X-Linked Hypophosphatemia: New Horizons

Marc K. Drezner

University of Wisconsin Medical School, Wisconsin, USA

Abstract. X-linked hypophosphatemia (XLH) is a hypophosphatemic rachitic disorder, which occurs in one of 20,000 live births. The disease phenotype is quite variable, and many patients do not have profound signs or symptoms including rickets. Thus, XLH is sometimes called “XLH rickets without rickets”. Such phenotypic variability makes a genetic examination of potentially affected patients inevitable for definite diagnosis. In most of the patients, hypophosphatemia, elevated serum alkaline phosphatase, and a lowered level of active vitamin D, as well as abnormalities in the bone mineralization, are seen. In hyp-mice, the model mouse of XLH, a hormonal abnormality that affects the Npt2 protein mediates the abnormal Na-dependent Pi transport in the renal tubules. The defect in vitamin D metabolism is the result of diminished 25(OH)D-1α-hydroxylase activity due to a post-transcriptional abnormality. There is a genetic abnormality of the PHEX gene causal of XLH. PHEX codes a protein from the cell membrane-bound endopeptidase family, which causes hypophosphatemia by decreasing the Npt2 in the kidneys; however, the localization of PHEX is limited to the bones and teeth. To clarify the pathophysiology of XLH, therefore, identification of the mechanism by which the diseased gene functions is urgently required.

Key words: Npt2, PHEX gene, phosphatonin/minhibin, vitamin D metabolism, X-linked hypophosphatemia

X-linked hypophosphatemia (XLH), or X-linked hypophosphatemic rickets/osteomalacia, is the archetypical disorder amongst the hypophosphatemic rachitic diseases in man (1). The disease is not infrequent, occurring in one of 20,000 live births. Recently, autosomal dominant hypophosphatemia, hereditary hypophosphatemic rickets/hypercalciuria (2), autosomal dominant hypophosphatemia (3), and Fanconi’s Syndrome Types I and II (4, 5) were described as disorders belonging to the genetic variants of this group. There are also acquired hypophosphatemic rachitic/osteomalacic disorders, including tumor-induced osteomalacia (TIO) (6) and a variant form of Fanconi’s syndrome Type I, which is caused by taking outdated tetracyclines.

XLH Phenotypes

Phenotypic abnormalities of XLH are growth retardation, osteomalacia, rickets and resistance to vitamin D therapy (7–12). Classic X-ray images often clearly show bowed legs and bone biopsy evidence of excessive unmineralized osteoid mixed with normal bone (13). Generally speaking, the phenotypic presentation may include variable findings, which are not limited to bowed legs (genu varum), but alternatively or in addition may include knock-knees (genu valgum), rachitic
rosary, and craniosynostosis (Fig. 1). Many patients with XLH, however, do not have such a profound presentation; indeed, in some patients with the disorder the disease is described as “XLH rickets without rickets” (14, 15). In these patients there is not demonstrable rickets at any of the epiphyseal plates in bone despite the definitive diagnosis of XLH. Such being the case, investigations of the genetic passage of the disorder in a particular patient is often critical for accurate diagnosis of XLH.

Classical biochemical abnormalities of XLH include hypophosphatemia, elevated alkaline phosphatase, inappropriately low levels of serum 1α,25-(OH)₂D (16-18) (despite normal levels of its precursor, 25-OH-D (19-21)), increased renal phosphorus wasting and decreased intestinal absorption of phosphorus. In addition, XLH patients often manifest decreased renal calcium excretion and intestinal calcium absorption.

Treatment of affected patients with vitamin D₂ and phosphorus, does not normalize the serum phosphorus level or correct the defect in vitamin D metabolism. Other biochemical abnormalities, such as the elevated serum alkaline phosphatase, likewise often remain unresolved. In addition, treatment with vitamin D₂ or combination treatment with vitamin D₂ and phosphorus does not induce normal bone mineralization (20, 22).

Despite the intensive investigation of XLH, which has revealed extensive information about the phenotypic and biochemical abnormalities of the disease, there had been limited progression in identifying and cloning the gene underlying the disorder. Although it was well known that this disease exhibited an X-linked dominant expression, the genetic defect had been localized only to the Xp22.1-22.3 region of the X-chromosome (23, 24) and further studies were stalled by the inability to identify close flanking markers around the hyp gene causing the hypophosphatemic disease.

**Pathophysiology of XLH**

Understanding the pathophysiology of XLH began with a clarification of the renal phosphate
transport defect. Normal renal phosphate transport in the proximal convoluted tubules occurs by sodium-dependent phosphate transport, mediated by a type IIa sodium-phosphate co-transporter (Npt2) protein (25). Investigation of this process in XLH was facilitated by the existence of the hyp-mouse, a murine homologue of the human disease, in which the 3'-terminus of the PHEX gene is deleted, resulting in a defect of sodium-dependent phosphate co-transport identical to that which exists in patients with XLH. Using this animal model, the first question studied was if this defect was caused by an intrinsic renal membrane defect or by a hormonal abnormality that was influencing the Npt2.

Initial investigations revealed that, phosphate transport in both the brush border membrane vesicles from the renal cortex of the hyp-mouse and in primary cell cultures of the hyp-mouse was decreased (26). In concert, the Npt2 mRNA and the protein were decreased in the kidneys of the hyp-mouse (27–29). These observations supported the existence of an intrinsic or nascent defect in the renal proximal tubule cell membrane. In contrast, parabiosis of normal and hyp-mice transmitted the renal transport defect from the mutants to the normal mice (30, 31). Similarly, in patients with XLH, renal transplantation from healthy donors resulted in no improvement in the phenotype of the patients. These observations paradoxically suggested that the renal phosphate transport defect in XLH is secondary to an hormonal abnormality, which influences the sodium-dependent phosphate transport by decreasing the membrane bound Npt2a. Further support for this possibility derived from studies of renal transplantation in normal and hyp-mice. Normal mice receiving kidneys from hyp-mice maintained normal serum levels of phosphorus, but hyp-mice transplanted with normal kidneys continued to have hypophosphatemia (32) (Fig. 2). These observations clearly established that there
exists a humoral defect in hyp-mice that affects the kidneys.

In subsequent studies, the mineralization defect in XLH was investigated in vitro using osteoblasts from normal and hyp-mice (33). Using alizarin red staining, it was confirmed that normal osteoblasts in plates were well mineralized, while osteoblasts from hyp-mice showed virtually no mineralization. In accord, the normal osteoblasts showed a remarkable increase in calcium incorporation as they mineralized, whereas the hyp-mouse osteoblasts had no calcium accrual. A co-culture of the normal and hyp-mouse osteoblasts was performed in a plate partitioned into upper and lower compartments by a semi-permeable membrane. When the normal osteoblasts were placed in both of the plates, normal mineralization was seen in the normal osteoblasts in the lower chamber. When normal osteoblasts were in the lower plate and osteoblasts from hyp-mice in the upper chamber, abnormal mineralization was induced in the normal osteoblasts, suggesting that some factors from the upper plate had crossed the semi-permeable membrane and induced abnormal mineralization in the normal osteoblasts. These results indicate that both the phosphate transport and osteoblast mineralization appear to be a hormonal abnormality.

**Abnormality in Vitamin D Metabolism and Role of Npt2**

Abnormal vitamin D metabolism in patients with XLH is characterized by: (1) a decreased circulating level of 1α,25-(OH)₂D, relative to the prevailing hypophosphatemia; (2) diminished responsivity of the serum 1α,25-(OH)₂D level to parathyroid hormone (PTH) stimulation, compared to age-matched controls (34); and (3) a subnormal increase of the serum 1α,25-(OH)₂D level upon dietary phosphorus deprivation (35).

We have investigated 25-(OH)D₃-1α-hydroxylase (1α-hydroxylase) which is responsible for the production of active vitamin D, 1α,25-(OH)₂D, in the kidneys. We found that there was no difference in the baseline 1α-hydroxylase activity of normal and hyp-mice, whereas phosphorus depleted (P-depleted) normal mice exhibited significantly increased enzyme activity (36). When the mice were stimulated with PTH, the renal 1α-hydroxylase was dramatically increased in the normal mice and the P-depleted normal mice, but in the hyp-mice, PTH failed to elevate the 1α-hydroxylase level. However, thyrocalcitonin (TCT), which allegedly affects 1α-hydroxylase only in the proximal straight tubules, significantly elevated the levels of 1α-hydroxylase in the normal, P-depleted normal, and hyp-mice. These data suggest that the defect in vitamin D metabolism occurs in the proximal convoluted tubules, the site for abnormal regulation of phosphate transport, as well as PTH stimulation of 1α-hydroxylase. Moreover, the absence of an apparent abnormality in vitamin D metabolism upon stimulation with TCT indicates that the defect is localized to the proximal convoluted tubules, since the site of TCT action is the proximal straight tubule.

Further characterization of the defect resulted from the studies of Dr. Ikuma Fujiwara, when he was in my laboratory. His work demonstrated that in hyp-mice stimulated with PTH, the mRNA for the 1α-hydroxylase was increased comparably to that in normal and P-depleted normal mice (37). These observations suggest that the defect in 1α-hydroxylase activity is a post-transcriptional abnormality (Fig. 3). We confirmed this conclusion through Western Blot analysis, when we found that 1α-hydroxylase protein in hyp-mice, despite hypophosphatemia, was no different from that in normal mice and did not increase upon PTH stimulation (38). In addition, consistent with previous observations regarding TCT stimulation, we observed that TCT treatment increased mRNA transcripts as well as protein comparably in normal and hyp-mice, thereby excluding the presence of a post-
transcriptional abnormality in the proximal straight tubules. To exclude the possibility that the post-transcriptional abnormality was enhanced degradation of 1α-hydroxylase, we treated normal and hyp-mice with protease inhibitors, which did not alter the presence or magnitude of the abnormality. Thus, we confirmed that the defect in vitamin D metabolism in the hyp-mouse is a failure to produce 1α-hydroxylase protein in response to mRNA.

**PHEX Defect, the Causative Abnormality**

A collaboration with other laboratories to clone the gene underlying XLH resulted in the analysis of data from 13 multigenerational pedigrees, refined mapping of the Xp22.1-p21 region of the X chromosome by restriction fragment polymorphism, identification of tightly linked flanking markers for the HYP locus, construction of a YAC contig spanning the HYP gene region, and eventual cloning and identification of the disease gene as PHEX, a Phosphate regulating gene with homologies to Endopeptidases located on the X chromosome (39–43). These studies ascertained a locus order on Xp22.1 of:

Xcen-DXS451-DXS41/DXS92-DXS274-DXS1052-DXS1683-HYP-DXS7474-
Moreover, the physical distance between the flanking markers, DXS1683 and DXS7474, was determined as 350 kb and their location on a single YAC ascertained. Subsequently, a cosmid contig spanning the HYP gene region was constructed and efforts directed at discovery of deletions within the HYP region. Identification of several such deletions permitted characterization of cDNA clones that mapped to cosmid fragments in the vicinity of the deletions. Database searches with these cDNAs detected homologies at the peptide level to a family of endopeptidase genes that includes neutral endopeptidase (NEP), endothelin-converting enzyme-1 (ECE-1) and the Kell antigen. These efforts clearly established PHEX as the candidate gene responsible for XLH (Fig. 4).

The tissue localization of PHEX is primarily in the bones and the teeth, but certainly not in the kidneys where some of the major physiological abnormalities occur (44, 45). Thus, it remains unknown the manner by which a genetic defect in a cell membrane-bound enzyme limited to the bones and teeth underlies a hormone-dependent disorder with a phenotypic abnormality secondary to defects in the kidney. Indeed, although abnormal PHEX is seen in 2/3 of XLH patients, we do not know how the PHEX is connected to the decrease in sodium-dependent phosphate co-transport protein in the kidney or for that matter the decreased bone mineralization and abnormal vitamin D metabolism. The confusion regarding the cascade of events that transfers the genetic abnormality to the hyp-mouse phenotype has been further heightened by the inability to rescue the phenotype. In in vitro studies, the transfection of osteoblasts failed to create normal mineralization in hyp osteoblasts (46). In addition, PHEX transfection using the osteocalcin and the collagen promoters failed to normalize both the hypophosphatemia and the abnormal mineralization of the bone (46, 47). Finally,
Miyamura et al. carried out a bone marrow transplantation attempting the rescue and, although the results were better, the mRNA for the renal sodium-dependent phosphate co-transporter did not reach that in normal mouse kidneys (48). In any case, a full understanding of the role that osteoblast PHEX plays depends on finding the substrate of PHEX, which translates the message of the gene defect throughout the body. Clearly, PHEX must have an interpolator that carries out its effects at the kidneys and at the osteoblasts, which have been called phosphatonin and ninhibin (49–55). However, it remains unknown if these presumptive proteins are substrates for PHEX, bind to PHEX, thereby precluding enzymatic activation, or merely serve as intermediary substrates, which in turn activate the putative phosphatonin or minhibin. Indeed, it is unclear if phosphatonin and minhibin are a single protein or a family of proteins. Currently, there are 3 so-called candidates for phosphatonin or minhibin that have been presented: 1) fibroblast growth factor 23 (FGF-23) (49–55); 2) matrix extracellular phosphoglycoprotein (MEPE) (56–62); and 3) Frizzled related protein 4 (FRP-4) (63, 64). Although each of these proteins may be members of the family of hormones that are critical to the pathogenesis of XLH, not any one of these factors satisfies the criteria for designation as “the” phosphatonin or minhibin (Table 1).

Even the presentation of new information about these candidate proteins at the recent ASBMR meeting in Seattle failed to clarify the enigmatic status of the factors. In this regard, FGF-23 knockout mice while having abnormalities in their bone mineralization typical of XLH had paradoxically immeasurably low circulating levels of PTH. Likewise conflicting were the observations that the serum FGF-23 levels in patients with XLH were variably elevated while hyp-mice had a 10-fold elevation and patients with various cancers had a 2.5-10-fold elevation in serum FGF-23 levels without any associated abnormalities of phosphorus, vitamin D, or bone metabolism. And finally, administration of FGF-23 to mice in vivo decreased 1α-hydroxylase activity by limiting transcription and not by altering translation as occurs in hyp-mice. Moreover, in contrast, the transgenic expression of FGF-23 variably resulted in suppression of the 1α-hydroxylase activity by an inhibition of transcription or translation.

With respect to MEPE, the MEPE knockout mice had no abnormalities in phosphate homeostasis and intact MEPE did not possess any discernable effects on renal phosphate transport. Further, the administration of MEPE to mice in vivo decreased 1α-hydroxylase activity by decreasing transcription and not by altering translation as occurs in hyp-mice. And perhaps most notably, transfection of the MEPE knockout genotype to the hyp-mouse did not alter the hypophosphatemia or abnormal bone mineralization of the phenotype.

In retrospect, several years ago, we designed a treatment regimen for XLH, including enough 1α,25-(OH)2D3 and phosphorus, to normally mineralize bone. However, the treatment cannot be regarded as the safest regimen. Thus, it is

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Activity of phosphatonin/minhibin candidate proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phex substrate</td>
<td>FGF-23</td>
</tr>
<tr>
<td>↑ Hyp osteoblast production</td>
<td>In vitro</td>
</tr>
<tr>
<td>↑ Circulating level</td>
<td>In vitro</td>
</tr>
<tr>
<td>Inhibits bone mineralization</td>
<td>In vitro</td>
</tr>
<tr>
<td>Inhibits phosphate transport</td>
<td>In vitro</td>
</tr>
<tr>
<td>Influences vitamin D metabolism</td>
<td>In vitro</td>
</tr>
<tr>
<td>Calcitriol/PI regulated production</td>
<td>+</td>
</tr>
</tbody>
</table>
fortunate that at present there are enough laboratories interested in clarifying the genetic and molecular-biological background of XLH that we will move closer and closer to achieving understanding of the disease and designing better treatment strategies. In any event, it is clear that following the discovery of PHEX, a perplexing (perPHEXing) trip from bench to bedside continues.

References

39. Francis F, Rowe PS, Econs MJ, See CG, Benham F,


57. Rowe PSN, Garrett IR, Schwarz PM, Carnes DL, Lafer EM, Mundy GR, et al. Direct binding of MEPE by PHEX via the ASARM-motif is confirmed by surface plasmon resonance: Implications for impairment of mineralization in X-linked rickets. J
