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Ovariectomy enhances mechanical load-induced solute transport around osteocytes in rat cancellous bone

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ABSTRACT

To test if osteoporosis alters mechanical load-induced interstitial fluid flow in bone, this study examined the combined effect of estrogen deficiency and external loading on solute transport around osteocytes. An in vivo tracer, FITC-labeled bovine serum albumin, was injected into anesthetized ovariectomized and control female Sprague–Dawley rats before the right tibia was subjected to a controlled, physiological, non-invasive sinusoidal load to mimic walking. Tracer movement through the lacunar–canalicular system surrounding osteocytes was quantified in cortical and cancellous bone from the proximal tibia using confocal microscopy, with the non-loaded tibia serving as internal control. Overall, the application of mechanical loading increased the percentage of osteocyte lacunae labeled with injected tracer, and ovariectomy further enhanced movement of tracer. An analysis of separate regions demonstrated that ovariectomy enhanced in vivo transport of the injected tracer in the cancellous bone of the tibial epiphysis and metaphysis but not in the cortical bone of the metaphysis. These findings show that bone changes due to reduced estrogen levels alter convectional transport around osteocytes in cancellous bone and demonstrate a functional difference of interstitial fluid flow around osteocytes in estrogen-deficient rats undergoing the same physical activity as controls. The altered interstitial fluid flow around osteocytes is likely related to nanostructural matrix–mineral level differences recently demonstrated at the lacunar–canalicular surface of estrogen-deficient rats, which could affect the transmission of mechanical loads to the osteocyte.

Introduction

Bone is a porous, dynamic cellular structure that can adapt to accommodate changes in its functional environment. The lacunar–canalicular porosity is a complex web of lacunae and canaliculi in the mineralized matrix that houses the osteocytes and their processes, forming a syncytium that connects osteocytes and cells located on bone surfaces [1]. The anatomical location of osteocytes and the extended connections throughout bone tissue make the osteocyte the ideal candidate to perceive and respond to mechanical stimuli [2,3].

When bone is mechanically loaded, fluid pressure gradients are induced in the bone pores, creating a load-induced interstitial fluid displacement in the lacunar–canalicular porosity [4,5]. Load-induced interstitial fluid movement affects osteocytes by enhancing solute transport via a convection mechanism that ensures the adequate metabolic function of bone cells, which is crucial for bone maintenance and adaptation [6–9]. Load-induced interstitial fluid movement is also believed to play a role in bone's mechanosensory system via its role in the transduction of whole-bone forces to the osteocyte process cytoskeleton via transmembrane links from the osteocyte to the canalicular wall [10–13]. To demonstrate bone's interstitial fluid pathway, we and several other groups have performed in vivo vascular injection of different tracers, such as microperoxidase, ferritin, reactive red, and procion red [14–20]. The same experimental approach has been used to demonstrate enhanced mass transport through the lacunar–canalicular system due to applied mechanical loading [21–24].

While weight-bearing exercise has been shown to be important in bone maintenance [2], estrogen also protects the skeleton from bone loss by suppressing bone turnover and maintaining a balance between formation and resorption [25]. A reduction of estrogen levels has been shown to induce osteocyte death via apoptosis in mice, rats, sheep, and humans [26–29]. This decrease in osteocyte viability could alter the interconnectedness of the osteocyte network, affecting interstitial fluid flow and altering the mechanical stimulus experienced by the bone cells [30–34]. Recent results from our lab analyzing changes in the osteocyte microenvironment due to ovariectomy (OVX) in the rat demonstrate nanostructural matrix–mineral level differences like loose collagen surrounding osteocyte lacunae and canaliculi [35]. These changes appear to make OVX bone tissue more permeable to small molecules at the lacunar–canalicular surface, which could potentially alter interstitial fluid flow around osteocytes during mechanical loading. The goal of the present study was to investigate the functional

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significance of a more permeable osteocyte microenvironment induced in the estrogen-deficient state.

This study tested the hypothesis that estrogen deficiency alters bone interstitial fluid movement through the osteocyte lacunar–canalicular network. Solute transport around osteocytes was measured in ovariectomized rats and compared to control rats undergoing a similar level of mechanical loading. A controlled, non-invasive, dynamic load that mimicked walking was applied to the right tibia immediately after the injection of a fluorescent tracer. Tracer distribution in the interstitial fluid space surrounding osteocytes was analyzed in cortical and cancellous bone from the proximal tibia, a region that undergoes bone loss when ovarian function is impaired in both humans and animal models [36,37].

Materials and methods

Permission for the study was granted by the Institutional Animal Care and Use Committees at the Hospital for Special Surgery and the City College of New York. A non-invasive device was used to apply mechanical loads to the rat hindlimb (ElectroForce TestBench, Bose Corp., Minnetonka, MN) similar to a tibial loading device previously developed in the mouse [38].

Loading device calibration

To calibrate the loading device, strain gages were applied to the tibia of female Sprague–Dawley rats (n = 3; 26 weeks old; 305 ± 18 g; Harlan Laboratories, Indianapolis, IN). The animals were anesthetized with isoflurane, and the right medial proximal diaphysis of the tibia was surgically exposed. This anatomical location was chosen for its lack of muscle attachments and flat surface, which could accommodate application of a strain gage. The tibial surface was scraped with a scalpel to remove the periosteal layer, and then it was cleaned and dehydrated with 100% ethanol and acetone. A single-element foil gage (CEA-032UW-120, Vishay Micro-Measurements, Raleigh, NC) was aligned with the tibial long axis and attached using cyanoacrylate. The rat's right lower limb was then placed between the knee and foot holder of the loading device and a compressive pre-load of 1 N was applied to maintain the limb's position (Fig. 1a). To produce physiological strains in the tibia [39,40], axial sinusoidal loads were applied at 1 Hz using four peak magnitudes ranging from 9 to 18 N to determine the load–microstrain relationship. The applied load of 14 N at 1 Hz used in the subsequent loading protocol to simulate slow walking generated approximately 500 microstrain at the medial proximal diaphysis of the tibia (Fig. 1b).

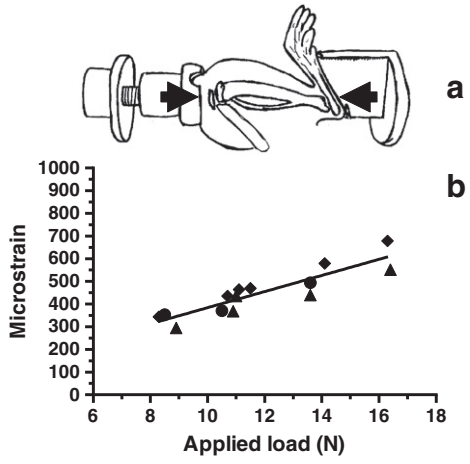

Fig. 1. (a) Sinusoidal loading was applied non-invasively to the rat lower hindlimb, which was constrained between a custom-made knee cup and foot holder. The lower limb was compressed in the direction indicated by the arrows. (b) Strain-gage calibration of the loading device using control rats (n = 3; each rat represented by a symbol, ●▲◆) illustrates the relationship between applied load (N) and measured deformation (microstrain) of the proximal tibia. After tracer injection, an applied load of 14 N, which engenders ~500 microstrain at the medial proximal diaphysis, was applied at 1 Hz to mimic slow walking.

Animal model and experimental design

To assess the effects of loss of ovarian function on mass transport due to mechanical loading, the rat ovariectomy model was utilized to induce osteopenia. Twenty-week-old rats were used to avoid the rapid growth stage associated with significant bone turnover in the proximal tibia [41]. Female Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) were divided into two groups at 20 weeks of age, with one group undergoing ovariectomy (OVX, n = 6), and the other group subjected to sham surgery (SHAM, n = 6), consisting of the surgical exposure of the ovaries without removal. After a one-week recovery period, the OVX group was pair-fed to the average food intake of the SHAM group for five weeks prior to load application to reduce weight gain caused by OVX [42]. Solute transport around osteocytes was assessed six weeks post-OVX to allow bone loss to develop in the proximal tibia [37].

Application of mechanical loading

One short-duration loading bout was applied to both SHAM and OVX groups six weeks post-surgery. After recording body weight, the rats were anesthetized via an intraperitoneal injection of a mixture of ketamine (75 mg/kg body weight), and xylazine (10 mg/kg body weight). The left jugular vein was exposed surgically and a 25G 5/8" needle attached to a 3-ml syringe was inserted into the vein to inject a bolus (~2 ml) of tracer solution of FITC-labeled bovine serum albumin (67 kDa, diameter of ~7 nm, dosage of 0.25 mg/g body weight; #A9771, Sigma-Aldrich, St. Louis, MO). Immediately after the tracer injection (injection time = 2 min) a sinusoidal load was applied to the right tibia using the non-invasive mechanical loading device. The applied loading magnitude of 14 N, and the loading duration and frequency (100 cycles at 1 Hz), were chosen to mimic a short period of slow walking activity (total loading time: 100 s) [39,40]. Immediately after load application the rats were sacrificed with carbon dioxide inhalation and the loaded and unloaded contralateral tibiae were harvested and put into fixative (0.5% gluteraldehyde, 2% paraformaldehyde in 0.05 M cacodylate-sodium buffer, pH 7.4) for 48 h. To confirm the effectiveness of the OVX procedure, ovary removal was visually verified, and the uterine horns were weighed.

Histological processing, imaging techniques, and data collection

Cortical and cancellous bone samples were analyzed from the metaphysis and epiphysis of the proximal tibia. Tibial blocks were cut 3 mm distal to the growth plate and embedded in PMMA after 48 h of fixation. Either sagittal or frontal tibial sections (400–600 μm) were cut with a diamond-blade saw. Sections were then ground down to a final thickness of 40–70 μm using Carbimet paper disks (800 and 1200 grit; Buehler, Lake Bluff, IL), dried in ascending graded ethanol (75%, 95%, and 100%, 5 min each), and coverslipped with mounting media (Richard-Allan Scientific, Kalamazoo, MI).

To visualize tracer labeling, 20 regions were analyzed for each tibia (both loaded and unloaded): 5 regions from the cancellous metaphysis, 5 from the cancellous epiphysis, and 10 from the cortical metaphysis (Fig. 2). The cortical regions were visualized using a confocal microscope with a 40× oil immersion lens (Leica TCS SP2, Germany, 1.25 numerical aperture, 630 gain, 2.4 offset, and pinhole set at 1 Airy unit, wavelength excitation of 488 nm, and laser intensity set to 15%); images were taken at a resolution of 2048 × 2048 pixels with a field of view of 375 μm × 375 μm. The cancellous regions were visualized using the same parameters used for the cortical regions with the only