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The kidney sodium–phosphate co-transporter alters bone quality in an age and gender specific manner

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ABSTRACT

Mutations in the kidney NaPiIIa co-transporter are clinically associated with hypophosphatemia, hyperphosphaturia (phosphate wasting), hypercalcemia, nephrolithiasis and bone demineralization. The mouse lacking this co-transporter system was reported to recover its skeletal defects with age, but the "quality" of the bones was not considered. To assess changes in bone quality we examined both male and female NaPiIIa knockout (KO) mice at 1 and 7 months of age using micro-computed tomography (micro-CT) and Fourier transform infrared imaging (FTIRI). KO cancellous bones at both ages had greater bone volume fraction, trabecular thickness and lesser structure model index based on micro-CT values relative to age- and sex-matched wildtype animals. There was a sexual-dimorphism in the micro-CT parameters, with differences at 7 months seen principally in males. Cortical bone at 1 month showed an increase in bone volume fraction, but this was not seen at 7 months. Cortical thickness which was elevated in the male and female KO at 1 month was lower in the male KO at 7 months. FTIRI showed a reduced mineral and acid phosphate content in the male and female KO's bones at 1 month with no change in acid phosphate content at 7 months. Collagen maturity was reduced in KO cancellous bone at 1 month. The observed sexual dimorphism in the micro-CT data may be related to altered phosphate homeostasis, differences in animal growth rates and other factors. These data indicate that the bone quality of the KO mice at both ages differs from the normal and suggests that these bone quality differences may contribute to skeletal phenotype in humans with mutations in this co-transporter.

Introduction

Control of phosphate homeostasis is a complex process dependent on a multitude of interacting factors [1–8]. In general, ingested phosphate enters the intestine via the activities of two sodium–phosphate co-transporters, NaPi IIa[1] and NaPi IIc. The activities of these co-transporters are regulated by vitamin D, PTH, and several newer regulatory factors [3,5,9] including FGF23 and Klotho. Once in the circulation, inorganic phosphate (Pi) levels are controlled primarily by the kidney via the NaPi IIa transporter, its cofactor, Klotho, PTH, and vitamin D through the action of its 1-hydroxlyase [6]. Other tissues important for Pi homeostasis are the intestines which control Pi absorption and excretion (action of co-transporter NaPi IIb) and the mineralized tissues (calcified cartilage, bones and teeth). These mineralized tissues express the intestinal co-transporter NaPi IIb, small amounts of NaPi IIa in osteoclasts and a type III co-transporter, Pit1 [10–12].

Heterozygous loss of function mutations of NaPi IIa in humans are associated with hypophosphatemia, hyperphosphaturia (phosphate wasting), hypercalcemia, nephrolithiasis (in males), and bone demineralization [13]. Mice, in which NaPi IIa is genetically ablated are smaller at birth than wild type animals, and are similarly hypophosphatemic and waste phosphate. The tibias of these KO mice, based on histomorphometric data, were shown to vary with age (21–184 days). This variance includes; (i) at 21 days reduced secondary ossification centers; (ii) at 45 days no appreciable differences; (iii) at 115 days increased metaphyseal trabeculae; and (iv) calcifications within the marrow cavity at older ages [14].

It is not clear if NaPi IIa has a direct effect on bone or if the observed global phenotype in the NaPi IIa KO and in patients with mutations (early hypercalcuria and osteomalacia) [15] is directly related to the observed phosphate wasting, to their hypercalcuria, or to their compensatory responses. The NaPi IIa KO animals are normophosphatemic

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[1] Abbreviations: BS/BV—bone surface/bone volume; BV/TV—bone volume fraction; Ca—calcium; FGF23—fibroblast growth factor-23; HYP—hypophosphatemic; KO—knockout; micro-CT—micro computed tomography; NaPi II—sodium–phosphate cotransporter type II; Pi—inorganic phosphate; PMMA—poly-methyl methacrylate; PTH—parathyroid hormone; SMI—structure model index; TMD—tissue mineral density; WT—wild type.

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but express elevated levels of NaPi IIb in the intestine. Because the process of bone formation is dependent on local Pi levels, we hypothesized that; were the bone phenotype in the NaPi IIa KO due to a decreased local Pi concentration (in the bone microenvironment) there would be a corresponding decrease in the mineral content in all areas of new bone formation, and newly formed crystals would grow to a lesser extent than those in the WT. However, if the bone phenotype occurred due to a general decrease in circulating Pi, then the ability of the osteoblasts to maintain a normal local Pi and Ca level in their microenvironment, would result in minimal changes in mineral properties. These changes would be commensurate with a smaller boney structure in the wild type. Since NaPi IIa deficient animals were reported to lack a bone phenotype when they were older [14], we predicted that according to these hypotheses, bone in older animals would appear normal in quality (composition, collagen cross links, micro-architecture). This quality would still be less in older mice compared with changes in younger mice.

As noted within, earliest radiographic and morphologic reports described a bone phenotype in young animals that disappeared as the animals aged [14]. To gain more insight into new bone formation and remodeling processes in the presence and absence of NaPi IIa, the specific aims of this study are to compare bone properties of 1 and 7 month old NaPi IIa KO mice to their age- and sex matched WT controls.

Material and methods

Study design

To characterize how bone mineral properties varied with age and genotype, male and female wild type (WT) and NaPi IIa knockout (KO) mice at 1 and 7 months of age were compared. The variables studied were the geometry, architecture, and density based on micro-CT. Bones of a 1 month old mouse would demonstrate mainly new bone formation. The 7 month old skeletally mature mouse would, similarly, exhibit changes in mature bone. Mineral compositional changes were based on Fourier transform infrared imaging microscopy. The covariates were age and sex. Based on a power-study, for an alpha = 0.05, beta = 0.20 (i.e. 80% power), four mice per group were needed for FTIRI and five per group for the micro-CT.

Animals

Four to six-month-old male and female B6 WT and Npt2a KO mice (acquired from Jackson laboratories) weighing 18 to 30 g were used as breeders at U. Fla Gainesville under IACUC approval and following NIH guidelines. The breeders and their pups were given regular powdered sterile rodent chow moistened with water and formed into small balls. Urinary and serum calcium and phosphate levels were measured as detailed previously [16,17]. NaPi IIa KO and WT pups (5 per genotype per sex) were sacrificed at 1 month and at 7 months, and their long bones stored in 90% ethanol were used for the analyses detailed below. Bones (5/group) from the following groups of mice were examined: 1 month old male WT, 1 month old female WT, 1 month old male KO, 1 month old female KO, 7 month old male WT, 7 month old female WT, 7 month old male KO, 7 month old female KO. There was a total of 40 animals.

Micro-CT

Femora, cleaned of soft-tissue and placed in 90% ethanol were used to determine the morphometry and density of cortical and cancellous bone by micro-Computed Tomography (micro-CT) using a Scanco μCT35 system operating at 55 kVp, 6 μm resolution (Scanco Medical, Basselsdorf, Switzerland). Three D-reconstructions were performed automatically with a user-defined beam hardening correction factor. Mineral density calibrations were done based on preset by Scanco algorithms for the 55 kVp during the reconstructions. Evaluation of the 3D reconstructed volumes was performed using the Scanco morphometry and densitometry software for open VMS on a Hewlett-Packard RAID server. The parameters evaluated [18] were bone volume fraction (BV/TV), porosity (%), cortical thickness (Ct.Th. (mm)), tissue mineral density (TMD (mg/cm^3)), and polar moment of inertia (pMOI (mm^4)) for cortical bone. The parameters for cancellous bone were BV/TV, TMD, trabecular number (Tb.N. (1/mm)), trabecular thickness (Tb.Th. (mm)) and trabecular separation (Tb.Sp. (mm)), the ratio of bone surface to bone volume (BS/BV), connectivity density (Conn-Dens.), and structure model index (SMI).

FTIR imaging

Following micro-CT femurs were embedded in polymethyl methacrylate (PMMA). Multiple 1–2 μm semi-thin sections were cut from each PMMA block using a heavy duty sledge microtome (Polycut 3500, Leica, Germany). These sections were examined by a Fourier Transform Infrared Spectroscopic Imaging System (Perkin Elmer model 100 imaging system) as detailed elsewhere [19]. For the one month old bones, cortical, cancellous and proximal growth plates were examined separately. In the 7 month bones only cortical and cancellous bone areas were examined. For each bone sample three areas (~100 μm × 200 μm) per section of trabecular or cortical bone were imaged with a 6.25 μm spatial and a 4 cm^{-1} spectral resolution. ISYS software (Spectral Dimensions, Olney, MD) was used to process the data, including a subtraction of the PMMA spectral contribution and base-lining the data. Five image parameters were calculated: mineral to matrix ratio, carbonate to phosphate ratio, crystallinity, acid phosphate substitution, and collagen maturity. Mineral to matrix which is linearly related to the tissue's ash content [20] was calculated as the relative integrated intensities of the ν_1,ν_3 phosphate band, ~900–1200 cm^{-1} to that of the protein amide I band (1585 cm^{-1}–1720 cm^{-1}). Carbonate (855 cm^{-1}–890 cm^{-1}) to phosphate band area ratio indicates the extent of carbonate substitution in the apatite lattice [21]. Crystallinity, related to crystallite size and perfection [20], was calculated based on the peak height ratios of stoichiometric (subband at 1030 cm^{-1}) and non-stoichiometric (subband at 1020 cm^{-1}) apatite [19]. Acid phosphate substitution was calculated from the intensity ratios of subbands at (1128 cm^{-1}/1096 cm^{-1}) [22]. Collagen maturity was calculated from the peak height ratios of subbands in the collagen amide I peak, related to reducible (1660 cm^{-1}) and non-reducible (1690 cm^{-1}) fibrillar collagen components [23], it does not reflect non-enzymatic cross links, but based on studies in osteonal bone is related to tissue age [24].

Statistics

Mean and standard deviations were calculated for each bone type, each gender, genotype and age. Linear regression was used to assess the effect of genotype while controlling for age and gender. Interaction effects between genotype, age, and gender were also considered in the regression. When an interaction effect was significant, we compared the two genotypes specifically for each age group or gender group. We conducted regression diagnostics including tests of collinearity, independence of residual errors, normality of residuals, and homogeneity of variance [25]. Specifically, variance inflation factor, Durbin–Watson test, Shapiro–Wilks test, and scatterplots of residuals versus predicted values were used to assess collinearity, independence, normality, and homogeneity, respectively. The criteria except for homogeneity were met in all regression analyses. Non-normality was found in regression analysis of three micro-CT parameters (porosity, Ct.Th., and TMD). However, linear regression has been proven to be very robust to the assumption of normality in statistical literature [26–28]. In addition, the test of normality is likely to be unreliable when the sample size is small. A $p<0.05$ was accepted as significant. These calculations were performed using SAS 9.2 (SAS Institute, Cary, NC). Significant results are shown in the table and figures.