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Review

Sex-related differences in the skeletal phenotype of aged vitamin D receptor global knockout mice

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ABSTRACT

The role of the vitamin D receptor (VDR) in maintaining skeletal health appears to be complex and dependent on the physiological context. Global *Vdr* deletion in a mouse model ($Vdr^{-/-}$) results in hypocalcemia, secondary hyperparathyroidism and bone features typical of vitamin D-dependent rickets type II. When weanling $Vdr^{-/-}$ mice are fed a diet containing high levels of calcium, phosphorus and lactose, termed the rescue diet, normalisation of serum calcium, phosphate and parathyroid hormone levels results in prevention of rickets at 10 weeks of age. However, 17 week old male $Vdr^{-/-}$ mice, fed the rescue diet, have been reported as osteopenic due to a decrease in bone formation when compared to *wild type* mice. We now report confirmation of this finding with further data on the effect of the rescue diet on appendicular and axial skeletal structures in male and female $Vdr^{-/-}$ mice at 26 weeks of age compared to $Vdr^{+/-}$ controls. All $Vdr^{-/-}$ mice were normocalcemic with no evidence of any mineralization defect. However, male $Vdr^{-/-}$ mice exhibited significantly reduced mineral in femoral and vertebral bones when compared to control littermate $Vdr^{+/-}$ mice, consistent with the previously reported data. In contrast, 26-week-old female $Vdr^{-/-}$ mice demonstrated significantly increased femoral trabecular bone volume although there was decreased vertebral trabecular bone volume, similar to males, and femoral cortical bone volume was unchanged. Thus, the $Vdr^{-/-}$ mouse model displays sex- and site-specific differences in skeletal structures with long-term feeding of a rescue diet. Although the global $Vdr^{-/-}$ ablation does not permit the determination of skeletal mechanisms producing these differences, these data confirm skeletal changes even when fed the rescue diet.

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1. Introduction

The vitamin D receptor (VDR) knockout ($Vdr^{-/-}$) mouse model fed standard chow diet demonstrate features typical of the human disease vitamin D-dependent rickets type II [1,2]. The inability for the 1,25 dihydroxyvitamin D_3 (1,25D) ligand to bind to the VDR results in gross changes to mineral ion homeostasis as a result of impaired intestinal calcium absorption and actions at the kidney and bone. At weaning, $Vdr^{-/-}$ mice develop hypocalcemia and secondary hyperparathyroidism giving rise to rickets with severe defects in bone growth and mineralization. Importantly, when weanling $Vdr^{-/-}$ mice are fed a diet containing high levels of calcium (2%), phosphorus (1.25%) and lactose (20%), normalization of plasma calcium, phosphate and parathyroid hormone (PTH) levels occurs, preventing the rickets bone phenotype and resulting in normal bone structure at least up to 10 weeks of age [3–5]. These data clearly demonstrate that the actions of 1,25D, acting through the VDR, are critical for maintaining plasma calcium and phosphate homeostasis, in order to adequately mineralize the skeleton in young mice.

Longer-term feeding of the rescue diet to $Vdr^{-/-}$ mice until adulthood, however, appears to be insufficient to maintain normal bone mineral volume levels. When weanling male $Vdr^{-/-}$ mice were fed the rescue diet until 17 weeks of age, a marked reduction in bone volume was observed compared to *wild type* mice, without the features of osteomalacia and akin to osteopenia [6]. These animals maintained normal serum calcium, phosphate and PTH levels. Furthermore, the osteopenia occurred without increased bone resorptive activity and was, at least in part, due to a significant reduction in mineral apposition rate (MAR). These data raise the possibility that VDR plays additional roles in the maintenance of bone homeostasis in adulthood beyond intestinal and renal transport of calcium. VDR-mediated activities in bone have been shown through numerous *in vitro* and *in vivo* studies to include regulation of proliferation, differentiation and mineralisation of osteoblasts [7,8], inhibition of mineralization in osteocytes [9] and the well-described bone resorption response via RANKL signalling [10,11].

In an effort to clarify the benefits and/or inadequacies of the rescue diet on calcium and skeletal homeostasis in $Vdr^{-/-}$ mice, we examined the bone phenotype of $Vdr^{-/-}$, compared to $Vdr^{+/-}$ mice following long-term feeding of rescue diet from weaning until 26 weeks of age using micro-computerised tomography to assess trabecular and cortical bone at axial and appendicular sites.

2. Materials and methods

2.1. Animals

Male and female $Vdr^{-/-}$ and $Vdr^{+/-}$ littermate mice were bred by mating $Vdr^{+/-}$ females with $Vdr^{-/-}$ males. Mice were group-housed, with 5 or fewer animals per cage. At 20 days of age, all mice were fed rescue diet containing 2% calcium, 1.25% phosphorus, 20% lactose diet based on the Teklad diet TD96348 (Specialty Feeds, WA, Australia). $Vdr^{+/-}$ littermate mice were used as control mice as it has been previously demonstrated that intestinal calcium absorption, serum PTH and serum 1,25D were all comparable to wild-type mice when fed the rescue diet [12]. All procedures were approved by the Institutional Animal Ethics Committee.

2.2. Serum biochemistry

Blood was collected via cardiac puncture at time of death. Serum levels of calcium, phosphate and alkaline phosphatase (ALP) were measured on the KoneLab 20XT Clinical Chemistry Analyser (ThermoScientific, MA, USA) using standard reagents (ThermoScientific, USA). Serum C-terminal telopeptide levels were determined using RatLaps EIA Kit (Immunodiagnostic Systems, UK). Serum PTH levels were determined using a mouse PTH ELISA Kit (Immutopics, San Clemente, CA).

2.3. Microcomputed tomographical analyses

Three-dimensional microcomputed tomography (μCT) was performed using the SkyScan 1174 (Bruker, Belgium). Acquired X-ray images were reconstructed using NRecon (v1.6.9, Bruker, Belgium) with an isotropic voxel size of 6.5 microns. A region beginning 0.5 mm proximal to the growth plate and encapsulating 10% of the metaphysis, adjusted for femur length, was isolated as a volume of interest from both the distal femoral and proximal tibial metaphyses of each mouse to quantify trabecular bone volume. A 1 mm segment of midshaft femoral and tibial cortical bone was used for volumetric analyses of cortical bone. The trabecular bone of the L1 vertebrae was isolated to quantify the trabecular bone volume in this region. Trabecular bone parameters (volume, thickness, number, and separation) and cortical parameters (volume, cortical width, periosteal and endosteal circumference) were all calculated using direct 3D and 2D approaches using CTan software (v1.7, Skyscan).

2.4. Histomorphometric analyses

Single injections of calcein (20 mg/kg BW) and xylenol orange (30 mg/kg BW) were administered intraperitoneally at 6 and 2 days, respectively, prior to death. Formalin-fixed femora were bisected in the sagittal plane with a diamond-tipped cutting blade using a slow speed saw and processed for resin embedding as previously described [11]. Sequential sagittal sections were cut to 5 μm thickness. Measures of mineral apposition rate (MAR, μm/day) and bone formation rate (BFR, $\mu m^3/\mu m^2$/day) were made from unstained fluorochrome labelled sections of the distal femoral metaphysis prepared by placing them in acetone for 15 min and protected from light. For assessment of bone resorption, sections were stained for tartrate-resistant acid phosphatase (TRAP) to identify bone-associated TRAP-positive osteoclasts to determine osteoclast number per bone surface (N.Oc/B.Pm). Briefly, resin-embedded sections were submerged in acetone for 15 min before 60 min incubation in Tris–HCL buffer (pH 9.4) at 37 °C followed by two washes in distilled water. Sections were then incubated at 37 °C for 60 min in an acid phosphatase (AcP) stain prepared by adding (A) 0.035 g of tartaric acid dissolved in 35 mls of sodium acetate (pH 5.2) to (B) 100 μl basic fuchsin in a 100 μl solution containing 0.4 mg of sodium nitrite. This solution was then added to a solution containing 0.04 g naphthol ASBI phosphate (Sigma–Aldrich, Missouri, USA) in 2 ml dimethylformamide before being counter-stained with haematoxylin. The assessment of osteoid was carried out by performing a Von Kossa stain and counter-staining with Toluidine Blue. Sequential drops of a 0.01% working solution of Toluidine Blue O (ProSciTech, QLD, AUS) and sodium tetraborate in distilled water were placed on sections for 2 min, prior to clearing and mounting. For formation period (FP) measurements, sections were decalcified in EDTA at 4 °C for 24 h followed by H&E staining to visualise cement lines within trabecular bone. FP measurements were derived from trabecular bone in the distal femoral metaphysis by following the protocol outlined [13]. Histomorphometric analyses of the distal femoral metaphysis were performed either using the OsteoMeasure histomorphometry system (Osteometrics, Atlanta, USA) or Nanozoomer and NDP.view (v2.0, Hamamatsu, Japan).