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NHERF1 Regulation of PTH-dependent Bimodal Pi Transport in Osteoblasts

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Abstract

Control of systemic inorganic phosphate (Pi) levels is crucial for osteoid mineralization. Parathyroid hormone (PTH) mediates actions on phosphate homeostasis mostly by regulating the activity of the type 2 sodium-phosphate cotransporter (Npt2), and this action requires the PDZ protein NHERF1. Osteoblasts express Npt2 and in response to PTH enhance osteogenesis by increasing mineralized matrix. The regulation of Pi transport in osteoblasts is poorly understood. To address this gap we characterized PTH-dependent Pi transport and the role of NHERF1 in primary mouse calvarial osteoblasts. Under proliferating conditions osteoblasts express Npt2a, Npt2b, PTH receptor, and NHERF1. Npt2a mRNA expression was lower in calvarial osteoblasts from NHERF1-null mice. Under basal conditions Pi uptake in osteoblasts from wild-type mice was greater than that of knockout mice. PTH inhibited Pi uptake in proliferating osteoblasts from wild-type mice, but not in cells from knockout mice. In vitro induction of mineralization enhanced osteoblast differentiation and increased osterix and osteocalcin expression. Contrary to the results with proliferating osteoblasts, PTH increased Pi uptake and ATP secretion in differentiated osteoblasts from wild-type mice. PTH had no effect on Pi uptake or ATP release in differentiated osteoblasts from knockout mice. NHERF1 regulation of PTH-sensitive Pi uptake in proliferating osteoblasts is mediated by cAMP/PKA and PLC/PKC, while modulation of Pi uptake in differentiated osteoblasts depends only on cAMP/PKA signaling. The results suggest that NHERF1 cooperates with PTH in differentiated osteoblasts to increase matrix mineralization. We conclude that NHERF1 regulates PTH differentially affects Na-dependent Pi transport at distinct stages of osteoblast proliferation and maturation.

Keywords

type 2 sodium-phosphate transporters; parathyroid hormone receptor; NHERF1; osteoblasts; inorganic phosphate

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Introduction

The vertebrate skeleton provides mechanical support and serves as a reservoir for the mineral ions calcium and phosphate. The majority of phosphorus is found in skeleton, where it is essential for normal bone mineralization. Bone growth is a sequential process with osteoblasts laying down osteoid that is then mineralized. Osteoid mineralization requires a high-phosphate environment that is derived from pyrophosphate, phosphoproteins, and circulating phosphate ions. Chronic hypophosphatemia or other phosphate-deficient conditions in humans result in mineralization defects including osteomalacia in adults and rickets in children. Bone mineral is deposited by regulated phosphate transport. Accumulation of inorganic phosphate (Pi)¹ by osteoblasts proceeds against a steep electrochemical gradient and is mediated by cell membrane phosphate transporters, principally Npt2a and Npt2b but also Pit1 [1, 2]. Npt2a-dependent Pi transport in kidney tubule cells is importantly regulated by NHERF1, a PDZ protein [3, 4]. In the absence of NHERF1, parathyroid hormone (PTH) [5] and dopamine [6] fail to inhibit Pi uptake, thereby increasing Pi excretion. NHERF1-null mice [7, 8] and humans [9] with NHERF1 mutations exhibit hypophosphatemia and exaggerated urinary Pi excretion and bone demineralization. It was thought that these effects on bone arose secondarily from mineral-ion wasting. However, recent work shows that mineralizing osteoblasts express NHERF1 and that there is an intrinsic osteoblast mineralization defect in NHERF1-null mice.

The phosphate transporter in osteoblasts has not been extensively studied than that in renal proximal tubule cells. The action of PTH on Pi transport by osteoblasts has been investigated but contradictory outcomes preclude a comprehensive understanding of the regulatory process or underlying mechanism. In osteosarcoma cell lines such as UMR-106, PTH stimulates Pi transport by a process that involves increased cAMP formation [10–13]. However, the regulation of sodium-dependent Pi transport by NHERF1 in osteoblastic cells has not been reported. In present study, we isolated calvarial osteoblasts from wild-type and NHERF1 knockout mice and found calvarial osteoblasts constitutively express NHERF1, as well as Npt2a and Npt2b. Using these nontransformed cells, we found that NHERF1 regulates Npt2a expression in proliferating and differentiated osteoblasts. Further, we show that PTH exerts opposite effects on Pi uptake in undifferentiated and differentiated osteoblasts from wild-type and NHERF1 knockout mice. This is the first study that NHERF1 regulation PTH-sensitive phosphate transport in osteoblasts has been demonstrated.

Materials and methods

Materials

NHERF1 polyclonal antibody and Npt2b polyclonal antibody were obtained from Santa Cruz Technology (Santa Cruz, CA). NHERF1 monoclonal antibody was purchased from Abcam (Cambridge, MA). Npt2a polyclonal antibody was obtained from Lifespan Biosciences, Inc. (Seattle, WA). NHERF2 polyclonal antibody was kindly provided by Dr. R. A. Frizzell (University of Pittsburgh). Polyclonal antisera targeted to the carboxyl-terminal tail of the mouse PTHR were generated by Gramsch Laboratories (Schwabhausen, Germany). H89, bisindolylmaleimide 1, and Protease inhibitor mixture Set I were from Calbiochem (San Diego, CA). Human PTH(1–34) was purchased from Bachem (Torrance,

¹Abbreviations:

PTHR, parathyroid hormone receptor; NHERF1, Na/H exchanger regulatory factor-1; Pi, inorganic phosphate; Npt2, type 2 sodium-phosphate cotransporter; ATP, adenosine triphosphate; Bis1, bisindolylmaleimide 1; PKA, protein kinase A; WT, wild-type; KO, knockout

CA). Alexa Fluor 488-tagged goat-anti-rabbit second antibody, Alexa Fluor 546-tagged donkey anti-mouse second antibody, Trizol, DNase, and L-glutamine were from Invitrogen (Carlsbad, CA). AccuScript high fidelity 1st strand cDNA synthesis kit was from Stratagene (La Jolla, CA). iTagTM SYBR Green Supermix with ROX was from Bio-Rad (Hercules, CA). Luminescence ATP detection assay kit was purchased from PerkinElmer (Waltham, MA). Other reagents were from Sigma-Aldrich (St. Louis, MO).

Animals

Osteoblasts were isolated from 8–12 week-old wild-type and NHERF1-null littermates weighing 26.6 ± 1.1 g and 21.0 ± 3.9 g, respectively. Mice were euthanized by CO₂ inhalation followed by cervical dislocation. Bone histology was performed on paired 25-week old littermates. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (protocol 0911025B-3).

Mice were genotyped by PCR amplification of genomic DNA from mouse tails. PCR primers used were as follows: primer 1 (5'- AGCCCAACCCGCACTTACCA), primer 2 (5'- TCGGGGTTGTT GGCTGGAGAC), and primer 3 (5'- AGGGCTGGCACTCTGTCG). PCR was performed under the following conditions: initial denaturation for 2 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 58 °C, and 1 min at 72 °C. The final step was followed by an additional extension at 72 °C for 10 min.

Isolation of calvarial osteoblasts

Calvarial osteoblasts were isolated from adult mice as described previously [14–16]. Briefly, calvaria were dissected and cut into small chips. Bone pieces were digested with 2mg/ml collagenase for 1h and then with 0.25% trypsin for 30 min. The digestion medium and released cells were discarded. Bone chips were incubated in minimum essential medium eagle (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Osteoblasts grew out of the bone chips at 5–7 days. After trypsinization of the confluent cells, osteoblasts were used in the experiments. Cells were not passaged but used exclusively as primary cultures. Proliferating cells were cultured in normal growth media; differentiating osteoblasts were cultured in the presence of 50 µg/ml ascorbic acid and 3 mM β-glycerolphosphate for 8 days.

Quantitative real-time PCR

RNA from calvarial osteoblasts was extracted with Trizol (Invitrogen) according to the manufacturer's directions. Total RNA was treated with DNase and converted to complementary DNA (cDNA) using the Accuscript high fidelity 1st strand cDNA synthesis kit. A StepOneTM Real-Time PCR System was used to measure mRNA expression. Primers were designed based on Gene bank, and amplicons of 50–250 base pairs with T_m between 55 °C and 60 °C were selected [17–19]. The primers utilized for real-time PCR to detect mRNA expression of Npt2a, Npt2b, Npt2c, osterix, osteocalcin, and PTHR genes are listed in Table 1. Aliquots of first-strand cDNA were amplified using iTagTM SYBR Green Supermix with ROX under the following conditions: initial denaturation for 10 min at 94 °C followed by 40 cycles of 15 s at 94 °C and 1 min at 60 °C, followed by melting curve analysis. The mRNA expression levels of the target gene were normalized to β-actin mRNA. Data are presented as fold induction.

Coimmunoprecipitation

Analysis of the interactions of NHERF1 with Npt2a or Npt2b was performed essentially as described previously [20]. Briefly, six-well plates of osteoblast-like ROS17/2.8 cells were transiently transfected with empty vector (pcDNA3.1), Flag-NHERF1, Flag-NHERF2, GFP-

Npt2a, or GFP-Npt2b (Provided by Dr. Jurg Biber, University of Zurich, Switzerland). 48 h later the cells were lysed with immunoprecipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, and 150 mM NaCl) supplemented with protease inhibitor mixture I (Calbiochem; San Diego, CA) and incubated for 15-min on ice. Solubilized materials were incubated with anti-Flag M2 affinity gel (Sigma-Aldrich) overnight at 4°C. Immunoprecipitated proteins were eluted by the addition of SDS sample buffer, and then resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted using polyclonal anti-Flag or anti-GFP antibody.

Western blotting

Immunoblotting was performed as described [21]. Osteoblasts were lysed with Nonidet P-40 (50 mM Tris, 150 mM NaCl, 5mM EDTA, 0.5% Nonidet P-40) supplemented with protease inhibitor mixture I and incubated for 15 min on ice. Solubilized materials were resolved on SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore) using the semidry method (Bio-Rad). Membranes were blocked overnight at 4 °C with 5% nonfat dried milk in Tris-buffered saline plus Tween 20 (TBST) and incubated with the indicated antibodies (polyclonal anti-NHERF1 antibody at 1:1000, anti-NHERF2 antibody at 1:2000, and antisera of mouse PTHR at 1:1000) for 2 hours at room temperature. The membranes were then washed and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase at a 1:5000 dilution for 1 hour at room temperature. Protein bands were visualized by Luminol-based enhanced chemiluminescence.

Immunofluorescence

Confocal fluorescence imaging was performed as described [22]. Briefly, primary osteoblasts were grown on poly-D-lysine-coated coverslips. After 48h incubation, the cells were rinsed in PBS, fixed on 4% paraformaldehyde for 10 min, and then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. Blocking was performed by incubating the cells for 1 hour at room temperature in 5% goat serum in PBS. Anti-Npt2a polyclonal antibody diluted 1:50, anti-Npt2b polyclonal antibody diluted 1:50 and anti-NHERF1 monoclonal antibody diluted (1:100) in blocking buffer were applied to the specimens for 1 hour at room temperature. Alexa Fluor 488-tagged goat anti-rabbit second antibody diluted 1:500 and Alexa Fluor 546-tagged donkey anti-mouse second antibody diluted 1:500 were applied under the same conditions as the primary antibody. Coverslips were mounted for immunofluorescence microscopy and analyzed using Olympus FluoView 1000 microscope with a 60× oil immersion objective.

Histology and histomorphometry

Lumbar vertebrae were collected and embedded in frozen sectioning embedding medium (Tissue-Tek O.C.T. compound, Sakura). Undecalcified 4 µm vertebrae frozen sections were cut on a Microm HM 505E cryostat using tape transfer (CryoJane, Instrumedics, St. Louis, Missouri). Frozen sections were fixed with cold acetone for 10 min at 4°C and permeabilized with 0.2% Triton X-100 for 10 min. Nonspecific binding was blocked with 10% goat serum in PBS for 20 min. After washing with PBS, polyclonal rabbit anti-Npt2a antibody (Lifespan Biosciences, LS-C37447) was applied at 1:25 for 2.5 hours. After washing with PBS, goat anti-rabbit Alexa 546 secondary antibody was applied (1:500) for 1.5 hour. The slides were washed and mounted with aqueous mounting medium for microscopy. For Npt2a and NHERF1 double staining, sections immunostained with Npt2a were further labeled with mouse monoclonal anti-NHERF1 antibody (Abcam, ab 9526) (1:50) and subsequent donkey anti-mouse Alexa 488 (1:500) as described above. All incubations were performed at room temperature unless otherwise specified. Slides were photographed using oil objectives. Images were acquired using a 14-bit 2048 × 2048 pixel charge-coupled detector array (Diagnostic Instruments, Sterling Heights, MI). Green

channel indicates signal from excitation at 450–490 nm using a 510-nm dichroic mirror and a 520-nm barrier filter; red signal represents excitation at 536–556 nm with a 580-nm dichroic mirror and a 590-nm barrier filter.

Matrix mineralization

Osteoblasts were seeded in 24-well plates and grown until confluent. The cells were then incubated with medium supplemented with 50 µg/ml ascorbic acid and β-glycerolphosphate (3 mM) for 21 days. Osteoblasts were then fixed in 70% ethanol. The fixative was removed and the cells were washed 3 times with Hank's Balanced Salt Solution and stained for 15 min at room temperature with a 2% (w/v) solution of Alizarin red S at pH 4.2. The stained samples were washed 5 times with water. Stained mineral depositions in cell cultures were dissolved in 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) [23]. Absorbance of the solubilized stain was measured spectrophotometrically at 560 nm.

Pi uptake

Phosphate uptake was measured as described [20, 24]. Confluent osteoblasts on 12-well plates were serum-starved overnight. The cells were incubated with vehicle or 100 nM hPTH(1–34) for 4 hours. Cells were then washed 3× with a Na-containing buffer (in mM: 140 NaCl; 4.8 KCl; 1.2 MgSO₄; 0.1 KH₂PO₄; 10 HEPES, pH 7.4) or Na-free buffer, where *N*-methyl-D-glucamine (NMDG) replaced sodium. Measurement of phosphate uptake was initiated by adding buffer containing 1 µCi/ml [³²P]orthophosphate for 8 min to triplicate wells. Uptake was stopped by washing 3× with ice-cold NMDG media. The cells were extracted overnight with 0.5% Triton X-100 and then counted by beta-scintillation spectrometry. Pi uptake was calculated as nanomoles of ³²P per milligram of protein taken up in 8 min.

ATP secretion

ATP release was measured luminetrically using the luciferin/luciferase assay described previously [25, 26]. Briefly, calvarial osteoblasts were set up in 6-well plates and treated as described above. The cells were serum starved overnight. The culture medium was replaced by 1 ml of fresh serum-free medium, and the preparation was allowed to equilibrate for 1 hour. 500 µl of serum-free medium was replaced by an equal volume of medium containing the reagents tested and incubated for 4 min. Medium (150 µl) was collected gently to minimize ATP release caused by mechanical stimulation of the cells. The concentration of ATP release in the collected medium was measured on a Victor³V plate reader (PerkinElmer) using an ATP assay kit (PerkinElmer) according to the manufacturer's protocol. Luminescence in test samples was quantified by using an ATP standard curve.

cAMP formation

Adenylyl cyclase activity was determined by assay of cAMP accumulation as before [27]. Briefly, osteoblasts on 24-well plates were labeled with 0.5 µCi of [³H]adenine for 2 hours. The cells were then treated with vehicle or 100 nM PTH(1–34) in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 1 mM) for 15 min. The reaction was terminated by addition of 1 M trichloroacetic acid. cAMP was isolated by the two-column method [28].

Statistical analysis

Data are presented as the mean ± S.E., where *n* indicates the number of independent experiments. Multiple comparisons were evaluated by analysis of variance with post-test repeated measures analyzed by the Bonferroni procedure (Prism; GraphPad). Differences greater than *p* < 0.05 were assumed to be significant.

Results

Calvarial osteoblasts express NHERF1

Osteoblasts isolated from genotyped adult wild-type and NHERF1-null mice (Fig. 1A) were grown in primary cell culture. Osteoblasts from wild-type mice expressed NHERF1 and NHERF2, whereas NHERF1 knockout mice expressed only NHERF2 (Fig. 1B). NHERF1 localized mostly to the cell membrane of osteoblasts from wild-type mice (Fig. 2A). This is the first evidence showing NHERF1 expression in primary osteoblasts. Induction of mineralization did not affect NHERF1 expression in osteoblasts from wild-type mice (Fig. 1B). NHERF2 protein levels were comparable in osteoblasts from wild-type and NHERF1-null mice and were unchanged under proliferating and differentiating conditions. Thus, the absence of NHERF1 is not compensated by an increase of NHERF2, and NHERF2 expression is not regulated by the state of osteoblast proliferation.

Matrix mineralization in calvarial osteoblasts requires NHERF1

In cell culture, as *in vivo*, primary osteoblasts form bone-like mineralized nodules by undergoing proliferation, extracellular matrix maturation, and mineralization [29]. NHERF1-null mice and humans with NHERF1 mutations exhibit bone demineralization [30, 31] but this effect is thought to be a secondary consequence of heightened urinary phosphate excretion. To characterize a direct role for NHERF1 on osteoblasts, we compared mineralization in osteoblast cultures from wild-type and NHERF1 knockout mice. The results in Fig. 3 show that matrix mineralization of osteoblasts from NHERF1 knockout mice was markedly decreased compared with that from wild-type animals.

NHERF1 regulates Npt2 expression in proliferating and differentiated osteoblasts

Inorganic phosphate, primarily in the form of hydroxyapatite, is the major anionic component in bone [32, 33]. The parathyroid hormone receptor (PTHr) mediates PTH actions on inorganic phosphate (Pi) homeostasis mostly by regulating the activity of the kidney type 2a sodium-phosphate cotransporter (Npt2a). Npt2a and Npt2b are expressed in preosteoblastic MC3T3-E1 cells, in osteoblast-like UMR-106 cells, and in odontoblasts [1, 34]. The expression of Npt2a, which interacts with NHERF1 and is required for PTH-dependent Npt2a sequestration and inhibition of Pi transport [4, 35], is significantly reduced in kidney tubule cells of NHERF1 knockout mice [30]. We found Npt2a expressed at the cell membrane of osteoblasts from wild-type mice, where it colocalized with NHERF1 (Fig. 2A). Npt2a staining was less abundant at the cell membrane of osteoblasts from NHERF1 knockout mice (Fig. 2B) though Npt2b location was similar in osteoblasts from wild-type and NHERF1-null mice (Fig. 2B). To verify that growing primary osteoblasts under cell culture conditions did not alter the expression or localization of Npt2a and NHERF1, we analyzed the expression and localization of endogenous Npt2a and NHERF1 in trabecular bone of 25-week old mice. As shown in Fig. 4 Npt2a (red) was prominently expressed in bone from wild-type animals but less so in trabeculae of NHERF1-null mice. NHERF1 (green) colocalized (merge) with Npt2a in wild-type mice. NHERF1 was undetectable in knock out mice. Because these findings suggested that NHERF1 and Npt2a extensively colocalized, further experiments were undertaken to determine if NHERF1 and Npt2a interact in bone cells. Here, ROS17/2.8 cells were transfected with Flag-NHERF1, Flag-NHERF2, GFP-Npt2a or GFP-Npt2b. NHERF1 interacted with Npt2a (Fig. 5) and also bound Npt2b but with lower apparent affinity than for Npt2a. NHERF2 associated with both Npt2a and Npt2b with apparently similar binding affinities.

We then asked if the presence of NHERF1 influences the expression or distribution of Npt2a and Npt2b under proliferating or differentiated conditions. The effects of NHERF1 on the differentiation of calvarial osteoblasts from wild-type and NHERF1-null mice by treating

cells with ascorbic acid and β -glycerolphosphate for 8 days. mRNA expression levels of both osterix and osteocalcin, markers of osteoblast differentiation [17–19, 36], significantly increased in differentiated cells (Fig. 6A and 6B). These effects were reduced in osteoblasts from NHERF1 knockout mice.

We next examined the effect of NHERF1 on Npt2a and Npt2b expression in undifferentiated and differentiated osteoblasts. The results in Fig. 7 show that proliferating osteoblasts express both Npt2a and Npt2b mRNA; Npt2c was undetectable (data not shown), consistent with Npt2a and Npt2b localization in osteoblasts (Fig. 2, Fig. 4). Npt2a mRNA expression was significantly lower in calvarial osteoblasts from knockout mice compared to that of wild-type animals (Fig. 7A), while there was no difference of Npt2b mRNA expression between wild-type and knockout mice (Fig. 7B). Upon induction of in vitro mineralization Npt2a and Npt2b expression significantly increased in osteoblasts from wild-type and knockout mice. Expression of Npt2a significantly decreased in osteoblasts from NHERF1-null mice compared with that in wild-type mice (Fig. 7A), whereas Npt2b expression was not affected (Fig. 7B).

NHERF1 regulates PTH-induced Pi uptake in undifferentiated and differentiated osteoblasts

We then inquired if NHERF1 modulates Pi uptake at different stages of osteoblast growth and differentiation. Under the basal conditions, Pi uptake in proliferating osteoblasts from wild-type mice was greater than that of knockout mice (Fig. 8A), consistent with the higher level of Npt2a mRNA expression. PTH inhibited Pi uptake in osteoblasts from wild-type mice, but had no measurable effect in cells from NHERF1-null mice (Fig. 8A). Notably, PTH treatment exerted an opposite action on differentiated osteoblasts, significantly increasing Pi uptake (Fig. 8A). PTH did not affect Pi uptake in osteoblasts from knockout mice.

We sought to identify the regulatory factors contributing to NHERF1 regulation of Pi uptake in differentiated osteoblasts. Elevated extracellular Pi upregulates Npt2a but not Npt2b in osteoblast-like cells [1]. The hydrolysis of extracellular ATP constitutes a source of inorganic phosphate, and plays an important role in the mineralization of the matrix [37, 38]. ATP can be secreted in response to mechanical or $1\alpha,25(\text{OH})_2$ vitamin D_3 stimulation in osteoblasts [25, 26]. We examined if PTH treatment affected ATP release in proliferating and differentiated osteoblasts. Under resting conditions, differentiated osteoblasts from wild-type mice secreted more ATP than did proliferating osteoblasts (Fig. 8B), consistent with a previous report that constitutive ATP secretion increases upon osteoblast differentiation [26]. No significant change was observed in osteoblasts from NHERF1 knockout mice. PTH(1–34) (100 nM) treatment for 4 min did not increase ATP release in proliferating osteoblasts, but significantly increased ATP secretion in differentiated osteoblasts from wild-type mice (Fig. 8B). The differential effect of PTH on Pi uptake and ATP release from differentiated and proliferating osteoblasts may explain the dual effects of PTH at different stages of osteoblast differentiation.

PKA and PKC differentially mediate NHERF1 regulation of PTH-sensitive Pi uptake

To explore the mechanism by which PTH exerts distinct actions on Pi uptake in osteoblasts from wild-type mice, we first examined the signaling pathway affecting PTH regulation of Pi transport at different stages of differentiation. Osteoblasts were pretreated with the PKA inhibitor H89 alone or together with the PKC inhibitor bisindolylmaleimide 1 (Bis1) and then challenged with PTH(1–34) for 4 hours. In proliferating osteoblasts H89 or Bis1 partially reversed PTH-inhibited Pi uptake, and in combination they abolished PTH-inhibitable Pi uptake (Fig. 9A). In contrast, in differentiated wild-type osteoblasts, H89

blocked PTH-stimulated Pi uptake, whereas Bis1 had no effect. These data imply that NHERF1 regulates PTH-sensitive Pi uptake in proliferating osteoblasts through a combination of PKA and PKC signaling pathways, while in differentiated osteoblasts PTH-stimulated Pi uptake proceeds exclusively through a PKA-sensitive mechanism.

The aforementioned results raised the hypothesis that NHERF1 regulates PTH-induced cAMP formation in osteoblasts. The results in Fig. 9B show that PTH (100 nM for 15 min) stimulated cAMP formation in proliferating osteoblasts from wild-type mice but much less in osteoblasts from NHERF1-null mice. In differentiated osteoblasts, PTH evoked greater increases of cAMP accumulation than in proliferating cells. Further, although PTH enhanced cAMP production in osteoblasts from knockout mice after PTH treatment, the magnitude of this effect was significantly lower than that of cell from wild-type mice. PTHR protein expression increased slightly in differentiated osteoblasts compared with proliferating cells from wild-type and knockout mice (Fig. 9C, 9D), while PTHR mRNA increased significantly (Fig 7E) but these changes were not affected by NHERF1 (Fig. 9C, 9D and 9E). Therefore, the extent of cAMP formed in response to PTH in osteoblasts is modulated by NHERF1 without a change of PTHR. In the absence of NHERF1, PTH-sensitive adenylyl cyclase activity is attenuated.

Discussion

Calcium and phosphate are the principal inorganic components of bone and are required for mineralization. Far more is known about the mechanism and regulation of skeletal calcium accumulation than of phosphate. Hypophosphatemia and phosphate-wasting disorders are associated with rickets in children and osteomalacia in adults. Notably, mineralization defects can arise in the face of normal calcium metabolism, underscoring the critical role played by phosphorous. Pi uptake by mineralizing cells is poorly studied and has been investigated almost exclusively in osteosarcoma cell lines, which express type II Na/Pi cotransporters Npt2a (SLC34A10) and Npt2b (SLC34A2) [1], and type III Na/Pi cotransporters PiT1 (SLC20A1) and PiT2 (SLC20A2) [39]. In the kidney, Npt2a localization and PTH sensitivity are governed by NHERF1[20, 40]. The goal of the present work was to characterize Pi transport in primary osteoblasts and define the role of NHERF1. Our data show that PTH inhibits Pi uptake in proliferating osteoblasts but stimulates Pi uptake in differentiated osteoblasts from wild-type mice. In NHERF1-null mice PTH-regulated Pi transport is absent.

Osteoblasts originate from mesenchymal progenitors that, with appropriate stimulation, undergo proliferation and differentiate into preosteoblasts, and then into mature and functional osteoblasts. Our data show that Npt2a and Npt2b expression significantly increased following in vitro differentiation and mineralization (Fig. 7). Expression of Npt2c in bone was not examined. During osteoblastic differentiation, both calcium and Pi are required for extracellular matrix assembly and subsequent mineralization. The increased extracellular Pi strongly stimulates the Npt2a expression in osteoblast-like cells [1]. Conversely, elevated Pi downregulates Npt2a in the kidney, thereby limiting phosphate reabsorption and facilitating normalization of serum Pi [41].

Pi availability in bone and cartilage depends upon Pi transporters, phosphatases such as nonspecific alkaline phosphatase, membrane bound ectophosphatase and an ectonucleotide phosphatase. Substrate for the alkaline phosphatase includes pyrophosphate that may be provided by the Ank pyrophosphate transporter [42, 43] though this has not been well studied and local pyrophosphate (in areas without alk phos) is important in preventing mineralization of joints, and Ank appears to be dispensable in production of phosphate for bone formation. In the genetic disease hypophosphatasia “rubber baby disease”

pyrophosphate accumulates in serum [44]. In the context of healthy bone, pyrophosphate is rapidly hydrolyzed with a rate constant of a few milliseconds [45]. Substrate for the other phosphatases is mainly ATP transported out of the osteoblast. Thus, under mineralizing conditions more ATP is secreted into the extracellular medium compared with proliferating osteoblasts (Fig. 8B). These data confirm and extend a previous report that constitutive ATP secretion increases with osteoblast differentiation [26]. The secreted extracellular ATP is rapidly hydrolyzed and provides Pi for matrix mineralization. The present data show that PTH stimulates ATP release selectively in differentiated osteoblasts but does not affect ATP secretion in proliferating osteoblasts (Fig. 8B). Recent evidence indicates that insulin-like growth factor-1 (IGF-1) is a mediator of the PTH anabolic function in osteoblasts [46, 47]. IGF-1 can directly stimulate Npt2a expression and increase Pi uptake [48]. PTH stimulates IGF-1 expression in differentiated osteoblasts [46] and this may increase Npt2a expression and Pi uptake. This stimulatory action in differentiated osteoblasts of PTH on Pi uptake and ATP secretion suggests that enhanced cellular Pi serves as the substrate for ATP synthesis. These findings may provide a physiological framework to explain how PTH exerts opposite effects on Pi uptake in proliferating and differentiated osteoblasts and the coupling of Pi transport and extracellular ATP with matrix mineralization.

PTH acts on phosphate homeostasis primarily by regulating the abundance of membrane delimited Npt2a in kidney. Here, PTH decreased Pi transport in proliferating wild-type cells expressing NHERF1. PTH can transiently decrease osteoblastic cell growth [49]. Thus, in principle the decreased Pi uptake by PTH could be secondary to an alteration in cell metabolism. Such an action would not account for the inhibitory effect of PTH in the absence of NHERF1 unless NHERF1 affected cell growth. However, this would contradict the finding that PTH increased Pi uptake by wild-type differentiated cells. Thus, we think it likely that the actions of PTH represent direct effects of PTH on Npt2a that are distinct in the presence and absence of NHERF1.

Upon occupancy of the PTHR, activated receptors initiate several parallel signaling pathways including Gs and Gq-mediated adenylyl cyclase and phospholipase C signaling. In osteoblasts, PTH increases Runx2, osterix and osteocalcin expression through the cAMP/PKA signaling pathway, and stimulates osteoblast differentiation [50–52], the outcome of which may enhance the expression of sodium phosphate transporters. Both PKA and PKC inhibitors blocked PTH-sensitive Pi uptake in osteoblasts, which is similar to that in renal proximal tubular cells. In contrast, PTH increased both Pi uptake and cAMP production in differentiated osteoblasts from wild-type but not NHERF1 knockout mice. The data are consistent with earlier work showing that the increase of Na-dependent Pi transport by PTH in osteoblast-like cells was mediated by cAMP formation [24].

The effect of NHERF1 on PTH-dependent regulation of Gs and cAMP production is controversial. As originally described by Mahon and Segre [53] NHERF2, a NHERF1 homolog, markedly inhibited adenylyl cyclase by stimulating inhibitory Gi proteins in PS120 cells transfected with the PTHR. In contrast, no differences of PTH-stimulated cAMP formation were noted between wild-type and NHERF1-null proximal tubule cells [54, 55] or in CHO-N10-R3 cells [21] in the presence or absence of NHERF1. In ROS17/2.8 cells NHERF1 increases PTH-stimulated cAMP accumulation [56]. The present data show greater PTH-induced cAMP formation in osteoblasts from wild-type mice than in cells from knockout mice, and in differentiated cells compared with proliferating osteoblasts (Fig. 8B). These observations are consistent with the effect of NHERF1 on the inhibitory effect of PTH on Pi uptake in proliferating osteoblasts, which is mediated by cAMP/PKA and PLC/PKC pathways, and the stimulatory action of PTH on Pi uptake by differentiated osteoblasts, which depends exclusively on cAMP/PKA signaling pathway.

Mutations in NHERF1 or its genetic removal contribute to osteopenia in patients [57, 58] and diminished bone mineral density in knockout mice [7], respectively. In both instances the skeletal effect was thought to be a secondary consequence of heightened urinary phosphate excretion. We show here that mouse primary osteoblasts constitutively express NHERF1 (Figs. 1, 2) as does trabecular bone (Fig. 4). We found that matrix mineralization was significantly impaired in the cultures of osteoblasts from NHERF1 knockout mice (Fig. 3). These findings support a direct role of NHERF1 in osteoblast differentiation and matrix mineralization that may exacerbate the effects of limited phosphate availability. NHERF2 is also expressed by mouse osteoblasts and interacts both Npt2a and Npt2b. NHERF2 expression was indistinguishable in proliferating and differentiated osteoblasts from wild-type and NHERF1 knockout mice (Fig. 1). Ablation of NHERF1 thus does not alter expression of NHERF2 in osteoblasts and the two proteins would seem to have non-redundant functions as in the kidney [7, 59]. Moreover, the results are consistent with the view that NHERF1 does not detectably modulate Npt2b function in osteoblasts. This contrasts with the regulatory role of NHERF1 on intestinal Npt2b function [60].

Cellular Pi entry is a carrier-mediated process that is driven by the transmembrane electrochemical sodium gradient, which is established by the Na^+, K^+ -ATPase [61]. Pi transport in osteoblasts is reduced by ouabain, an inhibitor of the Na^+, K^+ -ATPase. NHERF1 interacts with Na^+, K^+ -ATPase, which is necessary for PTH-mediated inhibition of Na^+, K^+ -ATPase in renal proximal tubular cells [62, 63]. NHERF1 may thus impinge on PTH-sensitive Pi uptake in osteoblasts at several steps in the transport process and its regulation.

Npt2a-null mice exhibit elevated urinary phosphate excretion that is accompanied by delayed skeletal ossification, under mineralization of bone, and disordered growth plate maturation [64]. Notably, after 1 month, the skeletal phenotype reverses [65]. This reversal requires the vitamin D receptor. Although Npt2c was undetectable in the primary osteoblasts studied here, its absence, when combined with Npt2a in double knockout mice led to exacerbation of the skeletal phenotype [66]. A high-phosphate diet rescued the bone phenotype in these double Npt2a/Npt2a double knockout mice. NHERF1-null mice display a similar rachitic phenotype but unlike Npt2a knockout animals, the bone phenotype does not spontaneously resolve, suggesting NHERF1 regulates other functions to defend skeletal integrity. Dietary phosphate supplementation of NHERF1-null mice restores serum phosphate levels but the skeletal defects persist [67]. The precise basis for these different actions is unclear but may have to do with the greater urinary phosphate wasting and attendant hypophosphatemia.

In summary, we explored the regulation of Na-dependent Pi transport by NHERF1 in calvarial osteoblasts. PTH exerted opposite effects on Pi transport in osteoblasts under proliferating and differentiated conditions. NHERF1 was required for PTH action in both conditions. PTH-induced Gas/cAMP signaling was significantly diminished in the absence of NHERF1, consistent with the critical role of osteoblast Gas signaling for the anabolic action of PTH on bone formation [68, 69]. These findings advance a model wherein PTH promotes physiological mineralization by basolateral vectorial NHERF1-dependent Pi transport mediated by Npt2a into osteoblasts, which fuels pyrophosphate (Ank) and ATP secretion for Pi production at the apical border. In conclusion, we propose that NHERF1 directly regulates bone development and enhances the anabolic actions of PTH.

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Highlights

1. NHERF1 and Npt2a are constitutively expressed by primary osteoblasts and intact bone.
2. Matrix mineralization in calvarial osteoblasts requires NHERF1.
3. NHERF1 regulates Npt2a expression in both proliferating and differentiated osteoblasts.
4. NHERF1 modulation of PTH-inhibited Pi uptake in proliferating osteoblasts is mediated by PKA and PKC signaling.
5. NHERF1 regulation of PTH-stimulated Pi uptake in differentiated osteoblasts is mediated by PKA.

Fig. 1. NHERF1 and NHERF2 expression in osteoblasts. (A) Genotype of wild-type and NHERF1 knockout mice. Mice were genotyped by PCR amplification of genomic DNA from mouse tails as described in Materials and Methods. After PCR the samples were resolved on a 2% agarose gel. The sizes of the upper band (303 bp) and lower band (231 bp) correspond with the predicted sizes for wild-type (WT) and knockout (KO) mice, respectively. (B) NHERF1 and NHERF2 expression in calvarial osteoblasts from wild-type (WT) and NHERF1 knockout (KO) mice. Calvarial osteoblasts were grown under proliferating (pro) conditions or differentiated (dif) in vitro as described in Materials and Methods. NHERF1 and NHERF2 expression levels were measured by immunoblotting with anti-NHERF1 or NHERF2 antibody. Actin expression was used as a loading control. A representative experiment from 3 independent experiments is shown.

Fig. 2.

Colocalization of Npt2 with NHERF1 in primary osteoblasts. Fluorescent staining was analyzed by confocal microscopy as described in Materials and Methods. Npt2a (A) and Npt2b (B) are shown in green, NHERF1 in red. Npt2 and NHERF1 are expressed at the cell membrane as shown by the z axis scan below each image. Merged images shown on the right indicate extensive colocalization of Npt2a with NHERF1 (A) but far less with Npt2b (B).

Fig. 3.

Mineralization of calvarial osteoblasts from NHERF1 wild-type and knockout mice. In vitro mineralization of osteoblasts was initiated by treatment with 50 mg/ml ascorbic acid and β -glycerolphosphate (3 mM) for 21 days. Mineralization was detected by Alizarin red S staining as described in Material and Methods (A). The extent of mineralization was quantified by densitometric scanning. Data are summarized as the mean $\pm$ S.E. of 3 independent experiments. **, $p < 0.01$, compared with wild-type control (B).

Fig. 4.

Npt2a expression and localization in wild- type (WT) and NHERF1 knockout mice (KO). Under phase illumination the mineralized bone appears as a grey region with a bright border at the mineral front surrounded by a layer of osteoblasts. Npt2a (red) was strongly expressed in WT bone at the osteoblast membrane adjacent to bone formation. NHERF1 (green) was likewise expressed at the mineralizing surface of WT bone and extensively colocalized with Npt2a staining (merge). In contrast, Npt2a labeling in the NHERF1 KO was weak in keeping with the in vitro results.

Fig. 5.

Direct interactions of NHERF1/2 and Npt2a/2b. Interaction of NHERF1 and NHERF2 with Npt2 was performed by coimmunoprecipitation on ROS17/2.8 cells transfected with pcDNA3.1 vector, Flag-NHERF1, Flag-NHERF2, GFP-Npt2a or GFP-Npt2b as described in Material and Methods. The figure is representative of three independent experiments. IP, immunoprecipitation; IB, immunoblot.

Fig. 6. Osteoblast markers in wild-type (WT) and NHERF1 knockout (KO) osteoblasts under proliferating (prolif) and differentiated (Diff) conditions. Osterix (A) and osteocalcin (B) were amplified from total RNA and analyzed by real-time PCR in untreated proliferating WT or KO calvarial osteoblasts or differentiated osteoblasts. Results are normalized to β -actin. Data are summarized as the mean $\pm$ S.E. of 3 independent experiments. **, $p < 0.01$, compared vs. wild-type control.

Fig. 7.

Npt2a and Npt2b expression in wild-type (WT) and NHERF1 knockout (KO) osteoblasts under proliferating (Prolif) and differentiated (Diff) conditions. Npt2a (A) or Npt2b (B) were amplified from total RNA and analyzed by real-time PCR in untreated proliferating WT or KO calvarial osteoblasts or differentiated osteoblasts. Results are normalized to β -actin and summarized as the mean $\pm$ S.E. of 4 independent experiments. *, $p < 0.05$, **, $p < 0.01$, compared with wild-type or knockout control.

Fig. 8.

Bimodal regulation of PTH-sensitive Pi uptake and ATP release in osteoblasts. Wild-type (WT) or knockout (KO) calvarial osteoblasts were grown under proliferating (Prolif) or differentiated (Diff) conditions as described. Treatment with 100 nM PTH for 4h decreased Pi uptake in proliferating WT cells but stimulated Pi uptake in differentiated WT cells (A). PTH failed to affect Pi uptake by NHERF1-null proliferating or differentiated osteoblasts. Data are summarized as the mean $\pm$ S.E. of 4 independent experiments. *, $p < 0.05$, **, $p < 0.01$, compared with vehicle control. (B) WT or KO osteoblasts were treated with PTH for 4 min. ATP release was measured with a luciferin/luciferase assay as described in the Material and Methods. Data are summarized as the mean $\pm$ S.E. of 4 independent experiments. *, $p < 0.05$, **, $p < 0.01$, compared with vehicle control or PTH treatment.

Fig. 9.

PTH-sensitive signaling in osteoblasts. Wild-type (WT) or knockout (KO) calvarial osteoblasts were grown under proliferating (Prolif) or differentiated (Diff) conditions for 8 days. (A) Cells were pretreated with H89 (10 μ M) and/or Bis 1 (10 μ M) for 15 min and then before addition of 100 nM PTH for 4h. Pi uptake in osteoblasts was measured as described. Data represent the mean $\pm$ S.E. of 4 independent experiments. *, $p < 0.05$, **, $p < 0.01$, compared with vehicle control or PTH treatment. (B) cAMP formation was measured under identical condition in WT or KO osteoblasts. Data are summarized as the mean $\pm$ S.E. of 4 independent experiments. **, $p < 0.01$, compared with vehicle control or PTH treatment. (C) PTHR expression levels were measured by immunoblotting with polyclonal antisera of mouse PTH1R. Actin expression was used as a loading control. (D) Quantitative expression of PTHR expression was determined by densitometric gel scanning and expressed as the fold change of PTHR normalized by actin is shown from 3 independent experiments. No statistically significant changes were noted. (E) PTHR was amplified from total RNA and analyzed by real-time PCR in untreated proliferating WT or KO calvarial osteoblasts or differentiated osteoblasts. Results are normalized to β -actin. Data are summarized as the mean $\pm$ S.E. of 4 independent experiments. *, $p < 0.05$, compared vs. wild-type control.

Table 1

Primer sequences for real-time PCR

Gene	Sequence	Accession number
Npt2a	Forward: 5'- AGACACAACAGAGGCTTC Reverse: 5'- CACAAGGAGGATAAGACAAG	NM_013030
Npt2b	Forward: 5'-CCTTGGCCCGAGTTGTTGGAAAAT Reverse: 5'- CTACAGGAGTCCCGTTGTCAT	NM_080854
Npt2c	Forward: 5'- CATCTTCAACTGGCTCAC Reverse: 5'- GGTATCACACTGCTATCC	NM_011402
osteocalcin	Forward: 5'- GCAATAAGGTAGTGAACAGACTCC Reverse: 5'- AGCAGGGTTAAGCTCACACTG	NM_007541
osterix	Forward: 5'-AGAGGTTCACTCGCTCTGACGA Reverse: 5'- TTGCTCAAGTGGTCGCTTCTG	NM_130458
PTHR	Forward: 5'-CAGGCGCAATGTGACAAGC Reverse: 5'-TTTCCCGGTGCCTTCTCTTTC	NM_011199
β -actin	Forward: 5'- AGCCATGTA CGTAGCCATCC Reverse: 5'- CTCAGCTGTGGTGGTGAA	NM_007393