Journal of Steroid Biochemistry and Molecular Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Absence of vitamin D receptor in mature osteoclasts results in altered osteoclastic activity and bone loss

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ARTICLE INFO

Keywords:
Osteoclast
Osteoclastogenesis
Vitamin D
Vitamin D receptor
CYP27B1

ABSTRACT

Mature osteoclasts express the vitamin D receptor (VDR) and are able to synthesise and respond to $1,25(OH)_2D_3$ via CYP27B1 enzyme activity. Whether vitamin D signalling within osteoclasts is necessary for the regulation of osteoclastic bone resorption in an *in vivo* setting is unclear. To determine the requirement for the VDR- and CYP27B1-mediated activity in mature osteoclasts, conditional deletion mouse models were created whereby either Vdr or Cyp27b1 gene was inactivated by breeding either $Vdr^{fl/fl}$ or $Cyp27b1^{fl/fl}$ mice with Cathepsin K-Cre transgenic mice ($Cstk^{Cre}$) to generate $Ctsk^{Cre}/Vdr^{-/-}$ and $Ctsk^{Cre}/Cyp27b1^{-/-}$ mice respectively. To account for potential $Ctsk^{Cre}$-meaited off-target deletion of Vdr, $Dmp1^{Cre}$ were also used determine the effect of Vdr deletion in osteocytes. Furthermore, $Ctsk^{Cre}/Vdr^{-/-}$ mice were ovariectomised (OVX) to assess the role of VDR in osteoclasts under bone-loss conditions and bone marrow precursor cells were cultured under osteoclastogenic conditions to assess osteoclast formation. Six-week-old $Ctsk^{Cre}/Vdr^{-/-}$ female mice demonstrated a 15% decrease in femoral BV/TV ($p < 0.05$). In contrast, BV/TV remained unchanged in $Ctsk^{Cre}/Cyp27b1^{-/-}$ mice as well as in $Dmp1^{Cre}/VDR^{-/-}$ mice. When $Ctsk^{Cre}/Vdr^{-/-}$ mice were subjected to OVX, the bone loss that occurred in $Ctsk^{Cre}/Vdr^{-/-}$ was predominantly due to a diminished volume of thinner trabeculae when compared to control levels. These changes in bone volume in $Ctsk^{Cre}/Vdr^{-/-}$ mice occurred without an observable histological change in osteoclast numbers or size. However, while cultured bone marrow-derived osteoclasts from $Ctsk^{Cre}/Vdr^{-/-}$ mice were marginally increased when compared to $VDR^{fl/fl}$ mice, elevated expression of genes such as Cathepsin K, Nfatc1 and VATPase was observed. Collectively, these data indicate that the absence of VDR in mature osteoclasts causes exacerbated bone loss in young mice and during OVX which is associated with enhanced osteoclastic activity and without increased osteoclastogenesis.

1. Introduction

1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is well known to regulate calcium homeostasis and skeletal health. In addition to the well-known activities of $1,25(OH)_2D_3$ in stimulating the intestinal absorption of calcium, we and others have identified roles for $1,25(OH)_2D_3$ in directly regulating serval bone cell types including osteoblasts, osteocytes and osteoclasts [1,2].

A current view is that $1,25(OH)_2D_3$ promotes both increasing the expression of the key osteoclast differentiation factor RANKL, and decreasing the expression of its antagonist, OPG [3]. While some controversy exists as to the presence of VDR in osteoclasts [4], overwhelming *in vitro* evidence supports the notion that vitamin D directly controls the resorptive activity of osteoclasts. $1,25(OH)_2D_3$ has been shown to facilitate adhesion of osteoclast precursors to stromal osteoblasts via induction of inter-cellular adhesion molecule, ICAM-1 [5] and osteoclast adhesion molecule, αVβ3 integrin [6]. As well, $1,25(OH)_2D_3$ directly supports RANKL-induced osteoclast formation from the mouse osteoclast precursor cell line, RAW 264.7 [7]. In addition, human osteoclast precursors can metabolise $25(OH)D_3$ into $1,25(OH)_2D_3$ by the enzyme CYP27B1 and this is required for the expression of a number of important osteoclast markers, and transcription factors, NFATc1 and c-

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http://dx.doi.org/10.1016/j.jsbmb.2017.10.022
Received 8 May 2017; Received in revised form 24 October 2017; Accepted 26 October 2017

FOS [2]. However, the resorptive activity of mature osteoclasts, was also shown to be inhibited by both 25(OH)D and $1,25(OH)_2D_3$ [2].

Consistent with these findings, splenocyte derived osteoclastic precursor cells derived from either VDR-*null* or CYP27B1-*null* mice demonstrate markedly reduced osteoclastogenesis [8,9]. While fewer osteoclasts form in these from VDR-*null* or CYP27B1-*null* mice, osteoclasts that do form exhibited greater resorptive activity per osteoclast and increased survival as indicated by lower BAX:BCL-2 ratio [8–10]. The findings from these studies suggest a role for the synthesis and activity of vitamin D being able to directly regulate both the formation and in turn the subsequent activity of osteoclasts.

Whether these *in vitro* and *ex vivo* observations translate in a role for vitamin D activity within osteoclasts in an *in vivo* setting is unclear. Thus, to assess the role for VDR- and CYP27B1-mediated activity in osteoclasts in *vivo*, osteoclast-specific VDR and CYP27B1 knockout mice, $Ctsk^{Cre}/Vdr^{-/-}$ and $Ctsk^{Cre}/Cyp27b1^{-/-}$, respectively, were generated. We demonstrate the relative impact of VDR and CYP27B1 deletion within mature osteoclasts on bone structure, osteoclast generation and subsequent bone resorption. These data expand the evidence that vitamin D activity contributes to the regulation of bone resorption via direct activities within osteoclasts.

2. Materials and methods

2.1. Animals

All animals used in the generation of both Osteoclast-specific mouse models ($Ctsk^{Cre}/Vdr^{-/-}$ $Ctsk^{Cre}/Cyp27b1^{-/-}$ and $Dmp1^{Cre}/Vdr^{-/-}$ were of *C57B6* background. Cathepsin-K-*Cre* ($Ctsk^{Cre}$) mice [11] were obtained from Assoc. Prof. R Davey (University of Melbourne, Australia). Dentin matrix protein-1-*Cre* $Dmp1^{Cre}$ mice were obtained from Prof L Bonewald (Indiana University) [12]. VDR-LoxP [13] (gift from Dr. S Kato, University of Tokyo) and CYP27B1-LoxP [14] (gift from Prof. R St-Arnaud, University of McGill) were mated with the Ctsk-*Cre* mice to generate $Ctsk^{Cre}/Vdr^{-/-}$ and $Ctsk^{Cre}/Cyp27b1^{-/-}$ mice respectively as well as VDR-LoxP or CYP27B1-LoxP litter-matched control mice. VDR-LoxP mice were mated with Dmp1-*Cre* mice to generate $Dmp1^{Cre}/Vdr^{-/-}$ in order to determine whether the $Ctsk^{Cre}/Vdr^{-/-}$ bone phenotype might be due to non-specific Vdr deletion in osteocytes. All animal procedures were approved by the University of South Australia, Animal Ethics Committee. Mice were housed in IVC caging, with a maximum 5 gender-matched animals/cage and a standard 12 hr light/dark cycle. Mice were provided *ad libitum* access to standard chow and tap water.

2.2. DNA and mRNA analyses

DNA and mRNA were extracted from whole tibia from littermate Vdrfl/fl and respective knockout mice by previously published procedures [15]. The presence of mutated Vdr DNA was amplified using specific primers (F: 5′-CCTTGGTGAGCTGAGTTTACTCTT-3′; R: 5′-CTTCCCACCACTTTGTACTACCA-3′). Messenger RNA was analysed using mRNA specific primers for Vdr, (F: 5′-TCGGATCTGTGGAGTGTGTGGAG-3′, R: 5′-TTGTCCTTGGTGATGCGGCAATCT-3′). These mRNA specific primers are design to specifically amplify mRNA in the region of exon 2. The floxed VDR mice have exon 2 of the vitamin D receptor gene flanked by short intronic loxP sites. Exon 2 encodes the 1 st zinc finger of the DNA binding domain of the in D receptor. Previously, the deletion of exon 2 was shown to result in a frame shift, resulting in complete deletion of the vitamin D receptor protein [16]. Other primers used include Cathepsin- K (5′-ggccaactcaagaagaaaactg-3′, R: 5′-tctctgtaccctctgcatttagc-3′); NFATc1 (5′-AGGACACCCCATTGTGCAGCT-3′, R: 5′- cgtcagccgtcccaatgaaca −3′), and VATPase (5′- GCTGCAGAGCGGGCTCAAG-3′, R: 5′- AAGGGGAATGTGATGATGGTGTAG-3′).

2.3. Ovariectomy

Female $Vdr^{fl/fl}$ and $Ctsk^{Cre}/Vdr^{-/-}$ mice (n = 4-5/group) were ovariectomised at 10 weeks of age. Briefly, mice received a bilateral ovariectomy (OVX) under isofluorane induced anaesthesia. A small incision (0.5 cm) on the mid-dorsal aspect of the mouse was created to allow blunt-nosed scissors to separate the skin from the peritoneal layer to cut through the peritoneal layer to retrieve and excise the ovary on each flank.

2.4. Biochemistry

Serum calcium (Ca), phosphate (P) and alkaline phosphatase (ALP) levels were measured using the KoneLab 20XT Clinical Chemistry Analyser (ThermoScientific, MA, USA), using standard protocols and reagents (ThermoScientific, USA). Serum C-terminal telopeptide (cross-laps, CTX) was measured using a RatLaps EIA Kit (Immunodiagnostic Systems Limited, UK).

2.5. Micro-computed tomography (μCT)

Right femora were subjected to *ex vivo* micro-computed tomography (μCT) using the 1174 μCT system (Bruker). Micro-architecture was analysed using high-resolution micro-CT in order to obtain multiple x-ray transmission images. The scanning resolution had a voxel size of 6.5 μm/pixel, with an x-ray tube potential of 50 kVp and 800 μA. To measure trabecular micro-architecture within the femur (metaphysis) a rotation step of 0.4 ° and a frame averaging of 2 was used. Cross-sectional images were reconstructed using the nRecon software (Version 1.6.9.18) (Bruker, BEL). Realignment of datasets was performed using Dataviewer software (v.1.5.1, Bruker, BEL). All bone quantification from reconstructed and realigned datasets was performed using CTan software (v.1.7, Bruker, BEL). A 2 mm metaphyseal region of interest was isolated from the distal femur. Parameters assessed include, bone volume (BV/TV, %), trabecular thickness (Tb.Th, mm) and trabecular number (Tb.N, #/mm). Analyses of trabecular volume parameters for Ovariectomy intervention studies were undertaken using the Skyscan 1076 (Bruker, BEL) for live *in vivo* scanning of femurs of ovariectomised animals (0.5 mm filter, 52 kVp and 112 μA, 9 μm pixel size, 5890 ms exposure time, rotation step of 0.80 and a frame averaging of 1). All analyses were done using the same software/programs as mentioned above.

2.6. Histology

Femora were then subjected to ethanol dehydration steps prior to being placed into a Methyl Methacrylate (MMA): Polyethylene Glycol 400 (PEG) solution (100% MMA: 10% PEG). Femurs were left in the MMA: PEG solution for 14 days, at which time polymerisation was induced using a solution containing MMA: PEG: Perkadox (0.4%). Trimmed resin blocks were sectioned in the sagittal plane at 5 μm thickness. Sections were prepared for TRAP staining or left unstained for fluorochrome label analyses. TRAP stained slides were used to measure metaphyseal osteoclasts numbers (N.Oc, #/mm), osteoclast surface per bone surface (Oc.S/BS, %), number of osteoclasts per bone perimeter (N.Oc/B.Pm, #/mm), and osteoclast size (nm^2). Unstained slides were used measurement of the mineralising surface (MS/BS) mineral apposition rate (MAR, μm/day) and bone formation rate/bone surface (BFR/BS, $\mu m^3/\mu m^2$/day). The All analyses were performed using OsteoMeasure™ Version 3.3.0.2 (OsteoMetrics, Inc. Decatur, GA, USA)

Expression of osteoclastic genes was analysed using real-time, quantitative PCR using iScript Reverse Transcription Supermix for RT-qPCR (BioRad). Relative gene expression was normalized to that of the beta-2-microglobulin (β2 M) housekeeping gene using the comparative cycle threshold (Ct) method (deltaCT).