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Effects of short-term recovery periods on fluid-induced signaling in osteoblastic cells

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Abstract

It is well known that cyclic mechanical loading can produce an anabolic response in bone. In vivo studies have shown that the insertion of short-term recovery periods (10–15 s) into mechanical loading profiles led to an increased osteogenic response compared to continuous cyclic loading of bone. Although this is suggestive of temporal processing at the bone cell level, there is little evidence to support such a hypothesis. Therefore, the current study investigated the cellular mechanism of bone's response to rest inserted vs. continuous mechanical loading. Cell responses to rest inserted mechanical loading were quantified by applying oscillatory fluid flow (OFF) to osteoblastic cells and quantifying real-time intracellular calcium $[Ca^{2+}]_i$, prostaglandin E_2 (PGE_2) release, and osteopontin (OPN) mRNA levels. Cells were exposed to OFF (1 Hz) at shear stresses of 1 and 2 Pa with rest periods of 5, 10, and 15 s inserted every 10 loading cycles. The insertion of 10 and 15 s rest periods into the flow profile resulted in multiple $[Ca^{2+}]_i$ responses by individual cells, increased $[Ca^{2+}]_i$ response magnitudes, and increased overall percent of cells responding compared to continuously loaded control groups. We determined the source of the multiple calcium responses to be from intracellular stores. In addition, rest inserted OFF led to similar levels of PGE_2 release and increased levels of relative OPN mRNA compared to cells exposed to continuous OFF. Our study suggests that the cellular mechanism of bone adaptation to rest inserted mechanical loading may involve modulation of intracellular levels of calcium (frequency, magnitude, percent of cells responding).

Keywords: Mechanotransduction; Osteoblast; Recovery; Calcium signaling; Bone adaptation; Oscillatory fluid flow; Osteopontin; PGE_2

1. Introduction

Bone adaptation to mechanical loading depends on the duration and the magnitude of the applied loads (Rubin and Lanyon, 1984; Forwood and Turner, 1994; Forwood et al., 1996). However, application of continuous dynamic mechanical loads in vivo in animal models suggests that the osteogenic response of bone saturates with continued long-term mechanical loading (Turner et al., 1994; Umemura et al., 1997; Turner, 1998). The mechanisms behind bone's osteogenic saturation in response to loading are not well understood. Several in vivo studies have shown that bone can recover its responsiveness and is able to respond to mechanical stimuli with the same magnitude as earlier exposures to loading with the insertion of appropriate length rest periods (Forwood et al., 1996; Robling et al., 2000). This suggests that recovery periods, during which mechanical loading is halted, would restore bone's sensitivity to mechanical stimuli. Modulating

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mechanical loading parameters such as duration and magnitude may be a key factor in allowing bone to recover its mechanosensitivity. Understanding the mechanism required to restore mechanosensitivity in bone will be important for optimizing the osteogenic potential of mechanically induced bone formation.

The recovery phenomenon has been explored in vivo by inserting rest periods of various lengths into mechanical loading protocols. When bone is loaded, the application of short-term rest periods (e.g. on the order of seconds) into loading profiles led to an increased osteogenic response compared to continuously loaded bone. Robling et al. (2000) found that, in a four-point bending model of the rat tibia, the insertion of a 14 s recovery period between loading cycles led to significantly higher relative bone formation rates compared to continuously loaded limbs. Using two distinct in vivo models of bone adaptation Srinivasan et al. (2002) found that, although continuous low-magnitude loading was a non-osteogenic stimulus, the insertion of a 10 s rest period between each loading cycle resulted in a potent anabolic response. These studies show that short-term recovery periods introduced into mechanical loading regimens in vivo can have an osteogenic effect at the tissue level. However, the cell-level response to rest inserted loading has not been characterized.

Data from a large number of experimental and theoretical studies have shown that bone cells residing in intact matrix may be activated to initiate bone adaptation processes by interstitial fluid flow through the lacuno-canalicular network (Weinbaum et al., 1994; Cowin et al., 1995; Knothe Tate et al., 2000). While arterial pressure may drive steady flow through the lacuno-canalicular network, mechanical loading induced fluid flow is oscillatory due to the cyclic nature of the applied loads in vivo. Therefore, in the present study, oscillatory fluid flow (OFF) was used to investigate the cellular mechanisms which may underlie bone's enhanced osteogenic response to rest inserted mechanical loading.

How bone cells temporally process mechanical stimuli is still largely unknown. Bone cells respond to mechanical stimuli with a variety of biological signals, and a number of these signals have been utilized as measures of their responsiveness to OFF (You et al., 2000, 2001; Kurokouchi et al., 2001). It is likely that second messenger systems are involved in filtering the wealth of mechanical information experienced by these cells in vivo (Brand and Stanford, 1994). These second messengers, which can act in a matter of seconds, then likely influence intermediate and late-stage downstream events (such as gene expression and protein synthesis) leading to a tissue-level response. In this study we examined the effects of rest inserted loading on osteoblastic cells by looking at early- (second messenger), intermediate-, and late-stage cellular events.

In the signaling cascade that occurs in bone cell mechanotransduction, one of the earliest events is intracellular calcium (Ca^{2+}) mobilization. Ca^{2+} is a ubiquitous second messenger molecule that plays a role in many physiological processes (Berridge et al., 1998). As a second messenger, it has the ability to transduce extracellular signals (i.e. physical changes) to the cell interior and potentially to the genome. Previous in vitro studies have shown the application of OFF to bone cells results in a rapid transient increase in intracellular calcium $[Ca^{2+}]_i$, and that this Ca^{2+} mobilization is required for the OFF-induced upregulation of osteopontin (OPN) mRNA (Jacobs et al., 1998; You et al., 2000, 2001).

An intermediate event in bone cell mechanotransduction is prostaglandin E_2 (PGE_2) synthesis and release. PGE_2 is released by osteoblastic cells in response to mechanical loading and has been implicated in regulating bone turnover in vivo (Nolan et al., 1983; Feyen et al., 1984; Rodan et al., 1986; Imamura et al., 1990). Prostaglandins can regulate bone turnover by both stimulating bone formation and regulating bone resorption (Dietrich et al., 1975; Raisz and Fall, 1990; Jee et al., 1991). Previous studies have shown that bone cells respond to OFF with increases in PGE_2 release (Donahue et al., 2003a, b; Saunders et al., 2003).

To examine the late-stage response of osteoblastic cells to rest inserted OFF, we used OPN mRNA levels as an osteogenic marker. OPN is an important non-collagenous bone matrix protein that is thought to be involved in mechanically induced bone remodeling in vivo (Terai et al., 1999). OPN is not only a structural protein, but also one that is involved in regulating bone cell attachment, osteoclast function, and bone remodeling. In osteoblastic cells, we have shown that OPN gene expression is upregulated in response to OFF via intracellular calcium mobilization (You et al., 2001).

In this study, bone cells were examined by quantifying intracellular calcium mobilization, PGE_2 release, and OPN mRNA levels in response to rest inserted OFF. Since in vivo experiments have shown that the insertion of short-term recovery periods into mechanical loading protocols enhanced osteogenesis, we inserted similar length recovery/rest periods ranging from 0 to 15 s into the OFF loading profiles of osteoblastic cells. In addition, we addressed the possibility that the effect of inserted rest periods might depend on the load magnitude by conducting experiments at shear stresses of both 1 and 2 Pa. Finally, we examined the effect of rest inserted OFF on events downstream of Ca^{2+} mobilization by quantifying PGE_2 release 1 h after OFF and OPN mRNA levels at 24 h post flow. We hypothesized that cells exposed to rest inserted OFF would have increased $[Ca^{2+}]_i$ signaling when compared to cells exposed to continuous OFF. Furthermore, rest inserted OFF would impact downstream events by modulating two osteoblastic markers of bone anabolism: PGE_2 release and relative OPN mRNA.

2. Methods

2.1. Cell culture

MC3T3-E1 osteoblastic cells were cultured in minimal essential alpha medium (MEM-α, Life Technologies Inc., Rockville, MD) containing 10% fetal bovine serum (FBS) (ATCC, Manassas, VA), 1% penicillin and streptomycin (Life Technologies, Inc.) and maintained at 37 °C and 5% CO_2 in a humidified incubator. For calcium imaging experiments, cells were subcultured on UV transparent quartz slides ($76 \times 26 \times 1.6$ mm) at 100,000 cells/slide. For PGE_2 and gene expression experiments, cells were subcultured on glass slides ($76 \times 48 \times 1$ mm) at 300,000 cells/slide. Fluid flow was applied 48 h following subculture such that cells were 80–90% confluent at the time of experimentation.

2.2. Oscillatory fluid flow (OFF)

A previously described fluid flow device was used to deliver laminar OFF to MC3T3-E1 osteoblastic cells (Jacobs et al., 1998). Flow was driven by a Hamilton glass syringe in series with rigid walled tubing, and a parallel plate flow chamber. The syringe was mounted in and driven by an electromechanical loading device (EnduraTec, Eden Prarie, MN). The flow rate was monitored with an ultrasonic flow meter (Transonic Systems Inc., Ithaca, NY). The flow rate was selected to yield peak sinusoidal shear stresses of 2 and 1 Pa at 1 Hz. During the calcium imaging experiments, cells were exposed to a total of 3 min of oscillating fluid flow at 2 Pa with rest periods of 0 (control), 5, 10, and 15 s inserted every 10 loading cycles (Fig. 1). These experiments were repeated at a lower shear stress of 1 Pa with all experimental groups having a minimum of $n = 149$ cells and three slides. For PGE_2 and OPN gene experiments cells were exposed to 1 h of oscillating fluid flow at 1 Pa with either 0 (control), or 10 s rest periods inserted into every 10 loading cycles.

2.3. PGE-2 release

Immediately following fluid flow exposure, cell seeded slides were removed from the flow chambers, placed in a sterile petri dish with 1 ml of fresh media, and returned to the incubator for 1 h. Next, media samples were collected and centrifuged at 12,000g to remove any particulate matter. Supernatant PGE_2 levels were measured using a commercially available enzyme immunoassay kit (Amersham Biosciences, Piscataway, NJ). PGE_2 levels were normalized to total protein as determined by a BCA assay (Pierce, Rockford, IL).

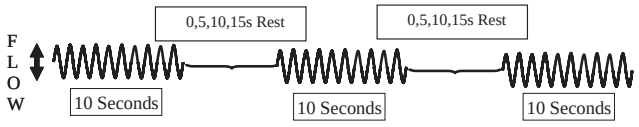

Fig. 1. Cells were exposed to a total of 3 min of OFF in 10 s bouts with rest periods of 0 (continuous flow), 5, 10, and 15 s inserted between bouts. Experiments were conducted using peak sinusoidal shear stresses of 1 and 2 Pa.

2.4. Osteopontin mRNA analysis

OPN mRNA levels were quantified using quantitative real-time reverse transcriptase polymerase chain reaction as described previously (You et al., 2000, 2001). Twenty-four hours following mechanical stimulation, total RNA was extracted from cells following lysis and homogenization (RNeasy mini kit, Qiagen Inc., Valencia, CA). Mouse OPN cDNA primers and probes were designed using sequence data from Miyazaki et al. (1990) and probe/primer design software (Primer Express, Applied Biosystems, Fostor City, CA). Relative changes in OPN mRNA and 18S rRNA were quantified using the ABI PRISM 7900 sequence detection system (Applied Biosystems, Fostor City, CA).

2.5. Calcium imaging

Real-time intracellular levels of calcium were quantified using a ratiometric imaging technique. Prior to exposure to OFF, cells were incubated with 10 μM Fura-2 AM (Molecular Probes, Eugene OR) for 30 min at 31 °C (to reduce dye compartmentalization), then washed with fresh MEM-α and 2% FBS. Following Fura-2 loading, the cell seeded quartz slides were mounted in a parallel plate flow chamber and fixed to the stage of a fluorescent microscope. For 30 min, the cells were left undisturbed. The flow media consisted of MEM-α and 2% FBS. All experiments were performed at room temperature to reduce Fura-2 AM dye compartmentalization. Ratio values were converted to $[Ca^{2+}]_i$ values using image analysis software (Metafluor, Universal Imaging, West Chester, PA) with a calibration curve derived from a series of standard $[Ca^{2+}]_i$ solutions. A cell response was defined as a transient increase in $[Ca^{2+}]_i$ of at least 4 times the maximum oscillation recorded during the 3 min preflow baseline period.

2.6. Data analysis

For calcium imaging experiments, we quantified the peak magnitude of response, the percentage of cells responding, and the percent of cells exhibiting multiple responses (two or more). PGE_2 data were normalized to total protein and OPN mRNA levels were normalized to 18 s rRNA levels. Data are expressed as mean ± SEM. To compare observations from continuous flow and rest inserted flow, a two-sample Student's t test was used

where sample variances were not assumed to be equal. To compare observations from more than two groups, statistical analysis using one-way ANOVA and Fisher's Protected Least Significant Difference was utilized (Statview, SAS Institute, San Francisco, CA). A significance level of 0.05 was employed for all statistical analyses.

3. Results

3.1. Early response-intracellular calcium mobilization

3.1.1. High flow (shear stress=2 Pa)

At the onset of flow, cells responded with a transient increase in intracellular calcium concentration ranging from 100–300 nM (Fig. 2A,B). Insertion of 10 and 15 s rest periods, but not 5 s rest periods, resulted in $[Ca^{2+}]_i$ response magnitudes that were significantly higher than control ($p<0.005$ for both 10 and 15 s vs. control) (Fig. 3A). A significantly higher percentage of cells responded to flow in the 15 s rest group vs. the control group, but not the 5 and 10 s groups vs. control ($p<0.05$ for 15 s vs. control) (Fig. 3B). The insertion of rest periods into the OFF profile allowed some cells to recover their mechanosensitivity and, as a result, many cells responded multiple times during the flow protocol. Typically, $[Ca^{2+}]_i$ transients that occurred after the initial response to flow were smaller in magnitude than the initial calcium transients (Fig. 2B). The percent of cells exhibiting multiple responses (2 or more) during

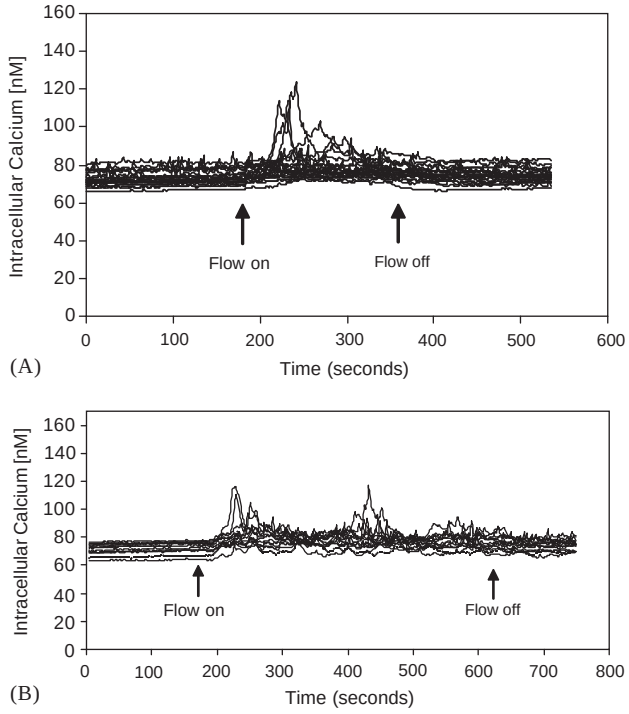

Fig. 2. Typical $[Ca^{2+}]_i$ time history traces for continuous oscillatory flow control cells (A) vs. cells exposed to oscillatory flow with a 15 s rest period inserted every 10 loading cycles (B). The duration of (B) is longer due to the inserted rest periods. Control cells (A) typically exhibited one initial response to flow then returned to baseline levels. However, cells exposed to rest inserted flow (B) exhibited multiple oscillations. In these cells the initial response to flow was the largest in magnitude and was followed by as many as six subsequent low-magnitude responses.

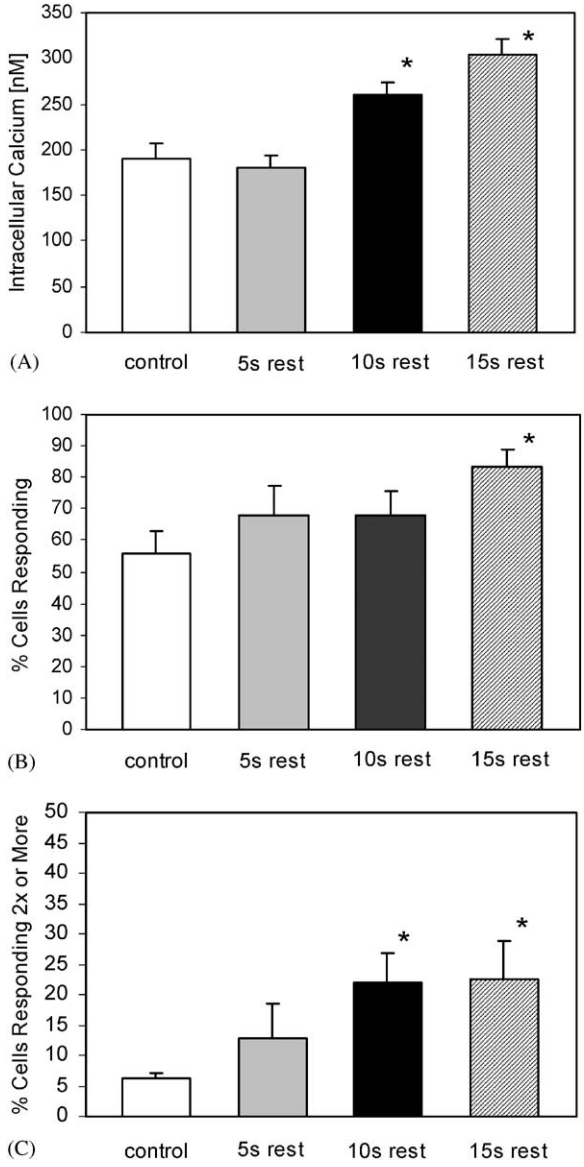

Fig. 3. High-flow results (shear stress = 2 Pa). (A) Insertion of 10 and 15 s rest periods into the loading profile resulted in higher peak response magnitudes compared to cells exposed to continuous oscillatory flow (0 s rest control). (B) Only the 15 s rest inserted group had a significantly greater percent of cells responding to flow than control. (C) Cells exposed to flow with 10 and 15 s rest periods inserted into the loading exhibited multiple responses to flow. This is quantified by taking the percent of cells that responded 2 or more times during exposure to the 3 min flow. A higher percentage of cells in the 10 and 15 s rest inserted groups exhibited multiple responses than the continuous flow controls. Data are mean ± SEM. $n \geq 5$ slides for all groups. (*) Significant difference from control ($p<0.05$).

flow was significantly higher in the 10 and 15 s rest groups but not the 5 s group compared to control ($p<0.001$ for 10 and 15 s vs. control) (Fig. 3C).

3.1.2. Low flow (shear stress=1 Pa)

Cells exposed to the low shear stress responded similarly to cells in the high-flow experiment with a transient increase in $[Ca^{2+}]_i$ following the onset of flow. However, the application of a lower shear stress resulted in response magnitudes that were generally lower than the high-flow experiments (50–300 nM). Only the insertion of a 10 s rest period resulted in a $[Ca^{2+}]_i$ response magnitude that was significantly greater than control ($p<.005$ for 10 s vs. control) (Fig. 4A). In contrast to the high-flow experiments, a significantly greater percentage of cells responded in *both* the 10 and 15 s rest groups compared to control ($p<0.01$ for 10 and 15 s vs. control) (Fig. 4B). In addition, 10 s rest inserted OFF resulted in significantly higher response magnitudes and percentage of cells responding compared to 15 s rest inserted OFF ($p<0.001$). The application of rest inserted OFF resulted in enhanced mechanosensitivity as many cells responded multiple times to flow with repeated transients that were smaller in magnitude than the initial response. The percent of cells exhibiting multiple responses (two or more) during flow was significantly higher in the 10 and 15 s rest groups but not the 5 s group compared to control ($p<0.05$ for 10 and 15 s vs. control) (Fig. 4C).

3.2. Source of intracellular calcium

To investigate the source of the multiple calcium responses that occurred in cells exposed to rest inserted flow, we used the pharmacological agent thapsagargin which empties intracellular calcium stores. Cells were pretreated with 50 nM thapsagargin 30 min prior to flow and then exposed to a 10 s rest inserted flow profile as described above. Thapsagargin treatment blocked cells from responding multiple times to rest inserted OFF as well as reduced the overall magnitude of the initial response (Fig. 5).

3.3. Intermediate response—PGE_2 release

Both 1 h of continuous OFF and 1 h of rest inserted OFF resulted in greater than 3-fold PGE_2 release compared to no flow controls. No significant difference in PGE_2 release was detected between continuous OFF and rest inserted OFF (Fig. 6).

3.4. Late-stage response—OPN mRNA

Relative OPN mRNA quantification at 24 h following 1 h of OFF indicated a 4-fold increase in OPN mRNA of cells exposed to 10 s rest inserted OFF compared to cells exposed to continuous OFF (Fig. 7).

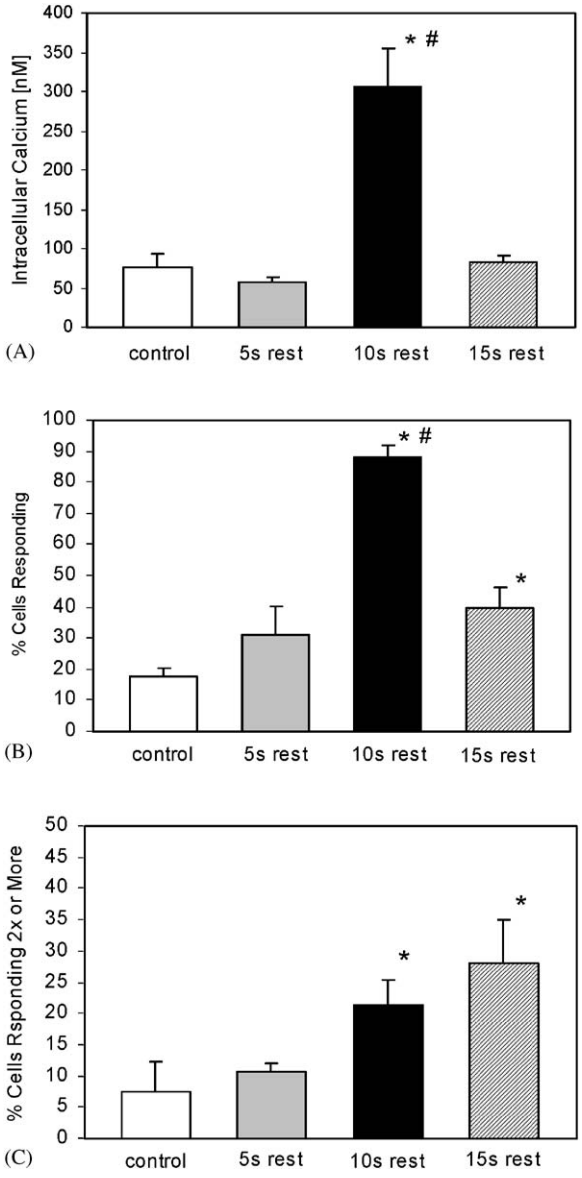

Fig. 4. Low-flow results (shear stress = 1 Pa). (A) The insertion of a 10 s rest period into flow resulted in response magnitudes that were 3-fold higher than continuous oscillatory flow controls. (B) Insertion of 10 and 15 s rest periods into the flow profile led to significantly higher percent of cells responding compared to control. (C) 10 and 15 s rest periods led to cells responding multiple times at a higher percentage than controls (similar to high-flow results). Data are mean ± SEM. $n \geqslant 3$ for all groups. (*) Significant difference from control ($p<0.05$), (#) significant difference from 15 s rest group ($p<0.001$).

4. Discussion

Previous in vivo studies have shown that bone adaptation to dynamic mechanical loading can potentially be enhanced by the insertion of short-term

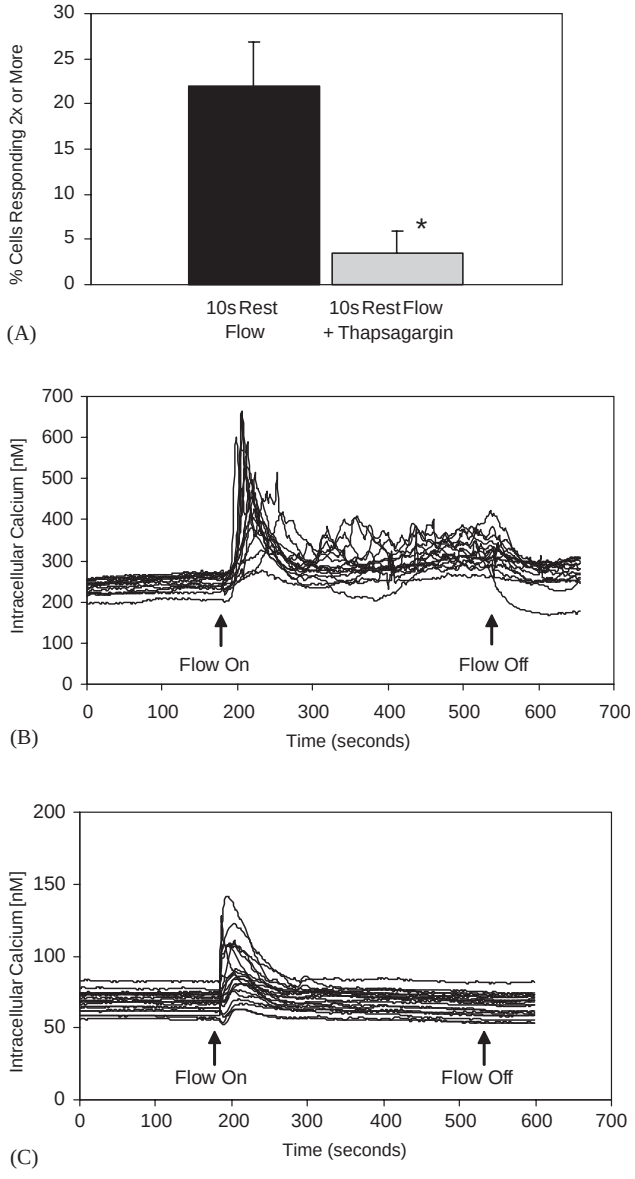

Fig. 5. (A) Pretreatment with 50 nM thapsagargin blocked cells exposed to 10 s rest inserted OFF (1 Pa shear stress) from exhibiting multiple $[Ca^{2+}]_i$ responses suggesting that the source of the multiple $[Ca^{2+}]_i$ oscillations observed under rest inserted flow is from intracellular stores. Typical $[Ca^{2+}]_i$ traces for cells exposed to 10 s rest inserted flow (B) vs. cells that were pretreated with thapsagargin prior to 10 s rest inserted flow (C). $n = 3$ for 10 s rest flow, $n = 2$ for thapsagargin + 10 s rest flow. Data are mean ± SEM. (*) Significant difference from 10 s rest flow ($p < 0.05$).

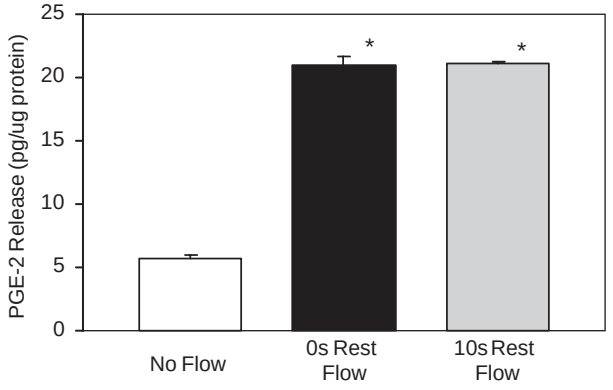

Fig. 6. Cells exposed to 1 h of 10 s rest inserted oscillatory flow released the same amount of PGE_2 as cells exposed to 1 h of continuous oscillatory flow. Media samples were collected 1 h following the completion of loading. Both flow groups released 4-fold greater PGE_2 than no flow control cells. Data are mean ± SEM. $n \geqslant 3$ for all groups. (*) Significant difference from no flow ($p < 0.05$).

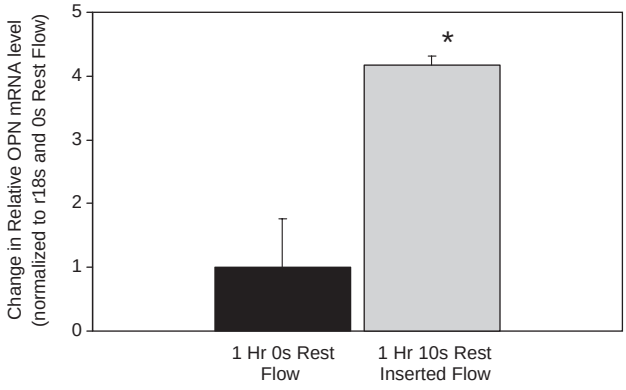

Fig. 7. Cells exposed to 1 h of 10 s rest inserted OFF had 4-fold higher levels of relative OPN mRNA compared to cells exposed to 1 h of continuous oscillatory flow. RNA was isolated 24 h following the completion of loading. Data are mean ± SEM. $n = 4$ for continuous flow, $n = 2$ for 10 s rest flow. (*) Significant difference from continuous (0 s rest) oscillatory flow ($p < 0.05$).

recovery/rest periods into loading regimes (Robling et al., 2001; Srinivasan et al., 2002). The current study was aimed at determining if a cellular-level difference in the response to rest inserted vs. continuous mechanical loading might contribute to the in vivo observations. The cell responses to rest inserted mechanical loading were quantified by applying OFF to osteoblastic cells and quantifying intracellular calcium mobilization, PGE_2 release, and OPN mRNA levels. These data show that there may indeed be a different cell-level response when bone is exposed to rest inserted loading compared to continuous sinusoidal loading.

Intracellular calcium, our primary outcome variable, is a ubiquitous signaling molecule that plays a critical role in a wide variety of physiological processes including cell proliferation, cell death, and cell response to mechanical stimuli (Berridge et al., 1998). In vitro studies have shown that osteoblast precursors, osteoblasts, and osteocytes all respond to OFF with increases in intracellular calcium (Jacobs et al., 1998; Yellowley et al., 2000; You et al., 2000). In addition, You et al. (2001) showed that $[Ca^{2+}]_i$ signaling in osteoblastic cells is required for the downstream upregulation of OPN mRNA induced by 2 h of OFF (2 Pa shear stress). Studies have also shown that osteocytes and osteoblasts

have the ability to communicate their calcium responses to mechanical loading via gap junctions (Yellowley et al., 2000). Within this context, the results of our in vitro study provide insight for understanding the enhanced osteogenic effect of rest inserted loading in vivo via its regulation of Ca^{2+} signaling.

Our results show that insertion of 10 and 15 s rest periods into an OFF profile resulted in greater $[Ca^{2+}]_i$ response magnitudes than those found in cells exposed to continuous OFF. This is an interesting result as peak oscillation magnitudes usually occurred as the initial response to flow (10–30 s after onset of flow, Fig. 2A,B). Since the flow rate is equivalent for all groups in each of the two experiments, we would expect the magnitude of the initial peak to be similar. However, because the initial $[Ca^{2+}]_i$ transient occurs while the first rest period is applied (10 s after onset of flow), the cellular mechanisms that allow for the release of Ca^{2+} may be extended, allowing for greater cytoplasmic concentrations of Ca^{2+}. Intracellular calcium magnitude has been shown to control cell processes in other cell types (Thomas et al., 1996; Dolmetsch et al., 1997; Berridge et al., 1998; Dolmetsch, 2003) and it may be a factor in the enhanced osteogenic effect of rest inserted loading on bone.

In addition, our data show that $[Ca^{2+}]_i$ response frequency increased with rest inserted OFF. A typical response consisted of a large initial transient followed by 1–6 subsequent low-magnitude transients. It has been established that the frequency of $[Ca^{2+}]_i$ oscillations in other cell types has an impact on downstream effects such as gene expression or protein modification (Putney and Bird, 1993; Nelson et al., 1995; De Koninck and Schulman, 1998; Dolmetsch et al., 1998; Li et al., 1998; Porter et al., 1998). Donahue et al (2003a, b) reported that continuous exposure to OFF for 15 min resulted in multiple $[Ca^{2+}]_i$ transients in primary rat osteoblastic cells. In the current study, as in Donahue et al. (2003a, b), the largest $[Ca^{2+}]_i$ transient was the initial response to flow while subsequent transients were lower in magnitude. Our study shows that short-term rest periods allow this repeated low-magnitude response to occur in a shorter time frame than continuous exposure to OFF.

We determined the source of the multiple low-magnitude transients observed under rest inserted OFF to be from intracellular stores. In cells exposed to 10 s rest inserted OFF (1 Pa), pretreatment with thapsagargin blocked the multiple low-magnitude Ca^{2+} responses (Fig. 5). In prior experiments we have shown that Ca^{2+} mobilization from intracellular stores due to continuous OFF occurs via IP_3 signaling due to PLC activity (You et al., 2001). Thus, it is likely that the secondary Ca^{2+} oscillations due to rest inserted OFF described here are due to PLC-IP_3 signaling; however, we did not test this hypothesis directly in this study.

Interestingly, the initial large $[Ca^{2+}]_i$ transient observed with the onset of flow was reduced but not eliminated by thapsagargin treatment. This suggests that the mechanism of the Ca^{2+} response to rest inserted OFF may be different than that to continuous OFF, perhaps involving extracellular calcium influx through stretch activated membrane cation channels. One concern with this interpretation is that the first 10 s of continuous and rest inserted OFF is identical. However, the large initial calcium transient begins 10–30 s after flow is applied. Thus, the calcium response occurs after the cells have experienced a different temporal pattern in the rest inserted flow group.

The current study also examined the effects of rest inserted OFF at both 2 and at 1 Pa. Both of these shear stresses lie within the range of shear stress levels predicted by theoretical models to be found in bone (0.5–3 Pa) (Weinbaum et al., 1994). Although there are currently no direct experimental measurements of the in vivo magnitudes of fluid flow in bone, the models have been used to accurately predict experimental measurements of mechanically induced streaming potentials (Cowin et al., 1995). In addition, the shear stresses in this range have been shown by a number of investigators to elicit intracellular calcium mobilization responses in osteoblastic and osteocytic cells (Hung et al., 1996; Jacobs et al., 1998; Yellowley et al., 2000; You et al., 2000, 2001; Donahue et al., 2003a, b).

Although this and other studies have only examined a few very specific rest inserted mechanical loading profiles, it appears that the potential to optimize these protocols to elicit the most osteogenic cell response pattern exists (Robling et al., 2001; Srinivasan et al., 2002). Our study confirms that a minimum duration of recovery exists, where OFF profiles with rest periods of 5 s or less do not result in significantly different cell responses than continuous OFF. We also observed that, in the low-flow experiments, the insertion of a 10 s rest period resulted in a 3-fold increase in response magnitudes and percent of cells responding compared to the insertion of 15 s rest periods. This result suggests that perhaps certain rest periods are more osteogenic than others. Donahue et al. (2003a, b) found that osteoblasts exhibit unique combinations of $[Ca^{2+}]_i$ oscillation characteristics called "Ca^{2+} fingerprints" which include magnitude, rise and fall times, duration, and overall shape (Prentki et al., 1988). It may be that particular $[Ca^{2+}]_i$ oscillation patterns activate more osteogenic processes than others, and that these $[Ca^{2+}]_i$ patterns are induced more by specific rest inserted mechanical loading regimes. Further in vitro experiments are needed to elucidate the roles of loading frequency, rest period duration, and loading magnitude.

In this study, we also sought to determine if the elevated levels of Ca^{2+} signaling in bone cells exposed to rest inserted OFF influences PGE_2 release and OPN

mRNA expression. We found no increase in the level of PGE_2 release due to OFF with the inclusion of rest periods. Since we found dramatically increased Ca^{2+} signaling with this same flow profile (Fig. 4), this suggests PGE_2 release due to OFF is not regulated by intracellular Ca^{2+} signaling. Indeed, previous experiments determined that PGE_2 release due to OFF in osteoblastic cells is independent of cytosolic calcium (Reilly et al., 2003; Saunders et al., 2003).

We examined the long-term effects of rest inserted OFF on osteoblast signaling by comparing relative mRNA levels of OPN in cells exposed to rest inserted OFF vs. continuous OFF. We observed a 4-fold increase in relative mRNA levels of cells exposed to 10 s rest inserted OFF compared to cells exposed to continuous OFF (Fig. 7). The fact that this same 10 s rest inserted flow profile led to elevated $[Ca^{2+}]_i$ responses (Fig. 4) suggests that rest inserted loading has the potential to change downstream cellular events through changes in intracellular calcium release. You et al. (2000, 2001) found that OPN mRNA levels were upregulated by OFF and that this increase was due to intracellular calcium mobilization. Our data are consistent with that finding because the larger calcium responses of cells exposed to 10 s rest inserted OFF lead to higher relative OPN mRNA levels compared to cells exposed to continuous OFF.

In summary, the insertion of short-term rest periods into a continuous OFF regime resulted in multiple calcium responses with significant increases in calcium response magnitudes and overall percentage of cells responding to flow compared to continuously loaded controls. Rest inserted OFF led to a 4-fold increase in relative OPN mRNA in cells when compared to continuously loaded controls. Our results suggest that mechanically induced modulation of intracellular levels of calcium in bone cells may be intricately linked to the ultimate downstream effect of whole bone adaptation to rest inserted mechanical loading. The dramatically enhanced osteogenic effect observed in vivo appears to be, at least in part, due to an increased cellular sensitivity to rest inserted loading.

Acknowledgements

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