SHORT TERM RECOVERY PERIODS LEAD TO MULTIPLE OSCILLATORY FLUID FLOW INDUCED INTRACELLULAR CALCIUM OSCILLATIONS IN OSTEOBLASTIC CELLS

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INTRODUCTION

The effect of mechanical loading on bone depends on the duration and magnitude of the applied load. Bone's mechanosensitivity tends to decline soon after the initiation of dynamic mechanical loading and as a result the osteogenic response of bone saturates [1]. This suggests the existence of a recovery period that would restore bone cell responsiveness to mechanical stimuli. Several studies have shown that bone does recover its responsiveness in vivo and is able to respond to stimuli with the same magnitude as earlier exposures to loading [2-4]. The application of mechanical stimulus, specifically fluid shear, to osteoblasts in vitro results in the instantaneous transient increase of intracellular Ca^{2+} through release of intracellular stores and opening of membrane ion channels [5-6]. Previously, we performed in vitro experiments that exposed MC3T3-E1 osteoblastic cells to rest inserted oscillatory fluid flow and found that short term recovery periods can enhance osteoblastic cells' ability to respond to fluid flow via intracellular calcium [7]. However the high shear stress (2 N/m^2) used in the previous studies resulted in no significant difference in the % of cells responding to flow between the rest-inserted groups and the control group. In this study we repeat our previous experiments with additional Low Flow experiments performed at half the shear stress (1N/m^2) in order to determine if the % of cells responding to oscillatory fluid flow is an important factor in the effect of rest inserted mechanical loading.

METHODS

CELL CULTURE – MC3T3-E1 osteoblastic cells were cultured in minimal essential alpha medium (MEM – a; Life Technologies, Rockville, MD) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 1% penicillin and streptomycin (Life Technologies, Rockville, MD) and maintained at 37ºC and 5% CO2 in a humidified incubator. Cells were subcultured on UV transparent quartz slides for 48 hrs prior to experiments at a seeding density of 1 x 10^5 cells/slide. The cells were 80-90% confluent at the time of experimentation.

CALCIUM IMAGING – Prior to exposure to oscillatory fluid flow, cells were incubated with 10 µM Fura-2 AM (Molecular Probes, Eugene OR) for 30 min. Following Fura-2 loading, the cells were washed with MEM -a containing 2% FBS, and the cell seeded quartz slides were mounted in a parallel plate flow chamber fixed to the stage of a fluorescent microscope. For 30 minutes the cells were left undisturbed. During this period a small but steady washout flow of 0.05 ml/min passed through the flow chamber providing the cells with fresh media. The flow media consisted of MEM -a and 2% FBS. A previously described flow system was used to expose the cells to oscillatory fluid flow [6] We were able to adjust the flow rate using the loading device to achieve peak sinusoidal shear stresses of 2 N/m^2 and 1 N/m^2 at 1 Hz. Intracellular levels of Ca^{2+} were quantified using a ratiometric imaging technique [6]. Cells were exposed to a total of 3 minutes of oscillating fluid flow at 2 N/m^2 with rest periods of 0 (control), 5, 10, and 15 seconds inserted every 10 loading cycles. These experiments were repeated at a lower shear stress of 1 N/m^2 with all experiments having a minimum of n=149 cells and 3 slides. Ratio images were acquired every 2 seconds for the duration of the loading as well as for 3 minutes prior to the onset of flow and for 3 minutes after flow had stopped. Ratio values were converted to $[Ca^{2+}]_i$ values using a calibration curve derived from a series of standard Ca^{2+} 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 pre-flow baseline period. Statistical analysis using one-way ANOVA and Fisher's Protected Least Significant Difference was utilized to detect significant differences between groups.

RESULTS

HIGH FLOW (SHEAR STRESS = 2 N/M^2) - Insertion of 10s and 15s rest periods but not 5s, resulted in $[Ca^{2+}]_i$ response magnitudes that were significantly higher than control (mean ± SE, 5s = 180.44nM ± 13.29%, 10s = 259.86nM ± 13.62%, 15s = 305.04nM ± 16.62 %, control = 185.168nM ± 17.82 %, $p < 0.005$ for both 10s vs. control, and 15s vs. control, $p = 0.68$ for 5s vs. control). A significant difference

in the percent of cells responding to flow was detected between the 15s vs. control groups but not the 5s and 10s groups (5s = 68.2% ± 9.4%, 10s = 68.1% ± 7.6%, 15s = 83.2% ± 5.6 %, control = 55.9% ± 6.9 %, p=.03 for 15s vs. control, p>0.1 for 5s and 15s vs. control). Among cells that did respond to flow, the percent of cells responding 2 or more times was significantly higher in the 10s and 15s but not the 5s group compared to control (5s = 12.9% ± 5.7 %, 10s = 21.9% ± 4.9 %, 15s = 22.5% ± 6.5%, control = 6.3 % ± 1.9 %, p < 0.05 for 10s and 15s vs. control, p = 0.38 for 5s vs. control). In addition, among cells that responded to flow, the average number of $[Ca^{2+}]_i$ responses during flow was significantly higher in the 10s and 15s groups but not the 5s group compared to control (5s = 1.16 ± 4.0 %, 10s = 1.27 ± 3.5 %, 15s = 1.28 ± 4.7 %, control = 1.07 ± 2.4 %, p < 0.001 for 10s and 15s vs. control, p = 0.15 for 5s vs. control).

LOW FLOW (SHEAR STRESS = 1 N/M^2) – For low flow experiments (Figure 1) only the insertion of a 10s rest period resulted in $[Ca^{2+}]_i$ response magnitude that was significantly greater than control (5s= 57.50 nM ± 5.88 %, 10s = 306.966 nM ± 50.32 %, 15s = 84.60 nM ± 16.75 %, control = 76.90 nM ± 16.75 %, p=.002 for 10s vs. control, p>0.8 for 5s, 15s vs. control). In contrast to the high flow experiments a significantly greater % of cells responded in *both* the 10s and 15s rest groups compared to control (5s= 31.0 % ± 9.3 %, 10s = 88.9 % ± 3.7 %, 15s = 39.6 % ± 2.7 %, p<0.01 for 10s and 15s vs. control, p = 0.13 for 5s vs. control). Among the cells that did respond to flow the % of cells that responded 2 or more times increased as length of rest period increased with the 15s group having a significantly greater percentage than control (5s = 10.6 % ± 1.5 %, 10s = 21.5 % ± 3.8 %, 15s = 28.0 % ± 7.1 %, control = 7.6 % ± 4.7 %, p<0.05 for 15s vs. control, p=0.72 for 5s vs. control, p=0.12 for 10s vs. control). Among cells that responded to flow the 1 N/m^2 applied shear stress resulted in significantly greater average # of $[Ca^{2+}]_i$ responses in the 10s and 15s groups compared to control (Figure 2); similar to what was found in the high flow experiments (5s = 1.09 ± 4.2 %, 10s = 1.26 ± 4.2 %, 15s = 1.43 ± 7.3 %, control = 1.07 ± 4.0, p<0.05 for 10s vs. control, p<0.0005 for 15s vs. control, p=.89 for 5s vs. control).

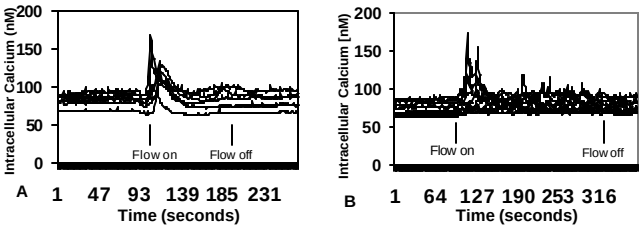

Figure 1. Example of Low Flow experiment time history traces for continuous flow Control (A) and 15s rest inserted flow (B) groups.

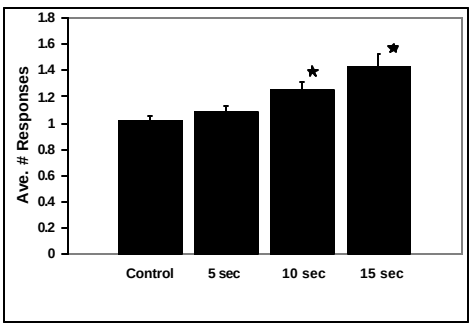

Figure 2. Average number of responses for Low Flow experiments. A star * indicates significant difference from Control group (p<0.05).

DISCUSSION

Previously we reported that the insertion of recovery periods of 10s and 15s in a continuous cyclic loading protocol significantly increases the average number of Ca^{2+} responses that a cell undergoes [7]. However, that data suggested that the % of cells responding to flow may not have been affected by the insertion of short term recovery periods. In this study we repeated the previous experiments performed at 2 N/m^2 and also performed experiments at 1 N/m^2. It seems that exposing cells to a shear stress of 2 N/m^2 resulted in the maximum number of cells responding in each group and as a result little difference was detected. Application of the lower shear stress yielded similar results compared to the high shear stress in terms of peak $[Ca^{2+}]_i$ oscillation magnitude, and multiple response data. Here we also report that the insertion of 10s and 15s recovery periods in the low flow groups results in increased % of cells responding to flow. This suggests that Ca^{2+} oscillation magnitude, % of cells responding to flow, and frequency of response may *all* be important factors in bone adaptation to mechanical loading. Recovery periods may not only increase the number of times certain cell processes are activated where Ca^{2+} oscillation frequency are important, but also the number of cells that are activated. Those activated processes may regulate gene expression and ultimately bone adaptation to mechanical loading.

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