Low Dietary Calcium Reduces 25-Hydroxycholecalciferol in Plasma of Rats[1]

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ABSTRACT We investigated whether dietary factors that are known to increase 1,25-$(OH)_2$-cholecalciferol production can deplete plasma 25-OH-cholecalciferol. Plasma concentration of 25-OH-cholecalciferol, its metabolism in vivo and activities of renal mitochondrial 25-OH-cholecalciferol 1-hydroxylase (1-OHase) and 25-OH-cholecalciferol 24-hydroxylase (24-OHase) were measured in rats fed various amounts of calcium (Ca) and phosphorus (P). All diets contained 5 μg (200 IU) cholecalciferol per 100 g. For rats fed the "normal" diet (0.7% Ca and 1.2% P) the mean plasma 25-OH-cholecalciferol level was 11.0 ± 0.8 nmol/L, and the mean 1-OHase activity was 30 ± 5 fmol/(mg·min). All rats fed the low Ca (0.014%) diet had 1-OHase activities above 200 fmol/(mg·min) and undetectable plasma 25-OH-cholecalciferol levels (<2.5 nmol/L). The chi-square test interrelating plasma 25-OH-cholecalciferol and dietary Ca showed statistical significance ($P < 0.001$). The high activity of 1-OHase that resulted from dietary Ca restriction increased utilization of 25-OH-cholecalciferol to the point of causing depletion of this metabolite in the circulation. *J. Nutr.* 117: 914–918, 1987.

INDEXING KEY WORDS:

• *dietary calcium* • *vitamin D requirement* • *25-OH-cholecalciferol 1-hydroxylase*

Vitamin D is acquired in mammals by exposure of the skin to ultraviolet light or by ingestion of preformed vitamin D in food. Most vitamin D in plasma exists as 25-hydroxycholecalciferol (25-OH-cholecalciferol), which circulates in nanomolar concentrations. In the vitamin D-sufficient state, 24,25-dihydroxycholecalciferol is also present in nanomolar concentrations. The only metabolite of 25-OH-cholecalciferol with undisputed bioactivity, 1,25-dihydroxycholecalciferol [1,25-$(OH)_2$-cholecalciferol], is present in picomolar concentrations. In normal humans, production of 1,25-$(OH)_2$-cholecalciferol by the renal enzyme 25-OH-cholecalciferol 1-hydroxylase (1-OHase) has been estimated by in vivo tracer-kinetic techniques to be 0.25 to 1 μg/d (1, 2). In an adult with calcium nephrolithiasis, 1,25-$(OH)_2$-cholecalciferol production was reported to metabolize as much as 6.9 μg vitamin D per day (2). This exceeds the U.S. adult recommended dietary vitamin D[3] allowance of 5 μg/d (3). There are many reports in the literature of patients with low plasma 25-OH-vitamin D and normal or high 1,25-$(OH)_2$-vitamin D (4–11). Although it seems reasonable that a high rate of 1,25-$(OH)_2$-vitamin D production may reduce 25-OH-vitamin D levels when vitamin D supply is marginal, we are not aware of previous studies designed specifically to demonstrate such a relation. In the present experiment, we set out to test the hypothesis that reduced plasma 25-OH-cholecalciferol levels in rats can result from increased activity of 1-OHase.

MATERIALS AND METHODS

Sixty male Wistar rats were obtained at 120 g wt and housed in hanging wire cages (two rats per cage) in a room lit by incandescent light. They were divided equally into five diet groups, each fed one of the following diets

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[3]For consistency and to conform with editorial policy the term "cholecalciferol" is used to denote vitamin D_3 when applied to our animal studies, but we use "vitamin D" to denote unspecified vitamin D (cholecalciferol and ergocalciferol) when applied to clinical reports cited in the text.

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TABLE 1

Composition of the low calcium, low phosphorus diet (A)[1]

Component	Amount
	g/kg
Casein[2]	240
Sucrose	684
Corn oil	50
Minerals[3]	22.972
Vitamins[4]	2.517

[1]The diet was prepared by Teklad Research Diets, Madison, WI.
[2]Casein was "vitamin-free" test.
[3]Minerals added (g/kg diet) were: KCl, 11.7; NaCl, 6.0; $NaHCO_3$, 1.6; $MgSO_4$, 2.1; ferric citrate, USP (16.7% Fe), 1.2; $MnSO_4 \cdot H_2O$, 0.192; KI, 0.034; $ZnCl_2$, 0.213; $CuSO_4 \cdot 5H_2O$, 0.013.
[4]Vitamins added (g/kg diet) were: *p*-aminobenzoic acid, 0.302; D-calcium pantothenate, 0.020; choline chloride, 1.0; inositol, 1.0; niacin, 0.025; pyridoxine·HCl, 0.0035; riboflavin, 0.0035; thiamin·HCl, 0.0035; retinyl palmitate (500,000 U/g), 0.037; DL-α-tocopheryl acetate (500 U/g), 0.11; menadione, 0.006; cholecalciferol, 0.005.

ad libitum for 4 wk. Diet A (TD78388, Teklad, Madison, WI) contained 0.014% elemental Ca, 0.18% P and 5 μg (200 IU) cholecalciferol per 100 g (**Table 1**). We prepared the remaining diets by adding inorganic phosphate ($NaH_2PO_4 \cdot H_2O$, "Baker Analyzed Reagent," J. T. Baker Chemical Co., Phillipsburg, NJ) and/or calcium (calcium carbonate, USP; J. T. Baker Chemical Co.) to diet A so that total diet Ca and P were as follows: diet B, 0.014% Ca and 1.2% P; diet C, 0.7% Ca and 1.2% P; diet D, 2% Ca and 1.2% P; diet E, 2% Ca and 0.18% P. To avoid spillage, powdered diets were tightly pressed into narrow-necked porcelain dishes. For each group the amount of diet eaten was weighed for all feedings cumulatively.

Twenty-five hours before they were killed, the rats were anesthetized with ether, a 1-mL blood sample was taken by heart puncture (for assay of 25-OH-cholecalciferol, calcium and phosphate) and each rat was injected slowly via the jugular vein with 0.1 mL ethanol containing 500,000 dpm 25-[^{3}H]hydroxycholecalciferol (10 Ci/mmol, TRK 396, Amersham, Oakville, Ontario). Blood was obtained by heart puncture under ether anesthesia for measurement of plasma 25-OH-[^{3}H]cholecalciferol, 1,25$(OH)_2$-[^{3}H]cholecalciferol and 24,25$(OH)_2$-[^{3}H]cholecalciferol concentrations and kidneys were removed for assay of 1-OHase and 24-OHase activities. Plasma 25-OH-cholecalciferol levels were measured by binding-protein assay subsequent to methanol/chloroform lipid extraction and Sephadex LH-20 minicolumn chromatography (12). Calcium was measured by atomic absorption spectrophotometry and phosphate by reaction with molybdic acid. 1-OHase and 24-OHase activities were measured as described previously (12). In summary, renal mitochondria were prepared in 150 mM KCl, 20 mM HEPES, 10 mM L-malic acid, 2 mM $MgSO_4$, 1 mM dithiothreitol and 25 nM EDTA. A 1-mL aliquot containing about 5 mg mitochondrial protein was incubated at 25°C for 15 min in the presence of 500 nmol/L nonradioactive 25-OH-cholecalciferol (courtesy of Dr. M. R. Uskokovic, Hoffmann-LaRoche Inc., Nutley, NJ) plus 50,000 dpm 25-OH-[^{3}H]cholecalciferol. The reaction was stopped and the lipid extraction procedure started by adding 2:1 methanol:chloroform. Extracted 25-OH-[^{3}H]cholecalciferol, 1,25$(OH)_2$-[^{3}H]cholecalciferol and 24,25$(OH)_2$-[^{3}H]cholecalciferol were separated by continuous-development thin-layer chromatography (TLC) on silica developed with 1:1 benzene:ethyl acetate and measured by scintillation counting. 1-OHase and 24-OHase activities, expressed in femtomoles per milligram mitochondrial protein per minute, were calculated from these measurements. Radioactive metabolites generated in vivo from 25-OH-[^{3}H]cholecalciferol were extracted from plasma and chromatographed by the same techniques.

In vivo metabolism of 25-OH-[^{3}H]cholecalciferol to its dihydroxy metabolites in plasma was expressed as the percentage of ^{3}H appearing as 1,25-$(OH)_2$-cholecalciferol and 24,25-(OH_2)-cholecalciferol. We validated the extraction and chromatographic procedures by using various known mixtures of radioactive 25-OH-cholecalciferol, 24,25-$(OH)_2$-cholecalciferol and 1,25-$(OH)_2$-cholecalciferol in bovine serum albumin solutions. The extraction and TLC procedures resulted in recoveries of the metabolites in the proportions expected. The relation of observed versus expected percent 1,25-$(OH)_2$-[^{3}H]cholecalciferol and 24,25-(OH_2)-[^{3}H]cholecalciferol in the mixtures gave slope 1.05 and intercept 0.08%; $r = 0.99$, $n = 12$. Samples produced under "blank" incubation conditions (using medium alone, liver mitochondria or renal mitochondria whose 1-OHase had been denatured with two short microwave bursts (to boiling)) were analyzed by high performance liquid chromatography or TLC. None of the blank samples yielded oxidation or metabolite peaks; only unchanged 25-OH-[^{3}H]cholecalciferol was present.

The chi-square test was carried out according to Dixon and Massey (14). Comparisons of the control diet group (C) with the other diet groups were conducted using formulas for the *t*-statistic and degrees of freedom that corrected for differences in variance (14). Decision values for the *t*-statistic were from the tables of Dunnett (15) for multiple comparisons with a control.

RESULTS

The mean daily consumption of diet for the groups ranged from 13.9 to 15.5 g/rat over the 4-wk period of feeding (**Table 2**), representing an intake of cholecalciferol of approximately 0.75 μg/d per rat. Animals in group C, fed normal Ca and normal P, had the greatest weight gain of all groups, the highest plasma 25-OH-cholecalciferol level and the highest mitochondrial 24-

TABLE 2

Effects of diet on 25-OH-cholecalciferol metabolism in vivo and in vitro[1]

	Diet				
	A	B	C	D	E
Diet					
Calcium, *g/100 g*	0.014	0.014	0.7	2.0	2.0
Phosphate, *g/100 g*	0.18	1.2	1.2	1.2	0.18
Amt. eaten, *g/(d·rat)*	14.4	14.6	15.5	13.9	14.5
Final rat wt, *g*	214 ± 5[2]	228 ± 3[2]	268 ± 5	215 ± 4[2]	216 ± 4[2]
Plasma					
Calcium, *mmol/L*	1.87 ± 0.11[2]	2.28 ± 0.09[2]	2.93 ± 0.03	3.08 ± 0.02[2]	3.24 ± 0.04[2]
Phosphate, *mmol/L*	2.38 ± 0.08	2.57 ± 0.04	2.56 ± 0.07	1.40 ± 0.08[2]	1.54 ± 0.07[2]
25-OHD_3, *nmol/L*[3]	<2.5[2]	<2.5[2]	11.0 ± 0.8	3.5 ± 0.2[2]	7.0 ± 0.7[2]
In vivo metabolism					
^3H as 1,25-$(OH)_2$-cholecalciferol, %	27.2 ± 1.7[2]	20.8 ± 1.2[2]	0.8 ± 0.1	1.2 ± 0.2	2.1 ± 0.2[2]
^3H as 24,25-$(OH)_2$-cholecalciferol, %	<0.3[2]	<0.3[2]	8.5 ± 0.5	8.6 ± 0.5	8.6 ± 0.5
Enzymes					
1-OHase, *fmol/(mg·min)*[4]	668 ± 33[2]	676 ± 89[2]	30 ± 5	20 ± 2	20 ± 3
24-OHase, *fmol/(mg·min)*[4]	14 ± 2[2]	32 ± 8[2]	150 ± 26	40 ± 6[2]	50 ± 8[2]

[1]There were 12 rats in each group. Results are means ± SE.
[2]Significantly different from the normal diet group C, $P < 0.01$.
[3]The undetectable values (<2.5) were assigned a value of 2.0 nmol/L.
[4]The undetectable values (<10) were assigned a value of 8 fmol/(mg·min).

OHase activity. Group A, fed the low Ca and low P diet, and group B, fed the low Ca and normal P diet, both had low plasma Ca and low plasma 25-OH-cholecalciferol in conjunction with high in vivo tracer metabolism to 1,25-$(OH)_2$-[^{3}H]cholecalciferol and high mitochondrial 1-OHase. The high dietary Ca groups, D (fed normal P) and E (fed low P), both had lower plasma P levels than group C, but mitochondrial 1-OHase results were not significantly different from those in group C; this is in contrast to the in vivo situation, where there was significantly greater synthesis of 1,25-$(OH)_2$-[^{3}H]cholecalciferol in the low P diet group E. Furthermore, plasma 25-OH-cholecalciferol for both high Ca groups was low in relation to the normal diet group but significantly higher than the undetectable levels of the low Ca diet groups, A and B. For all diet groups both in vivo and in vitro, no metabolites of 25-OH-[^{3}H]cholecalciferol were observed other than 1,25-$(OH)_2$-[^{3}H]cholecalciferol and/or 24,25-$(OH)_2$-[^{3}H]cholecalciferol.

Results for plasma 25-OH-cholecalciferol and renal 1-OHase activity for all rats are presented in **Fig. 1**. All rats fed low Ca diets (A and B) had undetectable levels of 25-OH-cholecalciferol and their 1-OHase activities were greater than 230 fmol/(mg·min). Rats fed adequate or high Ca diets (C, D, E) had 1-OHase activities below 60 fmol/(mg·min) and a wide range of plasma 25-OH-cholecalciferol levels.

To demonstrate statistically the relation between diet Ca and plasma 25-OH-cholecalciferol, we used the chi-square test (**Table 3**). This allowed us to combine, for a single statistic, data for all diet groups and made it unnecessary to use the default values to fill in for undetectable results. For this analysis, the rats were grouped according to plasma 25-OH-cholecalciferol level (measurable or undetectable) and diet Ca (low vs. normal or high Ca). We chose the detection limit for 25-OH-cholecalciferol to divide the sample population because it was a well-defined point and because there was no other

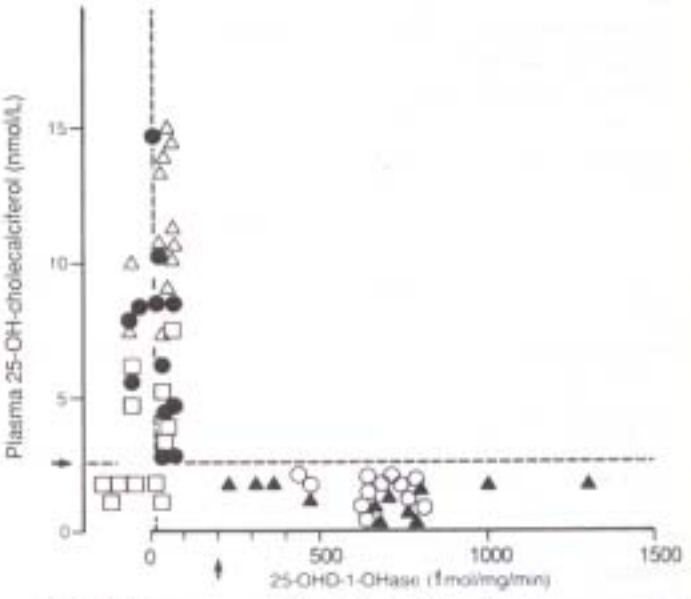

FIGURE 1 Plasma 25-OH-cholecalciferol level versus renal mitochondrial 1-OHase activities for rats fed diet (○) A, (▲) B, (△) C, (□) D, (●) E. Dashed lines indicate detection limits for 25-OH-cholecalciferol (horizontal line) and 1-OHase (vertical line). For clarity, points below detection limits are plotted away from the dashed lines. The arrows indicate the values of 25-OH-cholecalciferol and 1-OHase that divide the sample population into the four groups that coincide with the chi-square analysis (Table 3).

TABLE 3

Chi-square test showing number of rats observed with specified combinations of plasma 25-OH-cholecalciferol and diet calcium[1]

	Diet calcium[2]	
25-OH-cholecalciferol	0.7 or 1.2%	0.014%
nmol/L		
<2.5	6 (18)	24 (12)
>2.5	30 (18)	0 (12)

[1]Chi square = 36.7; $P < 0.001$.

[2]Values in parentheses are expected frequencies according to the null hypothesis. If there were no relation between 25-OH-cholecalciferol and diet calcium, the numbers in the table would be approximately those in parentheses.

25-OH-cholecalciferol level that clearly split the sample population. Since only the rats with low diet Ca had 1-OHase levels over 200 fmol/(mg·min), the chi-square groupings for diet Ca are identical to those that would have been obtained if instead the sample population had been divided according to 1-OHase value [over or under 200 fmol/(mg·min)] as indicated by the arrows in Fig. 1. Although the consumption of food and cholecalciferol was very similar for the five diet groups (Table 3), those ingesting a low Ca diet had undetectable plasma 25-OH-cholecalciferol whereas those ingesting normal or high Ca diets had higher plasma 25-OH-cholecalciferol levels.

DISCUSSION

The findings demonstrate that vitamin D requirement was increased when diet calcium was reduced. Low calcium intake stimulated 1-OHase activity in our rats to such a degree that the otherwise sufficient cholecalciferol intake was not enough to maintain plasma 25-OH-cholecalciferol at detectable levels.

The results show that 25-OH-cholecalciferol and 1-OHase are strongly interdependent. For a number of reasons we conclude that it was the low Ca-related increase in 1-OHase that depleted 25-OH-cholecalciferol: *1*) Vitamin D deficiency per se does not increase serum 1,25-$(OH)_2$-cholecalciferol concentrations (16). *2*) In humans, 1,25-$(OH)_2$-vitamin D synthesis can consume a large part of (1, 2) and may even exceed (2) the recommended daily dietary allowance for vitamin D. *3*) Our in vivo findings also support our argument. More than 20% of the tracer in circulation was 1,25-$(OH)_2$-[^{3}H]-cholecalciferol (Table 2). In view of the short in vivo half-life and large volume of distribution of 1,25-$(OH)_2$-cholecalciferol compared to 25-OH-cholecalciferol (17), this is conclusive evidence that 1-OHase activity was the major factor depleting 25-OH-cholecalciferol in our rats.

Although it is generally recognized that low dietary P stimulates 1,25-$(OH)_2$-cholecalciferol production, we did not observe effects of dietary P on 1-OHase in vitro. For low Ca groups A and B, we may not have observed P effects on 1-OHase activity because of the overriding effects of severe Ca depletion. For the two groups with high Ca intakes, the low P group (E) had significantly higher plasma 25-OH-cholecalciferol levels and higher in vivo metabolism to 1,25-$(OH)_2$-[^{3}H]cholecalciferol than the high P group (D). The effects of different levels of dietary P may also have been modified in rats fed high Ca diets by the formation of complexes of Ca and P either in the intestinal lumen or after absorption, with subsequent reduction in their biological availability.

Cholecalciferol intake was virtually identical for all diet groups because all the diets were prepared from the cholecalciferol-containing diet (A) and intake of diet was similar for all groups. Differences in plasma 25-OH-cholecalciferol could in theory be due to differences in the handling of cholecalciferol at a number of stages. For a given intake of cholecalciferol, variations in rates of intestinal absorption and hepatic 25-hydroxylation could affect 25-OH-cholecalciferol levels (18). Other enzymes such as 24-OHase could also influence plasma 25-OH-cholecalciferol levels. However, in the present study increased 24-OHase activity resulted from increased plasma 25-OH-cholecalciferol (Table 2) and was not the primary determinant of its concentration. Since plasma 25-OH-cholecalciferol concentrations varied widely among the groups in which diet Ca was adequate and 1-OHase activity was low, it follows that other undetermined mechanisms might also affect 25-OH-cholecalciferol levels.

Low serum 25-OH-vitamin D concentrations in association with high 1,25-$(OH)_2$-vitamin D have been attributed to feedback inhibition of hepatic cholecalciferol 25-hydroxylase by the hormonal product (19–21). Evidence for this is based on low serum 25-OH-vitamin D levels in patients receiving 1,25-$(OH)_2$-cholecalciferol therapy (20) and on in vitro inhibition of cholecalciferol 25-hydroxylase by 1,25-$(OH)_2$-cholecalciferol (21). More recent studies have concluded, however, that chronic 1,25-$(OH)_2$-cholecalciferol administration lowers serum 25-OH-cholecalciferol by increasing the metabolic clearance of 25-OH-cholecalciferol and not by decreasing its production (22, 23).

It is relevant to note that 1,25-$(OH)_2$-cholecalciferol is involved in a positive feedback loop whereby it can increase fourfold the levels of the cholecalciferol precursor 7,8-didehydrocholesterol in rat skin (24). Esvelt et al. (24) proposed that this feedback mechanism could protect animals with high 1-OHase activity from depleting their stores of 25-OH-cholecalciferol. However, this mechanism could not have helped our animals, which were kept away from ultraviolet light sources. If our animals had been exposed to ultraviolet light, their 25-OH-cholecalciferol concentrations would not have been as drastically reduced by calcium deprivation as we observed.

Our findings should be considered when interpreting plasma values of vitamin D metabolites in hypocalcemic and osteomalacic conditions. 25-OH-cholecalciferol depletion has classically been thought of as due to a low vitamin D supply; however, our study shows that it can also be secondary to increased metabolism of 25-OH-cholecalciferol to 1,25-$(OH)_2$-cholecalciferol. Thus, in a hypocalcemic patient with decreased plasma 25-(OH)-cholecalciferol and increased 1,25-$(OH)_2$-cholecalciferol concentrations, these apparently anomalous metabolite levels could be due to increased 1-OHase activity secondary to calcium deficiency, causing increased 1,25-$(OH)_2$-cholecalciferol synthesis and depletion of 25-OH-cholecalciferol stores. This sequence of adjustments might explain the metabolite values observed in many reported patients (4–11).

Gascon-Barré et al. (25) reported a study in which patients with urolithiasis were placed on diets containing either 1000 or 300 mg Ca daily. They found that with the low Ca intake, circulating 25-OH-vitamin D was significantly lower and 1,25-$(OH)_2$-vitamin D significantly higher than with the higher Ca intake. Since the rate of 1,25-$(OH)_2$-vitamin D synthesis was not estimated, changes in metabolite distribution volume or in 25-OH-vitamin D synthesis could not be excluded as possible explanations. In light of our findings, their observations could be attributed to increased 1,25-$(OH)_2$-vitamin D production.

We conclude that when calcium homeostasis is stressed, as by dietary calcium restriction, increased production of 1,25-$(OH)_2$-cholecalciferol causes a significant increase in 25-OH-cholecalciferol utilization. When intake of cholecalciferol is marginal, the added demand for 25-OH-cholecalciferol as substrate could result in vitamin D insufficiency.

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