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Dietary phosphorus restriction reverses the impaired bone mineralization in vitamin D receptor knockout mice

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ABSTRACT

Deficiency of vitamin D, which is required for calcium homeostasis, causes rickets with hypocalcemia and hypophosphatemia, resulting in growth retardation and impaired bone formation. Mice lacking the vitamin D receptor (VDR) develop the typical features of rickets, establishing that VDR plays a role in controlling the actions of vitamin D. Normalization of impaired mineral homeostasis in VDR KO mice fed a diet supplemented with high concentrations of calcium (2%) and phosphorus (1.25%) is reported to reverse the malformation of bone and the growth retardation as well. However, the relationship between mobilization of phosphorus and calcium and nuclear control of vitamin D actions remains unclear. The present study was undertaken to determine the effect of dietary phosphorus on mineral mobilization and bone mineralization. We report here that feeding a diet supplemented with a restricted amount of phosphorus (0.25%) and a normal amount of calcium (0.5%) for 4 weeks reverses the growth retardation and the impaired mineralization in VDR KO mice, as does a high-calcium and high-phosphorus diet (Ca: 2%; P: 1.25%). Thus, the present study suggests that mobilization of calcium and mobilization of phosphorus are differentially regulated through vitamin D-dependent and -independent systems, and that intake of calcium and phosphorus in the proper ratio is important for mineral homeostasis and bone mineralization.

Vitamin D plays a major role in mineral homeostasis by regulating intestinal (1) and renal (2) mineral transport. Bone mineralization is also stimulated by vitamin D through its regulation of serum mineral concentrations and its direct action on osteoblasts. Therefore, impaired actions of vitamin D due to either dietary vitamin D deficiency (3) or hereditary disease (4) results in rickets with hypocalcemia, hypophosphatemia and impaired bone mineralization. Most of the actions of vitamin D are exerted through transcriptional control of target genes by its nuclear receptor, VDR. VDR acts as a 1,25-dihydroxyvitamin D [1,25$(OH)_2$D]-inducible transcription factor, like other members of the nuclear receptor superfamily. To clarify the physiological role of VDR in controlling the actions of vitamin D, we generated mice lacking VDR (VDR KO) by homologous gene targeting (5). VDR KO mice exhibited features similar to those of patients with hereditary vitamin D resistant rickets (HVDRR), who have genetic mutations in the VDR gene. The growth of VDR KO mice is retarded, and these animals display hypocalcemia, hypophosphatemia and severely impaired bone mineralization, but only after weaning. Furthermore, supplementation of the diet with high concentrations of calcium and phosphorus, together with lactose, is reported to significantly reverse the impaired bone mineralization by normalization of the aberrant mineral homeostasis and hyperparathyroidism (6,7).

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The homeostasis of phosphorus as well as calcium is considered to be modulated by vitamin D, and phosphorus restriction is reported to improve calcium utilization (8). However, there is no report as to whether dietary phosphorus restriction improves the rachitic status. In this study we tested whether restriction of phosphorus intake could restore mineral homeostasis and bone mineralization in VDR KO mice. During our investigation of mineral utilization in the VDR KO mice, we found that feeding a diet supplemented with a restricted amount of phosphorus (0.25%) and a normal amount of calcium (0.5%) for 4 weeks resulted in remarkable reversal of impaired bone mineralization, hypophosphatemia, hypocalcemia and hyperparathyroidism, like a diet supplemented with high concentrations of calcium (2.0%) and phosphorus (1.25%) as previously reported. These results suggest that the proper ratio of calcium to phosphorus in the diet consumed is important for mineral mobilization and bone mineralization.

Materials and Methods

Experimental procedures

Wild type and VDRKO mice were bred and maintained on laboratory chow (CE-2 Clea, Japan). Null mutant mice were obtained by intercrossing a heterozygous VDRKO female and a heterozygous male, and the wild-type littermate mice were used for the analyses. After weaning at 3 weeks old, all mice were fed the control diet containing 0.5% calcium and 0.5% phosphorus with 20 % lactose for 1 week. Then, the wild type and VDRKO mice were each further divided into 2 dietary groups of five each, fed either the control diet described above or a low P diet containing half the phosphorus content (0.25%) but the same content

of calcium (0.5%) with 20% lactose. The mice were given free access to the assigned diet during the 4-week feeding period, during which samples of feces were collected from each mouse. The animals were housed individually in stainless-steel cages in a room maintained at 22°C with a 12-h light-dark cycle. The Tokyo University of Agriculture Animal Use Committee approved the study and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of the university. At the end of the feeding period, blood was collected from the inferior vena cava while the animal was anesthetized, and femurs were obtained for further analyses.

Blood and feces analyses

The blood samples were centrifuged at 3000g for 15 min and the supernatants were employed as serum samples. Feces samples were dried, ashed and then demineralized with 1N HCl solution. The calcium was analyzed by atomic absorption spectrometry (Shimazu AA 640-13) according to the method of Gimblet et al (9). Phosphorus was analyzed colorimetrically by the method of Gomori (10). The apparent absorption of calcium and that of phosphorus during the 4-week feeding period were determined as the amount of absorption (mg). The absorption ratio of calcium and that of phosphorus were each calculated as a percentage of the amount of intake. Serum parathyroid hormone (PTH) levels were assayed by means of a radioimmunoassay kit (Immutopics, Inc., San Clemente, CA). This method is a two-site immunoradiometric assay (IRMA) using two different goat antibodies to measure both intact rat PTH and its N-terminal fragments. Mayer et al (11) have reported that detection of mouse PTH with this kit has been validated.

Bone analysis

The femoral bones and connective tissues were carefully removed at the end of the feeding period. The left femur of each mouse was used for analysis of bone mineral levels and breaking force. The bone mineral content (BMC: mg) and bone mineral density (BMD: mg/cm^2) were measured by DXA (DCS-600A, Aloka, Tokyo) adapted for use in the case of small animals. The mineralization profiles of the specimens were stored as the monitoring images, and the BMC and BMD values for the femur were obtained. A three-point bending test was performed as previously described (12) using a three-point bending rheolometer (RX-1600, Aitechno, Japan). The left femur specimen was placed on a holding device with supports located at a distance of 5 mm, with the lesser trochanter proximal to and in contact with the proximal transverse bar. The midpoint served as the anterior (upper) loading point. A bending force was applied by the crosshead at a speed of 10 mm/min until fracture occurred. The breaking force (dynes) and breaking energy of the femoral diaphysis were obtained directly from the load-deformation curves that were recorded continually in the computerized monitor linked to the load tester. The right femur was fixed in 4% PBS-buffered paraformaldehyde for 3 days at 4°C. After decalcification with 10% buffered EDTA, the femur was embedded in paraffin, followed by Masson-trichrome staining before observation.

Statistical analysis

The data are presented as the mean±SE for each group of five mice. The effect of genotype and the effects of diets were analyzed by analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) for multiple comparisons. Scheffe's F-test was used whenever unequal sample sizes were observed. $P<0.05$ was considered significant (13).

Results

The retarded growth of VDR KO mice was restored by the restricted phosphorus diet.

The body weights of all of the mice and the weight gain calculated as the difference between the final weight at 8 weeks and the initial weight at 4 weeks, individually, are shown in Table 1.

Table 1. Growth and mineral metabolism analyses.

	wild type		VDRKO	
	control	low-P	control	low-P
initial weight (g)	13.3±1.2	13.2±1.3	10.5±0.4†	10.3±0.4†
final weight (g)	21.3±1.8	20.1±1.8	17.5±0.2†	19.5±1.6*
weight gain (g)	8.06±1.0	6.8±0.6	6.0±0.6	9.1±0.9*
Ca absorption (mg/4weeks)	127±20	134±11	91± 2	155± 5*
P absorption (mg/4weeks)	178±16	96± 6*	159± 7	108± 2*
Ca absorption (%)	29.8±1.6	30.4±1.2	22.2±0.9	34.8±1.7*
P absorption (%)	42.8±2.7	36.9±1.1*	42.7±1.6	40.9±1.0
serum Ca (mg/dl)	11.0±0.1	11.3±0.1	7.8±0.3†	10.7±0.1*
serum P (mg/dl)	6.8±0.5	7.5±0.3	6.6±0.2	7.9±0.4*
serum PTH(pg/ml)	14±1	16±2	2722±653†	88±37*

The data are presented as the mean±SE for a group of 5 mice. *Significant differences from control group in same genotype (effect of diet) at $P<0.05$. † Significant differences from wild type in same diet (effect of genotype) at $P<0.05$.

The initial weight of the VDR KO mice was the same as that of the wild type mice until weaning (3 weeks old), however, a weight difference between the wild type and VDR KO mice developed during the period when all mice were being fed the control diet (0.5% Ca: 0.5% P) for one week. The final weight of the VDR KO mice was significantly lower than that of the wild type mice fed the control diet (0.5% Ca: 0.5% P) for 4 weeks. The VDR KO mice fed the restricted phosphorus diet (0.5% Ca: 0.25% P) recovered from the retarded growth remarkably.

The restricted phosphorus diet normalized calcium mobilization.

The apparent Ca absorption after feeding the control diet for 4 weeks was decreased in the VDR KO mice as compared with the wild type mice, whereas it showed recovery in VDR KO mice as a result of intake of the restricted phosphorus diet and the degree of improvement

was statistically significant. However, unlike calcium absorption, the apparent phosphorus absorption decreased as a result of intake of the restricted phosphorus diet. As expected from the restored calcium absorption after feeding the restricted phosphorus diet, the serum calcium concentration in the VDR KO mice was elevated to a level nearly the same as that in the wild type mice, whereas in the wild type mice it was not affected by the phosphorus content of the diet. Hyperparathyroidism was seen in the VDR KO mice fed the control diet, however, this was mostly normalized as a result of intake of the restricted phosphorus diet (Table 1).

The impaired bone mineralization in the VDR KO mice was reversed by the restricted phosphorus diet.

There were significant decreases in femoral BMC, BMD and breaking force in the VDR KO mice fed the normal diet for 4 weeks. However, the restricted phosphorus diet significantly reversed the decreases in femoral BMC, BMD and breaking force in the VDR KO mice (Table 2).

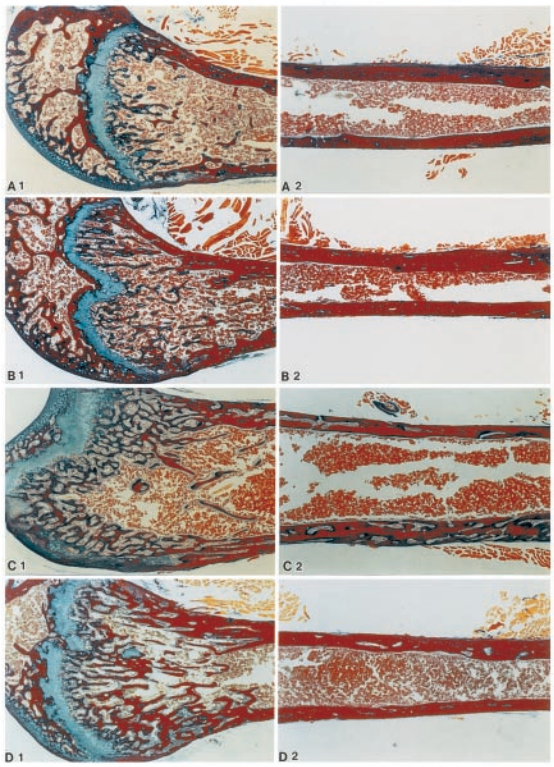

Figure 1. Masson-trichrome staining of the right femur. Osteoid seams were stained light blue. In the femoral metaphysis (1) and mid-shaft (2) of wild type mice fed either the control diet (A1, A2) or the low phosphorus diet (B1, B2) osteoid seams were scarcely observed on the periosteal and endocortical surfaces. In the femur of VDR KO mice fed the control diet (C1, C2), the occurrence of osteoid seams was markedly increased. In the femur of VDR KO mice fed the low phosphorus diet (D1, D2), normalization of osteoid seams was apparent and this observation confirmed that the restricted phosphorus diet reverses the impaired bone mineralization.

Table 2. BMC, BMD and breaking forces in femur

	wild type		VDRKO	
	control	low-P	control	low-P
BMC (mg)	13.7±0.8	11.9±1.1	8.7±0.5†	10.5±0.6
BMD (mg/cm^2)	34.8±0.4	33.9±1.2	30.5±0.6†	32.9±1.5
Breaking forces (10^6dyn)	2.0±0.1	2.1±0.1	1.3±0.2†	2.0±0.1*

The data are presented as the mean±SE for a group of 5 mice. *Significant differences from control group in same genotype (effect of diet) at $P<0.05$. † Significant differences from wild type in same diet (effect of genotype) at $P<0.05$.

Upon examination of the femur of mice fed the restricted phosphorus diet after Masson-trichrome staining, a decrease in occurrence of osteoid seams, which showed a marked increase in the case of VDR KO mice fed the control diet, was evident and this observation confirmed that the restricted phosphorus diet reverses the impaired bone mineralization. (Figure 1).

Discussion

The impaired bone mineralization, hypophosphatemia, hypocalcemia and hyperparathyroidism in the VDR KO mice were markedly improved as a result of feeding the mice a restricted phosphorus diet for 4 weeks. Interestingly, these effects of a restricted phosphorus diet closely resembled those of a diet supplemented with high levels of minerals (Ca: 2%; P: 1.25%) (R.M. et al., unpublished results), as previously reported (6,7). The degree of improvement of bone breaking force by the restricted phosphorus diet was statistically significant, and the impaired BMC and BMD were evidently improved, but the difference was not statistically significant. The appearance of the femur in the case of the VDR KO mice fed the restricted phosphorus diet showed that the impaired mineralization was reversed as normalized osteoid seams were evident, and the bone appeared normal like that in the case of their wild-type littermates. Thus, the mobilization of calcium from the diet appears to be dependent on the proper ratio of phosphorus to calcium (Ca:P=2:1) rather than being dependent simply on the concentration of calcium in the diet.

Serum PTH levels are considered to be elevated in the VDR KO mice, due to hypocalcemia and release from suppression of PTH secretion by vitamin D in the parathyroid gland. The impaired bone mineralization that occurs in the case of rickets is thus considered to be due, at least in part, to hyperparathyroidism. Therefore, patients with rickets are given diets supplemented with a high concentration of calcium to reduce the elevated serum PTH levels (14) and experimentally the hyperparathyroidism in VDR KO mice has been shown to be

reversed by feeding a high-calcium diet (Ca: 2%; P: 1.25%) (6,7). However, the present study clearly indicates that, under certain conditions, restricted phosphorus intake is effective to improve hyperparathyroidism, the same as supplementation of the diet with a high concentration of calcium. These findings are consistent with previous reports indicating that restricted phosphorus intake is beneficial for normalization of hypocalcemia in patients with hyperparathyroidism (15,16). Thus, it is likely that the elevated serum PTH levels are normalized by either diets with a restricted phosphorus content and a normal calcium content or diets with a high calcium content and a high phosphorus content. The dietary phosphorus restriction is not a suitable for the treatment of the disease since it is virtually impossible to lower phosphorus intake in a patient's diet. However, these apparently inconsistent results imply that a proper ratio of calcium to phosphorus in the diet, presumably reflecting serum levels of mobilized minerals, is more important than high calcium intake alone for mineral homeostasis and bone mineralization.

Serum calcium levels in the VDR KO mice were elevated as a result of intake of the restricted phosphorus diet, and this may be due to increased absorption of solubilized calcium ions derived from calcium phosphate salt in the intestine. These findings indicate that a vitamin D-independent system is physiologically important for intestinal absorption of calcium, as well as the vitamin D-dependent actions in regulating the intestinal absorption and renal excretion of calcium. On the other hand, serum phosphorus levels were not substantially affected in the absence of the action of vitamin D, despite feeding the restricted phosphorus diet. It is therefore likely that vitamin D is an indispensable factor for phosphorus mobilization. In conclusion, our findings indicate that calcium mobilization and phosphorus mobilization are differentially regulated by vitamin D -dependent and -independent systems, and the proper ratio of calcium to phosphorus in the diet consumed is important for mineral mobilization and bone mineralization.

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