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Research Article

The hypophosphatemic (Hyp) mouse, a murine homologue of human X-linked hypophosphatemic rickets, is characterized by renal defects in brush border membrane phosphate transport and vitamin D3 metabolism. The present study was undertaken to examine whether elevated renal 25-hydroxyvitamin D3-24-hydroxylase activity in Hyp mice is associated with increased degradation of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] by side chain oxidation. Metabolites of 1,25(OH)2D3 were separated by HPLC on Zorbax SIL and identified by comparison with standards authenticated by mass spectrometry. Production of 1,24,25-trihydroxyvitamin D3, 24-oxo-1,25-dihydroxyvitamin D3, and 24-oxo-1,23,25-trihydroxyvitamin D3 was twofold greater in mitochondria from mutant Hyp/Y mice than from normal +/Y littermates. Enzyme activities, estimated by the sum of the three products synthesized per milligram mitochondrial protein under initial rate conditions, were used to estimate kinetic parameters. The apparent Vmax was significantly greater for mitochondria from Hyp/Y mice than from +/Y mice (0.607 +/- 0.064 vs. 0.290 +/- 0.011 pmol/mg per protein per min, mean +/- SEM, P less than 0.001), whereas the apparent Michaelis-Menten constant (Km) was similar in both genotypes (23 +/- 2 vs. 17 +/- 5 nM). The Km for 1,25(OH)2D3 was approximately 10-fold lower than that for 25-hydroxyvitamin D3 [25(OH)D3], indicating that 1,25(OH)2D3 is perhaps the preferred substrate under physiological conditions. In both genotypes, apparent Vmax for 25(OH)D3 was fourfold greater than that for 1,25(OH)2D3, suggesting that side chain [...]

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Increased Renal Catabolism of 1,25-Dihydroxyvitamin D₃ in Murine X-Linked Hypophosphatemic Rickets

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Abstract

The hypophosphatemic (*Hyp*) mouse, a murine homologue of human X-linked hypophosphatemic rickets, is characterized by renal defects in brush border membrane phosphate transport and vitamin D₃ metabolism. The present study was undertaken to examine whether elevated renal 25-hydroxyvitamin D₃-24-hydroxylase activity in *Hyp* mice is associated with increased degradation of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] by side chain oxidation. Metabolites of 1,25(OH)₂D₃ were separated by HPLC on Zorbax SIL and identified by comparison with standards authenticated by mass spectrometry. Production of 1,24,25-trihydroxyvitamin D₃, 24-oxo-1,25-dihydroxyvitamin D₃, and 24-oxo-1,23,25-trihydroxyvitamin D₃ was twofold greater in mitochondria from mutant *Hyp/Y* mice than from normal +/Y littermates. Enzyme activities, estimated by the sum of the three products synthesized per milligram mitochondrial protein under initial rate conditions, were used to estimate kinetic parameters. The apparent V_{max} was significantly greater for mitochondria from *Hyp/Y* mice than from +/Y mice (0.607 ± 0.064 vs. 0.290 ± 0.011 pmol/mg per protein per min, mean \pm SEM, $P < 0.001$), whereas the apparent Michaelis-Menten constant (K_m) was similar in both genotypes (23 ± 2 vs. 17 ± 5 nM). The K_m for 1,25(OH)₂D₃ was ~ 10 -fold lower than that for 25-hydroxyvitamin D₃ [25(OH)D₃], indicating that 1,25(OH)₂D₃ is perhaps the preferred substrate under physiological conditions. In both genotypes, apparent V_{max} for 25(OH)D₃ was fourfold greater than that for 1,25(OH)₂D₃, suggesting that side chain oxidation of 25(OH)D₃ may operate at pharmacological concentrations of substrate. The present results demonstrate that *Hyp* mice exhibit increased renal catabolism of 1,25(OH)₂D₃ and suggest that elevated degradation of vitamin D₃ hormone may contribute significantly to the clinical phenotype in this disorder.

Introduction

The hypophosphatemic (*Hyp*)¹ mouse, a murine homologue of X-linked hypophosphatemia in man, is characterized by

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1. Abbreviations used in this paper: 24-hydroxylase, 25-hydroxyvitamin D₃-24-hydroxylase; *Hyp*, hypophosphatemic; K_m , Michaelis-

hypophosphatemia, rickets, and a specific renal defect in Na⁺-dependent phosphate transport at the brush border membrane (1–3). Recent studies have demonstrated that the regulation of renal 25-hydroxyvitamin D₃ [25(OH)D₃] metabolism is also impaired in the X-linked *Hyp* mouse (4–12). Mutant mice exhibit abnormal renal synthesis of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] in response to phosphate deprivation (4–6), PTH infusion (7), calcium restriction (7, 8), and vitamin D deficiency (9, 10). Moreover, the renal synthesis of 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃] is significantly elevated in *Hyp* mice when compared with normal littermates (9, 11, 12).

Although 24,25(OH)₂D₃ is the major renal metabolite produced by vitamin D-replete animals with normal calcium and phosphorus intake, its precise biological function is poorly understood. Accordingly, the significance of elevated renal mitochondrial 25(OH)D₃-24-hydroxylase (24-hydroxylase) activity in *Hyp* mice (9, 11, 12) and its contribution to the mutant phenotype are not clearly established. Recent studies have demonstrated that 24-hydroxylase serves the catabolism of 1,25(OH)₂D₃ in intestine, kidney, and perhaps other target tissues by converting 1,25(OH)₂D₃ to the biologically inactive metabolite, 1,24,25-trihydroxyvitamin D₃ [1,24,25(OH)₃D₃] (13, 14). The latter serves as a precursor for the synthesis of 24-oxo-1,25-dihydroxyvitamin D₃ [24-oxo-1,25(OH)₂D₃] (13, 14), which is then hydroxylated at position C-23, yielding 24-oxo-1,23,25-trihydroxyvitamin D₃ [24-oxo-1,23,25(OH)₃D₃] (13, 14). Recently, it has been demonstrated that in rat kidney the metabolite 24-oxo-1,23,25(OH)₃D₃ undergoes oxidative side chain cleavage to yield 1,23-dihydroxy-24,25,26,27-tetranor vitamin D₃ (15). This side chain cleaved metabolite may be converted (15) to the final inactivation product of 1,25(OH)₂D₃, namely 1 α -OH-24,25,26,27-tetranor-23-COOH-D₃ (calcitric acid) (16).

In view of the importance of the side chain oxidation pathway in the degradation of 1,25(OH)₂D₃ (13–16), the present study was undertaken to determine whether elevated renal 24-hydroxylase activity in *Hyp* mice contributes significantly to the catabolism of 1,25(OH)₂D₃. We report here that renal degradation of the vitamin D₃ hormone is twofold greater in *Hyp* mice than in normal littermates.

Methods

Mice. Normal male (+/Y) and hemizygous mutant male (*Hyp/Y*) mice were bred and raised in our laboratory. The initial breeding pairs (C57BL/6J males and *Hyp*/+ females) were obtained from Jackson Laboratories (Bar Harbor, ME) and from R. A. Meyer, Marquette University (Milwaukee, WI). The mice were maintained on Wayne Lab Blox (Allied Mills Inc., Chicago, IL) containing 1.2% calcium,

Menten constant; 1,24,25(OH)₃D₃, 1,24,25-trihydroxyvitamin D₃; 24-oxo-1,25(OH)₂D₃, 24-oxo-1,25-dihydroxyvitamin D₃; 24-oxo-1,23,25(OH)₃D₃, 24-oxo-1,23,25-trihydroxyvitamin D₃.

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0.99% phosphorus, and 4.41 IU vitamin D₃/g. Mice were killed at 3–4 mo of age by decapitation.

Renal metabolism of vitamin D₃ metabolites. Mitochondria were prepared from renal cortex of individual or groups of mice according to the method of Vieth and Fraser (17). In our standard assay procedure, aliquots (1 ml) of oxygenated mitochondria containing ~1–2 mg protein in 125 mM KCl, 20 mM Hepes, 10 mM malic acid, 2 mM MgSO₄, 1 mM dithiothreitol, and 0.25 mM EDTA, adjusted to a pH of 7.42 were incubated at 25°C with 500 nM ³H-1,25(OH)₂D₃ (~200 cpm/pmol) for 5–15 min as described previously (9, 10). To estimate kinetic parameters, mitochondria were incubated under initial rate conditions with ³H-1,25(OH)₂D₃ (2–500 nM) or ³H-25(OH)D₃ (20–2,000 nM) and enzyme activity was estimated by the sum of the three products synthesized. Reaction mixtures in which boiled mitochondria were incubated with ³H-substrate at each concentration examined served as background in the quantitation of product formation. The reactions were stopped by the addition of 3.75 ml chloroform-methanol (1:2) and samples were stored at –20°C under N₂ until extraction. ³H-Vitamin D₃ metabolites were purchased from Amersham, Oakville, Ontario, Canada, and New England Nuclear, Boston, MA. Crystalline 25(OH)D₃ and 1,25(OH)₂D₃ were gifts from Hoffman-La Roche Ltd., Etobicoke, Ontario, Canada.

Extraction and assay of vitamin D₃ metabolites. Extraction of reaction mixtures was performed as described by Bligh and Dyer (18). Recovery of ³H-label from incubations with either intact or boiled mitochondria (where no significant conversion of substrate is apparent) was similar and ranged from 85 to 95%, indicating that all reaction products could be accounted for under the conditions of our experiments. Because the ³H-vitamin D₃ substrates used in the present study were labeled in the C-26 and C-27 positions, we would not detect the production of ³H-labeled side chain cleavage products from 1,25(OH)₂D₃ and 25(OH)D₃, i.e., 1,23(OH)₂-24,25,26,27-tetranor-D₃ and 23(OH)24,25,26,27-tetranor-D₃. However, at high substrate concentrations where products were detectable by ultraviolet absorption, no evidence was obtained for the formation of unlabeled side chain cleavage products. Note that recovery of ³H-label with intact mitochondria fell to 60–80% of that from boiled mitochondria in those reaction mixtures containing 2 nM 1,25(OH)₂D₃. Accordingly, kinetic parameters for side chain oxidation of 1,25(OH)₂D₃ were estimated both without and with the data obtained at 2 nM substrate. Exclusion of the data derived at 2 nM 1,25(OH)₂D₃ did not significantly alter the kinetic parameters that were estimated by the Eadie-Hofstee transformation. The estimated kinetic constants are a measure of the first reaction in the sequence 1,25(OH)₂D₃ → 1,24,25(OH)₃D₃ → 24-oxo-1,25(OH)₂D₃ → 24-oxo-1,23,25(OH)₃D₃.

Vitamin D₃ metabolites were separated on Zorbax CN or SIL as described by Jones (19, 20). The identity of products was confirmed by co-chromatography with standards authenticated by mass spectrometry.

Statistical methods. Effect of genotype on metabolite production was analyzed by Student's *t* test.

Results

The separation of side chain oxidation products, derived from incubation of renal mitochondria with ³H-1,25(OH)₂D₃, is illustrated in Fig. 1. The identity of each metabolite was confirmed by comparison with standards authenticated by mass spectrometry. A clear separation of ³H-1,25(OH)₂D₃ (peak 2), 24-oxo-1,25(OH)₂D₃ (peak 3), 24-oxo-1,23,25(OH)₃D₃ (peak 4), and 1,24,25(OH)₃D₃ (peak 5) was achieved on Zorbax SIL (Fig. 1). The material which eluted in peak 1 represents an impurity in the ³H-1,25(OH)₂D₃ substrate and was present in all mitochondrial extracts, irrespective of whether mitochondria were intact or boiled.

Fig. 2 depicts metabolite production from 500 nM

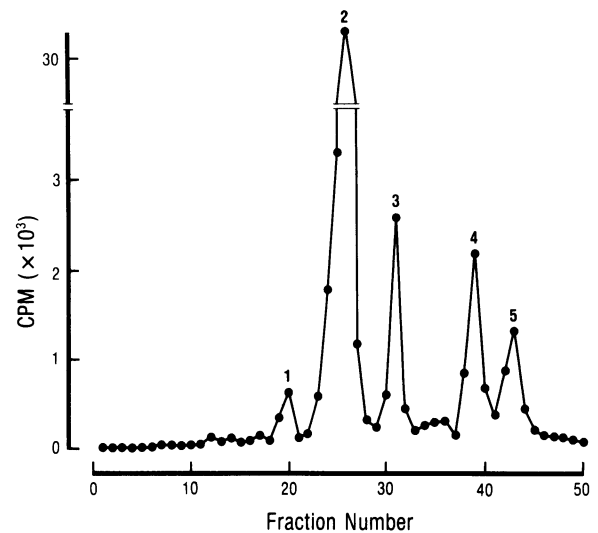


Figure 1. Chromatographic separation of ³H-1,25(OH)₂D₃ (peak 2), ³H-24-oxo-1,25(OH)₂D₃ (peak 3), ³H-24-oxo-1,23,25(OH)₃D₃ (peak 4), and ³H-1,24,25(OH)₃D₃ (peak 5) on Zorbax SIL. Metabolites were derived from an extract of renal mitochondria that had been incubated with ³H-1,25(OH)₂D₃ at 25°C as described in Methods. The material in peak 1 which represents an impurity in the ³H-1,25(OH)₂D₃ substrate was present in all mitochondrial extracts irrespective of whether mitochondria were intact or boiled. Conditions: Zorbax SIL (6.2 mm × 25 cm); hexane-isopropanol-methanol (88:10:2); 2 ml/min. Standard compounds: 1,25(OH)₂D₃ (12.8 min), 24-oxo-1,25(OH)₂D₃ (15.3 min), 24-oxo-1,23,25(OH)₃D₃ (19.3 min), and 1,24,25(OH)₃D₃ (21.2 min).

³H-1,25(OH)₂D₃ by renal mitochondria derived from +/Y and Hyp/Y mice. The formation of all three products is twofold greater in mitochondria from mutant mice. Under these conditions, 1,24,25(OH)₃D₃, 24-oxo-1,25(OH)₂D₃, and 24-oxo-1,23,25(OH)₃D₃ comprised 36, 44, and 20%, respectively, of the total products synthesized by renal mitochondria derived

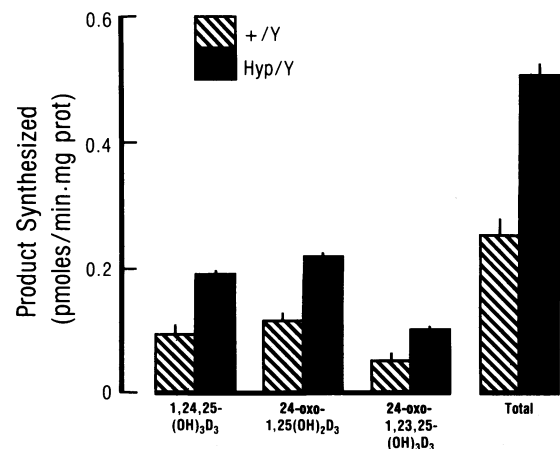


Figure 2. Effect of the Hyp mutation on side chain oxidation of 1,25(OH)₂D₃. Renal mitochondria from +/Y and Hyp/Y mice were incubated with 500 nM ³H-1,25(OH)₂D₃ for 15 min at 25°C. Extraction and HPLC of vitamin D₃ metabolites were performed as described in Methods. Each bar depicts mean ± SEM and are based on values derived from six individual mice of each genotype. Total depicts the sum of the three metabolites. Genotype differences were significant for each product (*P* < 0.001) by Student's *t* test.

from normal mice ($n = 7$, SEM < 2%). In spite of a twofold increase in the rate of product formation by renal mitochondria from *Hyp* mice, the relative proportion of each metabolite was identical to that of normal mice.

To understand the mechanism for the observed increase in 1,25(OH)₂D₃ catabolism by *Hyp* mouse kidney, the effect of substrate concentration on side chain oxidation was examined. We estimated enzyme activity, at each concentration of 1,25(OH)₂D₃, from the sum of the three products synthesized per milligram protein under initial rate conditions (see Total, Fig. 2). Fig. 3 A shows that total enzyme activity is saturable in both +/Y and *Hyp*/Y mice and is higher in the mutants at all substrate concentrations examined. Kinetic parameters, estimated from Eadie-Hofstee transformation of the data, indicate that the apparent Michaelis-Menten constant (K_m) for 1,25(OH)₂D₃ is not significantly different in normals and mutants (17±5 vs. 23±2 nM, respectively), whereas the V_{max} is significantly greater in *Hyp*/Y mice relative to +/Y littermates (0.607±0.064 vs. 0.290±0.011 pmol/mg per protein per min, respectively) (Table I).

We also examined the side chain oxidation of 25(OH)D₃, as a function of substrate concentration, in similar preparations of renal mitochondria derived from +/Y and *Hyp*/Y

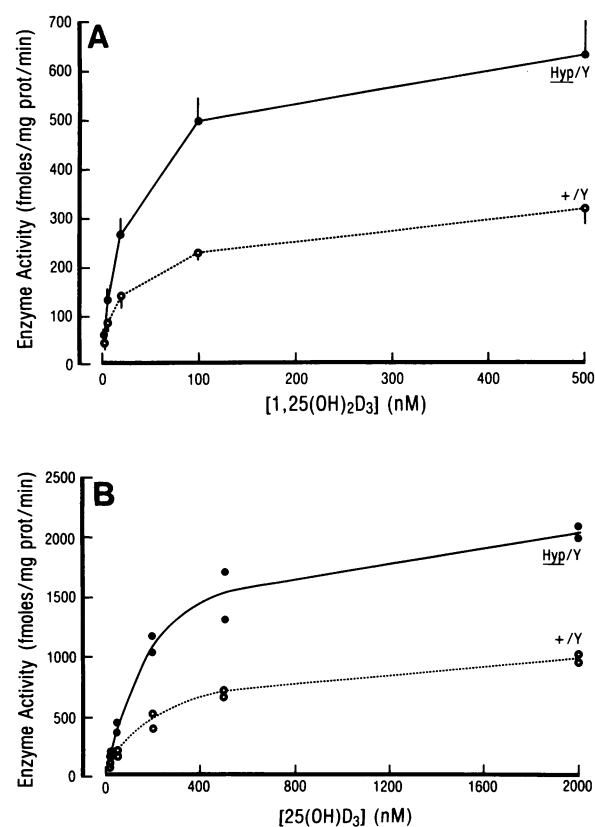


Figure 3. (A) Effect of 1,25(OH)₂D₃ concentration on its side chain oxidation by renal mitochondria from +/Y (○) and *Hyp*/Y (●) mice. Incubations were carried out under initial rate conditions as described in Methods. Data represent mean±SEM of product formation from three separate experiments in the case of +/Y and *Hyp*/Y mice, respectively. (B) Effect of 25(OH)D₃ concentration on its side chain oxidation by renal mitochondria from +/Y (○) and *Hyp*/Y (●) mice. Incubations were carried out under initial rate conditions as described in Methods. Data from two experiments in each genotype are shown. Notice that the scales of the x and y axes differ in A and B.

Table I. Apparent Kinetic Constants for Side Chain Oxidation of 1,25(OH)₂D₃ and 25(OH)D₃ by Renal Mitochondria of +/Y and *Hyp*/Y Mice

	+/Y	<i>Hyp</i> /Y
1,25(OH) ₂ D ₃ : K_m * V_{max} ‡	17±5 0.290±0.011	23±2 0.607±0.064§
25(OH)D ₃ : K_m * V_{max} ‡	152; 215 0.992; 1.011	195; 217 2.058; 2.360

Experimental details for kinetic experiments are described in Methods and in the legend to Fig. 3.

* Nanomolar.

‡ Picomoles per milligram protein per minute.

§ Effect of mutation is significant ($P < 0.001$).

|| Data are shown from two experiments with the same mitochondrial preparations used above with 1,25(OH)₂D₃ as substrate. Data are consistent with previously reported kinetic parameters for renal 24-hydroxylase in +/Y and *Hyp*/Y mice (12).

mice. Total enzyme activity is saturable in both genotypes and is significantly higher in *Hyp*/Y mice (Fig. 3 B). Kinetic parameters, estimated as described above are shown in Table I. In both genotypes, the apparent K_m for 25(OH)D₃ is similar and is ~ 10-fold higher than that for 1,25(OH)₂D₃. V_{max} for side chain oxidation of 25(OH)D₃ is twofold greater in mitochondria from *Hyp* mice relative to normal mice and is fourfold higher for 25(OH)D₃ than for 1,25(OH)₂D₃ in both genotypes.

Discussion

The present investigation was undertaken to establish whether elevated renal 24-hydroxylase activity in *Hyp* mice contributes to accelerated renal degradation of 1,25(OH)₂D₃ by the side chain oxidation pathway. We show that the renal synthesis of side chain oxidation products derived from 1,25(OH)₂D₃, namely 1,24,25(OH)₃D₃, 24-oxo-1,25(OH)₂D₃, and 24-oxo-1,23,25(OH)₃D₃, is twofold greater in *Hyp*/Y mice than in +/Y littermates (Fig. 2). We suggest that increased catabolism of 1,25(OH)₂D₃ may play an important role in the pathophysiology of both murine and human X-linked hypophosphatemic rickets.

Side chain oxidation is a major catabolic pathway for 1,25(OH)₂D₃ in kidney (13, 15) and intestine (13, 14) under physiological conditions. The pathway involves sequential 24-hydroxylation, 24-oxidation, 23-hydroxylation, and eventual side chain cleavage of 1,25(OH)₂D₃. It provides an important mechanism whereby the physiological concentration of hormone, and thus the biological response, can be controlled. Accordingly, increased renal side chain oxidation of 1,25(OH)₂D₃ in *Hyp* mice would decrease the effective concentration of 1,25(OH)₂D₃ available for biological action in the kidney and perhaps other target tissues. Our demonstration of increased renal catabolism of 1,25(OH)₂D₃ may account, in part, for the inappropriate plasma levels of 1,25(OH)₂D in *Hyp* mice (4) and in patients with X-linked hypophosphatemia (21, 22), and may explain why supraphysiological doses of 1,25(OH)₂D₃ (and phosphate supplementation) are required for correction of bone lesions in these patients (23). Moreover, our results are consistent with the previous demonstration that plasma clearance of high doses of

exogenous $1,25(\text{OH})_2\text{D}_3$ is more rapid in *Hyp* mice than in normal littermates (24). Whether other target tissues in the mutant strain exhibit increased degradation of the vitamin D hormone requires further study.

Increased renal catabolism of $1,25(\text{OH})_2\text{D}_3$ by the side chain oxidation pathway may also account, in part, for inappropriate renal production of $1,25(\text{OH})_2\text{D}_3$ in *Hyp* mice compared with normal mice with comparable hypophosphatemia, achieved by feeding a low phosphate diet (5, 6). It is well documented that phosphate deprivation is associated not only with a stimulation in renal 1-hydroxylase activity and vitamin D hormone production (25, 26), but also with a marked inhibition of renal 24-hydroxylase activity (11, 25). Accordingly, one would predict that catabolism of $1,25(\text{OH})_2\text{D}_3$ via the side chain oxidation pathway would be markedly reduced in phosphate-deprived normal mice when compared with *Hyp* mice or normal mice. Recent studies have demonstrated > 50% reduction in renal catabolism of $1,25(\text{OH})_2\text{D}_3$ via the side chain oxidation pathway 24 h after phosphate deprivation of normal rats and guinea pigs (Simboli, M., and G. Jones, unpublished observations). The above considerations question the validity of estimating $1,25(\text{OH})_2\text{D}_3$ production in renal preparations containing enzymes capable of its degradation. Moreover, a comparative study of $1,25(\text{OH})_2\text{D}_3$ synthesis in renal preparations with different catabolic potential, i.e., *Hyp* mice greater than normal mice greater than phosphate-deprived normal mice, may not be appropriate (5, 6). Note that increased side chain oxidation cannot account for the blunted 1-hydroxylase response to vitamin D and calcium deficiency reported in *Hyp* mice (9, 10), since these experiments were performed under conditions where renal 24-hydroxylase is completely inhibited (9, 12). Moreover, no evidence for the production of $1,24,25(\text{OH})_3\text{D}_3$ was found in vitamin D and calcium-deprived *Hyp* mice (12).

The present study demonstrates that renal side chain oxidation of both $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ is increased in the *Hyp* mouse relative to normal littermates and that the relative proportion of products synthesized from $1,25(\text{OH})_2\text{D}_3$ is similar in both genotypes. Other studies have shown, in rat and mouse, that renal side chain oxidation of both $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ can also be increased by prior treatment with vitamin D_3 or $1,25(\text{OH})_2\text{D}_3$ (13, 20, 27, 28). These findings raise two questions:

(i) Are the three reactions in this pathway, namely 24-hydroxylation, 24-oxidation, and 23-hydroxylation, mediated by a single enzyme or by three distinct enzymes whose regulation is coordinated?

(ii) Are $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ both metabolized by the same enzyme system?

The demonstration that catabolism of both substrates is increased by the *Hyp* mutation (Table I) as well as by prior treatment with $1,25(\text{OH})_2\text{D}_3$ (13, 20, 27, 28), suggests the existence of one multienzyme complex that mediates destruction of both $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$. Clearly, information regarding the molecular structure and the regulation of the enzyme(s) involved in the renal mitochondrial side chain oxidation pathway will require further study. As is the case for the enzymes that catalyze the production of $24,25(\text{OH})_2\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$, the side chain oxidation system may involve cytochrome P-450 mixed function oxidases that are tightly associated with the inner mitochondrial membrane of renal

proximal tubular cells (29). Direct evidence for one cytochrome P-450 enzyme exhibiting several catalytic activities has been presented in the adrenal cortex (30).

The present study demonstrates that the apparent affinity of the side chain oxidation enzyme system is 10-fold greater for $1,25(\text{OH})_2\text{D}_3$ than that for $25(\text{OH})\text{D}_3$. Although the apparent K_m values for $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ are approximately 200 and 20 nM, respectively (Table I), in both cases they are significantly higher than their reported plasma concentrations (29). The reason for this discrepancy is not clear but may be attributed to lower than predicted concentrations of vitamin D_3 substrate available for metabolism by *in vitro* mitochondrial preparations. This could arise from disruption of vitamin D_3 metabolite transport systems, differential solubility of vitamin D_3 metabolites, and/or contamination of mitochondrial fractions with vitamin D binding protein and $1,25(\text{OH})_2\text{D}_3$ receptor (29). The 10-fold difference in affinities for the two vitamin D_3 metabolites may indicate that the side chain oxidation pathway is designed to degrade $1,25(\text{OH})_2\text{D}_3$ and not $25(\text{OH})\text{D}_3$ at physiological concentrations. In addition, the fourfold greater V_{max} for $25(\text{OH})\text{D}_3$ suggests that side chain oxidation of this metabolite probably operates at pharmacological concentrations of substrate.

In summary, we have demonstrated increased renal side chain oxidation of $1,25(\text{OH})_2\text{D}_3$ in mice bearing the X-linked *Hyp* mutation. Renal side chain oxidation of $25(\text{OH})\text{D}_3$ is also significantly elevated in *Hyp* mice. The enzyme system has a 10-fold greater affinity for $1,25(\text{OH})_2\text{D}_3$ than for $25(\text{OH})\text{D}_3$. We suggest that increased renal catabolism of $1,25(\text{OH})_2\text{D}_3$ by *Hyp* mice leads to reduced availability of the vitamin D hormone, thereby contributing to the clinical phenotype in this disorder.

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