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Novel anatomic adaptation of cortical bone to meet increased mineral demands of reproduction

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ABSTRACT

The goal of this study was to investigate the effects of reproductive adaptations to mineral homeostasis on the skeleton in a mouse model of compromised mineral homeostasis compared to adaptations in control, unaffected mice. During pregnancy, maternal adaptations to high mineral demand include more than doubling intestinal calcium absorption by increasing calcitriol production. However, calcitriol biosynthesis is impaired in HYP mice, a murine model of X-linked hypophosphatemia (XLH). In addition, there is a paucity of mineralized trabecular bone, a primary target of bone resorption during pregnancy and lactation. Because the highest density of mineral is in mature cortical bone, we hypothesized that mineral demand is met by utilizing intracortical mineral reserves. Indeed, analysis of HYP mice revealed dramatic increases in intracortical porosity characterized by elevated serum PTH and type-I collagen matrix-degrading enzyme MMP-13. We discovered an increase in carbonate ion substitution in the bone mineral matrix during pregnancy and lactation of HYP mice, suggesting an alternative mechanism of bone remodeling that maintains maternal bone mass during periods of high mineral demand. This phenomenon is not restricted to XLH, as increased carbonate in the mineral matrix also occurred in wild-type mice during lactation. Taken together, these data suggest that increased intracortical perilacunar mineral turnover also contributes to maintaining phosphate levels during periods of high mineral demand. Understanding the mechanisms of skeletal contribution to mineral homeostasis is important to improving the treatment and prevention of fracture risk and bone fragility for female patients with XLH, but also provides important insight into the role and unique adaptations of the maternal skeleton to the demands of fetal development and the needs of postnatal nutrition.

1. Introduction

Pregnancy and lactation require mobilization of mineral to provide to the offspring and several physiologic and metabolic adaptations occur to supply the required mineral [1,2]. Bone resorption provides much of the mineral during lactation. However, the effects on maternal bone metabolism during pregnancy have been largely unexplored and may involve short-term fragility and depletion of skeletal mineral content. During pregnancy, maternal adaptations to high mineral demand include more than doubling intestinal calcium absorption [2]. This process is mediated by $1,25(OH)_2D$ and possibly other factors [1]. Total $1,25(OH)_2D$ levels more than double during the first trimester and levels remain elevated until term [3]. However, free $1,25(OH)_2D$ levels do not increase until the third trimester when 80% of the calcium gained by the fetal skeleton is actively transferred across the placenta [2–4]. PTH falls to the lower end of the normal range [5–8], potentially protecting the maternal skeleton from excessive bone resorption [9]. Therefore, PTH is not the source driving the increase in $1,25(OH)_2D$. Instead, other regulators of 1α-hydroxylase must account for most of the circulating $1,25(OH)_2D$ during pregnancy [10].

In women with low dietary intake of calcium and vitamin D, PTH concentrations do not drop during pregnancy [11]. Thus, in certain cases, the maternal skeleton may contribute substantial amounts of calcium to the fetus. While the reliance on mineral from the maternal skeleton during pregnancy normally does not cause long-term changes in skeletal calcium content or strength [12], the effects of high mineral demand during pregnancy on maternal bone metabolism in patients with an already compromised mineral metabolism are not fully understood. Specifically, studies on the effects of pregnancy on mineral and bone

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metabolism in patients with X-linked hypophosphatemia (XLH) have not been conducted. XLH, an X-linked dominant disorder, is the most common form of familial hypophosphatemia, affecting an estimated 1 in 20,000 [13]. Patients with XLH suffer from aberrant regulation of $1,25(OH)_2D$ production leading to impaired intestinal calcium and phosphorus absorption and skeletal abnormalities characterized by defective calcification of cartilage and bone [14–16]. In addition, despite therapy with phosphate salts and active vitamin D, osteomalacia is not resolved and persists throughout adulthood [17]. Further, fibroblastic growth factor-23 (FGF-23), which is predominantly expressed in bone, stimulates renal phosphate wasting and impairs production of $1,25(OH)_2D$ in vivo [18].

In the current study, we investigated these reproductive adaptations to mineral homeostasis in a mouse model of XLH and control, unaffected mice. The HYP mouse of the C57BL/6 strain is a murine model that genocopies and phenocopies human XLH, making HYP mice ideal for studying the effects of impaired mineral homeostasis [17,18]. Mating of HYP females to C57BL/6 males produces both HYP and skeletally normal wild-type pups. Enormous mineral demand during pregnancy combined with the impaired intestinal phosphorous and calcium absorption in HYP mice could potentially increase the risk of fracture and contribute to the long-term compromise of the adult skeleton [14] as the skeleton would appear to be a reservoir by which pregnant and lactating patients with XLH maintain mineral metabolism. Further, because a murine model of XLH has decreased number of osteoclasts [19] and osteoclast activity [20], alternative forms of bone remodeling may be required to mobilize mineral [21]. Thus, the HYP mouse model offers two benefits in studying the skeletal contributions to mineral homeostasis: 1) improving our understanding on the impact of pregnancy and lactation on bone quality in patients with XLH for whom there are no definitive protocols for management and 2) studying alternative mechanisms for bone remodeling using the unusual combination of high mineral demand and impaired mineral homeostasis. By studying mineral and bone metabolism in both HYP and control mice, we aim to better our understanding of the interactions of vitamin D, mineral homeostasis and PTH on bone strength, providing an evidence-based rationale for improved clinical management of XLH during the reproductive years.

2. Materials and methods

Mice were fed standard chow ad libitum and tap water. All mice were maintained in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals and procedural protocol was approved by Yale's Institutional Animal Care and Use Committee. The study was split into 2 parts — pregnancy and lactation.

2.1.1. Generation of pregnant mice

HYP mice were obtained from colonies maintained by our laboratory. At 12 weeks of age, female C57BL/6J wild-type (WT) and HYP mice (n = 8/group) were bred to WT males. With HYP mice, litters are smaller in number. However, mating of HYP females to WT males produces both HYP and skeletally normal wild-type pups. Pregnant females were identified by the first appearance of a vaginal plug (designated as embryonic day 0.5 [E0.5]). Dynamic changes in femoral bone mineral density (BMD, g/cm^2) were measured by dual-energy X-ray absorptiometry using a PIXImus densitometer (Lunar, Madison, WI) 2 days before mating (baseline) and on E3, E10, E16, and after giving birth (E18.5). Immediately after giving birth, mice were killed, the femora and tibiae were harvested, cleaned of soft tissue, and analyzed as described below.

2.1.2. Generation of lactating mice

At 12 weeks of age, female WT and HYP mice were separated into the following groups (n = 4–6/group): 1) Lactating HYP, 2) Virgin HYP, 3) Lactating WT, 4) Virgin WT. Mice in the lactating groups were paired with WT males for breeding. At the end of a 21-day lactation period post-partum, mice were killed, the femora and tibiae were harvested, cleaned of soft tissue and analyzed as described below.

2.2. High-resolution micro-computed tomography (HR micro-CT)

The mid-diaphysis of the right femur was scanned via phoenix nanotom® nano-CT (GE Measurement & Control Solutions, Longmont, CO, USA) at 1.55 μm resolution. At the selected resolution, both large pores such as vascular canals and small pores such as osteocyte lacunae are readily visible. To avoid potential bias associated with differences in the degree of mineralization between groups, images were individually thresholded to segment bone tissue from porosity and soft tissue via a standard thresholding algorithm [22], rather than using a global threshold. The resulting binarized images were then inverted for the quantification of porosities. To decrease errors associated with the limits of spatial resolution, pores with a volume less than 5 μm^3 were excluded. Cannular structures representing vasculature and/or bone remodeling units within cortical bone were classified as objects greater than 1000 μm^3 in volume [23]. Parameters measured included cannular volume (Ca.V) and cannular volume density (cannular volume per total cortical bone volume; Ca.V/TV). Porosity comprising the osteocyte lacunar system was classified as objects 100–1000 μm^3 in volume. Osteocyte lacunar indices included number of lacunae (Lc.N), lacuna number density (number of lacunae per total cortical bone volume; Lc.N/TV), total lacuna volume (Lc.V), lacuna volume density (Lc.V/TV), and average lacuna volume (<Lc.V> = Lc.V/Lc.N). Percent porosity was defined as the total pore volume per total cortical bone volume.

2.3. Histology and immunohistochemistry

To explore potential mechanisms for bone remodeling, histological assessment of the bone was done to describe changes in cellular activity. Histological preparation was conducted at the Yale Orthopaedics Histology Lab. At death, right tibiae were rapidly dissected and fixed in 4% buffered paraformaldehyde for immunohistochemistry (IHC) staining for tartrate-resistant acid phosphatase (TRAP). IHC staining for alkaline phosphatase (ALP) activity was also used to detect and confirm the presence of intracortical vascular channels as described previously [24]. MMP-13 is a collagen matrix-degrading enzyme shown to be important in bone remodeling and in lactation-induced osteocytic remodeling [25]. We therefore assessed evidence of mineral mobilization by osteocytes by IHC staining of matrix metalloproteinase-13 (MMP-13) with a mouse anti-rat MMP-13 monoclonal antibody, as previously described [14]. After HR micro-CT, the right femora were embedded in poly(methyl methacrylate) (PMMA) and stained using von Kossa stain as previously described [14]. Measurement of areas of unmineralized matrix (osteoid volume/total volume; OV/TV) was performed on von Kossa stained slides of diaphyseal cortical bone and analyzed using Osteomeasure software (Osteometrics, Atlanta, GA).

2.4. Serum biochemical measurements

Blood was collected from the retro-orbital sinus of all pregnant mice at baseline, E3, E10, E16, and after giving birth (E18.5). Serum phosphorus was measured using a commercially available Liqui-UV kit (Stanbio, Boerne, TX). Serum phosphorous was not measured at in WT mice at E16 because of inadequate amounts to perform all assays. Serum calcium was assessed using Sigma kits employing a plate reader (Titertek Multiscan, Huntsville, AL). $1,25(OH)_2D$ was measured using a commercially available RIA kit (DiaSorin, Stillwater MN). Parathyroid hormone (PTH) was measured using a two site ELISA that employs antimouse PTH antibodies and intact rat PTH standards (Alpco, Salem, NH) [26].