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Bone status of acetylcholinesterase-knockout mice

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ABSTRACT

Acetylcholinesterase (AChE) hydrolyzes acetylcholine (ACh) to acetate and choline and thereby terminates nerve impulse transmission. ACh is also expressed in bone tissue and enhances here proliferation and differentiation of osteoblasts, which makes it interesting to investigate effects of AChE deficiency on bone. To our knowledge, this is the first study that analyzed bone of heterozygous acetylcholinesterase-knockout (AChE-KO) mice.
Tibia, femur, thoracic and lumbar vertebrae of 16-week-old female heterozygous AChE-KO mice and their corresponding wildtypes (WT) were analyzed using real-time RT-PCR, dual-energy X-ray absorptiometry, biomechanics, micro-computed tomography, histology and histomorphometry.
Our data revealed that heterozygous AChE-KO did not cause negative effects upon bone parameters analyzed. In contrast, the number of osteoclasts per perimeter was significantly reduced in lumbar vertebrae. In addition, we found a significant decrease in trabecular perimeter of lumbar vertebrae and cortical area fraction (Ct.Ar/Tt.Ar) in the mid-diaphysis of femurs of AChE-KO mice compared to their WT. Therefore, presumably a local homozygous knockout of AChE or AChE-inhibitor administration might be beneficial for bone formation due to ACh accumulation. However, many other bone parameters analyzed did not differ statistically significantly between AChE-KO and WT mice. That might be reasoned by the compensating effect of butyrylcholinesterase (BChE).

1. Introduction

Acetylcholinesterase (AChE) is commonly known for terminating the transmission of nerve impulses by hydrolyzing acetylcholine to acetate and choline [1]. However, there is evidence that AChE has many additional functions [2] since it was also detected in several non-neuronal tissues [3]. More important for our studies, AChE is also active in bone cells, like osteoblasts, mesenchymal cells and it was found in hematopoietic cells [1].

AChE nullizygous (−/−) mice are able to live up to 21 days after birth [3]. Although nursing of $AChE^{-/-}$ mice was observed, they weighed less in comparison to WT littermates and appeared dehydrated and emaciated shortly before death [3]. This might be reasoned by the tremor of $AChE^{-/-}$ mice, which demands higher intake of calories [3]. Surprisingly, several internal organs examined did not show abnormalities [3]. However, AChE deficiency does not hinder embryonic organogenesis, but damps development after birth.

The substrate of AChE, ACh and its receptors are expressed in bone too [4,5]. ACh is known for enhancing proliferation and differentiation of many cell types, including osteoblasts [4,5] and thus contributes to bone formation. Inhibition of ACh release by botulinum neurotoxin decreased bone mineral content and impaired bone healing [6]. ACh was also shown to up-regulate apoptosis of osteoclasts by binding to nicotinic acetylcholine receptors (nAChR) and therefore decreases bone resorption [7]. Especially signaling via the α_2nAChR seems to inhibit osteoclastic activity since knockout of this receptor caused increased bone resorption in mice [7].

In osteoporosis, muscarinic acetylcholine receptor expression decreased due to loss of osteoblasts [8]. Bone of muscarinic acetylcholine receptor 3 (M3 mAChR) knockout mice resembled an osteoporotic bone phenotype showing a significant decrease in bending stiffness, trabecular number, trabecular thickness, bone surface density and relative bone volume. Additionally, a significant increase in trabecular separation and structure model index was detected [9]. That implicates that M3 mAChR and ACh are important for physiological bone remodeling.

Therefore, we investigated for the first time, consequences of AChE-KO for bone remodeling and osteoporosis in heterozygous AChE-KO mice, generated as previously described [10]. Due to the knockout, AChE activity was reduced by half in AChE heterozygous (+/−) mice compared to WT mice [3]. We used 16-week-old female AChE heterozygous mice, which in contrast to nullizygous (−/−) AChE mice were

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reproductive and had no decreased life expectancy [3]. This age was important since murine bone starts to mature at the age of 12 weeks but becomes osteopenic after 42 weeks [11].

2. Materials and methods

2.1. Animals

Sixteen-week-old female heterozygous AChE-KO mice (JAX Mice Database — 005987 129-Achetm1Loc/J; n = 8) and their corresponding WT (129S6/SvEvTac; n = 8) were deposited in the Jackson Laboratory in Bar Harbor, Maine, USA and kindly provided by Prof. Oksana Lockridge. All animal experiments were approved by the local animal welfare representative as well as the regional commission (455-M, 88/2009) and performed in accordance with the National Institutes of Health (NIH) principles of laboratory animal care and the current version of the German law on protection of animals. Heterozygous AChE-KO mice were generated by knocking out one allele of the AChE gene [10].

Mice were bred and granted free access to chow and water under a 12-hour light–dark cycle in the local breeding facility of the University of Giessen. For the determination of heterozygous AChE-KO and WT mice, genotyping was performed using qualitative PCR with gene specific primers for AChE-KO (forward: AACATTGGCCGCCTCCAG CTC, reverse: TGGAAGGTGCAACTCCAACTG, 319 bp, Accession No. NC_000071.6) and WT (forward: AATGACACCGAGCTGATAGCC, reverse: CCAGTATTGATGAGAGCCTCC, 164 bp, Accession No. NC_ 000071.6) before sacrificing mice.

At the age of 16 weeks mice were euthanized. Tibia, femur, thoracic vertebra (Th10) and lumbar vertebrae (L1–4) were manually extracted and surrounding tissue completely removed. Samples for real-time RT-PCR were stored in RNA later at −80 °C. Bone mineral density (BMD) of femur and spine was analyzed immediately after euthanasia by dual-energy X-ray absorptiometry (DEXA). Samples for biomechanical analysis were stored at −20 °C in NaCl-soaked gauze bandages. For micro-CT and histological analyses samples were stored in 4% paraformaldehyde (Roth, Karlsruhe, Germany) or in 0.1 M phosphate buffered (pH = 7.3) yellow-fix solution consisting of 2% paraformaldehyde (Roth), 2% glutaraldehyde (Roth) and 0.02% picrinic acid (Fluka AG, Buchs SG, St. Gallen, Switzerland) at 4 °C.

2.2. Real-time RT-PCR

RNA was isolated by homogenizing Th10 in QIAzol Lysis Reagent (Qiagen, Hilden, Germany) using a Mixer Mill MM400 (Retsch, Haan, Germany) and the RNeasy Lipid Tissue Mini Kit (Qiagen) following the manufacturer's protocol. Subsequently, 750 ng of RNA were reverse transcribed into cDNA using a cycler (TC-3000, Techne, Bibby Scientific, USA) and the Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Real-time RT-PCR was performed using the Quantifast SYBR Green PCR Kit (Qiagen) and a LightCycler 2.0 (Roche, Basel, Switzerland). Primers for the detection of alkaline phosphatase (Alp) [forward: TCAGCTAATGCACAATATCAAGG reverse: TCCACATCAGTTCTGTTCTTCG, 87 bp, Accession No. NM_007431.2], cathepsin K (Ctsk) [forward: GAGGCGGCTATATGACCACT, reverse: CTTTGCCGTGGCGTTATACA, 119 bp, Accession No. NM_007802.3] and connexin 43 (Cx43) [forward: TGCTTCCTCTCACGTCCCAC, reverse: CGCGATCCTTAACGCCCTTG, 127 bp, Accession No. NM_010288.3] were used. To verify the purity of the RT-PCR product, a melting curve was conducted. Samples, which were not reverse transcribed or received water instead of cDNA served as negative controls. Evaluation of results was performed by determination of delta threshold cycle (CT) values using the reference gene beta-actin (forward: TGTTACCAACTGGGAC GACA, reverse: GGGGTGTTGAAGGTCTCAAA, 165 bp, Accession No. NM_007393.3).

2.3. Dual-energy X-ray absorptiometry (DEXA)

Mice were euthanized and immediately placed ventrally on the lying surface of the DEXA machine (Lunar Prodigy, GE Healthcare, Germany). The whole body was scanned to determine the bone mineral density (BMD) in g/cm^2. Regions of interest (ROI) were left femur and spine. Analyses were conducted with the enCORE software (GE Healthcare, v. 13.40) using the small animal mode. The instrument was calibrated using a phantom.

2.4. Biomechanics

Bone bending stiffness of femurs was analyzed using a three-point bending test. Femurs were positioned horizontally on a testing device (Z 10, Zwick, Ulm, Germany) with condyles resting on a metal plate and proximal ends fixed in a retainer. The distance between the metal plate and the retainer was 20 mm. Specimens were preconditioned by two load cycles with a maximum bending force of 4 N, which was applied vertically on the diaphysis of femurs. Finally, bones were loaded until fracturing occurred. Bending stiffness was calculated as described previously [9].

2.5. Micro-computed tomography (micro-CT)

Right femur, L1 and a calibrating phantom were fixed together in a 4% paraformaldehyde filled straw and scanned with a SkyScan 1173 micro-CT (Bruker microCT, Kontich, Belgium). Scanning was performed by rotating samples about 180° around the vertical axis in 0.3° rotation steps. The X-ray source voltage was set to 40 kV and source current to 200 μA, resulting in 8 W of power and 5 μm spot size. Images were captured with a flat panel sensor (2240 × 2240 pixels, 12-bit), an exposure time of 1700 ms and a resolution of 6.05 μm isotropic voxel side length. Cross sectional images with a $2^8 = 256$ (8 bit) gray scale range were reconstructed by application of a Feldkamp cone-beam algorithm. ROI were the trabecular and cortical area of bone. ROI were defined according to the guidelines published by Bouxsein et al. [12]. Trabecular ROI of femur was the distal metaphysis which comprised a total of 290 axial slices. Taking the metaphyseal growth plate as reference level, the ROI started with a distance of 0.22 mm and ended at 1.97 mm above the reference level. Regarding L1, the whole vertebra was considered comprising of 311 axial slices. ROI was drawn manually with a short distance to the endocortical surface as described in the guidelines mentioned before [12].

ROI of cortical bone were selected in the distal- and mid-diaphyseal region of femur. Distal diaphyseal and mid-diaphyseal ROI were drawn 2.2 mm or 5.5 mm respectively, above the metaphyseal growth plate, which served as reference level. Both cortical ROI comprised of an axial length of 0.45 mm correlative with 74 slices.

Analyzed parameters were chosen according to the manual “Structural parameters measured by the Skyscan™ CT-analyzer software” and guidelines published in JBMR [12]. Trabecular bone parameters considered were bone volume fraction (BV/TV), bone specific surface (BS/BV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N) and trabecular bone pattern factor (Tb.Pf). Analyzed cortical bone parameters were total cross-sectional area inside the periosteal envelope (Tt.Ar), cortical bone area (Ct.Ar), cortical area fraction (Ct.Ar/Tt.Ar) and average cortical thickness (Ct.Th).

2.6. Histology and histomorphometry

2.6.1. Alkaline phosphatase (ALP) histochemistry

After decalcification, 5 μm thin paraffin sections of L3 were deparaffinized in xylene and rehydrated. Subsequently, sections were kept in distilled water and rinsed in 0.1 M Tris buffer (pH 9.4) for 10 min. ALP detection was achieved after incubation of sections in substrate solution [5-bromo-4-chloro-3-indolyphosphate/nitroblue