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Bone status of adult female butyrylcholinesterase gene-deficient mice

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ABSTRACT

Butyrylcholinesterase (BChE) degrades acetylcholine in addition to acetylcholinesterase (AChE) which is involved in embryonic development of limbs. Since BChE is expressed by osteoblast-like cells we asked whether it is functional in adult bone remodeling. We addressed this issue by analyzing BChE gene-deficient mice (BChE-KO).

Bones were extracted from 16-week old female BChE-KO and corresponding wild type mice (WT). Femoral bones were used for biomechanical testing and μCT evaluation of cancellous and cortical bone. Also vertebrae Th12 and L1 were investigated with μCT while L3 was used for tartrate-resistant acidic phosphatase (TRAP) histomorphometry and Th10 for gene expression analysis by means of real-time RT-PCR.

BChE-KO did not reveal significant differences in biomechanical bone strength and bone mineral density determined by μCT. Microarchitecture of cancellous and cortical bone showed an increase in μCT parameters like trabecular thickness, trabecular separation, and relative cortical bone area of femoral BChE-KO bone compared to WT. In vertebrae no changes of microstructure and mRNA expression were detected. However, osteoclast histomorphometry with TRAP stained sections demonstrated a significant increase in relative osteoclast number. In conclusion, in adult murine bone the role of BChE is limited to bone specific changes in microarchitecture and to an increase in relative number of bone resorbing osteoclasts whereas the main collagen resorbing enzyme Cathepsin-K (CtsK) was stably expressed. Besides, AChE might be able to compensate the lack of BChE. Thus, further analyses using bone tissue specific AChE BChE cre–lox double knockout mice would be helpful.

1. Introduction

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are known to be involved in skeletal development [1,2]. AChE is a highly specific enzyme which degrades acetylcholine (ACh) very fast while BChE often named pseudocholinesterase is less restricted to ACh and much slower [3]. Besides enzymatic effects, cholinesterases have "non-classical" functions in the non-neuronal cholinergic system as non-synaptic signaling molecules [1]. During limb development embryonic cholinesterases were localized at the limb tip and in cells that were accumulated in the central limb core and formed the chondrogenic blastema [4,5]. Alber et al. demonstrated a spatiotemporal distribution pattern of AChE and BChE, where high amounts of BChE were expressed in regions with high proliferation rates whereas AChE was increased in differentiating non-proliferative cells [2]. It was, for example, reported that embryonic limb chondroblasts expressed cholinesterases whereas fully differentiated chondrocytes stopped generation of esterases [5,6]. Interestingly, in hematopoietic stem cells inhibition of BChE by application of antisense oligonucleotides (AS) suppressed megakaryocyte formation, and interfered with platelet production while enhancing myeloidogenesis [7]. During the developmental stage of fiber growth AChE was linked to migrating cells that where moving and growing towards higher concentrations of BChE [2]. In early chick limb development AChE activity was increased threefold [8]. BChE also increased during development. However, a smaller amount of BChE was found and it increased more rapidly compared to AChE [8]. Interestingly, Layer et al. demonstrated increased body size of AChE knockout mice (KO) at embryonic stage E13.5 compared to wild type mice (WT) [1]. In an in-vitro system that mimics endochondral ossification Layer et al. measured an increase in AChE activity during cartilage condensation and mineralization after inhibition of BChE activity [1]. Surprisingly, Layer et al. reported that around birth no differences in the skeletal morphology existed [1]. In male mice (strain C57BL/6 J) bone development

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is completed at the age of 12 weeks after birth. Then bone mass, mineralization, and strength remain constant up to week 42 because of continuous bone remodeling. After 42 weeks the bone of mice becomes osteopenic [9]. The main players in bone remodeling are the bone forming osteoblasts and the bone resorbing osteoclasts [10]. Regulation of this process is complex and still not fully understood since cross-talk between osteoblasts and osteoclasts, mechanosensitivity of osteocytes, several hormones (e.g. estrogen), blood calcium and phosphate content as well as the nervous system is involved [10]. In addition, cholinergic mechanisms contribute to bone regulation [11,12]. Osteoblasts, osteoblast-like cells and mesenchymal stem cells that are able to differentiate into osteoblast have been shown to express AChE [13–15]. In addition, Sato et al. suggested that the expression of AChE is up-regulated during osteoblastic differentiation [14]. This regulation seemed to be correlated with the alkaline phosphatase (ALP) activity of osteoblasts [11]. Typically, ALP activity rises during maturation of osteoblasts. Furthermore, it was described that AChE colocalizes with Golgi apparatus in osteoblasts, and is involved in osteoblastic cell adhesion and is therefore supposed to be involved in regulation of cell-matrix adhesion in bone [11]. Moreover AChE was found in fetal chondrocytes and is discussed to be responsible for the balance of proliferation and differentiation in human osteogenesis [12]. However, our recent study by Kauschke et al. ([16], this volume) showed that the bone of 16-week old female AChE heterozygote KO presented only small changes compared to the corresponding WT. In our previous results we only detected BChE mRNA but not AChE expression in osteoblast-like osteosarcoma cells SAOS-2 [13]. Furthermore Alber et al. described that during late stages of chick limb and wing maturation AChE activity was not found in bone whereas BChE was located in bone-forming centers of several bones [2]. Thus we asked in the present study whether depletion of BChE in bone of adult mice leads to substantive alterations in the bone. In contrast to AChE the knowledge about BChE in adulthood is restricted. Up to now, the expression of BChE in osteoblast-like and mesenchymal stroma cells could be demonstrated [13,15]. Therefore, we used BChE-KO and the corresponding BChE-WT generated in the institute of Oksana Lockridge who kindly endowed us mice breeding pairs. Mice were bred and grown to 16-weeks of age, bones were extracted, and their strength, microarchitecture, expression of bone markers and relative number of osteoclast were determined by radiological, cell-, and molecular biological methods.

2. Material and methods

2.1. Animals

Animal procedures were performed in full compliance with the institutional and German protection laws and approved by the local animal welfare committee. Female 16-week old BChE-KO ($n = 10$) [17] and the corresponding WT ($n = 10$) were euthanized by CO_2-inhalation, bone samples were harvested, stored in 4% phosphate buffered paraformaldehyde (right femur, thoracic (Th) vertebrae Th12, lumbar vertebrae L1, and L3), liquid nitrogen (Th10), or frozen at −20 °C enwrapped in a 0.9% NaCl impregnated compress (left femur).

2.2. Biomechanical test

The bone strength of the left femora was assessed by a three point bending test. Therefore, the proximal end of the femora was secured with dental cement (iCem, Heraeus Kulzer, Hanau, Germany) in an aluminum cylinder in the testing machine (Zwick Z010, Zwick, Ulm, Germany). The distal end of the femora rested on a metal support. The bending load was applied to the center of the femoral diaphysis until destruction. The load-deformation curve was continuously recorded (testXpert II Version 3.2, Zwick) and the maximum bending force (Fmax) and the bending stiffness assessed from the linear region of the load-deformation curve, were determined as described previously [18].

2.3. 3D microtomography (μCT)

Vertebrae Th12, L1, and right femur as well as the calcium hydroxylapatite phantoms with 0.25 and 0.75 g CaHA/cm^3 (Bruker μCT, Kontich, Belgium) were scanned with the micro-CT (1173, Bruker μCT) using the following parameters: 40 kV tube voltage, 200 μA tube current, 1.6 s exposure time/frame, 4 averaging frames, 5.66 μm isotropic voxel resolution side length, 0.28° rotation steps, and 115 min scan duration. Reconstruction of the images and quantitative analysis was performed by using the CT-analyzer software (Bruker μCT). Afterwards bone mineral density (BMD) was calculated using the images of CaHA-phantoms. The following cortical parameters were assessed for proximal and distal femur: a) cortical thickness (Ct.Th), b) relative cortical bone area (Ct.Ar/Tt.Ar), and c) total cortical porosity (Ct.Po). Spongious bone was determined using the following parameters: bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), structure model index (SMI), and bone surface density (BS/TV).

2.4. Histology, enzyme histochemistry, and histomorphometry

After fixation vertebrae L3 were decalcified with 10% ethylenediaminetetraacetic acid for approximately 14 days, dehydrated and embedded in paraffin. Sections of 3–5 μm were cut with a rotating microtome (Leica RM2155, Wetzlar, Germany), deparaffinized with xylene and processed for standard hematoxylin–eosin staining and enzyme histochemical staining of tartrate-resistant acidic phosphatase (TRAP) to identify osteoclasts and macrophages as described earlier [19]. In brief, after washing in sodium acetate buffer (pH 5.2) sections were incubated in Naphtol AS-TR phosphate (Sigma-Aldrich, Taufkirchen, Germany), NN-dimethylformamid (Sigma-Aldrich), sodium tartrate (Merck, Darmstadt, Germany), and fast red TR-salt (Sigma-Aldrich). Accordingly, sections were processed to a nuclei staining with hematoxylin (Shandon Scientific Ltd, Cheshire, UK) and cover slipped. Stained sections were digitalized with the Axioplan-2 Microscope (Zeiss, Jena, Germany) and the DC500 camera (Leica, Wetzlar, Germany). Osteoclasts were identified by red staining and content of at least three nuclei and osteoclast number/bone surface (N.Oc/BS) and osteoclast number/bone volume (N.Oc/BV) was calculated.

2.5. Real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR)

RNA was isolated from Th10 by QIAzol Lysis Reagent (Qiagen, Hilden, Germany), reverse transcribed with Quantitect Reverse Transcription Kit (Qiagen) including decontamination of genomic DNA, and processed for real-time RT-PCR with the Quantifast SYBR Green PCR Kit (Qiagen) and 0.2 μl of a 20 μM primer solution in a LightCycler 2.0 (Roche, Basel, Switzerland) according to the manucfacturer's protocols. PCR included 40 cycles with annealing and elongation for 30 s at 60 °C. Purity of PCR product was verified by a melting curve, and control runs without reverse transcriptase. Primers (MWG Eurofins, Ebersberg, Germany) were established by a dilution series and standard curve. The following primers were used: a) Alkaline phosphatase (ALP) 5′-TCAGCTAATGCACAATATCAAGG-3′, 3′-TCCACATCAGTTCTGTTCTTCG-5′, length of 87 base pairs (bp), accession number: NM_007431.2, b) cathepsin-K (CtsK) 5′-GAGGCGGCTATATGACCACT-3′, 3′-CTTTGCCGTGGCGTTATACA-5′, 119 bp, NM_007802.3, c) connexin-43 5′-TGCTTCCTCTCACGTCCCAC-3′, 3′-CGCGATCCTTAACGCCCTTG-5′, 127 bp, NM_010288.3, and d) beta-actin 5′-TGTTACCAATGGGACGACA-3′, 3′-GGGGTGTTGAAGGTCTCAAA-5′, 165 bp, NM_007393.3. Expression was calculated using the ΔΔ Ct method with beta-actin as reference gene.