Intracellular and Endoplasmic Reticulum Calcium Dynamics in Osteocyte Mechanobiology

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Introduction: Due to their abundance and unique arrangement within bone tissue, osteocytes are considered the primary mechanosensing cells in bone. One of the earliest responses of bone cells to mechanical stimulation is a rise in intracellular calcium ([Ca$^{2+}$]), and previous studies have demonstrated that osteocytes exhibit unique [Ca$^{2+}$] patterns. Both in vitro [1] and ex vivo [2], osteocytes exhibit spike-like oscillations in [Ca$^{2+}$], in response to mechanical stimuli, whereas osteoblasts demonstrate fewer, weaker responses. Inhibitor studies have implicated the release of Ca$^{2+}$ from endoplasmic reticulum (ER) stores as being critical to subsequent responses. Thus, it is possible the mechanisms of Ca$^{2+}$ release and reuptake by the ER are important to osteocyte mechanobiology. The differentiation of osteoblasts to osteocytes is characterized by many phenotypic changes, including changes in expression of membrane calcium channels. Osteoblasts express both T- and L-type voltage-sensitive calcium channels (VSCC), whereas L-type VSCC are barely detectable in osteocytes. We hypothesized that the predominant expression of T-type channels in osteocytes may contribute to their unique [Ca$^{2+}$] patterns and further speculated that T-type VSCC in osteocytes may interact with ER stores. We sought to visualize the dynamics of Ca$^{2+}$ signaling within bone cells under fluid flow by simultaneously monitoring Ca$^{2+}$ separately in the cytosolic ([Ca$^{2+}$]) and endoplasmic ([Ca$^{2+}$]$_{ER}$) spaces in osteocytes and osteoblasts. The purpose of this study is to determine the mechanisms by which osteocytes generate [Ca$^{2+}$] oscillations and their dependence on VSCC.

Methods: The osteocyte-like MLO-Y4 and osteoblastic MC3T3-E1 cell lines were maintained at 37°C and 5% CO$_2$ in serum-supplemented medium. For flow experiments, cells were grown to ~80% confluency onto fibronectin-coated glass slides to establish cell-cell contact. To visualize intracellular calcium changes, cells were incubated with 15um Fluo-8 AM and assembled into a parallel plate flow chamber. Cells were pre-treated with the T-type inhibitor NNC 55-0396 or the L-type inhibitor nifedipine for 15 minutes prior to flow. Time-lapse images were collected at 20x magnification for 10 minute periods of fluid flow stimulation at 35 dynes/cm$^2$. To simultaneously visualize intracellular and ER calcium, cells were transiently transfected with the D1ER plasmid [3] using Fugene 6. Thirty-six to forty-eight hours post transfection, cells were incubated with Fura Red-AM to indicate intracellular calcium. Time-lapse images were collected at 40x magnification. Single excitation (430/24) was used to excite the dye and FRET biosensor. Fluorescence emissions of YFP (530/30), CFP (470/28), and Fura Red (641/75) were captured separately and simultaneously using a quadview beamsplitter and custom quad-band polychroic [4]. To monitor ER depletion, transfected cells were imaged for 20 minutes following the addition of VSCC inhibitors. The FRET ratio was calculated on a pixel-by-pixel basis using image registration of the FRET and donor emissions. All images underwent background subtraction and median filtering.

Results: Osteocytes exhibited numerous [Ca$^{2+}$] oscillations under fluid flow, and these multiple responses were abolished by treatment with the T-type inhibitor NNC 55-0396 (Fig. 1A). The L-type inhibitor had no significant effect on the number of [Ca$^{2+}$] responses in osteocytes, consistent with the
limited expression of L-type VSCC in these cells (Fig. 1A) [5]. We previously reported that osteoblasts demonstrated fewer responses under fluid flow, and these responses were not affected by T- or L-type inhibition [1]. Treatment of D1ER transfected cells with thapsigargin depleted the ER stores, and depletion times were not significantly different between MLO-Y4 and MC3T3-E1 (Fig. 1B). Inhibition of VSCC had no effect on ER depletion times in osteoblasts, and L-type inhibition had no effect on osteocytes. However, inhibition of T-type VSCC resulted in faster depletion of ER stores in osteocytes, suggesting an interaction between T-type channels and the ER in osteocytes. In D1ER transfected osteocytes stained with Fura Red-AM, elevations of [Ca^{2+}]_{i} coincided with depression of [Ca^{2+}]_{ER}, with subsequent peaks occurring after recovery of [Ca^{2+}]_{ER} levels (Fig. 2A). In osteoblasts, only a single [Ca^{2+}]_{i} response was observed, and while the ER contributed to this response, it did not refill in the time course of the experiment (Fig. 2B).

Discussion: These studies demonstrate the ER is more intimately involved in osteocyte calcium signaling than previously considered and support the hypothesis that osteocytes are capable of generating characteristic multiple responses by an ability to refill the ER stores. Future studies will further explore the role of VSCC and other channels on release and reuptake dynamics of ER Ca^{2+} trafficking in osteocytes under flow, which may contribute to their mechanobiological function.

Significance: This study aims to determine the mechanisms by which osteocytes sense and respond to mechanical stimuli. A greater understanding of osteocyte mechanobiology may contribute to our ability to prevent or treat bone degeneration in diseases such as osteoporosis.
Figure 1. Effects of T- and L-type VSCC inhibitors on intracellular and ER calcium. *p<0.01
Figure 2. Simultaneous monitoring of intracellular and ER calcium levels under fluid flow.

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