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Rapid alterations of avian medullary bone material during the daily egg-laying cycle

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ABSTRACT

Bone is a dynamic tissue which is continuously adapting not only to external mechanical stimuli but also to internal metabolic calcium demands. During normal bone remodeling, bone-resorbing osteoclasts release calcium from the bone and digest the collagenous bone matrix, after which bone-depositing osteoblasts form unmineralized collagen matrix, which subsequently mineralizes. The detailed mechanism by which calcium is deposited at the site of mineralization and removed from it during bone resorption is largely unknown. Experimental studies are difficult to conduct because in adult bone only a small fraction of bone tissue is remodeled at any moment in time. Thus, one promising approach is to study mineral deposition and resorption in model systems in which a large fraction of the bone mineral is mobilized in a relatively short period of time. We investigated the microscopic and nanoscopic alterations of avian medullary bone architecture during the egg-laying (oviposition) cycle of hens. Medullary bone forms a labile calcium reservoir for eggshell production and is characterized by an extremely rapid and high-flux calcium metabolism. It thus, provides the unique opportunity to study processes of bone remodeling in their most intensive form. We used a combination of synchrotron X-ray tomography together with small angle X-ray scattering (SAXS), wide angle X-ray diffraction (WAXD) and X-ray fluorescence (XRF) to correlate microscopic medullary bone attributes such as the mineral content, medullary bone volume fraction and medullary bone trabecular thickness with nanoscopic alterations in the mineral particle size (thickness parameter T and length parameter L) during the oviposition cycle. To identify the timing of the different stages of the cycle, ionic calcium, phosphorus and PTH concentrations in the blood of the layers were monitored.

We found that the microscopic and nanoscopic architecture of avian medullary bone material changes rapidly during the oviposition cycle. During eggshell calcification, the mineral content and the size of trabeculae of medullary bone decrease markedly. Furthermore, the average mineral particle size increases during resorption, suggesting that the smaller mineral particles are preferentially removed. Medullary bone thus formes a fast-responding system exhibiting rapid alterations of the material at the micron and nano scale. Those mechanisms are crucial to provide calcium for the high metabolic calcium demand during eggshell mineralization.

Introduction

Bone material appears to be designed to function under varying environmental conditions that go far beyond the need to serve protection and load bearing purposes. It also fulfills metabolic functions such as maintaining body's calcium reservoir [1]. During growth and maturation, bone constantly undergoes (re)modeling. The rates of bone formation and resorption together with the kinetics of bone mineralization determine the distribution of mineral in the bone tissue [2] and thus, are characteristic for bone tissue in both health and disease [3].

During the process of bone formation, osteoblasts deposit an organic matrix – consisting of collagen and a series of non-collagenous proteins and lipids – which subsequently mineralizes. The mineral which is in a disordered calcium phosphate phase is translocated to the mineralization front where it is later transformed into more ordered phases [4–7]. After nucleation, hydroxyapatite crystals grow longitudinally to form platelets with an average length and width of 50 × 25 nm [8,9] and subsequently

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in thickness of 1.5 nm to 4.5 nm — depending on species, age and tissue type [10,17]. During bone resorption, osteoclasts form a sealed highly acidic zone at the bone surface, dissolving bone mineral and decomposing the collagenous bone matrix. Bone resorption products are removed from the resorption site via transcytosis [11,12] to the basolateral region of the osteoclast where they are eventually released [13].

The most favorable circumstances to study the processes of bone formation and resorption during bone remodeling are when they occur under extreme conditions. Such a situation can be found during the daily egg-laying (oviposition) cycle in hens, in which calcium metabolism is extraordinarily intense. Commercial layers transfer about 10% of their total body calcium volume daily to the shell producing machinery in their oviducts, of this only about 50% originates from dietary sources. They are therefore the most efficient calcium transporters among all vertebrates [14]. Such an intensive transport mechanism imposes severe demands on ionic calcium homeostasis. During production of the eggshell which mainly consists of calcium carbonate, considerable amounts of the required calcium (20–40%) must be derived from skeletal reserves [15]. To accommodate this, the function of the osteoblasts changes from laying down cortical bone to producing a spongy bone termed medullary bone [22]. This bone type, unique to birds and dinosaurs [18], has no major mechanical function [19] but serves as a labile calcium source for eggshell formation. Medullary bone is laid down within the medullary cavity of the mid-diaphysis of the long bones, particularly those of the legs [19,22]. Thus, medullary bone is distinct from cancellous bone which is concentrated towards the metaphysis/epiphysis having important mechanical functions for the integrity of the whole bone structure [20,21].

During the 24 h oviposition cycle, medullary bone builds up rapidly throughout the inactive stage of egg shell mineralization at night [23,22] and during the early stages in the morning after oviposition when hens consume calcium-rich food. At the onset of mineralization of the new eggshell calcium from medullary bone reserves is released and transported to the oviducts, where it forms the calcium carbonate eggshell [15]. To date there are no studies describing the alteration of microscopic and nanoscopic mineral properties of medullary bone material in relation to the oviposition cycle. Thus, it is unclear how such high rates of calcium storage and mobilization can be facilitated and if medullary bone material shows certain adaptations in its architecture to meet these requirements.

In this work we attempt to answer these questions by investigating the dynamics of microscopic and nanoscopic structural changes in avian medullary bone material during the oviposition cycle, observed in commercial layers. We monitored serum concentrations of ionic calcium and phosphorus as well as parathyroid hormone (PTH) during the different stages of the oviposition cycle. We studied the 3D architecture and mineral content of representative samples of medullary bone by synchrotron X-ray tomography. Furthermore we used synchrotron small angle X-ray scattering (SAXS), wide angle X-ray diffraction (WAXD) and X-ray fluorescence (XRF) to characterize the ultrastructure of medullary bone material together with alterations in the mineral particle size (thickness parameter T and length parameter L) during the oviposition cycle.

Materials and methods

Samples

Egg laying hens were euthanized at the Hebrew University of Jerusalem, Israel. Ethics approval for all procedures to be carried out was obtained from the Committee for Ethics in Research of the Hebrew University of Jerusalem. At predetermined time points as detailed below, hens were euthanized by intravenous injection of an anesthetic overdose (80 mg/Kg, pentobarbital sodium (200 m/ml), CTS, Israel). Both femur bones were collected at 13 different points in time according to the oviposition cycle, starting from 0 h (directly after oviposition), and every 2 h thereafter up to 24 h after oviposition ($n = 2$ per time point). We thus obtained bones from a total of $n = 26$ animals with precisely timed bone states following oviposition. Blood samples were obtained from each hen before euthanasia, to determine serum calcium, phosphorus ion and PTH concentrations during the oviposition cycle. To improve statistics for blood analysis, blood samples from additional animals were obtained, but no bone samples were tested. Statistical significance was tested using ANOVA with subsequent post hoc tests. A statistical significant difference ($P < 0.05$) is denoted with an asterisk.

After harvesting, bone samples were stored dry in a freezer at −20 °C until further anhydrous processing, except for samples taken immediately for fresh IR analysis. Transverse slices ~1 cm thick were cut with a low speed diamond saw (IsoMet, Buehler GmbH, Düsseldorf, Germany) from the mid-diaphysis of each bone and were embedded in PMMA. Samples were dehydrated in 100% ethanol (3 times per day) for a total of 2 days and subsequently immersed in methyl methacrylate (MMA). Finally, the samples were embedded in covered plastic containers with PMMA solution containing 400 ml MMA, 100 ml nonylphenyl-polyethyleneglycol acetate (NPA) and 10 g dibenzoyl peroxide (BPO). During embedding the samples were placed in an oven at 42 °C for 12 h, 48 °C for an additional 12 h and finally at 58 °C for 24 h for hardening.

Electron microscopy

Thin transverse cross sections with a thickness of approximately 200 μm were cut from the embedded bone blocks and polished to a final thickness of approximately 50 μm. Samples were glued on an object holder with double-sided tape and mounted on aluminum stubs. A FEI-Quanta 600FEG electron microscope (FEI Company, Oregon, USA) was used in low vacuum mode (0.75 Torr) at a working distance of 10 mm. Images were taken with a solid state detector (SSD) at 15 kV acceleration voltage measuring the back-scattered-electron signal.

IR-spectroscopy

The freshly dissected pieces of medullary bone were washed with acetone to remove fatty tissue components. Samples were subsequently crushed in an agate mortar with sodium hypochlorite solution (6%) added for 5 min at room temperature. The suspension was then transferred into Eppendorf tubes and centrifuged at 14,000 rpm for 3 min in a micro centrifuge (Eppendorf 5417C, Hamburg, Germany) to remove the supernatant. The pellet was washed three times with double distilled water saturated with calcium and phosphate and twice with 100% ethanol. The pellet was resuspended in ethanol and sonicated (Ultrasonicprocessor W-380; Heat Systems Ultrasonics, Newtown, USA). The re-suspended medullary bone mineral particles were subsequently exposed to a heat lamp in order to remove the remaining ethanol. The residual bone mineral was lightly crushed in an agate mortar, mixed with potassium bromide (KBr) and a 7-mm pellet was prepared. The IR-spectra were measured with a Nicolet 380 FTIR spectrometer (Thermo Scientific, USA). The splitting factor of the phosphate υ_4 peak describing the crystallinity of the bone material was calculated following Weiner and Bar-Yosef [24].

Synchrotron X-ray scattering measurements

Thin transverse bone cross-sections with a thickness of approximately 50 μm were measured at the μSpot beamline at BESSY II (Helmholtz Zentrum Berlin für Materialien und Energie, Berlin, Germany) with a monochromatic X-ray beam of 15 keV and a beam size of $30 \times 30\ \mu m^2$. In order to obtain high q-resolution a silicon-111 double monochromator was used. A sample to detector distance of ~300 mm was used for diffraction measurements with a 2D position-sensitive CCD-detector (MarMosaic 225, Evanston, USA) with 3072×3072 pixels and a