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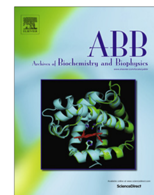

Dynamic fluid flow induced mechanobiological modulation of *in situ* osteocyte calcium oscillations

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ABSTRACT

Distribution of intramedullary pressure (ImP) induced bone fluid flow has been suggested to influence the magnitude of mechanotransductory signals within bone. As osteocytes have been suggested as major mechanosensors in bone network, it is still unclear how osteocytes embedded within a mineralized bone matrix respond to the external mechanical stimuli derived from direct coupling of dynamic fluid flow stimulation (DFFS). While *in vitro* osteocytes show unique Ca^{2+} oscillations to fluid shear, the objective of this study was to use a confocal imaging technique to visualize and quantify Ca^{2+} responses in osteocytes *in situ* under DFFS into the marrow cavity of an intact *ex vivo* mouse femur. This study provided significant technical development for evaluating mechanotransduction mechanism in bone cell response by separation of mechanical strain and fluid flow factors using ImP stimulation, giving the ability for true real-time imaging and monitoring of bone cell activities during the stimulation. Loading frequency dependent Ca^{2+} oscillations in osteocytes indicated the optimized loading at 10 Hz, where such induced response was significantly diminished via blockage of the Wnt/β-catenin signaling pathway. The results provided a pilot finding of the potential crosstalk or interaction between Wnt/β-catenin signaling and Ca^{2+} influx signaling of *in situ* osteocytes in response to mechanical signals. Findings from the present study make a valuable tool to investigate how *in situ* osteocytes respond and transduce mechanical signals, e.g. DFFS, as a central mechanosensor.

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Introduction

Biophysical stimuli derived from mechanical loading are proven essentials for bone tissue modeling, remodeling and regeneration. Changes in the pressure or velocity of bone fluid flow (BFF)[1] act as a communication medium that connects external loading signals and internal cellular activities in bone, which ultimately regulate the bone remodeling process [1–6]. On the other hand, discontinuation of BFF can result in higher bone loss, leading to conditions such as osteopenia [7–10]. Intramedullary pressure (ImP) is one of the influential factors for BFF and osteogenic signals within bone, which eventually influences bone growth [8].

In vivo studies on turkey ulna and mouse femur models have demonstrated the independent effect of ImP on inductions of potent osteogenic and adaptive responses in bone [8,11]. Demonstrating in a functional disuse rat model, oscillatory electrical muscle stimulation (MS) was able to induce non-linear ImP and bone strain to mitigate disuse bone loss [12–14]. Adaptation of skeletal nutrient vasculature was also found to be interrelated with ImP alteration [15]. More recently, our group has developed a dynamic hydraulic stimulation (DHS) that meant to directly couple an externally compressive load with internal BFF, which was able to non-invasively distinguish the anabolic role of the ImP factor and the bone deformation factor of BFF in an *in vivo* setting, as well as to establish the translational potential of ImP. As shown in a 4-week hindlimb suspension (HLS) rat study, DHS was able to mitigate disuse trabecular [16] and cortical bone loss [17]. In addition, direct measurements of ImP and bone strain via an operated *in vivo* study showed that DHS generated local ImP that acted independently from simultaneous bone strain. Moreover, the generated ImP was found to fall in a non-linear interrelationship with DHS loading frequencies, and yet in a directly proportional interrelationship with DHS loading magnitudes. Altogether, DHS was

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[1] *Abbreviations used:* BFF, bone fluid flow; DFFS, dynamic fluid flow stimulation; DHS, dynamic hydraulic stimulation; Dkk-1, Dickkopf-related protein 1; DMSO, dimethyl sulfoxide; FFT, Fast Fourier Transform; HLS, hindlimb suspension; ImP, intramedullary pressure; MSC, mesenchymal stem cell; PGE2, prostaglandin E2; Sost, sclerostin.

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suggested as a novel and non-invasive method to isolate the ImP and bone strain factors in an *in vivo* rodent model [18,19].

Elucidating downstream cellular and molecular effects of BFF to enhance bone quality has gained strong research interests. Subsequent *in vivo* studies of DHS further demonstrated the potential functional process of DHS-derived mechanical signals in bone metabolism. A longitudinal *in vivo* study using HLS rats was designed to evaluate mesenchymal stem cell (MSC) populations within the bone marrow in response to daily DHS loading over a course of 21-day [20]. In addition, alterations in gene expressions of osteogenic growth factors and transcription factors in response to DHS as a function of time were also evaluated [17]. A strong time-dependent manner of inductions of bone marrow MSCs as well as osteogenic gene expressions was observed in both studies.

As the most abundant cells in bone, osteocytes' critical role has been shown by targeted ablation of them, which led to altered bone modeling/remodeling with defective mechanotransduction [21]. Therefore, osteocyte mechanotransduction has been gaining significant amount of research attention for its great clinical potential in diseases involving dysfunctional bone remodeling, such as osteoporosis. While their cell bodies embedded within the fluid-filled mineralized bone matrix, the cell processes of osteocytes contact each other and possibly other cell types, allowing small signaling molecules to be transported between cells. BFF into the osteocyte canaliculi also triggers this cell–cell communication. This essential network acts as the central mechanosensor and aids in regulating bone modeling/remodeling and coordinating the adaptation of bone to the mechanical stimuli applied to the skeleton through BFF [22–24].

External mechanical stimuli promote bone formation via activation of intracellular signaling pathways that converge with growth factors to express osteogenic transcription factors. Bone cells perceive such external signals that trigger numerous intracellular responses including the release of prostaglandin E2 (PGE2) into the lacunar-canalicular fluid, where binding of PGE2 to its receptors (EP2 and/or EP4) leads to inhibition of GSK-3β and intracellular accumulation of free β-catenin. Nuclear translocation of β-catenin alters the expressions of a number of key target genes including the reduced expressions of sclerostin (Sost) and Dickkopf-related protein 1 (Dkk-1) and the increased expression of Wnt. After all, these changes facilitate the binding of Wnt to LRP5-Fz and amplify the load signal [25]. Recognized as an important regulator of bone mass and bone cell functions, Wnt/β-catenin signaling pathway may transmit the mechanical signals sensed by osteocytes to bone surface [26,27]. Moreover, potential crosstalk between Wnt/β-catenin and prostaglandin signaling pathways in response to loading may also reduce the expressions of Sost and Dkk-1 [27,28].

In response to external physical signals, escalation of intracellular Ca^{2+} has been observed as one of the earliest biochemical events in bone cells [29]. In change of mechanical environment, cellular functions may be regulated by triggered biochemical signaling cascades in response to changes in the upstream intracellular Ca^{2+} concentration [30]. Furthermore, in response to *in vitro* and *in situ* mechanical stimulations, osteocytes seem to be more sensitive than osteoblasts, in terms of Ca^{2+} oscillations. Recent studies have observed the real-time Ca^{2+} oscillations in response to fluid shear on *ex vivo* bone segments, as well as a direct mechanical stimulation on an intact *ex vivo* mouse tibia [31–33]. However, these studies either investigated the effect of fluid shear on osteocyte Ca^{2+} oscillations only on *ex vivo* bone segment surfaces that were not yet adapted for dynamic mechanical events within bone, or studied the effect of osteocyte Ca^{2+} oscillations of intact mouse long bones by deformable bone loading that the recording was done only at the resting period with time delay. In addition, the Ca^{2+} oscillation pattern of an intact *ex vivo* mouse long bone has not been studied with a direct dynamic fluid flow stimulation (DFFS) into the marrow cavity, which is highly related to the ImP/BFF mechanism. Therefore, the objective of this study was to provide a novel, non-bone-deforming experimental approach to real-time monitor the effect of DFFS into the bone marrow cavity of a fresh, intact mouse long bone on *in situ* osteocyte Ca^{2+} oscillations. Furthermore, the role of Wnt/β-catenin signaling pathway in the induced Ca^{2+} oscillations was also evaluated.

Materials and methods

Preparation of mouse femur samples

All animal protocols were approved by Stony Brook University IACUC. Female C57BL/6J mice of 3-month-old (Jackson Laboratory, Bar Harbor, ME, United States) were used to obtain fresh, intact femur samples immediately after euthanasia by CO_2 inhalation. The experimental groups were (1) 1 Hz DFFS, (2) 5 Hz DFFS, (3) 10 Hz DFFS, (4) 20 Hz DFFS and (5) Wnt/β-catenin inhibition + 10 Hz DFFS ($n = 5$ per group). The surrounding soft tissues of the femur samples were gently removed, maintained with periosteum. The bone samples were then incubated in DMEM with 5% FBS and 1% penicillin/streptomycin. A hole was drilled into the marrow cavity of each bone sample from the distal end using a 24-gauge micro drill bit.

Inhibition of Wnt/β-catenin signaling pathway and Ca^{2+} fluorescent staining

ICG-001 (Selleck Chemicals, United States and Europe) was used to antagonize Wnt/β-catenin-TCF-mediated transcription. For Group 5 (Wnt/β-catenin inhibition + 10 Hz), the femur samples were incubated in 25 μM of ICG-001 in culture medium for 1 h at room temperature. Meanwhile, the samples of the other groups (1 Hz, 5 Hz, 10 Hz and 20 Hz of DFFS) were incubated in culture medium for 1 h at room temperature.

For Ca^{2+} fluorescent labeling, the bone samples were incubated for 2 h in 15 μM Fluo-8 AM (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) dissolved in dimethyl sulfoxide (DMSO) and culture medium before confocal imaging [31].

DFFS loading

A custom-made experimental setup was built for imaging *in situ* osteocyte Ca^{2+} response in mouse femurs (Fig. 1). For DFFS, the drilled hole of each femur sample was tightly sealed with a 24-gauge catheter that was connected to a water-filled syringe pump. The syringe pump was controlled by a function generator that set the loading frequency and amplitude. DFFS with loading frequencies of 1 Hz, 5 Hz, 10 Hz, and 20 Hz and a constant loading magnitude of 1 V was applied to the bone samples for 10 s of baseline – 30 s of loading – 10 s of post-loading. Real-time confocal imaging (Zeiss LSM 510 META NLO Two-Photon Laser Scanning Confocal Microscope System) with 40× objective, 488-nm laser excitation, and 2 frames/s (512 × 512 pixel images) was performed to capture the Ca^{2+} signals of the osteocytes within each bone that was subjected to DFFS.

Intramedullary pressure measurements

To quantify DFFS-induced ImP within the marrow cavity in response to various loading frequencies, a separate set of experiments were performed on five long bone samples from 3-month-old female C57BL/6J mice to directly measure the ImP values during DFFS. A 24-gauge micro drill bit was used to drill a

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