Bone 90 (2016) 15–22

Contents lists available at ScienceDirect

Bone

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

Full Length Article

Regional differences in oxidative metabolism and mitochondrial activity among cortical bone osteocytes

Dorra Frikha-Benayed, Jelena Basta-Pljakic, Robert J. Majeska, Mitchell B. Schaffler *

Department of Biomedical Engineering, The City College of New York, United States

ARTICLE INFO

Article history:
Received 22 January 2016
Revised 26 May 2016
Accepted 27 May 2016
Available online 31 May 2016

Keywords:
Osteocytes
Oxidative metabolism
Mitochondrial function
In situ imaging
Multiphoton microscopy
Cortical bone

ABSTRACT

Metabolic oxidative stress has been implicated as a cause of osteocyte apoptosis, an essential step in triggering bone remodeling. However, little is known about the oxidative behavior of osteocytes in vivo. We assessed the redox status and distribution of total and active mitochondria in osteocytes of mouse metatarsal cortical bone in situ. Multiphoton microscopy (MPM) was used to measure fluorescence of reduced pyridine nucleotides (NADH) under normoxic conditions and acutely following extreme (postmortem) hypoxic stress. Under non-hypoxic conditions, osteocytes exhibited no detectable fluorescence, indicating rapid NADH re-oxidation. With hypoxia, NADH levels peaked and returned to near baseline levels over 3 h. Cells near the periosteal surface reached maximum NADH levels twice as rapidly as osteocytes near the mid-cortex, due to the time required to initiate NADH accumulation; once started, NADH accumulation followed a similar exponential relationship at all sites. Osteocytes near periosteal and endosteal bone surfaces also had higher mitochondrial content than those in mid-cortex based on immunohistochemical staining for mitochondrial ATPase-5A (Complex V ATPase). The content of active mitochondria, assessed in situ using the potentiometric dye TMRM, was also high in osteocytes near periosteum, but low in osteocytes near endocortical surfaces, similar to levels in mid-cortex. These results demonstrate that cortical osteocytes maintain normal oxidative status utilizing mainly aerobic (mitochondrial) pathways but respond to hypoxic stress differently depending on their location in the cortex, a difference linked to mitochondrial content. An apparently high proportion of poorly functional mitochondria in osteocytes near endocortical surfaces, where increased apoptosis mainly occurs in response to bone remodeling stimuli, further suggest that regional differences in oxidative function may in part determine osteocyte susceptibility to undergo apoptosis in response to stimuli that trigger bone remodeling.

1. Introduction

Stress on osteocytes leading to apoptotic cell death appear to play essential roles in triggering bone remodeling due to diverse stimuli that include microdamage, disuse, ovariectomy and aging [1–7]. Yet the specific nature of those stresses, and the ways in which osteocytes respond to them, are frequently unclear. In estrogen loss, aging and disuse, for example, osteocyte apoptosis occurs where there is no obvious local trauma such as bone microcracks, and even at bone microdamage sites, apoptosis is more widespread than can be accounted for by direct physical injury to osteocytes by microcracks.

Local metabolic stress has been posited to be a major contributor to osteocytes apoptosis in all of these instances. Verborgt et al. [8] suggested that microdamage in fatigued bone might disrupt canalicular integrity, altering fluid flow and reducing metabolite access to osteocyte, leading to apoptosis; further, Tami et al. [9] confirmed experimentally that microcracks impaired fluid and solute transport through the lacunar-canalicular system (LCS). They also observed that this poorly perfused region around microcracks coincided with the area where osteocyte apoptosis occurs and where many osteocytes showed acutely elevated levels of Hypoxia Inducible Factor-1α (HIF-1α). Thus, impaired metabolite transport and consequent oxidative and metabolic stress appear to be major triggers of osteocyte apoptosis in microdamage regions. In disuse, lack of mechanical loading broadly impairs fluid and solute transport in the LCS [10], causing potentially similar oxidative and metabolic stresses on osteocytes [11].

Estrogen loss also causes osteocyte apoptosis [4–6], but does not alter the LCS and fluid transport in cortical bone [12]. However, Emerton et al. [4] in our laboratory demonstrated that the osteocytes that underwent apoptosis after estrogen loss were the oldest osteocytes in cortical bone, that they resided in regions of tissue with poor baseline perfusion and that they exhibited elevated levels of pre-existing oxidative damage. As a result, the authors speculated that estrogen was protective against oxidative stress and apoptosis in osteocytes. Indeed, estrogen administration has been shown to exert anti-oxidant effects and to protect cells after acute brain and heart injury [13,14]. Recent data also indicate that estrogen directly affects mitochondrial function,

* Corresponding author at: Department of Biomedical Engineering, The City College of New York, 160 Convent Avenue, New York, NY 10031, United States.

http://dx.doi.org/10.1016/j.bone.2016.05.011
8756-3282/

principally by regulating the biogenesis of electron transfer complex components [15].

Finally, oxidative stress has been proposed as a pivotal mechanism contributing to age-related bone loss [7,16] as it has been implicated in the tissue degeneration associated with aging [17]. Thus the potential for impaired oxidative metabolism and mitochondrial stress appears to be a common denominator in all the physiological challenges that cause osteocyte apoptosis.

The oxidative metabolism of osteocytes in bone is poorly understood. Electron microscopy studies by Belanger [18] and others [19,20] established that osteocytes have numerous mitochondria, but their mitochondrial content is reduced as they age. Recently, Guo et al. [21] demonstrated by immunohistochemistry that osteocytes located deep in cortical bone express high levels of glycolytic enzymes, along with the ORP150 protein which suppresses hypoxia-induced apoptotic cell death, while osteocytes near bone surfaces did not. These data suggest that the metabolic profiles of osteocytes are complex and may be functionally specialized by location within bone. However, the oxidative metabolism of osteocytes in bone remains poorly studied.

Direct assessment of cellular oxidative metabolism was pioneered in the 1950s by Chance and others, through monitoring the intrinsic fluorescence of the reduced form of the pyridine nucleotide NADH; the oxidized form (NAD+) is not fluorescent [22,23]. Subsequent advances in fluorescence microscopy have greatly improved the ability to monitor NADH levels in real time, both in cell culture and in vivo and ex-vivo at precise locations. Recently, multiphoton fluorescence microscopy (MPM) has seen wide use for monitoring oxidative metabolism via NADH fluorescence in brain [24,25], muscle [26] and other soft tissues [27,28]. MPM has proven particularly advantageous as the low energy photons in this approach allow prolonged observations without phototoxicity to living cells and tissues, while the longer wavelength light used for MPM excitation reduces tissue scattering and allow deeper tissue penetration into tissues. However, the efficacy of such approaches to study metabolism in osteocytes in situ in fully mineralized bone is not known.

The objective of the current study was to examine the metabolic state of osteocytes in bone in situ under normal conditions and in response to stress. To do so, we developed a novel approach to monitor real time osteocyte cellular NADH levels in situ in mouse long bone cortices.

2. Materials and methods

2.1. Visualization of mouse osteocytes in vivo by multiphoton microscopy (MPM)

All experiments were performed on adult female C57BI/6 mice (15–17 weeks old, Jackson Lab, Bar Harbor, ME) and were IACUC approved. In vivo observations were made on cortical bone osteocytes in the mid-diaphysis of the 2nd and 3rd metatarsal bones (MT-2 and MT-3). This anatomic site is advantageous for direct visualization of cortical osteocytes in living animals. The dorsal surfaces of MT-2 and MT-3 are effectively subcutaneous, allowing easy access for study with minimal surgery. Mice were anesthetized using isoflurane inhalation (0.5–2%). The skin and extensor aponeurosis on the dorsal MT surfaces were incised longitudinally with a scalpel to expose the bone; the periosteum was left intact. The surgical approach does not alter the major metatarsal blood supply, which comes from the arterial arcade located on the volar surface; the dorsalis pedis artery, which is located along the dorsal surface of MT-2, primarily supplies the digits rather than metatarsals and can be retracted from the surgical field. For observations, the mouse was placed on a warming blanket and the foot was positioned in a Petri dish and submerged in Hanks Balanced Salt Solution at 37 °C. The foot was stabilized with a custom-made holder to prevent movements caused by the animal's heartbeat and respiration. The entire apparatus was set onto the stage of a multiphoton imaging system (Ultima, Bruker Instruments, Inc., Middleton WI) for observation. The microscope light source was a Ti-sapphire laser tunable from 680 to 1080 nm and focused on the tissue by a 40× magnification water immersion objective (Olympus LUMPLFLN 40XW, NA = 0.8; working distance = 3.3 mm).

2.2. NADH fluorescence detection

Fluorescence excitation and emission wavelengths were established to permit the specific measurement of NADH independent of other fluorescent materials in cells and extracellular matrix. The cellular NADH fluorescence spectrum is clearly separate from other cellular fluorophores (e.g. flavins) [27]. However, the emission spectra of NADH, partially overlaps with that of collagen [29,30]. To resolve the fluorescence of osteocyte NADH from that of the collagen-rich matrix, we carried out an excitation spectrum study following the methods described by Hillman et al. [31]. In brief, we used MPM to scan a mouse metatarsal diaphysis containing both cells and matrix. Scans were carried out 30 min after animal death – a period sufficient to induce NADH accumulation in cells, based on pilot studies. Excitation wavelengths were varied between 700 nm and 960 nm in 20 nm increments; emitted fluorescent signals were collected from each scan with a 460 ± 25 nm band-pass filter. The emission light intensities were measured using ImageJ. We found that dual photon excitation at 833 nm elicited NADH fluorescence in osteocytes but not the surrounding matrix (Fig. 1a). In contrast, excitation at 920 nm resulted in matrix fluorescence but not in cells or lacunar spaces, which remained dark (Fig. 1b). These wavelengths were used for subsequent experiments to image NADH and bone matrix respectively.

2.3. Assessment of oxidative state: NADH measurement in osteocytes in situ

The responsiveness of osteocyte oxidative metabolism was calibrated by measuring NADH fluorescence following hypoxia induction [23]. Mice (n = 4) were anesthetized using isoflurane and prepared for imaging as described above. Baseline in vivo osteocyte images were acquired over 2–4 h under anesthesia, after which mice were euthanized by anesthesia overdose to induce hypoxia. This approach is a well-validated, widely used model to induce and study complete hypoxia in tissues and organs [32]. During the development of hypoxia from the postmortem tissue ischemia, osteocyte images were acquired over observation periods of 3 h.

NADH fluorescence images were acquired beginning at the periosteal surface and sequentially captured to depths (z-axis) of 90 μm below the periosteal surface, which is approximately half the cortical width for adult mouse metatarsals. Fluorescence signals became degraded beyond that depth. For each study, a time (t-) series of a z series images (1 μm steps) were captured every 5 min. At the end of each NADH imaging session for a bone, the complete z-stack was imaged again at 920 nm (the collagen excitation wavelength) to visualize the bone matrix. With the 40× magnification objective used and the 90 μm depth limit for the z-axis, we typically visualized 20–30 osteocytes per bone in a single experiment. NADH fluorescence signal intensity was measured in arbitrary intensity units as follows: Integrated fluorescence intensity was determined from 3D reconstructions of individual osteocytes at each time point using ImageJ software (http://rsb.info.nih.gov/ij/). Changes in integrated NADH fluorescence intensity were normalized to peak fluorescence in each osteocyte in order to facilitate inter-animal comparisons.

2.4. Measurement of osteocyte active mitochondrial content using a potentiometric dye

Imaging studies were also performed using the membrane potential sensitive dye Tetramethylrhodamine methyl ester (TMRM, Invitrogen, T-668) to assess the content of active mitochondria in live osteocytes.