. The urinary calcium/creatinine ratio varied in direct relation to dietary calcium –so much so mean calcium/creatinine molar ratios approximated the numerical values for grams of calcium/100-g diet (upper panels of Fig. 3). Despite minor variations among the groups, urinary calcium/creatinine ratios did not differ significantly.

The number of days it took for serum $1,25(OH)_2D$ concentrations to respond to the changes in diet calcium were studied (Fig. 3). Within each group of rats, after every change in diet calcium, there was no difference between the $1,25(OH)_2D$ levels of d 3 and d 7. There was also no difference when results for the two 20 nmol/d groups were pooled and compared to the pooled results of the lower vitamin D intake groups. After a change in diet calcium, the 100-fold range of vitamin D nutrition (from 0.2 to 20 nmol/d) had no effect on the rate at which $1,25(OH)_2D$ reached new set points.

Higher vitamin D nutrition had a consistent suppressive effect on $1,25(OH)_2D$ concentrations (Fig. 3, lower panels). Because of the sample size per group and the low statistical power of ANOVA to detect individual differences, the differences in $1,25(OH)_2D$ were not consistently significant for the single-day comparisons among all four groups. To overcome this, we pooled $1,25(OH)_2D$ levels of the 14 rats provided vitamin D at 20 nmol/d to compare with the 14 rats given 0.2 or 2 nmol/d. In those given 20 nmol/d, $1,25(OH)_2D$ levels were lower ($P < 0.05$) on every day of the protocol except on the three sampling days when the rats were fed the 1.5 g of calcium/100-g diet. Furthermore, for each rat, the log of serum $1,25(OH)_2D$ during calcium deprivation (average of levels on d 3 and d 7 of weeks fed 0.01 g/100 g) was subtracted by the log of serum $1,25(OH)_2D$ during calcium repletion (average of baseline and d 3 and d 7 of the week fed on 1.5 g/100 g). This was done to reflect the amplitudes of the log changes shown for the groups in Figure 3. There was no significant difference in these log or ratio changes in $1,25(OH)_2D$ either by ANOVA among all four groups ($P = 0.67$), or by *t* test comparing the pooled 20 nmol/d groups vs. the pooled rats given 0.2 or 2 nmol/d of vitamin D ($P = 0.28$)

DISCUSSION

In contrast to most rat studies in which vitamin D nutrition is manipulated, serum 25(OH)D levels were maintained within the human normal range. The vitamin D intakes provided here caused neither deficiency nor excess, based on their lack of effect on serum calcium, phosphate, alkaline phosphatase, urine calcium, pyridinoline excretion or weight gain. Nonetheless, the higher 25(OH)D supply caused large reductions in both $1,25(OH)_2D$ and PTH (Table 1). These changes were accompanied by an increase in the VDR mRNA level in kidneys. This agrees with results of Sandgren and DeLuca (1990) who showed that vitamin D supply increases renal VDR as measured by immunoassay (Sandgren and DeLuca 1990). Conventional theories of calcium regulation predict that lower levels of both $1,25(OH)_2D$ and PTH will lower serum calcium concentrations, and lessen urine calcium excretion. Because these other parameters were not affected by vitamin D supply, mechanisms responding to higher 25(OH)D must have offset the reductions in $1,25(OH)_2D$ and PTH.

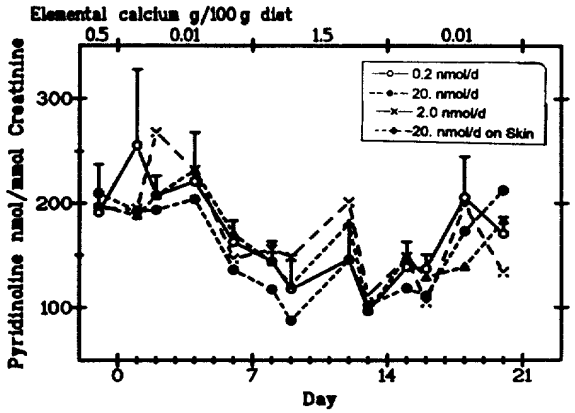

FIGURE 2 Effect of changes in diet calcium on urine pyridinoline/creatinine ratio in groups of rats maintained on the indicated daily vitamin D intakes. Since there were no differences among groups, representative ± SEM are indicated, based on only the first group of rats.

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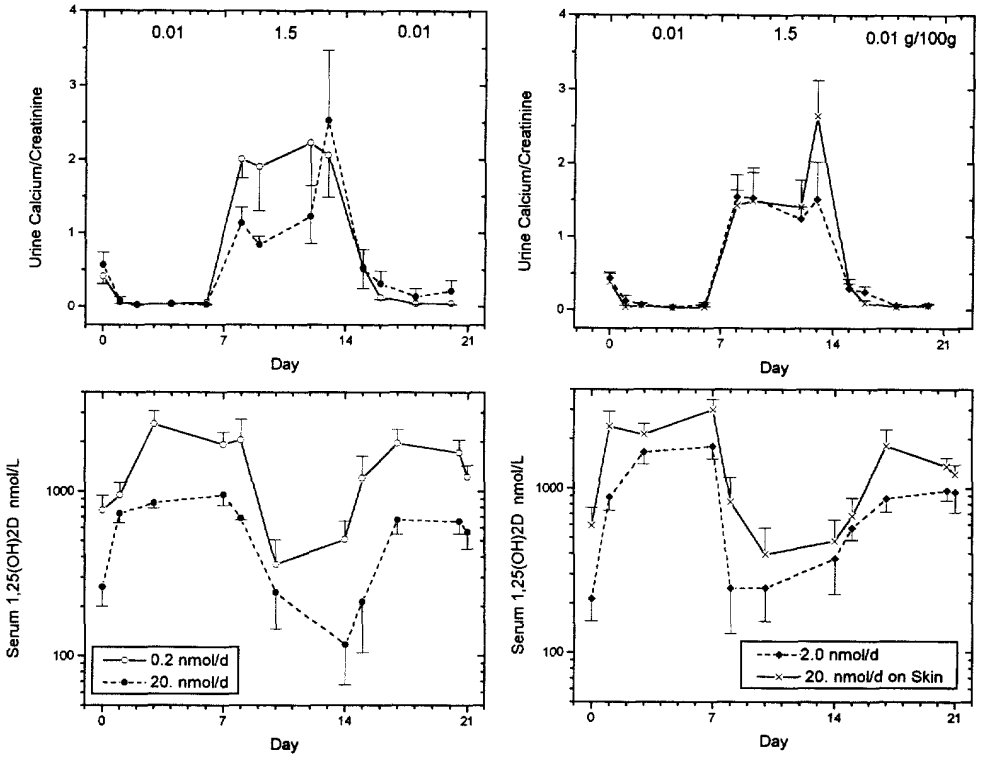

FIGURE 3 Dynamic responses of urine calcium/creatinine ratio and serum 1,25-dihydroxycholecalciferol [1,25$(OH)_2$D] to changes in dietary calcium content. Up to d 0, the diet contained 0.5 g of elemental calcium/100-g diet. Starting on d 0 the amount of calcium in the diet fed the rats was adjusted every 7 d to the amounts shown by the numbers across the top of the figure, in units of g/100-g diet. Groups of rats differed according to the amount and route of vitamin D nutrition, as indicated in the figures. The error whiskers indicate ± SEM The graphs show that urine calcium excretion paralleled diet calcium content, and that on the log scales of the bottom panels the serum 1,25$(OH)_2$D levels of groups given 20 nmol/d of vitamin D were shifted lower by a constant amount at all calcium intakes, which indicates greater responsiveness to circulating 1,25$(OH)_2$D.

One of our aims was to find out whether moderate differences in vitamin D supply would affect the way rats adapt to changes in diet calcium. During the experiment, a reasonable average of 0.5% diet calcium was maintained (Reeves et al. 1993) but with periods of excess and deficiency that required adaptation. The inclusion of 1 wk with 1.5% calcium provided an additional insight about the role of 1,25$(OH)_2$D during calcium repletion. The urinary calcium/creatinine ratio during the period of 1.5% dietary calcium intake averaged three times the ratio with 0.5% calcium intake. We interpret this as evidence that renal calcium excretion paralleled dietary calcium, and that it reflects passive intestinal calcium absorption (Heaney 1991). There were no differences within any group, in 1,25$(OH)_2$D levels during 0.5% vs. 1.5% calcium intake (Fig. 2). This implies that serum 1,25$(OH)_2$D was not functioning to regulate intestinal calcium absorption while rats were fed the calcium-replete diets. It was during consumption of calcium-deficient diet that differences in 1,25$(OH)_2$D due to vitamin D supply were largest, and this is expected if target tissue sensitivity differs.

The urinary excretion of the collagen crosslinks, pyridinoline and deoxypyridinoline, reflects bone resorption. In rats, the crosslink ratio with urinary creatinine adjusts for body mass and is preferable to results with 24-h excretion (Jerome et al. 1992). Pyridinoline and deoxypyridinoline were not affected by calciferol supply. This lack of a bone-marker response to diet calcium is consistent with a previous study of PTH infusion. It took 8 d of infusion to demonstrate a statistically higher pyridinoline/creatinine ratio (Jerome et al. 1992). Our reason for measuring the crosslinks was to determine whether they were affected by the degree of vitamin D sufficiency. The 100-fold range in calciferol supply among our rats was maintained for a total of 6 wk. This would have been long enough to manifest differences in the crosslinks if they existed. Consistent with this, there were no effects of the calciferol supply on serum calcium, phosphate or alkaline phosphatase (Tables 1 and 2).

Vitamin D supply influenced 25(OH)D levels and the renal hydroxylases appropriately for the doses given. Dermal treatment produced a marginally lower level of 25(OH)D than achieved with the equivalent oral dose. If one uses the 25(OH)D levels of the group receiving the lowest vitamin D intake as a reference point, the 20 nmol/d dermal dose generated an increase in 25(OH)D that was 85% of the increase obtained with the same dose given orally. Vitamin D intake lowered 1α-hydroxylase and increased 24-hydroxylase, as expected. These enzyme changes show that dermal and gastric vitamin D were comparable in the rats, with minor differences attributable to lower vitamin D absorption efficiency from the skin than the gut. There is no support here for the hypothesis that there is some fundamental difference in metabolism or biological effect between dermal and gastrically acquired vitamin D (Fraser 1983).

Dermal application of vitamin D has potential advantages over oral vitamin D intake because it obviates the need for efficient intestinal absorption, and it avoids the requirement for ultraviolet exposure to the skin while delivering vitamin D via its physiological route of entry.

In every group, the proportional increase in serum 1,25$(OH)_2$D concentration reached within 3 d of calcium restriction was essentially the same as the ratios of 1,25$(OH)_2$D in previous comparisons of long-term calcium-deficient vs. calcium-replete diets (Gray 1981, Hughes et al. 1975). We were unable to detect any effects of the 100-fold range of vitamin D supply on the speed with which 1,25$(OH)_2$D changed in response to dietary calcium.

Vitamin D nutrition did influence serum PTH, 1,25$(OH)_2$D and kidney VDR mRNA. Moderately elevated 25(OH)D suppresses 1,25$(OH)_2$D in rats (Hsu and Patel 1990, Vieth and Milojevic 1995). Sometimes, this is also the case in

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humans (Bell et al. 1988, Reasner 1990, Reasner et al. 1990). To explain the phenomenon, Reasner et al. hypothesized that 25(OH)D would occupy some receptors for $1,25(OH)_2D$ in bone and intestine and exert its effects on calcium metabolism even when the concentration of the metabolite is in the normal range (Reasner 1990). The present results suggest another mechanism. Previous reports have highlighted up-regulation of VDR with administration of $1,25(OH)_2D$ or its analogs and imply a positive relationship between $1,25(OH)_2D$ and VDR in vivo (Gensure et al. 1998, Goff et al. 1993, Solvsten et al. 1997, Yao et al. 1998). In contrast, we produced opposite changes in VDR mRNA and $1,25(OH)_2D$ under the nutritionally relevant conditions of expt. 1 (Fig. 1). The negative relationship is what should be expected if higher renal VDR mRNA content results in higher tissue VDR and higher tissue sensitivity.

Higher end-organ sensitivity to $1,25(OH)_2D$ in vivo is consistent with the results of expt. 2, showing lower set-point concentrations of serum $1,25(OH)_2D$ in groups with higher vitamin D nutrition (Fig. 3). These rats were adapting to dynamic changes in diet calcium. At all vitamin D intakes, $1,25(OH)_2D$ concentration responded to diet calcium restriction in a proportional manner, shown by the constant offsets on the log-scales. The further analysis which combined all rats given 20 nmol/d vs. the lesser intakes showed that serum $1,25(OH)_2D$ concentrations were proportionally less at all calcium intakes compared to rats given less vitamin D. This proportionality would not occur if the constant 25(OH)D concentrations in each group were simply competing with $1,25(OH)_2D$ at the VDR.

It is relevant to note that vitamin D nutrition did not affect circulating vitamin D binding protein (DBP). Without this result, it could have been argued that lower $1,25(OH)_2D$ levels were due to lower DBP levels, and a greater proportion of $1,25(OH)_2D$ in the biologically active, "free" state when 25(OH)D concentration was higher (Vieth 1990).

In short, we have shown that higher serum 25(OH)D concentrations which would have been considered of little consequence do have substantial biological effects not attributable to a higher $1,25(OH)_2D$ concentration. The apparent efficacy of $1,25(OH)_2D$ in vivo increased with greater vitamin D nutrition. This had been implied by the effect of vitamin D intake to increase renal VDR (Sandgren and DeLuca 1990), which we have confirmed at the level of mRNA. We found that higher 25(OH)D was associated with suppression of $1,25(OH)_2D$ regardless of calcium intake, as well as suppression of PTH concentration without effects on other variables of mineral homeostasis. These results support views derived from clinical studies, that 25(OH)D has mechanisms that have yet to be explained (Heaney et al. 1997, Rasmussen et al. 1980, Reasner et al. 1990). We showed that in rats these mechanisms do not involve changes to vitamin D binding protein or a first-pass action at the gut.

When viewed from a perspective that starts with higher vitamin D nutrition, the results indicate that low vitamin D nutrition may bring about a form of resistance to $1,25(OH)_2D$. This situation would explain why, in humans, nutritional rickets and osteomalacia are commonly associated with normal or increased levels of $1,25(OH)_2D$ (Chesney et al. 1981, Eastwood et al. 1979, Garabedian et al. 1983, Rasmussen et al. 1980)—these are not like the low hormone levels associated with any other endocrine—deficiency disorder. A connection between lower vitamin D nutrition and vitamin D resistance helps to explain why the supposedly inactive compound 25(OH)D is more relevant in diagnosing nutritional rickets than is the active hormone $1,25(OH)_2D$.

If the features of improved vitamin D nutrition shown here were demonstrated for any newly synthesized compound, the compound would be classified as a noncalcemic $1,25(OH)_2D$ analogue (Brown et al. 1989, Finch et al. 1999, Goff et al. 1993, Koshizuka et al. 1999). Thus, we contend that 25(OH)D or a metabolite of it other than $1,25(OH)_2D$ exists as a physiological and biologically-active noncalcemic vitamin D metabolite whose effects require further examination, particularly in relationship to studies involving the synthetic analogs of $1,25(OH)_2D$.

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