International Immunopharmacology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

International Immunopharmacology

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

Altered ultrastructure, density and cathepsin K expression in bone of female muscarinic acetylcholine receptor M3 knockout mice

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ARTICLE INFO

Article history:
Received 13 February 2015
Received in revised form 4 May 2015
Accepted 7 May 2015
Available online xxxx

Keywords:
Transmission electron microscopy
TRAP
Bone mineral density
Biomechanics
Osteocyte
Cholinergic receptor

ABSTRACT

High frequency of osteoporosis is found in postmenopausal women where several molecular components were identified to be involved in bone loss that subsequently leads to an increased fracture risk. Bone loss has already been determined in male mice with gene deficiency of muscarinic acetylcholine receptor M3 (M3R-KO). Here we asked whether bone properties of female 16-week old M3R-KO present similarities to osteoporotic bone loss by means of biomechanical, radiological, electron microscopic, cell- and molecular biological methods.
Reduced biomechanical strength of M3R-KO correlated with cortical thickness and decreased bone mineral density (BMD). Femur and vertebrae of M3R-KO demonstrated a declined trabecular bone volume, surface, and a higher trabecular pattern factor and structure model index (SMI) compared to wild type (WT) mice. In M3R-KO, the number of osteoclasts as well as the cathepsin K mRNA expression was increased. Osteoclasts of M3R-KO showed an estimated increase in cytoplasmic vesicles. Further, histomorphometrical analysis revealed up-regulation of alkaline phosphatase. Osteoblasts and osteocytes showed a swollen cytoplasm with an estimated increase in the amount of rough endoplasmatic reticulum and in case of osteocytes a reduced pericellular space.
Thus, current results on bone properties of 16-week old female M3R-KO are related to postmenopausal osteoporotic phenotype. Stimulation and up-regulation of muscarinic acetylcholine receptor subtype M3 expression in osteoblasts might be a possible new option for prevention and therapy of osteoporotic fractures. Pharmacological interventions and the risk of side effects have to be determined in upcoming studies.
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1. Introduction

The highest frequency of osteoporosis is found in postmenopausal women where the decreasing level of estrogen is identified as one of the reasons for bone loss that subsequently leads to an increased fracture risk. During the last years several factors, signaling molecules and molecular components were proven to be involved in osteoporotic bone loss. Thus, the options of pharmacological treatment increased but still there are high requirements for new preventive and therapeutic tasks without detrimental side effects.

Kliemann et al. [1] demonstrated that male mice with gene deficiency for the muscarinic acetylcholine receptor M3 (M3R-KO) revealed a bone loss that was assumed to be similar to osteoporotic bone fragility by means of µCT, biomechanical, and collagen type 1 expression analyses. The bone loss determined by µCT confirmed the results of Shi et al. [2] who further reported about increased number of osteoclasts and less osteoblasts in M3R-KO whereas the amount of apoptotic cells was not changed [2]. Using neuron specific M3R-KO Shi et al. [2] showed

Abbreviations: ALP, alkaline phosphatase; BCIP/NBT, 5-bromo-4-chloro-3-indolyphosphate/nitro blue tetrazolium chloride; BMC, bone mineral content; BMD, bone mineral density; BS/TV, relative bone surface; BV/TV, relative bone volume; CaHA, calcium hydroxyapatite; CtsK, cathepsin-K; DXA, dual-energy X-ray absorptiometry; EI, bending stiffness; F_U, ultimate bending load; HE, hematoxylin-eosin; L, lumbar vertebrae; mAChR, muscarinic acetylcholine receptor; M3R, muscarinic acetylcholine receptor subtype M3; M3R-KO, muscarinic acetylcholine receptor M3 knockout mice; min, minutes; nAChR, nicotinic acetylcholine receptors; N/TbS, osteoclast number per trabecular surface; RANK, receptor activator of nuclear factor κB; RANKL, receptor activator of nuclear factor κB ligand; real-time RT-PCR, real-time Reverse-Transcriptase polymerase chain reaction; ROI, region of interest; SMI, structure model index; TbN, trabecular number; TbTh, trabecular thickness; TbPf, trabecular pattern factor; TEM, transmission electron microscopy; Th, thoracic vertebrae; TMD, tissue mineral density; TRAP, tartrate-resistant acidic phosphatase; WT, wild type.

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http://dx.doi.org/10.1016/j.intimp.2015.05.012
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Please cite this article as: K.S. Lips, et al., Altered ultrastructure, density and cathepsin K expression in bone of female muscarinic acetylcholine receptor M3 knockout mice, Int Immunopharmacol (2015), http://dx.doi.org/10.1016/j.intimp.2015.05.012

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that the bone loss also occurred if neurons lacked muscarinic acetylcholine receptor (mAChR) subtype M3 (M3R) but when osteoblasts expressed the receptor. Therefore it was postulated that bone loss resulted from an over activity of sympathetic tone, which was not dampened by cholinergic action of ACh via M3R [2].

Acetylcholine is the most prominent neurotransmitter of the parasympathetic nervous system. It acts via binding to two different classes of receptors: a) nicotinic acetylcholine receptors (nAChR) and b) mAChR. The G-protein coupled mAChR are a group of 5 different receptors which provoke second messenger signal transduction systems that include the regulation of cGMP and intracellular calcium concentrations. In doing so the physiological effects depend on the cell type, mAChR subtype and the molecular structure of the receptor [3]. Expression of M3R in osteoblast-like cells was first shown in vitro [4,5] and afterwards in femur of rats and mice and in human ribs [5]. In vertebrae of rats with induced osteoporosis M3R mRNA was down-regulated compared to sham operated control rats [6]. Thus, M3R expressed by murine osteoblasts was assumed to be functionally important for bone homeostasis especially proliferation of osteoblasts. Stimulation of osteoblasts is mostly followed by increased bone mass. Humans bone mass is usually determined by measurement of bone mineral density (BMD) [7] that had initially not been determined in M3R-KO. Shi et al. [2] determined the bone loss in M3R-KO by means of 2D and 3D histomorphometry. In our previous study on male M3R-KO we expanded the analyses of Shi et al. [2] by mRNA expression and biomechanical testing [1].

Since these investigations were restricted to males, we conducted the current study to determine the BMD, biomechanical strength, cortical thickness, mRNA expression, and transmission electron microscopic evaluation on 16-week old female mice with systemic gene-deficiency of M3R concerning all cell types including osteoblasts, osteocytes, and osteoclasts.

2. Material and methods

2.1. Animals

Homozygous female M3R-KO (n = 21) [8] and the corresponding C57BL/6N wild type mice (n = 20) were kept under standard laboratory conditions. All animal procedures were performed according to the German animal protection act and were approved by the regional council of Giessen. At the age of 16 weeks mice were euthanized by CO_2-inhalation, dual-energy X-ray absorptiometry (DXA) scans were performed and the bone samples were harvested.

2.2. Dual-energy X-ray absorptiometry (DXA)

Immediately after sacrificing of the mice bone mineral density (BMD) as well as bone mineral content (BMC) of the whole skeleton, vertebral column, and femur was measured by DXA (Lunar Prodigy, GE Healthcare, Germany) using the encore software for small-animals (GE Healthcare, version 13.40).

2.3. 3D μCT

Specimens (vertebra Th12 and right femur) as well as calcium hydroxyapatite containing phantoms (0.25 and 0.75 g CaHA/cm^3, Bruker μCT (former SkyScan), Kontich, Belgium) were batched in a plastic tube and vertically fixed on the rotation stage of the micro-CT (1173, Bruker μCT (former SkyScan), Kontich, Belgium). Scan parameters were set as described earlier [1,9]. Reconstruction of cross-sectional images was followed by post processing for quantitative analysis. Briefly, three regions of interest (ROI) were defined: i) spongiosa of the vertebral body, ii) spongiosa of the distal femoral metaphysis and iii) mid-diaphyseal corticalis of the femur. An offset of 0.5 mm between metaphyseal growth plate and volume of interest (total of 600 slices) was chosen for the assessment of spongiosa. For the assessment of vertebral spongiosa, an offset 0.25 mm distant to the terminal plates was chosen. Manual exclusion of the cortical shell was performed in Th12 and femoral metaphysis. Cortical parameters were obtained from a stack of 200 slices with an offset of 8 mm proximal to the intercondylar notch. To avoid artificial inclusion of artefacts or exclusion of spongiosal structures, thresholding was carried out using an adaptive technique. BMD was assessed using the calcium hydroxyapatite CaHA-Phantoms of each individual scan for exact calibration. Tissue mineral density (TMD) was assessed using the threshold-demarcated structures as region of interest.

2.4. Biomechanical analysis of femoral bones

A three point bending test was used to assess femoral bone strength as described previously [1,9]. In brief, half distance of femoral length was marked. The proximal end of the left femur was fixed in an aluminum cylinder while the distal condyles rested on a metal support of the test machine (Zwick Z010, Zwick, Ulm, Germany). The bending load was applied at the previously marked position until failure. The load-deformation curve was continuously recorded (testXpert II Version 3.2, Zwick, Ulm, Germany) and ultimate bending load F_U and bending stiffness (EI) were determined. All measurements were carried out at room temperature (23 °C) and samples were kept in saline solution to prevent dehydration.

2.5. Histology, enzyme histochemistry

Specimens of vertebrae L3 were fixed in 4% phosphate-buffered paraformaldehyde (Merck, Darmstadt, Germany), decalcified with a solution of 10% ethylenediaminetetraacetic acid for approximately 14 days, dehydrated with an increasing series of ethanol and embedded in paraffin. Sections were cut at a thickness of 3–5 μm and routinely stained with hematoxylin–eosin (HE) or used for enzyme histochemical incubations. Therefore, sections were deparaffinized with xylene 2 × 5 min and rehydrated with a descending series of ethanol. For detection of osteoblasts, enzyme histochemistry for alkaline phosphatase (ALP) was conducted. Sections were first washed in TRIS-buffer, then incubated in the substrate solution 5-bromo-4-chloro-3-indolyphosphate/nitro blue tetrazolium chloride (BCIP/NBT; KLP, Gaithersburg, MD, USA) for 1 hour (h) at 37 °C and afterwards in nuclear fast red-aluminum sulfate (Roth, Karlsruhe, Germany) for 10 min at 37 °C to counterstain the nuclei. Osteoclasts and macrophages were determined by tartrate-resistant acidic phosphatase (TRAP) histochemistry for which sections were washed in sodium acetate buffer (pH 5.2), incubated in a solution of Naphtol AS-TR phosphate (Sigma-Aldrich, Taufkirchen, Germany), NN-dimethylformamide, (Sigma-Aldrich), sodium tartrate (Merck, Darmstadt, Germany) and fast red TR-salt (Sigma-Aldrich) for 50 min at 37 °C. Sections were counterstained with hematoxylin (Shandon Scientific Ltd, Cheshire, UK) and coverslipped with Kaisers Glyceringelatine (Merck).

2.6. Histomorphometry

Osteoclasts (TRAP positive multinuclear cells located on bone surface) and osteoblasts (ALP positive cells on bone surface) were counted in the trabecular bone of vertebrae L3 with help of quantitative histomorphometrical evaluations (for ALP n = 10 M3R-KO, n = 9 M3R-WT; for TRAP n = 11 M3R-KO, n = 13 M3R-WT, always one bone (vertebrae L3), and one section per bone). The ALP and TRAP stained sections were imaged with the Axioplan-2 light microscope with photo module (Zeiss, Jena, Germany) and a digital camera (Leica DC 500, Bensheim, Germany). Trabecular surface, relative number of TRAP positive osteoclasts, and ALP positive trabecular surface were calculated with the software programs GIMP 2.8.2 and Inkscape 0.48. Therefore a ROI was determined that encompassed the trabecular

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