Cellular and molecular mechanism underlying the anti-resorptive effect of low-magnitude, high-frequency vibrations on bone

+[1]Lau, E; [2]Pang, Q; [1,2]You, L
+[1]IBBME, [2]Dept. of Mechanical and Industrial Engineering, University of Toronto, Toronto, ON, Canada
esther.lau@utoronto.ca

INTRODUCTION. Osteocytes are well believed to be the major mechanosensors in bone, responsible for sending signals to the effector cells (osteoblasts and osteoclasts) that carry out bone formation and resorption. Consistent with this hypothesis, it has been shown that osteocytes release various soluble factors (e.g. transforming growth factor-β, nitric oxide, and prostaglandins) that influence osteoblastic and osteoclastic activity when subjected to a variety of mechanical stimuli, including fluid flow, hydrostatic pressure, and stretching[1,2]. Recently, low-magnitude (<1*g*, where *g*=9.98 m/s^2), high-frequency (20-90 Hz) vibration has gained interest as studies show that such a mechanical signal can positively influence skeletal status in animals and humans[3,4]. Although the anabolic and anti-resorptive potential of low-magnitude, high-frequency vibration is becoming apparent, the underlying cellular and molecular regulation of this mechanosensitivity is currently unknown. We hypothesize that osteocytes are the mechanosensors responsible for detecting the vibration stimulation and producing soluble factors that modulate the activity of effector cells. Here, we applied low-magnitude (0.3*g*) vibrations at varying frequencies (30-90 Hz) to osteocyte-like MLO-Y4 cells, after which we studied differential changes in mRNA expression of several bone regulatory genes and explored the potential of conditioned media collected from vibrated MLO-Y4 cells to inhibit the formation of osteoclasts (OC).

METHODS. ***Cell culture:*** MLO-Y4 cells (gift from Dr. Lynda Bonewald, University of Missouri–Kansas City) were maintained in α-MEM supplemented with 2.5% FBS, 2.5% CS, and 1% P/S on type I rat tail collagen-coated plates at 37 ºC and 5% CO_2. For vibration experiments, MLO-Y4 cells were plated in 12-well plates at 7.5x10^3 cells/cm^2. After 48 h, wells were completely filled with supplemented media and tightly sealed with gas permeable sealing film (Aeraseal, Excel Scientific) immediately prior to vibration. This prevented fluid flow within the wells when vibrations were applied, thus delineating any vibration effects from fluid flow shear stress effects. ***Low-magnitude, high-frequency vibration:*** Multi-well culture plates containing MLO-Y4 cells were placed securely onto a custom-made vibration plate attached to a vertically oriented shaker (Labworks Inc). The amplitude, wave shape, and frequency of the vibration provided by the shaker were controlled with VibeLab computer program. MLO-Y4 cells in the experimental group were subjected to 30, 60, or 90 Hz of sinusoidal vibrations at 0.3*g* for 15 minutes. The chosen frequency and acceleration amplitude were based on various animal and human studies that reported positive bone remodeling as a result of vibration loading. Cells in the no vibration group were placed on the same but stationary vibration plate for 15 minutes. Immediately following the completion of vibration procedure, the media in multi-wells was replaced with fresh supplemented α-MEM (5% FBS, 5% CS, 1% PS) and incubated for 24 hours, and was collected as conditioned media (CM) and stored at -20ºC until subsequent analysis. ***mRNA quantification*:** Total RNA was isolated from MLO-Y4 cells 2 h after vibration and treated with DNase I (Fermentas). Reverse transcription was performed on 0.5 μg RNA. The resulting cDNA samples were subjected to quantitative PCR (qPCR) using gene-specific primers and SYBR Green I in LightCycler 480 (Roche). Standards and samples were run in triplicate. mRNA levels of each gene of interest were normalized to 18S levels. ***Osteoclast formation:*** RAW264.7 cells were maintained in DMEM supplemented with 10% FBS. To induce osteoclast formation, 20 ng/ml sRANKL (R&D Systems) was added to RAW264.7 cells seeded at 5x10^3 cells/cm^2 in a 24-well plate. The effect of soluble factors released by osteocytes on osteoclastogenesis was studied using CM from MLO-Y4 cells collected 24 h after vibration. On day 3 (starting from RAW264.7 plating), CM was added to RAW264.7 cells to replace 50% of the original culture medium. This procedure was repeated every subsequent day until day 6, when cultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP). Osteoclasts were identified as TRAP-positive cells containing three or more nuclei. ***Statistical analysis:*** ANOVA was used to compare means of more than three groups, followed by Tukey post-hoc test (p=0.05).

RESULTS. There is a trend of increasing COX-2 and RANKL mRNA levels in MLO-Y4 cells from 0 to 60 Hz. At 90 Hz, mRNA expression dropped to a level comparable to no vibration condition. Cells that were subjected to 60 Hz vibration showed a significantly higher expression in COX-2 and RANKL (3.4 and 2.5-fold increase, respectively; p<0.05) as compared to non-vibrated controls. In addition, the effect of CM collected from vibrated MLO-Y4 cells on OC formation was evaluated. Cultures containing CM from vibration conditions had fewer OCs, but the decrease was not statistically significant.

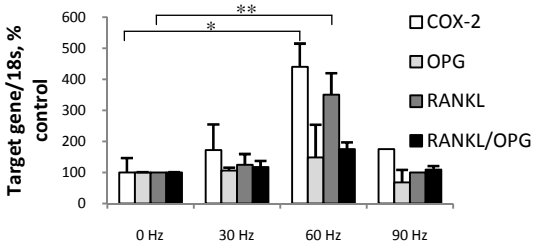

Figure 1. Effect of vibration on mRNA levels in MLO-Y4 cells. Data were normalized to 0 Hz control (n=2; *p=0.005, **p=0.01).

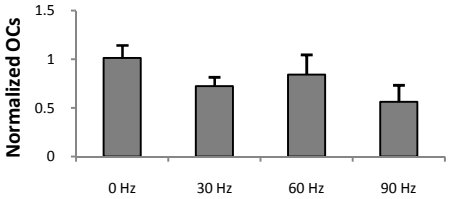

Figure 2. Effect of MLO-Y4 conditioned media on OC formation. The average value corresponding to normalized value 1 is 199 cells (n=4).

DISCUSSION. Our data suggest that MLO-Y4 cells respond to the vibration signal at least at the transcript level, with vibration at 60 Hz being the most potent signal. This corresponds partially with animal studies, where vibrations at 90 Hz were more anabolic than a 45 Hz signal in ovariectomized rats[5]. The seemingly contradictory observation that MLO-Y4 cells showed a lower response to the 90 Hz signal may be explained by the viscoelasticity of cells, where cell stiffness defines an upper limit of loading rate that causes cell deformation[6]. Similar to previous studies in which bone cells were stimulated with fluid flow[7], vibration stimulation caused an increase in the expression of COX-2, an enzyme that is critical for the production of prostaglandins and has been implicated in promoting bone formation. Vibration also resulted in increased expression of RANKL, an important molecule for osteoclast formation. Interestingly, however, our preliminary data from the CM study indicate a decreased number of OCs in vibration conditions. While the preliminary data does not show a statistically significant difference, future experiments involving larger sample size and quantitative protein analysis may confirm whether osteocytes release soluble factors that inhibit OC formation in the presence of vibration stimulation. This study is the first to identify the regulatory biochemical agents associated with the vibration signal in osteocytes, which serve as potential targets for pharmacological interventions of osteoporosis.

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