# Abnormal Regulation of Renal 25-Hydroxyvitamin D-1$\alpha$-Hydroxylase Activity in the X-Linked Hypophosphatemic Mouse

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ABSTRACT Abnormal vitamin D metabolism has been suspected in patients with X-linked hypophosphatemic rickets (XLH) and X-linked hypophosphatemic mice (*Hyp*-mice), the murine homologue of the human disease. We compared 25(OH)D-1$\alpha$-hydroxylase activity in the *Hyp*-mouse kidney to that in normal and phosphate-depleted mouse kidney. Weanling normal and *Hyp*-mice were fed a 0.6% phosphorus diet; phosphate-depleted mice received a 0.02% phosphorus diet. At 8–10 wk of age the serum phosphorus values in *Hyp* (3.35±0.12 mg/dl) and phosphate-depleted mice (3.83±0.56) were not significantly different. Despite the similar magnitude of phosphate depletion, however, the maximum levels of 25(OH)D-1$\alpha$-hydroxylase activity were disparate: phosphate-depleted mouse kidney had profoundly increased activity compared to normal (17.04±.104 vs. 4.96±0.23 fmol $1,25(OH)_2D_3$ produced/mg kidney per min) while *Hyp*-mouse kidney had a fourfold lesser increment (8.18±0.62). These data indicate that phosphate depletion is a potent stimulus of 25(OH)D-1$\alpha$-hydroxylase activity in the (C57BL6J) mouse. Moreover, the results show that abnormal regulation of 25(OH)D-1$\alpha$-hydroxylase activity is manifest in the *Hyp*-mouse.

An abstract of this work appeared in 1982. *Clin. Res.* 30: 399A.

Dr. Drezner is a recipient of a Research Career Development Award from the National Institute of Arthritis, Metabolic, Digestive Diseases, and Kidney (AM-00643). Address reprint requests to Dr. Drezner.

*Received for publication 17 August 1982 and in revised form 20 October 1982.*

## INTRODUCTION

Patients with X-linked hypophosphatemic rickets/osteomalacia (XLH)[1] and X-linked hypophosphatemic mice (*Hyp*-mice), the murine homologue of the human disease, have a normal or moderately decreased serum 1,25-dihydroxyvitamin D ($1,25[OH]_2D$) concentration despite manifesting hypophosphatemia and phosphate depletion, which allegedly stimulate $1,25(OH)_2D$ production (1–5). The inappropriately low circulating $1,25(OH)_2D$ levels could result from a primary or acquired defect in the renal 25(OH)D-1$\alpha$-hydroxylase enzyme system, accelerated degradation of $1,25(OH)_2D$ or increased uptake of $1,25(OH)_2D$ by target tissues. No evidence is currently available that supports or refutes either of these possibilities.

In the present study we examined whether phosphate deficiency enhances 25(OH)D-1$\alpha$-hydroxylase activity in normal mouse kidney and if, in the *Hyp*-mouse kidney, abnormal regulation of enzyme activity is apparent. We measured 25(OH)D-1$\alpha$-hydroxylase activity in normal and phosphate-depleted mouse kidney and compared these values to that in *Hyp*-mouse kidney. In addition, we correlated the results with serum phosphorus and $1,25(OH)_2D$ levels in these animal models.

[1] *Abbreviations used in this paper*: *Hyp*, hypophosphatemic; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; $V_{max}$, maximal velocity; XLH, X-linked hypophosphatemic rickets/osteomalacia.

## METHODS

Normal C57BL6J mice were mated with C57BL6J *Hyp*-mouse heterozygous females as previously described (6). An equal number of normal male and female littermates were weaned at 4 wk of age and randomly divided into two groups fed a normal (0.6% calcium; 0.6% phosphorus) or matched phosphorus-deficient (0.6% calcium; 0.02% phosphorus) diet. Male and female *Hyp*-mice that were identified and selected for study (6) were likewise weaned at 4 wk and received a normal diet.

In initial studies we assessed optimal assay condition for measurement of 25(OH)D-1$\alpha$-hydroxylase activity (see Results). Subsequently, we performed studies in the normal, P-depleted and *Hyp*-mice at 8–10 wk of age. In each of seven experiments we collected blood in three to four mice from each group for measurement of calcium, phosphorus, and 25(OH)D by previously described techniques (1). Subsequently, we dispatched the animals and removed the kidneys for measurement of 25(OH)D-1$\alpha$-hydroxylase activity. Aliquots of the kidneys were distributed in three to four flasks per group for assay in each experiment. In 10–14 additional animals from each group we obtained sufficient blood to assay plasma 1,25$(OH)_2$D in three pooled samples (1).

We measured 25(OH)D-1$\alpha$-hydroxylase activity by a modification of methods reported by Tanaka and DeLuca (7). This assay uses (*a*) sufficient nonradioactive 25$(OH)D_3$ to saturate the inhibitory factor present in mammalian (murine) renal homogenates while maintaining an optimum concentration for 1$\alpha$-hydroxylation; (*b*) a simple sample purification scheme consisting of chloroform/methanol [1:2] extraction of kidney homogenate, Sephadex LH-20 chromatography of lipid extract, and straight-phase high-pressure liquid chromatography (hexane/isopropanol [90:10]) of purified lipid extract, on a microporasil column, to achieve resolution of the 1,25$(OH)_2D_3$ produced in vitro; and (*c*) a competitive binding protein assay for quantifying the 1,25$(OH)_2D_3$. The sensitivity of the assay, when corrected for fractionation and recovery (50–60%) allows measurement of <0.5 fmol 1,25$(OH)_2$D produced/mg kidney tissue per min, a 10-fold enhancement over that detected by any previously reported method. The intra- and interassay coefficients of variation of the assay average 6.2 and 11.0%, respectively.

*Materials.* We obtained normal and phosphorus-deficient diets from the Teklad Co., Madison, WI. J. Alan Campbell, Upjohn Pharmaceuticals, Kalamazoo, MI, provided authentic 25$(OH)D_3$ and Dr. Milan K. Uskokovic, Hoffman-La Roche, Nutley, NJ, authentic 1,25$(OH)_2D_3$ for our studies. We purchased [$^3$H]1,25$(OH)_2D_3$ (92 Ci/mmol) from Amersham Searle, Arlington Heights, IL.

*Statistical methods.* We performed statistical analysis of the data using one-way analysis of variance and appropriate a posteriori multiple range tests (8).

## RESULTS

*Biochemistries.* At the time of study P-depleted and *Hyp*-mice had serum phosphorus concentrations of 3.83±0.56 and 3.35±0.12 mg/dl, values significantly less ($P < 0.001$) than that of normals (7.15±1.00 mg/dl), but not statistically different from each other. In contrast, the *Hyp*-mice had a serum calcium level (8.3±0.10 mg/dl) that was significantly less ($P < 0.001$) than that of both normals (9.5±0.19 mg/dl) and P-depleted mice (11.1±0.13 mg/dl). Moreover, the value

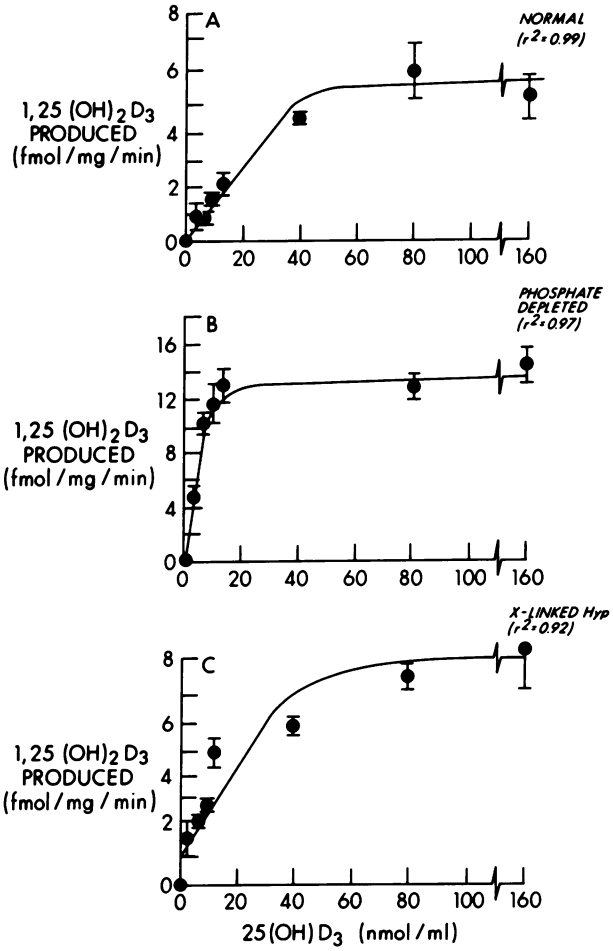


FIGURE 1 Dependency of in vitro production of 1,25$(OH)_2D_3$ on concentration of 25$(OH)D_3$ provided as substrate in A normal, B phosphate-depleted, and C *Hyp* mouse kidney homogenates. Each point shown represents the mean±SE of three individual determinations. All flasks were incubated for 20 min at 37°C.

of the P-depleted mice was significantly greater ($P < 0.001$) than that of normals.

*Vitamin D metabolites.* The P-depleted mice had a serum 1,25$(OH)_2$D level (72.2±7.0) that was significantly greater ($P < 0.01$) than that of normals (39.5±3.9 pg/ml) while the 1,25$(OH)_2$D concentration of the *Hyp*-mice (45.0±4.1 pg/ml) was not statistically different from normal. The discordant values were not due to differences in vitamin D stores since serum 25(OH)D levels were similar in normal, P-depleted and *Hyp*-mice (20.8±2.9; 22.6±1.6; 24.4±1.2 ng/ml, respectively).

*25(OH)D-1$\alpha$-Hydroxylase activity in mouse kidney.* To determine optimal assay conditions for these studies we assessed the effects of substrate concentration (0-160 nmol/ml) and incubation time on the

amount of $1,25(OH)_2D_3$ produced by normal, P-depleted and *Hyp*-mouse renal homogenates. A measurable conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ occurred at the lowest concentration of substrate employed (4 nmol/ml) in each case and increased in a linear fashion until a plateau was reached (Fig. 1). In each group, the plateau, corresponding to the maximal velocity ($V_{max}$) of enzyme activity, was manifest at 80 nmol/ml, the concentration of substrate that we chose for subsequent experiments.

In time course studies we observed that the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ by normal, P-depleted and *Hyp*-mouse renal homogenates is linear from 0 to 30 min (Fig. 2). Thereafter, no increase in enzyme activity is apparent. Consequently, we used a 20-min incubation in further studies to permit measurement of the initial rate of enzyme activity.

We then compared 25(OH)D-1$\alpha$-hydroxylase activity in the kidneys of normal, P-depleted and *Hyp*-mice. In seven experiments renal homogenates from P-depleted mice repeatedly had profoundly increased (259%) enzyme activity compared to that of normals (Fig. 3). In contrast, enzyme activity in the *Hyp*-mouse kidney was far below that in the kidneys of P-depleted mice, although significantly increased (65%) above that in normal kidneys (Fig. 3).

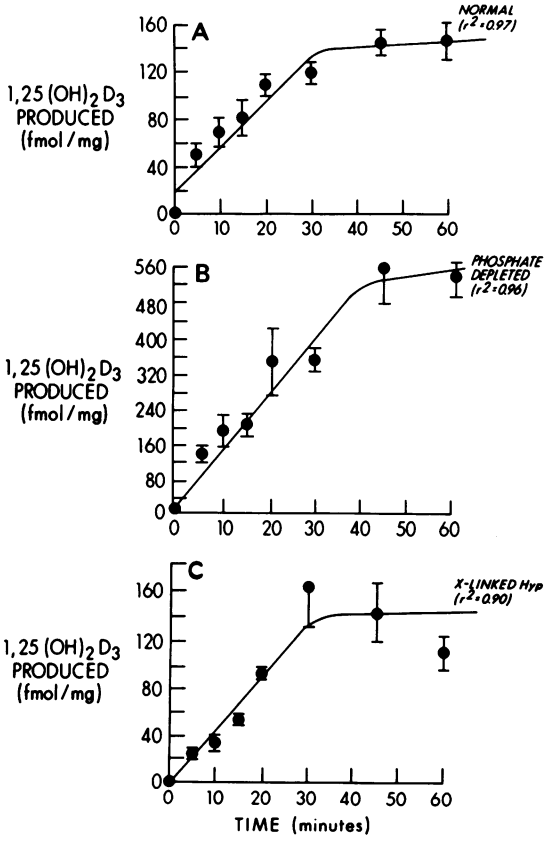


FIGURE 2 Effect of incubation time on $1,25(OH)_2D_3$ production by A normal, B phosphate-depleted, and C *Hyp* mouse kidney homogenates. Each point shown represents the mean±SE of three individual determinations. All flasks were incubated at 37°C with 80 nmol/ml $25(OH)D_3$ provided as substrate.

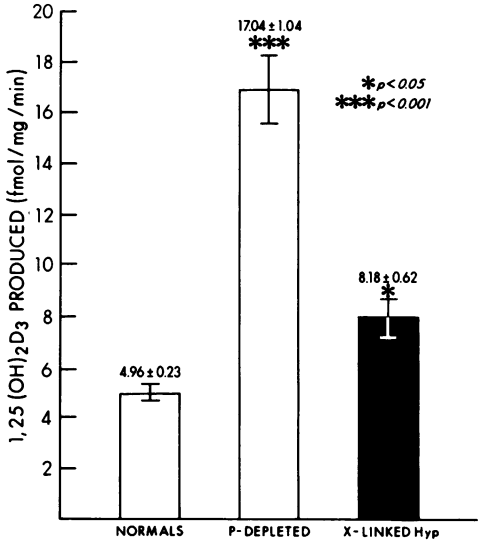


FIGURE 3 $25(OH)D_3$-1-$\alpha$-hydroxylase activity in normal, phosphate-depleted, and *Hyp* mouse kidney homogenates. Each bar represents the mean±SE of 26 individual determinations obtained over the course of seven separate experiments. In each experiment individual reaction vessels contained 100 mg of kidney cortex, approximately one-third to one-fourth of the pooled kidney homogenate from three to four mice in each group. All samples were incubated at 37°C with 80 nmol/ml $25(OH)D_3$ provided as substrate.

## DISCUSSION

Our studies indicate that phosphate deficiency is a potent stimulus of renal 25(OH)D-1$\alpha$-hydroxylase activity in the normal (C57BL6J) mouse. In contrast, the results show that the enzyme activity in *Hyp*-mouse kidney, while significantly elevated above that in normal kidneys, is substantially less than that in the P-depleted mouse kidney. Indeed, the increment above normal of enzyme activity in the *Hyp*-mouse is fully fourfold less than that in the P-depleted mouse. These data indicate that abnormally regulated 25(OH)D-1$\alpha$-hydroxylase activity is manifest in the *Hyp*-mouse kidney.

The diminished responsiveness of 25(OH)D-1$\alpha$-hydroxylase activity in the *Hyp*-mouse kidney does not appear to be the result of an attentuated biological stimulus. The serum phosphorus concentration in the P-depleted and *Hyp*-mice is similar, consistent with phosphate depletion of equal magnitude. Moreover,

the depressed enzyme responsiveness in the *Hyp*-mouse is evident in spite of a significant decrease below normal of the serum calcium and an increase of the serum parathyroid hormone level (9), which should increase the enzyme activity (10).

Consistent with our observations, P-depleted mice had a significantly greater serum $1,25(OH)_2D$ concentration than that of normal mice. In contrast, the increase in enzyme activity (65%) in the *Hyp*-mice is not associated with a significant measurable increase of the serum $1,25(OH)_2D$ concentration.

The decreased enzyme activity in the *Hyp*-mouse kidney is most likely representative of a similar defect in humans with XLH. The mice manifest biochemical abnormalities that are remarkably similar to those expressed in the human disease. Indeed, several observations in affected human subjects suggest that regulation of vitamin D metabolism is abnormal. Among these are: (*a*) the presence of serum $1,25(OH)_2D$ levels in the normal range (1–4); and (*b*) subnormal responsiveness of serum $1,25(OH)_2D$ levels to administration of parathyroid hormone (11).

Measurement of 25(OH)D-1$\alpha$-hydroxylase activity in mammalian kidney homogenates is complicated by the presence of a 25(OH)D binding protein that reduces the availability of substrate to the enzyme and variably inhibits measurable activity (7). In order to obviate this problem we used an assay designed to measure enzyme $V_{max}$. Utilization of a saturating concentration of substrate (Fig. 1) and measurement of the initial rate of enzyme activity (Fig. 2) in preparations from each animal model assures uniform expression of maximal enzyme activity without regard to the amount of binding protein present. Variations in enzyme activity due to alterations in the $K_m$, however, cannot be determined. Thus, the differences in enzyme activity that we observed are most likely the result of a change in the amount of enzyme. In any case, it remains unclear whether the decreased enzyme activity in the *Hyp*-mouse kidney represents diminished responsiveness to hypophosphatemia alone or a more generalized derangement. The recent demonstration of inappropriately increased 25(OH)D-24-hydroxylase activity in the *Hyp*-mouse kidney, suggests, however, that a complex disturbance of vitamin D metabolism is present (12).

The results of previous studies on the role of hypophosphatemia in the regulation of 25(OH)D-1$\alpha$-hydroxylase activity have been controversial. Increased activity in response to this stimulus as well as unaltered activity have been reported in both the avian and mammalian species (10). We have no explanation for these varied findings. However, we do feel that our studies of the P-depleted mouse kidney, in which we observed increased enzyme activity, are an appropriate control for investigation of the *Hyp*-mouse kidney.

Thus, our data represent the first firm evidence that the suspected abnormality of vitamin D metabolism in the *Hyp*-mouse is real and due to defective regulation of 25(OH)D-1$\alpha$-hydroxylase activity. We anticipate a similar defect is present in the kidneys of human subjects with XLH.

## ACKNOWLEDGMENTS

Grants from the National Institute of Arthritis, Digestive Diseases and Kidney (AM-27032), the National Cancer Institute (CA 11265), and from the March of Dimes (5-279) supported these studies.

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