

CHAPTER 6

BONE CELL RESPONSES TO HIGH-FREQUENCY VIBRATION STRESS: DOES THE NUCLEUS OSCILLATE WITHIN THE CYTOPLASM?

Rommel G. Bacabac¹, Theo H. Smit², Jack J.W.A. Van Loon^{1,3}, Jolanda M.A. de Blicck-Hogervorst¹, Cor M. Semeins¹, Behrouz Zandieh Doulabi¹, Marco Helder¹, Jenneke Klein-Nulend¹

¹Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam-Universiteit van Amsterdam and Vrije Universiteit, Amsterdam, The Netherlands

²Department of Clinical Physics and Informatics, VU University Medical Center, Amsterdam, The Netherlands

³Dutch Experiment Support Center, Vrije Universiteit, Amsterdam, The Netherlands

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ABSTRACT

Mechanosensing by cells directs changes in bone mass and structure, in response to the challenges of mechanical loading. Low-amplitude, high-frequency loading stimulates bone growth by enhancing bone formation and inhibiting disuse osteoporosis. However, how bone cells sense vibration stress is unknown. Hence, we investigated bone cell responses to vibration stress at a wide frequency range (5-100 Hz). We used nitric oxide (NO) and prostaglandin E₂ (PGE₂) release, and COX-2 mRNA expression, as parameters for bone cell response, since these molecules regulate bone adaptation to mechanical loading. NO release positively correlated, while PGE₂ release negatively correlated to the maximum acceleration rate of the vibration stress. COX-2 mRNA expression increased in a frequency-dependent manner, which relates to increased NO release at high frequencies confirming our previous results. The anti-correlated release of NO and PGE₂ suggests that these signaling molecules play different roles in bone adaptation to high-frequency loading. The maximum acceleration rate is proportional to ω^3 (frequency = $\omega/2\pi$), which is commensurate with the Stokes-Einstein relation for modeling cell nucleus motion within the cytoplasm due to vibration stress. Correlations of NO and PGE₂ with the maximum acceleration rate then relate to nucleus oscillations, providing a physical basis for cellular mechanosensing of high-frequency loading.

INTRODUCTION

Bone is an obvious biological system that exhibits the interplay of mechanical stress and adaptive response both at the tissue and cellular levels(1-3). Bones adapt their mass and structure in response to the demands of mechanical loading (4). Furthermore, it has been suggested that the rate rather than the magnitude alone of the applied loading stimulus correlates to bone formation *in vivo* (5, 6). This suggests that both the frequency and amplitude of applied stresses are important for the osteogenic response of bone.

We have recently found in another study that the NO production by MC3T3-E1 cells was linearly dependent on the rate of fluid shear stress, which depended on both the amplitude and frequency of stress (7). In that study however, the applied fluid shear stress was only up to a maximum of 9 Hz. Nitric oxide (NO) and prostaglandin E₂ production are essential for the induction of new bone formation in response to mechanical loading *in vivo* (8-10). The constitutional, endothelial form of nitric oxide synthase (eNOS), one of the three NOS enzyme isoforms responsible for the synthesis of NO, is prominently expressed in osteocytes and upregulated by mechanical loading (11). Blocking of either cyclooxygenase-2 (COX-2), the key enzyme for mechanically-induced PG production, or NOS could prevent mechanically induced bone formation (8, 12, 13). Since osteoblasts respond to fluid shear stress as osteocytes, although to a lesser extent, osteoblasts could provide a practical model for osteocyte response to stress.

Using animal models, low magnitude ($< 10 \mu\epsilon$) high-frequency (10 - 100 Hz) mechanical stimuli have been shown to be capable of stimulating bone growth by doubling bone formation rates and inhibiting disuse osteoporosis (14). Thus, it would seem that higher frequencies are also stimulatory to bone cells. Much is unknown however, about how high frequency loading might permeate bone despite the presence of soft tissues. We have shown earlier that external loading from exercise could involve frequencies reaching 9 Hz (7). Frequencies experienced by cells beyond 10 Hz might occur at much lower

amplitudes considering the damping effects of soft tissue. Regardless of frequency range, the applied rate of loading, which is dependent on both frequency and magnitude, seems to be a decisive factor in bone formation and maintenance.

Paradigms for understanding mechanosensing by cells include models for the mechanical properties of the cytoplasm as predominantly a continuum (15) or as composed of linked polymers that transfer forces through the cytoskeleton to the nucleus (16). Cellular activation by mechanical loads in general or vibration in particular, leading to a biochemical cascade requires some form of cellular deformation as a mechanism for sensing forces. Although there is evidence that bone cells respond to dynamic loading, how bone cells might sense mechanical vibration is unknown. Therefore, we studied the response of bone cells to mechanical vibration over a wide frequency range (5 Hz up to 100 Hz), at different magnitudes. We tested whether vibration stress applied with varying frequencies and amplitudes affects the nitric oxide (NO) and prostaglandin E₂ (PGE₂) production, and mRNA expression for COX-2 by MC3T3-E1 osteoblastic cells. Based on the results, we empirically derived a model to propose an alternative mechanism by which cells might sense high-frequency mechanical loading.

MATERIALS AND METHODS

Bone cell cultures

MC3T3-E1 cells (Kodama, et al, 1981; kindly provided by Dr. Kumegawa, Mekai University School of Dentistry, Sakado, Saitama, Japan) were cultured up to near-confluency in 75 cm² cell culture flasks (Nunc, Roskilde, Denmark), using α -Modified Eagle's Medium (α -MEM; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Gibco), ascorbate (50 μ g/ml; Merck, Darmstadt, Germany), β -glycerophosphate disodium salt hydrate (10 mM; Sigma, St. Louis, MO, USA), l-glutamine (300 μ g/ml; Merck),

gentamycine (50 $\mu\text{g/ml}$; Gibco), fungizone (1.25 $\mu\text{g/ml}$; Gibco), at 37°C with 5% CO_2 in air. Cells were then harvested using 0.25% trypsin (Difco Laboratories, Detroit, MI, USA) and 0.1% EDTA (Sigma) in PBS, and seeded at 0.7×10^5 cells per well in a 24-well plate, then incubated overnight in α -MEM with 10% FBS to promote cell attachment prior to vibration stress treatment as described below.

Application of vibration stress

For vibration stress treatment, the culture medium was changed to CO_2 -independent medium (Gibco, USA) with 2% FBS, and incubated for 5 min in the presence of mechanical vibration at varying frequencies and amplitudes (see Table 1). Vibration stress was implemented on attached cells by sinusoidal displacement of the 24-well plate along the cells' plane of attachment using a voltage controlled linear actuator (fig. 1A). Conditioned medium was sampled after 5 min of vibration stress treatment to measure accumulated NO in medium produced by MC3T3-E1 cells.

Table 1. *Data of applied vibration stress*

Regime frequency	5 Hz	30 Hz	60 Hz	100 Hz
Amplitude (mm)	5	4.5	1.75	0.75
Maximum acceleration rate (km/s^3)	0.15	30.1	93.8	186
Maximum velocity (m/s)	0.15	0.85	0.66	0.47

$\omega = 2\pi \times \text{frequency}$; Maximum velocity = amplitude $\times \omega$;

Maximum acceleration = amplitude $\times \omega^3$

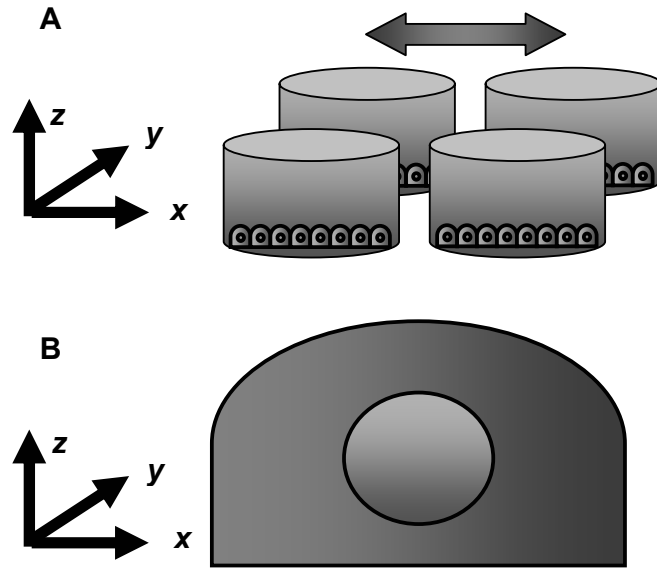


Figure 1. Vibration stress application. A. Cells were seeded onto the bottom surface of the 24-wells plate (as described in Methods). Sinusoidal motion is along the x-axis (arrow). B. Simplified model of a cell with an approximately rigid spherical nucleus compared to its viscoelastic cell body.

Nuclear oscillations induced by vibration stress

We take a minimalist approach to understand how the cells are possibly receiving stress by translational oscillation. We consider the nucleus to be spherical and embedded in the viscoelastic medium of the cell body. The source of mechanical stimulus is then attributed to the possible motion of the cellular nucleus (modeled to be a sphere, figure 1B) inside the cell. Based on the Generalized Stokes-Einstein relation we estimated the displacement of the nucleus in the cell $x_n(\omega)$, modeled as a rigid sphere compared to the cytoplasm, as proportional to the applied force F :

$$x_n(\omega) \propto \frac{F(\omega)}{R_n G(\omega)} \quad [1]$$

where $G(\omega)$, is here considered to be the elastic modulus of the cell cytoplasm (at the order of 100 Pa) (17), and the acting force is due to the mass of the nucleus $\rho_n V_n$, with a volume V_n , and radius R_n , considering a relative acceleration of the nucleus with respect to the cell body. In this approximation, we neglect the time dependence of the modulus, so, $G(\omega) \rightarrow G_o$. The nucleus is considered to be four times more rigid compared to the cytoplasm (e.g., the elastic modulus of the nucleus is at the order of 400 Pa) (18). The force acting on the nucleus is due to the acceleration it experiences due to the applied sinusoidal vibration, with amplitude x_o and frequency $= \omega/(2\pi)$:

$$F = -\rho_n V_n \frac{d^2}{dt^2} [x_o \sin(\omega t)] = \rho_n V_n x_o \omega^2 \sin(\omega t) \quad [2]$$

Thus, the absolute maximum force due to the nucleus F_{max} , is related to the maximum acceleration of the entire plate:

$$F_{max} = \rho_n V_n x_o \omega^2 \quad [3]$$

where $x_o \omega^2$ is the maximum acceleration of the plate. The maximum velocity of the plate is related to nuclear velocity v_n , which in turn is related to ω :

$$v_n(\omega) \propto \left(\frac{\rho'_n V_n}{R_n G_o} \right) x_o \omega \quad [4]$$

where ρ'_n is the density difference between the cell nucleus and the surrounding cytoplasm. Here, we approximate the elastic modulus to be constant G_o . The maximum rate of acceleration by the plate is related to the rate of acceleration ra_n by the nucleus at ω :

$$ra_n(\omega) \propto \left(\frac{\rho'_n V_n}{R_n G_o} \right) x_o \omega^3 \quad [5]$$

Using this *in vitro* system, for a non-negligible density difference between the cell nucleus and the surrounding cytoplasm, we are able to mechanically stimulate cells by inducing body forces by vibration stress. Hence, by correlating the amount of released signaling molecules to the maximum

velocity or rate of acceleration of the plate we characterized the effect of vibration stress to bone cell mechansosensitivity.

Nitric Oxide and Prostaglandin E₂ determination

The conditioned medium was assayed for NO, which was measured as nitrite (NO₂⁻) accumulation in the conditioned medium, using Griess reagent (1% sulfanilamide, 0.1% naphthylethelene-diamine-dihydrochloride, and 2.5 M H₃PO₄). The absorbance was measured at 540 nm. NO concentrations were determined using a standard curve derived from known concentrations of NaNO₂ in non-conditioned culture medium. PGE₂ was measured in conditioned medium by an enzyme immunoassay (EIA) system (Amersham, Buckinghamshire, UK) using an antibody raised against mouse PGE₂. The detection limit was 16 pg/ml. Absorbance was measured at 450 nm.

Total DNA, RNA and total protein determination

DNA, RNA and protein were isolated from the bone cell cultures using Trizol reagent according to the manufacturer's instructions. The amount of protein was determined using a BCA protein Assay Reagent Kit (Pierce, Rockford, IL, USA), the absorbance was read at 570 nm. The RNA and DNA content were determined by measuring absorbance in water at 260 nm using an Ultraspec III spectrophotometer (Amersham).

RNA isolation and Reverse transcription

Total RNA from the cells was isolated using Trizol[®] reagent with one modification; 5 µg of glycogen (Roche Diagnostics, Mannheim, Germany) was added to RNA and isopropanol solution prior to centrifuge step to increase RNA yield. Total RNA concentration was quantified spectrophotometrically. cDNA synthesis was performed using 0.5-1 µg total RNA in a 20 µl reaction mix consisting of 5 Units of Transcriptor Reverse Transcriptase according to the manufacturer's instructions (Roche Diagnostics) with 0.08 A₂₆₀ units random primers (Roche Diagnostics), 1 mM of each dNTP (Invitrogen), and

Transcriptor RT reaction buffer. cDNA was diluted 5 times and stored at -80°C prior to real-time PCR.

Real-time PCR

Real-time PCR reactions were performed using the SYBRGreen reaction kit according to the manufacturer's instructions (Roche Diagnostics) in a LightCycler (Roche Diagnostics). cDNA (2 μl each) was diluted to a volume of 20 μl with PCR mix (Light Cycler DNA Master Fast start^{plus} Kit, Roche Diagnostics) containing a final concentration of 0.2 pmol of primers. Relative housekeeping gene expression (18S; which its expression was not subjected to time and or treatment related variations) and relative target gene expression (COX-2) were determined. Primers (Invitrogen) used for real-time PCR are listed in Table 2 were designed using Clone manager suite software program version 6 (Scientific & Educational Software, NC, USA) and the amplified PCR fragment had extension over at least one exon-border except for 18S which gene is encoded only by one exon. Values of relative target gene expression were normalized for relative 18S housekeeping gene expression.

Table 2. Primers used for real time PCR

Target gene	Oligonucleotide sequence	Expected product size, bp
18s forward	5'-gtaaccggtgaacccatt-3'	151
18s reverse	5'-ccatccaatcggtagtagcg-3'	
COX-2 forward	5'-gcattcttgcccagcaact-3'	299
COX-2 reverse	5'-agaccaggcaccagaccaaaga-3'	

Real Time PCR Data analyses

With the Light Cycler software (version 2), the crossing points were assessed and plotted versus the the serial dilution of known concentrations of the standards derived from each gene. PCR efficiency (E) was obtained by the formula: $E=10^{-1/\text{slope}}$ and the data were used if and only if the PCR efficiency was calculated between 1.85-2.0.

Statistics

Data were pooled from the results of at least 5 experiments for each vibration stress regime. The effects of vibration stress regimes were analyzed with the non-parametric Wilcoxon signed rank sum test of the S-Plus 2000 package (release 1). Differences between total DNA, RNA, and protein expressions for different vibration stress regimes were tested using one-way ANOVA. The relation between the release of NO or PGE₂ against the peak rate acceleration by the vibration stress, and against each other, was characterized by linear regression. Significant differences were considered at a p-value < 0.05.

RESULTS

Application of vibration stress for 5 min to the MC3T3-E1 cells did not result in visible changes in cell shape or alignment of the cells to any orientation (data not shown). No cells were removed by any of the vibration stress regimes, as assessed by visually inspecting the cultures before and after vibration stress treatment, and by measuring the total amount of DNA, RNA and protein (table 3).

Table 3. Total DNA, RNA, and Protein.

	5 Hz	30 Hz	60 Hz	100 Hz
DNA ($\mu\text{g}/\mu\text{l}$)	0.045 ± 0.021	0.035 ± 0.012	0.029 ± 0.009	0.041 ± 0.012
RNA ($\mu\text{g}/\mu\text{l}$)	0.200 ± 0.064	0.163 ± 0.038	0.170 ± 0.048	0.203 ± 0.055
Protein ($\mu\text{g}/\text{ml}$)	108 ± 5	98 ± 11	84 ± 10	94 ± 8

One-way ANOVA test indicated that the means are not significantly different at $p < 0.05$.

The rapid response to vibration stress by bone cells was measured as the accumulation of NO released in the medium after 5 min of treatment with the different vibration stress regimes (table 1). NO production in rapid response to treatment with vibration stress linearly correlated with the applied maximum

acceleration rate, (fig. 2A; $p < 0.05$, $R = 0.95$). However, this response did not correlate linearly to the applied maximum velocity. The highest response was due to the 100 Hz regime (see table 1), which was significantly larger than the response to 5 Hz and 60 Hz regimes (fig. 2A and B, $p < 0.03$).

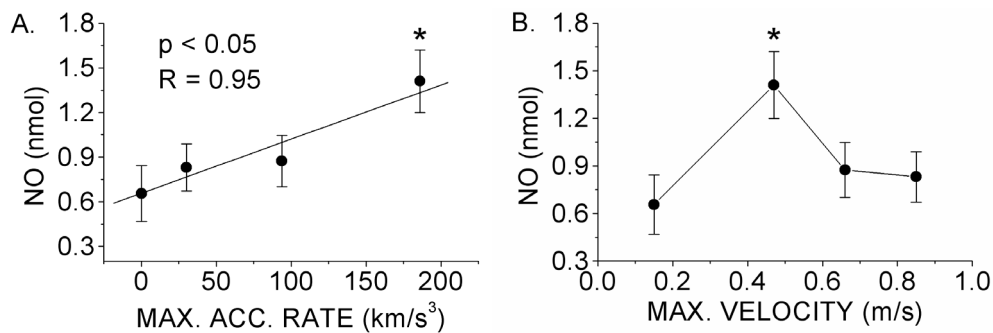


Figure 2. Effect of vibration stress on NO production by bone cells. A. Bone cells respond in positive correlation to the applied maximum acceleration rate (max. acc. rate) of vibration stress immediately after 5 min ($p < 0.05$). B. The response to mechanical vibration does not correlate linearly to the applied maximum velocity (max. velocity). The response to vibration stress of 100 Hz was significantly larger than the response to 5 Hz and 60 Hz ($*p < 0.03$). Values are mean total amount \pm SEM.

The late response to vibration stress by bone cells was measured as the accumulation of PGE₂ released in the medium after 5 min of treatment with the different vibration stress regimes (table 1). PGE₂ was assayed in the conditioned medium after 30 min of post incubation at 37°C without vibration stress. The PGE₂ released by bone cells in response to vibration stress anti-correlated with the applied peak acceleration rate (fig. 3A, $p < 0.006$). However, the response to mechanical vibration did not correlate linearly to the applied maximum velocity (fig. 3B). The highest response to vibration stress was due to the 5 Hz regime (see table 1), which was significantly larger than the response to 100 Hz (fig. 3, $p < 0.003$).

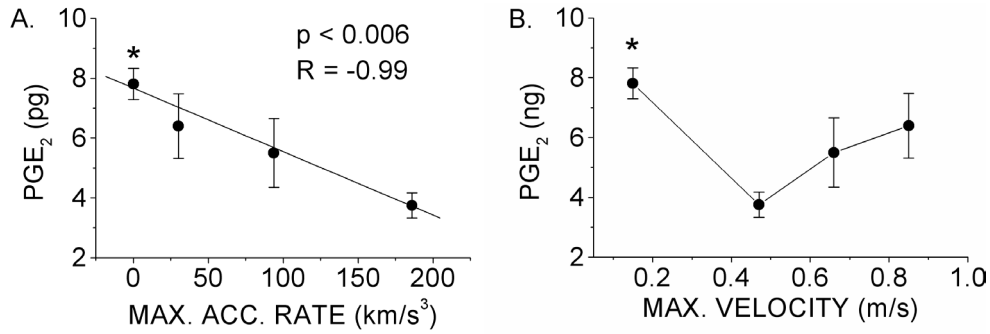


Figure 3. Effect of vibration stress on PGE₂ production by bone cells. A. Bone cells respond in negative correlation to the applied maximum acceleration rate (max. acc. rate) of vibration stress ($p < 0.006$). B. The response to mechanical vibration does not correlate linearly to the applied maximum velocity (max. velocity). The response to vibration stress of 5 Hz was significantly larger than the response to 100 Hz ($*p < 0.003$). PGE₂ was assayed from medium harvested after 30 min of post-incubation subsequent to 5 min of vibration stress. Values are mean total amount \pm SEM.

To investigate whether the production of NO and PGE₂ was related, the measured release of these signaling molecules to corresponding increasing frequencies of the vibration stress were correlated by linear regression. The NO and PGE₂ released by bone cells in response to vibration stress at varying frequencies, were found to be anti-correlated (fig. 4, $p < 0.013$, $R = -0.99$).

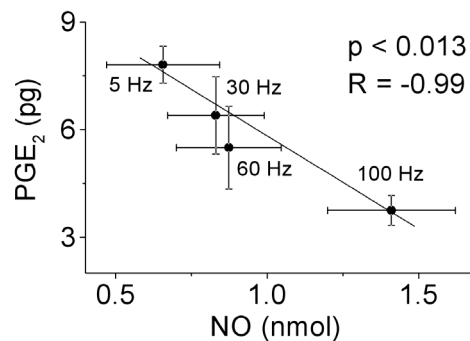


Figure 4. Anti-correlated release of NO and PGE₂ by bone cells in response to vibration stress.

To investigate the long-term effect of vibration stress on bone cells, the mRNA expression for COX-2 was measured in relation to PGE₂ release, at 30 min subsequent to 5 min vibration stress. The bone cells were harvested for COX-2 mRNA expression after 2.5 hours of post-incubation subsequent to 5 min of vibration stress. The mRNA expression for COX-2 was found most upregulated in response to 100 Hz vibration stress, which was significantly higher than the response to all the other regimes (fig. 5). The mRNA expression for COX-2 in response to 100 Hz was 2-fold higher than the response to 5 and 30 Hz. Also, the mRNA expression for COX-2, in response to 60 Hz vibration was found to be 1.5 times higher than the response to the 30 Hz regime (fig. 5, $p < 0.047$).

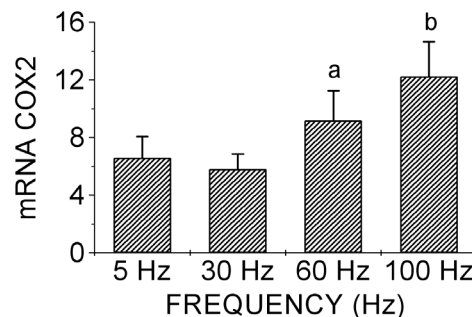


Figure 5. Vibration stress upregulates mRNA for COX-2 expression by bone cells. mRNA expression for COX-2, was 2-fold regulated in response to 100 Hz vibration stress (b, higher than the response to 5 Hz, $p < 0.028$, higher than 30 Hz, $p < 0.011$, and higher than 60 Hz, $p < 0.05$). mRNA expression for COX-2, in response to 60 Hz vibration was 1.5 times higher than the response to 30 Hz ($^a p < 0.047$). Cells were harvested for measuring mRNA expression for COX-2 after 2 hours of post-incubation subsequent to 5 min of vibration stress. Values are mean total amount \pm SEM. mRNA values were normalized relative to the 18S housekeeping gene expression.

DISCUSSION

The membrane-bound soluble enzyme called endothelial cell NOS (ecNOS or NOS III, one of three members of the NOS family) is involved in the NO response of bone cells to mechanical stress (19, 20). NO production is an essential step for mechanical loading-induced bone formation as observed in rats *in vivo* (8). We have found earlier that mRNA expression for ecNOS in bone cells (osteocytes isolated from chicken calvaria) is upregulated in response to fluid shear stress (21). Prostaglandins are generated by the release of arachidonic acid from phospholipids in the cell membrane, followed by conversion of arachidonic acid into prostaglandin G₂ and subsequently H₂. Prostaglandin H₂ is further isomerized to the biological active prostanoids, prostaglandin E₂ (PGE₂). The *in vivo* acute prostaglandin production by loading-stimulated bone cells seems to be more important than the sustained prostaglandin release (10, 13). Hence, NO and PGE₂ production, in response to fluid shear stress, are meaningful parameters for measuring bone cell activation.

NO and PGE₂ production by bone cells linearly correlated at opposing signs, with the maximum applied acceleration rate, at opposing signs, which is third order or cubic in frequency dimension (see Table 1). This suggests that the bone cell response to vibration stress treatment is highly dependent on the applied frequency of loading. The response however, did not linearly correlate with the applied maximum velocity, which is a joint effect of the applied amplitude and frequency, both at first order. The most likely effect of the vibration stress might be the movement of the cell nucleus through the cell body induced by the acceleration of the plate. NO release and PGE₂ release correlated with the rate of acceleration, which is proportional to the rate of the force on the nucleus. The rate of force is proportional to the rate of stress experienced by the cell through nuclear motion directly. This supports our earlier finding that bone cell response is linear to the applied fluid shear stress rate (7). In our model for the mechanical effect of vibration stress, the

acceleration rate corresponds to the rate of the force acting on the cell nucleus. Since stress is the amount of force about a cross-sectional area, the consistent signature for bone cell response to mechanical loading seems to be linear stress-rate dependence. Fluid shear stress, which is contact stress primarily acting on the cell membrane, might be linearly rate-dependent only for low frequencies (< 10 Hz), since fluid flow might attenuate at high frequencies (> 10 Hz). Thus, our model for the motion of the cell nucleus for vibration stress at high frequencies, involving body forces, explains the different way how bone cells might sense loading at high frequencies (> 10 Hz) or at high impact loading.

The anti-correlation of NO and PGE₂ release is closely linked to the rate of stress experienced by the bone cells at a wide range of frequencies induced by vibration. These opposing trends of molecular release suggest different roles for NO and PGE₂ in regulating the mechanical adaptation of bone. It has been shown *in vitro* that osteoclasts migrate away from NO (22, 23). PGE₂ increases mRNA levels of osteoprotegerin ligand (OPG-L)/osteoclast differentiation factor (ODF), from osteoblastic lineage cells. OPG-L/ODF stimulates osteoclast differentiation and activity, further inhibiting osteoclast apoptosis (22). NO and PGE₂ regulate the activity of osteoclasts (23, 24). Since at higher frequencies, MC3T3-E1 tends to increase NO release but tends to decrease PGE₂ release, this suggests an acute tendency for bone cells to oppose the presence of osteoclasts at high frequency loading. It is possible that *in vivo*, osteoblasts enhance their rejection of osteoclasts when stimulated with high frequencies. This might in turn, stimulate osteoblast activity in the absence of osteoclasts. Indirectly, osteoclasts are possibly recruited onto regions not experiencing high frequency loading. Since materials of higher density are expected to favor force transfer at high frequencies, it is possible that in bone, regions that are less dense (hence not responsive to high frequencies) are more prone to being degraded by osteoclasts, while regions of higher densities are strategically maintained. Furthermore, since the release of signaling molecules correlates strongly to ω^3 , the absence of high strains in normal daily activities

does not necessarily correspond to bone loss. High frequencies in the spectrum of loading are expected during movements of high impact activity, as in exercise and sports, which have been shown to be beneficial for bone health (25-27). Thus, it would seem that the loss in strain amplitude can be compensated by the application of higher frequency loading.

Our results might also provide further insight on the effects of a broad band of frequencies on bone formation (6, 28). Our model for predicting the motion of the nucleus through the cell body during vibration stress application, might relate to the reported osteogenic benefits of vibration-related stimulation. For example, the use of low-intensity ultrasound has been shown to stimulate a higher occurrence of spinal fusion compared to cases without low-intensity ultrasound application (29). There is much unknown on the mechanisms responsible for the transfer of forces in bone as imparted by loading of minute magnitudes, at high frequencies. It is possible that high frequency vibration attenuate through soft-tissue surrounding the bone mineralized matrix. However, it is also likely that high frequencies are able to survive in the denser mineralized matrix of bone. In this case, our model for the motion of the cell nucleus is a possible mechanism for bone cells to sense high frequency loading.

The increased mRNA expression for COX-2, in relation to increasing frequencies of vibration stress suggests a memory response for high frequency loading. In this study, the vibration stress was applied for only 5 min, however, after 2.5 hours of post-incubation without stress, the upregulation of mRNA expression for COX-2 occurred. Despite a decreased PGE₂ release at high frequencies, after 30 min of post-incubation, subsequent to the 5 min vibration stress, bone cells maintain the capacity for producing PGE₂. This suggests that bone cells compensate for a short-term PGE₂ production (after 30 min) by increasing mRNA levels for COX-2 for a possible delayed response (or memory effect).

Interestingly, bone cells do respond to high frequency vibration stress (i.e., 100 Hz) although fluid shear stresses *in vivo* might involve lower frequencies (7). In this study we have not considered in detail the physical differences of

the effects of fluid shear stress or vibration stress on bone cell deformation. However complicated the transfer of forces at the cellular level might be, in terms of fluid shear stress or vibration stress, our results imply that the joint effect of the frequency and amplitude of loading might play similar roles for different types of stresses on bone cells. Our results suggest that the joint effect of the frequency (at third order) and amplitude (at first order) of loading correlates to the biochemical response of bone cells that contribute to sustained bone metabolism. Furthermore, this response might involve mechanisms that contribute to a specific behavior of bone cells in response to vibration stress enabling recognition of high frequency loading.

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