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Response of jejunal phosphate absorption to 1,25-dihydroxyvitamin D_3 stimulation *in vivo* in young X-linked hypophosphatemic (*Hyp*) mice

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Young *Hyp* mice malabsorb phosphate from the jejunum at 4 weeks of age. This has been attributed to both low plasma levels of 1,25-dihydroxyvitamin D and to intestinal resistance to stimulation by 1,25-dihydroxyvitamin D. To differentiate between these two hypotheses, 4 week old normal and *Hyp* mice were treated with 0, 17, 50, or 150 ng/kg/day of 1,25-dihydroxyvitamin D_3 by Alzet osmotic mini pumps ($n = 10-12$/group). After 4 days, the jejunum was isolated by sutures and 0.5 ml 2 mM Na_2HPO_4 in 150 mM NaCl with 1.0 μCi $^{32}PO_4$ was injected into the lumen. After 8 min, plasma, jejunal tissue and lumenal contents were measured for ^{32}P content. Absorption was measured as counts removed from the lumen. Both normal and *Hyp* mice responded to the 1,25-dihydroxyvitamin D_3 with increased absorption, increased tissue ^{32}P and increased plasma ^{32}P. *Hyp* mice responded less than normal mice to the 50 ng/kg/day dose in plasma ^{32}P levels (significant dose by genotype interaction, $P < 0.05$). Plasma was pooled by genotype and dose for the measurement of plasma 1,25-dihydroxyvitamin D. This yielded 13 samples (7 normal and 6 *Hyp*). Absorption of ^{32}P ($r = 0.75$, $p = 0.002$) and jejunal tissue content of ^{32}P ($r = 0.66$, $p = 0.02$) were correlated to plasma 1,25-dihydroxyvitamin D. Analysis of covariance revealed a significant difference in phosphate absorption between normal and *Hyp* mice ($p = 0.02$). In conclusion, there is a partial resistance of intestinal phosphate absorption to 1,25-dihydroxyvitamin D stimulation.

Keywords: mice; Hyp; phosphate; jejunum; 1,25-dihydroxy-vitamin D_3; malabsorption

Introduction

X-linked hypophosphatemia is the most common cause of metabolic bone disease in children (Rasmussen & Tenenhouse, 1989). Similar mutations also occur in mice. In mice, two closely-linked loci on the X chromosome, *Hyp* (Eicher *et al.*, 1976) and *Gy* (Lyon *et al.*, 1986), cause low plasma phosphate, impaired renal tubular reabsorption of phosphate, and rachitic and osteomalacic bone disease which resemble the human disease.

The etiology of the bone disease in X-linked hypophosphatemia is not understood. It has been variously ascribed to effects of the low plasma phosphate (Rasmussen & Tenenhouse, 1989), to intestinal malabsorption of calcium and phosphate (Meyer *et al.*, 1987; Brault *et al.*, 1988), and to abnormal bone cell metabolism (Ecarot-Charrier *et al.*, 1988). Our laboratory has noticed a close correlation between the exacerbation of the rachitic bone disease in growing, young *Hyp* mice and the occurrence of the intestinal malabsorption of calcium and phosphate (Kay *et al.*, 1985).

The cause of the intestinal malabsorption of calcium and phosphate is controversial. One hypothesis implicates the low levels of plasma 1,25-dihydroxyvitamin D which occur in young *Hyp* mice (Meyer *et al.*, 1987). Alternatively, the reduced binding of 1,25-dihydroxy-vitamin D to intestinal receptors may make the intestine resistant to stimulation (Yamamoto *et al.*, 1985; Yamamoto *et al.*, 1988). The reduction in duodenal calbindin D_{9K} and absorption of ^{45}Ca from an oral test meal seem to be caused by low plasma 1,25-dihydroxyvitamin D with no signs of intestinal resistance (Meyer *et al.*, 1987). However, the absorption of ^{32}P-phosphate from an oral test meal was partially resistant to 1,25-dihydroxyvitamin D action (Meyer *et al.*, 1987). To test whether phosphate absorption in the intestine is indeed resistant to stimulation, normal and *Hyp* mice were treated with 1,25-dihydroxy-vitamin D_3. Phosphate absorption was then measured in isolated intestinal segments *in vivo* as a more specific assay for intestinal transport of phosphate.

Results

Plasma inorganic phosphate

The *Hyp* mice were hypophosphatemic at each dose level studied (Figure 1). Only the highest dose significantly increased the plasma phosphate level of the *Hyp* mice.

Jejunal ^{32}P absorption and tissue ^{32}P

Absorption of ^{32}P from the jejunum follows the typical curve for first order kinetics as shown in Figure 2. Eight minutes was chosen as the time which would give approximately 50% absorption in normal mice. This would allow us to detect both depressed absorption in *Hyp* mice as well as stimulated absorption following 1,25-dihydroxyvitamin D_3 treatment.

The jejunal absorption of ^{32}P (Figure 3A) and the jejunal tissue content of ^{32}P (Figure 3B) were lower in the *Hyp* mice than the normal mice as shown by the significant main effects of genotype (Table 1) for both

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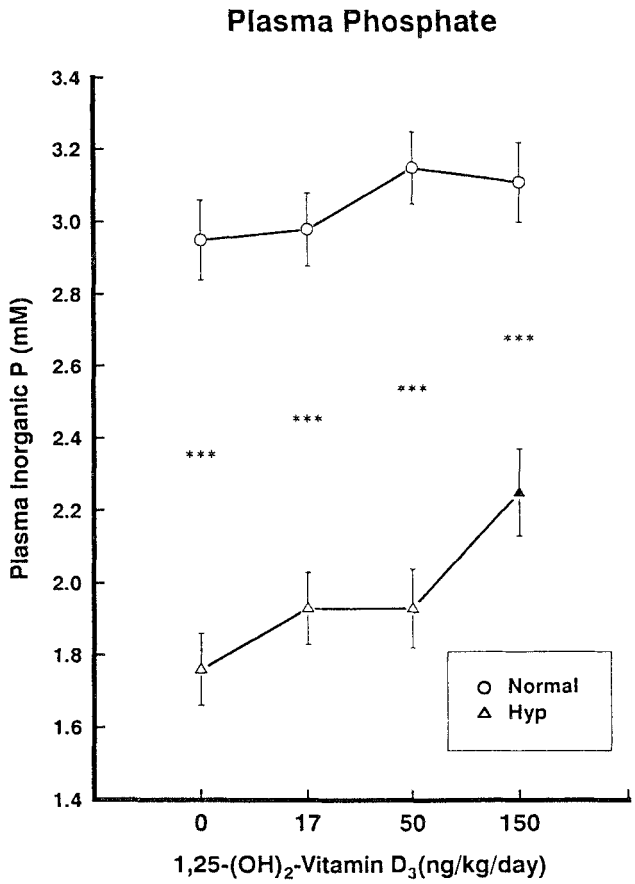

Figure 1 Dose response curves for plasma inorganic phosphate. The asterisks denote significant differences between normal and *Hyp* mice at the same dose level (*, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$). Filled symbols denote means significantly different from the 0 dose group of the same genotype. Data are mean ± SE with $n = 10-12$/group. Samples were collected after 4 days of continuous infusion with 1,25-dihydroxyvitamin D_3 by osmotic mini pump at the indicated doses

variables. Both variables were increased by increasing doses of 1,25-dihydroxyvitamin D. However, the response of both to 50 ng of 1,25-dihydroxyvitamin D_3 was significant for the normal mice, but nonsignificant for the *Hyp* mice. However, this did not produce a significant interaction between dose and genotype in the factorial analysis of variance (Table 1).

Plasma ^{32}P

Plasma ^{32}P was significantly lower in *Hyp* mice than in normals at the lower dose levels (Figure 3C). In both *Hyp* and normal mice, the highest dose caused a significant increase in plasma ^{32}P. However, at the

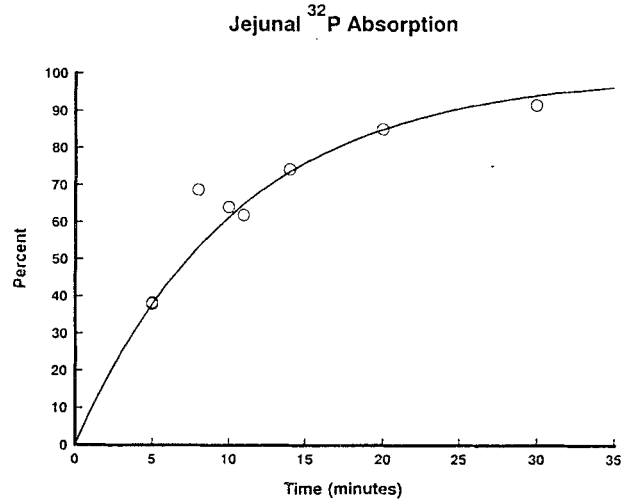

Figure 2 Absorption of ^{32}P from isolated jejunal segments *in vivo* at various times after isotope administration to genetically normal mice. The curved line ($Y = 100\ (1\text{-}e^{-0.095X})$) was empirically fitted to the data

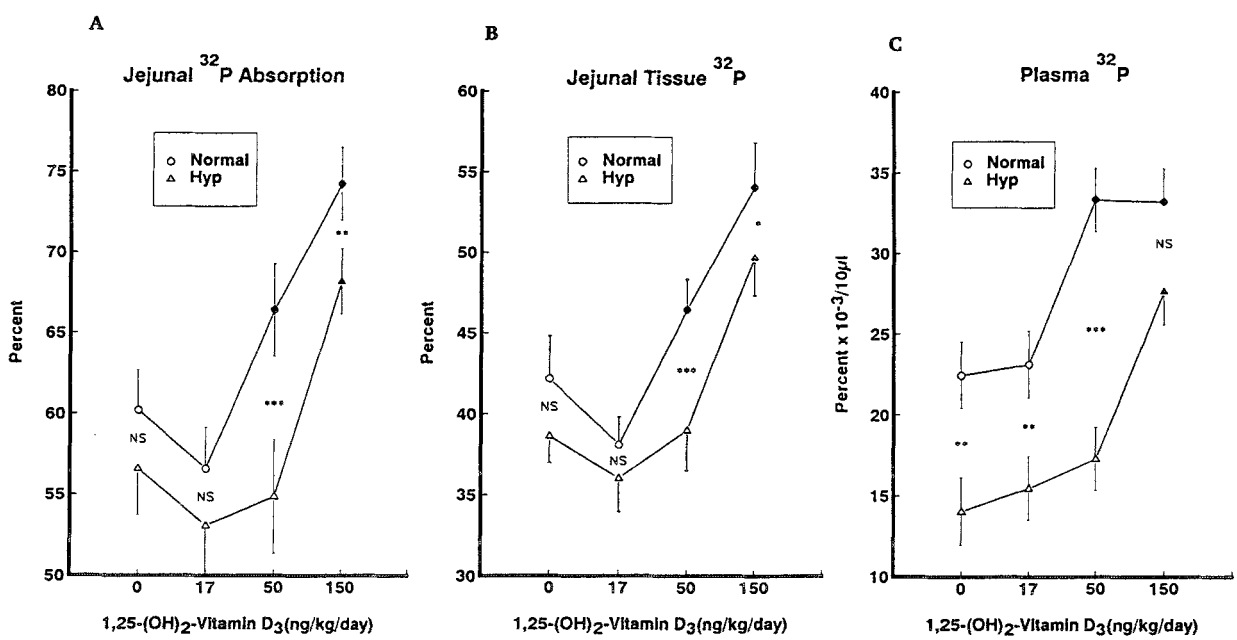

Figure 3 (A) Dose response curves for the absorption of ^{32}P from the isolated jejunum *in vivo*. Absorption was measured 8 min after ^{32}P was injected into the jejunal segments. The data are the percent of the administered ^{32}P which was absorbed from the lumen of the segment and are presented as in Figure 1. (B) Dose response curves for the percent of the administered ^{32}P found in the jejunal tissue. (C) Dose response curves for the percent of the administered ^{32}P found in the plasma ($\times 10^{-3}$) per 10 µl

50 ng dose, the normal mice responded significantly, but the *Hyp* mice did not. This greater response by the normal mice produced a significant interaction between dose and genotype (Table 1, $p = 0.03$).

Plasma 1,25-dihydroxyvitamin D

The plasma samples remaining from these mice were pooled between mice of the same dose, sex and genotype to create pools which were used for the analysis of 1,25-dihydroxyvitamin D. The data are shown in Figure 4. The higher doses of 1,25-dihydroxyvitamin D resulted in higher plasma levels of 1,25-dihydroxyvitamin D (Table 1, significant main effect of dose, $p = 0.01$). There was no significant difference between normal and *Hyp* mice in their response to administered 1,25-dihydroxyvitamin D (Table 1, non-significant effect of genotype). However, as we have noted before (Meyer *et al.*, 1987), female mice treated with 1,25-dihydroxyvitamin D_3 had a smaller rise in plasma 1,25-dihydroxyvitamin D than similarly treated male mice (significant effect of sex as a covariate, $p = 0.025$).

Plasma 25-hydroxyvitamin D

Plasma 25-hydroxyvitamin D was also measured in the plasma pools (Figure 4). As we have noted before (Meyer *et al.*, 1980), *Hyp* mice have lower 25-hydroxyvitamin D levels than do normals (Table 1, main effect of genotype, $p = 0.004$). Administration of 1,25-dihydroxyvitamin D_3 resulted in lower plasma levels of 25-hydroxyvitamin D (Table 1, main effect of dose, $p = 0.01$).

Jejunal ^{32}P absorption vs *plasma 1,25-dihydroxyvitamin D*

The jejunal ^{32}P absorption rates were averaged among the mice that went into each pool. These averaged values were then plotted against the measured levels of plasma 1,25-dihydroxyvitamin D, and the results are shown in Figure 5A. There was a significant correlation between plasma 1,25-dihydroxyvitamin D and the percent of ^{32}P absorbed from the jejunum ($r = 0.748$; $p = 0.002$). When these data were analysed by analysis of covariance, there was a significant difference between normal and *Hyp* mice at $p = 0.006$. This indicated that the *Hyp* mice were responding somewhat less than the normal mice to equivalent plasma levels of 1,25-dihydroxyvitamin D.

Table 1 Analysis of variance for the effect of 1,25-dihydroxyvitamin D on jejunal phosphate absorption

Main effects				*Interaction*	
Dose		*Genotype*		*Dose × Genotype*	
F[a]	*P*	*F*	*P*	*F*	*P*
Plasma Inorganic Phosphate					
2.89	0.04	230.98	<0.0001	0.92	NS
Absorption of ^{32}P					
21.51	<0.0001	20.03	<0.0001	1.68	NS
Jejunal Tissue ^{32}P					
29.64	<0.0001	15.52	0.0002	1.01	NS
Plasma ^{32}P					
14.37	<0.0001	40.43	<0.0001	3.23	0.03
Plasma 1,25-Dihydroxyvitamin D					
14.26	0.01	1.13	NS	1.92	NS
Plasma 25-Hydroxyvitamin D					
17.15	0.01	37.86	0.004	7.85	0.04

[a]F statistics and their associated probability values (P) are shown for each factorial analysis of variance. The first four analyses of variance were run with a complete block design and with jejunal length as a covariate (both block and covariate were not significant for plasma inorganic phosphate). The two main effects were the dose of 1,25-dihydroxyvitamin D (0, 17, 50 or 150 ng/kg/day) and genotype (normal or *Hyp*). The last two analyses were run without blocks and with sex as the covariate. Sex of the mouse was a significant covariate for plasma 1,25-dihydroxyvitamin D ($p = 0.025$)

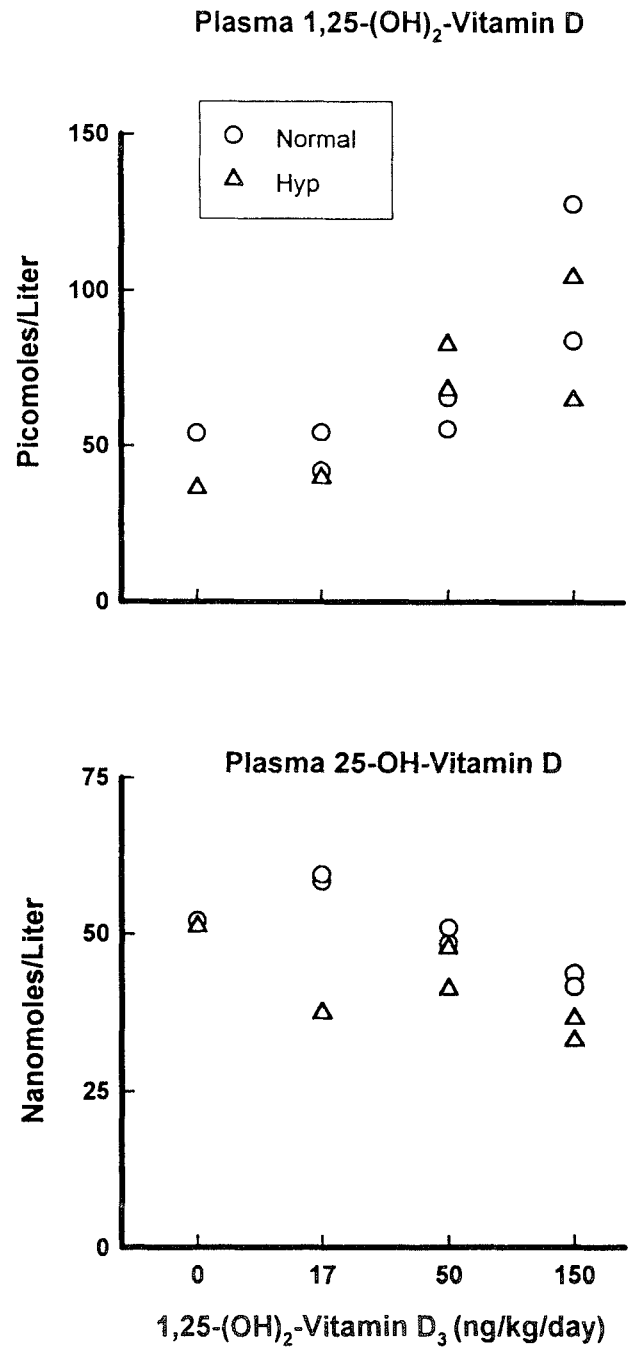

Figure 4 Plasma 1,25-dihydroxyvitamin D (top) and 25-hydroxyvitamin D (bottom) levels after 4 days of continuous infusion by osmotic mini pumps with 1,25-dihydroxyvitamin D_3. Each point is the plasma level of a pool of 4 mice of the indicated dose and genotype

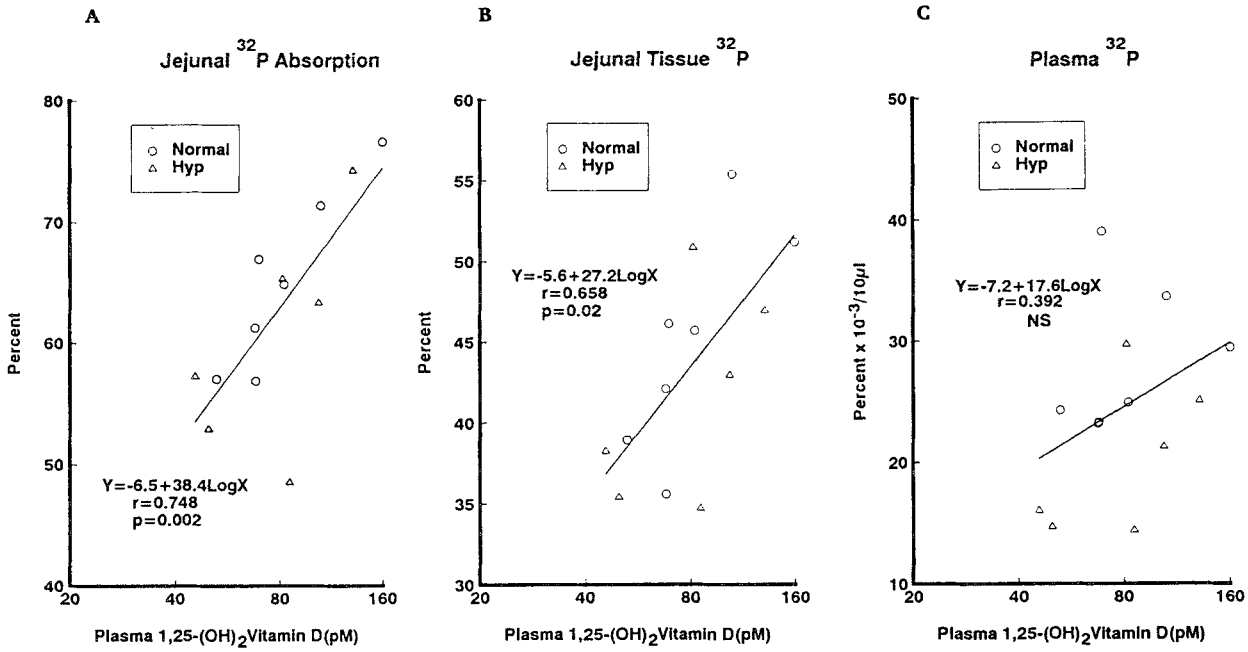

Figure 5 (A) Plasma 1,25-dihydroxyvitamin D *vs* the absorption of ^{32}P. The ^{32}P absorption data for the mice in each plasma pool shown in Figure 4 were averaged and plotted against the pool level of 1,25-dihydroxyvitamin D. Each point is the plot of one pool. The indicated regression line is for all points. Analysis of covariance revealed a significant difference between normal and *Hyp* mice at $p = 0.006$. (**B**) Plasma 1,25-dihydroxyvitamin D *vs* jejunal tissue ^{32}P. The jejunal tissue data were similarly averaged and plotted against the pool level of 1,25-dihydroxyvitamin D. Analysis of covariance showed no significant difference between normal and *Hyp* mice. (**C**) Plasma 1,25-dihydroxyvitamin D *vs* plasma inorganic ^{32}P. The plasma inorganic ^{32}P data were also averaged and plotted against the pool level of 1,25-dihydroxyvitamin D. Analysis of covariance showed no significant difference between normal and *Hyp* mice

Jejunal tissue ^{32}P vs *plasma 1,25-dihydroxyvitamin D*

The data for the jejunal tissue ^{32}P were treated similarly to the absorption data. The regression of tissue ^{32}P against the measured plasma 1,25-dihydroxyvitamin D levels is shown in Figure 5B. There was a significant correlation between the two variables ($r = 0.658$; $p = 0.02$). Analysis of covariance showed no significant difference between normal and *Hyp* mice for this variable.

Plasma ^{32}P vs *plasma 1,25-dihydroxyvitamin D*

The plasma ^{32}P data was also plotted against plasma 1,25-dihydroxyvitamin D (Figure 5C). There was no significant correlation between the two variables ($r = 0.392$,NS). Analysis of covariance also failed to find a significant difference between normal and *Hyp* mice in this variable.

Discussion

Young 4-week old *Hyp* mice have low intestinal absorption of calcium (Meyer *et al.*, 1986) and phosphate (Brault *et al.*, 1988) and low levels of intestinal calbindin-D_{9K} (Bruns *et al.*, 1984). Young 4-week old *Hyp* mice also have significantly lower levels of plasma 1,25-dihydroxyvitamin D (Meyer *et al.*, 1987). This reduced plasma level of 1,25-dihydroxyvitamin D was sufficient to explain the malabsorption of calcium and the low values of duodenal calbindin-D_{9K} found in the 4-week old *Hyp* mice. However, the phosphate absorption data were not as clear cut. Phosphate absorption from an oral test meal was increased by raising plasma 1,25-dihydroxyvitamin D levels. Nevertheless, low plasma 1,25-dihydroxyvitamin D in the untreated *Hyp* mice would only account for 72% of the malabsorption of phosphate in these young *Hyp* mice (Meyer *et al.*, 1987). There remained a significant portion of the malabsorption of phosphate which could not be explained by the low 1,25-dihydroxyvitamin D levels.

The partial resistance of intestinal phosphate absorption to 1,25-dihydroxyvitamin D (Meyer *et al.*, 1987) was puzzling since such resistance was not seen in the calbindin-D_{9K} data nor in the intestinal calcium absorption data. However, these latter data were from the duodenum while much of the phosphate absorption occurs in the jejunum (Walling, 1977). These problems led us to the present experiment to try to clarify this issue. We employed the isolated jejunal segment as a more specific measure of intestinal phosphate absorption.

In the present experiment, the *Hyp* mice showed significantly lower intestinal phosphate absorption than did the normal mice. Both *Hyp* and normal mice responded to administered 1,25-dihydroxyvitamin D_3 with increased phosphate absorption as judged by removal of ^{32}P from the intestinal segments, increased tissue ^{32}P content, and increased plasma ^{32}P content. When these indices of phosphate absorption were related to circulating plasma 1,25-dihydroxyvitamin D levels, there was significant correlation of plasma 1,25-dihydroxyvitamin D to intestinal phosphate absorption and to tissue phosphate content. There was evidence for intes-

tinal resistance to stimulation by 1,25-dihydroxyvitamin D_3: (1) there was the reduced response of plasma ^{32}P to the 50 ng/kg/day dose of 1,25-dihydroxyvitamin D_3; (2) when the jejunal phosphate absorption was subjected to analysis of covariance, there was a significant difference between normal and *Hyp* mice; and (3) *Hyp* mice failed to respond to the 50 ng/kg dose with increased ^{32}P absorption and increased tissue ^{32}P. Thus, we conclude that the jejunal absorption of phosphate is partially resistant to stimulation by 1,25-dihydroxyvitamin D.

A possible basis for this resistance of the *Hyp* intestine to stimulation by 1,25-dihydroxyvitamin D has been explored. There is decreased binding of 1,25-dihydroxyvitamin D to its receptor in the intestine of the *Hyp* mice (Yamamoto *et al.*, 1985). This is caused by the low plasma phosphate (Yamamoto *et al.*, 1988). Normalization of the plasma phosphate in these young *Hyp* mice led to normalization of the nuclear binding of 1,25-dihydroxyvitamin D_3 (Yamamoto *et al.*, 1988). We speculate that there may be a physiological correlation for this effect. Only the highest dose (150 ng/kg/day) both elevated plasma phosphate in the *Hyp* mice and also caused a significant increase in intestinal phosphate absorption.

This period of intestinal malabsorption of calcium and phosphate between 4 and 7 weeks of age correlates with a period of an exacerbation of the bone disease (Meyer *et al.*, 1984; Kay *et al.*, 1985). Thus, it is our hypothesis that intestinal malabsorption of calcium and phosphate in young, rapidly growing *Hyp* mice is a significant factor exacerbating the bone disease in these animals. Young, nursing *Hyp* mice prior to this age range, while rachitic, are capable of making well-mineralized bone (Mostafa & Meyer, 1981; Kay *et al.*, 1985). Also, adult *Hyp* mice, when they have ovarian steroids available to them, will remineralize their skeleton despite continued hypophosphatemia (Brault *et al.*, 1987; Soener *et al.*, 1988).

In summary, phosphate absorption by the jejunum of *Hyp* mice is partially resistant to stimulation by 1,25-dihydroxyvitamin D. This may be a physiological consequence of impaired binding of 1,25-dihydroxyvitamin D to its nuclear receptors. This intestinal malabsorption of calcium and phosphate may contribute to the severity of the hypophosphatemic bone disease.

Materials and methods

Mice

All mice used in this investigation were bred in our laboratory. Normal C57BL/6J male mice (+/Y) were bred to heterozygous *Hyp* female mice (*Hyp*/+). This gave rise to litters containing half normal and half hemizygous *Hyp* male pups (*Hyp*/Y). In addition, half of the female pups were normal (+/+), and half were heterozygous *Hyp*. Littermate normal mice were used as controls for the *Hyp* mice in this study. The breeders were fed Wayne Breeder Blox (Allied Mills, Inc., Chicago, IL) and tap water *ad lib*. The pups were weaned at 3 weeks of age and were switched to a diet of Wayne Rodent Blox (1.2% Ca, 1.0% P, 0.2% Mg, and 4.5 IU vitamin D/g diet). *Hyp* mice were differentiated from normal mice by their characteristic low plasma phosphate, low body weight and short tails (Meyer *et al.*, 1979).

1,25-Dihydroxyvitamin D_3 Treatment

1,25-Dihydroxyvitamin D_3 was administered to 4 week old normal and *Hyp* mice by Alzet osmotic mini pumps (model 2001, Alza Corporation, Palo Alto, CA) implanted subcutaneously along the back. The pumps contained a vehicle of 1,2 propanediol with 1,25-dihydroxyvitamin D_3 (generously donated by Dr Uskokovic, Hoffman-La Roche, Inc., Nutley, NJ) dissolved at a concentration of 0, 8, 25 or 75 pg/μl vehicle. The pumps delivered a dose of 1 μl/hr. At this age, the normal mice (13.7 ± 0.3 g) were similar in body weight to the *Hyp* mice (11.8 ± 0.2), so that the mice received a similar dose per g body weight. For each experimental day, one mouse was done from each of the eight treatment groups.

Surgery

Four days after the initiation of 1,25-dihydroxyvitamin D_3 therapy, each mouse was anesthetized with sodium pentobarbital. A mid-ventral incision was made, and the jejunum was exposed. Sutures were placed around the intestine 3 cm distal to the pylorus, and another suture was placed 8 cm proximal to the ileocecal valve. Then 0.5 ml of 2.0 mM Na_2HPO_4 in 150 mM NaCl was injected into the lumen with 1 μCi $Na_2H^{32}PO_4$ at pH = 7.2. After 8 min, the mice were exsanguinated from the carotid artery into heparinized microfuge tubes (Meyer *et al.*, 1980) and the jejunal segments were collected and averaged 12.3 ± 0.2 cm in length. The lengths of the duodenum and ileum were recorded and did not differ significantly between the various treatment groups.

Assays

Plasma inorganic phosphate was measured by the method of Chen *et al.* (Chen *et al.*, 1956). Jejunal segments were rinsed and ashed (Meyer & Meyer, 1976). The ash was dissolved in 1 ml 1 N HCl, and a portion was counted for ^{32}P content. The ^{32}P contents of the plasma and the lumenal fluid were also measured. Absorption was calculated as the amount of ^{32}P administered minus the amount of ^{32}P present in the rinse from the lumen of the segment, expressed as a percent.

Plasma was pooled among animals of the same sex, genotype and dose group to provide pools of 1.0 ml each. Plasma 25-hydroxyvitamin D (Hollis, 1984) and 1,25-dihydroxyvitamin D (Reinhardt *et al.*, 1984) were measured in each pool.

Statistics

Dose response curves were subjected to factorial analysis of variance with a multiple comparison test for individual treatment group means. A complete block design was used with each day's results as a block. This was computed with the aid of the GLM procedure from Release 5.16 of the SAS package of statistical programs (SAS Inc, Cary, NC). In addition, the data from the mice in each pool used for 1,25-dihydroxyvitamin D analysis were averaged, and the averages were tested for significant correlation to the plasma 1,25-dihydroxyvitamin D level by Release 3.1 of the SPSSX package of statistical programs (SPSSX, Inc., Chicago, IL). All data are reported as mean ± SEM for 10–12 mice per treatment group.

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