Low-magnitude, high-frequency vibration inhibits osteoclast formation via soluble factors from MLO-Y4 osteocytes

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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 (LM; <1*g*, where *g*=9.98 m/s^2), high-frequency (HF; 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 LMHF vibration is becoming apparent, the underlying cellular and molecular regulation of this mechanosensitivity is currently unknown. We have previously shown that MLO-Y4 osteocyte-like cells respond to LMHF vibration at the transcript level. To further investigate the influence of LMHF vibration on the expression of osteocytic soluble factors that are known to play a role in bone remodeling, we applied low-magnitude (0.3*g*) vibrations at varying frequencies (30-90 Hz) to MLO-Y4 cells, and measured the level of prostaglandin E_2 (PGE_2), osteoprotegerin (OPG), and soluble RANKL (sRANKL) release. We also investigated the potential of conditioned media (CM) from vibrated MLO-Y4 cells to influence osteoclast (OC) formation.

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 at 30,000 cells/well in 6-well plates. After 48 h, wells were completely filled with working media (1% FBS, 1% CS, 1% P/S) and tightly sealed with gas permeable sealing film immediately prior to vibration. This prevented fluid flow within the wells when vibrations were applied. ***Low-magnitude, high-frequency vibration:*** Multi-well culture plates containing MLO-Y4 cells were placed onto a custom-made vibration plate attached to a vertically oriented shaker (Labworks Inc) 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 1 h, while cells in the no vibration group were placed on the same but stationary vibration plate. At the end of vibration, the media was replaced with fresh supplemented α-MEM (5% FBS, 5% CS, 1% PS) and collected as conditioned media (CM) at various time points. ***mRNA quantification*****:** Total RNA was isolated from MLO-Y4 cells immediately after vibration and treated with DNase I (Fermentas). Reverse transcription was performed on 2 µg RNA. The resulting cDNA samples were subjected to quantitative PCR (qPCR) using gene-specific primers and SYBR Green I in Realplex^2 (Eppendorf). mRNA levels of each gene of interest were normalized to 18S levels. ***PGE_2, OPG & sRANKL quantification:*** Supernatant levels of PGE_2, OPG & RANKL were measured using enzyme-linked immunoassay (Cayman Chemicals), Quantikine Mouse RANKL Immunoassay and OPG Immunoassay (both from R&D Systems), respectively. Values were normalized to total protein content. ***Osteoclast formation:*** RAW264.7 cells were maintained in DMEM supplemented with 10% FBS and 1% P/S. For OC formation, RAW264.7 cells were seeded at 5x10^3 cells/cm^2 in a 24-well plate (day 0). After 24 h, the media was replaced with a 1:1 mixture of MLO-Y4 CM (collected 30 min after vibration) and growth media containing 20 ng/ml sRANKL (R&D Systems). This procedure was repeated every subsequent day until day 5, when cultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP). OCs were identified as TRAP-positive cells containing ≥3 nuclei and enumerated in 5 fields of view under 10x objective. ***Statistical analysis:*** Student t-test was used to compare experimental and control mean values. ANOVA was used to compare means of ≥3 groups, followed by Tukey post-hoc test (p=0.05).

RESULTS. ***COX-2 expression and PGE_2 release*****:** COX-2 gene expression increased significantly as frequency of LMHF vibration increased (3.4-fold at 90 Hz; p<0.01) (Fig. 1a). There were no significant changes in the amount of PGE_2 released at 30 minutes post-vibration (data not shown), but at 6 hour post-vibration there is a similar trend of increasing PGE_2 level from 0 to 90 Hz (Fig. 1b). ***RANKL gene and protein expression:*** Vibration caused RANKL gene expression to decrease in MLO-Y4 cells at all frequencies, and most significantly at 60 Hz (-55%, p<0.01) (Fig. 2a). The amount of sRANKL released by MLO-Y4 cells vibrated at 60 Hz decreased by 53% (Fig. 2b) at 30 minutes post-vibration and returned to control levels as early as 2 hours. At all time points, no measurable amount of OPG was detected in CM of MLO-Y4 cells. ***Effect of CM on osteoclast formation:*** Cultures containing CM from MLO-Y4 cells vibrated at 60 Hz had moderately fewer OCs (Fig. 3a). However, the population of OCs containing ≥10 nuclei was significantly decreased (-36%, p<0.05) (Fig. 3b).

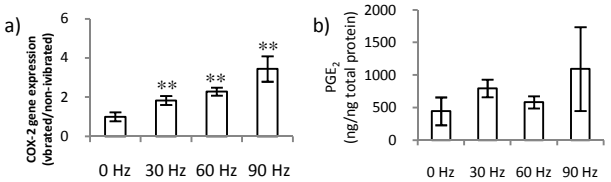

Fig.1. LMHF vibration increased COX-2 expression (a) and PGE_2 release (b). **p<0.01 compared to 0 Hz, n=5

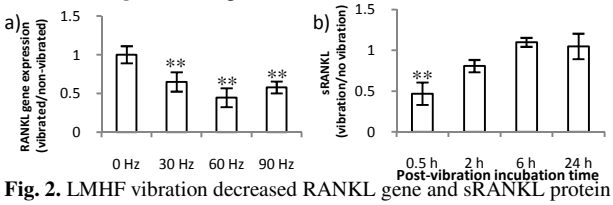

Fig. 2. LMHF vibration decreased RANKL gene and sRANKL protein expression at 30 min post-vibration. **p<0.01 compared to 0Hz, n=5

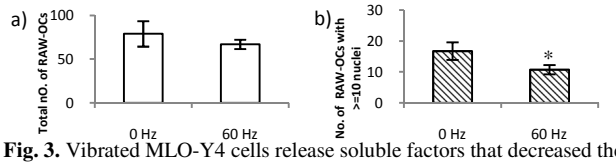

Fig. 3. Vibrated MLO-Y4 cells release soluble factors that decreased the formation of large osteoclasts (≥10 nuclei).*p<0.05, n=4

DISCUSSION. The observed vibration-induced changes in gene expression were frequency-dependent; COX-2 expression peaked at 90 Hz, while RANKL expression decreased most significantly at 60 Hz, suggesting there is an optimal frequency at which the signal is most anabolic/anti-resorptive. As COX-2 is a critical enzyme that catalyzes the biosynthesis of PGE_2, we next investigated the amount of PGE_2 secreted by MLO-Y4 cells under vibration loading. Differential levels of PGE_2 release were observed at 6 hours post-vibration. This is in contrast to the temporal response of MLO-Y4 cells under fluid flow, where PGE_2 levels were significantly increased within 10 minutes[5]. The decrease in sRANKL expression occurred more rapidly (at 30 min post-vibration), suggesting that vibration loading may inhibit ectodomain shedding of RANKL from the membrane as opposed to *de novo* protein synthesis. As PGE_2 has been implicated in promoting bone formation while RANKL promotes osteoclast formation and survival, our data suggest that vibration regulates bone remodeling via soluble factors released by osteocytes. Indeed, when we induced OC formation in the presence of CM from vibrated MLO-Y4 cells, the number of large OCs formed (≥10 nuclei per cell) decreased. As large OCs are associated with higher resorbing capability, vibrated MLO-Y4 osteocytes may secrete soluble signals that inhibit OC formation and activity. This study is the first to identify the regulatory biochemical signals associated with the vibration signal in osteocytes, which serve as potential targets for pharmacological interventions of osteoporosis.

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