Differential regulation of PHEX expression in bone and parathyroid gland by chronic renal insufficiency and 1,25-dihydroxyvitamin D₃

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Brewer, Angela J., Lucie Canaff, Geoffrey N. Hendy, and Harriet S. Tenenhouse. Differential regulation of PHEX expression in bone and parathyroid gland by chronic renal insufficiency and 1,25-dihydroxyvitamin D₃. Am J Physiol Renal Physiol 286: F739–F748, 2004. First published December 23, 2003; 10.1152/ajprenal.00321.2003.—Mutations in the PHEX gene are responsible for X-linked hypophosphatemia, a renal phosphate-wasting disorder associated with defective skeletal mineralization. PHEX is predominantly expressed in bones and teeth and in the parathyroid gland of patients with chronic renal failure and tertiary hyperparathyroidism. The purpose of the present study was to examine the effects of renal insufficiency and 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] on the regulation of PHEX expression in rat tibia and parathyroid gland. In rats fed a high-phosphate (Pᵢ) diet, 5/6 nephrectomy elicited a significant increase in the serum parathyroid hormone (PTH) concentration that was associated with a significant increase in the abundance of PHEX mRNA and protein in the tibia and a significant increase in PHEX mRNA in the parathyroid gland. In contrast, 1,25(OH)₂D₃ administration to intact rats fed a control diet elicited a significant decrease in the serum PTH concentration that was accompanied by a significant decrease in PHEX mRNA and protein abundance in the tibia and a significant decrease in PHEX mRNA in the parathyroid gland. In addition, the increases in serum PTH levels and PHEX mRNA in the tibia and parathyroid gland in 5/6 nephrectomized rats fed a high-Pᵢ diet were blunted by 1,25(OH)₂D₃. Serum PTH concentration was positively and significantly correlated with tibial PHEX mRNA and protein abundance. In summary, we demonstrate that PHEX expression in the tibia and parathyroid gland is increased by chronic renal insufficiency and decreased by 1,25(OH)₂D₃ administration and suggest that PTH status may play an important role in mediating these changes in PHEX expression.

Parathyroid hormone; hyperphosphatemia; endopeptidase

MUTATIONS in PHEX, a phosphate-regulating gene with homology to endopeptidases on the X chromosome, are responsible for X-linked hypophosphatemia (XLH) (16), the most prevalent form of inherited rickets in humans (38). XLH is a dominant disorder of phosphate (Pᵢ) homeostasis characterized by growth retardation, rickets and osteomalacia, hypophosphatemia, and renal defects in Pᵢ reabsorption and the regulation of vitamin D metabolism (38). The PHEX gene exhibits significant homology to the M13 family of zinc metallopeptidases, type II integral membrane glycoproteins involved in the activation or inactivation of a variety of peptide hormones (40). Although PHEX exhibits endopeptidase activity (4), the nature of its endogenous substrates and their role in the regulation of skeletal function and renal Pᵢ transport and vitamin D metabolism have not been elucidated. Accordingly, the mechanism whereby loss of PHEX function elicits the clinical phenotype in XLH patients is not understood.

PHEX mRNA is expressed predominantly in bones and teeth (2, 8, 33), and PHEX protein was localized to osteoblasts, osteocytes, and odontoblasts both prenatally and postnatally (27, 34, 39). These findings are compatible with the skeletal and dental abnormalities that characterize XLH and the murine hyp and Gy homologs of XLH (11, 17, 37). Of interest is the finding that PHEX is not expressed in the kidney (2, 33, 34). The latter is consistent with the demonstration that the renal Pᵢ leak in the murine hyp homolog of XLH is not intrinsic to the kidney but rather is dependent on a circulating factor (25, 26, 30). On the basis of the above findings, it was suggested that PHEX is involved in the activation or degradation of autocrine or paracrine factors involved in bone mineralization and circulatory factors that influence renal Pᵢ reabsorption and vitamin D metabolism (37).

Several studies have examined the regulation of PHEX mRNA and protein expression. The active vitamin D hormone, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], decreases PHEX mRNA and protein abundance in primary osteoblast cultures derived from newborn mouse calvaria as well as in MC3T3-E1 cells, a mouse osteoblastic cell line (10). In contrast, both growth hormone and IGF-I increase PHEX mRNA expression in bone when administered to hypophysectomized rats (44). Glucocorticoids also increase PHEX mRNA abundance in mouse calvaria and rat osteogenic sarcoma (UMR-106) cells, and an atypical glucocorticoid-response element was identified in the promoter region of the mouse PHEX gene (13). In addition, the chondrogenic factor SOX9 was found to stimulate PHEX promoter activity, suggesting a role for PHEX in cartilage as well as bone (22).

PHEX expression has also been detected in the parathyroid gland derived from two patients with tertiary hyperparathyroidism (3), one with chronic renal insufficiency and the other with XLH. The second patient exhibited significant hyperparathyroidism before initiation of treatment with Pᵢ and 1,25(OH)₂D₃ and eventually developed tertiary hyperparathyroidism after treatment (3). The function of PHEX in the parathyroid gland is not known. However, based on the demonstration that membrane preparations of PHEX-transfected cells could degrade exogenous parathyroid hormone (PTH) (21), it has been suggested that PHEX in the parathyroid gland may serve to regulate the concentration of PTH.

Given that PHEX is involved in the regulation of Pᵢ homeostasis and is expressed in the parathyroid gland under conditions
where PTH synthesis and secretion are increased, we hypothesized that PTH may be a regulator of PHEX gene expression. Moreover, because patients with chronic renal failure develop hyperphosphatemia and severe hyperparathyroidism, we hypothesized that PHEX expression may be increased under these conditions. As a first step in addressing these issues, the present study was undertaken to examine the regulation of PHEX expression in the tibia and parathyroid gland in a rat model of chronic renal failure. We also examined the effect of 1.25(OH)2D3 on the same parameters in intact and nephrectomized rats to determine whether inhibition of PTH synthesis and secretion can reverse the effects of renal insufficiency on PHEX expression.

MATERIALS AND METHODS

Animal models. Sprague-Dawley male rats weighing 180–200 g were purchased from Charles River Laboratories (St. Constant, Quebec). The rats were either intact, sham operated (sham), or nephrectomized. Two diets were used in the present study: a control diet (Ralston Purina, LaSalle, Quebec), containing 1.01% calcium, 0.74% phosphorus, and 3.3 IU vitamin D3/g, and a high-Pi diet (TD.94238, Ralston Purina, LaSalle, Quebec), containing 1.01% calcium, 1.4% phosphorus, and 1.0 IU vitamin D3/g. Spot urine was collected before death and stored at −20°C. Blood was obtained by cardiac puncture, and the serum was separated and stored at −20°C. All rats were killed using CO2. Microdissected parathyroid glands (36) and tibiae were placed immediately in liquid N2 and kept at −80°C until analyzed.

The following groups of rats were studied: (1) sham and nephrectomized rats fed either the control or high-Pi diet for 5 wk; (2) intact rats fed the control diet for 3 days and then injected with mineral oil or 1.25(OH)2D3 (Rocaltril, 10 pmol/g body wt ip, Roche, Laval, Quebec) 48 and 24 h before death; and (3) sham and nephrectomized rats fed the high-Pi diet for 5 wk and then injected with 1.25(OH)2D3 (10 pmol/g body wt) or vehicle as in group 2. Each experimental protocol was repeated three times, and essentially identical results were obtained. Results of a representative experiment with 6–16 rats/group are shown.

Analysis of serum and urine parameters. Serums were analyzed for Pi, creatinine, urea, and calcium concentrations by the Royal Victoria Hospital Clinical Biochemistry Department. Serum PTH was measured using a Rat Intact PTH Elisa kit (Immutopics, San Clemente, CA). Urine was analyzed for calcium, Pi, and creatinine concentrations using Stanbio kits (San Antonio, TX).

RNA isolation. Total RNA was prepared from the parathyroid gland and tibia using the standard TRIzol method as recommended by the manufacturer (GIBCO BRL, Burlington, Ontario). For the extraction, 1 ml TRIzol/35 mg bone was used. The tibial ends, proximal to the knee and tibia using the standard TRIzol method as recommended by the manufacturer (Stanbio kits, San Antonio, TX). Once the bone was added to the TRIzol, it was further crushed for 30 s using a Polytron. The samples were frozen overnight at −80°C before completion of the extraction. When the TRIzol-containing bone was thawed, an additional spin was performed to remove the bone fragments before the addition of chloroform. In the case of the parathyroid gland, two microdissected glands from an individual rat were homogenized in TRIzol using a Kontes Pestle Pestle Mixer (VWR, Ville Mont-Royal, Quebec), after which the standard extraction method was used.

Semiquantitative RT-PCR. RT-PCR was used to estimate the abundance of PHEx and PTH mRNAs, relative to GAPDH mRNA, in the parathyroid gland as was previously described (7). The primer sequences used for the PHEX transcripts were as follows: forward primer 5'-AGAATTGATTGAGGTCCTGAC-3' and reverse primer 5'-TCGAGACCTCACCATTACAGA-3'; for the PTH transcripts, the sequences were forward primer 5'-TTTTGGCTCTTACCGAGATGGGAAACTTGTGTTAG-3' and reverse primer 5'-TAAATACCTCACCTTTGTCCTCCCCGTCAAGACT-3'; and for the GAPDH transcripts, the sequences were forward primer 5'-CCCTTCTATT-GACCTCAACTACATG-3' and reverse primer 5'-CAGAAAGACTGTTGATGGCCCTCC-3'.

RNaase protection assay. PHEX mRNA expression was determined and compared with that of β-actin mRNA using PHEX and β-actin riboprobes, respectively, as described by Beck et al. (2). All cDNA inserts were generated by RT-PCR and confirmed by sequencing. For rat 5′-PHEX, a 742-bp cDNA insert (nucleotides 142–884) was subcloned into the pCR2.1 vector in the 5′- to 3′- direction using the EcoRI restriction site. The plasmid was linearized with the restriction enzyme Acc1. The antisense riboprobe created was 392 bp in length, and the protected fragment contained 325 bp. As an internal standard, we used an 833-bp mouse β-actin cDNA insert (nucleotides 1–833) subcloned into the pGEM3 vector in the 5′- to 3′- orientation between the HindIII and KpnI restriction sites. The plasmid was linearized with the restriction enzyme CvnI. The antisense riboprobe generated was 160 bp in length, and the protected fragment contained 137 bp, as previously described (2).

Protein extraction. Tibia midshafts were lyophilized for 4 days, crushed in liquid N2 with a mortar and pestle, and shaken in 0.05% Triton-PBS at 4°C for 3 days. Samples were then centrifuged at 4°C for 30 min at 14,000 g. The supernatants were analyzed for protein concentration using the Lowry method (24). When necessary, samples were concentrated using YM-30 Centriprep centrifugal filters (Millipore, Mississauga, Ontario) and reassayed for protein concentration. Samples were stored at −80°C until used for Western blot analysis.

Western blot analysis. The protein extracts were incubated in the presence or absence of PNGaseF (New England Biolabs, Mississauga, Ontario) for 1 h as specified by the manufacturer. The PHEX protein was susceptible to deglycosylation by PNGaseF as described previously (4). Moreover, deglycosylation of PHEX led to the appearance of cleaner and sharper bands for quantitation. Thus the results presented were generated from deglycosylated protein samples. The samples were boiled for 3 min in 6× Laemmli sample buffer before fractionation on SDS-PAGE (10% [vol/vol] gel). The proteins in the gel were transferred to a nitrocellulose filter for 1 h using standard methods. PHEX proteins were detected by immunoblotting with PHEX 13B12 monoclonal antibody (1,200 dilution) (34). This was followed by incubation with peroxidase-conjugated anti-mouse IgG (PI-2000, Vector Laboratories, Burlingame, CA) at 1:3,000 dilution. Actin, which was detected using an actin antibody (A2066, Sigma, Oakville, Ontario) at 1:2,000 dilution, was used as a loading marker and detected using peroxidase-conjugated anti-rabbit IgG at 1:3,000 dilution (PI-1000, Vector Laboratories). An ECL detection kit (Amersham Pharmacia, Baie D’Urfé, Quebec) was used to visualize the bands according to the manufacturer’s instructions. The abundance of deglycosylated PHEX protein, relative to actin, was quantitated using FujiScan software, as described (14).

Statistical analysis. Data were analyzed using a t-test assuming unequal variances or ANOVA as needed. Significance was determined as P < 0.05. All values are given as means ± SE.

RESULTS

Effect of % nephrectomy on serum and urine parameters. Serum and urine parameters in sham and % nephrectomized rats fed the control and high-Pi diets for 5 wk are shown in Table 1. In rats fed either diet, % nephrectomy elicited significant increases in the serum concentration of creatinine, urea, Pi, and PTH compared with sham-operated controls (Table 1). However, the increases in serum parameters were more pronounced in the % nephrectomized rats fed the high-Pi diet than in % nephrectomized rats fed the control diet (Table 1). In addition, the high-Pi diet elicited a modest but significant increase in the serum PTH concentration in sham-operated rats compared with sham animals fed the control diet (Table 1). In
rats fed both the control and high-Pi diets compared with sham-operated rats, % nephrectomy generally led to a modest decrease in the serum calcium concentration.

In rats fed the control diet, % nephrectomy had no apparent effect on the urine Pi/creatinine and urine calcium/creatinine ratios (Table 1). However, in rats fed the high-Pi diet, the urine Pi/creatinine ratio was significantly higher in % nephrectomized rats than in sham-operated animals (Table 1). In addition, the high-Pi diet elicited a marked increase in urinary Pi excretion in both sham and % nephrectomized rats compared with their counterparts fed the control diet (Table 1). Finally, % nephrectomy elicited changes in the urine cAMP/creatinine ratio (data not shown) that were consistent with changes in the serum PTH concentration (Table 1).

Effect of % nephrectomy on PHEX expression in tibia and parathyroid gland. In rats fed the control diet, % nephrectomy elicited a 46% increase in the abundance of PHEX mRNA, relative to that of β-actin, in the tibia (Fig. 1A). PHEX protein abundance was also significantly increased in % nephrectomized rats maintained on the normal diet compared with their sham-operated counterparts (Fig. 1B). In rats fed the high-Pi diet, % nephrectomy also elicited a significant increase in PHEX mRNA abundance (Fig. 2A) and protein abundance (Fig. 2B) in tibia compared with sham-operated rats on the same diet.

In the parathyroid gland of rats fed the control diet, % nephrectomy led to a significant increase in PTH mRNA abundance (Fig. 3A). However, there was no effect of % nephrectomy on PHEX mRNA abundance in the parathyroid gland of rats under these conditions (Fig. 3B). In contrast, in rats fed the high-Pi diet, % nephrectomy elicited a significant increase in both PTH and PHEX mRNA expression in the parathyroid gland (Fig. 4, A and B). Moreover, the parathyroid gland responses to % nephrectomy were more robust in rats fed the high-Pi diet than in rats on the control diet (Fig. 3 vs. Fig. 4).

Effect of 1,25(OH)2D3 on serum and urine parameters. Table 2 summarizes the effects of 1,25(OH)2D3 on serum and urine parameters in intact rats fed the control diet. Serum creatinine and urea concentrations were similar in the vehicle-and 1,25(OH)2D3-treated groups (Table 2). Serum Pi, and serum calcium concentrations increased significantly in the 1,25(OH)2D3-treated rats compared with the vehicle-injected rats (Table 2), whereas serum PTH concentrations decreased significantly with 1,25(OH)2D3 treatment (Table 2), as expected.

Urinary Pi and calcium excretion, relative to urinary creatinine excretion, both increased significantly in response to 1,25(OH)2D3 treatment (Table 2). This is most likely due to the increase in serum concentrations of both ions in 1,25(OH)2D3-treated rats. The urinary cAMP concentration, relative to that of creatinine, was significantly decreased in response to 1,25(OH)2D3 treatment (data not shown), consistent with the changes in serum PTH concentration (Table 2).

Effect of 1,25(OH)2D3 on PHEX expression in tibia and parathyroid gland. 1,25(OH)2D3 administration to intact rats

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**Table 1. Effect of 5/6 nephrectomy on serum and urine parameters in rats fed control and high-Pi diets**

<table>
<thead>
<tr>
<th></th>
<th><strong>Control</strong></th>
<th><strong>High Pi</strong></th>
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<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Nephrectomy</td>
</tr>
<tr>
<td></td>
<td>High Pi</td>
<td>Nephrectomy</td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine, mmol/l</td>
<td>18.9 ± 2.0</td>
<td>20.25 ± 1.6</td>
</tr>
<tr>
<td>Urea, mmol/l</td>
<td>3.9 ± 0.9</td>
<td>3.35 ± 0.3</td>
</tr>
<tr>
<td>Phosphate, mmol/l</td>
<td>2.6 ± 0.3</td>
<td>2.44 ± 0.1</td>
</tr>
<tr>
<td>Calcium, mmol/l</td>
<td>2.48 ± 0.1</td>
<td>2.45 ± 0.1</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pi/creatinine</td>
<td>2.7 ± 0.6</td>
<td>23 ± 2.6†</td>
</tr>
<tr>
<td>Ca/creatinine</td>
<td>0.1 ± 0.02</td>
<td>0.2 ± 0.03†</td>
</tr>
</tbody>
</table>

Values are means ± SE (serum parameters, n = 8–16/group; urine parameters, n = 6–7/group). PTH, parathyroid hormone. *P < 0.05, effect of nephrectomy. †P < 0.05, effect of diet.

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**Fig. 1. Effect of % nephrectomy on PHEX mRNA (A) and protein abundance (B) in tibia of rats fed the control diet. A: abundance of PHEX mRNA, relative to β-actin mRNA, in tibia of sham-operated (Sham) and % nephrectomized rats (Nx) fed the control diet was determined by RNase protection assay, as described in MATERIALS AND METHODS. Autoradiographs of representative RNase protection analysis signals depicting the 325-bp protected PHEX fragment and the 137-bp protected β-actin fragment are shown. Each bar represents the mean ± SE obtained from 12–14 rats/group. B: abundance of deglycosylated PHEX protein, relative to actin, in tibia extracts prepared from the same groups of rats was determined by Western blot analysis using PHEX and actin antibodies, as described in MATERIALS AND METHODS. Representative immunoblots depicting the ~87-kDa deglycosylated PHEX protein and the 42-kDa actin protein are shown. Each bar represents the mean ± SE obtained from 12–14 rats/group. *P < 0.05, effect of % nephrectomy.**
elicited a significant decrease in PHEX mRNA (Fig. 5A) and protein abundance (Fig. 5B) in the tibia. Administration of 1,25(OH)₂D₃ also significantly decreased PTH and PHEX mRNA expression in the parathyroid gland (Fig. 6, A and B).

**Effect of % nephrectomy and 1,25(OH)₂D₃ on serum parameters and PHEX expression in tibia and parathyroid gland.**

We sought to determine whether the administration of 1,25(OH)₂D₃, at 1 and 2 days before death, would attenuate the increase in PHEX mRNA abundance that occurs with % nephrectomy in rats fed the high-P̄ diet.

The serum creatinine, urea, P̄, and PTH concentrations were significantly increased in both vehicle- and 1,25(OH)₂D₃-treated, % nephrectomized rats compared with their sham-operated counterparts (Table 3). 1,25(OH)₂D₃ appeared to blunt the increase in serum P̄ and PTH in % nephrectomized rats (Table 3). The serum calcium concentration was significantly decreased in both vehicle- and 1,25(OH)₂D₃-treated, % nephrectomized rats compared with their sham-operated counterparts and was increased in the 1,25(OH)₂D₃-treated groups compared with their vehicle-treated counterparts (Table 3).

In the tibia of both vehicle-treated and 1,25(OH)₂D₃-treated rats, % nephrectomy elicited a significant increase in PHEX mRNA abundance (Fig. 7). Tibial PHEX mRNA expression was significantly lower in the 1,25(OH)₂D₃-treated groups than in their corresponding vehicle-treated counterparts (Fig. 7).
Thus treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3} appeared to blunt the increase in tibial PHEX expression induced by \% nephrectomy.

In parathyroid glands of both vehicle-treated and 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated rats, \% nephrectomy also elicited a significant increase in PTH and PHEX mRNA abundance (Fig. 8). In addition, expression of both PTH and PHEX mRNA was significantly lower in the 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated groups than in their corresponding vehicle-treated counterparts (Fig. 8). Thus 1,25(OH)\textsubscript{2}D\textsubscript{3} appeared to blunt the increase in PTH and PHEX expression in parathyroid glands induced by \% nephrectomy.

Correlation between tibial PHEX expression and parathyroid gland status. We showed that both serum PTH and tibial PHEX mRNA and protein expression are increased in response to \% nephrectomy and decreased after the administration of 1,25(OH)\textsubscript{2}D\textsubscript{3}. Using the data generated from all experiments, we demonstrated that the serum PTH concentration is significantly and positively correlated with PHEX mRNA in parathyroid glands induced by \% nephrectomy (Fig. 8). Thus treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3} appeared to blunt the increase in PTH and PHEX expression in both tissues is decreased.

Effect of \% nephrectomy and 1,25(OH)\textsubscript{2}D\textsubscript{3} on neutral endopeptidase 24.11 (NEP) mRNA expression in rat tibia. We also assessed neutral endopeptidase 24.11 (NEP) mRNA expression in rat tibia to determine whether the regulation of this related metalloendopeptidase correlated with that of PHEX. We found that neither \% nephrectomy nor 1,25(OH)\textsubscript{2}D\textsubscript{3} elicits comparable changes in PHEX and NEP mRNA, indicating that PHEX and NEP expression are not coordinately regulated in these models (data not shown).

**DISCUSSION**

We report that PHEX expression in bone and parathyroid gland are differentially regulated by chronic renal insufficiency and the administration of 1,25(OH)\textsubscript{2}D\textsubscript{3}. In the \% nephrectomized rat model, PHEX expression in the tibia and parathyroid gland is increased, whereas in intact rats treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} PHEX expression in both tissues is decreased. Furthermore, the increase in PHEX expression in the tibia and parathyroid gland of \% nephrectomized rats is blunted by treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3}. Analysis of the data from all rats reveals a positive and significant correlation between circulating PTH levels and tibial PHEX mRNA and protein abundance. The present findings in \% nephrectomized rats are consistent with previous studies of PHEX expression in the parathyroid gland of patients with chronic renal failure and tertiary hyperparathyroidism (3) and suggest that PTH is an important regulator of PHEX expression and that PHEX may play a role in modulating PTH status and the skeletal abnormalities associated with renal insufficiency (15, 28).

An important question relates to the biological significance of the observed changes in tibial and parathyroid gland PHEX expression in response to \% nephrectomy and 1,25(OH)\textsubscript{2}D\textsubscript{3}. In this regard, it is important to consider the results of studies in humans and mice with X-linked hypophosphatemia, a dominant disorder of Pi homeostasis associated with impaired skeletal mineralization and caused by inactivating mutations in the \textit{PHEX}/\textit{Phex} gene (37). The disease phenotype is as severe in affected males and females (31, 43), as well as in female mice carrying either one or two copies of the mutant allele (31, 32, 41, 42, 43).
These findings, which provide evidence for haploinsufficiency of PHEX (41), indicate that PHEX protein produced from the wild-type allele in affected heterozygous females does not reach the threshold level necessary for normal function and that the disease phenotype is manifest in the face of a 50% decrease in PHEX expression. Accordingly, it is likely that a smaller decrement or increment in PHEX expression may also be associated with significant clinical and biochemical consequences. Thus the differences in PHEX mRNA and protein expression reported in our studies are almost certain to be of functional importance.

Previous work demonstrated that 1,25(OH)\(_2\)D\(_3\) significantly decreases PHEX expression in mouse calvarial osteoblast cultures in vitro (10), thereby providing evidence for the direct action of 1,25(OH)\(_2\)D\(_3\) on PHEX regulation in bone. The present demonstration that in vivo administration of 1,25(OH)\(_2\)D\(_3\) reduces tibial PHEX expression in intact and \(\frac{5}{6}\) nephrectomized rats is consistent with the in vitro data (10). Given that 1,25(OH)\(_2\)D\(_3\) directly modulates PHEX expression in bone, the increase in PHEX mRNA and protein in the tibia of \(\frac{5}{6}\) nephrectomized rats may be attributed to either the increase in serum PTH, the 50% or more (Ref. 36 and unpublished data) decrease in serum 1,25(OH)\(_2\)D\(_3\) concentration reported in this model, or both. Thus additional studies are necessary to investigate the consequences of elevated serum PTH levels on PHEX expression in bone and parathyroid gland.
in the absence of changes in serum 1,25(OH)2D3. The calcitomin-treated rat, in which the serum PTH concentration is increased acutely without a concomitant fall in serum 1,25(OH)2D3 (7), will serve as a suitable model for addressing this question.

The decrease in PHEX expression in the tibia and parathyroid gland of 1,25(OH)2D3-treated rats demonstrated here may be ascribed to either the direct action of 1,25(OH)2D3 on bone, a 1,25(OH)2D3-mediated decrease in serum PTH levels, or both. In any case, our data in % nephrectomized and 1,25(OH)2D3-treated rats demonstrate a direct and significant relationship between tibial PHEX expression and circulating PTH levels, suggesting a role for PTH in the regulation of PHEX expression.

PHEX expression in bone is localized to osteoblasts and osteocytes (27, 34, 39). Although previous studies reported that PHEX mRNA is expressed in stromal cells derived from bone marrow of young mice (9, 29), we did not detect PHEX mRNA or protein in adult rat tibial bone marrow (unpublished observations). Our data thus reflect PHEX expression in mature tibial osteoblasts and osteocytes of adult rats. In addition, previous studies reported that PHEX expression is significantly higher in the femur and calvaria of newborn mice than in their adult counterparts (33, 34), suggesting that PHEX is involved in early events associated with bone growth and mineralization (34). The present findings support the notion that PHEX also plays an important physiological role in adult rat bone.

It is well known that increasing dietary Pi intake results in a further increase in serum PTH levels in the % nephrectomized rat model of renal insufficiency (5). In addition, parathyroid gland PTH mRNA levels positively correlate with dietary Pi intake in the rat (19). Thus the 50% increase in serum PTH levels in % nephrectomized rats fed the high-Pi diet, relative to counterparts fed the control diet (see Table 1), is consistent with results of earlier studies (5, 19). In addition, we show that the increase in serum PTH levels is associated with a corresponding increase in parathyroid PTH mRNA levels (see Figs. 3 and 4). Furthermore, while no increase in parathyroid PHEX mRNA levels was noted in % nephrectomized rats fed the control diet, a doubling in transcript abundance was observed after they were fed a high-Pi diet. Therefore, it appears that after a threshold level has been reached, PHEX expression in the parathyroid gland increases to match the increase in PTH.

In vivo and in vitro data indicated that transgenic expression of PHEX in osteoblasts of Hyp mice was not sufficient to rescue the mutant Hyp phenotype completely (1, 23). While partial improvement of the mineralization defect was observed (1), neither hypophosphatemia nor disordered regulation of vitamin D metabolism, characteristic of this disorder, was affected by the transgene in osteoblasts (1, 23). These findings suggest that other sites of PHEX expression are likely to be important in controlling P, homeostasis and vitamin D metabolism. One nonosseous site of PHEX expression, which was examined in detail in the present study, is the parathyroid gland (3). While the function of PHEX at this site is not clear, it was demonstrated that recombinant PHEX can degrade PTH (21) and suggested that PHEX may regulate the concentration of PTH.

The present finding that PHEX expression in the tibia and parathyroid gland is upregulated by % nephrectomy may reflect a role for this endopeptidase in the inactivation of PTH, and perhaps other factors that accumulate in the serum of patients with renal insufficiency, to limit their circulating concentrations and, in doing so, their bone resorptive and phosphaturic actions. In this regard, recent studies demonstrated that serum (12) and dialysates (20) from patients with end-stage renal disease specifically inhibit Na-Pi cotransport in cultured renal cells. While little is known about the nature of the circulating phosphaturic factors (referred to as phosphonins), a possible candidate is FGF-23, a novel secreted peptide that is elevated in the serum of patients with renal failure (18). Of relevance to the present study are the recent findings that serum FGF-23 levels are markedly increased in patients with end-stage renal disease (42) and that a COOH-terminal fragment of FGF-23 is degraded by recombinant

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**Table 3. Effect of 1,25(OH)2D3 on serum parameters in sham and 5/6 nephrectomized rats fed the high-Pi diet**

<table>
<thead>
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<th>Serum</th>
<th>Sham</th>
<th>Nephrectomy</th>
<th>Sham</th>
<th>Nephrectomy</th>
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</thead>
<tbody>
<tr>
<td>Creatinine, μmol/l</td>
<td>28.2±0.03</td>
<td>49.5±4.5*</td>
<td>22.2±1.2</td>
<td>38.2±1.1*</td>
</tr>
<tr>
<td>Urea, mmol/l</td>
<td>4.09±0.2</td>
<td>8.8±0.3*</td>
<td>3.8±0.2</td>
<td>8.3±0.4*</td>
</tr>
<tr>
<td>Phosphate, mmol/l</td>
<td>2.9±0.1</td>
<td>4.3±0.1*</td>
<td>2.7±0.06</td>
<td>3.2±0.08**</td>
</tr>
<tr>
<td>Calcium, mmol/l</td>
<td>2.5±0.03</td>
<td>2.3±0.03*</td>
<td>3.1±0.07†</td>
<td>2.9±0.05†</td>
</tr>
<tr>
<td>PTH, pg/ml</td>
<td>39.1±1.3</td>
<td>103.4±5.8*</td>
<td>31.4±2.0</td>
<td>53.9±1.5†</td>
</tr>
</tbody>
</table>

Values are means±SE; n=6/group. *P<0.05, effect of 5/6 nephrectomy. †P<0.05, effect of 1,25(OH)2D3.

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**Fig. 7. Effect of % nephrectomy and 1,25(OH)2D3 on PHEX mRNA abundance in rat tibia. Abundance of PHEX mRNA, relative to β-actin, in tibia from sham and % nephrectomized rats fed the high-Pi diet and injected with either vehicle or 1,25(OH)2D3 (10 pmol/g body wt) was determined by RNase protection assay, as described in MATERIALS AND METHODS. Autoradiographs of representative RNase protection analysis signals depicting the 325-bp protected PHEX fragment and the 137-bp protected β-actin fragments are shown. Each bar represents the mean±SE obtained from 6 rats/group. *P<0.05, effect of % nephrectomy. †P<0.05, effect of 1,25(OH)2D3.**
PHEX (6). Clearly, further work is necessary to establish the overall contribution of PHEX in the clearance of PTH and other circulating phosphaturic factors under conditions of normal and compromised renal function.

NEP and PHEX belong to the same family of zinc metallopeptidases (40) and, therefore, could be regulated in a similar fashion. We therefore examined the effects of 5/6 nephrectomy and 1,25(OH)2 D3 on NEP mRNA abundance in rat tibia. Overall, there was no correlation of NEP expression with either PHEX expression or serum PTH concentrations (data not shown). Thus we found no evidence for coordinate regulation of NEP and PHEX expression in bone. The latter is consistent with the demonstration that the two endopeptidases exhibit different substrate specificity, with the substrate pocket of NEP accommodating hydrophobic amino acid residues and that of PHEX exhibiting strict specificity for hydrophilic acidic amino acid residues (4, 6).

In summary, the present data support an emerging picture of regulation of PHEX expression in bone and parathyroid gland by PTH and 1,25(OH)2 D3 and its potential role in the skeletal manifestations of chronic renal failure. The notion that PHEX expression at nonosseous sites may contribute significantly to mineral ion homeostasis is reinforced. At present, the precise functions of PHEX are not known, but these will become clearer as our understanding of interactions between PHEX and circulating phosphaturic factors advances.

NOTE ADDED IN PROOF

While our manuscript was under review, Vargas et al. (Endocrinology 144: 4876–4885, 2003) reported that high concentrations (10−7 and 10−6 M) of PTH-related protein (1–34) decreased Phex expression in UMR-106 rat osteosarcoma cells and in fetal rat calvarial cultures. The relationship between these in vitro results and the present in vivo data remains to be determined, given that the Phex response to PTH and related peptides may depend on the peptide used, the dose, and the site and differentiation state of the particular skeletal target cell involved.

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