The Regulation of PHEX Expression by 5/6 Nephrectomy and 1,25-dihydroxyvitamin D_3

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ABSTRACT

Mutations in the PHEX gene are responsible for X- linked hypophosphatemia, the most prevalent form of inherited rickets in humans. PHEX is an endopeptidase that is expressed in bone and parathyroid gland and shown to cleave parathyroid hormone [PTH]. To determine whether PHEX is regulated by PTH we used two models: (1) 5/6 nephrectomized [5/6 Nx] rats which exhibit significant hyperparathyroidism and (2) 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$D]-treated rats, which are characterized by marked hypoparathyroidism. We demonstrate that 5/6 Nx elicits an increase in PHEX mRNA and protein abundance in rat tibia whereas 1,25$(OH)_2$D causes a decrease in both tibial parameters. In addition, administration of 1,25$(OH)_2$D to 5/6 Nx rats blunts the increase in PHEX gene expression in bone. We also observe a significant correlation between serum PTH concentration and tibial PHEX expression. Our data thus provides evidence for a role of PTH in the regulation of PHEX expression.

SOMMAIRE

Des mutations dans le gène *PHEX* sont responsables de l'hypophosphatémie liée au chromosome X, la forme la plus commune de rachitisme héréditaire chez l'humain. PHEX est une endopeptidase qui est exprimée dans l'os et la glande parathyroide dont la capacité à couper l'hormone parathyroide [PTH] a été démontrée. Pour déterminer si la PTH a un effet régulateur sur PHEX, nous avons utilisé deux modéles: (1) des rats 5/6 néphrectomizés [5/6 Nx] qui souffrent d'hyperparathyroidisme et (2) des rats injectés avec de la 1,25-dihydroxyvitamine D_3 [1,25(OH)D] , qui présentent de l'hypoparathyroidisme très marqué. Nous démontrons que la 5/6 Nx induit une augmentation des niveaux d'ARNm et de protéine de PHEX dans le tibia du rat tandis que la 1,25(OH)D cause une diminution des deux paramètres dans le tibia. De plus, l'administration de la 1,25(OH)D à des rats 5/6 Nx inhibe l'augmentation de l'expression du gène PHEX observée dans l'os. Nous observons aussi une correlation significative entre la concentration de la PTH dans le sérum et l'expression du PHEX dans le tibia. Nos résultats fournissent des preuves pour un rôle joué par la PTH dans la régulation de l'expression de PHEX.

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ABBREVIATIONS

$1,25(OH)_2D$	1,25-dihydroxyvitamin D_3
ADHR	Autosomal Dominant hypophosphatemic rickets
bp	Base pair
BW	Body weight
Ca	Calcium
cAMP	Cyclic adenosine 3',5'-monophosphate
CaR	Calcium-sensing receptor
cDNA	Complementary deoxyribonucleic acid
Cl	Chlorine
Cr	Creatinine
CRI	Chronic Renal Failure
dCTP	Deoxy-cytosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
ECE-1	Endothelin converting enzyme type 1
ECE-2	Endothelin converting enzyme type 2
FGF-23	Fibroblast growth factor 23
Fig.	Figure
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoids
Gcm2	Glial cells missing 2
Gy	Gyro
Hyp	Hypophosphatemic
kb	Kilobases
kDa	KiloDalton
KO	Knockout
L	Liter
MEPE	Matrix extracellular phosphoglycoprotein
mM	MilliMolar
Mg	Magnesium
mRNA	Messenger ribonucleic acid
N_2	nitrogen
Na	Sodium
NEP	Neutral endopeptidase 24.11
ng	nanograms
Npt2	Na/Pi cotransporter type 2
Nx	Nephrectomy
OHO	Oncogenic hypophosphatemic osteomalacia
OK	Opossum kidney
PCR	Polymerase chain reaction
PHEX	Phosphate regulating gene with homologies to endopetidases on the X chromosome
Pi	Phosphate
pmol	Pico moles

PNGaseF	Peptide:N-glycosidase F
PTG	Parathyroid gland
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related protein
RT	Reverse transcriptase
RVH	Royal Victoria Hospital
SDS	Sodium dodecyl-sulfate
SEM	Standard error of the mean
SSC	Sodium-sodium citrate
TBS	Tris buffered saline
TIO	Tumour induced osteomalacia
U	Units
UTP	Uridine triphosphate
UTR	Untranslated region
VDR	Vitamin D receptor
XLH	X-linked hypophosphatemia

INTRODUCTION

Mutations in the PHEX[1] gene are responsible for X-linked hypophosphatemia (XLH), the most prevalent form of inherited rickets in humans. The treatment for this disease, which consists of oral phosphate supplements and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$), is far from ideal. Moreover, there is little information on the regulation of PHEX expression or the mechanism whereby loss of PHEX function elicits the clinical phenotype. The aim of this study was to examine the regulation of PHEX mRNA and protein in response to changes in parathyroid hormone (PTH) status. To this end, rat models involving 5/6 nephrectomy and $1,25(OH)_2D$ treatment were used. Background information is provided below.

X-linked Hypophosphatemia

X-linked hypophosphatemia (XLH) is a dominant disorder of phosphate (Pi) homeostasis. It is characterized by rickets accompanied by growth retardation and bone pain, hypophosphatemia resulting from a defect in renal reabsorption of Pi as well as a defect in vitamin D metabolism (Tenenhouse, 1999; Econs, 1999; Drezner, 2000). XLH has a prevalence of 1 in 20,000. The Hyp consortium identified, through positional cloning, the gene that is mutated in XLH (HYP Consortium, 1995). The gene was initially termed PEX to depict a phosphate regulating gene with homology to endopeptidases on the X chromosome. This was later changed to PHEX to avoid confusion with the genes involved in peroxisomal disorders. At present, 167 different

[1] Phex denotes mouse gene. PHEX denotes rat and human gene

mutations in the PHEX gene have been identified and can be found in the PHEX database (http://data.mch.mcgill.ca/phexdb) (Sabbagh et al., 2000) but no genotype-phenotype correlations have been discovered and variation in expression of the disease is common even among family members who presumably have the same mutation (Holm et al., 2001).

There are two mouse models available that mimic the phenotype of XLH. The Hyp mouse contains a deletion beginning between exons 15 and 16 of the Phex gene that is 18 to 33kb in length (Beck et al., 1997). The Gy mouse model contains a large deletion spanning the spermine synthase gene, the Phex promoter and the first three exons of the Phex gene (Lorenz et al., 1998; Meyer, Jr. et al., 1998). As a contiguous gene deletion syndrome, Gy mice present additional characteristics to those found in Hyp mice and XLH patients.

Hyp and Gy mice are visually distinguished from their wild type littermates by their shortened tail and hind legs and their lower body-weight (Eicher et al., 1976; Meyer, Jr. et al., 1979). The mice in both models also exhibit the reduced serum Pi and increased urinary Pi excretion (Eicher et al., 1976; Lyon et al., 1986) that is characteristic of the human disease. The Pi leak is caused by a 50% reduction in the mRNA and protein of the type II Na/Pi co-transporter (Npt2) (Tenenhouse et al., 1994; Beck et al., 1996; Tenenhouse and Beck, 1996) and a 20% reduction in the mRNA of the type I Na/Pi cotransporter (Tenenhouse et al., 1998) in the brush border membrane of the renal proximal tubular cells.

Hyp and Gy mice, like patients with XLH, exhibit inappropriately normal levels of $1,25(OH)_2D$ (Tenenhouse et al., 1992; Meyer, Jr. et al., 1980; Tenenhouse and Jones,

1990). The relationship of serum Pi to serum $1,25(OH)_2D$ in these mice are in opposition to that of the relationship in wild type littermates (Tenenhouse et al., 1992; Tenenhouse and Jones, 1990). Serum $1,25(OH)_2D$ is not elevated in Hyp mice in the face of hypophosphatemia which in normal mice is a stimulus for increased renal production of the $1,25(OH)_2D$ hormone (Tenenhouse et al., 1992; Tenenhouse and Jones, 1990). This change in $1,25(OH)_2D$ regulation in Hyp mice has been partially explained by the down regulation of $1,25(OH)_2D$ synthesis (Tenenhouse, 1983; Tenenhouse, 1984a; Tenenhouse, 1984b) and up regulation of $1,25(OH)_2D$ catabolism (Tenenhouse et al., 1992; Tenenhouse and Jones, 1990; Roy and Tenenhouse, 1996; Roy et al., 1994).

The gene that is mutated in XLH and Hyp mice is expressed mainly in the osteoblasts, osteocytes and odontoblasts which is consistent with the bone mineralization defect and tooth abnormalities (Beck et al., 1997; Ruchon et al., 1998; Ruchon et al., 2000a). PHEX is not, however, found in the kidney where the defect in Pi reabsorption takes place. It has been suggested that the loss of PHEX expression in XLH affects the activation or degradation of a humoral/circulatory factor which in turn results in the down regulation of the Na/Pi cotransporters (Fig. 1). Experiments in parabiosis (Meyer, Jr. et al., 1989b; Meyer, Jr. et al., 1989a) and cross-transplantation (Nesbitt et al., 1992) lend support to this hypothesis. Wild type mice parabiosed to Hyp mice began to develop hypophosphatemia and to show evidence of a phosphate leak suggesting that a humoral factor from the Hyp mice traveled through the shared blood circulation to the wild type mouse kidney (Meyer, Jr. et al., 1989b; Meyer, Jr. et al., 1989a). In the cross-transplantation experiment, kidneys from Hyp mice were placed in wild type mice and vice versa. Normal kidneys placed in the Hyp mice began to leak Pi and Hyp kidneys

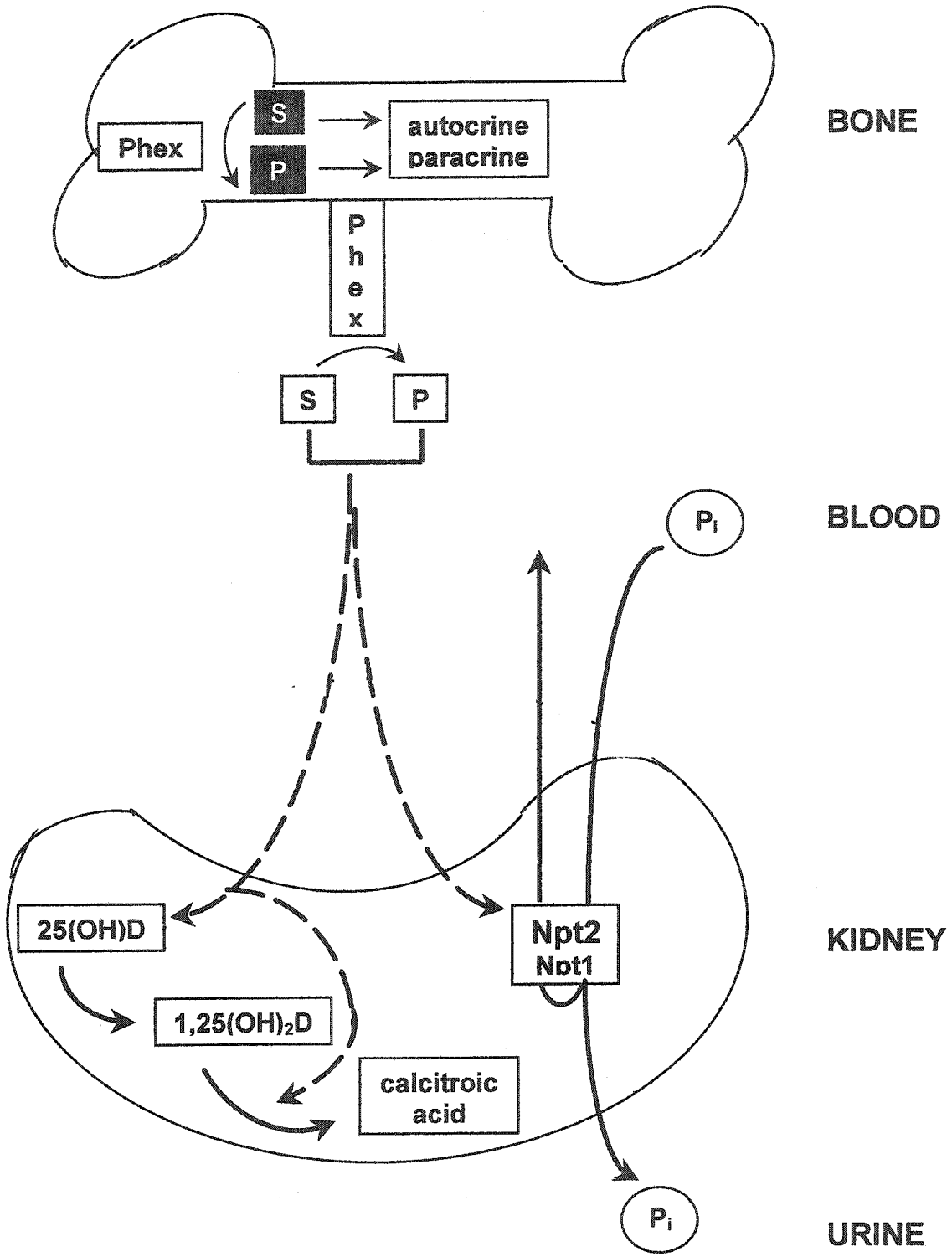


Fig. 1 Schematic of the PHEX model. S - substrate, P- product. PHEX cleaves peptide substrate (s) that are either synthesized in bone or circulate to bone following their production in other tissues. The substrates and/or products down-regulate Npt2 protein in the kidney thereby decreasing the renal reabsorption of Pi. The substrate/products also play a role in the regulation of $1,25(OH)_2D$ synthesis and catabolism. (Tenenhouse, 1999)

placed in wild type mice functioned normally (Nesbitt et al., 1992) again suggesting that the hypophosphatmia and hyperphosphaturia are due to a humoral factor.

Endopeptidases

PHEX demonstrates a high degree of homology at the amino acid level to a group of membrane bound metalloendopeptidases (Turner and Tanzawa, 1997). This family, the M13 family, includes neutral endopeptidase (neprilysin, NEP), the prototype for the family, and endothelin converting enzyme –1 and –2 (ECE-1, ECE-2). These enzymes are type II integral membrane glycoproteins and are characterized by a short N-terminal cytoplasmic domain, a short transmembrane domain, and a long extracellular domain. Studies of NEP revealed that the extracellular domain contains 10 conserved cysteine residues and a zinc-binding motif that are essential for conformational integrity and catalytic activity, respectively.

NEP

Neutral endopeptidase is comprised of approximately 700 amino acid residues (90-100 kDa) and functions as a non-covalently associated homodimer. There are three isoforms of the enzyme found in the rat and each are abundantly expressed in most tissues (Li et al., 1995), such as the kidney, lung and testes, and the brain. The active site of NEP recognizes the N-terminal side of hydrophobic amino acids (Turner et al., 2001). The protein inactivates the various peptides it cleaves. For example, NEP inactivates substance P and enkephalins in the brain, and atrial natriuretic peptide. It also limits

inflammation in the lung (Turner et al., 2001; Turner et al., 2000) by inactivating proinflammatory peptides like endothelins and bradykinins (Turner and Tanzawa, 1997). The NEP knockout mouse is developmentally normal but is at least 100 times more susceptible to septic shock than wild-type littermates (Lu et al., 1995).

ECE-1

Endothelin converting enzyme-1 or ECE-1 is a covalently-bonded homodimer (Shimada et al., 1996) that converts the inactive big endothelin to its active form. It is a 130 kDa protein under reducing conditions and 300 kDa under non-reducing conditions (Turner and Tanzawa, 1997). ECE-1 is most abundant in the epithelial cells and it is also found in some exocrine cells, smooth muscle cells, and in the neurons and glia of the brain (Turner et al., 2001). There are four human isoforms (a to d) which differ in the N-terminal cytoplasmic domains and are variants of the same gene (Turner and Tanzawa, 1997). They also differ in cellular localization. Type 1b is found only intracellularly, and the others are found mainly at the cell surface. It has been suggested that intracellular ECE-1 is involved in the processing of endothelins while the cell surface proteins could be involved in cleaving other peptides (Hoang and Turner, 1997). ECE-1 knockout mice have craniofacial and cardiac defects (Yanagisawa et al., 2000) which are similar to the effects shown in endothelin -1 and -3 knockout mice demonstrating that ECE-1 and the two endothelin isoforms are part of the same pathway.

PHEX

The PHEX gene is 243 Kb in length and contains 22 exons which encode a 749 amino acid protein (Fig. 2) (Tenenhouse, 1999; Econs, 1999). As mentioned earlier, PHEX is mainly expressed in the osteoblasts, odontoblasts, and osteocytes of bone (Ruchon et al., 1998; Ruchon et al., 2000a). Expression has also been reported in the lung (Beck et al., 1997; Grieff et al., 1997), human ovary (Grieff et al., 1997), and in the human parathyroid gland (Blydt-Hansen et al., 1999). PHEX mRNA abundance appears to decrease with age in the mouse (Ruchon et al., 2000a; Meyer et al., 2000). PHEX is one of a few percent of genes that does not have a Kozak sequence – there is no purine at the –3 position before the ATG initiation sequence. Generally, this implies that PHEX would be post-transcriptionally regulated (Econs, 1999; Zoidis et al., 2000).

Several studies have examined the regulation of PHEX mRNA and protein in response to various hormones. Experiments show that $1,25(OH)_2D$ down-regulates PHEX mRNA and protein abundance in primary osteoblast cultures and MC3T3-E1 cells (a mouse osteoblastic cell line) (Ecarot and Desbarats, 1999) and that IGF-1 and GH elicit an increase in PHEX mRNA expression in hypophysectomized rats (Zoidis et al., 2002). The most recent data on PHEX regulation examined the effect of glucocorticoid (GC) on PHEX gene expression in mouse bone (Hines et al., 2002). Glucocorticoids increase PHEX mRNA abundance in mouse bone and in rat osteogenic sarcoma (UMR-106) cells treated with the hormone. The putative GC response element was also located in the promoter and was an atypical sequence.

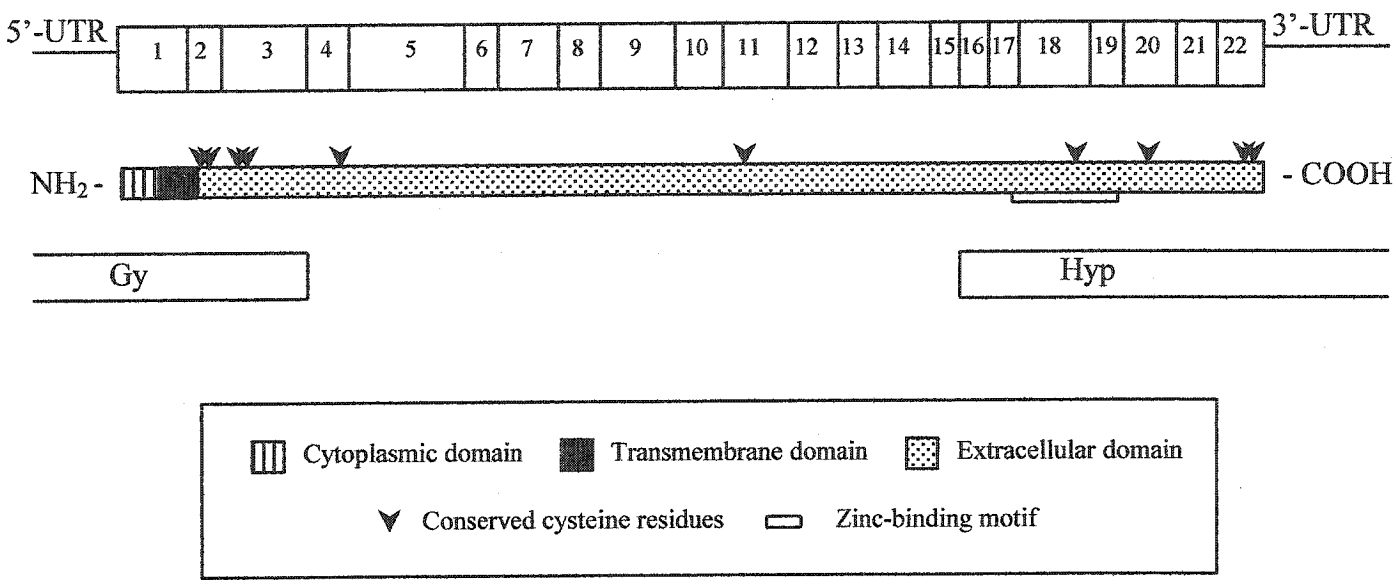


Fig. 2 PHEX coding sequence and protein motifs. (Tenenhouse, 1999)

The physiological substrate(s) of PHEX has not yet been identified. Since other members of the metalloendopeptidase family are involved in the activation or degradation of various peptides and since the loss of PHEX function is associated with renal Pi wasting, it was hypothesized that PHEX either inactivates a phosphaturic factor or activates a phosphate conserving peptide (Tenenhouse, 1999; Econs, 1999; Drezner, 2000). Many potential substrates have been tested. The first report of a peptide being cleaved by PHEX was provided by Lipman et al (Lipman et al., 1998) where PTH was reported to be cleaved by a membrane preparation derived from PHEX-transfected cells. PTH was examined because it is a phosphaturic factor that could be involved in regulating Pi reabsorption. However, these data were not confirmed in other studies (Guo et al., 2001). Casein, human stanniocalcin-1, [leu]enkephalin and a peptide of FGF-23 (172-186) were also tested as substrates but they were not cleaved by PHEX (Guo et al., 2001). MEPE, osteopontin, and dentin matrix protein were also tested as PHEX substrates but, again, none of these proteins were cleaved by PHEX (Bowe et al., 2001). In another study, a soluble and secreted form of PHEX was genetically engineered, purified and tested with several peptides, including [leu]enkephalin, PTH, substance P, brakykinin, endothelin-1, calcitonin, osteocalcin, and osteogenic growth peptide and PTHrP (107-139) (Boileau et al., 2001). Only the PTHrP (107-139) was degraded by PHEX. While the role of PTHrP (107-139) is unknown, it is expressed in tissues where PHEX is expressed.

FGF-23 was another substrate tested for PHEX cleavage. These studies are of interest because FGF-23 inhibits renal phosphate uptake (Bowe et al., 2001). FGF-23 is the gene that, when mutated, causes Autosomal Dominant Hereditary Rickets (ADHR)

(The ADHR Consortium, 2000). ADHR presents many of the same symptoms as XLH. It is a rare syndrome where patients exhibit hypophosphatmia, inappropriately low or normal $1,25(OH)_2D$ concentration, bone defects, muscle weakness, and bone pain (Jan and Levine, 2002). Patients with ADHR can also exhibit incomplete penetrance, delayed onset or reversion of the symptoms after puberty (Jan and Levine, 2002). Missense mutations involving one of two arginines in the FGF-23 sequence were found in four unrelated families (The ADHR Consortium, 2000). These mutations interrupt a furin processing site that renders the mutant FGF-23 protein resistant to cleavage (Bowe et al., 2001; White et al., 2001).

The full-length FGF-23 protein, both wild type and a mutant form, were tested for cleavage by PHEX (Bowe et al., 2001). Whereas, the wild type protein was degraded by PHEX, the mutant FGF-23 was not cleaved. The earlier experiment testing FGF-23 as a substrate, mentioned above, used only a peptide (amino acids 172-186) of the total protein which included the furin processing site (Guo et al., 2001). They could find no PHEX cleavage of FGF-23. Therefore, it is possible that the full-length protein is needed for recognition by PHEX.

Oncogenic hypophosphatemic osteomalacia (OHO) or tumour induced osteomalacia (TIO) is another phosphate wasting disorder that is acquired rather than hereditary (Tenenhouse and Sabbagh, 2002). A variety of benign, mesenchymal tumours that can present anywhere in the body have been shown to produce an excess of phosphaturic factors. Upon removal of the tumour(s), all symptoms of hypophosphatemia, Pi wasting and osteomalacia are reversed. OHO tumors over express several proteins that are candidates for the phosphaturic factor including FGF-23

(Shimada et al., 2001; Bowe et al., 2001). Moreover, conditioned media from *in vitro* cultures of COS-7 cells transfected with FGF-23 inhibit phosphate transport in renal proximal tubular (OK) cells (Bowe et al., 2001).

The above two syndromes and the knowledge that PHEX cleaves FGF-23 have led to an intriguing hypothesis (Fig. 3) (Tenenhouse and Sabbagh, 2002; Jan and Levine, 2002). It is possible that in an optimally functioning environment PHEX cleaves FGF-23 to help maintain phosphate homeostasis. When PHEX is malfunctioning (XLH), FGF-23 accumulates in the system and down-regulates renal Pi reabsorption perhaps through Npt2. In ADHR, FGF-23 mutations prevent the processing of FGF-23 and there is an accumulation of the intact protein in the circulatory system again causing a down regulation of Pi reabsorption. In OHO, FGF-23 is over-produced and overwhelms the degradation capability of PHEX. This, again, results in increased concentrations of circulating FGF-23 and a down regulation of Pi reabsorption.

Loss of PHEX function also appears to cause a primary defect involving osteoblast mineralization. This was first noted in experiments involving the transplantation of Hyp bone cells into wild type mice (Ecarot-Charrier et al., 1988; Ecarot et al., 1992b; Ecarot et al., 1992a; Ecarot et al., 1995). These cells did not form normal bone tissue in a normal Pi and PHEX environment. The bone in Hyp animals demonstrates enlarged osteoid areas (Ruchon et al., 2000a) that are not corrected by phosphate supplementation or $1,25(OH)_2D$ administration (Ecarot-Charrier et al., 1988; Ecarot et al., 1992b; Ecarot et al., 1992a; Ecarot et al., 1995). Mineralization of Hyp osteoblasts *in vivo* and *ex vivo* is reduced in comparison to wildtype counterparts (Miao et al., 2001) and immortalized Hyp osteoblast cultures demonstrate a defective

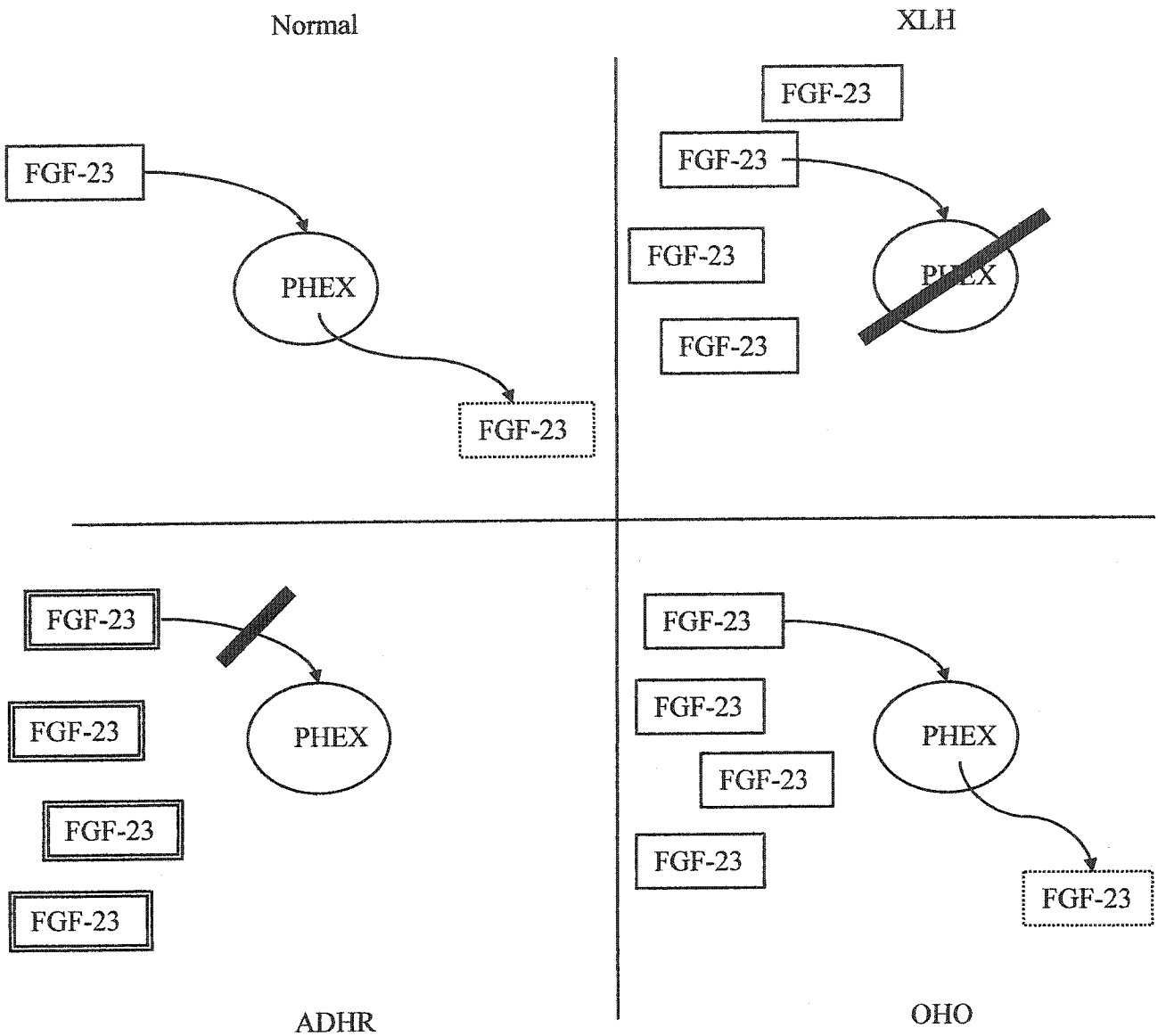


Fig. 3 Hypothesis of PHEX action on FGF-23 in normal and disease states. XLH - X-linked hypophosphatemia, ADHR - autosomal dominant hereditary rickets, OHO - oncogenic hypophosphatemic osteomalacia, FGF-23 (solid line) - intact wild type protein, FGF-23 (dotted line) - cleaved wild type protein, FGF-23 (double line) - mutated protein that can not be cleaved due to mutations in the furin cleavage site. In each of the three disorders, intact FGF-23 accumulates in the circulatory system and decreases Pi reabsorption.

mineralization process (Xiao et al., 1998). Further evidence for a role of PHEX in the mineralization process was examined using a human osteoblast cell line, MG-63, that was stably transfected with a PHEX antisense vector (Shih et al., 2002). The resulting cell culture had reduced ^{45}Ca incorporation and a lower level of nodule formation while the sense controls had normal mineralization patterns. Moreover, the conditioned media from antisense- but not sense- transfected cells inhibited ^{32}P uptake by OK cells (Shih et al., 2002). These findings suggest that PHEX expression in the osteoblast is necessary for normal mineralization and the proper development of the bone matrix (Ruchon et al., 1998).

Parathyroid gland

The parathyroid glands (PTG), located above the thyroid, mediate the homeostasis of calcium through the release of parathyroid hormone. Humans have 4 glands and rats have two. When calcium levels deviate from normal, cellular functions are modified and PTH production is either up-regulated or down-regulated. The changes in the serum calcium levels are detected by a calcium-sensing receptor (CaR) in the parathyroid gland.

PTH

Parathyroid hormone is produced almost exclusively by chief cells in the parathyroid gland (Hendy, 1997). In the nucleus, mRNA encoding preproPTH is produced. Exon 1 encodes the 5'UTR, exon 2 the prepro sequence, and exon 3 the

hormone and the 3'UTR (Hendy et al., 1981). The pre- sequence allows the protein to enter the endoplasmic reticulum where it is then removed. In the Golgi apparatus, the pro- sequence is removed by furin and the active peptide is packaged in secretory granules (Hendy, 1997). Once released from the gland, the hormone interacts with receptors largely in kidney and bone. PTH stimulates bone resorption leading to the release of calcium (and Pi) from mineralized bone to the blood (Kilav et al., 2001). When PTH is not needed, the hormone is completely degraded through intraglandular metabolism and inactive fragments are released (Hendy, 1997). Degradation in the PTG is reduced when PTH release is stimulated.

Low serum calcium stimulates PTH secretion and PTH mRNA abundance (Naveh-Many and Silver, 1990) and, if the decrease in calcium is prolonged, PTG cell proliferation can occur (Naveh-Many et al., 1995). Increased serum Pi also increases PTH secretion and PTH mRNA abundance (Estepa et al., 1999). PTH, in turn, decreases serum Pi by increasing renal phosphate excretion (Almaden et al., 1996; Slatopolsky et al., 1996; Nielsen et al., 1996). Low serum phosphate can lead to a decrease in serum PTH, PTH mRNA abundance, and PTG cell proliferation (Kilav et al., 1995).

Elements within the 3'UTR of PTH mRNA have been shown to bind proteins in the PTG when hypocalcemia is sensed (Moallem et al., 1998). The binding of the proteins to the PTH 3'UTR leads to an increase in the stability and abundance of PTH mRNA. Degradation of the PTH protein can occur within the secretory granules and in hypercalcemic states. Up to 50% of newly synthesized PTH is degraded, whereas, in hypocalcemic states less than 10% of the PTH is degraded (Habener et al., 1975).

A second source of PTH was discovered when a knockout (KO) mouse for the

glial cells missing2 (Gcm2) gene was created (Gunther et al., 2000). Removal of this gene led to the complete absence of the PTG. It was demonstrated that, of those *Gcm2* deficient mice that survived the first few weeks, the animals were viable, fertile, had mild hypocalcemia and hyperphosphatemia as well as low bone turnover. The KO mice also had serum PTH concentrations similar to that of the wild-type littermates. Amplification of mRNA from various tissues determined that the additional PTH made by the *Gcm2* deficient mice was produced by the thymus. A cluster of cells under the thymic capsule were responsible for the PTH production and since the PTH levels were not increased in these mice, though they are hypocalcemic, these cells may be at a maximal output. This alternate source of PTH is not available in animals that have viable PTGs.

CaR

The calcium-sensing receptor (CaR) mediates changes in the signaling pathways of the parathyroid gland that regulate PTH secretion (Ye et al., 1996). Regulation of Ca^{++} homeostasis and CaR expression occurs mainly in chief cells of the parathyroid gland (Brown and Hebert, 1997). CaR down-regulates PTH secretion when circulating Ca^{++} levels increase, by a complex mechanism that has not been completely elucidated. It is known that CaR activates the phospholipase C, A_2, and D pathways, the MAP kinase pathway as well as inhibits adenylate cyclase (Brown and MacLeod, 2001). However, how these modulations in turn affect PTH secretion remains speculative. The receptor is a member of the G-protein coupled receptor super family. As such, it has a large amino-terminal extracellular domain, 7 transmembrane loops, and a carboxyl terminal

intracellular region. The human gene is located on chromosome 3p (Chou et al., 1992), and in the rat and mouse, it is found on chromosomes 11 and 16 (Janicic et al., 1995), respectively. The regulatory regions of the gene have not yet been characterized. Several splice variants have been found; one insertion (Garrett et al., 1995), and two deletions in the exon regions (Bradbury et al., 1998; Oda et al., 1998), and an alternative 5'UTR (Garrett et al., 1995). The variants have not been fully evaluated to determine whether or not these changes have effects on activity, regulation or tissue expression patterns.

The effects of various conditions on the regulation of CaR have been studied. In rats with renal insufficiency, the level of CaR decreases (Mathias et al., 1998). Whether this reduction is due to the decrease in $1,25(OH)_2D$ concentrations that occur during development of renal insufficiency (Stewart and Broadus, 1987) or due to the rise in PTH concentrations (Mathias et al., 1998) has yet to be determined. Hyperparathyroidism also diminishes the level of CaR expression (Brown et al., 1999). Renal failure in rats and humans is associated with a drop in sensitivity of CaR to calcium (Schwartz et al., 1994) i.e. there is an elevation in the 'set-point' in the inverse relationship between PTH production and Ca^{++} concentration. Increased levels of $1,25(OH)_2D$ in normal rats will up-regulate CaR mRNA expression and in vitamin D-deficient rats CaR is down-regulated (Brown et al., 1996). This effect is mediated through the vitamin D receptor (VDR). High Ca^{++} concentration raises both CaR and VDR abundance (Emanuel et al., 1996; Russell et al., 1993). The action of Ca^{++} on VDR abundance has not yet been shown to be CaR-mediated but this would be a reasonable assumption as the VDR does influence CaR abundance (Brown and MacLeod, 2001).

Hyperparathyroidism

There are three types of hyperparathyroidism. Primary hyperparathyroidism is caused by an intrinsic defect in the gland such as a genetic defect or an adenoma or carcinoma. Secondary hyperparathyroidism arises from defects in calcium homeostasis that cause a marked increase in parathyroid gland function. However, this condition can be reversed with treatment by normalizing serum Ca levels. An example of this would be hyperparathyroidism caused by renal failure. Tertiary hyperparathyroidism occurs when secondary hyperparathyroid conditions have existed for so long that the parathyroid gland can no longer be down-regulated. At this point, surgical removal of the gland is necessary. Further issues related to hyperparathyroidism will be discussed in the section on 5/6 nephrectomy.

The parathyroid gland consists of low turnover, long living cells (Drueke, 2000) that have a life span of approximately 2 years in the rat (Wang et al., 1996) and 20 years in humans (Wang et al., 1997). This is why there are no PTG cell lines available for study. The increase in PTG mass during hyperparathyroidism is due to increased cell proliferation (Parfitt, 1997). There is also an increase in PTG volume due to cell hypertrophy (Wang et al., 1996). When serum PTH levels are reduced to normal by lowering Pi intake, hyperplasia of the PTG persists (Slatopolsky et al., 1999). Injection of $1,25(OH)_2D$ before hyperplasia occurs can be preventative but cannot reverse the effects (Szabo et al., 1989). During periods of hyperparathyroidism and hyperplasia, the set-point for calcium-regulated PTH secretion is elevated (Brown et al., 1982). In other words, control of PTH secretion by extracellular Ca concentrations becomes more

difficult. There is a resistance to Ca that is not present during normal conditions.

PHEX in the Parathyroid Gland

Recently, it has been shown that PHEX is expressed in the PTG (Blydt-Hansen et al., 1999). PHEX expression was examined in patients who had either tertiary hyperparathyroidism resulting from chronic renal insufficiency, tertiary hyperparathyroidism due to an imbalance in Pi and $1,25(OH)_2D$ treatment of XLH and a child with XLH who had significant hyperparathyroidism before treatment began. After parathyroidectomy, each of the glands was assessed for PHEX mRNA levels by ribonuclease protection assay. PHEX mRNA abundance, relative to β-actin, was present at levels several-fold higher than that of fetal calvaria (Blydt-Hansen et al., 1999). The function of PHEX in the parathyroid gland is unknown at this time. PHEX may be expressed in the PTG to help regulate PTH concentrations. This idea was put forward by Lipman et al who demonstrated that membrane preparations of PHEX-transfected cells could degrade exogenous PTH (Lipman et al., 1998).

1,25-dihydroxyvitamin D_3

$1,25(OH)_2D$ is the active hormonal form of vitamin D. It affects mineral homeostasis by acting on various target organs such as the intestine, kidney, bone and parathyroid gland. $1,25(OH)_2D$ is the end product of a multi-step pathway (Fig 4). 7-dehydrocholesterol is converted to previtamin D_3 and then into vitamin D_3 by photolysis

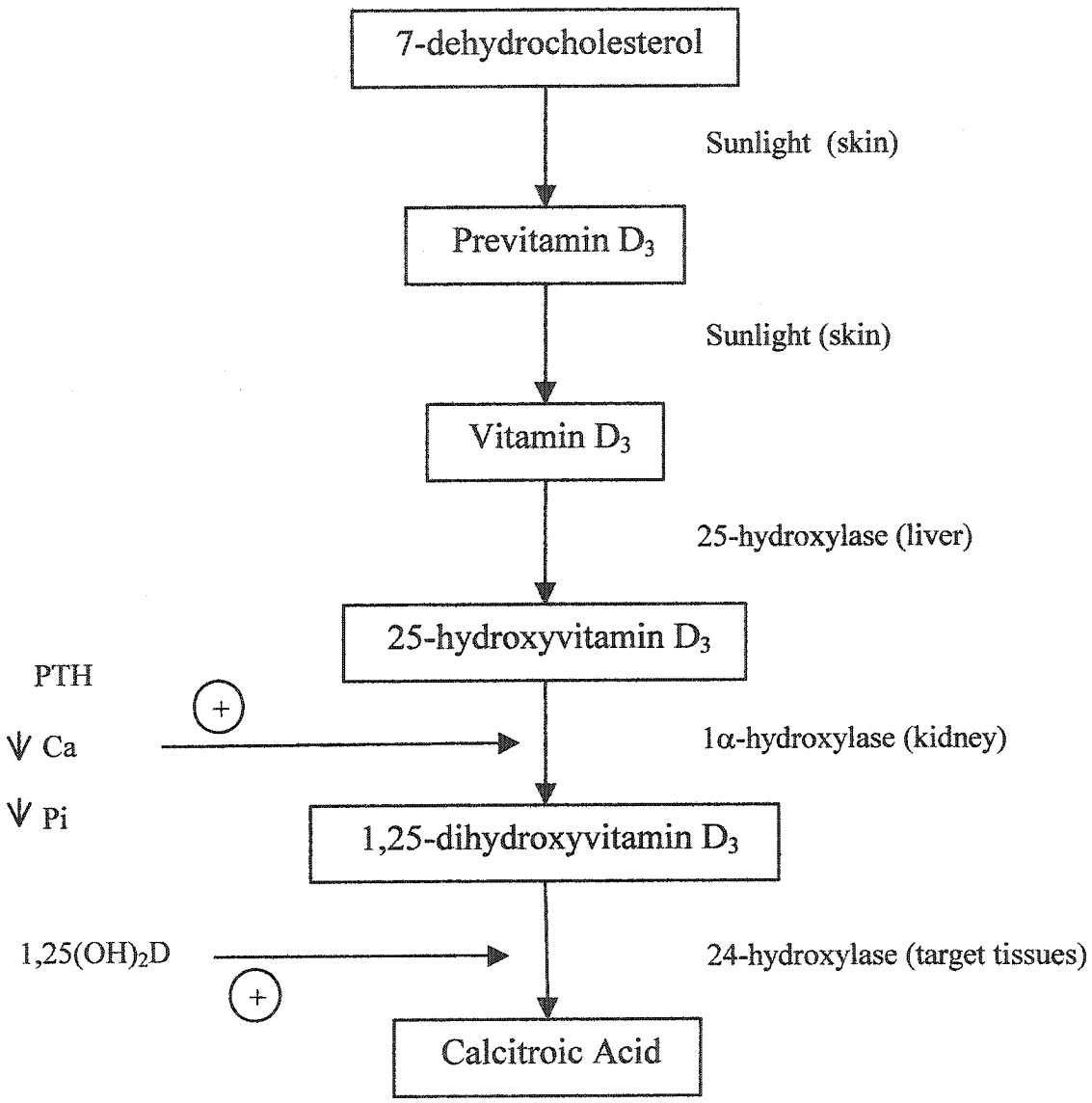


Fig. 4 Schematic of vitamin D_3 synthesis and catabolism. The flowchart presents each vitamin D_3 metabolite in the pathway, depicts the enzyme that processes each metabolite, and indicates in which tissues the reactions have been localized. Some of the regulators controlling synthesis versus catabolism are also presented.

in the skin (Hendy, 1997; Brown et al., 1999). Vitamin D_3 is hyroxylated in the liver into 25-hydroxyvitamin D_3. This is the most abundant vitamin D metabolite present in serum. 25-hydroxyvitamin D_3 is again hydroxylated, this time in the kidney, to produce $1,25(OH)_2D_3$. The enzymes responsible for the hydroxylation, vitamin D-25-hydroxylase (CYP27) and 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1), are members of the cytochrome P450 super family. $1,25(OH)_2D_3$ is catabolized by 25-hydroxyvitamin D-24-hydroxylase (CYP 24) via a multi-step pathway to calcitroic acid.

Serum $1,25(OH)_2D$ concentrations are regulated by PTH, calcium, phosphate, and $1,25(OH)_2D$ itself. PTH increases $1,25(OH)_2D$ production by increasing 1α-hydroxylase and decreasing 24-hydroxylase (Henry, 1981). Hypercalcemia and hyperphosphatemia down-regulate $1,25(OH)_2D$ abundance by suppressing 1α-hydroxylase activity (Bland et al., 1999; Hughes et al., 1975; Tanaka and DeLuca, 1973). Feedback regulation of $1,25(OH)_2D$ is also important. When $1,25(OH)_2D$ concentrations are elevated, catabolism of this metabolite is increased (Henry, 1979).

$1,25(OH)_2D$ action in target tissues is mediated by the vitamin D receptor (VDR), discovered in 1968 (Haussler et al., 1968). It displays a high affinity for $1,25(OH)_2D$ as well as varying lower affinities for the other vitamin D metabolites that correspond to their potency *in vivo*. The receptor is a member of the steroid hormonal receptor super family and as such has two zinc finger motifs in a DNA-binding domain (Burmester et al., 1988) and is a transcription factor. The VDR is expressed in many tissues.

$1,25(OH)_2D$ increases the efficiency of calcium and phosphate absorption in the intestine (Li et al., 1998) and it is essential for proper bone maintenance. The hormone induces bone formation by up regulating bone matrix proteins and increasing serum

calcium and phosphate (Li et al., 1998). $1,25(OH)_2D$ can also enhance bone resorption. $1,25(OH)_2D$ inhibits growth of chief cells and PTH synthesis in the PTG. Other roles for $1,25(OH)_2D$ include immunosuppression of lymphocytes, antiproliferative and prodifferentiation of various cell types, and neuronal regeneration (Brown et al., 1999).

Calcitonin

Calcitonin was first discovered in 1962 (Copp et al., 1962) and is produced by the thyroid gland (Kumar et al., 1965). It is a fast acting hormone that leads to decreased bone resorption (Friedman and Raisz, 1965) by inhibiting the activity of the osteoclasts (Chambers and Magnus, 1982; Chambers, 1982). For this reason, osteoporosis, Paget's Disease and skeletal complications in certain cancers are all treated by calcitonin administration.

The precursor for calcitonin is 136 amino acids in length. A leader sequence is cleaved once the peptide enters the endoplasmic reticulum where it is glycosylated (O'Neil et al., 1981). The active form of calcitonin is a 32 amino acid peptide with high homology among various species in the first 7 amino acids but differing in carboxyterminal regions. The first 7 amino acids form a loop via a sulfide bond between amino acids 1 and 7 (Sexton et al., 1999). In the kidney, this hormone appears to increase 1α-hydroxylation of 25-hydroxyvitamin D_3 (Kawashima et al., 1981). In target tissues, calcitonin binds to its receptor and increases camp concentrations (Nicholson et al., 1986) by activating the adenylate cyclase pathway (Chabardes et al., 1980).

Renal Failure and Animal Models Thereof

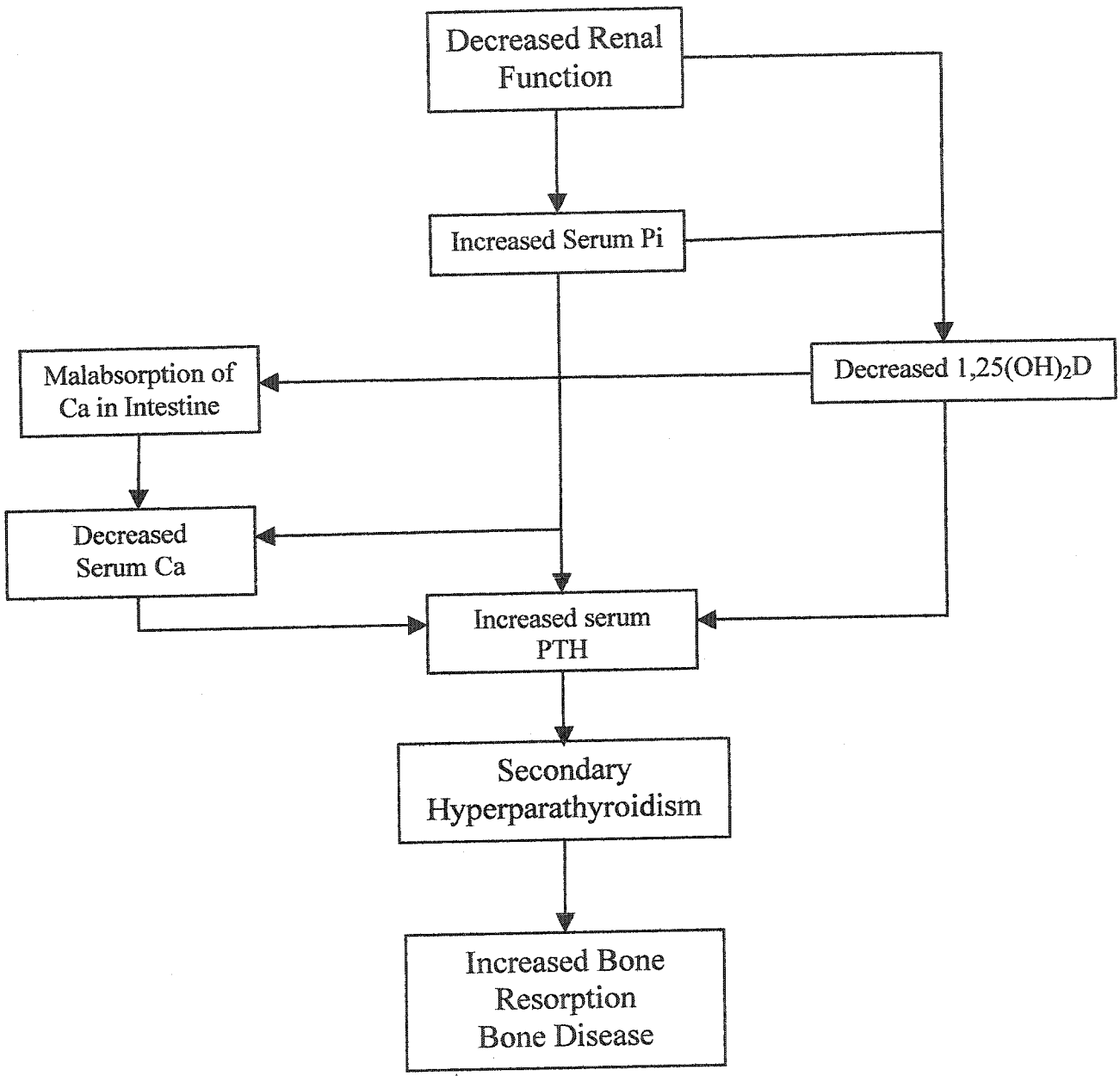
The three leading causes of kidney failure in humans are diabetes (31%), renal vascular disease including high blood pressure (21%), and glomerulonephritis (15%) (Kidney Foundation of Canada). In 1999, there were over 23, 000 Canadians on renal replacement therapy and this number is expected to double over the next ten years. Hyperphosphatemia, due to Pi retention, is a prominent finding in patients with renal failure and persists in approximately 50% of patients on dialysis (Block et al., 1998). Hyperphosphatemia is one of the most important factors in the development of secondary hyperparathyroidism and in its later stages is responsible for soft tissue calcification and cardiovascular disease. Greater than 45% of dialysis patients die from cardiac arrest and acute myocardial infarction. In addition, hyperparathyroidism, secondary to hyperphosphatemia, causes renal osteodystrophy – another cause of morbidity. Efforts to reduce serum phosphate are difficult at best. Dialysis does not remove all of the excess Pi and reducing dietary Pi intake by decreasing protein intake (Block et al., 1998) leads to inadequate nourishment. Phosphate binders are also used in an effort to reduce serum Pi, however, this can also lead to other health risks.

The parathyroid gland, together with kidney, bone and intestine plays an important role maintaining serum Ca and phosphate homeostasis. Under normal conditions, when serum Ca is decreased, PTH secretion is stimulated. This leads to stimulation of $1,25(OH)_2D$ synthesis in the kidney and to elevated serum concentrations of the vitamin D hormone. This in turn increases intestinal Ca and Pi absorption. PTH also stimulates bone resorption and the release of Ca and Pi into the circulation. In the

kidney, PTH increases Ca reabsorption and decreases renal phosphate absorption. All of these steps combine to increase the overall serum Ca concentration and maintain normal Pi concentrations.

As kidney function becomes impaired, clearance of Pi from the serum into the urine is decreased leading to Pi retention, hyperphosphatemia, and the development of hyperparathyroidism (Fig 5). Increased serum PTH decreases the reabsorption of Pi to try to maintain serum Pi concentrations (Brenner BM and Herbert SC, 1994). However, when the glomerular filtration rate drops below 20-25 mL/min, the Pi excretion to reabsorption ratio cannot be maintained and serum Pi concentations increase (Slatopolsky and Bricker, 1973). Reduced kidney function and elevated serum Pi leads to a decrease in the renal synthesis, and therefore serum concentration, of $1,25(OH)_2D$. This results in decreased intestinal Ca absorption. Supplements of $1,25(OH)_2D$ increase serum Ca concentration by stimulating intestinal absorption and help control serum PTH levels by inhibiting PTH gene transcription in the PTG. Another factor responsible for reducing serum Ca concentrations is the precipitation of Ca with phosphorus into the skin and soft tissues. These factors all contribute to and exacerbate secondary hyperparathyroidism further by increasing PTH production and secretion.

Bone is in a constant state of turnover. Resorption is balanced by the formation of new bone matrix under normal conditions. In the case of secondary hyperparathyroidism, elevated serum PTH elicits an acceleration of both bone resorption and formation. Instead of the proper development of lamellar bone, a less structured woven bone replaces it and, if hyperparathyroidism is prolonged, fibrosis will occur (osteitis fibrosa cystica). During this process, bones become resistant to the actions of



F ig 5. Schematic of chronic renal failure and the steps leading to secondary hyperparathyroidism and bone disease.

PTH (Bover et al., 1994).

Approximately four times the amount of serum PTH in normal rats is needed to maintain bone turnover rates in chronic renal failure. High turnover osteodystrophy is associated with increased serum abundance of PTH, alkaline phosphatase, osteocalcin, and phosphorus (Torres et al., 1995).

Alkaline phosphatase and osteocalcin are bone markers expressed by the osteoblast and are generally used in the clinical setting to monitor bone metabolism (Calvo et al., 1996). Serum concentrations of these proteins increase as bone turnover accelerates and a significant correlation has been reported between bone formation rates and these bone formation markers (Hamdy et al., 1994).

Regulation of alkaline phosphatase expression is influenced by PTH and $1,25(OH)_2D$. Incubation of rat osteoblasts with $1,25(OH)_2D$ down-regulates alkaline phosphatase mRNA production (Atmani et al., 2002). In humans and mice, alkaline phosphatase activity is elevated during chronic renal failure i.e. during periods of increased PTH (Magnusson et al., 2001; Gagnon and Gallimore, 1988). Another study compared alkaline phosphatase activity in mouse and rat osteoblast-like cells derived from fetal calvaria. When the cells from both species were incubated with PTH (1-34), alkaline phosphatase activity was increased in the mouse but decreased in the rat (Jongen et al., 1993). Further examination of PTH administration in rat osteoblastic cells isolated from the calvaria showed that the manner in which cells were exposed to PTH was important. Intermittently exposed cells demonstrated increased alkaline phosphatase activity and mRNA associated with stimulated osteoblast differentiation. In constrast, cells continuously exposed to PTH, similar to the conditions found in the nephrectomized

rat, exhibited decreased alkaline phosphatase activity and mRNA, associated with an inhibition of osteoblast differentiation (Ishizuya et al., 1997).

Regulation of osteocalcin expression is controlled by factors such as PTH and $1,25(OH)_2D$. Osteocalcin mRNA abundance is down-regulated by PTH administration in a mouse osteoblastic cell line with maximal inhibition occurring after 24 hours (Gopalakrishnan et al., 2001). Both human and rat osteocalcin promoters contain a vitamin D response element (Kerner et al., 1989; Demay et al., 1990) and $1,25(OH)_2D$ has been shown to induce osteocalcin expression in humans and rats *in vivo* and in primary osteoblast cultures *in vitro* (Viereck et al., 2002; Tsuruoka et al., 2000; Hauschka et al., 1989; Lian and Gundberg, 1988). Osteocalcin mRNA is also increased in 5/6 nephrectomized rats injected with $1,25(OH)_2D$ (Tsuruoka et al., 2002).

To gain some insight into the pathophysiology of renal failure and to improve its treatment, animal models were developed for its study. The 5/6 nephrectomy (5/6 Nx) model has been used over the past few decades, including by our collaborators (Soliman et al., 1997; Canaff et al., 1999). This is a well-established model that mimics the human disease. All of the factors that cause hyperparathyroidism in humans with renal failure occur in the 5/6 Nx rat as well. This model permits the examination of the steps involved in the development of renal failure, the consequences of such failure as well as steps to correct the deficiency in renal function.

A recent paper (Green et al., 2001) questioned why patients who have recently undergone kidney transplantation still maintained a Pi leak, that is independent of PTH status, for a period extending anywhere from 1 week to 3 months post-transplant. Serum from chronic renal failure patients as well as from recent renal transplant patients were

examined for the serum's effect on Na/Pi cotransport in opossum kidney (OK) cells, a model for the proximal tubular cells. They found that serum from these patients significantly decreased Na/Pi cotransport in OK cells. They hypothesized that a phosphaturic factor is expressed during chronic renal failure (CRF) to limit the development of hyperphosphatemia and that it takes several weeks before the newly grafted kidneys can revert the patients system towards normal Pi levels by reducing the release of the phosphaturic factor. The factor responsible for the Pi leak in these CRF patients is most likely one of a family of phosphaturic factors that could be cleaved by PHEX.

The above paper, the knowledge that PHEX is expressed in the parathyroid gland under conditions of increased PTH expression, and that PHEX is involved in Pi homeostasis has led to the following hypothesis: serum PTH status is a regulator of PHEX mRNA and protein abundance and that the cleavage of the PHEX substrate(s) is necessary for attempting to maintain Pi homeostasis under conditions of chronic renal failure.

Aims

Experiments evaluating the regulation of PHEX mRNA and protein expression in response to changes in PTH status were examined in the rat bone. To this end, established animal models for hyper- and hypo-parathyroidism were chosen. Five-sixth nephrectomized rats and rats injected with $1,25(OH)_2D$ were the models selected for this study. We monitored serum and urine parameters to determine the degree of hyper- or

hypo- parathyroidism induced in the rat models. In addition, we examined the expression of PHEX mRNA and protein in the tibia, and occasionally calvaria, of these rat models. Increased knowledge of PHEX and its regulation will hopefully lead to more appropriate therapies for treatment of chronic renal failure and possibly other related diseases such as XLH , ADHR and OHO.

MATERIALS AND METHODS

Animal Models, Rats

Sprague-Dawley male rats weighing 180 - 200g were purchased from Charles-River (St-Constant, Quebec). The rats were either intact, sham operated or 5/6 nephrectomized (Nx) by the company. Two diets were used over the course of the experiments. A control diet (Ralston Purina Co., LaSalle, Quebec, Canada) containing 1.01% calcium, 0.74% phosphorus, and 3.3 IU vitamin D_3/g and a high Pi diet (TD.94238, Harlan Teklad, Madison, WI) containing 0.6% calcium, 1.4% phosphorus, and 1.0 IU vitamin D_3/g. Spot urine was collected from each rat either the morning before or the morning of sacrifice and stored at -20°C. All rats were sacrificed using CO_2. Blood was extracted by cardiac puncture, and the serum separated and stored at -20°C (Canaff et al., 1999). Parathyroid glands (PTG), tibia and calvaria were collected, placed immediately in liquid N_2 and then transferred to -80°C freezer until analysis.

The following groups of rats were studied. (1) Sham and Nx rats were fed either the control or high Pi diet for 5 weeks and then sacrificed. (2) Intact rats were fed the control diet for a few days to give the rats time to acclimate to their new surroundings and then were injected i.p. with $1,25(OH)_2D$ (10 pmol/g BW) 48 and 24 hours before sacrifice. $1,25(OH)_2D$ was purchased from the Montreal Children's Hospital pharmacy in the form of 0.5 μg Rocaltrol capsules (Roche). A needle was used to puncture the capsule and remove the liquid. Mineral oil was used as the vehicle. (3) Sham operated and Nx rats were fed the high Pi diet for approximately 5 weeks and then injected with 10

pmol/g BW of $1,25(OH)_2D$ as in group 2. (4) Rats were injected i.p. with synthetic salmon calcitonin (Rhone-Poulence Rorer Canada Inc, Montreal, Quebec, Canada) as follows: Group i) was injected with 25ng/g BW, group ii) with 50ng/g BW and both groups were sacrificed 6 hours later. Group iii) was injected with 25ng/g BW and sacrificed after 3 hours. Group iv) was vehicle injected with the same volume as the other groups.

Animal Models, Mouse

Dr. Raymonde Gagnon provided our group with mice in which chronic renal failure was induced as described previously (Gagnon and Gallimore, 1988). 5 week old female C57B1/6 inbred mice (Canadian Breeding, St. Constant, Quebec) were fed a commercially available standard diet (Ralston Purina Co., St. Louis, Mo, USA) and renal failure was induced by a two step procedure. The right renal cortex was electro-coagulated, and this was followed two weeks later by removal of the left kidney. The mice were sacrificed at two week intervals for 6 weeks following removal of the left kidney. Mice were anesthetized with ketamine/xylasine and blood was collected by cardiac puncture. Tibia, calvaria, and thyroid/parathyroid tissue were collected and analyzed as in the rat model.

Mutant Hyp and wildtype male mice were used in this study. The mice were raised in our laboratory; the original breeding pairs were obtained from the Jackson Laboratory (Bar Harbor, ME) (Beck et al., 1997).

All animal experiments were carried out in compliance with, and were approved by, the institutional Animal Care and Use Committee.

Analysis of Serum and Urine

Sera were analyzed for phosphate, creatinine, urea, and calcium concentrations by the Royal Victoria Hospital Clinical Biochemistry Dept. Serum alkaline phosphatase activity was measured using 5 mM p-nitrophenylphosphate substrate in 1M diethanolamine, 2.5 mM $MgCl_2$, 10 mmol/L l-phenylalanine (to inhibit intestinal ALP), pH 10.0 as described (Dimai et al., 1998). Incubations were performed at 30°C for 10 minutes and the absorbance at 410 nm recorded. Values were presented as nmol/ml/min. Alternatively, the RVH Clinical Biochemistry Dept also produced some alkaline phosphatase data. These values are presented as U/L. PTH levels were measured using the Rat Intact PTH Elisa kit (Immutopics, San Clemente, CA). Urines were analyzed for calcium, phosphate, and creatinine concentrations using Stanbio kits (San Antonio, Texas). Urine cAMP concentration was measured using a cyclic AMP (^3H) assay system from Amersham Biosciences (TRK 432, Baie d'Urfé, Quebec).

Bone Marrow Flushing

Immediately after removal of the muscle from around the rat tibia, the proximal and distal ends of the bone were cut and the ends placed in liquid N_2. 1ml of cold DMEM was flushed through the midshaft using 1cc syringe and collected in a 15 ml

conical tube on ice. The marrow samples were centrifuged for 10 minutes at 2000xg at 4°C (Beckman J6-HC centrifuge, JS-4.2 rotor). The supernatant was discarded and red blood cells in the pellet were hemolyzed by resuspending the cells in 1 ml 0.017M Tris-HCL pH 7.5 containing 0.8% ammonium chloride. The samples were then centrifuged for 10 minutes at 3000 rpm at 4°C. The supernatant was discarded and the pellet resuspended in PBS. RNA and protein prepared from the resuspended pellet. The tibia, immediately after flushing, was frozen in liquid N_2 and RNA and protein were prepared as described in other sections.

RNA Isolation

Total RNA was prepared from the PTG, tibia and calvaria using the standard Trizol method recommended by the manufacturer (Gibco-BRL). One mL of Trizol per 35 mg of bone was used in the extraction. The tibial ends, proximal to the knee, and tibial midshafts were cut from the rest of the long bone and crushed in liquid N_2 using a mortar and pestle. Once the bone was added to the Trizol, it was further crushed for approximately 30 seconds using a Polytron (Brinkmann Homogenizer PT-10/35 with a PTA-10S generator) set at 5. The samples were frozen at -80°C overnight before completing the extraction. When the Trizol-containing bone was thawed, before adding chloroform, an additional spin was performed to remove the bone fragments. In the case of PTG, two microdissected glands from an individual rat were homogenized in Trizol using the Kontes Pellet Pestle Mixer (VWR) after which the standard extraction method was used. RNA concentrations were determined by taking the OD at 260 nm and

multiplying by 0.04 μg/μl and correcting for the dilution factor. To test for the integrity of total RNA isolated from rat bone, randomly selected aliquots were fractionated on formaldehyde-1% agarose gels run in 1xMOPS. The gels were exposed to ethidium bromide and placed in the gel doc 2000 for visualization of the 18S and 28S bands in each of the samples.

Time Course RT-PCR

Reverse transcriptase (RT) -PCR was used to qualitatively assess the abundance of PHEX and PTH mRNAs, relative to β-actin or GAPDH mRNA, in the PTG. Table 1 depicts the sequences of the primers used. The protocol outlined below was described by Dr. Hendy's lab (Canaff et al., 1999). Briefly, PTG mRNA was reverse transcribed using Omniscript (Qiagen) or Superscript II (Gibco-BRL) for 60 min at 37°C followed by 5 min at 93°C. 10μl of the RT reactions (total volume 20μl) were diluted 1/10 for the PCR reaction (*Taq*, Gibco-BRL). The PCR reaction followed a cycle of 35 sec at 94°C, 35 sec at 57°C and 1min at 76°C for thirty-five cycles. Aliquots (10μl) were taken at cycles 18, 22, 26, 30, 35 (chosen by the level of expression of the gene) for β-actin, at cycles 16, 20, 24, 28, 35 for GAPDH, at cycles 26, 28, 30, 32, 35 for PHEX and NEP and cycles 14, 20, 25, 30, 35 for PTH. The aliquots were run on a 1% agarose gel with the appropriate controls and a DNA mass ladder (Gibco-BRL, cat# 10496-016). The resulting bands were either quantitated using a gel doc 2000 imaging program, Quantity One, from BioRad or transferred to a nitrocellulose membrane for probing (see Southern analysis below). The values were plotted on a graph and the slope of the line (linear portion) used

in the calculations.

Table 1. Primers used in RT-PCR experiments

Primer pairs	Strand	Predicted cDNA size (bp)
PHEX (rat)		
5' AGAATTGATTGAGGGTGTTCGC 3'	Forward	
5' TCGAAGACCTCCATTATCAGCA 3'	Reverse	645
PTH (rat)		
5' TTTATCTCCTTACCCAGACGGATGGGAAACCCGTT 3'	Forward	
5' ATCCACATCAGCTTTGTCCCCCTCGCCAAGACT 3'	Reverse	272
NEP (rat)		
5' GTGATGGGAAGATCAGAAAGTCA 3'	Forward	
5' CTGGGTAGAATTCTTATCATCAG 3'	Reverse	644
GAPDH (human)		
5' CCCTTCATTGACCTCAACTACATG 3'	Forward	
5' CAGAAGACTGTGGATGGCCCCTC 3'	Reverse	529
β-ACTIN (rat)		
5' AACCGCGAGAAGATGACCCAGATCATGTTT 3'	Forward	
5' AGCAGCCGTGGCCATCTCTTGCTCGAAGTC 3'	Reverse	370

Southern Analysis

The PHEX reactions from the time course RT-PCR were fractionated on a 1% agarose gel and transferred to nitrocellulose membranes overnight using a standard protocol (Ausubel et al., 1995). The membranes were washed using 2xSSC and dried for 2 hours at 80°C. The blots were probed with a PHEX cDNA, prepared by RT-PCR of total rat bone RNA, using the same primers as above (Table 1). The probe was labeled using [αP^{32}]-dCTP (ICN, Mississauga, Ontario) and the RadPrime DNA labeling system (Gibco-BRL). The blots were hybridized overnight at 42°C and washed twice for 30

minutes each with 2xSSC, 0.1% SDS at room temperature and again twice for 30 minutes with 0.1% SSC, 0.1% SDS at 65°C. The membranes were exposed overnight on KODAK film and for 4 hours on a phosphoimager plate for quantification.

cDNA Template and Riboprobe Preparation

All cDNA inserts were generated by RT-PCR and confirmed by sequencing. The enzyme-digested plasmids containing the inserts were gel purified using the Qiaex II gel purification kit (Qiagen). The riboprobes were labeled with αP^{33}-UTP (ICN, Mississauga, Ontario) using the RNA polymerase T3 for NEP and T7 for PHEX and β-actin for 1 hour and then treated with DNase1 (2U/μl) for 15 minutes before fractionation on a polyacrylamide gel and purification.

Rat 5'PHEX. A 742 bp cDNA insert (nucleotides 142-884) was placed into the pCR2.1 plasmid in the 5' to 3' direction using EcoRI. The plasmid was linearized with ACC1. The antisense riboprobe created (protocol described above) was 392 bp in length and the protected fragment was 325 bp. *Rat NEP.* A 645 bp cDNA insert (nucleotides 294-651) was placed into the pBluescript-Ks plasmid in the 3' to 5' direction using EcoRI and EcoRV. The plasmid was linearized with PflmI (Biolabs, Inc). The antisense riboprobe generated was 470 bp in length and the protected fragment was 426 bp. *Rat Osteocalcin.* A 327 bp cDNA insert (nucleotides 143 - 480) was placed into the pT7T3-19U plasmid in the 3' to 5'direction using EcoRI. The plasmid was linearized with KpnI. The antisense riboprobe generated was 354 bp in length and the protected fragment was 327 bp. *Mouse β-actin.* As an internal standard, we used an 833 bp cDNA insert

(nucleotides 1-833) placed into the pGEM3 plasmid in the 5' to 3' orientation using HindIII and KpnI. The plasmid was linearized with CVNI. The antisense riboprobe generated was 160 bp in length and the protected fragment was 137 bp as previously described (Beck et al., 1997).

Ribonuclease Protection Assay

PHEX and NEP mRNA expression were determined and compared to β-actin mRNA using the PHEX, NEP, and β-actin riboprobes as described by Beck et al (Beck et al., 1997). After gel purification (described in previous section), the labeled riboprobes described above were hybridized in 40mM Pipes pH 6.4, 0.4M NaCl, 1mM EDTA, 80% formamide overnight at 50°C with 20 ug total RNA isolated from rat bone. The hybridized samples were then treated with RNase T1(~200U/μl) for 1 hour at 30°C, precipitated with ethanol on dry ice, resuspended in 5μl loading buffer, and heat-denatured for 5 min at 100°C. The samples were run on a 6% denaturing polyacrylamide gel and the gel was dried for 1 hour. The dried gel was exposed to a phosphoimager for quantification and to film for photography. The abundance of PHEX or NEP mRNA, relative to β-actin mRNA, was determined under conditions where linearity was achieved.

Protein Extraction

Protein was extracted from tibia and calvaria. Tibia midshafts were lyophilized for 4 days, crushed in liquid N_2 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 minutes at 14,000xg. The supernatants were analyzed for protein concentration using the Lowry method (Lowry et al., 1951). Samples were concentrated using YM-30 Centriprep centrifugal filter devices (Millipore, Mississauga, Ontario) and re-assayed for protein concentration if necessary. Samples were stored at -80°C until used for western analysis.

Western Analysis

The protein extracts were incubated in the presence or absence of 1μl PNGaseF (New England Biolabs, Mississauga, Ontario) for 1 hour as specified by the manufacturer. The PNGaseF treatment reduced background and confirmed that PHEX was glycosylated. Moreover, deglycosylation led to cleaner and sharper bands for quantitation. Thus, the results presented were generated from samples that had been deglycosylated. The samples were boiled for 3 min in 6x Laemmli sample buffer before fractionation on SDS/PAGE (10% (v/v) gel). The proteins in the gel were transferred to a nitrocellulose filter for 1 hour using standard methods. PHEX proteins were detected by immunoblotting with a PHEX 13B12 monoclonal antibody (1:200 dilution) (Ruchon et al., 2000a). This was followed by incubation with peroxidase-conjugated anti-mouse IgG at a 1:3000 dilution. NEP was detected using a monoclonal antibody (18B5, 1:2000 dilution) (Ruchon et al., 2000b). Actin, which was detected using an actin antibody (Sigma, A2066, 1:2000 dilution), was used as a loading marker and detected using a peroxidase-conjugated anti-rabbit IgG at a 1:3000 dilution.

An ECL detection kit (Amersham Pharmacia) was used to visualize the bands as

per the manufacturers instructions.

Statistical analysis

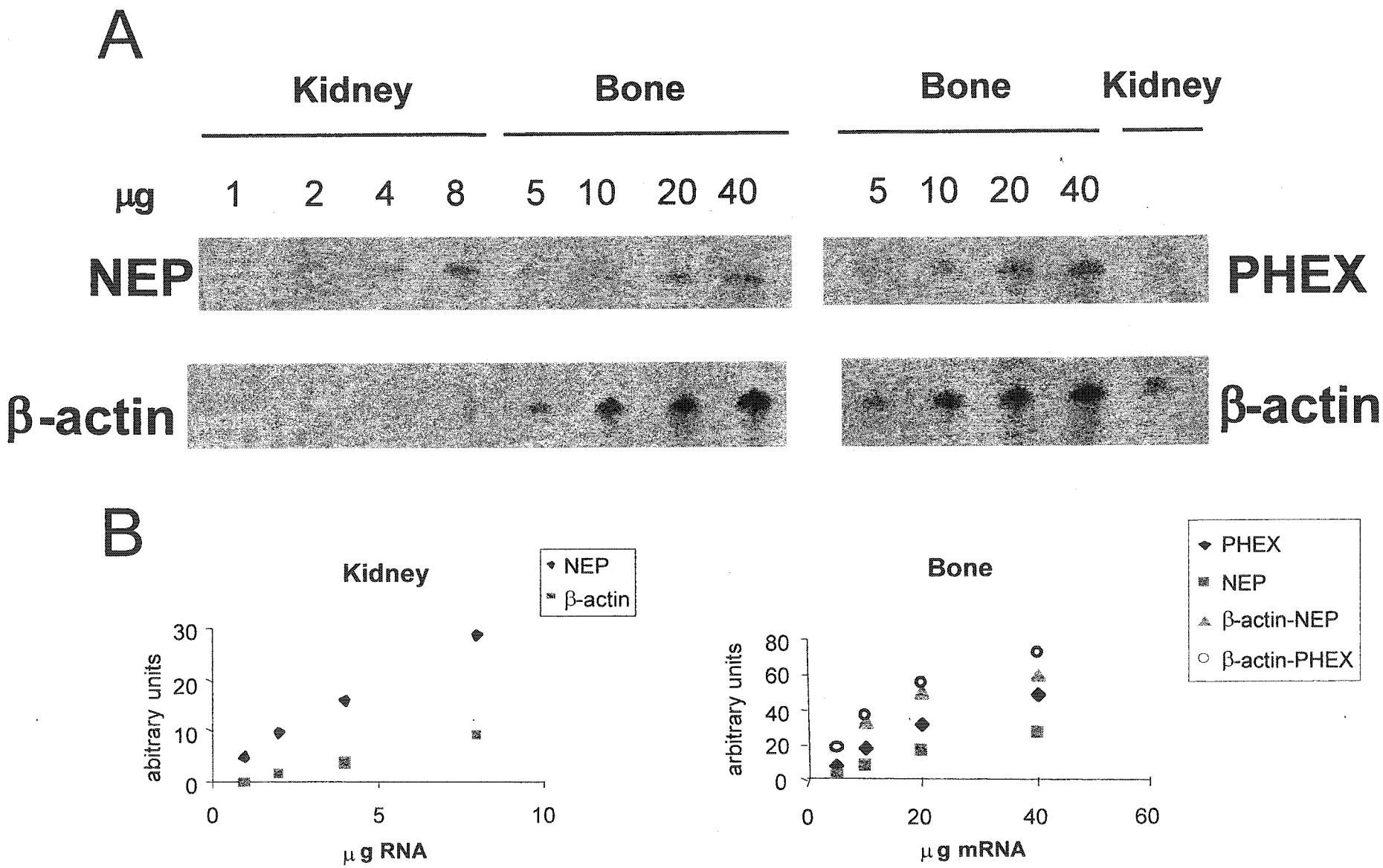
Data was analyzed using a t-test assuming unequal variances or ANOVAS as needed. Significance was determined as $P<0.05$. All values are given as means ± SEM unless otherwise noted.

RESULTS

Validation of Methodology

Ribonuclease Protection Assay. Varying amounts of total RNA, extracted from rat kidney and tibia, were hybridized to rat PHEX and NEP riboprobes as well as a mouse β-actin riboprobe. A single band of the appropriate size was detected for all three transcripts (Fig. 6A). The PHEX, NEP and β-actin signals increased with increasing amounts of RNA (Fig. 6B). The increase was linear and 20μg of tibial mRNA was chosen for all further experiments.

Western Analysis. The PHEX antibody, 13B12, was validated by probing lysates prepared from various mouse tissues (Fig. 7). Lung, brain, kidney and bone protein from wildtype and Hyp mice were incubated in the absence and presence of PNGaseF before fractionation on a polyacrylamide gel (see Materials and Methods). Phex expression was only detected in the bone extracts from wildtype mice. The undigested PHEX protein was 97 kDa and deglycosylation reduced the molecular weight of the protein to approximately 86 kDa, consistent with the predicted amino acid sequence. An actin antibody was used as a loading marker and the expected 42 kDa band was detected (Fig. 7). The absence of Phex expression in Hyp bone is consistent with the large deletion in the Phex gene identified in Hyp mice (Beck et al., 1997)


A
Kidney
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abitrary units
30
20
10
0
0
5
10
µg RNA
Bone
PHEX
NEP
β-actin-NEP
β-actin-PHEX
arbitrary units
80
60
40
20
0
0
20
40
60
µg mRNA

Fig.6 Validation of ribonuclease protection assay. Increasing amounts of total RNA, isolated from rat kidney and tibia, were hybridized with rat PHEX and NEP, and mouse β-actin, riboprobes as described in Materials and Methods. (**A**) The hybridization signals, visualized by autoradiography, are depicted for each transcript. (**B**) Band intensity for each transcript, determined by phosphoImager analysis, was proportional to the quantity of RNA used in the hybridization reaction.

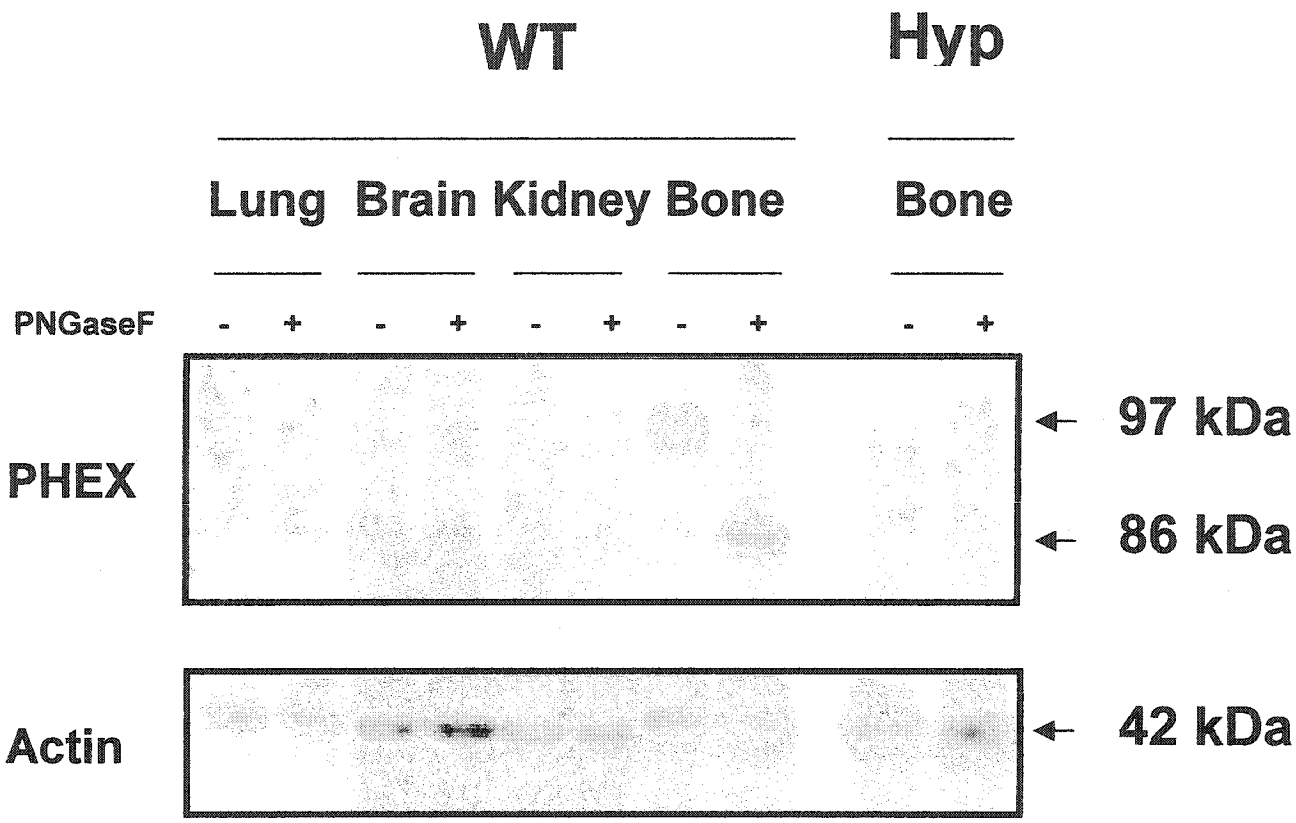

Fig. 7 Validation of PHEX antibody. Various mouse tissues including kidney and bone from both a wildtype and Hyp mouse, were either mock digested or PNGaseF digested before loading on a polyacrylamide gel as stated in the Materials and Methods. An actin antibody was used as a control. Fifty μg protein was loaded in each lane.

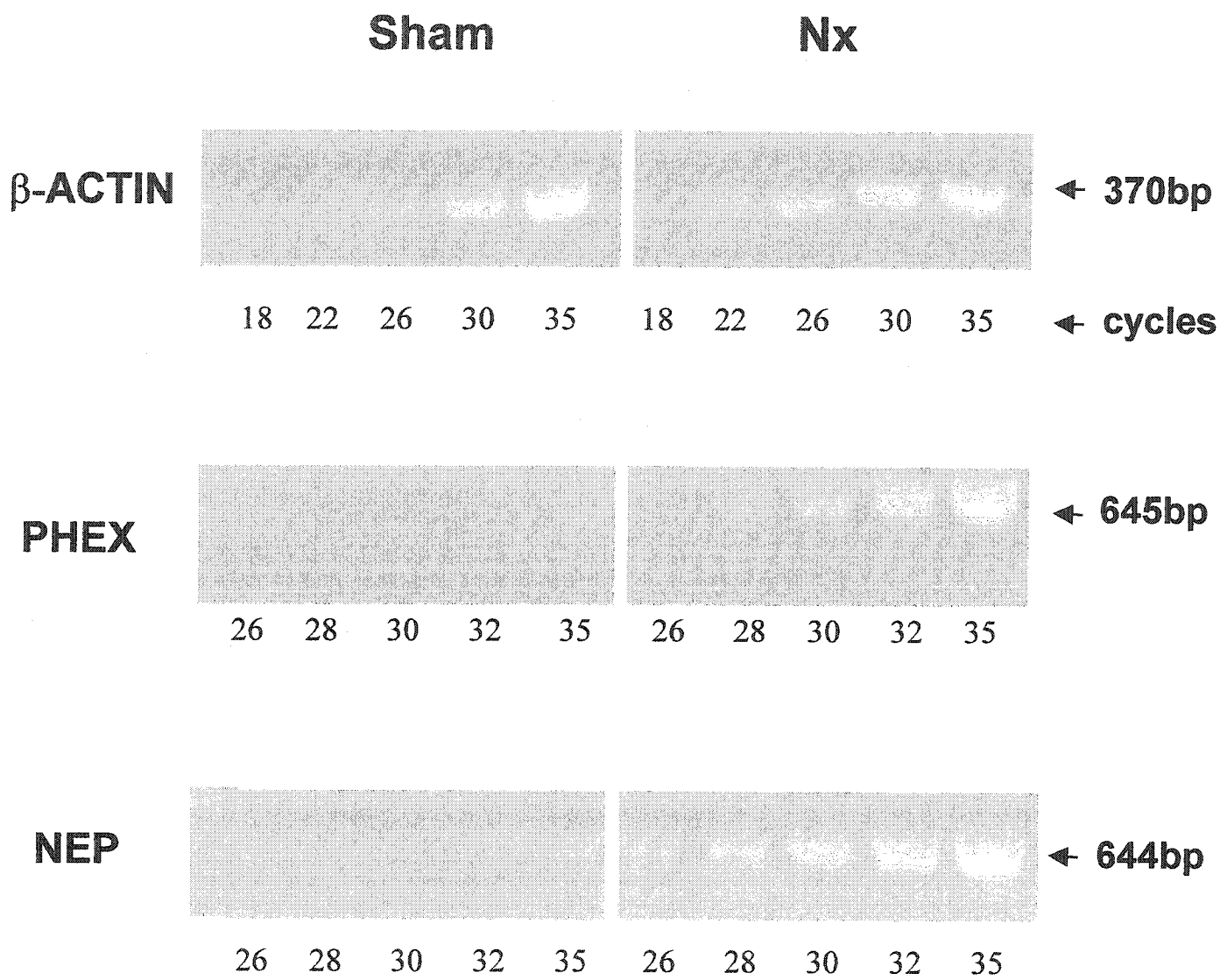

Fig. 8 Validation of time course RT-PCR. RNA, isolated from tibia of sham and Nx rats, were reverse transcribed and PCR amplified as stated in the Materials and Methods. During the PCR amplification of βactin, PHEX and NEP, 10 μl aliquots were removed at the cycles specified above and fractionated on an agarose gel.

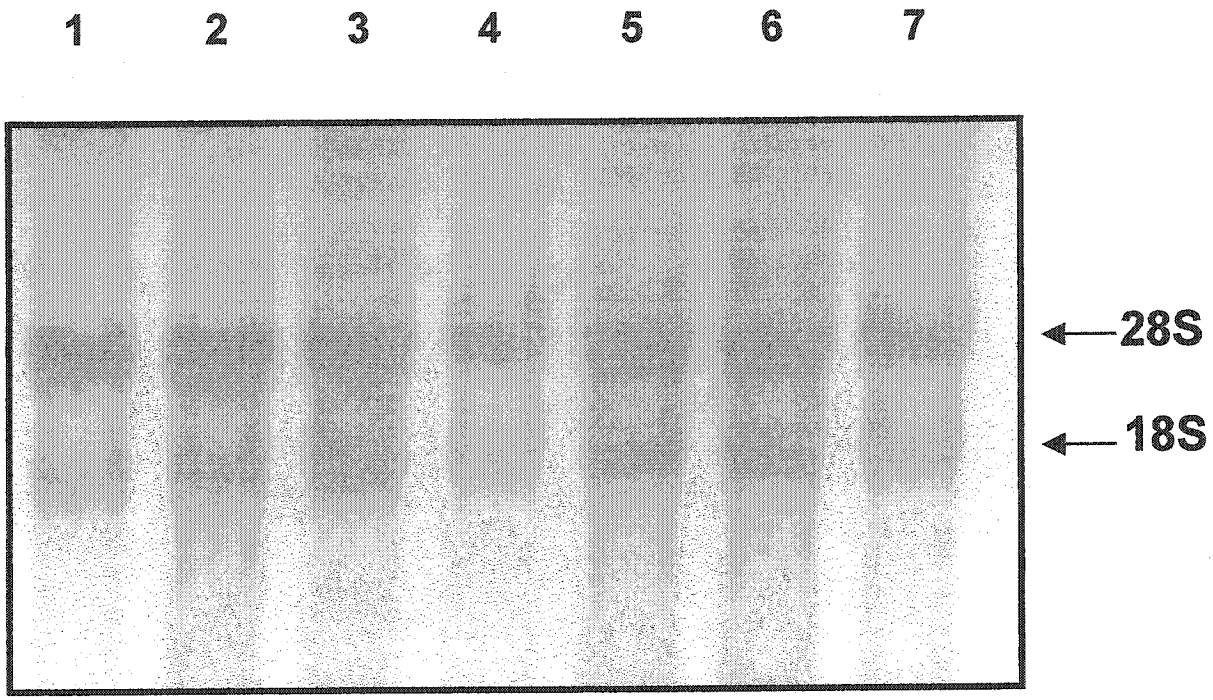

Fig. 9 Determination of total RNA integrity. A random selection of RNA samples extracted from rat tibia were fractionated on a formaldehyde gel (see Materials and Methods). The bands depicting the 18S and 28S ribosomal RNA are shown.

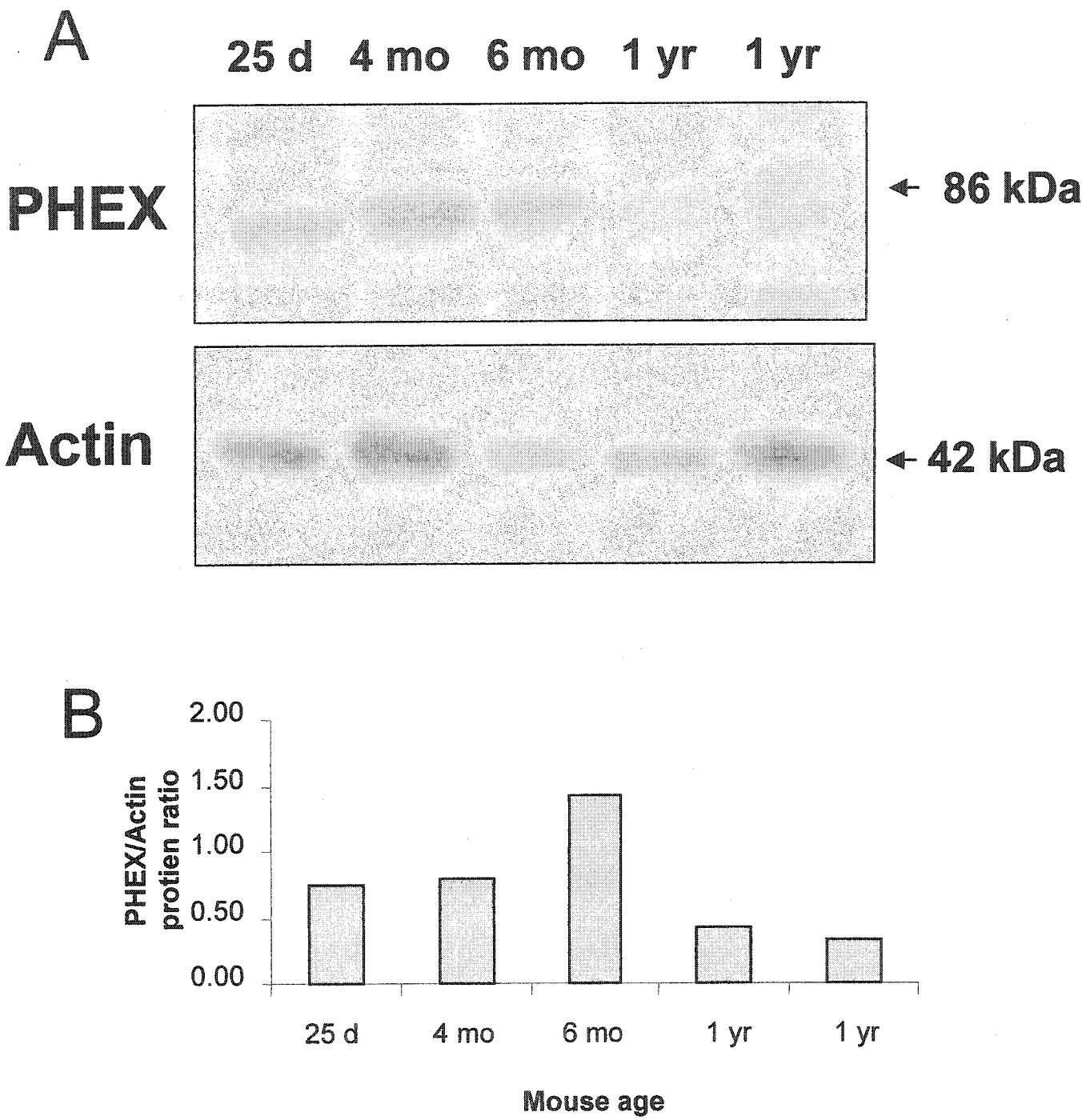

Fig. 10 Effect of age on PHEX protein expression in mouse tibia. (**A**) Normal mice were sacrificed at the ages indicated and protein extracts were prepared from tibia. Western analysis was performed on aliquots of PNGaseF-digested protein (50 μg), using PHEX and actin antibodies, as described in Materials and Methods. (**B**) The abundance of PHEX protein, relative to actin, as a function of age is depicted.

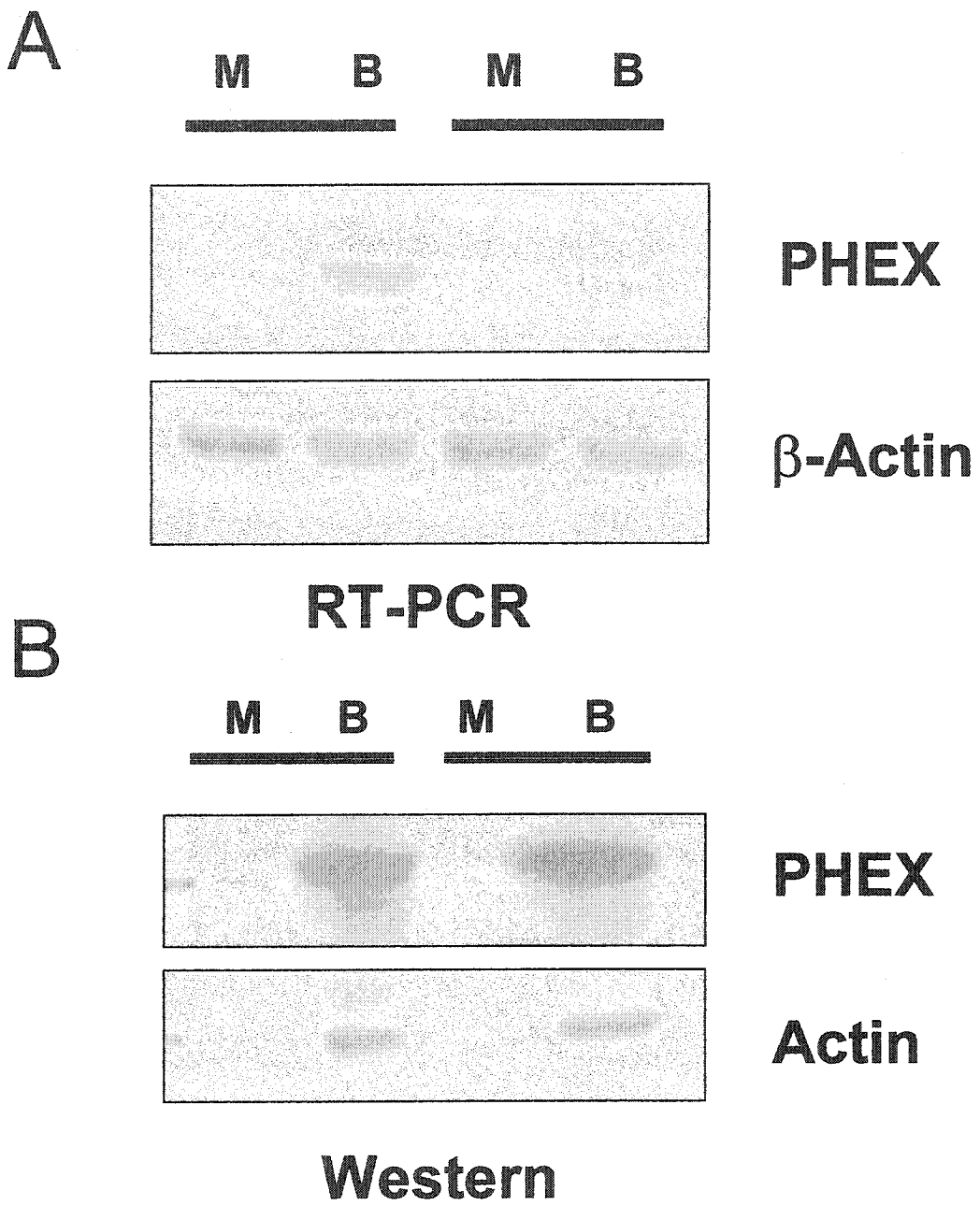

Fig. 11 PHEX mRNA and protein expression in marrow and calcified portion of bone. Rat tibia was cut at both ends and marrow was flushed out. (A) Total RNA, prepared from the marrow and residual bone, was reverse-transcribed and PCR amplified with PHEX and β-actin primers, and the PCR products fractionated on agarose gels, as described in Materials and Methods. (B) Western analysis was performed on 50 μg of protein, extracted from marrow and residual bone and digested with PNGaseF, using PHEX and actin antibodies, as described in Materials and Methods. (M, marrow; B, calcified portion of bone)

Time course RT-PCR. RNA extraction from microdissected rat parathryoid glands (PTG) generally yielded, at most, 2 ug of total RNA. This is an insufficient quantity for ribonuclease protection assay. Thus, an alternate method for transcript quantification was evaluated. For this purpose, total RNA from sham-operated and 5/6 nephrectomized rat tibia were reverse transcribed and PCR amplified (Fig. 8) using PHEX, NEP, and βactin specific primers (Table 1). Aliquots taken at various cycles were loaded on an agarose gel and fractionated. In all cases, the intensity of the PCR product increased with the number of cycles. Abundance of PHEX and NEP mRNA, relative to β-actin mRNA, was determined as described in the Materials and Methods (Fig. 8). We showed that PHEX and NEP mRNA abundance in the Nx rat tibia were elevated in comparison to the sham-operated rat. The results of the time course RT-PCR were confirmed by ribonuclease protection assay. Therefore, it is expected that results from using this procedure with PTG RNA will also yield quantitative data.

Determination of RNA Integrity. A random selection of RNA samples (n = 7) prepared from rat tibia were examined for RNA integrity. The samples were run on a formaldehyde gel and stained with ethidium bromide. Illumination with UV light showed that the two ribosomal bands, 18S and 28S, were clear and bright in each of the samples examined and the relative abundance of 28S is double of that of 18S (Fig. 9). This suggests that the RNA samples were not degraded to any large extent.

Effect of Age on PHEX Protein Expression in Tibia. Tibial protein expression was examined in the mouse and not the rat because of the availability of mice of the

appropriate ages. Protein was extracted from the tibia of mice aged 25 days to 1 year. Fifty microgram aliquots were PNGaseF digested and run on a polyacrylamide gel. The anti-actin antibody confirmed that protein was loaded into each lane. PHEX protein expression was robust in samples up to six months of age (Fig. 10A). The PHEX/actin ratios for each of the samples are depicted in Fig. 10B and indicated that after 6 months and before 1 year, PHEX expression declined to a minimum.

Is PHEX Expressed in Marrow or in the Calcified Portion of Tibia? Bone marrow was flushed from rat tibia and both RNA and protein were extracted from the marrow as well as the remaining calcified bone. Total RNA was reverse transcribed and PCR amplified and, as shown in Fig. 11A, PHEX was expressed in the calcified tissue only. β-actin was used as a loading marker to show that the absence of PHEX expression in marrow was not due to a technical error.

Protein from the bone marrow and rat tibia (after flushing) were deglycosylated using PNGaseF, run on a polyacrylamide gel and transferred to a membrane. The membrane was probed with the anti-PHEX and anti-actin antibodies. As in the RT-PCR results, PHEX protein was only detected in the remaining calcified tissue (Fig. 11B). A second membrane, prepared using 4 times the amount of bone marrow protein, confirmed that there was no PHEX protein expression in the bone marrow (data not shown). The actin levels, in this preparation, were comparable between the marrow samples and the calcified bone tissue.

Effect of Nephrectomy on PHEX and NEP Expression in Rat Bone

Previous studies (Denda et al., 1996) have shown that 5/6 nephrectomy results in the development of hyperparathyroidism. To ascertain whether or not increasing dietary Pi intake would further stress the rats and elicit a further increase in serum PTH levels, experiments were performed with rats fed either a control (0.74% Pi) diet or a high Pi (1.4% Pi) diet for 5 weeks (see Materials and Methods).

In rats fed the control diet, nephrectomy elicited a significant increase in serum creatinine and urea concentrations in comparison to the sham-operated rats (Table 2). Serum Pi increased in Nx rats fed the control diet (Table 2). This increase was also observed in Nx rats fed the high Pi diet. The high Pi diet led to an even greater increase in serum Pi in the sham-operated rats in comparison to the sham rats fed the control diet. Serum calcium concentrations were unchanged in the Nx rats on the control diet and were decreased in Nx rats fed the high Pi diet when compared to the sham-operated controls. Changes in Pi intake appeared to have no effect on the serum calcium concentration. Serum alkaline phosphatase remained unchanged in Nx rats fed the control diet in comparison to the sham-operated group (Table 2). However, the Nx rats fed the high Pi diet demonstrated a significant decrease in the serum alkaline phosphatase levels when compared to the sham-operated group. Nx rats fed the control and high Pi diets exhibited an increased serum PTH concentration in comparison to the sham-operated groups. In addition to nephrectomy eliciting an increase in serum PTH, the high Pi diet further elevated the serum concentrations in both the sham-operated and Nx rats (Table 2).

Urine Pi excretion, relative to urine creatinine excretion, was unchanged by Nx in rats fed the control diet (Table 2). Urine Pi/creatinine increased in Nx rats fed the high Pi diet in comparison to the sham-operated rats. The introduction of the high Pi diet significantly increased Pi/creatinine excretion in both the sham-operated and Nx rats when compared to their control diet counterparts. Urine calcium excretion, normalized by urine creatinine concentrations, remained unchanged by Nx or the high Pi diet. Urine cAMP, normalized by urine creatinine concentrations, by and large mirrored those of serum PTH, with the exception of rats fed the control diet, where nephrectomy elicited no change in concentration (Table 2). In the rats fed the high Pi diet, Nx elicited an increase in cAMP in comparison to sham-operated rats. The high Pi diet elicited a significant increase in cAMP concentrations in both the sham-operated and Nx rats. The cAMP/creatinine ratios for the rats on the control diet did not change with Nx. This maybe due to the small increase in serum PTH concentration of the Nx rats fed the control diet.

Table 2. Effect of 5/6 Nx on serum and urine parameters in rats fed control and high Pi diets

		Control diet		High Pi	
		sham	Nx	Sham	Nx
	n	18	18	25	26
Serum	Creatinine (μmol/L)	29.9 ± 3.6	49.2 ± 3.7*	28.2 ± 3.0	49.5 ± 4.5*
	Urea (mmol/L)	4.2 ± 0.2	8.8 ± 0.5*	4.1 ± 0.2	8.8 ± 0.3*
	Phosphate (mmol/L)	3.8 ± 0.1	4.5 ± 0.1*	2.9 ± 0.1	4.3 ± 0.1*
	Calcium (mmol/L)	2.4 ± 0.05	2.4 ± 0.05	2.5 ± 0.03	2.3 ± 0.03*
	Alkaline phosphatase (U/L)	249 ± 5.5	237 ± 10.8	244 ± 11.5	190 ± 7.05*^{9}
	PTH (pg/ml)	34.4 ± 1.4	68.4 ± 3.5*	39.1 ± 1.3^{9}	103.4 ± 5.8*^{9}
Urine	Pi/Creatinine	2.7 ± 0.6	2.7 ± 0.5	28.3 ± 2.6^{9}	39.7 ± 3.0*^{9}
	Ca/Creatinine	0.1 ± 0.02	0.2 ± 0.06	0.2 ± 0.02	0.3 ± 0.04
	cAMP/Creatinine	23.5 ± 1.0	19.6 ± 1.8	67.3 ± 14.6^{9}	105.6 ± 10.3*^{9}

Data depicted mean ± SEM

* Effect of nephrectomy, $p<0.05$ ^{9} Effect of diet, $p<0.05$

The effects of nephrectomy and dietary Pi on PHEX mRNA and protein expression in the tibia are depicted in Fig. 12. On a control Pi diet, nephrectomy elicited a 46% increase in the abundance of PHEX mRNA, relative to β-actin mRNA (100% ± 9 vs 146 ± 13, $p<0.05$). On the high Pi diet, nephrectomy also exhibited an increase in PHEX mRNA abundance when compared to the sham-operated counterparts (87% ± 4 vs 184 ± 21, $p<0.05$). There was no significant effect of dietary Pi in the sham-operated and Nx groups.

PHEX protein abundance also increased in Nx rats fed the control diet when compared to the sham-operated counterparts (100% ± 11 vs 139 ± 10). In addition, the high Pi diet significantly increased the overall abundance of PHEX protein in both the sham and Nx rats by almost 3-fold. The Nx rats fed the high Pi diet had approximately 80% more PHEX protein than the sham rats (293% ± 24 vs 378 ± 49, $p<0.05$) on the same diet.

NEP mRNA expression in tibia of sham and Nx rats fed the control and high Pi diets was also examined to determine whether the regulation of this related metallo-endopeptidase was correlated to that of PHEX. In rats fed the control diet, NEP mRNA abundance increased by 54% (100% ± 13 vs 154 ± 19, $p<0.05$) and in the rats fed the high Pi diet there was no significant effect of nephrectomy (139% ± 8 vs 178 ± 34) (Fig. 12). In the sham rats, the high Pi diet caused a significant increase of 39% in NEP mRNA abundance in comparison to the sham-operated rats on the control diet.

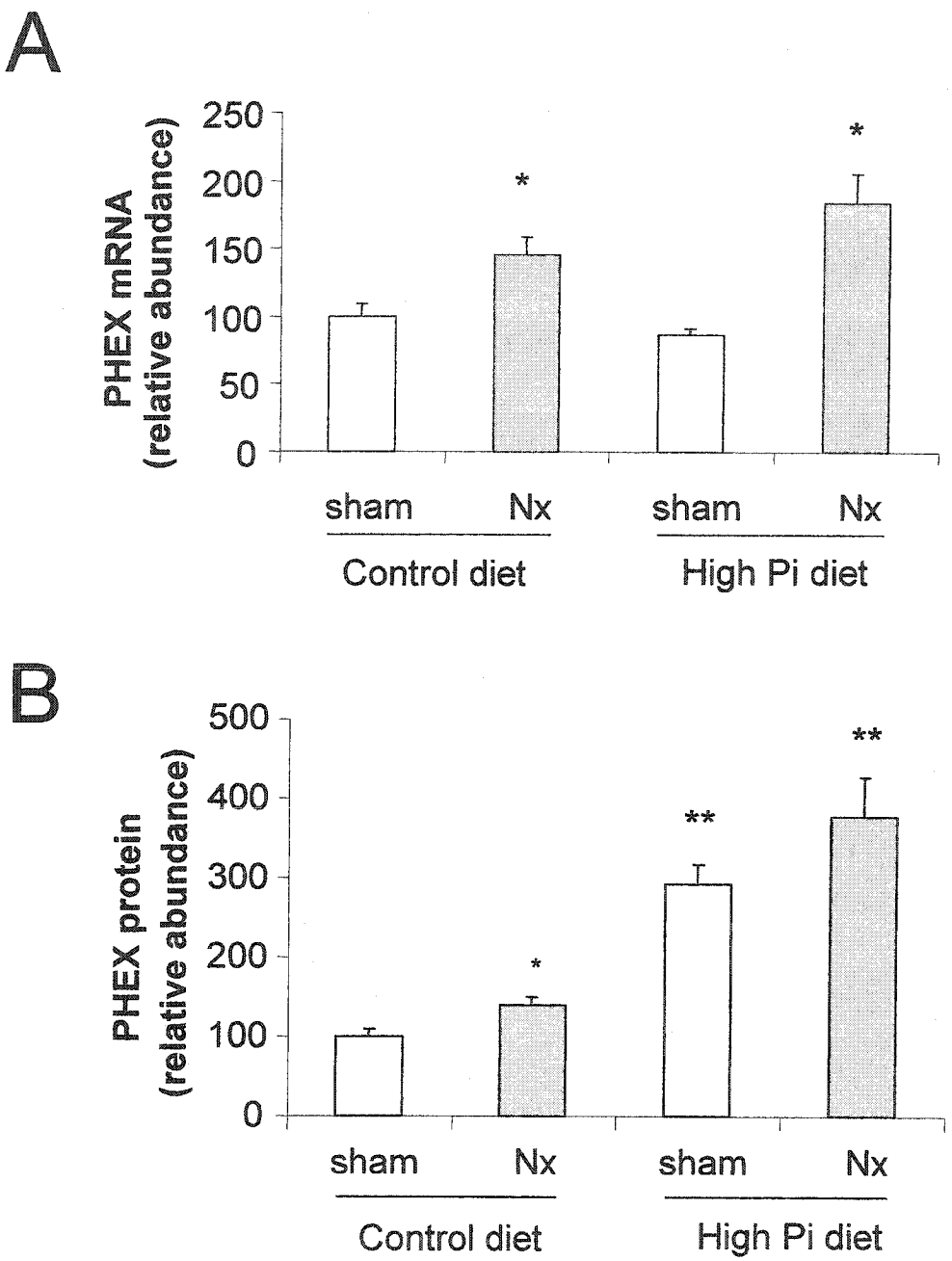

Fig. 12 Effect of Nx and dietary Pi on PHEX mRNA and protein abundance in rat tibia. (A) The abundance of PHEX mRNA, relative to β-actin mRNA, in tibia from sham and Nx rats fed control (0.76% Pi) and high Pi (1.4% Pi) diets was determined by ribonuclease protection assay as described in Materials and Methods (n = 10-20/group). (B) The abundance of PHEX protein, relative to actin, in tibial extracts prepared from the same groups of rats, was determined by western analysis using PHEX and actin antibodies, as described in Materials and Methods.

* Effect of Nx, $p<0.05$

** Effect of diet, $p<0.05$

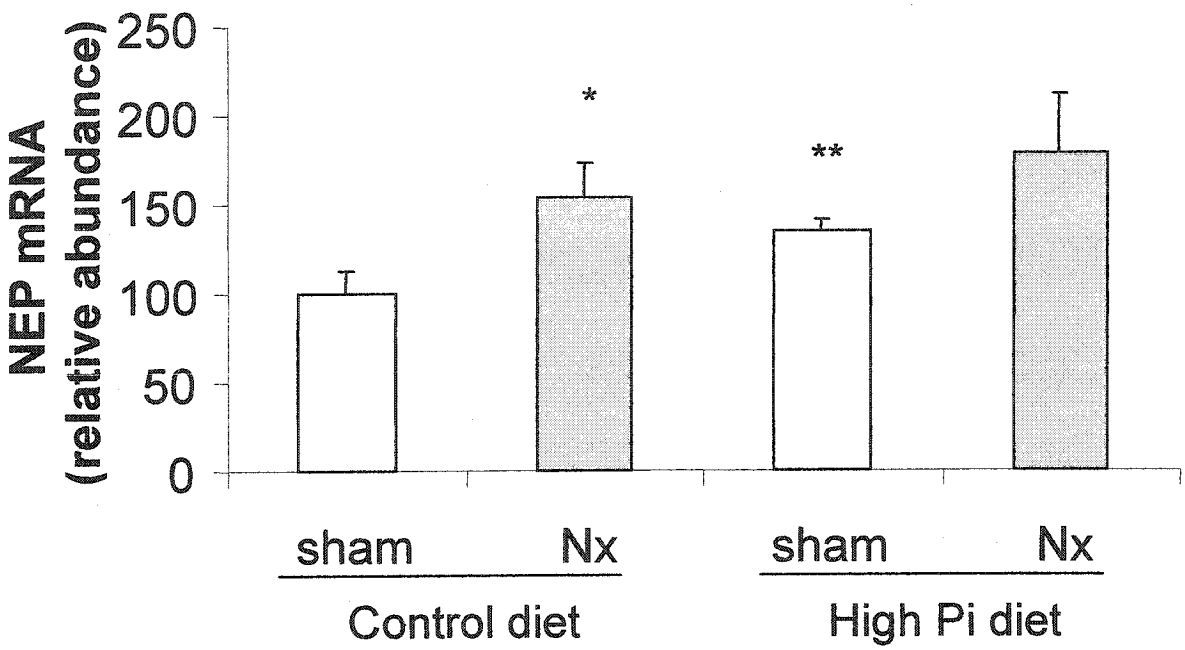

Fig. 13 Effect of Nx and dietary Pi on NEP mRNA abundance in rat tibia. The abundance of NEP mRNA, relative to β-actin mRNA, in tibia from sham and Nx rats fed control (0.76% Pi) and high Pi (1.4% Pi) diets was determined by ribonuclease protection assay as described in the Materials and Methods (n = 8-25/group).

* Effect of Nx, $p<0.05$

** Effect of diet, $p<0.05$

Osteocalcin is an osteoblastic bone marker that is up-regulated when bone turnover is increased. A ribonuclease protection assay was conducted, measuring osteocalcin mRNA, relative to β-actin. Neither nephrectomy nor diet had any effect on osteocalcin mRNA abundance (data not shown).

Effect of 1,25(OH)$_2$D on PHEX and NEP Expression in Rat Bone

It is well known that 1,25(OH)$_2$D is an inhibitor of PTH synthesis and secretion (Canaff et al., 1999). Thus, to examine the effect of hypoparathyroidism on PHEX expression, normal rats fed the control diet were treated with a pharmacalogical dose of 1,25(OH)$_2$D (10 pmol/g BW).

Serum creatinine levels were similar in the vehicle and 1,25(OH)$_2$D-treated groups (Table 3) as expected since kidney function was not compromised by short term treatment with 1,25(OH)$_2$D. Serum urea concentration was significantly increased in the 1,25(OH)$_2$D-treated group when compared to the vehicle-injected rats. The increase in serum urea, however, was small when compared to the increase elicited by nephrectomy (Table 2). Serum phosphate and serum calcium concentrations increased significantly in the 1,25(OH)$_2$D treated rats in comparison to the vehicle-injected rats (Table 3). Serum alkaline phosphatase concentrations remained unchanged when the rats were treated with 1,25(OH)$_2$D. PTH concentrations in the serum decreased significantly (35.14 pg/ml ± 1.91 vs 7.08 ± 0.53, $p<0.05$) with 1,25(OH)$_2$D treatment, as reported previously (Canaff et al., 1999).

Table 3. Effect of $1,25(OH)_2D$ on serum and urine parameters in normal rat fed the control diet.

		Vehicle	$1,25(OH)_2D$
	n	11	11
Serum	Creatinine (μmol/L)	20 ± 0.6	22.4 ± 1.3
	Urea (mmol/L)	3.2 ± 0.1	3.7 ± 0.1*
	Phosphate (mmol/L)	2.6 ± 0.05	3.3 ± 0.1*
	Calcium (mmol/L)	2.4 ± 0.06	3.1 ± 0.03*
	Alkaline phosphatase (nmol/ml/min)	7.4 ± 1.4	6.7 ± 0.5
	PTH (pg/ml)	35.1 ± 1.9	7.1 ± 0.5*
Urine	Pi/Creatinine	3.0 ± 0.7	13.2 ± 1.3*
	Ca/Creatinine	0.7 ± 0.1	4.6 ± 0.4*
	cAMP/Creatinine	19.1 ± 1.9	12.4 ± 1.2*

Data depicted mean ± SEM
* Effect of $1,25(OH)_2D$, $p<0.05$

Urine phosphate and calcium excretion, relative to urinary creatinine concentration, both increased significantly in response to $1,25(OH)_2D$ treatment (Table 3). This is most likely due to the increase in serum concentrations of both these minerals in $1,25(OH)_2D$-treated rats. Urine cAMP, relative to urinary creatinine, decreased in $1,25(OH)_2D$-treated rats when compared to the vehicle-treated rats, although the magnitude of the decrease was not as great as the one that occurred in the serum PTH concentration.

The effect of $1,25(OH)_2D$ treatment on PHEX mRNA and protein expression in the tibia differed from that of nephrectomy. PHEX mRNA abundance significantly decreased from 100% ± 12 in vehicle-treated rats to 63% ± 11 in $1,25(OH)_2D$-treated rats (Fig. 14A). This was mirrored by the PHEX protein abundance which significantly decreased from 100% ± 13 in vehicle-treated rats to 56% ± 11 in $1,25(OH)_2D$-treated rats (Fig. 14B). PHEX protein abundance in the calvaria of $1,25(OH)_2D$-treated rats (Fig. 15,

100% ± 38 vs 68 ± 16, $p<0.05$) demonstrated a significant decrease in comparison to the vehicle-treated rats. This data is in accordance to that of the tibia PHEX protein expression.

NEP mRNA abundance responded in a different manner than PHEX to $1,25(OH)_2D$ treatment. In the nephrectomy experiment discussed above, NEP and PHEX mRNA abundance were both increased in Nx rats in comparison to sham-operated rats. In the $1,25(OH)_2D$ injected rats, however, PHEX expression was decreased while NEP expression increased (100% ± 11 vs 143 ± 10, $p<0.05$) when compared to vehicle-treated rats (Fig. 16). This suggests that PHEX and NEP mRNA expression are independently regulated by $1,25(OH)_2D$.

Osteocalcin mRNA, relative to β-actin, was not affected by $1,25(OH)_2D$ administration in comparison to vehicle-treated rats (data not shown).

Effect of Nx and 1,25(OH)$_2$D on PHEX and NEP expression in Bone

We then compared the effect of $1,25(OH)_2D$ on PHEX expression in sham and Nx rats fed the high Pi diet. This was to determine whether the administration of $1,25(OH)_2D$ during the last two days before sacrifice would attenuate the increase in PHEX mRNA and protein levels that occur during 5/6 Nx.

Serum creatinine concentrations increased significantly only in Nx rats that were vehicle-treated when compared to sham-operated counterparts (Table 4). The increase in serum Cr concentration in $1,25(OH)_2D$-treated Nx rats was not significant but this was

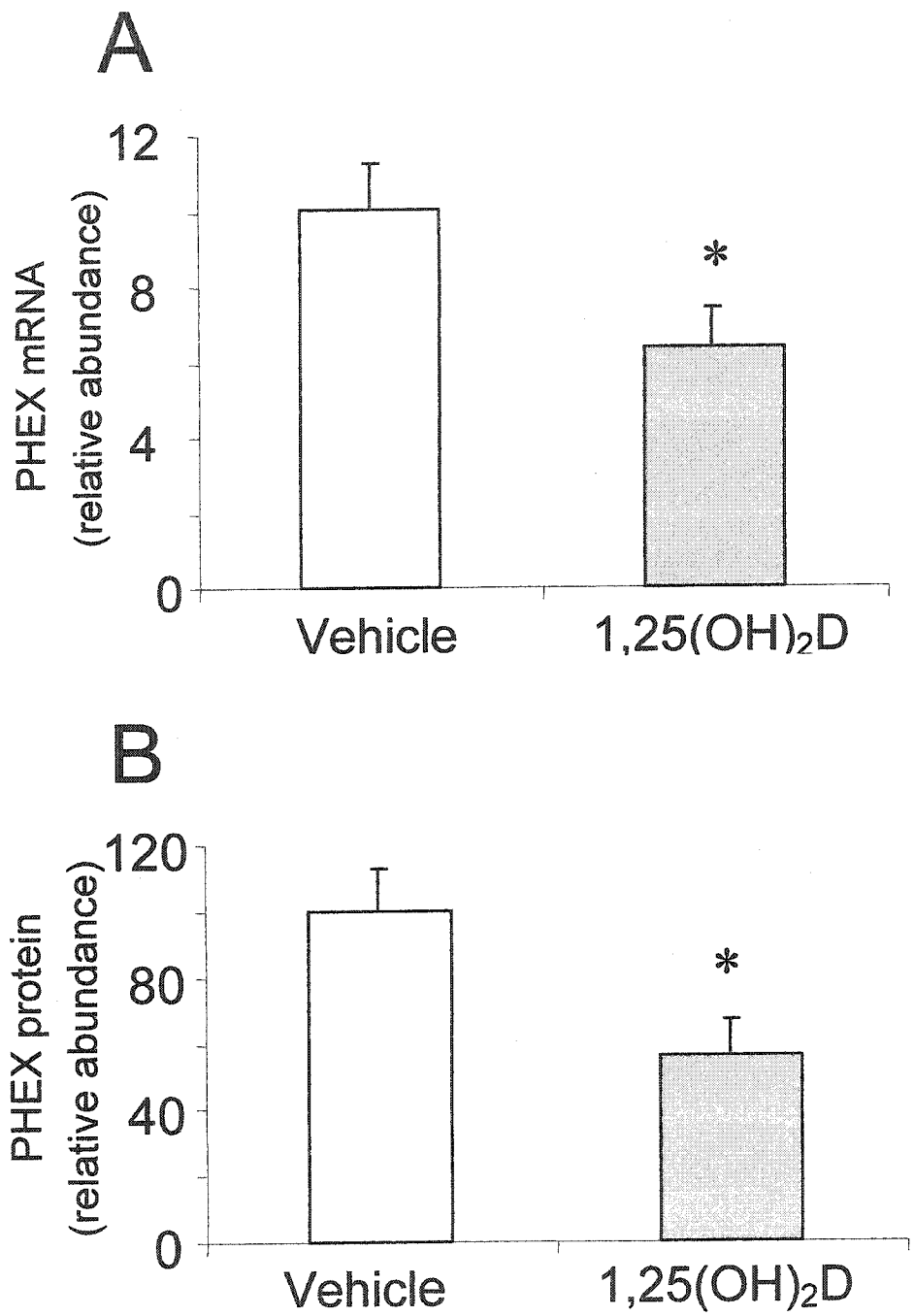

Fig. 14 Effect of $1,25(OH)_2D$ on PHEX mRNA and protein abundance in tibia of rats fed a control diet. (A) The abundance of PHEX mRNA, relative to β-actin mRNA, in tibia of rats injected with vehicle or $1,25(OH)_2D$ (10 pmol/g BW) was determined by ribonuclease protection assay as described in Materials and methods (n = 11/group). (B) The abundance of PHEX protein, relative to actin, in tibial extracts prepared from the same groups of rats, was determined by western analysis using PHEX and actin antibodies, as described in Materials and Methods.

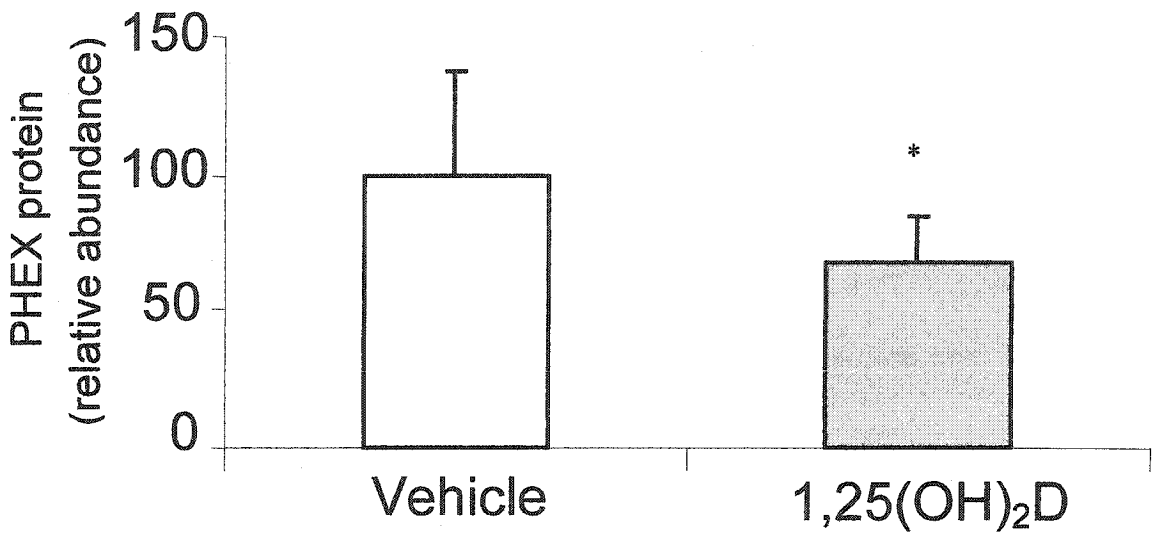

Fig. 15 Effect of $1,25(OH)_2D$ on PHEX protein abundance in rat calvaria. The abundance of PHEX protein, relative to actin, in calvaria of rats fed the control diet and injected with vehicle or $1,25(OH)_2D$ (10 pmol/g BW) was determined by western analysis (n = 9/group).

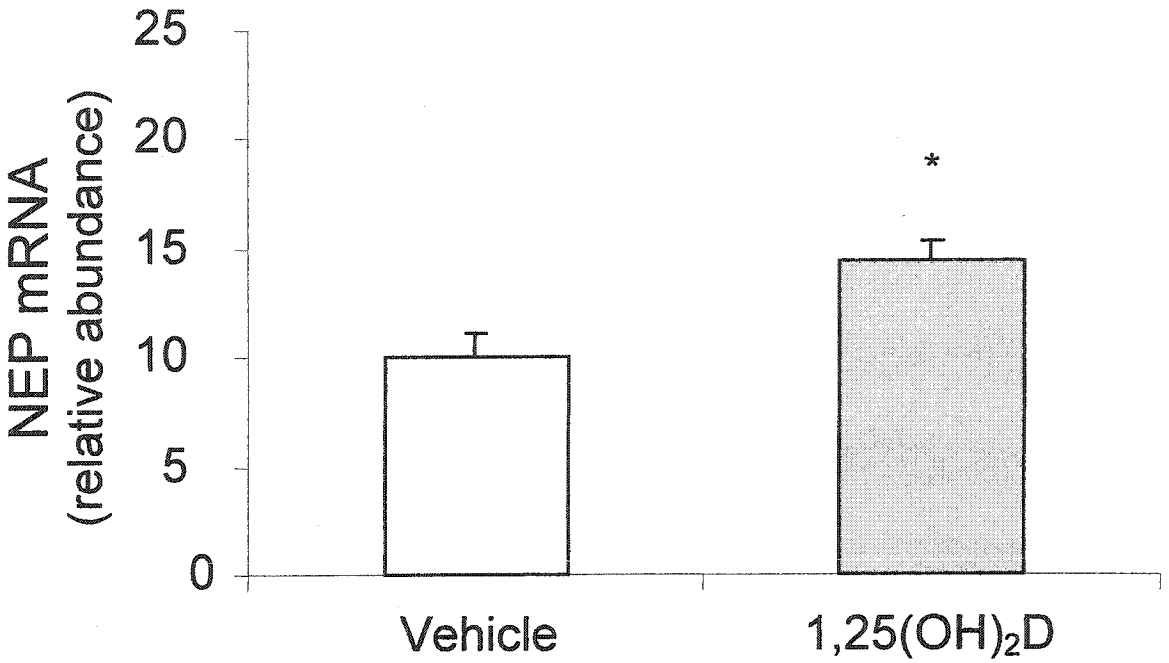

Fig. 16 Effect of $1,25(OH)_2D$ on NEP mRNA abundance in rat tibia. The abundance of NEP mRNA, relative to β-actin, in tibia of rats fed the control diet and injected with vehicle or $1,25(OH)_2D$ (10 pmol/g BW) was determined by ribonuclease protection assay (n = 11/group).

most likely due to the small number of rats used inthis study. $1,25(OH)_2D$ treatment did not affect the magnitude of increase in serum creatinine in the Nx rats when compared to vehicle-injected rats. Serum urea concentrations were significantly increased in Nx rats that were either vehicle- or $1,25OH)_2D$-treated when compared to the sham-operated counterparts (Table 4). $1,25(OH)_2D$ treatment significantly decreased the urea concentration in the sham-operated rats. However, this decrease is not repeated in either the Nx or normal rats injected with $1,25(OH)_2D$ and may be an artefact of the statistical analysis. Nephrectomy led to a significant increase in serum Pi concentration in the vehicle-treated rats. $1,25(OH)_2D$ treatment significantly blunted the increase of serum Pi in the Nx rats when compared to the vehicle-treated Nx rats (Table 4). Therefore, the serum Pi concentrations in the $1,25(OH)_2D$-treated Nx rats did not increase significantly in comparison to the $1,25(OH)_2D$-treated Nx rats. Nephrectomy caused a significant decrease in serum Ca concentration in the vehicle-treated rats in comparison to the vehicle-treated sham-operated rats. $1,25(OH)_2D$ led to an increase in serum calcium concentration in both the sham and Nx rats (Table 4). Alkaline phosphatase concentrations in the serum remained unchanged in response to nephrectomy and $1,25(OH)_2D$ treatment. Nephrectomy significantly increased serum PTH in the vehicle-treated group only. $1,25(OH)_2D$ treatment blunted the increase caused by nephrectomy leading to an insignificant increase in PTH concentration in the $1,25(OH)_2D$-treated Nx rats in comparison to the $1,25(OH)_2D$-treated sham-operated rats (Table 4).

Urine Pi excretion, relative to urine creatinine, increased in the vehicle-treated Nx rats (Table 4). Urine Pi/Creatinine values are not available for the $1,25(OH)_2D$-treated

animals. Urine calcium excretion, relative to urine creatinine, increased significantly in the vehicle-treated Nx rats in comparison to the sham-operated counterparts. Treatment

Table 4. Effect of $1,25(OH)_2D$ on serum and urine parameters in sham and Nx rats fed the high Pi diet

		Vehicle		$1,25(OH)_2D$	
		sham	Nx	sham	Nx
	n	25	26	5	5
Serum	Creatinine (μmol/L)	28.2 ± 3.0	49.5 ± 4.5*	22.2 ± 1.2	38.2 ± 1.1
	Urea (mmol/L)	4.1 ± 0.2	8.8 ± 0.3*	3.8 ± 0.2[9]	8.3 ± 0.4*
	Phosphate (mmol/L)	2.9 ± 0.1	4.3 ± 0.1*	2.7 ± 0.06	3.2 ± 0.08[9]
	Calcium (mmol/L)	2.5 ± 0.03	2.3 ± 0.03*	3.1 ± 0.07[9]	2.9 ± 0.05[9]
	Alkaline phosphatase (nmol/ml/min)	7.8 ± 0.3	6.5 ± 0.3	7.3 ± 0.4	7.3 ± 0.3
	PTH (pg/ml)	39.1 ± 1.3	103.4 ± 5.8*	31.4 ± 2.0	53.9 ± 1.5[9]
Urine	Pi/Creatinine	39.4 ± 3.3	45.6 ± 3.9	-	-
	Ca/Creatinine	0.1 ± 0.02	0.3 ± 0.06*	1.8 ± 0.4[9]	2.1 ± 0.9[9]
	cAMP/Creatinine	23.3 ± 5.2	36.0 ± 5.4*	11.4 ± 0.4[9]	11.7 ± 1.0[9]

Data depicted mean ± SEM - data not available

* Effect of Nephrectomy, $p<0.05$ [9] Effect of $1,25(OH)_2D$, $p<0.05$

with $1,25(OH)_2D$ increased Ca/creatinine excretion in both the sham and Nx rats in comparison to the vehicle-treated groups. cAMP excretion in the urine, relative to urine creatinine concentration, increased in the vehicle-treated Nx rats. In the $1,25(OH)_2D$-treated sham and Nx rats, the cAMP/creatinine concentrations were both much lower than the vehicle-treated sham-operated rats. This is consistent with the suppressive effect of $1,25(OH)_2D$ on PTH synthesis and secretion.

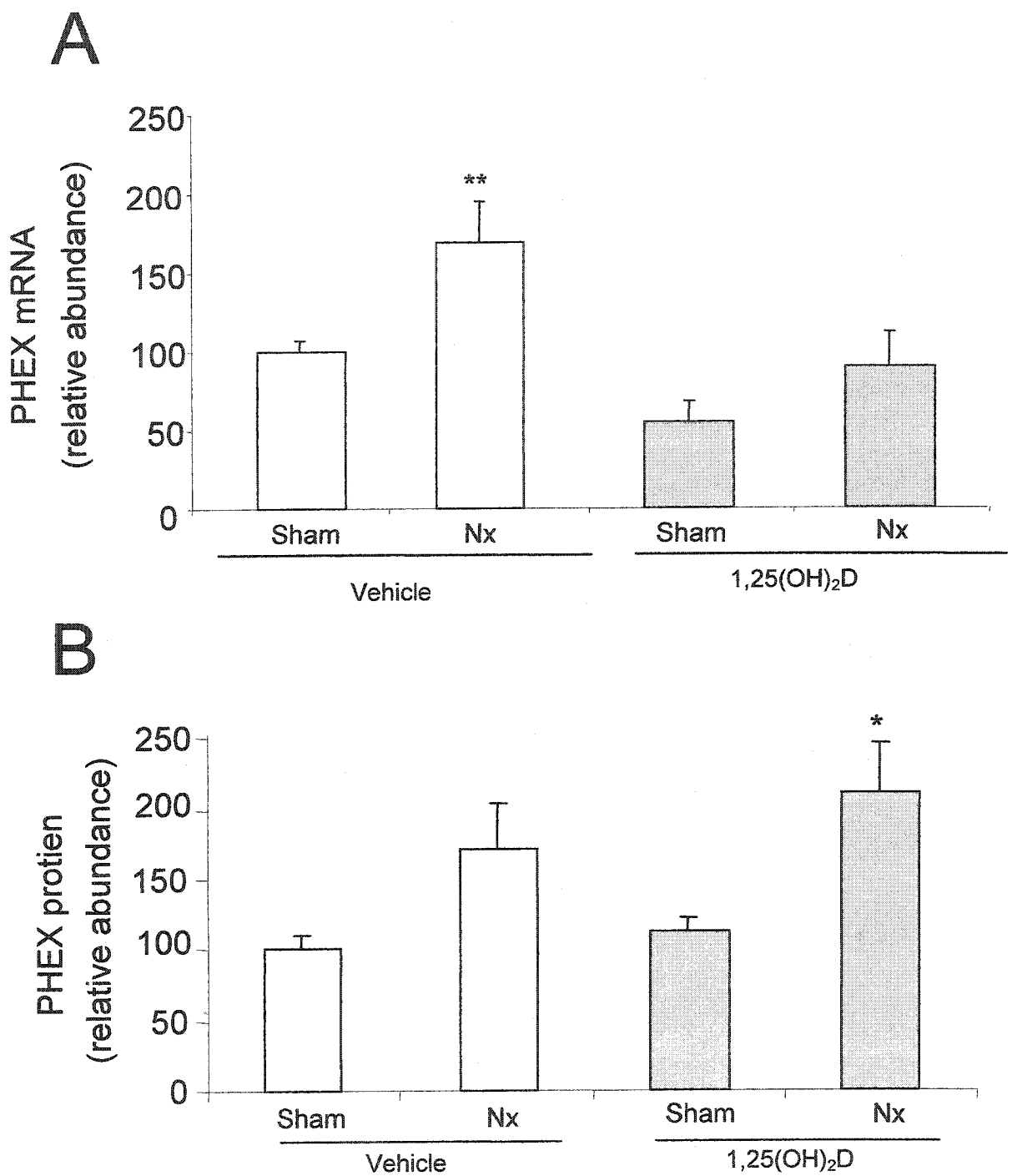

Fig. 17 Effect of Nx and $1,25(OH)_2D$ on PHEX mRNA and protein abundance in rat tibia. (**A**) Abundance of PHEX mRNA, relative to β-actin, in tibia from sham and Nx rats fed a high Pi (1.4% Pi) diet and injected with either vehicle or $1,25(OH)_2D$ (10 pmol/g BW) was determined by ribonuclease protection assay (n = 4-20/group). (**B**) Abundance of PHEX protein, relative to actin, by western analysis for the same groups.

* Effect of $1,25(OH)_2D$, $p<0.05$

** Effect of Nx, $p<0.05$

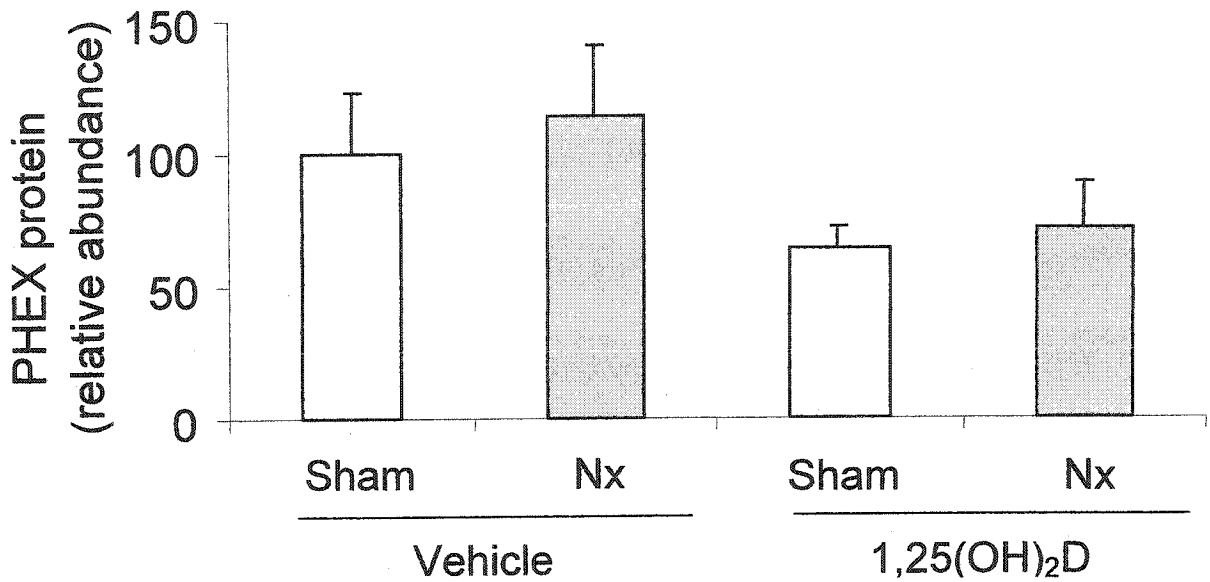

Fig. 18 Effect of Nx and $1,25(OH)_2D$ on PHEX protein abundance in rat calvaria. Abundance of PHEX protein, relative to actin, in sham and Nx rats fed a high Pi diet and injected with either vehicle or $1,25(OH)_2D$ was determined by western analysis (n = 3-7/group). No significant effects were observed.

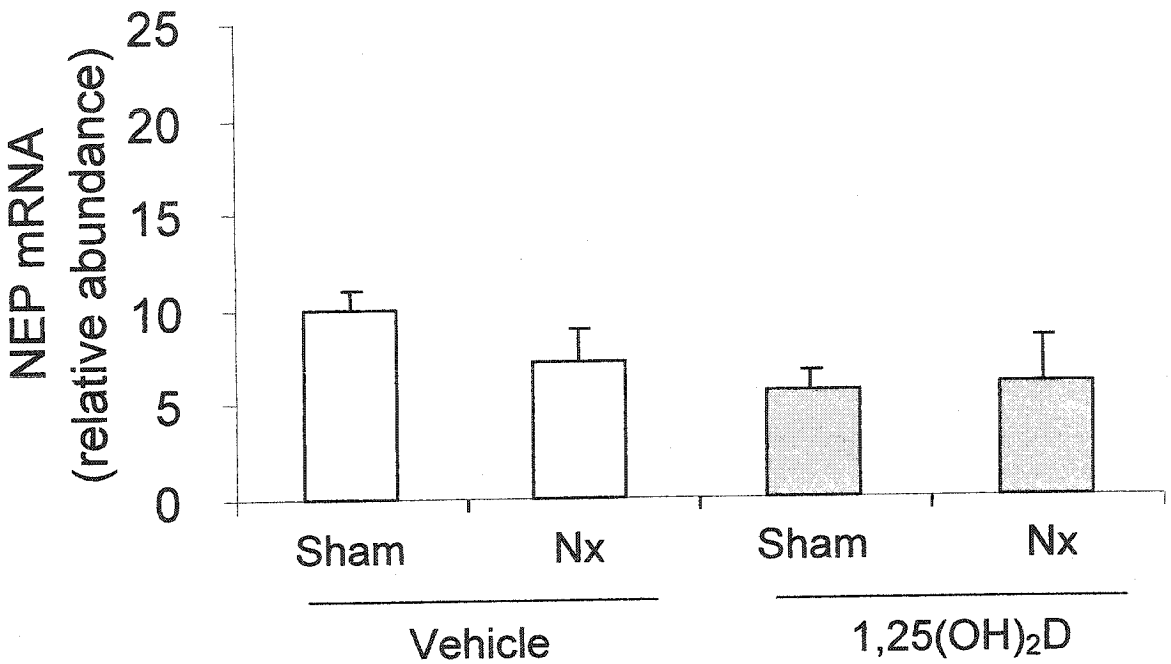

Fig. 19 Effect of Nx and $1,25(OH)_2D$ on NEP mRNA abundance in rat tibia. Abundance of PHEX mRNA, relative to β-actin, in sham and Nx rats fed a high Pi (1.4% Pi) diet and injected with either vehicle or $1,25(OH)_2D$ (10 pmol/g BW) was determined by ribonuclease protection assay (n = 4-8/group). No significant effects were observed.

PHEX mRNA and protein abundance in tibia were affected by both Nx and/or $1,25(OH)_2D$ injection. Nephrectomy caused a significant increase in PHEX mRNA expression in the vehicle-treated rats (Fig. 17A, 100% ± 7 vs 169 ± 26, $p<0.05$). The increase in PHEX mRNA in the $1,25(OH)_2D$-treated Nx rats was not significant (Fig. 17A, 55% ± 13 vs 89 ± 22). This is most likely due to the small number of rats used in the study. $1,25(OH)_2D$ injection resulted in an apparent decrease in PHEX mRNA expression in both the sham-operated and Nx rats in comparison to the respective vehicle-treated groups but this decrease was not significant. Nephrectomy caused a significant increase in PHEX protein expression in the tibia of $1,25(OH)_2D$-injected rats when compared to the $1,25(OH)_2D$-injected sham-operated rats (Fig. 17B). Nephrectomy in vehicle treated rats caused an apparent increase in PHEX protein expression but it was not significant. $1,25(OH)_2D$ did not affect PHEX protein abundance in either the sham or Nx groups in comparison to the vehicle-treated rats. PHEX protein abundance in the calvaria was not changed in response to nephrectomy or $1,25(OH)_2D$ injection (Fig. 18). Similarly, changes in NEP mRNA abundance in tibia was not significantly different in response to nephrectomy and $1,25(OH)_2D$ treatment (Fig. 19).

Correlation Between Tibial PHEX Expression and Parathyroid Gland Status

We showed that PHEX mRNA and protein expression in tibia appears to be regulated by the state of the parathyroid gland. During periods of increased PTH production, (e.g. in 5/6 nephrectomy rats) an increase in PHEX mRNA and protein abundance is elicited. When circulating PTH concentrations are reduced, (e.g. in

1,25(OH)2D-treated rats), PHEX mRNA and protein abundance in the tibia are decreased. Our findings, however, do not rule out the possibility that PHEX mRNA and protein levels are regulated by serum calcium concentrations since the osteoblasts where PHEX is expressed also contain a calcium sensing receptor. We thus performed regression analysis between either serum PTH or serum calcium and PHEX mRNA and protein to discern which of these two mechanisms is more likely. All samples from each of the previously discussed experiments were used for these analyses.

Initially, we showed that the serum PTH concentration correlated with the urine cAMP/creatinine ratio, thereby confirming the positive correlation that exists between these two parameters (Fig. 20, $p<<0.0001$, $R^2 = 0.5323$). We also show that serum PTH concentration was positively correlated to PHEX mRNA abundance (Fig. 21, $p<0.001$, $R^2 = 0.3028$) and to PHEX protein (Fig. 22, $p<<0.0001$, $R^2 = 0.5501$) in rat tibia, as expected from the results described above.

The negative correlation between serum PTH and serum Ca concentrations was confirmed (Fig. 23, $p<<0.0001$, $R^2 = 0.3011$). There appeared to be a segregation of data points between the sham/vehicle rats and the Nx/1,25$(OH)_2$D treated rats. When the regression is calculated separately for these two possible groupings, the p value for the sham/vehicle group is 0.0005 and the R-squared is 0.8984. The second grouping, the Nx/1,25$(OH)_2$D group, has a p value of lower than 0.0001 and an R-squared value of 0.7998. The increased R-squared value when the groups are calculated separately rather than together suggests that there is a difference in the PTH/calcium relationship following the insult of nephrectomy or 1,25$(OH)_2$D-injection. In contrast to the PHEX mRNA and protein correlations with serum PTH, the relationship between PHEX mRNA and protein

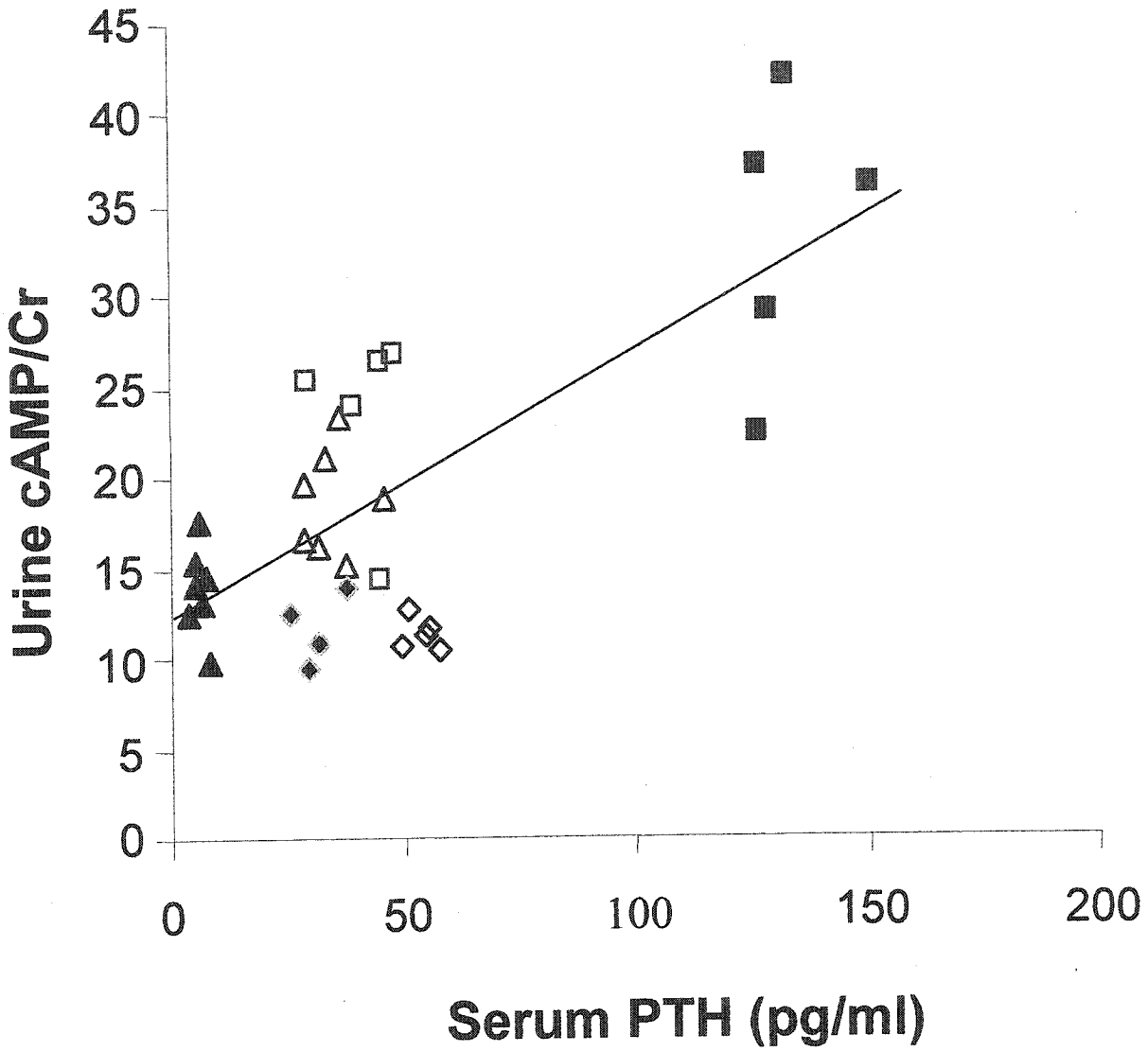

Fig. 20 Relationship between serum PTH concentration and urine cAMP/creatinine. Data were obtained from rats that were sham-operated (white square), nephrectomized (black squares), vehicle-treated (white triangles), $1,25(OH)_2D$-treated (black triangles), sham-operated and $1,25(OH)_2D$-treated (white diamonds), and nephrectomized and $1,25(OH)_2D$-treated (black diamonds). Solid line represents the trendline. The serum PTH concentration and urine cAMP/creatinine were determined as described in Methods and Materials. $p<0.0001$, $R^2 = 0.5323$

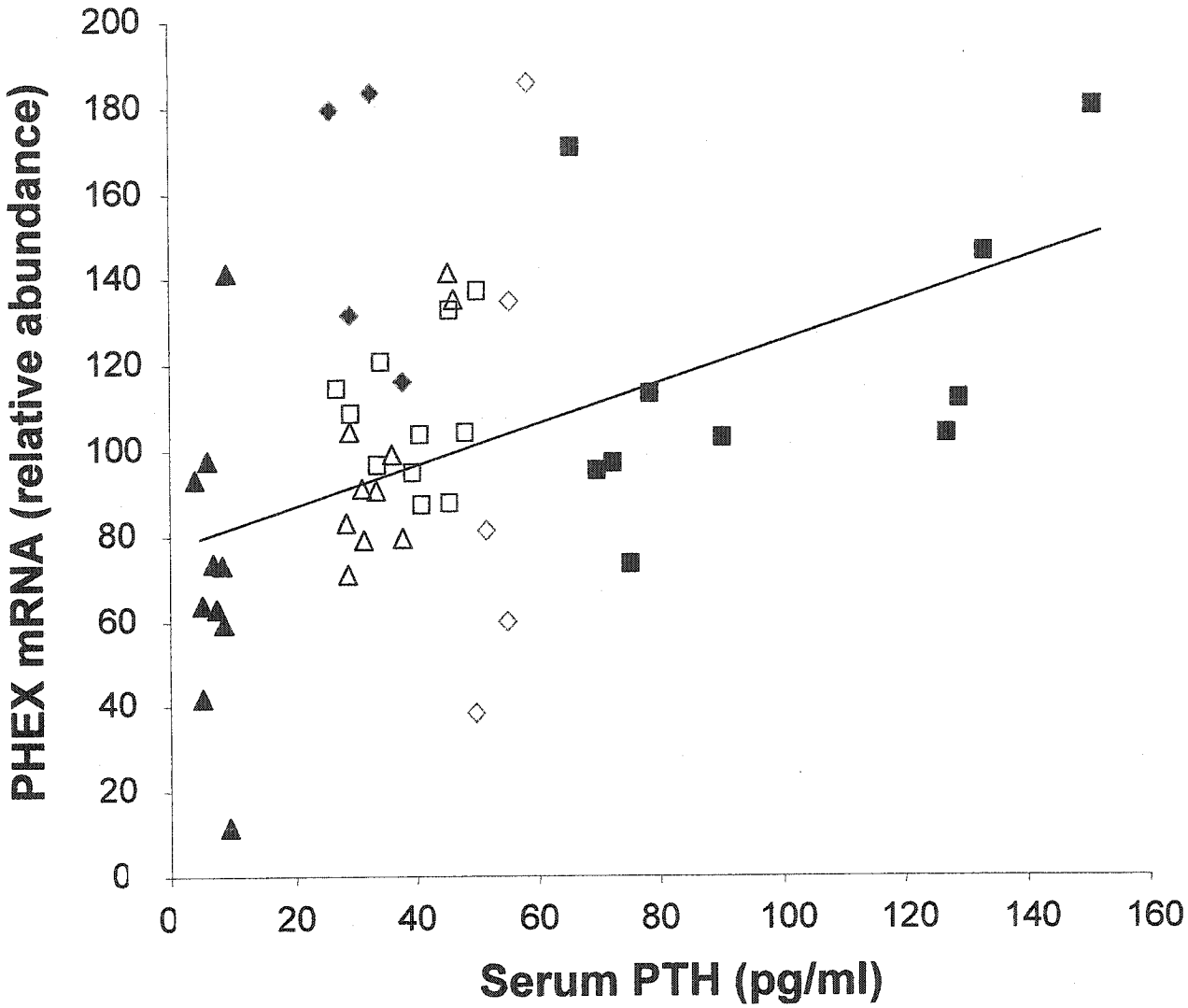

Fig. 21 Relationship between relative PHEX mRNA abundance in tibia and serum PTH concentration. Data were obtained from rats that were sham-operated (white square), nephrectomized (black squares), vehicle-treated (white triangles), $1,25(OH)_2D$-treated (black triangles), sham-operated and $1,25(OH)_2D$-treated (white diamonds), and nephrectomized and $1,25(OH)_2D$-treated (black diamonds). Solid line represents the trendline. PHEX mRNA abundance and serum PTH concentration were determined as described in Methods and Materials. $p<0.0001$, $R^2 = 0.3028$

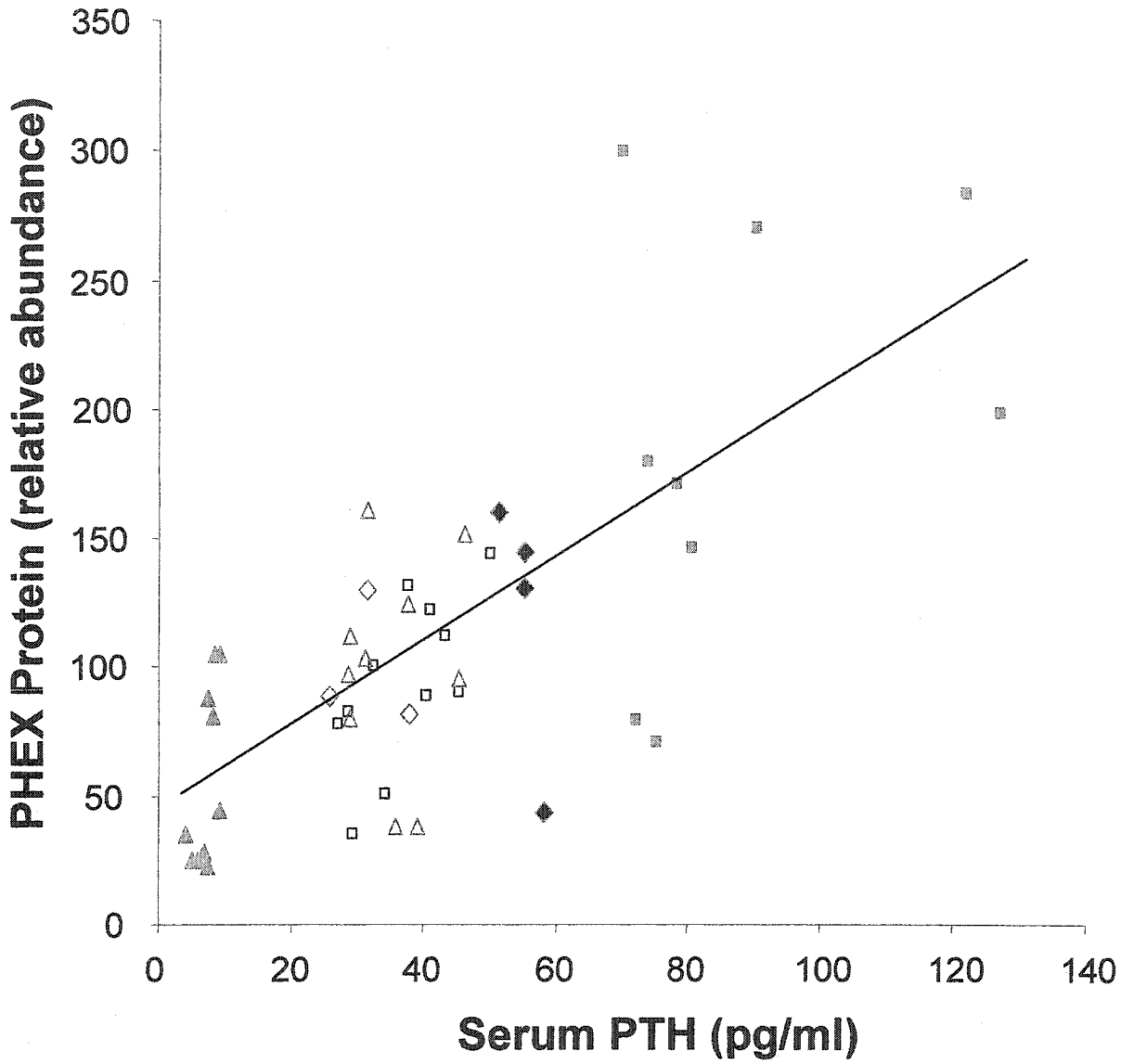

Fig. 22 Relationship between relative PHEX protein abundance in tibia and serum PTH concentration. Data were obtained from rats that were sham-operated (white square), nephrectomized (black squares), vehicle-treated (white triangles), 1,25$(OH)_2$D-treated (black triangles), sham-operated and 1,25$(OH)_2$D-treated (white diamonds), and nephrectomized and 1,25$(OH)_2$D-treated (black diamonds). Solid line represents the trendline. PHEX protein abundance and serum PTH concentration were determined as described in Methods and Materials. $p<0.0001$, $R^2 = 0.5501$

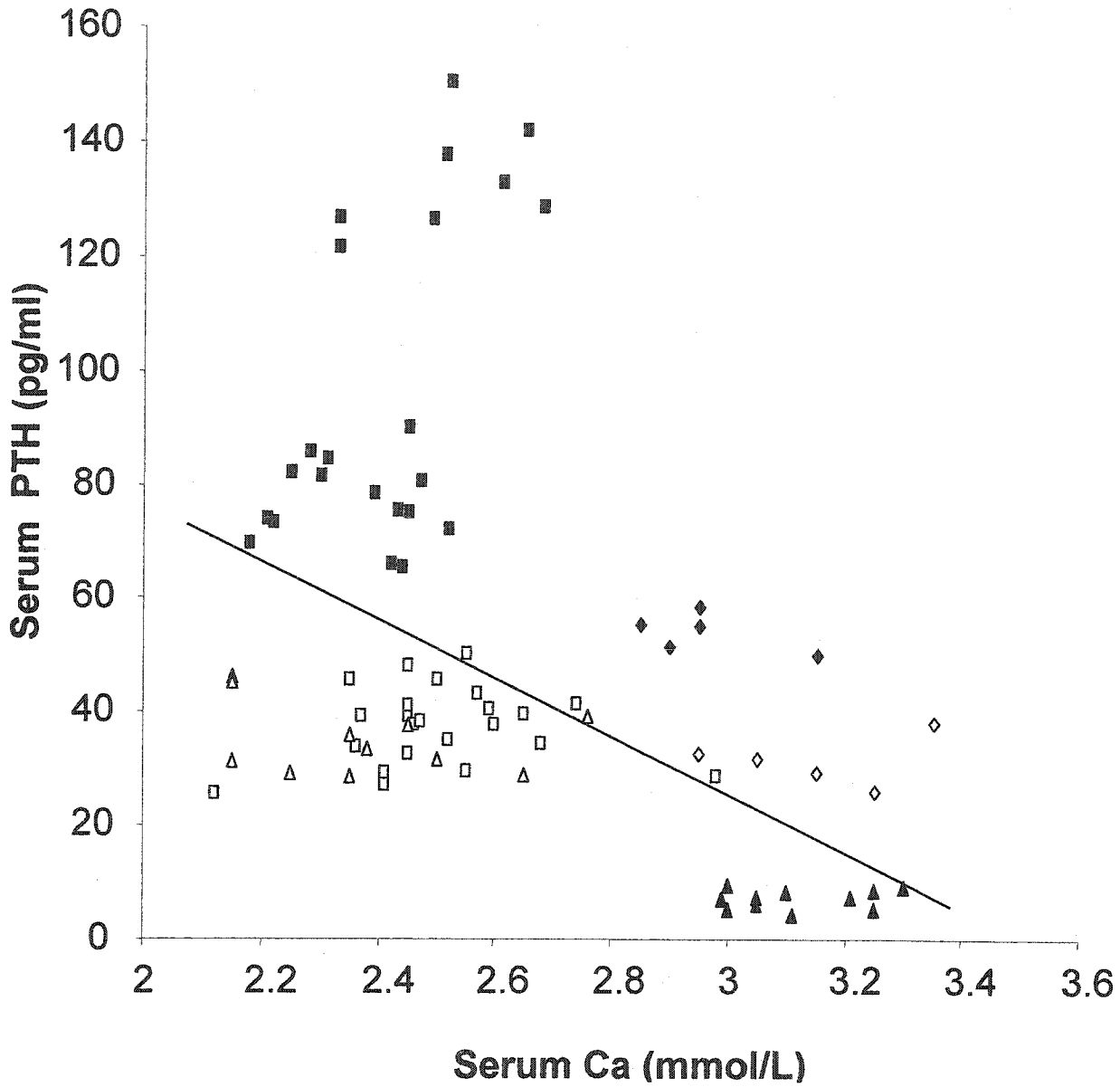

Fig. 23 Relationship between serum PTH concentration and serum calcium concentration. Data were obtained from rats that were sham-operated (white square), nephrectomized (black squares), vehicle-treated (white triangles), 1,25$(OH)_2$D-treated (black triangles), sham-operated and 1,25$(OH)_2$D-treated (white diamonds), and nephrectomized and 1,25$(OH)_2$D-treated (black diamonds). Solid line represents the trendline. The serum PTH concentration and serum calcium concentration were determined as described in Methods and Materials. $p<0.0001$, $R^2 = 0.3011$

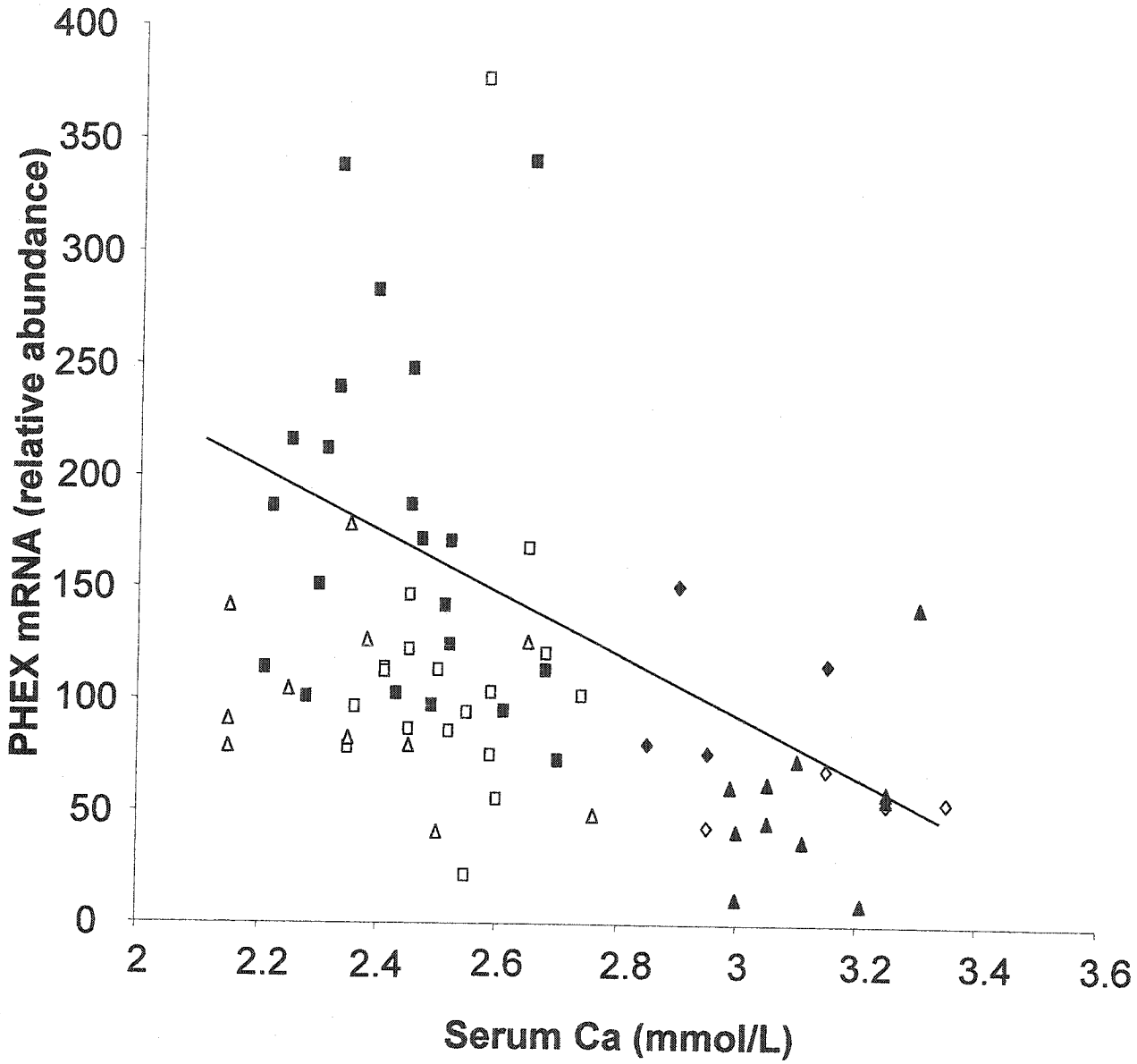

Fig. 24 Relationship between relative PHEX mRNA abundance in rat tibia and serum calcium. Data were obtained from rats that were sham-operated (white square), nephrectomized (black squares), vehicle-treated (white triangles), $1,25(OH)_2D$-treated (black triangles), sham-operated and $1,25(OH)_2D$-treated (white diamonds), and nephrectomized and $1,25(OH)_2D$-treated (black diamonds). Solid line represents the trendline. PHEX mRNA abundance and serum calcium concentration were determined as described in Methods and Materials. $p<0.0002$, $R^2 = 0.1811$

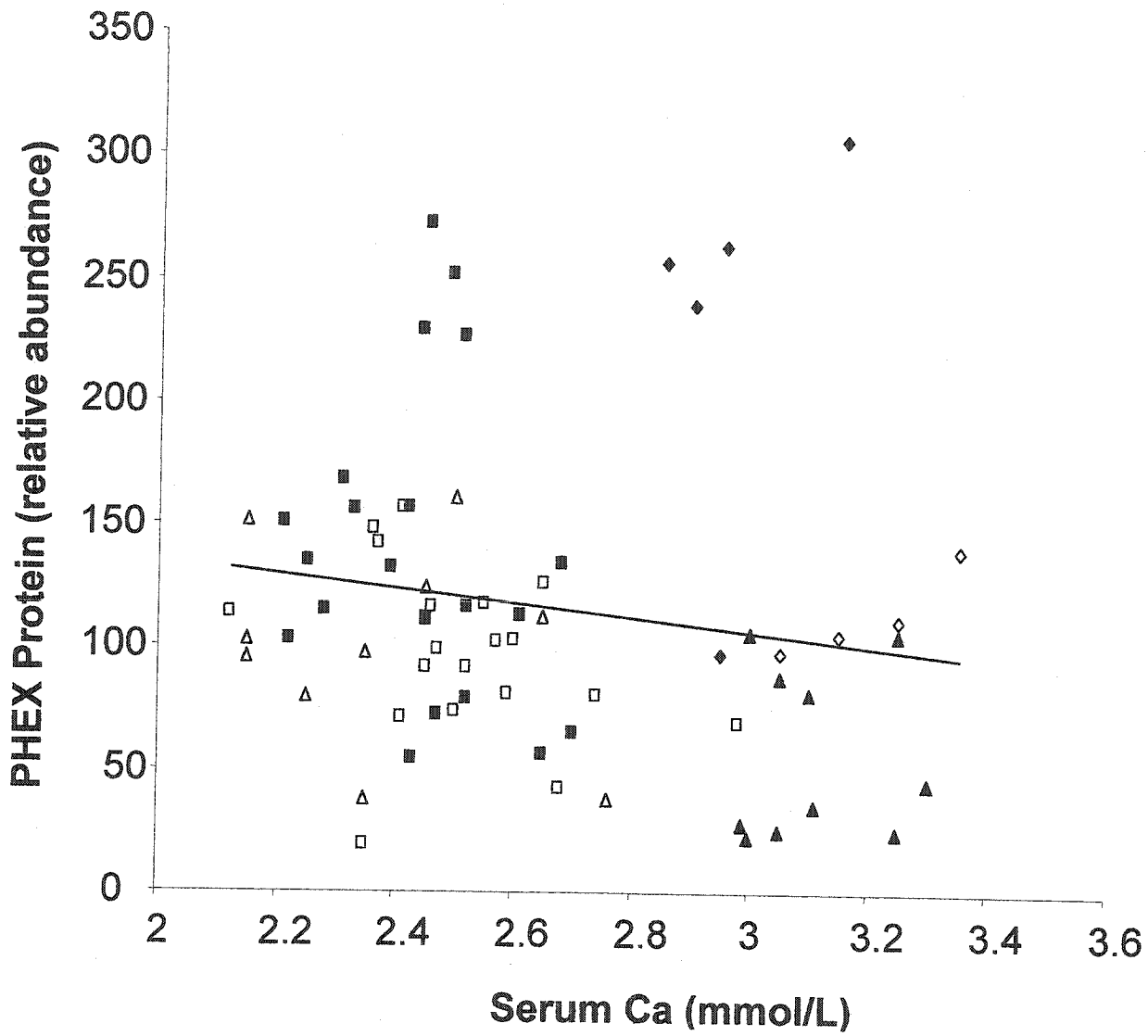

Fig. 25 Relationship between relative PHEX protein abundance in rat tibia and serum calcium. Data were obtained from rats that were sham-operated (white square), nephrectomized (black squares), vehicle-treated (white triangles), $1,25(OH)_2D$-treated (black triangles), sham-operated and $1,25(OH)_2D$-treated (white diamonds), and nephrectomized and $1,25(OH)_2D$-treated (black diamonds). Solid line represents the trendline. PHEX protein abundance and serum calcium concentration were determined as described in Methods and Materials. $p<0.3114$, $R^2 = 0.0150$

and serum calcium is not as tightly correlated (Fig. 24, $p<0.001$, $R^2 = 0.1811$), as evidenced by the lower R-squared value. PHEX protein abundance appears not to be significantly correlated with serum calcium (Fig. 25, $p= 0.3114$, $R^2 = 0.0150$).

Regression analysis was also performed on NEP mRNA abundance in comparison with serum PTH concentrations. The sham-operated and Nx rats fed control and high Pi diets, the normal rats treated with $1,25(OH)_2D$ as well as the sham-operated and Nx rats treated with $1,25(OH)_2D$ were used in the analysis. NEP mRNA abundance does not appear to be correlated to serum PTH status (*data not shown*, $R^2= 0.071$).

Effect of Calcitonin

A pilot study examining an alternate method for inducing hyperparathyroidism was conducted. To achieve this goal, a small group of rats (n= 2/group) were injected i.p. with calcitonin (25 or 50 ng/g) and sacrificed at 3 or 6 hours after injection to examine the effects of calcitonin on PHEX mRNA and protein abundance. Due to the small number of animals in each group, no statistical analysis was possible.

Serum creatinine and urea appear to have increased in the three calcitonin-injected groups in comparison to the vehicle-injected group (Table 5). Serum phosphate concentrations were moderately increased and serum calcium decreased in the three calcitonin-treated groups when compared to the vehicle-treated group. Alkaline phosphatase remained unchanged. PTH in the serum increased in each of the injected groups (39.95 pg/ml ± 0.95 vs 50.25 ± 1.25, 57.15 pg/ml ± 1.35, and 49.05 ± 0.3) when compared to vehicle-injected rats.

Urine Pi excretion, relative to urine creatinine, was unchanged by calcitonin injection and urine calcium excretion, relative to urine creatinine, was elevated in the calcitonin-treated groups when compared to vehicle-injected rats. cAMP, relative to urine creatinine, was increased in the calcitonin-treated groups in comparisn to vehicle-treated rats as expected from the serum PTH results.

PHEX mRNA abundance in the tibia increased with injection of 25 ng/g of calcitonin in comparison to the vehicle-injected rats (Fig. 26A). A change was seen after three hours (100% ± 19 vs 162 ± 42) and increased even more after 6 hours (244% ± 56). The injection of 50 ng/g of calcitonin, however, appears to have a diminished effect on the PHEX mRNA levels (137% ± 25) compared to vehicle injected rats. PHEX protein abundance did not appear to be different in the vehicle-treated and calcitonin-treated rats (Fig. 26B) although six hours may not be enough time to see a change in the protein abundance. PHEX mRNA and protein abundance in rat calvaria remained unchanged (Fig. 27 A and B).

NEP mRNA abundance in the tibia followed the same pattern as the PHEX mRNA. The expression level increased to 198% in the 25 ng/g calcitonin-treated group that was sacrificed at 3 hours, to 486% in the 25 ng/g rats sacrificed after 6 hours and in the 50 ng/g sacrificed after 6 hours group the NEP mRNA increased to 232% in comparison to the vehicle-treated rats (Fig. 28).

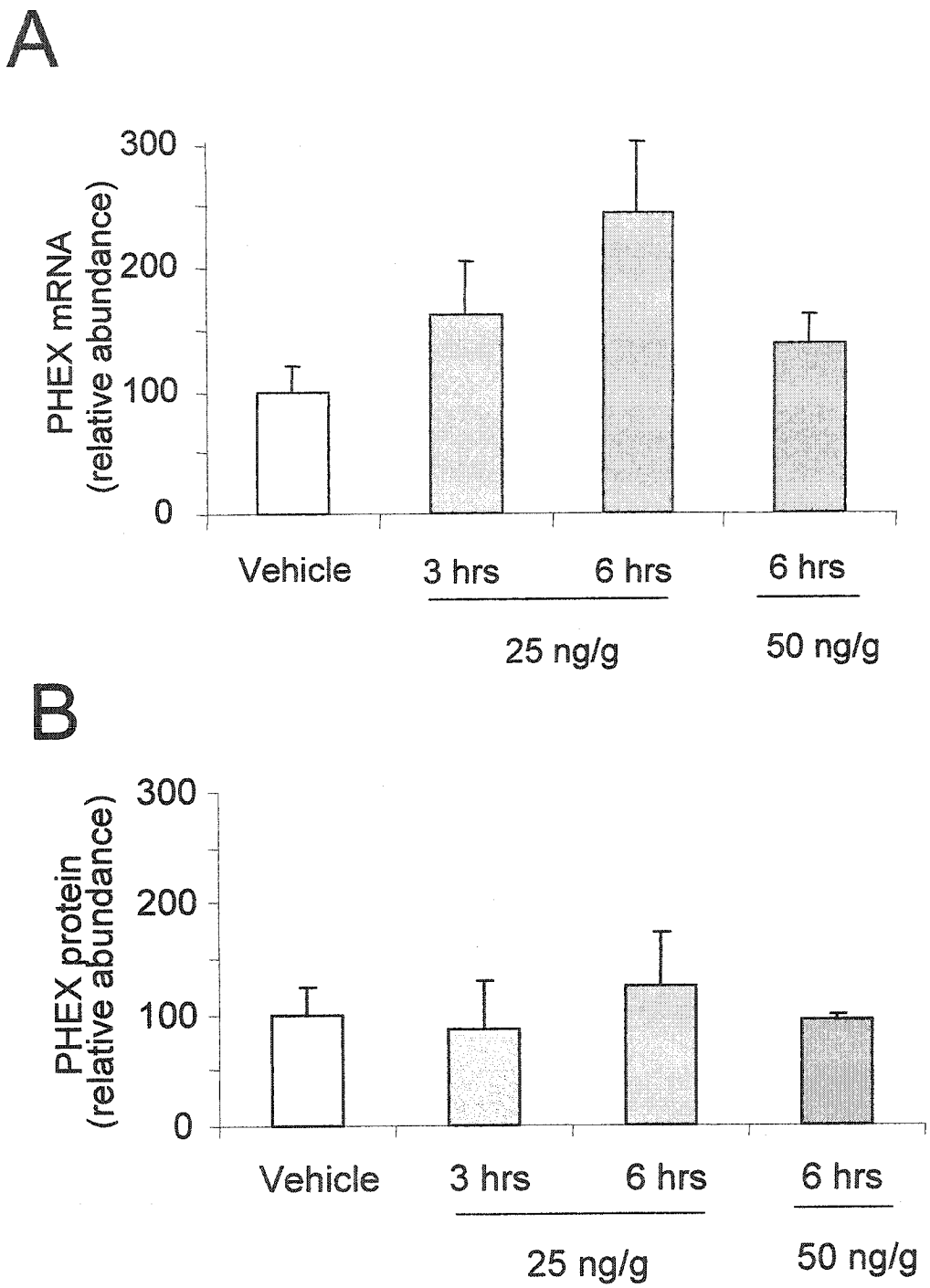

Fig. 26 Effect of calcitonin on PHEX mRNA and protein abundance in rat tibia. (**A**) Abundance of PHEX mRNA, relative to β-actin, in normal rats injected i.p. with vehicle or calcitonin (25–50 ng/g BW) and sacrificed at 3 or 6 hours was determined by ribonuclease protection assay (n= 2/group). (**B**) Abundance of PHEX protein, relative to actin, of the same groups was determined by western analysis.

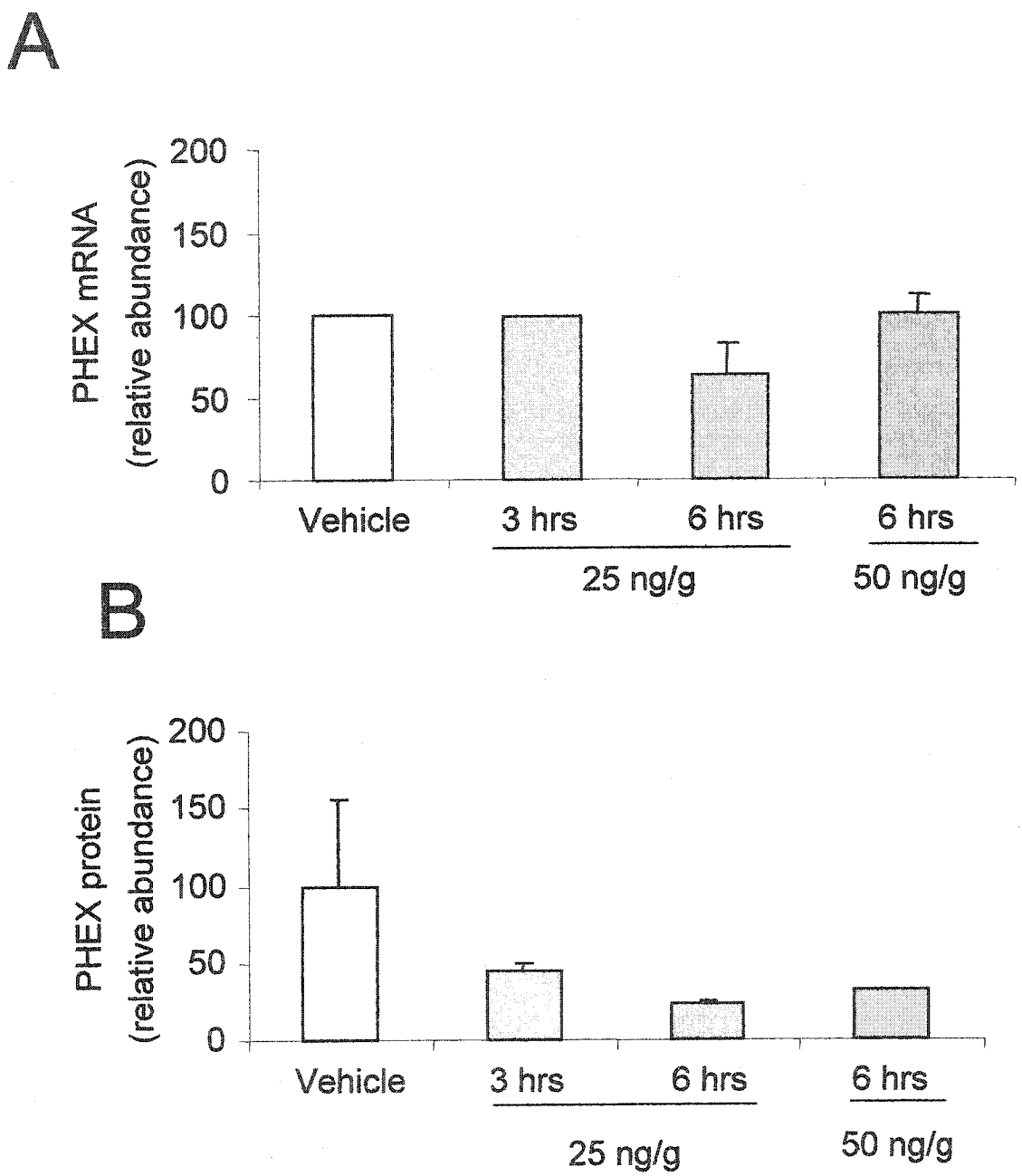

Fig. 27 Effect of calcitonin on PHEX mRNA and protein abundance in rat calvaria. (**A**) Abundance of PHEX mRNA, relative to β-actin, in normal rats injected i.p. with vehicle or 25-50ng/g BW calcitonin was determined by ribonuclease protection assay (n= 1-2/group). (**B**) Abundance of PHEX protein, relative to actin, by western analysis for the same groups.

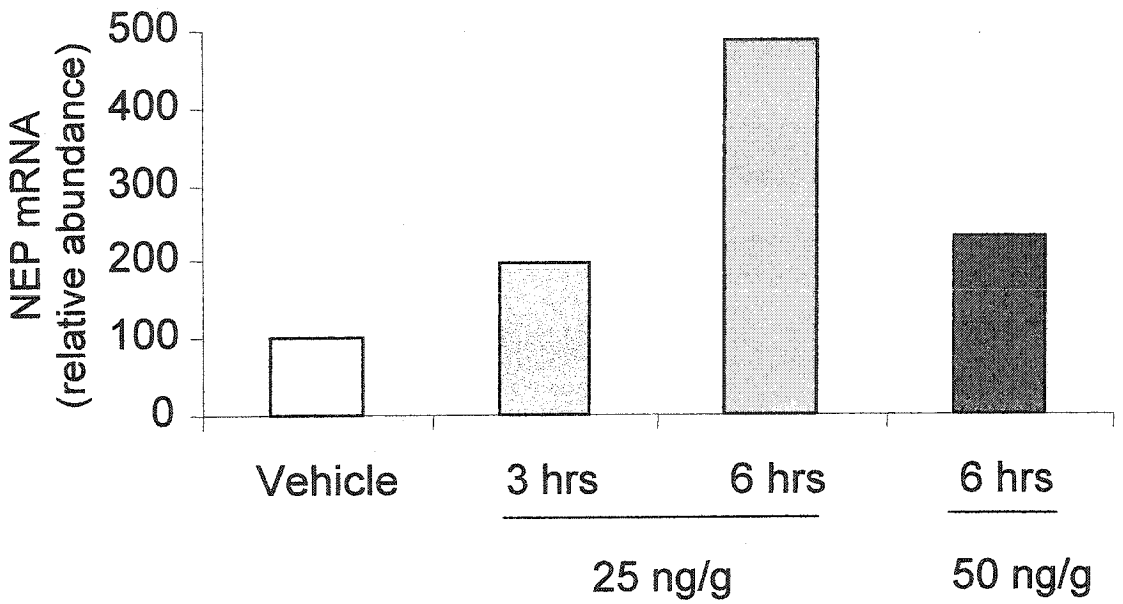

Fig. 28 Effect of calcitonin on NEP mRNA abundance in rat tibia. Abundance of NEP mRNA, relative to β-actin, in normal rats injected i.p. with vehicle or 25-50ng/g BW calcitonin was determined by ribonuclease protection assay (n= 2/group).

Table 5. Effect of calcitonin on serum and urine parameters in normal rats fed the control diet

		Vehicle	25ng/g 3hrs	25ng/g 6hrs	50ng/g 6hrs
	n	2	2	2	2
Serum	Creatinine (μmol/L)	14 ± 1	21 ± 1	19 ± 0	20.5 ± 2.5
	Urea (mmol/L)	2.7 ± 0.3	3.5 ± 0.1	2.9 ± 0.2	3.0 ± 0.05
	Phosphate (mmol/L)	2.3 ± 0.03	2.4 ± 0.04	2.6 ± 0.1	2.5 ± 0.03
	Calcium (mmol/L)	2.5 ± 0.03	2.1 ± 0.1	2.4 ± 0.1	2.2 ± 0.02
	Alkaline phosphatase (nmol/ml/min)	6.9 ± 0.9	6.7 ± 2.0	5.2 ± 1.0	6.5 ± 1.9
	PTH (pg/ml)	39.9 ± 0.9	50.3 ±1.	57.1 ±1.3	49.0 ± 0.3
Urine	Pi/Creatinine	1.1 ± 0.3	-	0.4 ± 0.1	1.9 ± 0.8
	Ca/Creatinine	0.2 ± .05	-	0.5 ± 0.3	0.4 ± 0.1
	cAMP/Creatinine	15.2	-	29.7 ± 1.1	36.4 ± 1.8

Data depicted mean ± SEM
- , information not available

PTH and PHEX mRNA Expression in the Parathyroid Gland

In collaboration with Lucie Canaff in Dr. Hendy's laboratory, we examined PTH and PHEX mRNA expression in the parathyroid gland (PTG).

Five-sixth nephrectomy led to a significant increase in PTH mRNA abundance in the PTG of rats fed either the control or high Pi diets (Fig. 29A). The high Pi diet had no effect on PTH mRNA expression in the PTG of sham-operated rats. However, nephrectomy elicited a greater increase in PTH mRNA abundance in rats fed the high Pi diet. In contrast, 5/6 nephrectomy did not increase PHEX mRNA abundance in the PTG of rats fed the control diet but Nx rats fed the high Pi diet demonstrated a significant increase in PHEX mRNA expression in the PTG (Fig 29B).

Administration of $1,25(OH)_2D$ to normal rats significantly decreased PTH and PHEX mRNA expression in the PTG in comparison to the vehicle-injected rats (Fig. 30A and B).

Calcitonin elicited comparable increases in PTH mRNA and PHEX mRNA abundance in the PTG of rats injected with vehicle or calcitonin (Fig 31A and B).

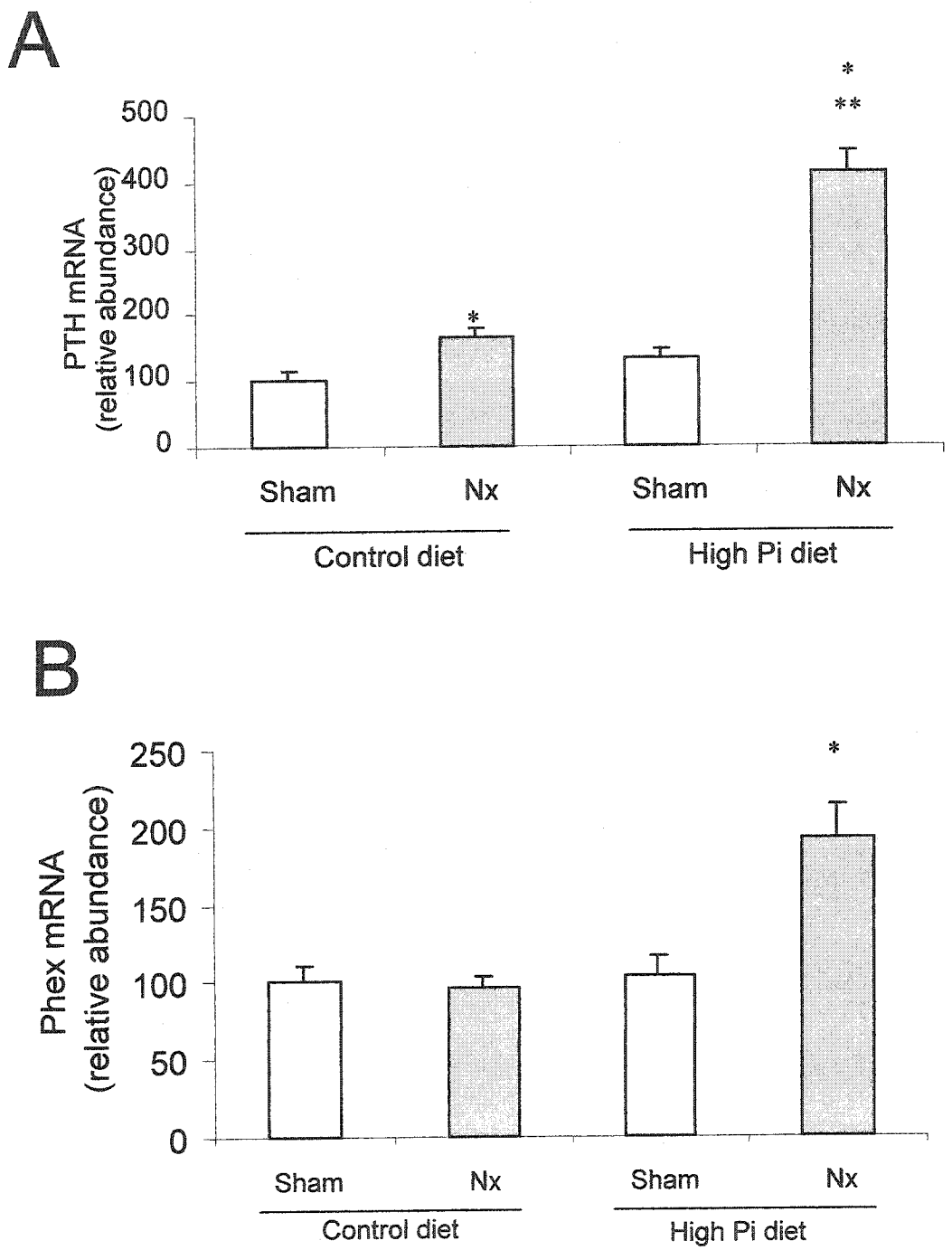

Fig. 29 Effect of Nx and dietary Pi on PTH and PHEX mRNA abundance in rat PTG. The abundance of (A) PTH mRNA, relative to GAPDH mRNA and (B) PHEX mRNA, relative to GAPDH mRNA in PTG from sham and Nx rats fed control (0.76% Pi) and high Pi (1.4% Pi) diets was determined by time course RT-PCR as described in the Materials and Methods. (n = 6/group).

* Effect of Nx, $p<0.05$

** Effect of high Pi diet, $p<0.05$

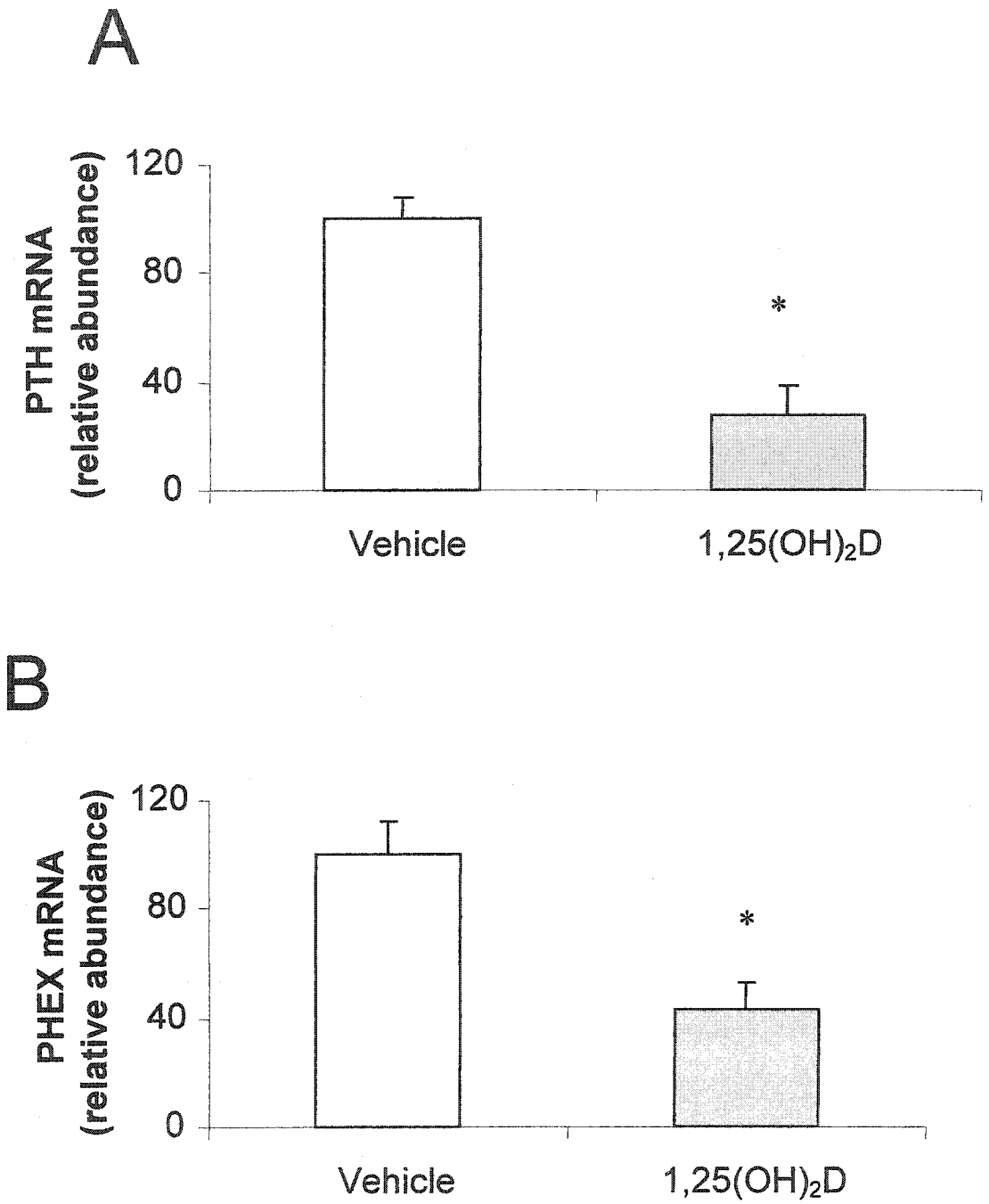

Fig. 30 Effect of $1,25(OH)_2D$ on PTH and PHEX mRNA abundance in rat PTG. Abundance of (**A**) PTH mRNA, relative to GAPDH and (**B**) PHEX mRNA, relative to GAPDH in normal rats fed the control diet and injected with either vehicle or $1,25(OH)_2D$ was determined by time course RT-PCR. (n = 6/group).

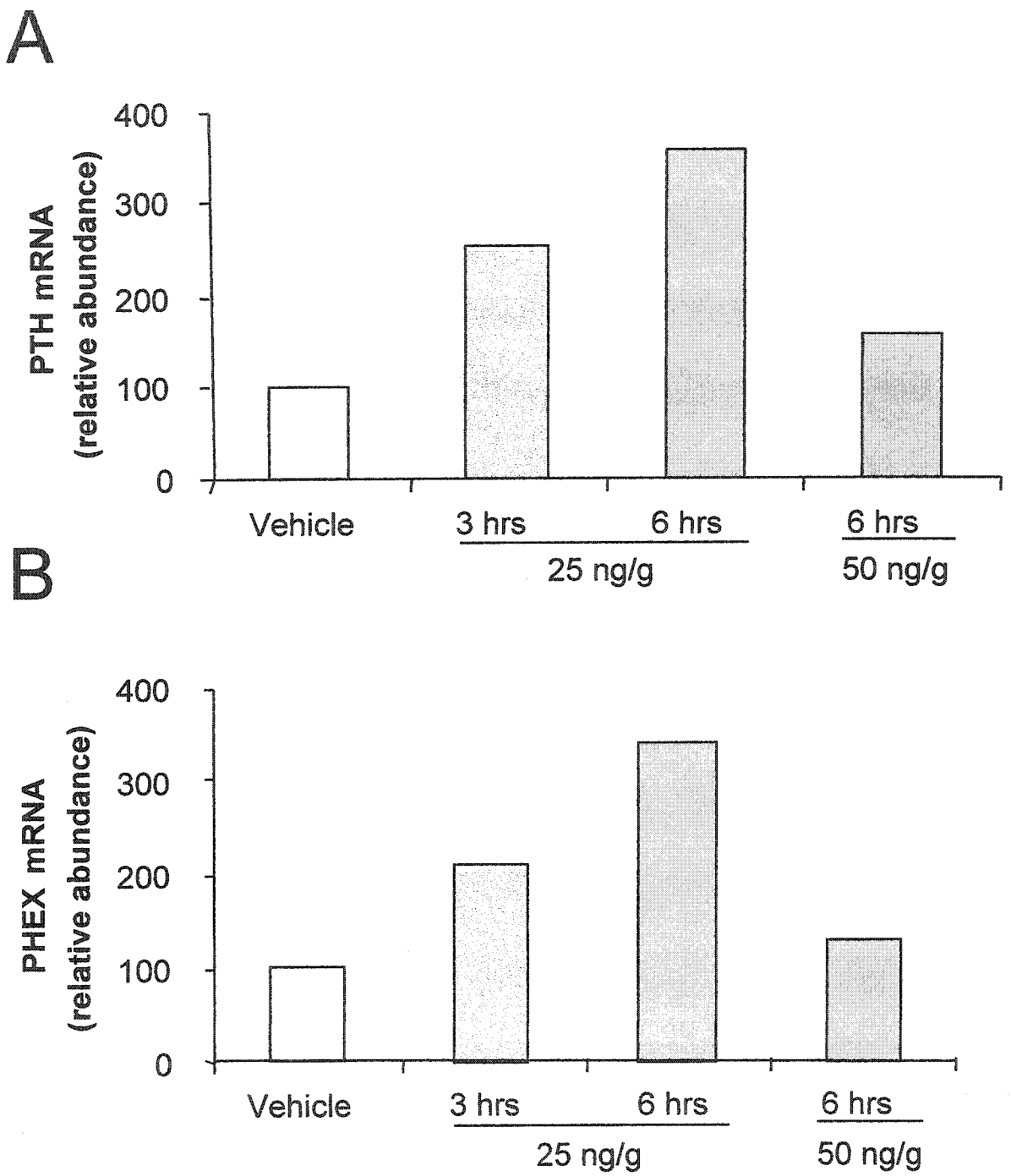

Fig. 31 Effect of calcitonin on PTH and PHEX mRNA abundance in rat PTG. Abundance of (**A**) PTH mRNA, relative to GAPDH and (**B**) PHEX mRNA, relative to GAPDH in normal rats injected i.p. with either vehicle or 25-50 ng/g BW calcitonin was determined by time course RT-PCR (n = 1-2/group).

DISCUSSION

The aim of this study was to examine the effect of changes in PTH status on PHEX expression in bone and parathyroid gland. Serum (Pi, Ca, creatinine, urea, alkaline phosphatase, and PTH) as well as urine (Pi, Ca, and cAMP relative to creatinine) parameters were measured to confirm that our animal models were responding appropriately to the nephrectomy or $1,25(OH)_2D$ treatment. NEP and osteocalcin mRNA abundance in tibia were also determined. In addition, the ontogeny of PHEX expression in mouse bone was investigated and experiments were performed to determine the distribution of PHEX in the marrow and calcified portion of bone.

PHEX Expression

Changes in serum PTH concentrations caused by different factors and the subsequent effects on PHEX mRNA and protein abundance in rat bone were investigated in this study. Determination of the degree of hyper- or hypo- parathyroidism was confirmed by measuring serum PTH and urine cAMP concentrations. We demonstrated that PHEX mRNA and protein expression in rat tibia are positively and significantly correlated with serum PTH levels, namely that PHEX mRNA and protein abundance in the tibia were increased with increases in serum PTH concentration and vice versa. The correlation between serum PTH and PHEX protein exhibited a greater significance than the correlation between serum PTH and PHEX mRNA. We also showed that PHEX

protein expression in rat calvaria and tibia appears to be differentially regulated (see section below).

Effect of Nephrectomy and Diet

Nephrectomy increased both PHEX mRNA and protein abundance in the tibia of rats fed the control Pi diet (Fig. 12A & B). The high Pi diet appeared to further increase PHEX mRNA expression in Nx rats although this increase was not statistically significant. Moreover, in rats fed the high Pi diet, Nx increased PHEX mRNA but not PHEX protein. This was mainly due to the fact that the high Pi diet alone increased PHEX protein abundance (i.e. sham-operated rats). The high Pi diet did not affect PHEX mRNA abundance in the sham-operated rats but did increase PHEX protein abundance in the same rats. This finding implies that PHEX is regulated at the post-transcriptional /translational level.

In a preliminary study, it was demonstrated that PTH administration decreased PHEX mRNA and protein expression in an osteoblastic cell line, primary osteoblast cultures from mouse calvaria and in calvaria from mice infused with PTH (Alos and Ecarot, 2000). These findings are in contrast to the results reported in the present study. The study by Alos and Ecarot examined PHEX mRNA and protein expression in calvaria, whereas tibial PHEX gene expression was examined in the current study. Moreover, the present study shows that PHEX expression in calvaria and tibia are differentially regulated (see below) which may account for the difference between our studies. Another factor that may effect PHEX response to increased PTH concentrations

is time. Alos and Ecarot examined PHEX expression after a 3-day infusion with PTH and our study examined PHEX expression after 5 weeks of hyperparathyroidism. It has been demonstrated that VDR and CaR receptors are down-regulated under long-term conditions of hyperparathyroidism and skeletal resistance to PTH develops (Slatopolsky et al., 1999). This may also account for the discrepencies between this study and Alos and Ecarot.

We also examined the effect of 5/6 nephrectomy on the expression of PTH and PHEX mRNA in PTG of rats treated with control or high Pi diets (Fig. 29). The PTH mRNA levels in the PTG of these groups of rats mimic, to some extent, the PTH concentrations in the serum (Table 2). The increase in serum PTH concentration in Nx rats fed the control diet was not as large as the increase observed in Nx rats fed the high Pi diet. This is reflected by the changes in PTH mRNA abundance. Unlike PHEX mRNA abundance in tibia, there was no difference in PHEX mRNA expression in the PTG of Nx rats fed the control diet. However, PHEX mRNA abundance was significantly increased in both the tibia and PTG of Nx rats fed the high Pi diet (Fig. 12 & 29). It is possible that a larger increase in PTG PTH mRNA or serum PTH concentration is necessary for up-regulation of PHEX in the PTG.

Effect of 1,25(OH)$_2$D

In the present study, we also examined the effect of 1,25(OH)$_2$D in normal rats. 1,25(OH)$_2$D decreased serum PTH concentration, which was associated with a decrease in PHEX mRNA and protein abundance in tibia (Fig. 14A & B). Moreover, the

expression of both PHEX mRNA and protein in the tibia was significantly and positively correlated to serum PTH concentrations.

The down-regulation of PHEX gene expression by $1,25(OH)_2D$ has been reported in mineralizing primary osteoblast cultures as well as MC3T3 cells, a mouse osteoblastic cell line (Ecarot and Desbarats, 1999). In addition to the time-dependent down-regulation of PHEX mRNA and protein abundance, $1,25(OH)_2D$ also inhibited mineralization in both the primary osteoblast and MC3T3 osteoblastic cell cultures. These data are consistent with the data in this study involving the *in vivo* administration of $1,25(OH)_2D$.

Although our data demonstrate a positive correlation between serum PTH and PHEX mRNA and protein expression, we cannot conclude that the *in vivo* effect of $1,25(OH)_2D$ on the regulation of PHEX expression in bone is an effect of decreased serum PTH concentration. As discussed above, $1,25(OH)_2D$ has been shown to down-regulate PHEX mRNA expression *in vitro*. Since $1,25(OH)_2D$ status in the serum was not measured in this study, the correlation of PHEX mRNA and protein to this hormone could not be determined. Further studies involving the *in vitro* examination of PTH and $1,25(OH)_2D$ in osteoblastic cell cultures from tibia as well as calvaria will be required.

Administration of $1,25(OH)_2D$ to normal rats significantly decreased PTH and PHEX mRNA expression in the PTG in comparison to vehicle-injected rats (Fig. 30A and B). These data are consistent with the serum PTH concentration and tibial PHEX mRNA abundance in these rats and suggest a significant relationship between PTH status and PHEX expression in bone and PTG (Table 3).

Effect of Nephrectomy and 1,25(OH)$_2$D

The effect of 1,25(OH)$_2$D on PHEX mRNA and protein abundance was also assessed in the tibia of Nx rats. Administration of 1,25(OH)$_2$D lowered PHEX mRNA expression in both sham-operated and Nx rats (Fig. 17) consistent with the effect of 1,25(OH)$_2$D in normal rats (Fig. 14). These findings suggest that 1,25(OH)$_2$D blunted the increase in PHEX mRNA expression inducible by nephrectomy.

1,25(OH)$_2$D administration had no effect on PHEX protein abundance in sham-operated and Nx rats (Fig. 17B). This was surprising since we observed a decrease in PHEX mRNA and protein expression in tibia of normal rats injected with 1,25(OH)$_2$D (Fig. 14) as well as a decrease in PHEX mRNA expression in the sham-operated and Nx rats injected with 1,25(OH)$_2$D (Fig. 17A). In the Nx experiment, however, the rats were fed a high Pi diet for a period of 5 weeks over and above the effect of nephrectomy, whereas the normal rats were fed the control diet. The difference in diet and degree of kidney function may take precedence over the effect of 1,25(OH)$_2$D administration. It is possible that if 1,25(OH)$_2$D was administered over a longer period of time, the effect of the high Pi diet and nephrectomy would be overcome and PHEX protein abundance in bone would reflect the expression of PHEX at the mRNA level.

Correlation of PHEX Expression with Serum PTH and Ca Concentrations

The relationship between PHEX mRNA and protein expression in tibia of individual rats and their serum PTH and Ca concentrations was examined. We

hypothesized that PHEX may be regulated by PTH status since PHEX is expressed in the parathyroid gland and may function to cleave PTH. Alternatively, PHEX may be regulated by changes in serum Ca concentration via the calcium-sensing receptor, which is expressed in bone and the PTG (Brown and MacLeod, 2001). To determine which scenario was more likely, regression analysis of PHEX mRNA and protein expression in bone versus serum PTH and serum Ca concentrations was conducted. Serum PTH status was significantly correlated with PHEX mRNA and protein expression in bone under all conditions, whereas serum Ca was modestly correlated with PHEX mRNA and did not correlate with PHEX protein. These data suggest that serum PTH is the important regulator of PHEX expression and that the calcium-sensing receptor may not be involved.

Ontogeny of Phex Expression in Mouse

Phex gene expression has been shown to be age-dependant. Adult mice had lower Phex protein expression in the femur in comparison to 4-day-old mice (Ruchon et al., 2000a) and Phex mRNA expression in mouse calvaria was shown to decrease immediately after birth (Meyer et al., 2000). In the present study, Phex protein expression was examined in tibia from 25-day, 4-month, 6-month, and 1-year-old mice and was found to decrease dramatically after six months. The difference between the present results in mouse tibia and those in mouse calvaria reported by Meyer et al could not be due to the sensitivity of the method used by the latter, namely RT-PCR. More likely, however, is that the ontogeny of PHEX expression may differ in both bone types, intramembranous (calvaria) versus endochondral (tibia).

PHEX Protein Expression in Calvaria

In the present study we initially examined for PHEX protein expression in calvaria as well as tibia since protein extraction from calvaria is significantly more efficient. However, we have found that the regulation of PHEX protein expression appears to differ in calvaria and tibia. In tibia, PHEX protein expression showed definite changes in response to PTH status, whereas in calvaria, PHEX protein abundance either decreased or remained unchanged. The only condition where PHEX protein expression was similarly regulated in calvaria and tibia was in response to $1,25(OH)_2D$ administration.

The different patterns of PHEX expression may be due to the different origins and functions of the two types of bone. Long bones (tibia) are created through endochondral ossification and flat bones (calvaria) are created through intramembranous ossification (Baron, 1999). The difference in the two types of bone begins in the developmental process. A cartilaginous matrix is produced in the long bones before osteoblast differentiation and bone matrix formation. In contrast, osteoblasts in flat bones are produced directly in the embryonic connective tissue and bypass this cartilaginous step. Bone formation in the tibia occurs at the growth plate where cartilage is replaced by osteoblasts that form a layer of woven bone, which is then replaced by the final lamelar bone. Calvaria bone matrix is formed in irregular bundles and calcification is delayed and occurs in irregularly distributed patches.

One reason why PHEX regulation might be different in these two types of bone could be related to PHEX having two different functions – maintenance of Pi

homeostasis and proper bone mineralization. The long bones are the source for hematopoeitic bone marrow and as such have more contact with the circulation and with the circulating PHEX substrate. Therefore, in tibia, PHEX could function as a regulator of Pi homeostasis as well as of bone matrix mineralization whereas, in calvaria, the more important function of PHEX would be for proper bone mineralization.

Examination of PHEX Expression in Bone Marrow

There has been some question as to whether PHEX is expressed in the stromal cells of the bone marrow. Therefore, we examined PHEX mRNA and protein expression in the bone marrow of rats and were unable to detect expression by both RT-PCR and western analysis. However, PHEX mRNA and protein expression were robust in the calcified portion of the tibia from which the marrow was harvested. In addition, both β-actin and actin, used as loading markers for mRNA and protein, respectively, were expressed. Both results demonstrate that the absence of PHEX gene expression in the bone marrow was not due to technical difficulties. These data assure us that the bone marrow does not contribute to, or confound the data on PHEX mRNA and protein expression profiles in rat tibia reported in the present study.

A previous study examined the effect of bone marrow transplantation from wild type into Hyp mice on several phenotypic parameters (Miyamura et al., 2000). Bone marrow from the long bones of male wild type mice was injected into female Hyp mice. This allowed for the determination of the bone marrow source through the male-specific *Sry* gene. Eight weeks post-transplantation, the Hyp recipients were sacrificed and

serum and several tissues were analyzed for the presence of donor cells and any effects thereof. The time between injection of the bone marrow and collection of mouse tissues would be of sufficient time for the bone marrow to differentiate into Phex-producing osteoblasts. Miyamura et al found that injection of wild type bone marrow into Hyp mice elicited an increase in serum Pi, renal Na/Pi cotransport, renal 24-hydroxylase expression, and bone mineral density. The efficiency of the engraphment in this study was 5%. Therefore, the results are surprising because of the apparent haploinsufficiency seen in XLH patients. The authors suggest that the important difference between their results and what would previously have been expected is that the wild-type donor cells have full enzymatic acitivity and heterozygote cells only have half of the activity. The heterozygotes would therefore have more difficulty inactivating the circulating phosphaturic factor.

Miyamura et al also examined Phex expression in bone marrow from 8-week-old male wild type mice using RT-PCR (Miyamura et al., 2000). Phex expression was positive in the marrow of wild type mice and negative in Hyp mice which is contrary to the results found in this study. It is possible that the bone marrow extractions performed on the mice in this study contained osteoblasts that would express Phex and lead to a positive Phex signal.

There was another study examining PHEX mRNA expression in bone marrow stromal cells (Dubois et al., 2002). This study isolated bone marrow stromal cells and then cultured them in media. RT-PCR was then performed on the cultured cells and PHEX mRNA was present. The culturing of the cells would be sufficient time to cause

the differentiaton of the stromal cells into preosteoblasts and osteoblasts whereas the experiment performed in this study used stromal cells directly from the marrow.

Calcitonin

Our pilot study to examine the effects of calcitonin on PHEX expression in the tibia and PTG was conducted to investigate an alternative approach to induce hyperparathyroidism. In this model, hyperparathyroidism occured in hours whereas in the 5/6 nephrectomy model more time was required to achieve this state. The small number of animals used in this study precluded the use of statistics for analysis of the data. However, the results suggest that calcitonin was able to decrease serum Ca and increase concentrations of serum PTH and PHEX mRNA abundance in the tibia. The protein data for these groups did not show as clear a response as the mRNA data, which may be due to the small sample size.

Calcitonin effects on PTH and PHEX mRNA in the PTG were also examined. The hormone elicited comparable increases in PTH and PHEX mRNA abundance in the PTG of rats (Fig 31A and B). This pattern of expression parallels that of serum PTH concentration (Table 5) and PHEX expression in tibia (Fig. 31), again suggesting a positive correlation between serum PTH and PHEX expression in bone and PTG.

Bone Markers

Alkaline phosphatase and osteocalcin are synthesized by osteoblasts and released into the circulation during osteoblast mineralization (Anh et al., 1998). In humans with

bone disorders, such as osteoporosis or renal osteodystrophy, these two proteins are used as markers to assess levels of bone formation and remodeling.

Serum Alkaline Phosphatase

Previous reports in humans (Magnusson et al., 2001) and mice (Gagnon and Gallimore, 1988) with chronic renal failure (CRF) have shown that serum alkaline phosphatase activity increases when compared to normal conditions. However, there is evidence to show that the opposite occurs in the rat. *In vitro* studies have shown that when PTH exposure is continuous, rat osteoblast differentiation determined by von kossa staining and nodule formation, is inhibited and alkaline phosphatase activity and mRNA abundance (northern analysis) are decreased (Ishizuya et al., 1997; Jongen et al., 1993). In the present study, we demonstrated a decrease in serum alkaline phosphatase concentration in Nx rats fed the high Pi diet when compared to sham-operated rats (Table 2). In addition, we found by visual examination that tibia from the Nx rats were shorter in length, had wider epiphyses, and were more brittle than the sham-operated counterparts (data not shown) suggestive of increased bone resorption.

Our study showed that $1,25(OH)_2D$ did not elicit a change in serum alkaline phosphatase activity (Table 3). However, a previous *in vitro* study showed that treatment of osteoblasts derived from rat bone marrow stromal cells with $1,25(OH)_2D$ led to decreased alkaline phosphatase mRNA levels and activity (Atmani et al., 2002). The absence of a similar response in intact rats in this study may be due to the different environment found *in vivo* or to the fact that the serum concentration of $1,25(OH)_2D$ in

the rats was not high or long enough (i.e. catabolism reducing concentration) to have an effect.

Bone Osteocalcin mRNA

Osteocalcin, another osteoblast marker, was also examined in the current study as a possible way to determine bone turnover status. It was previously demonstrated that osteocalcin is up-regulated at the mRNA level in human primary osteoblast cell cultures treated with $1,25(OH)_2D$ (Viereck et al., 2002). In addition, serum concentrations of osteocalcin have been shown to be increased in normal (Tsuruoka et al., 2000) as well as 5/6 Nx (Tsuruoka et al., 2002) rats treated with $1,25(OH)_2D$. In contrast, PTH decreased osteocalcin mRNA expression in osteoblast-like MC3T3-E1 cells (Gopalakrishnan et al., 2001). In our study, osteocalcin mRNA abundance was examined in the tibia of 5/6 Nx rats fed control and high Pi diets and in normal rats treated with $1,25(OH)_2D$. However, the expression pattern for this bone marker remained unchanged at the mRNA level in the groups tested. It is possible that the degree of bone turnover evident in 5/6 Nx rats and $1,25(OH)_2D$-injected normal rats may not be large enough to alter the transcript expression of this osteoblast-specific bone marker however we don't understand the basis for the lack of change in expression.

NEP mRNA Expression in Tibia

NEP is a member of the same metalloendopeptidase family as PHEX. As such, there is the possibility that NEP could also cleave PHEX substrate(s) or their products.

To determine whether NEP and PHEX were subject to the same regulatory factors, we also examined the effects of 5/6 Nx, dietary Pi and $1,25(OH)_2D$ on NEP mRNA abundance in rat tibia. While PHEX mRNA abundance was correlated with serum PTH concentrations, NEP mRNA was significantly increased or demonstrated no change (Fig. 13,16,19,28) and regression analysis indicated that NEP mRNA abundance and serum PTH are not correlated (data not shown). Thus regulation of NEP expression in bone was not similar to that of PHEX and the possibility that NEP and PHEX are coordinately regulated and that NEP is involved in the processing of the PHEX substrates is unlikely. This contention is supported by the fact that the substrate pocket of NEP accommodates hydrophobic residues whereas that of PHEX accommodates acidic amino acid residues (Boileau et al., 2001).

Phosphaturic factors in the Rat

In the present study we showed that PHEX expression in bone is positively correlated to serum PTH concentrations. We postulate that the upregulation of PHEX by serum PTH may be related to its role in the degradation and inactivation of a phosphaturic factor(s), which like PTH, are upregulated by hyperphosphatemic states. Under normal conditions, a transitory increase in serum Pi, which may occur in response to an increase in dietary Pi intake, elicits an increase in serum PTH, which in turn promotes increased Pi excretion by the kidney (Fig. 32). This is accomplished by PTH-mediated endocytosis of Npt2 protein from the brush-border membrane of proximal tubular cells and its lysozomal degradation (Pfister et al., 1998). This increase in renal Pi

excretion would serve to normalize the serum Pi concentration and subsequently serum PTH levels (Kempson et al., 1995). With time the abundance of Npt2 protein in the BBM would be restored to normal.

Under more extreme hyperphosphatemic conditions, such as those observed in the first stages of renal disease, phosphaturic factors other than PTH may also be elaborated and released to augment renal Pi excretion in order to control serum Pi levels (Green et al., 2001). (Fig. 32). We hypothesize that the release of PTH and other phosphaturic factors, in response to hyperphosphatemia, would elicit an increase in PHEX mRNA and protein abundance, which in turn would serve to degrade and inactivate PTH and other phosphaturic factors and thereby prevent their accumulation in the serum.

In Nx rats, which have chronic renal failure and severe hyperparathyroidism, serum PTH and serum Pi concentrations remain high due to the reduced number of nephrons available for Pi clearance. This would imply that the serum abundance of the phosphaturic factors would also be elevated. Indeed, Green et al recently demonstrated that serum from human patients with chronic renal failure can inhibit Pi uptake in OK cells, independently of PTH status, consistent with the presence of phosphaturic factors (Green et al., 2001). Thus, we would predict that under these conditions, a sustained up regulation of PHEX mRNA and protein is necessary to control the serum concentrations of PTH and other phosphaturic factors.

In the current study, we also showed that PHEX expression in bone is decreased in rats injected with pharmacological doses of $1,25(OH)_2D$ which also significantly decreases the serum concentrations of PTH (Table 3). The elevated serum $1,25(OH)_2D$ concentration leads to increased bone resorption and intestinal Ca and Pi absorption.

Both these actions of $1,25(OH)_2D$ would result in an elevated serum Pi concentration. However, in this case, serum PTH is decreased (Table 3) and the renal expression of Npt2 is not down-regulated (Taketani et al., 1998). We postulate that the phosphaturic factors discussed above would also be down-regulated under these conditions. Accordingly, in this scenario, there would not be a need to upregulate PHEX expression in bone. This action of $1,25(OH)_2D$ on PHEX expression in bone may be secondary to the reduction in the serum concentration of PTH or may be attributable to a direct action of $1,25(OH)_2D$ on bone. Indeed, Ecarot and Desbarats (1999) demonstrated that $1,25(OH)_2D$ inhibited PHEX expression directly in osteoblast cultures in vitro.

As discussed earlier in this thesis, recent studies have identified a novel fibroblast growth factor, FGF-23, that is cleaved by PHEX (Bowe et al., 2001) and inhibits Pi uptake in OK cells (Bowe et al., 2001). These two findings have made FGF-23 the leading candidate for the phosphaturic factor, and a physiological substrate for PHEX. Abstracts at the 24th Annual Meeting of the Society for Bone and Mineral Research have suggested that FGF-23 is cleared by the kidney and that serum FGF-23 concentrations are elevated in OHO, XLH, and chronic renal failure patients. This is relevant to our rat model of CRF because hyperphosphatemia is present in chronic renal failure and an increase in a phosphaturic factor or FGF-23 would, presumably, be necessary for increased Pi clearance from the circulation. FGF-23 has also been shown to be positively correlated to serum Pi status (*personal communication*). Therefore, under conditions of

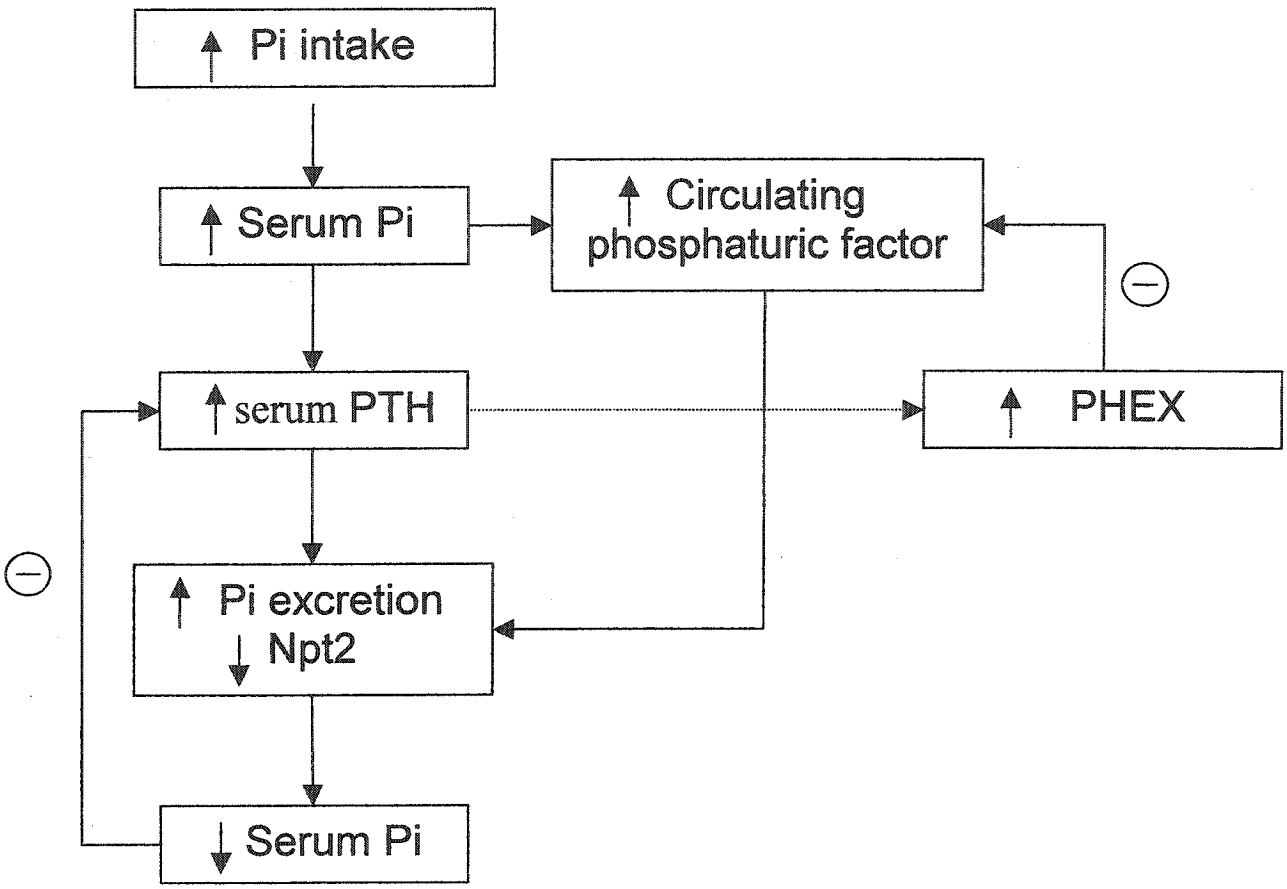

Fig. 32 Schematic of a phosphaturic factor activity during a transient increase in serum Pi. Dotted line represents unknown direct or indirect connection. Minus sign represents down-regulation.

hyperphosphatemia (as in the experimental models used here), FGF-23 would be elevated.

This study provides new insight into the regulation of PHEX mRNA and protein expression. This is the first time PHEX expression has been examined *in vivo* in the 5/6 nephrectomy and $1,25(OH)_2D$-treated animal models. The increased understanding of how PHEX responds to a given experimental condition *in vivo* provides further knowledge into how PHEX is integrated into the regulation of Pi homeostasis. The data presented here may also contribute to our understanding of the etiology of chronic renal failure and lead to improved treatment of this disorder as well as XLH and ADHR.

Future Studies

- To further characterize the role $1,25(OH)_2D$ administration on PHEX expression in nephrectomized rats by measuring serum 1,25(OH)2D concentrations and repeating the experiment with a larger number of animals.
- To examine the direct action of $1,25(OH)_2D$ with and without PTH on PHEX expression in osteoblast cultures *in vitro*
- To examine the effects of the hyper- and hypoparathyroidism on PTH receptors in bone.
- To confirm the calcitonin effects on PHEX expression in bone and compare the response of calcitonin with 5/6 nephrecomy
- To futher elucidate the differences in PHEX expression in tibia versus calvaria

APPENDIX A

Effect of 3/6 Nephrectomy

Although this study was not intended, due to an error by the company supplying us with sham and 5/6 Nx rats, we received and studied rats in which only one kidney was removed (3/6 Nx). There are some items that were worth noting from this experiment.

Serum creatinine and urea levels were only slightly elevated in the 3/6 Nx rats in comparison to the sham-operated rats, on either the control or high Pi diet (Table 6). The modest increases in urea were significant only in the 3/6 Nx rats on the control diet. Serum Pi was similar in sham and 3/6 Nx rats, but the high Pi diet elicited an increase in serum Pi concentration in both sham and 3/6 Nx groups. Serum calcium values did not change in response to diet or 3/6 nephrectomy. Alkaline phosphatase decreased significantly in the 3/6 Nx rats on the high Pi diet only. Serum PTH was significantly elevated in the 3/6 Nx rats compared to sham-operated rats on both the control and high Pi diets. Three-sixth Nx rats on the high Pi diet exhibited a greater increase in serum PTH levels than the 3/6 Nx rats on the control diet (Table 6). It is of interest that the increases in PTH levels seen in the 3/6 Nx rats were not as high as those of the 5/6 Nx rats (Table 2).

Urine Pi excretion, relative to urine creatinine, did not change in response to 3/6 nephrectomy on either the control or high Pi diet (Table 6). However, rats fed the high Pi diet exhibited an increase in Pi/creatinine excretion in comparison to the rats fed the control diet. Urine calcium excretion, relative to urine creatinine, did not change in response to 3/6 nephrectomy. However, sham-operated rats fed the high Pi diet had a

significantly lower calcium excretion in comparison to the sham-operated rats on the control diet. Three-sixth Nx did not elicit an increase in urine cAMP, relative to urine creatinine, in rats fed the control diet. However, in rats fed the high Pi diet, 3/6 Nx elicited an increase in cAMP/creatinine in comparison to the sham-operated rats. The high Pi diet elicited a significant increase in urine cAMP/creatinine in 3/6 Nx rats in comparison to the sham and 3/6 Nx rats fed the control diet.

Table 6. Effect of normal and high Pi diets on serum and urine parameters in 3/6 Nx rats fed control and high Pi diets

		Control diet		High Pi	
		sham	Nx	sham	Nx
	n	7	6	8	6
Serum	Creatinine (μmol/L)	52.4 ± 2.2	62.6 ± 2.2	53.2 ± 2.6	60.7 ± 8.6
	Urea (mmol/L)	6.5 ± 0.4	8.3 ± 0.2*	5.8 ± 0.6	7.9 ± 0.9
	Phosphate (mmol/L)	4.3 ± 0.2	4.4 ± 0.2	5.0 ± 0.2[9]	5.2 ± 0.2[9]
	Calcium (mmol/L)	3.1 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	2.9 ± 0.2
	Alkaline phosphatase (U/L)	298 ± 34	295 ± 22	231 ± 16	176 ± 10*[9]
	PTH (pg/ml)	36.7 ± 1.8	43.1 ± 1.7*	38.1 ± 1.9	75.5 ± 1.2*[9]
Urine	Pi/Creatinine	10.5 ± 1.2	9.8 ± 0.6	27.3 ± 4.2[9]	25 ± 1.2[9]
	Ca/Creatinine	0.3 ± 0.1	0.4 ± 0.1	0.1 ± 0.03[9]	0.3 ± 0.1
	cAMP/Creatinine	45.5 ± 5.3	15.2 ± 1.1*	51.6 ± 4.1	92.5 ± 5.2*[9]

Data depicted mean SEM
* Effect of nephrectomy, $p<0.05$
[9] Effect of diet, $p<0.05$

PHEX mRNA abundance in the tibia of 3/6 Nx rats fed the control diet was increased in comparison to the sham-operated rats fed the same diet (100% ± 10 vs 156 ± 25, $p>0.05$, fig not shown). Three-sixth nephrectomy had no effect on PHEX mRNA abundance in rats fed the high Pi diet when compared to the sham-operated rats (197% ± 18 vs 211 ± 16). However, PHEX mRNA abundance, in both sham-operated and 3/6 Nx

Effect of Nephrectomy in Mouse Model

Preliminary studies were initiated in a nephrectomized mouse model as an alternative to the rat model. These mice have one kidney removed and the other kidney electrocoagulated over its entire surface. The mice develop the classic bone phenotype of chronic renal failure (CRF) (Gagnon and Gallimore, 1988). A total of 43 normal and 44 CRF mice were examined. There were no apparent trends of the effect of CRF on serum phosphate and calcium (data not shown). Alkaline phosphatase concentrations in the CRF mice were also erratic when compared to the sham-operated mice. This is in contrast to the increase in alkaline phosphatase concentration in CRF mice reported by Gagnon et al (Gagnon and Gallimore, 1988). Moreover, ribonuclease protection assays showed no increase in the PHEX mRNA abundance, in relation to β-actin, in the tibia or calvaria of the CRF mice in comparison to sham-operated mice (data not shown). These data are in contrast to the bone histological changes reported consistent with CRF in this mouse model (Gagnon and Gallimore, 1988). Therefore, this was not a useful model to study the effect of CRF on regulation of PHEX expression.

rats, fed high Pi diet was increased when compared to sham-operated and 3/6 Nx rats fed the control diet. Neither 3/6 nephrectomy nor diet elicited an effect on PHEX protein abundance (100% in each group).

NEP mRNA abundance increased in the 3/6 Nx rats when compared to the sham-operated rats fed the control diet (100% ± 20 vs 151 ± 22, $p<0.05$). An increase in NEP mRNA abundance was also seen in 3/6 Nx rats fed the high Pi diet in comparison to the sham-operated rats although this increase is not significant (237% ± 22 vs 298 ± 28). The high Pi diet elicited an increase in NEP mRNA abundance in comparison to rats fed the control diet.

The serum and urine data suggest that 3/6 Nx elicits a more moderate form of renal failure than 5/6 Nx (see Table 2). Nevertheless, serum PTH concentrations were elevated in response to the 3/6 nephrectomy and urine cAMP/creatinine mirrored this change in PTH status except in the 3/6 Nx rats on the control diet where a decrease was seen for reasons that are not understood. PHEX mRNA expression in 3/6 Nx rats on the control diet are similar to the data found in the 5/6 Nx rats (see Fig. 12). In these rats, the high Pi diet elicits an overall increase in PHEX mRNA levels as seen in the 5/6 Nx rats but it also abolishes the effect of nephrectomy. This is not seen in 5/6 Nx rats fed the high Pi diet. PHEX protein abundance is not affected by 3/6 Nx on either the control or high Pi diets as it is by 5/6 Nx on control and high Pi diets.

APPENDIX B

McGill University
Animal Use Protocol – Research

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Protocol #: 3650
Investigator #: 354
Approval End Date: Feb 28, 2003
Facility Committee: MCH

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 3650

Title (must match the title of the funding source application): Function and regulation of PHEX, a candidate gene for X-linked hypophosphatemia

1. Investigator Data:

Principal Investigator: Harriet S. Tenenhouse **Office #:** 412-4400, X22342

Department: Pediatrics and Human Genetics **Fax#:** 412-4331

Address: MUHC-MCH,4060 Ste. Catherine St West, Room 222, Montreal H3Z 2Z3 **Email:** mdht@debelle.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Harriet S. Tenenhouse	**Work #:**	412-4400, X22342	**Emergency #:**	937-4297
Name:	Stephanie Aubin	**Work #:**	412-4400, X22761 or 22502	**Emergency #:**	299-3969 (mobile) or 351-9243 (residence)

3. Funding Source:

External ☒
Source (s): CIHR
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: 3/2000 to 3/2003

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

ACTION	✓	DATE
P.I.	✓	Mar 15'02
FACC	✓	"
RGO	✓	"
VET	✓	"
DB		

Approved

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: Harriet S Tenenhouse **Date:** Feb. 15, 2002

Approval Signatures:

Chair, Facility Animal Care Committee:		**Date:** Feb 22 / 2002
University Veterinarian:	RH Tait	**Date:** 3/05/02
Chair, Ethics Subcommittee(as per UACC policy):		**Date:**
Approved Period for Animal Use	**Beginning:** MARCH 1, 2002	**Ending:** Feb 28, 2003

☐ This protocol has been approved with the modifications noted in Section 13.

FEB 28 2002

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #:
Investigator #:
Approval End Date:
Facility Committee:

__ Pilot __ New Application __ Renewal of Protocol # 1744

Title (must match the title of the funding source application): Molecular Genetics of the Calcium-sensing Receptor (new ti

1. Investigator Data:

Principal Investigator:	Dr. Geoffrey N. Hendy	**Office #:**	843-1632
Department:	Calcium Research Lab., Medicine	**Fax#:**	843-1712
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Name:	G.N. Hendy	**Work #:**	843-1632 or 842-1231 ext. 35165 or 35697	**Emergency #:**	937-6458
Name:	L. Canaff	**Work #:**	842-1231 ext 35697	**Emergency #:**	276-9331

3. Funding Source:

External x **Source (s):** Kidney Foundation of Canada	**Internal** __ **Source (s):**
Peer Reviewed: x YES __NO**	**Peer Reviewed:** __ YES __ NO**
Status : x Awarded __ Pending	**Status:** __ Awarded __ Pending
Funding period: July 1, 2002 to June 30, 2004	**Funding period:**

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y):	**or ongoing** x
Expected Date of Completion of Animal Use (d/m/y):	**or ongoing** x

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: GN Hendy	**Date:** 11 April 2002

Approval Signatures:

Chair, Facility Animal Care Committee:		**Date:**
University Veterinarian:		**Date:**
Chair, Ethics Subcommittee(as per UACC policy):		**Date:**
Approved Period for Animal Use	**Beginning:**	**Ending:**

__ This protocol has been approved with the modifications noted in Section 13.

Centre universitaire de santé McGill
McGill University Health Centre

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Permis interne 7-2003-03

Détenteur de permis: Harriet S. Tenenhouse Téléphone: 22342

Département: Pediatrics and Human Genetics Bureau : PT-223

(A) Localisation

Local	Classification
PT-222	Élémentaire

(B) Activité autorisées

Isotopes	Activités (MBq)
H3	160
P32	37
P33	20

(C) Personnel autorisé à manipuler des radioisotopes

Nom	Prénom	P32	P33	S35	H3	C14	I125	Ca45	Co57	Cr51	Ni63
Brewer	Angela	☑	☑	☐	☑	☐	☐	☐	☐	☐	☐
Chau	Hien	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐
Gauthier	Claude	☑	☑	☐	☐	☐	☐	☐	☐	☐	☐
Martel	Josée	☑	☐	☐	☑	☐	☐	☐	☐	☐	☐
Sabbagh	Yves	☑	☐	☐	☐	☐	☐	☐	☐	☐	☐
Soumounou	Youssouf	☑	☑	☐	☐	☐	☐	☐	☐	☐	☐
Haque	Rivana	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

7-2003-03

Par la présente, le détenteur de permis interne ainsi que toutes les personnes mentionnées en (C) sont autorisés à manipuler dans les laboratoires indiqués en (A), les isotopes listés en (B). L'importation, l'entreposage, l'utilisation et la disposition de ces substances radioactives doivent se faire en conformité avec les directives du Service de radioprotection, les exigences du manuel de radioprotection, les conditions du permis émis par la Commission de contrôle de l'énergie atomique (CCEA) ainsi que les lois et règlements en vigueur. Une copie du permis valide émis par la CCEA est affiché au secrétariat de la recherche et une autre est disponible au service de radioprotection (RVH local S4.79 ext. 6133).

Approuvée par: Maureen McQueen
Chef du Service de radioprotection (6133)

Date d'entrée en vigueur: March 13, 2002
Date d'expiration: March 31, 2003

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