Increased Renal Catabolism of 1,25-Dihydroxyvitamin D_3 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 D_3 metabolism. The present study was undertaken to examine whether elevated renal 25-hydroxyvitamin D_3–24-hydroxylase activity in *Hyp* mice is associated with increased degradation of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] by side chain oxidation. Metabolites of $1,25(OH)_2D_3$ were separated by HPLC on Zorbax SIL and identified by comparison with standards authenticated by mass spectrometry. Production of 1,24,25-trihydroxyvitamin D_3, 24-oxo-1,25-dihydroxyvitamin D_3, and 24-oxo-1,23,25-trihydroxyvitamin D_3 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±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 $1,25(OH)_2D_3$ was ~ 10-fold lower than that for 25-hydroxyvitamin D_3 [$25(OH)D_3$], indicating that $1,25(OH)_2D_3$ is perhaps the preferred substrate under physiological conditions. In both genotypes, apparent V_{max} for $25(OH)D_3$ was fourfold greater than that for $1,25(OH)_2D_3$, suggesting that side chain oxidation of $25(OH)D_3$ may operate at pharmacological concentrations of substrate. The present results demonstrate that *Hyp* mice exhibit increased renal catabolism of $1,25(OH)_2D_3$ and suggest that elevated degradation of vitamin D_3 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 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 D_3 [$25(OH)D_3$] metabolism is also impaired in the X-linked *Hyp* mouse (4–12). Mutant mice exhibit abnormal renal synthesis of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] 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 D_3 [$24,25(OH)_2D_3$] is significantly elevated in *Hyp* mice when compared with normal littermates (9, 11, 12).

Although $24,25(OH)_2D_3$ 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 $25(OH)D_3$–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 $1,25(OH)_2D_3$ in intestine, kidney, and perhaps other target tissues by converting $1,25(OH)_2D_3$ to the biologically inactive metabolite, 1,24,25-trihydroxyvitamin D_3 [$1,24,25(OH)_3D_3$] (13, 14). The latter serves as a precursor for the synthesis of 24-oxo–1,25-dihydroxyvitamin D_3 [24-oxo-$1,25(OH)_2D_3$] (13, 14), which is then hydroxylated at position C-23, yielding 24-oxo-1,23,25-trihydroxyvitamin D_3 [24-oxo-$1,23,25(OH)_3D_3$] (13, 14). Recently, it has been demonstrated that in rat kidney the metabolite 24-oxo-$1,23,25(OH)_3D_3$ undergoes oxidative side chain cleavage to yield 1,23-dihydroxy-24,25,26,27-tetranor vitamin D_3 (15). This side chain cleaved metabolite may be converted (15) to the final inactivation product of $1,25(OH)_2D_3$, namely 1α-OH-24,25,26,27-tetranor-23-COOH-D_3 (calcitroic acid) (16).

In view of the importance of the side chain oxidation pathway in the degradation of $1,25(OH)_2D_3$ (13–16), the present study was undertaken to determine whether elevated renal 24-hydroxylase activity in *Hyp* mice contributes significantly to the catabolism of $1,25(OH)_2D_3$. We report here that renal degradation of the vitamin D_3 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,

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1. *Abbreviations used in this paper:* 24-hydroxylase, 25-hydroxyvitamin D_3–24-hydroxylase; *Hyp,* hypophosphatemic; K_m, Michaelis-Menten constant; $1,24,25(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3; 24-oxo-$1,25(OH)_2D_3$, 24-oxo-1,25-dihydroxyvitamin D_3; 24-oxo-$1,23,25(OH)_3D_3$, 24-oxo-1,23,25-trihydroxyvitamin D_3.

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0.99% phosphorus, and 4.41 IU vitamin D_3/g. Mice were killed at 3–4 mo of age by decapitation.

Renal metabolism of vitamin D_3 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 $MgSO_4$, 1 mM dithiothreitol, and 0.25 mM EDTA, adjusted to a pH of 7.42 were incubated at 25°C with 500 nM ^{3}H-1,25(OH)$_2$D$_3$ (~ 200 cpm/pmol) for 5–15 min as described previously (9, 10). To estimate kinetic parameters, mitochondria were incubated under initial rate conditions with ^{3}H-1,25(OH)$_2$D$_3$ (2–500 nM) or ^{3}H-25(OH)D$_3$ (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 ^{3}H-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 N_2 until extraction. ^{3}H-Vitamin D_3 metabolites were purchased from Amersham, Oakville, Ontario, Canada, and New England Nuclear, Boston, MA. Crystalline 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ were gifts from Hoffman-La Roche Ltd., Etobicoke, Ontario, Canada.

Extraction and assay of vitamin D_3 metabolites. Extraction of reaction mixtures was performed as described by Bligh and Dyer (18). Recovery of ^{3}H-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 ^{3}H-vitamin D_3 substrates used in the present study were labeled in the C-26 and C-27 positions, we would not detect the production of ^{3}H-labeled side chain cleavage products from 1,25(OH)$_2$D$_3$ and 25(OH)D$_3$, i.e., 1,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ and 23(OH)24,25,26,27-tetranor-D$_3$. 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 ^{3}H-label with intact mitochondria fell to 60–80% of that from boiled mitochondria in those reaction mixtures containing 2 nM 1,25(OH)$_2$D$_3$. Accordingly, kinetic parameters for side chain oxidation of 1,25(OH)$_2$D$_3$ were estimated both without and with the data obtained at 2 nM substrate. Exclusion of the data derived at 2 nM 1,25(OH)$_2$D$_3$ 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)$_2$D$_3$ → 1,24,25(OH)$_3$D$_3$ → 24-oxo-1,25(OH)$_2$D$_3$ → 24-oxo-1,23,25(OH)$_3$D$_3$.

Vitamin D_3 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 ^{3}H-1,25(OH)$_2$D$_3$, is illustrated in Fig. 1. The identity of each metabolite was confirmed by comparison with standards authenticated by mass spectrometry. A clear separation of ^{3}H-1,25(OH)$_2$D$_3$ (peak 2), 24-oxo-1,25(OH)$_2$D$_3$ (peak 3), 24-oxo-1,23,25(OH)$_3$D$_3$ (peak 4), and 1,24,25(OH)$_3$D$_3$ (peak 5) was achieved on Zorbax SIL (Fig. 1). The material which eluted in peak 1 represents an impurity in the ^{3}H-1,25(OH)$_2$D$_3$ substrate and was present in all mitochondrial extracts, irrespective of whether mitochondria were intact or boiled.

Fig. 2 depicts metabolite production from 500 nM

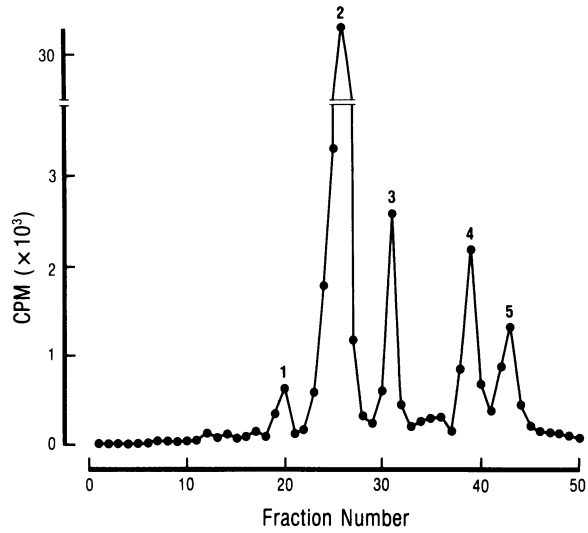

Figure 1. Chromatographic separation of ^{3}H-1,25(OH)$_2$D$_3$ (peak 2), ^{3}H-24-oxo-1,25(OH)$_2$D$_3$ (peak 3), ^{3}H-24-oxo-1,23,25(OH)$_3$D$_3$ (peak 4), and ^{3}H-1,24,25(OH)$_3$D$_3$ (peak 5) on Zorbax SIL. Metabolites were derived from an extract of renal mitochondria that had been incubated with ^{3}H-1,25(OH)$_2$D$_3$ at 25°C as described in Methods. The material in peak 1 which represents an impurity in the ^{3}H-1,25(OH)$_2$D$_3$ 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)$_2$D$_3$ (12.8 min), 24-oxo-1,25(OH)$_2$D$_3$ (15.3 min), 24-oxo-1,23,25(OH)$_3$D$_3$ (19.3 min), and 1,24,25(OH)$_3$D$_3$ (21.2 min).

^{3}H-1,25(OH)$_2$D$_3$ 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)$_3$D$_3$, 24-oxo-1,25(OH)$_2$D$_3$, and 24-oxo-1,23,25(OH)$_3$D$_3$ comprised 36, 44, and 20%, respectively, of the total products synthesized by renal mitochondria derived

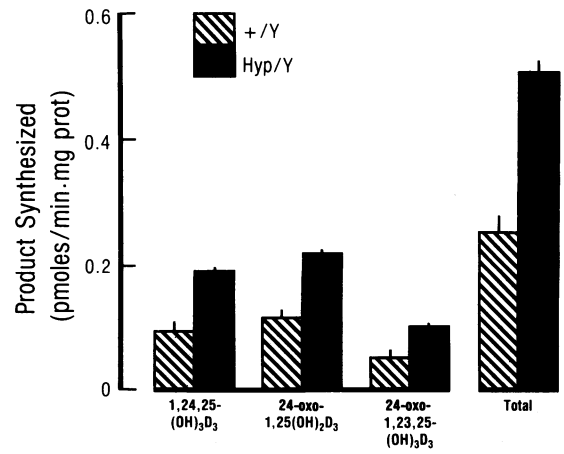

Figure 2. Effect of the *Hyp* mutation on side chain oxidation of 1,25(OH)$_2$D$_3$. Renal mitochondria from +/Y and *Hyp*/Y mice were incubated with 500 nM ^{3}H-1,25(OH)$_2$D$_3$ for 15 min at 25°C. Extraction and HPLC of vitamin D_3 metabolites were performed as described in Methods. Each bar depicts 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 $1,25(OH)_2D_3$ catabolism by *Hyp* mouse kidney, the effect of substrate concentration on side chain oxidation was examined. We estimated enzyme activity, at each concentration of $1,25(OH)_2D_3$, 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 $1,25(OH)_2D_3$ 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 $25(OH)D_3$, as a function of substrate concentration, in similar preparations of renal mitochondria derived from +/Y and *Hyp*/Y 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 $25(OH)D_3$ is similar and is ~ 10-fold higher than that for $1,25(OH)_2D_3$. V_{max} for side chain oxidation of $25(OH)D_3$ is twofold greater in mitochondria from *Hyp* mice relative to normal mice and is fourfold higher for $25(OH)D_3$ than for $1,25(OH)_2D_3$ in both genotypes.

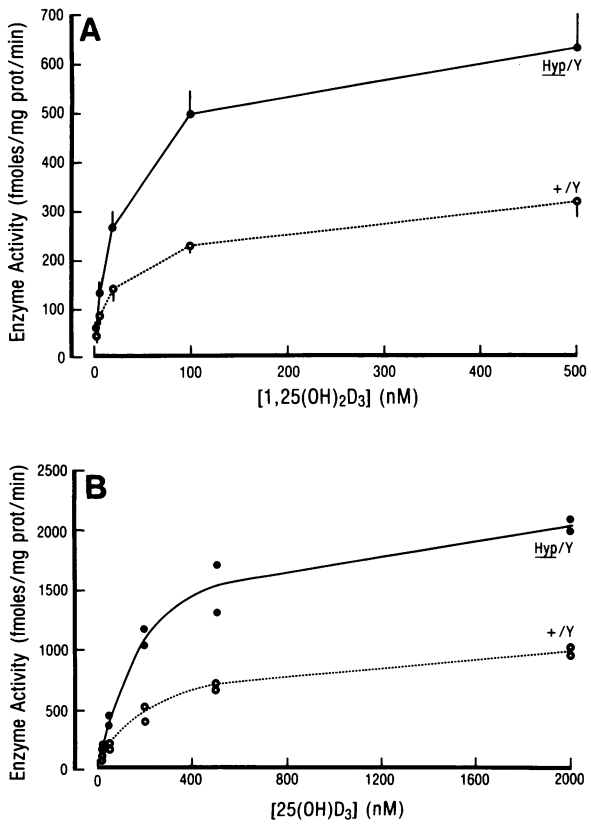

Figure 3. (*A*) Effect of $1,25(OH)_2D_3$ concentration on its side chain oxidation by renal mitochondria from +/Y (○) and *Hyp*/Y (●) mice. Incubations were carried out under initial rate conditions as described in Methods. Data represent mean±SEM of product formation from three separate experiments in the case of +/Y and *Hyp*/Y mice, respectively. (*B*) Effect of $25(OH)D_3$ 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 $1,25(OH)_2D_3$ and $25(OH)D_3$ by Renal Mitochondria of +/Y and Hyp/Y Mice

	+/Y	*Hyp*/Y
$1,25(OH)_2D_3$: K_m*	17±5	23±2
V_{max}‡	0.290±0.011	0.607±0.064§
$25(OH)D_3$:‖ K_m*	152; 215	195; 217
V_{max}‡	0.992; 1.011	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 $1,25(OH)_2D_3$ as substrate. Data are consistent with previously reported kinetic parameters for renal 24-hydroxylase in +/Y and *Hyp*/Y mice (12).

Discussion

The present investigation was undertaken to establish whether elevated renal 24-hydroxylase activity in *Hyp* mice contributes to accelerated renal degradation of $1,25(OH)_2D_3$ by the side chain oxidation pathway. We show that the renal synthesis of side chain oxidation products derived from $1,25(OH)_2D_3$, namely $1,24,25(OH)_3D_3$, 24-oxo-$1,25(OH)_2D_3$, and 24-oxo-$1,23,25(OH)_3D_3$, is twofold greater in *Hyp*/Y mice than in +/Y littermates (Fig. 2). We suggest that increased catabolism of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$. 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 $1,25(OH)_2D_3$ in *Hyp* mice would decrease the effective concentration of $1,25(OH)_2D_3$ available for biological action in the kidney and perhaps other target tissues. Our demonstration of increased renal catabolism of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ (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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ by the side chain oxidation pathway may also account, in part, for inappropriate renal production of $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ production in renal preparations containing enzymes capable of its degradation. Moreover, a comparative study of $1,25(OH)_2D_3$ 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 $1,24,25(OH)_3D_3$ was found in vitamin D and calcium-deprived *Hyp* mice (12).

The present study demonstrates that renal side chain oxidation of both $25(OH)D_3$ and $1,25(OH)_2D_3$ is increased in the *Hyp* mouse relative to normal littermates and that the relative proportion of products synthesized from $1,25(OH)_2D_3$ is similar in both genotypes. Other studies have shown, in rat and mouse, that renal side chain oxidation of both $25(OH)D_3$ and $1,25(OH)_2D_3$ can also be increased by prior treatment with vitamin D_3 or $1,25(OH)_2D_3$ (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 $25(OH)D_3$ and $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ (13, 20, 27, 28), suggests the existence of one multienzyme complex that mediates destruction of both $25(OH)D_3$ and $1,25(OH)_2D_3$. 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 $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$, 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 $1,25(OH)_2D_3$ than that for $25(OH)D_3$. Although the apparent K_m values for $25(OH)D_3$ and $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ and not $25(OH)D_3$ at physiological concentrations. In addition, the fourfold greater V_{max} for $25(OH)D_3$ 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 $1,25(OH)_2D_3$ in mice bearing the X-linked *Hyp* mutation. Renal side chain oxidation of $25(OH)D_3$ is also significantly elevated in *Hyp* mice. The enzyme system has a 10-fold greater affinity for $1,25(OH)_2D_3$ than for $25(OH)D_3$. We suggest that increased renal catabolism of $1,25(OH)_2D_3$ 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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