CHAPTER 3

NITRIC OXIDE PRODUCTION BY BONE CELLS IS FLUID SHEAR STRESS RATE DEPENDENT

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ABSTRACT

Shear stress due to mechanical loading-induced flow of interstitial fluid through the lacuno-canalicular network is a likely signal for bone cell adaptive responses. Moreover, the rate (determined by frequency and magnitude) of mechanical loading determines the amount of bone formation. Whether the bone cells' response to fluid shear stress is rate dependent is unknown. Here we investigated whether bone cell activation by fluid shear stress is rate dependent. MC3T3-E1 osteoblastic cells were subjected for 15 min to fluid shear stress of varying frequencies and amplitudes, resulting in peak fluid shear stress rates ranging from 0 to 39.6 Pa-Hz. Nitric oxide production, a parameter for bone cell activation, was found to be linearly dependent to the fluid shear stress rate; the slope was steepest at 5 min (0.11 Pa-Hz^{-1}), and decreased to 0.03 Pa-Hz^{-1} at 15 min. We conclude that the fluid shear stress rate is an important parameter for bone cell activation.

INTRODUCTION

Bone as a living tissue has long been recognized to be capable of adapting its mass and structure in response to the demands of mechanical loading (Wolff's Law) (1, 2). However, the underlying cellular processes remain poorly understood. It has been postulated that due to loading and unloading, extracellular fluid flows through the lacuno-canalicular network to the bone surface and back (3). Streaming potentials measured on loaded bone confirmed the transfer of ions due to fluid movement in bone (4). The loading-induced movement of labeled molecules of varying sizes directly demonstrated fluid flow in the mineralized matrix of bone, both *in vivo* and *ex vivo* (5, 6). The flow of interstitial fluid through the lacuno-canalicular network induces shear stress on the membrane of bone cells. This shear stress is a likely candidate signal for bone cell adaptive responses (7-10). Bone cells respond to fluid flow stimulation *in vitro* (11-18). They are sensitive to fluid shear stress (16, 18), rather than to streaming potentials mediated by the transport of ions with the flow (18). Bone cells are also more responsive (in terms of nitric oxide (NO) and prostaglandin E_2 production) to shear stress by fluid flow stimulation than to direct mechanical strain by substrate stretching (19, 20). However, how fluid shear stress activates bone cells is not well understood.

The fluid shear stress amplitudes and frequencies in bone can be determined theoretically from known physiological loading parameters. By applying Biot's theory of poroelasticity to bone, the predicted range of *in vivo* fluid shear stress ranges from 0.8 – 3 Pa due to loading-induced strains ranging between 1000 – 3000 με (21, 22). That this range of fluid shear stress magnitudes is enough to stimulate bone cells, was confirmed by *in vitro* studies (11-17).

Several studies have suggested that the rate (determined by the frequency and amplitude) rather than the magnitude alone of the applied loading stimulus correlates to bone formation (23, 24). This implies that bone formation is essentially enhanced by dynamic loading. Thus, both the magnitude (or

amplitude) and the frequency of loading are important parameters for bone formation. Indeed, it has been shown that low magnitude (< 10 με), high frequency (10 – 100 Hz) loading is capable of stimulating bone growth and inhibiting disuse osteoporosis (25). Furthermore, it has been shown that high-amplitude, low-frequency stimuli are rare in the activities of daily life, whereas high-frequency, low-amplitude stimuli are common (26). High rates of loading, as in high impact physical activity including jumps in unusual directions, have a great osteogenic potential in humans (27) and in osteopenic ovariectomized rats (28). High stress rate, as in step-wise increased fluid shear stress, was also shown to stimulate neonatal rat calvarial bone cells (29). Therefore, the osteogenic response to high impact activity might be related to the response of bone cells to a sudden increase (i.e., high rate) in fluid shear stress. The rate of loading seems to be a decisive factor in bone formation and maintenance. However, how bone cells respond to the rate of loading is not well understood.

In this study, we investigated the response of bone cells to varying rates of fluid shear stress. We used NO production as a parameter for bone cell activation since it is an early mediator of mechanical loading-induced bone formation (30). Furthermore, NO has been shown to be essential to adaptive bone formation *in vivo* (31). To our knowledge this is the first study that aimed at a quantitative relationship between bone cell activation and the rate of fluid shear stress.

MATERIALS AND METHODS

Bone cell cultures

MC3T3-E1 cells were cultured up to near-confluency in 75 cm^2 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 μg/ml; Merck, Darmstadt, Germany), β-

glycerophosphate disodium salt hydrate (10 mM; Sigma, St. Louis, MO, USA), l-glutamine (300 μg/ml; Merck), gentamycine (50 μg/ml; Gibco), fungizone (1.25 μg/ml; Gibco). Cells were then harvested and seeded at $2x10^5$ cells per polylysine-coated (50 μg/ml; poly-L-lysine hydrobromide; Sigma) glass slide (5 cm^2), and incubated overnight in α-MEM with 10% FBS to promote cell attachment, at 37°C with 5% CO_2 in air, prior to fluid shear stress experimental treatment as described below.

Calculation of frequency spectra after loading

To determine the precise flow regimes to stimulate bone cells (as described under Fluid shear stress), we first calculated the frequency spectra after loading of bone (Fig. 1). We used the results from the studies of Bergmann et al. (32), in which forces on a human hip were measured using strain sensors. The frequency spectra of the resultant forces on the hip showed a rich harmonic content ranging between 1-3 Hz for walking cycles, and reaching 8-9 Hz for running cycles (Fig. 1).

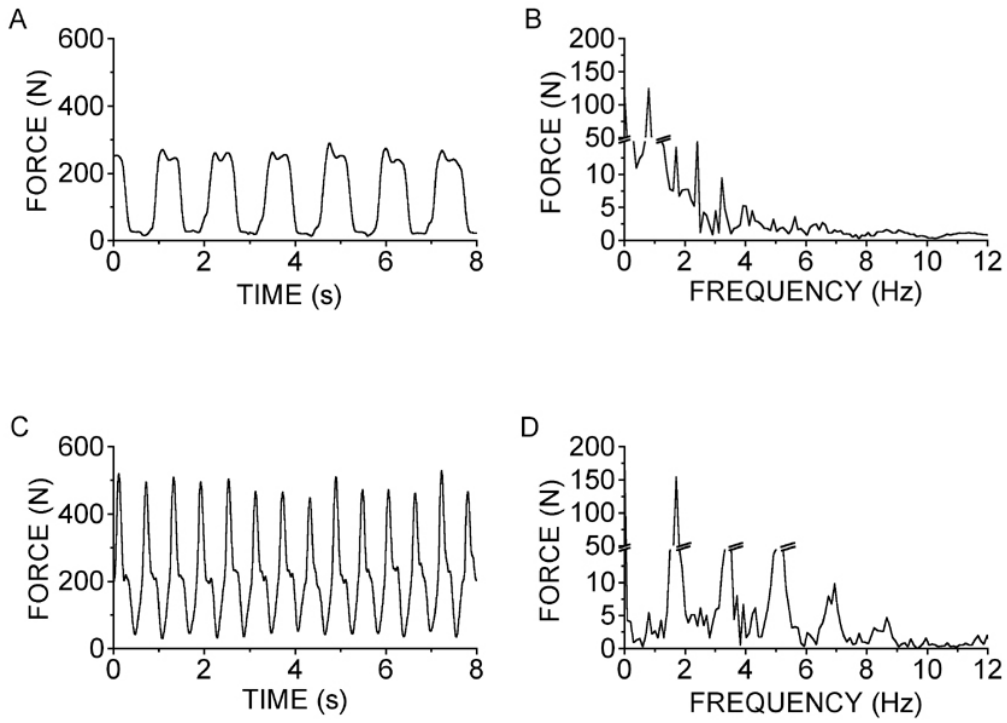

Figure 1. Hip joint forces. A. Resultant force on the left hip joint of a male human subject (62 kg,) as a function of time, measured by implanted sensors inside a femur at normal walking on a treadmill (2 km/h). B. The frequency spectrum of the resultant hip joint force due to normal walking. C. Resultant force on the hip as a function of time at jogging pace on a treadmill (8 km/h). D. The frequency spectrum of the resultant force due to jogging pace. Data taken with permission from Bergmann and co-workers (25).

Fluid shear stress

Pulsating fluid shear stress was generated using a flow apparatus containing a parallel plate flow chamber (PPFC) as described earlier (14, 33, 34). Fluid shear stress was induced on the monolayer of cells attached to the polylysine-coated glass slide serving as the bottom of the parallel plate flow chamber, by circulating 9 ml of α-MEM containing 2% FBS plus supplements as described above using a computer-controlled micro annular gear pump (developed by HNP Mikrosysteme GmbH, Parchim, Germany). Precise flow regimes (see Table 1 and Fig. 2) were implemented by controlling the pump using

computer-mediated instrumentation by LabView™ (National Instruments Corp., Austin, Texas, USA). The flow through the PPFC was monitored online using a small animal blood flowmeter (T206, Transonic Systems Inc., Ithaca, New York, USA).

Table 1. *Data of applied pulsating fluid shear stress (PFSS) regimes*

Regime	5 Hz lo	5 Hz hi	9Hz lo	9Hz hi	steady
mean shear stress (Pa)	0.70	0.70	0.70	0.70	0.70
pulse amplitude (Pa)	0.31	0.70	0.31	0.70	0
*peak fluid shear stress rate (Pa-Hz)	9.70	22.0	17.5	39.6	0
average flow (ml/min)	8.30	8.30	8.30	8.30	8.30

lo, low amplitude; hi, high amplitude; steady, steady flow.
*peak fluid shear stress rate = pulse amplitude x frequency x (2π).

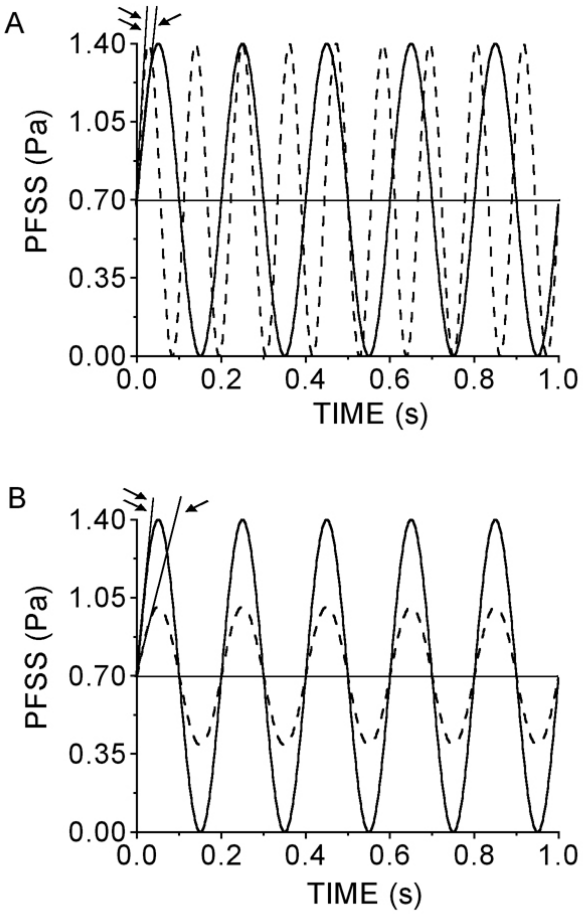

Figure 2. Fluid shear stress regimes applied to bone cells for 15 min. Only 1 s duration of the regimes are shown. A. The slope of the lines equals the peak fluid shear stress rate. The rate increases with higher flow regime frequency (double arrows, 9 Hz high amplitude; single arrow, 5 Hz high amplitude; solid line, 5 Hz; dashed line, 9 Hz). The steady flow regime is constant at 0.7 Pa, hence having zero rate. B. The peak fluid shear stress rate is also varied by changing the amplitude from a 0.31 Pa to 0.70 Pa (double arrows, 5 Hz high amplitude; single arrow, 5 Hz low amplitude; solid line, high amplitude; dashed line, low amplitude).

During the shear stress experiment the flow apparatus was placed in a 37°C incubator, and connected to a gassing system that maintained a pH of 7.4 in the medium using 5% CO_2 in air. For the PFFS experiments, the culture medium was changed to α-MEM with 2% FBS plus supplements as described above (fluid viscosity = 0.69 x 10^{-4} Pa-s). Cells were incubated for 15 min in the

presence of: 1) steady fluid shear stress (0.70 Pa), 2) low amplitude pulsating fluid shear stress (PFSS, 0.70±0.31 Pa) at two different frequencies (5 Hz or 9 Hz), and 3) high amplitude pulsating fluid shear stress (0.70±0.70 Pa, 5 Hz or 9 Hz), 4) no shear stress (static controls) (Table 1). Medium samples were taken at 5, 10 and 15 min for measuring the response to fluid flow parameterized as production of NO in the medium.

The PPFC was designed such that it is capable of inducing high frequency fluid shear stress on the cells (33). The PPFC geometry and the flow frequencies that were utilized allowed for the use of the formula: $\tau = 6\mu Q/(bh^2)$ under dynamic flow. This formula is used to calculate the peak fluid shear stress τ (acting on the cells), due to a flow Q, in a chamber of width b and plate separation h, with a fluid of viscosity μ.

Nitric Oxide determination

The conditioned medium was assayed for NO, which was measured as nitrite (NO_2^-) accumulation in the conditioned medium, using Griess reagent (1% sulfanilamide, 0.1% naphtylethelene-diamine-dihydrochloride, and 2.5 M H_3PO_4). The absorbance was measured at 540 nm. NO concentrations were determined using a standard curve derived from known concentrations of $NaNO_2$ in non-conditioned culture medium. Data from separate experiments were collected and normalized with respect to static controls, and expressed as treatment-over-control (T/C) ratios, or as absolute values where indicated.

Statistics

Data were pooled from the results of at least five experiments for each fluid shear stress regime tested (Table 1). The effects of treatment with fluid shear stress were analyzed with the non-parametric Wilcoxon rank sum test of the S-Plus 2000 package (release 1). Differences were considered significant at a p value <0.05. The relation between NO production and the peak rate of fluid shear stress was analyzed by linear regression.

RESULTS

Application of fluid flow for 15 min to the MC3T3-E1 cells did not result in visible changes in cell shape or alignment of the cells in the direction of the fluid flow (data not shown). No cells were removed by any of the fluid shear stress regimes, as assessed by visually inspecting the cultures before and after fluid shear stress treatment, and by measuring the total amount of DNA (control, 261 ± 19 ng; PFSS, 279 ± 27 ng; mean ± SEM of 6 experiments).

The NO response of MC3T3-E1 cells to a fluid shear stress with zero rate was first studied separately, by culturing cells under steady flow conditions (Table 1, Fig. 3). MC3T3-E1 cells treated with a steady fluid shear stress of 0.70 Pa showed a significantly increased NO release as compared to static control cultures within the first five minutes of treatment (Fig. 3A), but not thereafter. The 2-fold increase in NO production in response to steady shear stress remained significant during the 15 minute treatment period (Fig. 3B).

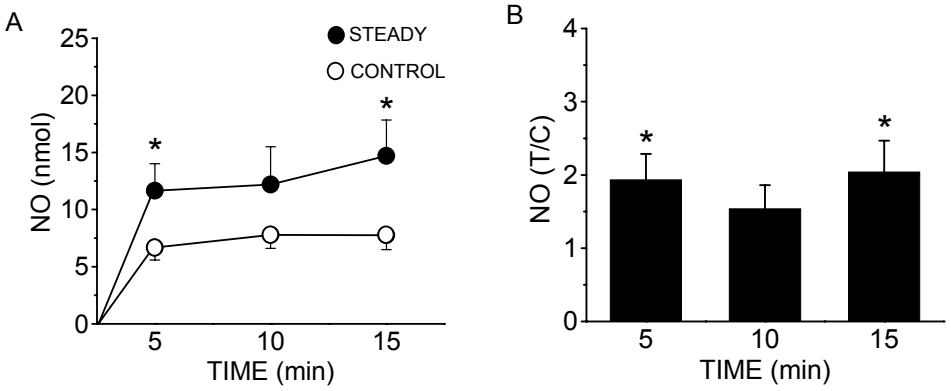

Figure 3. Effect of steady fluid shear stress on the NO production by MC3T3-E1 bone cells. A. The absolute amount of NO production by bone cells subjected to steady fluid shear stress, increased after 5 min and continued thereafter as compared to control cultures not subjected to flow. B. Treatment-over-control ratios showed that the response to steady shear stress increases 2-fold throughout 15 min. *$p < 0.05$.

To study the effect of the fluid shear stress amplitude and frequency on the NO response of the bone cells, they were subjected to varying pulsating fluid shear stress (PFSS) regimes (see Table 1). The NO response to PFSS became higher with increasing shear stress amplitude after 5, 10 and 15 min of treatment (Fig. 4). In addition, the NO response was more pronounced at higher frequencies (Fig. 4). Treatment with 5 Hz lo (low amplitude; see Table 1) elicited a comparable 2-fold increase in NO response by the cells as steady fluid shear stress within the first 5 min (Fig. 4). Treatment with 9 Hz lo caused a rapid 3-fold increase of NO after 5 min, as did treatment with 5 Hz hi (high amplitude; see Table 1). Treatment with 9 Hz hi elicited the highest, 5-fold, increase in NO production after 5 min (Fig. 4). So, high amplitude PFSS (5 Hz or 9 Hz hi) elicited a higher level of NO production than low amplitude PFSS (Fig. 4). Similarly, high frequency PFSS (9 Hz) treatment elicited a higher NO response by bone cells than low frequency PFSS (5 Hz), despite having the same fluid shear stress average. Thus, both the applied frequency and amplitude of PFSS affected the response of MC3T3-E1 bone cells.

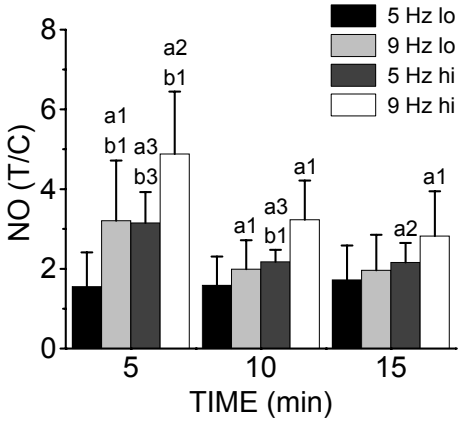

Figure 4. Effect of PFSS frequency and amplitude on the NO production by MC3T3-E1 bone cells during 15 min. NO production was between 0.31 to 0.90 μM per $2x10^5$ cells for all cell cultures without fluid shear stress treatment. The effect of fluid shear stress on NO production is maximal within 5 min of treatment for all PFSS regimes. Treatment with high amplitude (or frequencies) elicited higher NO production than low amplitude (or frequencies). Values are mean treatment-over-control ratios (T/C)±SEM. lo, low amplitude (0.31 Pa); hi, high amplitude (0.70 Pa). [a]Significantly different from 1.0: a1, $p < 0.05$; a2, $p < 0.01$; a3, $p < 0.006$. [b]Significantly different from 5 Hz low amplitude: b1, $p < 0.05$; b2, $p < 0.01$; b3, $p < 0.006$.

Next we tested whether there was a correlation between the fluid shear stress rate and the NO response of the bone cells (Fig. 5). At 5 min MC3T3-E1 bone cells reacted to the application of a fluid shear stress rate of 21.99 Pa-Hz (5 Hz hi) with a 3-fold enhanced NO production ($p<0.02$), similar to the bone cell's response to a comparable shear rate of 17.53 Pa-Hz (9 Hz lo) (Fig. 5A). At 5 and 10 min the NO production was linearly related with the peak rate of the PFSS (Fig. 5A,B). At 15 min there was still a trend of a linear relationship (Fig. 5C). This means that a higher PFSS rate induced a higher NO production.

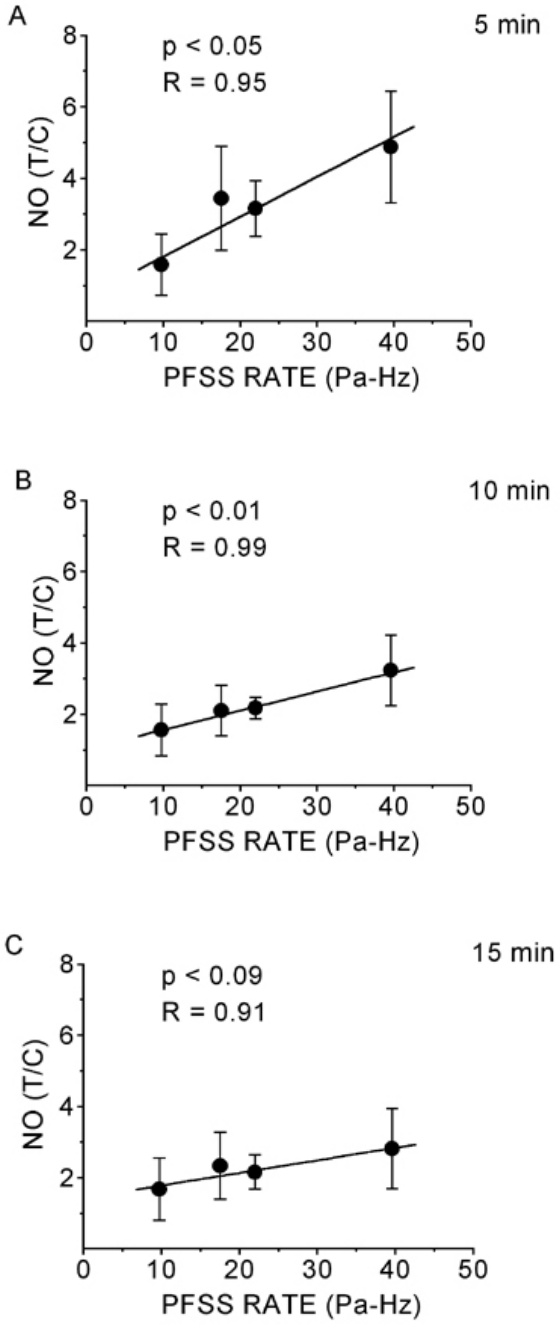

Figure 5. Nitric oxide production by bone cells is linearly proportional to the rate of fluid shear stress. The steepest slope was found at 5 min (0.11 Pa-Hz^{-1}), indicating that the highest bone cell response to fluid shear stress rate occurs rapidly. At 10 and 15 min, NO levels were lower than those found at 5 min. Values are mean treatment-over-control ratios (T/C)±SEM.

The slope of the linear curve relating the NO production to the PFSS rate indicates the magnitude of the bone cell response (Fig. 5A-C). The slope was 0.11 Pa-Hz^{-1} (or 2-fold per 20 Pa-Hz) at the 5-min time point (Fig. 5A), and decreased to 0.05 Pa-Hz^{-1} and 0.03 Pa-Hz^{-1} at the 10-min and 15-min time points respectively (Fig. 5B,C). Therefore, the NO production response to PFSS by bone cells was highest immediately after 5 min after the onset of flow (Fig. 5).

DISCUSSION

The present study suggests that the bone cell response to fluid shear stress is rate- dependent. Increasing either shear stress amplitude or frequency, without increasing the average stress, enhanced NO production by bone cells *in vitro*. Bone cells responded similarly to similar rates of fluid shear stress, despite different frequencies (5 Hz, 21.99 Pa-Hz or 9 Hz, 17.53 Pa-Hz). Hence, a useful parameter for predicting a proportional increase of NO production of bone cells by fluid flow is the peak rate of fluid shear stress, which is proportional to the product of the fluid shear stress amplitude and frequency.

Our results support the notion that bone formation is stimulated by dynamic rather than static loads (34), and that low magnitude, high frequency mechanical stimuli may be as stimulatory as high-amplitude, low frequency stimuli. Low amplitude, high frequency loading in bone has been shown to occur more often in normal daily activities *in vivo* (26). Since we showed that bone cells are responsive to the rate of loading, high frequency, low amplitude strain-derived fluid shear stresses might be enough to stimulate the cells. This could then result in adaptive bone formation. Hence, the finding that the bone cell's response to fluid shear stress is rate dependent provides an explanation why adaptive bone formation can occur despite the sporadic occurrence of high amplitude strains in daily life (26). The application of vibration on bone *in vivo*

and bone cells *in vitro* has been shown to elicit adaptive responses (35, 36). Vibration, or low amplitude, broad frequency spectrum stimulation, was shown to enhance the osteogenic response of bone *in vivo* (35). In addition, osteoblasts cultured on collagen gels, were shown to be more sensitive to low amplitude, broad frequency vibration than sinusoidal strain alone (36). In these studies vibratory stimulation on bulk specimen did not account for the direct forces acting on bone cells (35, 36), while in our current investigation we directly applied a homogenous fluid shear stress on a monolayer of bone cells. Therefore our study provides a cellular basis for explaining the role of the rate of loading on bone formation as observed *in vivo* (23, 24, 37).

Steady, non-dynamic shear stress provides a zero shear rate, but in the present study it elicited, like dynamic fluid shear stress regimes, also a significant NO response by bone cells. However, when the steady shear stress was applied, the cells experienced a sudden increase of fluid shear stress from 0 to 0.70 Pa by switching on the pump. This sudden increase in stress gave a very high rate of stress, which probably was sufficient to elicit a strong response by the bone cells. This also confirms our finding that the cellular response to shear stress is rate-dependent.

The NO production by bone cells in response to fluid shear stress was rapid (i.e., within 5 min). The slope relating bone cell activation and fluid shear stress rate was highest after 5 min of stimulation, and decreased afterwards. The slope of NO production against the peak PFSS rate indicates how much NO is produced per unit of peak PFSS rate. Since this slope was highest within the first 5 min of stimulation (2-fold increase of NO production per 20 Pa-Hz), bone cells were responsive to PFSS quite rapidly and the response declined thereafter. This suggests that a continuous bone cell stimulation might not be necessary to maintain bone mass and structure. Hence, the rare occurrence of high strains *in vivo* is not necessarily predictive of bone loss. The rapid NO response by bone cells to fluid shear stress indicates a possible mechanism by

which bone can make use of rarely occurring high amplitude strains to maintain proper metabolism.

Based on both the rapid and rate dependent response of bone cells to fluid shear stress, bone loss after prolonged bed rest (37) or microgravity (38) might depend on other factors aside from the lack of loading itself. Our results might suggest that if that bone cells do not require continuous loading to maintain bone mass and structure, then sporadic bouts of exercise should fully counteract bone loss under conditions of prolonged unloading. However, full recovery from bone loss does not always occur (39, 40). Thus, the observation of bone loss after prolonged bed rest or under microgravity suggests another cellular mechanism that might be impaired under such conditions (41).

Our finding that the bone cell response to fluid shear stress, in terms of NO production, is rapid and rate-dependent, might further the description and prediction of bone loss by existing theoretical models (42, 43). Although these models considered cellular activity, a truly dynamic simulation of adaptive bone formation, incorporating how fast bone cells produce signaling molecules, and build or resorb bone has yet to be attempted. Our results might provide a basis for a dynamic simulation of adaptive bone formation for predicting bone loss under environments of unloading as in prolonged bed rest or microgravity.

The transfer of forces from the effects of loading on the whole bone tissue, down to the cellular level might be complex, however our results clearly indicate that dynamic loading plays an essential role for the activation of bone cells. We conclude that NO production by bone cells subjected to fluid flow is linearly dependent on the rate of fluid shear stress. Both the amplitude and frequency of stress contribute to the flow-induced activation of bone cells.

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