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PHOSPHO1 is essential for mechanically competent mineralization and the avoidance of spontaneous fractures☆,☆☆

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ABSTRACT

Phosphatases are essential for the mineralization of the extracellular matrix within the skeleton. Their precise identities and functions however remain unclear. PHOSPHO1 is a phosphoethanolamine/phosphocholine phosphatase involved in the generation of inorganic phosphate for bone mineralization. It is highly expressed at sites of mineralization in bone and cartilage. The bones of *Phospho1*$^{-/-}$ mice are hypomineralized, bowed and present with spontaneous greenstick fractures at birth. In this study we show that PHOSPHO1 is essential for mechanically competent mineralization that is able to withstand habitual load. Long bones from *Phospho1*$^{-/-}$ mice did not fracture during 3-point bending but deformed plastically. With dynamic loading nanoindentation the elastic modulus and hardness of *Phospho1*$^{-/-}$ tibiae were significantly lower than wild-type tibia. Raman microscopy revealed significantly lower mineral:matrix ratios and lower carbonate substitutions in *Phospho1*$^{-/-}$ tibia. The altered dihydroxylysinonorleucine/hydroxylysinonorleucine and pyridinoline/deoxypyridinoline collagen crosslink ratios indicated possible changes in lysyl hydroxylase-1 activity and/or bone mineralization status. The bone formation and resorption markers, N-terminal propeptide and C-terminal telopeptide of Type I collagen, were both increased in *Phospho1*$^{-/-}$ mice and this we associated with increased bone remodeling during fracture repair or an attempt to remodel a mechanically competent bone capable of withstanding physiological load. In summary these data indicate that *Phospho1*$^{-/-}$ bones are hypomineralized and, consequently, are softer and more flexible. An inability to withstand physiological loading may explain the deformations noted. We hypothesize that this phenotype is due to the reduced availability of inorganic phosphate to form hydroxyapatite during mineralization, creating an undermineralized yet active bone.

Introduction

During bone growth, formation and development, the mineralization of the extracellular matrix (ECM) of both chondrocytes and osteoblasts involves the deposition of crystalline hydroxyapatite (HA) within the interior of membrane-limited matrix-vesicles (MVs) [1–3]. This process is instigated by the accumulation of Ca^{2+} and inorganic phosphate (Pi) within MVs resulting in the formation of HA crystals. This initial phase is followed by MV membrane rupture/breakdown and the modulation of ECM composition to further promote propagation of HA outside of the MVs [1–3]. ECM mineralization is a highly regulated process and chondrocytes, osteoblasts and their derived MVs accomplish this by expressing Pi-transporters for Pi uptake [4,5], annexin V for Ca^{2+} influx [6] and regulators of inorganic pyrophosphate (PPi) metabolism [7]. Extracellular PPi is a recognized potent mineralization inhibitor in biological fluids [8] and its concentration is regulated by tissue-nonspecific alkaline phosphatase (TNAP) which hydrolyzes PPi in the ECM to establish a Pi/PPi ratio permissive for the initial formation of HA crystals within MVs [9–12]. Also, nucleotide pyrophosphatase phosphodiesterase 1 (NPP1)

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ectoplasmically generates PPi from nucleoside triphosphates [13], and the multiple-pass transmembrane protein ANK mediates intracellular to extracellular channeling of PPi [14,15].

In addition to its PPi hydrolase activity, TNAP also has recognized ATPase activity [16] and disruption of PPi and/or ATP hydrolysis which may contribute to hypophosphatasia (HPP), an inborn error of metabolism resulting in rickets and osteomalacia [17]. Mice deficient in TNAP function (*Akp2*$^{-/-}$) phenocopy infantile HPP *i.e.* their skeleton at birth is mineralized normally but hypomineralization rapidly ensues within 1–2 weeks of postnatal life before death at postnatal day 20 [18,19]. The failure of bone to mineralize properly after birth in *Akp2*$^{-/-}$ mice has been associated with PPi accumulating within the ECM and blocking the propagation of HA in the ECM beyond the confines of the MV membrane [20,21].

An explanation as to why the skeleton of *Akp2*$^{-/-}$ mice are normally mineralized at birth has focused on the existence of other phosphatases responsible for MV-mediated ECM mineralization and PHOSPHO1 which was identified 10 years ago is a strong candidate for this missing phosphatase. Since its discovery and characterization [16,22–27] we have proposed that PHOSPHO1 is, in part, responsible for Pi accumulation (and HA formation) within the MV through its phosphohydrolase activity towards the membrane phospholipids, phosphoethanolamine and phosphocholine [26,28]. Consequently, due to its cytosolic localization and its known presence and activity within MVs [24,25], PHOSPHO1 is likely to be partly responsible for the intravesicular HA formation noted in MVs derived from HPP and *Akp2*$^{-/-}$ chondrocytes and osteoblasts [20,21]. The critical importance of PHOSPHO1 for skeletal mineralization has been recently suggested by the use of small molecule compounds to inhibit PHOSPHO1 activity in MVs and developing embryonic chick limbs *in vivo* [25,27]. Definitive evidence for a mineralization role of PHOSPHO1 was obtained in a comparison of the bone phenotype of *Phospho1*$^{-/-}$, *Akp2*$^{-/-}$ and *Phospho1*$^{-/-}$; *Akp2*$^{-/-}$ double knockout mice [29]. The *Akp2*$^{-/-}$ and *Phospho1*$^{-/-}$ mice are both characterized by lower skeletal mineralization whereas the double ablation of PHOSPHO1 and TNAP leads to the complete absence of skeletal mineralization. These data are strongly supportive of independent, non-redundant mechanisms of action of both phosphatases in the mineralization process [18,29].

Whilst the functional importance of PHOSPHO1 in regulating skeletal mineralization has now been clearly demonstrated [29] it is still unclear as to how PHOSPHO1 fully contributes to the maintenance of bone quality and ultimately, bone strength. Our aim was to analyze the role of PHOSPHO1 during this developmental phase and not at adulthood where any alterations noted in skeletal integrity may be secondary and a consequence of earlier developmental cues. Such information is essential if we are to understand fully the physiological role of PHOSPHO1 in the maintenance of skeletal integrity and explain the pathological long bone bowing and spontaneous greenstick fractures noted in *Phospho1*$^{-/-}$ mice [29]. In this paper we conclusively demonstrate that PHOSPHO1 is essential for the proper formation of mechanically competent bones able to withstand habitual load.

Materials and methods

Mice and tissues

Phospho1-R74X-null mutant (*Phospho1*$^{-/-}$) mice were generated by N-ethyl-N-nitrosourea mutagenesis (ENU) as previously described [29]. We chose to study mice at one month of age as the skeletal abnormalities previously described by us [29] present immediately after birth and during juvenile development. For the study of material and mechanical properties and collagen cross-link analysis, 7 wild-type (WT) and 9 *Phospho1*$^{-/-}$ 30-day old male mice were euthanized and their right tibia and right femur were removed and stored in distilled H_2O at −20 °C. The left tibia was removed and fixed in 4% paraformaldehyde (PFA) for static histomorphometric analysis. In further studies, dynamic histomorphometry was completed in calcein labeled male mice (8 WT and 8 *Phospho1*$^{-/-}$). Mice were injected i.p. with calcein (10 mg/kg in 1.4% w/v $NaHCO_3$) at 19-days and then again at 29 days of age. Mice were sacrificed at 30 days of age and the left tibia from each mouse was dissected and processed as described by Rawlinson et al. [30]. Blood was collected by cardiac puncture from 30-day old male mice (14 WT and 16 *Phospho1*$^{-/-}$) and serum separated using serum-clotting-activator tubes (Starstedt Ltd., UK). All animal studies were approved by the Institutional Animal Users' Committees of the Sanford-Burnham Medical Research Institute, La Jolla, CA and The Roslin Institute, UK.

3-point bending for the determination of bone stiffness and breaking strength

An Instron 5564 materials testing machine (Instron, High Wycombe, UK) fitted with a 2 kN load cell was used to determine bone stiffness and breaking strength [31]. The span was fixed at 5.12 mm for femora and at 6.95 mm for tibiae. The cross-head was lowered at 1 mm/min and data were recorded after every 0.2 N change in load and every 0.1 mm change in deflection. Each bone was tested to fracture. Failure and fracture points were identified from the load–extension curve as the point of maximum load and where the load rapidly decreased to zero, respectively. The maximum stiffness was defined as the maximum gradient of the rising portion of this curve, and the yield point, the point at which the gradient reduced to 95% of this value. Both values were calculated from a polynomial curve fitted to the rising region of the load–extension curve in Mathcad (Mathsoft Engineering and Education Inc., Cambridge, MA, USA).

Raman microscopy

Raman microscopy and nanoindentation (see below) were conducted on cortical bone fragments of both tibia and femur after the completion of the 3-point bending analysis. A ~2 mm transverse section of the diaphyses were analyzed by Raman microscopy as previously described [32]. The collected data were processed as described by Goodyear et al. [32]. Intensities of bands representing mineral (phosphate ν_4), matrix (amide III), carbonate and acid phosphate (HPO_4^{2-}) were measured and ratios calculated. The full width at half maximum height of the phosphate symmetric stretching vibration (ν_1) was measured to estimate the crystallinity of the bone mineral [33].

Nanoindentation

Bone slices were embedded in epoxy resin (EPO-SET, MetPrep Ltd, Coventry, UK) for nanoindentation. The blocks were then ground using sand paper of successively decreasing grid size. Once the whole cross-section was visible at the surface final polishing was done with 5 μm and then 1 μm diamond suspension and finalized with 0.05 μm γ-alumina slurry (MetPrep Ltd, Coventry, UK). Nanoindentations were performed using a G200 nanoindenter (Agilent Technologies) fitted with a Berkovich shaped diamond tip having a Poisson's ratio of 0.07 and elastic modulus of 114 GPa. The indenter was controlled with Testworks 4 version 4.10 (MTS System Corporation) to produce 60 indentations using both quasistatic and dynamic loading schemes [34]. Indentations for tibiae were done in the anterior–medial aspect of the transverse diaphyseal bone, while indentations in the femur were done on an anterior section. Both regions corresponded to areas previously analyzed by Raman microscopy. All indentations were inspected with a optical microscope (Nikon Eclipse ME600) equipped