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Media Calcium Attenuates Mitochondrial 1,25(OH)$_2$D Production in Phosphorus or Vitamin D-Deprived Rats

THOMAS O. CARPENTER AND BRUCE ELLIS

Department of Pediatrics (Endocrinology), Yale University School of Medicine, New Haven, Connecticut 06520-8064

ABSTRACT

Although PTH and hypophosphatemia are the best known stimulators of 25-hydroxyvitamin D-1α-hydroxylase, 1,25(OH)$_2$D$_3$ production in rats and humans can be modulated by circulating calcium, independent of PTH. To test whether calcium modulates this function directly in mitochondria, we examined effects of calcium on 1α-hydroxylase in isolated mitochondrial preparations under basal and stimulated conditions. Rats were fed a low phosphorus (or matched control) diet for 4 or 7 d or a vitamin D-deficient (or matched control) diet for 2, 4, or 7 wk. Renal mitochondria were isolated and assayed for 1α-hydroxylase activity in the presence or absence of added calcium. Calcium did not alter 1α-hydroxylase in rats on control diets. After 4 d of low phosphorus diet, 1α-hydroxylase was increased 2-fold over basal activity; media calcium prevented this stimulatory response. By 7 d the calcium effect was not evident. After 4 wk of vitamin D deprivation, activity was ~30-fold greater than controls; calcium reduced this response significantly (15-fold). A significant, but less marked inhibition of activity by calcium was present in rats subjected to 7 wk of vitamin D deprivation. Extramitochondrial calcium can directly modulate 1,25(OH)$_2$D$_3$ production, but this effect appears to be secondary to the primary physiologic regulators of this function. The calcium effect can be overcome after longer term exposure to phosphorus deprivation, but is sustained in the presence of long term vitamin D deprivation. (***Pediatr Res* 37: 726–730, 1995**)

Abbreviation

1α-OHase, 25-hydroxyvitamin D-1α-hydroxylase

The best known active metabolite of vitamin D is 1,25(OH)$_2$D, which in humans and other mammals, effects numerous physiologic events. Production of 1,25(OH)$_2$D is tightly controlled at the final step of its biosynthetic pathway, performed in renal tubular mitochondria by a P_{450} enzyme system, the renal 1α-OHase (1). Although several factors have been shown to be trophic for this reaction (*e.g.* IGF-I, estrogen, thyroid hormone, calcitonin), the predominant physiologic regulators of this reaction in the intact organism are hypophosphatemia and PTH (2). The stimulatory effect of hypocalcemia on 1,25(OH)$_2$D production has generally been attributed to resultant secondary hyperparathyroidism (3). In contrast with the view that calcium regulation of 1α-OHase is entirely dependent on PTH effects, more recent work has shown that extracellular calcium is able to regulate this reaction in rats (4, 5) and humans (6) independently of PTH. In view of this finding, we examined the ability of calcium to modulate *1*) basal enzyme activity and *2*) stimulated enzyme activity (using two standard dietary provocations) directly at the mitochondrial level.

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Correspondence: Thomas O. Carpenter, M.D., Yale University School of Medicine, Department of Pediatrics, P.O. Box 208064, 333 Cedar Street, LMP 3103, New Haven, CT 06520–8064.
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METHODS

Animals. Male Holtzman rats were obtained from Harlan (Madison, WI) at weanling. In experiments examining effects of phosphorus deprivation, rats were housed in our animal facilities and placed on either a low phosphorus diet (Teklad, Madison, WI), containing 0.02% phosphorus, 0.6% calcium and 2200 U of vitamin D/kg, or a control diet containing 0.6% phosphorus, and otherwise matched to the experimental diet. Animals were killed after 4 or 7 d of diet. Several experiments were performed using rats at different weights and ages, varying between 100 and 400 g; each group consisted of two to four rats.

In experiments examining the effects of vitamin D deprivation, rats were housed in rooms shielded from UV light upon arrival at our institution. They were placed on either the control diet described above, or an identical diet with no added vitamin D. An experiment after 2 wk showed no effect of vitamin D deprivation on 1α-OHase activity, therefore other experiments

were performed after 4 and 7 wk of vitamin D deprivation. Each dietary group consisted of four to six rats.

Mitochondrial preparations. Rats were killed by decapitation after intraperitoneal pentobarbital anesthesia (50 mg/kg). Animals were segregated as to dietary grouping. One kidney from each animal was designated for preparation and incubation in calcium buffer, whereas the contralateral kidney from the animal was designated for calcium-free buffer. All kidneys from animals exposed to the same diet and designated for calcium presence were pooled and minced. Minced renal cortices were homogenized on ice in 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-0.19 M sucrose-2 mM $MgCl_2$-0.5 mM EGTA-0.5 mM $CaCl_2$ (homogenization buffer) (5% wt/vol), then centrifuged at 1000 × *g* for 10 min at 4°C. The supernatant was centrifuged at 10,000 × *g* for 10 min at 4°C. The resultant pellet was washed once and resuspended in incubation buffer (identical to homogenization buffer with the addition of 25 mM sodium succinate). A free calcium concentration ($[Ca^{2+}]$) of approximately 100 μM was estimated from calcium-buffer curves (7). Calcium-free mitochondria were prepared from the contralateral kidney of the same animal with 0.5 mM EGTA, but in the absence of any added calcium.

1α-OHase assay. Mitochondrial preps were incubated in two to three replicates in the presence of 80 μM unlabeled 25-OHD_3 (a generous gift of Organon, West Orange, NJ). Samples were oxygenated, and incubated at 37°C for 20 min. The reaction was stopped by the addition of 3 mL acetonitrile. Samples were centrifuged at 1,500 × *g* at 4°C for 10 min. Supernatant was decanted, combined with 1.5 mL 0.4 M K_2HPO_4 (pH 10.6), and loaded into Sep-Pak C_{18} columns (Waters, Milford, MA), then eluted with 3 mL acetonitrile. Eluant was dried under nitrogen gas and resuspended in 1 mL hexane-2-propanol (96:4, vol/vol), and loaded onto Sep-Pak silica columns (Waters), washed with 20 mL hexane-2-propanol (96:4, vol/vol), 8 mL hexane-2-propanol (94:6, vol/vol), and eluted with 10 mL hexane-2-propanol (85:15, vol/vol).

Samples were further purified using an isocratic HPLC column, with a mobile phase of 97% methylene chloride-3% 2-propanol as described (8). Fractions co-eluting with 1,25$(OH)_2D_3$ were collected, dried under nitrogen, resuspended in 2-propanol, and stored for 1,25$(OH)_2$D determination.

The quantity of 1,25$(OH)_2$D in the HPLC-purified samples was determined with a nonequilibrium protein binding assay using highly purified 1,25$(OH)_2$D receptor from calf thymus as described by Reinhardt *et al.* (9). Recovery of 1,25$(OH)_2$D was monitored by addition of 5,000 cpm of ^{3}H-1,25$(OH)_2D_3$ to each sample before extraction and chromatography. Average recovery for each assay set-up varied from 37 to 39% and the final value for each sample was corrected for recovery determined for that sample.

Analytic techniques. The protein concentration of mitochondrial preps was determined using the method of Lowry. Calcium and phosphorus concentrations were determined by colorimetric assay as described elsewhere (8).

Statistical analysis. Analysis of variance and *post hoc* Fisher's least mean square difference tests were performed with the Systat version 5.03 statistical software package for IBM (Evanston, IL) compatible computers. All data are presented as mean ± SEM.

RESULTS

Phosphorus deprivation. Serum calcium and phosphate levels in phosphorus deprivation experiments are shown in Table 1. Phosphorus deprivation had a significant effect on serum calcium levels ($p < 0.0005$), however there was no difference in serum calcium between rats subjected to 4 and 7 d of phosphorus deprivation ($p = 0.85$). Serum phosphorus levels were significantly lower in the phosphorus deficient groups ($p < 0.0005$), but did not differ between phosphorus deprivation of 4 and 7 d duration ($p = 0.06$).

The effect of media calcium on 1α-OHase in the phosphorus replete control rats for these experiments is summarized in Figure 1. The experiments were performed in four different age groups, as noted by the average weights of the animals as shown along the abscissa. Two-way analysis of variance revealed no effect of media calcium ($p = 0.45$), however there was a significant effect of weight of the animal ($p < 0.0005$) on 1α-OHase. The youngest rats (96 g, approximately 4 wk of age) had greater 1α-OHase activity than all other groups; no differences were apparent between the older groups. This finding is consistent with earlier reports of the age dependence of this enzyme (10). Figure 2 shows the effect of both media calcium and the duration of phosphorus deprivation on renal 1α-OHase activity. Two-way analysis of variance revealed a significant effect of media calcium ($p = 0.02$), and of duration of diet ($p < 0.0005$) on 1α-OHase activity. Media calcium attenuates the stimulation of the 4-d period of phosphorus deprivation ($p < 0.0005$), but does not maintain this capacity to dampen activity when the animals have been subjected to 7 d of phosphorus deprivation ($p = 0.62$).

Vitamin D deprivation. Serum calcium and phosphorus levels in vitamin D deprivation experiments are shown in Table 2. As with phosphorus deprivation, vitamin D deprivation had a significant effect on serum calcium levels ($p = 0.02$). Serum phosphate levels were slightly, but not significantly lower in the vitamin D-deficient group at 4 wk.

The effect of media calcium on 1α-OHase in the vitamin D-replete control rats is summarized in Figure 3. There was no effect of media calcium ($p = 0.29$) on 1α-OHase in control animals. All experiments were done beyond the early period of

Table 1. *Serum calcium and phosphorus levels in phosphorus-deprived and control rats*

		Duration of phosphorus deprivation	
		4 d	7 d
Ca (mM)	+P	2.52 ± 0.10	2.58 ± 0.08
	−P	3.42 ± 0.08	3.32 ± 0.15
P (mM)	+P	3.42 ± 0.19	2.84 ± 0.16
	−P	1.45 ± 0.06	1.35 ± 0.19

Analysis of variance reveals a significant effect of dietary phosphorus status on serum calcium ($p < 0.0005$) and on serum phosphorus ($p < 0.0005$). To convert to metric units (mg/dl) multiply Ca value by 4, and P value by 3.1.

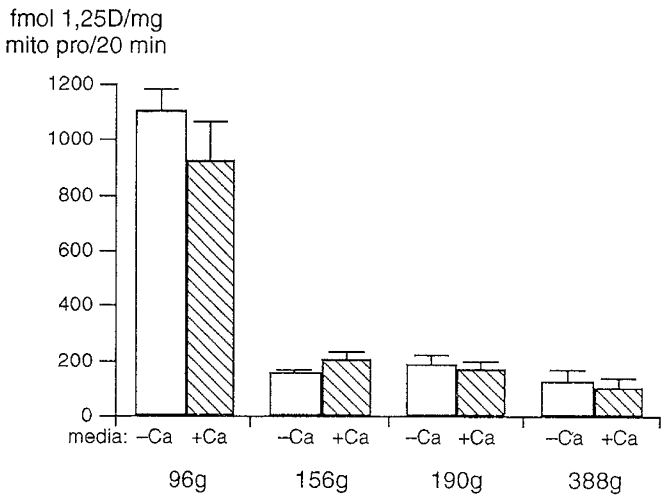

Figure 1. 1α-OHase activity (expressed as femtomoles of $1,25(OH)_2D$ produced per milligram of mitochondrial protein per 20 min) in mitochondria from control animals used in the phosphorus deprivation experiments. Experiments were performed in various weight animals, as noted along the abscissa. *Open columns*, mitochondria incubated without calcium; *hatched columns*, mitochondria incubated with calcium. Two-way analysis of variance revealed no effect of media calcium on 1α-OHase activity ($p = 0.45$), but a significant effect of weight of the animal ($p < 0.0005$). *Post hoc* testing revealed that the youngest (96 g) rats were different from all other groups ($p < 0.0005$); there was no difference among all older groups ($0.39 < p < 0.93$).

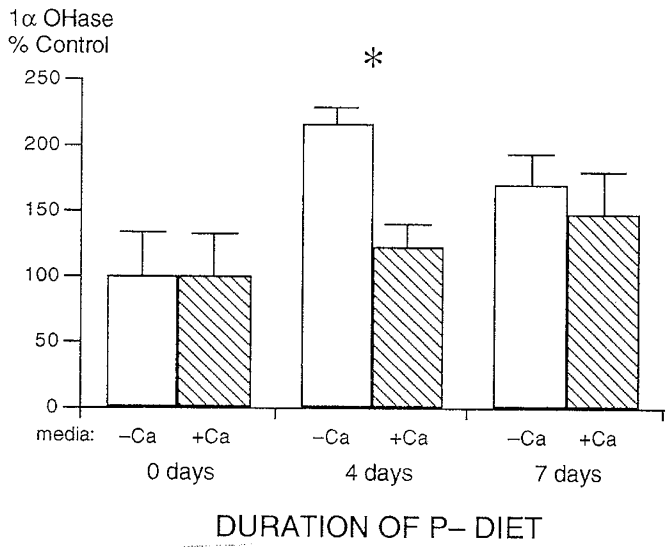

Figure 2. 1α-OHase activity (expressed as percent of activity in control animals, see Fig. 1) in phosphorus-deprived rats. Duration of diet is indicated on the abscissa (controls are shown as 0 d of diet). *Open columns*, mitochondria incubated without calcium; *hatched columns*, mitochondria incubated with calcium. Two-way analysis of variance revealed a significant effect of media calcium on 1α-OHase activity ($p = 0.02$) and a significant effect of duration of low phosphorus diet ($p < 0.0005$). *Difference between −Ca and +Ca groups was present only after 4 d of dietary phosphorus deprivation ($p < 0.0005$).

increased enzyme activity noted in the phosphorus deprivation experiments; therefore there was no effect of age on 1α-OHase in control animals in the vitamin D deprivation experiments ($p = 0.28$). Figure 4 shows the effect of both media calcium and the duration of vitamin D deprivation on renal 1α-OHase activity. There was a markedly significant effect of media calcium ($p < 0.0005$), and of diet ($p < 0.0005$) on 1α-OHase activity. Media calcium attenuated the stimulatory response of vitamin D deprivation of both 4- and 7-wk duration.

Ruthenium red. The mitochondrial calcium transport inhibitor, ruthenium red (3 μM) was added to preparations in several experiments. No consistent effect of ruthenium red was seen in vitamin D deficiency experiments. However in the phosphorus deficiency experiments (in both control and phosphorus-deprived animals), the addition of ruthenium red to preparations incubated in the presence of calcium resulted in a slight, but significantly insignificant increase in enzyme activity (data not shown).

DISCUSSION

Although these experiments are the first to address direct calcium regulation of 1α-OHase activity in mammalian mitochondrial preparations, several early studies examined this issue in chick mitochondria (11–18). Limitations of these earlier reports include *1*) relatively insensitive and nonspecific methods of purification of $1,25(OH)_2D$; *2*) studies restricted to animals subjected to vitamin D-deficient diets; *3*) use of media calcium concentrations in supraphysiologic millimolar ranges; and *4*) inconsistencies in the methods and results among the various reports. For example, several studies demonstrated that a discreet range of media calcium concentrations resulted in stimulation of 1α-OHase activity, but the ranges were variable in the different studies (11, 12, 16, 18). Other studies reported inhibition of activity with addition of calcium to the media (13–15, 17). We therefore elected to examine a mammalian model on standard diet, after vitamin D deprivation, and phosphorus deprivation, with specific and sensitive methods of $1,25(OH)_2D$ determination; the effect of free calcium was tested at a concentration that did not alter enzyme activity in normally fed animals.

Phosphorus deprivation. The effect of phosphorus deprivation resulted in an expected increase in 1α-OHase activity. Young rats demonstrated greater activity than older rats. Media calcium did not affect enzyme function in control animals at any of the various weight groups examined. However, the presence of calcium in the media prevented expression of the 2-fold stimulation of 1α-OHase activity seen with 4 d of phosphorus deprivation. Stimulation was completely inhibited when rats greater than 100 g were examined, although the

Table 2. *Serum calcium and phosphorus levels in vitamin D-deprived and control rats*

		Duration of vitamin D deprivation	
		4 wk	7 wk
Ca (mM)	+D	2.45 ± 0.02	2.52 ± 0.04
	−D	2.40 ± 0.01	2.32 ± 0.03
P (mM)	+D	3.06 ± 0.02	ND
	−D	2.81 ± 0.04	ND

Analysis of variance revealed a significant effect of vitamin D status on the serum calcium level ($p = 0.02$). ND, not determined. To convert to metric units (mg/dl) multiply Ca value by 4, and P value by 3.1.

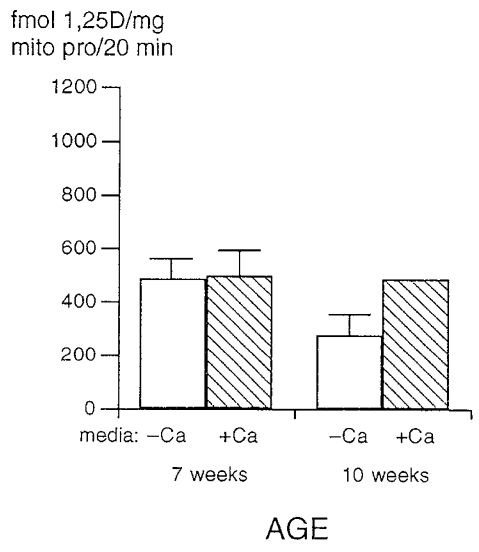

Figure 3. 1α-OHase activity (expressed as femtomoles of $1,25(OH)_2D$ produced per milligram of mitochondrial protein per 20 min) in mitochondria from control animals used in the vitamin D deprivation experiments. Experiments were performed in various age animals, as noted along the abscissa. *Open columns*, mitochondria incubated without calcium; *hatched columns*, mitochondria incubated with calcium. Two-way analysis of variance revealed no effect of media calcium on 1α-OHase activity ($p = 0.29$) and no significant effect of age of the animal ($p = 0.28$).

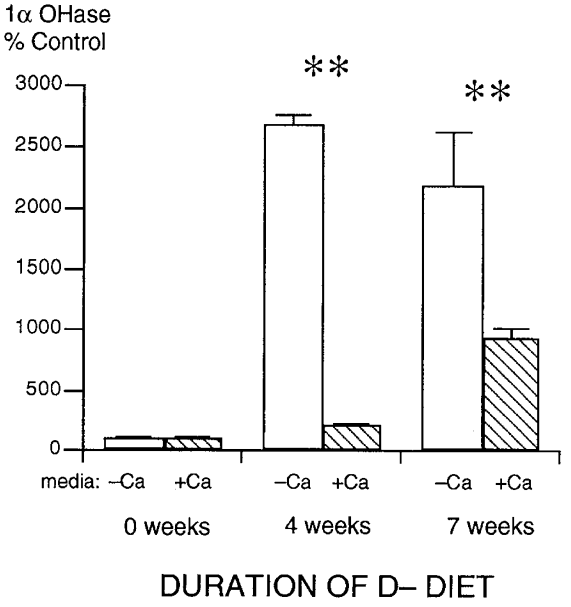

Figure 4. 1α-OHase activity (expressed as percent of activity in control animals, see Fig. 3) in vitamin D-deprived rats. Duration of diet is indicated on the abscissa (controls are shown as 0 wk of diet). *Open columns*, mitochondria incubated without calcium; *hatched columns*, mitochondria incubated with calcium. Two-way analysis of variance revealed a significant effect of media calcium on 1α-OHase activity ($p < 0.0005$) and a significant effect of vitamin D-deficient diet ($p < 0.0005$). **+Ca mitochondria were significantly less active than −Ca mitochondria after 4 and 7 wk ($p < 0.0005$), but not at baseline ($p = 1.0$).

effect was significant for the youngest (96 g) group as well (data not shown). When rats were deprived of dietary phosphorus for 7 d, approximately 2-fold stimulation of 1α-OHase was again evident, but there was no effect of media calcium. These data indicate that although calcium can modulate enzyme activity, it plays a secondary role in this regard. Further, there appears to be an adaptation to phosphorus deprivation such that after prolonged exposure (in this case 7 d), the calcium-modulating effect is overcome.

Vitamin D deprivation. All vitamin D deprivation experiments were performed on older animals than the young phosphorus-deprived group; this eliminated the effect of age on enzyme activity in these experiments. Vitamin D deprivation, like phosphorus deprivation, resulted in an expected increase in 1α-OHase activity, however the vitamin D deprivation stimulus was far more potent than the hypophosphatemic stimulus, resulting in up to 20–30-fold increases over 1α-OHase activity in age-matched rats on control diet. However, 4 wk of vitamin D-deficient diet was required to effect this change; no stimulation was evident by 2 wk of this dietary treatment (data not shown). Furthermore, the dietary regimen resulted in only modest changes in serum calcium, indicating that these animals were not severely depleted of vitamin D stores and that hypocalcemia, *per se*, was probably not responsible for the stimulation. The magnitude of the decrease in serum phosphorus levels in vitamin D-deprived animals was also modest. Although we were not able to perform PTH measurements in these experiments, the dominant stimulus in these experiments is most likely secondary hyperparathyroidism. Our previous studies demonstrate an approximate 10-fold increase in immunoreactive PTH when weanling rats are placed on a vitamin D-deficient diet for 3 wk (19). In the current vitamin D deprivation experiments, as in the phosphorus deprivation experiments, there was no effect of media calcium on basal (control) enzyme activity. In rats subjected to 4 wk of vitamin D deprivation; however, where enzyme activity is enhanced 20–30 fold, media calcium resulted in a marked reduction in 1α-OHase activity (Fig. 4). Rats deprived of vitamin D for 7 wk showed a marked stimulation of 1α-OHase, comparable to that seen in rats subjected to the 4-wk period of deprivation. Although media calcium maintained the capacity to attenuate this stimulation, only a 2-fold reduction in a 1α-OHase activity was apparent. These data indicate that a direct modulatory effect of calcium occurs when vitamin D deprivation is the primary stimulus for 1α-OHase, as when phosphorus deprivation is the primary stimulus. In both cases media calcium plays a secondary modulating role in the regulation of the enzyme. A concentration of calcium was used in which no effect was ascertained when animals with normal basal enzyme activity are examined; however, the exposure of stimulated mitochondria to the same media calcium concentration resulted in marked attenuation of the activity. This secondary modulation by calcium appears to effect the hypophosphatemic and vitamin D deficient stimuli for 1α-hydroxylase slightly differently: the effect on the hypophosphatemic stimulus can be overcome by exposure to phosphorus deprivation for 7 d, whereas the effect when vitamin D deprivation is the primary stimulus persists for the long term (up to 7 wk). Finally, it is possible that the earlier studies in which chick mitochondria demonstrated increased 1α-OHase activity in the presence of calcium were affected by the degree of vitamin D deficiency.

It is worth noting that earlier investigators noted a direct correlation between extracellular calcium concentration and intracellular calcium in renal cells (20); this is consistent with the notion that the described inhibitory effect of extracellular calcium upon this enzyme system (4–6) is mediated by an increase in intracellular calcium, which may directly effect the mitochondrial enzyme system. Moreover similar studies demonstrated an increase in intracellular calcium occurring with an increase in extracellular phosphorus concentration (21), consistent with intracellular calcium, mediating, in part, the effects of extracellular phosphorus on this enzyme system.

Our studies confirm that both hypophosphatemia and vitamin D deprivation are major regulators of renal 1α-OHase activity. In these studies vitamin D deficiency appears to be a more potent mechanism of activation than phosphorus deprivation. Both means of stimulation can be modulated by an effect of media calcium at the calcium level; it is possible that a calcium entry effect into mitochondria, which may be slightly inhibited by ruthenium red, plays a role in mediating this phenomenon. At present the precise mechanism(s) of stimulation by these factors is unknown, however the mechanisms differ with respect to the duration that the calcium-modulating effect at the mitochondrial level can be sustained.

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