The Effect of Mechanical Stimulation on Osteocyte Chemo-
Sensitivity

by

Jia Ning Zhang

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Institute of Biomaterials and Biomedical Engineering

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Abstract

Osteocytes are believed to be the mechanosensory cells that detect and respond to mechanical loading. Physiological loading by oscillatory fluid flow (OFF) activates osteocytes to increase intracellular calcium concentration and release prostaglandin E2 (PGE₂). Osteocytes are also sensitive to chemical stimulations such as serotonin, which can also increase PGE₂ release. However, it is unclear whether mechanical stimulation can influence osteocyte sensitivity towards serotonin. In this thesis, MLO-Y4 osteocyte-like cells were subjected to serotonin with or without precondition by OFF, the responses of intracellular calcium and PGE₂ release were measured. Serotonin increased intracellular calcium and PGE₂ release in osteocytes. The effects were significantly reduced by OFF precondition, suggesting mechanical precondition by OFF can reduce osteocyte sensitivity towards serotonin. In terms of mechanisms, OFF- and serotonin-induced calcium responses depended on intra- and extracellular calcium stores. ATP was found to partially mediate OFF modulation of serotonin-induced PGE₂ release but not calcium.
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Chapter 1: Introduction

1.1 Motivation

Osteoporosis is a major health concern affecting more than 3 million Canadians [1], with symptoms of decreased bone mineral density and increased risk of bone fracture. One out of every two women and one in four men over the age of 50 will break a bone in their lifetime due to osteoporosis. Roughly 25 to 30% of women who suffer a hip fracture will die within one year of the injury. This accounts for more deaths than does breast cancer [2].

Mechanical loading has been shown to play a critical role in maintaining bone mineral density, thereby can be potentially used to treat and prevent osteoporosis [3]. Osteocytes embedded in the bone mineralized matrix are believed to be the mechanosensory cells responsible for converting mechanical stimuli into biochemical signals, which regulate bone adaptation to mechanical loading [4-8]. Mechanical loading results in oscillatory fluid flow (OFF) of interstitial fluid through the lacunae-canalicular networks [9]. In response to physiological levels of OFF, osteocyte respond immediately by increasing intracellular calcium mobilization [10], and up-regulating the synthesis and release of prostaglandin E2 (PGE$_2$), a key molecule in the regulation of bone remodeling [5, 11, 12].

It is believed that OFF activates intracellular calcium by acting on various types of calcium channels such as L-type, T-type and a mechanosensitive cation channel [13], leading to the entry of extracellular calcium and the release of calcium from intracellular stores [14]. In addition, ATP diffusion through the hemichannels also plays an important role in mediating intracellular calcium signalling [14, 15]. In this regard, mechanical stimulation by
OFF initiates the release of ATP in bone cells [16, 17], and the ATP can act on ATP receptors on membrane to induce intracellular calcium mobilization. On the other hand, the mechanism of OFF stimulation of PGE$_2$ release is more complex and not fully understood. Recently, P2X7 receptor has been identified in MLO-Y4 osteocyte-like cells, which is believed to partially mediate the PGE$_2$ release initiated by OFF [17, 18]. Since P2X7 is an ATP receptor, this suggests the involvement of ATP receptors in OFF regulation of PGE$_2$ release in osteocytes.

Aside from mechanical stimulation, osteocytes are also sensitive to chemical stimulation, such as ATP, vitamin K, phosphate and hormones [17, 19-22]. Recently, neurotransmitter serotonin has been heavily studied on its potential role in bone metabolism. While in vivo studies suggest catabolic effects of serotonin on bone mass, in vitro studies have shown that serotonin causes rapid release of PGE$_2$ in MLO-Y4 osteocyte-like cells. Although the mechanism of serotonin-induced PGE$_2$ release is not known for osteocytes, it has been shown in Hamster ovary cells that ATP activation of P2Y receptors reduced 5-HT1A (serotonin receptor 1A) responsiveness to agonist [23]. In bone cells, since serotonin functions mainly through the serotonin receptors [24], it is possible for ATP to play a role in mediating serotonin-induced PGE$_2$ release in osteocytes.

Therefore, evidences suggest that osteocytes are able to respond to both mechanical and chemical stimulations. However, the combined effect of mechanical and chemical stimulation on osteocytes has not been characterized. Specifically, it is not clear whether mechanical stimulation can affect the osteocyte sensitivity towards chemical stimuli. Since osteocytes experience both mechanical and chemical stimulation in their physiological conditions, an exploration into the combined effect of mechanical and chemical stimulation
is a necessary step towards fully understanding the osteocyte. I hypothesize that chemo-sensitivity of osteocytes is affected by mechanical stimulation. Since it is known that osteocyte can respond to mechanical stimulation with intracellular calcium mobilization and PGE\textsubscript{2} release, and serotonin can trigger the same responses in osteocyte and other cells, I decided to look at the mechanical stimulation effect on osteocyte sensitivity towards serotonin. Overall, a deeper understanding of the cellular response of osteocyte to biochemical signals under mechanical loading may lead to novel therapeutic treatment of musculoskeletal diseases such as osteoporosis.

1.2 Hypothesis

I hypothesize that chemo-sensitivity of osteocytes is affected by mechanical stimulation, specifically

1. OFF affects osteocyte response to serotonin in triggering calcium mobilization.
2. OFF regulates serotonin-induced intracellular calcium response through calcium release from intracellular stores.
3. OFF also regulates serotonin-induced intracellular calcium response via ATP release and signaling through P2 receptors.
4. OFF affects osteocyte response to serotonin in releasing PGE\textsubscript{2}.
5. OFF regulates serotonin-induced PGE\textsubscript{2} release through ATP release and signaling through P2 receptors.

1.3 Objectives

The main objective of this study is to investigate the effect of mechanical stimulation on the osteocyte sensitivity towards chemical stimulation. The specific objectives are:
1. Investigate OFF modulation of osteocyte response to serotonin in triggering calcium mobilization.

2. Investigate the calcium pathway of OFF regulating serotonin-induced calcium mobilization.

3. Investigate ATP receptor inhibition on OFF modulation of serotonin-induced calcium response.

4. Investigate OFF modulation of osteocyte response to serotonin in releasing PGE$_2$.

5. Investigate ATP receptor inhibition on OFF modulation of serotonin-induced PGE$_2$ release.

1.4 Thesis Organization

This document consists of seven chapters, chapters 3-5 are based on a manuscript to be submitted to *Bone*. Chapter 1 introduces the study by describing its motivation, objectives and hypothesis. Chapter 2 contains relevant background knowledge for the study. Chapter 3 describes the study’s experiment approach in detail. Chapter 4 summarizes the experimental results. Chapter 5 discusses the experimental results. Chapter 6 provides recommendations for future work. Chapter 7 concludes the study. Finally, references are listed at the end along followed by appendices.
Chapter 2: Background

2.1 Osteoporosis and Mechanical Loading

Osteoporosis is a musculoskeletal disease characterized by decreased bone mineral density and increased risk of bone fracture [1]. Osteoporosis is prevalent in elderly population and affects more than 3 million Canadians [2]. According to the National Institute of Health, one out of every two women and one in four men over the age of 50 will break a bone in their lifetime due to osteoporosis. In addition, roughly 25% to 30% of women who suffer a hip fracture will die within one year of the injury. This is astonishing because it accounts for more deaths than does breast cancer [2].

In terms of treatment, weight-bearing exercise in the form of mechanical loading on bone has been shown to play a critical role in maintaining bone mineral density, thereby can be potentially used to treat and prevent osteoporosis [3]. It was found that sports producing significant impact loading (at least 3 times body weight) on the bone resulted in greater bone density than sports producing loads to bone mainly through muscular contraction (i.e. swimming) [25]. It was also shown that splitting the exercise regime into smaller sessions separated by recovery periods resulted in greater bone mass than exercise applied in a single session [26]. Therefore, optimal exercise consists of short burst of impact loading on bone with long rest inserted between each loading. However, human exercise programs for maintaining or improving bone health are often tedious and compliance is usually low [27]. The most practical advice for patients is to walk a minimum of 30 to 60 minutes per day [27]. Exercises like swimming or cycling that rely on muscle have less effect on bone, but could reduce fracture risk indirectly by maintaining muscle mass and force [27].
Other chemical based treatment methods include bisphosphonates (alendronate, risedronate, ibandronate, zoledronic acid), parathyroid hormone, teriparatide, calcitonin, estrogen (for women), raloxifene (selective estrogen receptor modulator), and denosumab [28]. In addition, dietary supplement of calcium and vitamin D have also been shown to preserve bone mass and decrease the risk of osteoporotic fracture [28].

2.2 Mechanical Loading and the Bone Cells

Bone is a dynamic tissue that continuously undergoes modelling and remodelling to meet its mechanical demands. This is best summarized by Wolff’s Law, coined by Julius Wolff in the 19th century, which states that the structure and mass of bone adapts at the function level [29]. Bone tissue remodelling is ultimately governed by the cells in bone. The three major types of cells in bone are: osteoblasts, osteoclasts, and osteocytes. The first two cell types are antagonists in that the osteoblasts are responsible for depositing new bone matrix, whereas the osteoclasts work to resorb bone. The least understood of the three are the osteocytes. Osteocytes are the most abundant cell type in bone, making up 90-95% of the total bone cells. These are formally osteoblast cells and as bone grows they become trapped inside the lacunae of the mineralized bone matrix [30]. The osteocytes show physically distinct processes that stick out of the ovular shaped cell bodies. These processes form an extensive communication network within the narrow canaliculi channels of the bone matrix (Figure 2-1) [31].

2.3 Osteocytes Function as Possible Mechanosensory Cells

Although the concept of loading induced bone remodeling is well accepted, much less is known about the underlying cellular signaling processes. There is growing evidence that
osteocytes are the primary mechanosensitive cells in the bone, which not only directly modify bone structure but also regulate remodeling activities of other cell types in the bone (i.e. the osteoblasts and osteoclasts [30, 32]). The notion of the osteocyte to sense mechanical loading and then transducing the signals to other bone cells is called osteocyte mechanotransduction. Previous studies have indicated that the dynamic fluid flow in the osteocytic network of lacunae-canaliculi is one of the physiological mechanical stimuli that osteocytes respond to in vivo [8]. In other studies, the flow-induced cellular signals elicited in one osteocyte have been shown to propagate to other cells directly through cell-cell contacts at the osteocytic processes and/or through paracrine mediators.

**Figure 2-1:** Diagram depicting transverse section of the decalcified fibula at x250 magnification. (Reprinted from *Gray’s Anatomy of the Human Body*, 1918; Fig. 77 [33].)
transported in the lacunar-canalicular system [6, 34]. These evidences reinforce the notion of osteocytes being the mechanosensitive and mechanotransductive cells in the bone remodelling process.

2.4 Mechanical Stimulation on Osteocytes *in vitro*

From the theory of mechanotransduction, osteocytes are able to sense fluid flow shear stress in their local environment and transduce the signals to other bone cells in bone remodelling. Physiological loading induces interstitial fluid flow and hydraulic pressure in the lacunar-canalicular porosity, which are the two main stimulations experienced by osteocytes [29]. Under physiological loading conditions (*e.g.* walking), hydraulic pressure is cyclic in nature [35]. Cyclic hydraulic pressure (CIP) has been shown to stimulate osteocytes *in vitro* by increased intracellular calcium, altered gene expressions and reduced apoptosis [36]. These findings support the potential role of loading-induced CIP on osteocyte in the regulation bone remodeling. On the other hand, interstitial fluid flow has been demonstrated to be a potent regulator of osteocyte mechanotransduction [37]. To study this phenomenon *in vitro*, Oscillatory Fluid Flow (OFF) is commonly used to model the osteocyte response to fluid flow *in vivo*. It has been predicted that physiological loading on bone experienced by osteocytes is equivalent to OFF producing shear stresses of 0.8-3 Pa [29]. Numerous studies have reported the effects of OFF on osteocytes, which are described in the following section. In addition, strains caused by physiological loading have also been proposed to mediate osteocyte mechanotransduction *in vivo* [29]. Although physiological levels of strains are too low (below 0.1% [38]) to stimulate osteocytes, recent evidences show that osteocytes may experience amplified local strains [31, 39]. This amplification may be caused by the
ultrastructure of the osteocyte processes [31], or the strain contractions occurring at the lacunae [39].

2.5 Effects of OFF on Osteocytes in vitro

In vitro studies on osteocytes were done using the MLO-Y4 osteocyte-like cell line, which has been shown to have marker expressions very similar to primary osteocytes [40]. In response to OFF at 1 Pa and 1 Hz, MLO-Y4 osteocytes respond by increasing intracellular Ca$^{2+}$, which is believed to be the immediate response [10]. Increase in intracellular calcium concentration has been linked to a number of gene expression changes, such as osteopontin (OPN), c-fos, cyclooxygenase-2 (COX-2), and transforming growth factor-β (TGF-β) [13]. Increase in nitric oxide synthesis has also been shown to be downstream of calcium [41]. In response to OFF, osteocytes also release the protein prostaglandin E2 (PGE$_2$) [11], an important regulator of bone remodelling. Both bone formation and bone resorption are stimulated by PGE$_2$, and intermittent PGE$_2$ was shown to have anabolic effect on bone [42]. PGE$_2$ is regulated by COX-2 [43], which is the gene that encodes prostaglandin-endoperoxide synthase 2, a key enzyme in the production of PGE$_2$. It has been shown that OFF induces increased COX-2 gene expression in osteocytes in vitro [29]. In addition, OFF-induced PGE$_2$ release requires an intact cytoskeleton in osteocytes, but not in pre-osteoblasts [29]. Another set of important intercellular signalling molecules are receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) and osteoprotegerin (OPG) [5]. OFF has been shown to result in decreased RANKL/OPG gene ratio, which repressed osteoclast formation in RAW264.7 cells in direct cellular contact with the osteocytes [5]. RAW264.7 is a pre-osteoclast cell line. In addition, the conditioned media from osteocytes exposed to OFF
also inhibited osteoclastogenesis [5], suggesting that osteocytes have the potential to prevent bone resorption by regulating soluble factors like RANKL and OPG.

Other molecules shown with possible roles in OFF-mediated mechanotransduction in osteocytes include: sclerostin, osteopontin (OPN), and nitric oxide (NO). Sclerostin, encoded by the Sost gene, is highly expressed in osteocytes in adult bone and inhibits bone formation by blocking Wnt signalling. Cyclic loading in mice reduced Sost and sclerostin levels in a loading-dependent manner [29]. OPN knockout in mice prevented bone loss in response to tail suspension, suggesting its vital role in load-induced bone remodelling [29]. Fluid flow decreased OPN protein expression but increased OPN release in MLO-Y4 cells [29]. NO is a small, short-lived second messenger molecule that is mechanically regulated. Poking of a single osteocyte with a micro-needle increased intracellular NO production by 94%, and increased NO in the surrounding osteocytes by 31-150% [29].

2.5.1 Mechanism of OFF-induced Calcium Mobilization

It is believed that OFF activates intracellular calcium by acting on various types of calcium channels such as L-type, T-type and a mechanosensitive cation channel [13], leading to the entry of extracellular calcium and the release of calcium from intracellular stores. [14]. Recently, it has been shown that ATP diffusion through the hemichannels also plays an important role in mediating intercellular calcium signalling [14, 15]. In this regard, mechanical stimulation by OFF initiates the release of ATP in bone cells [16, 17], and the ATP can act on two types of ATP receptors on membrane to induce calcium mobilization. P2X receptor, a type of ligand gated ion channel, can respond to the binding of extracellular ATP by opening and introducing calcium influx into cytosol [44, 45]. P2Y receptors, a type of G-protein coupled receptor, can also respond to the binding of ATP by synthesizing and
releasing inositol trisphosphate (IP3) into cytosol. IP3 activates IP3 receptor (IP3R) on the endoplasmic reticulum (ER) to induce calcium release from ER calcium store [45-49], which is the largest intracellular calcium store. In addition, emptying of the ER calcium store can activate store-operated calcium channels on cell membrane leading to calcium influx from extracellular environment [50]. These pathways are encapsulated in the schematic drawing shown in Figure 2-2.

2.5.2 Mechanism of OFF-induced PGE₂ Release

The mechanism of OFF stimulation of PGE₂ release is more complex and not fully understood. It has been reported that the increase of intracellular calcium concentration contributes to increased PGE2 release by enhancing the expression of cyclooxygenase-2 (COX-2) [51], which is a gene important in the synthesis of PGE2. OFF-induced PGE₂ is synthesized from Arachinodic Acid (AA) liberated from G-protein [52]; this process is mediated by Phospholipase A2 (PLA2) and Protein Kinase C (PKC). PKC also mediates the opening of hemichannels in osteocytes in response to OFF [17, 53]. Recently, P2X7 receptors have also been identified in MLO-Y4 osteocyte-like cells, which can lead to increased PGE₂ release [17, 18]. Since activation of P2 receptors can lead to intracellular calcium, and calcium can lead to PGE₂ changes via COX-2, it is speculated that P2X7 activation increases PGE2 release via up-regulation of COX-2.

2.6 Chemical Stimulation on Osteocytes

Aside from mechanical stimulation, osteocytes are also sensitive to chemical stimulation, ranging from serotonin, ATP, vitamin K, phosphate, hormones to hypoxia. It was shown that 100 µM of serotonin could significantly increase the PGE₂ release in osteocytes, the effect
Figure 2-2: Schematic drawing of the calcium signaling pathways in bone cells. (Reprinted from X. L. Lu et al.: JBMR 2012;27(3):563-74 [14], with permission form John Wiley and Sons.)

was 3-fold increase after one hour [24]. ATP was found to stimulate the PGE2 release in osteocytes dose-dependently [17]. Vitamin K affects osteocyte mineralization and gene expression [19] by decreasing RANKL expression and increasing E11, an osteocyte marker involved in cell process extension. Phosphate has been shown to lower the osteocyte expression of osteocalcin (OCN), which is a marker for bone formation [20]. Hyperphosphatemia, or elevated levels of phosphate in blood, induces OCN production and nuclear export of Cbfa1, which is a gene for bone formation and regulates OCN expression. The voltage-operated calcium channels (VOCC) in osteocytes were found to be hormonally
linked [21], and estrogen was able to enhance the inhibitory effect on osteoclasts by osteocytes [22]. All of these were interesting results obtained from MLO-Y4 cellular studies.

Hypoxia, or reduced oxygen levels are in direct physical relevance to osteocytes. When the bone surrounding tissue is damaged, blood vessels are disrupted and this decreases the availability of oxygen and nutrient transport to the cells. Several studies have been done on osteocytes in hypoxic conditions (1-2% O₂) compared with normal conditions (21% O₂). It was shown that hypoxia had no effect on osteocyte viability, but the hypoxic media from osteocytes induced MSC migration through OPN and CD44 mediated pathway [54]. OPN is secreted by osteocytes and interacts with the cell surface receptor CD44. Hypoxia induced increase in OPN gene expression after 3 hours but the level of OPN returned to normal after 24 hours of re-oxygenation [55]. In addition, hypoxia increased HIF-1alpha expression by 2-fold in 4 hours [55], reduced mineralization and alkaline phosphatase activity[56], and made osteocytes more resistant to apoptosis through down-regulation of p53 gene and up-regulation of Bax gene [57]. Overall, lots of separate experiments have been done on osteocytes in hypoxic conditions, and yet no conclusion is reached.

A number of animal studies were also reported on the effect of osteocytes to chemical stimulation. The results from these studies could be easily confirmed in vitro in MLO-Y4 osteocytes. In mouse calvariae, isolated osteocytes showed increased sclerostin expression by calcitonin stimulation [58]. In transgenic mice, PTH stimulation increased bone mass and bone remodelling, reduced expression of sclerostin, increased Wnt signalling, and decreased osteoblast apoptosis [59]. In chick calvaria osteocytes, fluorescence recovery was rapidly reduced by GJIC inhibitor and pH reduction [60]. Lastly, glucocorticoid increased osteocyte
lacunae size, with demineralization around the osteocyte and reduced elastic modulus in mice [61].

2.7 Serotonin as the Target Chemical Stimulation of This Study

Among the multitude of chemical stimulation studies on osteocytes, serotonin is the most interesting because it has recently been shown to be linked to bone metabolism and has raised tremendous interest. Current *in vivo* studies have suggested a theory by which serotonin can act on osteoblasts to inhibit bone formation leading to bone loss [62], but the mechanisms are unclear and under debate [63]. *In vitro* studies have also shown a potential role of serotonin acting on osteocytes, in which serotonin induced rapid release of PGE2 in MLO-Y4 osteocytes [24]. Since PGE2 is an important molecule in bone remodelling, this suggests that serotonin may also act on osteocytes to influence bone remodelling. Better understanding of the effect of serotonin on osteocytes would be required to fully understand the link between serotonin, bone cells, and bone metabolism.

2.7.1 Serotonin and Bone Metabolism

Serotonin, 5-hydroxytryptamin (5-HT), is a neurotransmitter produced either by neurons in the brain stem or by duodenal cells in the gut [63]. Since serotonin does not cross the brain-blood barrier, these two sources of serotonin function in relative isolation. Serotonin in brain influences a broad range of behavioural, cognitive, and physiologic functions. Gut-derived serotonin traditionally acts as paracrine factors to stimulate peristalsis and mucus secretion, but has recently being found to play a major role in bone metabolism. Serotonin is mediated by seven families of membrane-bound receptors (5-HTRs), and can also be taken into the cell via a plasma membrane serotonin transporter (5-HTT) that actively
transports serotonin using transmembrane ion gradients [63]. Interestingly, a null mutation in the 5-HTT gene resulted in bone phenotype \textit{in vivo} characterized by decreased bone formation, altered architecture, and inferior mechanical properties [62], suggesting the negative role of serotonin on bone mass. The mechanism was believed to be gut-derived serotonin travelling through the blood stream to function as endocrine signalling molecule acting on osteoblast. A schematic drawing of the proposed mechanism is shown in Figure 2-3, adapted from [62]. In addition, it was proposed that serotonin acted as a downstream mediator of LDL Receptor-Related Protein 5 (LRP5).

\textbf{Figure 2-3:} Model of Lrp5-dependent regulation of bone formation through serotonin synthesis in endochromaffin cells. 5-HT (serotonin) binds to Htr1b receptor in osteoblasts and inhibits cyclic AMP response element binding protein (Creb) expression and function, resulting in reduced osteoblast proliferation. Lrp5 favors bone formation and bone mass accrual through this pathway. (Reprinted from V. K. Yadav \textit{et al:} \textit{Cell} 2008;135(5):825-37 [62], with permission from Elsevier.)
However, another study showed that deletion of LRP5 gene did not produce a bone phenotype and did not cause any difference in blood serotonin levels [64]. Overall, there is still much debate about the effects of serotonin on bone metabolism, and the serotonergic pathways are still unclear. Nevertheless, the presence of the role of serotonin on bone metabolism has been confirmed, which raised tremendous interest, and progress have been made to identify the exact effects and mechanisms. Therapies targeting LRP5 and serotonin have become a new area of study for treating osteoporosis [65].

2.7.2 Serotonin Stimulates PGE₂ Release in Osteocytes *in vitro*

Most studies on serotonin’s effect in bone have been *in vivo* studies. To the best of my knowledge, there’s only one *in vitro* cellular study which looks at the potential role of serotonin in osteocytes [24]. These authors demonstrated the presence of serotonin synthesis and production pathways in MLO-Y4 osteocytes, which express specific markers very similar to osteocytes *in vivo* [24]. The gene expressions of serotonin transporters and receptors were identified in osteocytes cultured in tissue culture dish and *in vivo* in rat tibiae. In addition, PGE₂ release was shown to be stimulated in response to 100 uM serotonin injection, and the effect was 3-fold increase after one hour (Figure 2-4). The team also identified tryptophan hydroxylase, the initial and rate-limiting enzyme in serotonin synthesis, thus proving the existence of a functional serotonin synthesis mechanism in osteocytes. Overall, the team identified the existence of serotonergic pathways and successfully provided functional evidence of serotonin on osteocytes *in vitro*. 
Figure 2-4: Serotonin stimulates the release of PGE₂ from MLO-Y4 cells represented in time course. MLO-Y4 or MC3T3-E1 cells were treated for the indicated times with serotonin (100 μM) or vehicle and the amount of PGE₂ in the medium following treatment was measured using a PGE₂ EIA kit (Cayman Chemical) according to the manufacturer's instructions. Data shown were derived from three experiments performed in triplicate. Bars represent mean ± SEM. Each data point represents treated minus vehicle control for that time point. Baseline (untreated) PGE₂ concentrations were 643±196 pg/ml for MLO-Y4, and 554±224 for MC3T3-E1. *p<0.01; **p<0.001 compared to MC3T3-E1. (Reprinted from M. Bliziotes et al.: Bone 2006;39(6):1313-21 [24], with permission from Elsevier.)

2.7.3 Mechanism of Serotonin-Induced PGE₂ Release

Serotonin in bone is mainly mediated via the serotonin receptors on the cell membrane, the ones found in osteocytes are receptors 1A and 2A. Serotonin can also mediate its function through the transporter; and tryptophan hydroxylase is the rate-limiting enzyme for serotonin synthesis. Current studies in bone suggest that serotonin function in bone cells
is entirely mediated via the serotonin receptors. However, no data has yet to support the roles of the transporter or endogenous production of serotonin as active mechanisms [63].

There is limited research available on the study of serotonin in osteocytes, most of the work are done on osteoblasts [62]. It was shown that serotonin binds to Htr1b receptor in osteoblasts and inhibits the expression and function of Creb (cAMP response element binding protein), resulting in reduced osteoblast proliferation [62].

There are, however, published studies on the mechanism of serotonin in non-bone cells. In mesangial (smooth muscle like) cells, serotonin increased PGE2 release, and this effect was abolished by serotonin receptor antagonist ketanserin [66]. Intracellular calcium inhibitor 8-(N,N-diethylamine)-octyl-3,4,5 trimethoxybenzoate (TMB-8) and not extracellular calcium inhibition abolished the effect, suggesting the role of intracellular calcium in serotonin-induced PGE2 release. In neurons, PGE2 and serotonin each induced calcium response, and the effects were abolished by removing extracellular calcium. In addition, calcium response induced by PGE2 and serotonin were not additive [67]. Taken together, these studies pinpoint the role of calcium as a potential mediator of serotonin-induced PGE2 release. Nevertheless, no studies have reported the mechanism of serotonin in osteocytes.

2.7.4 Possible ATP Involvement in OFF Regulation of Serotonin-Induced PGE2 Release

In Hamster ovary cells, ATP activation of P2Y receptor reduced 5-HT1A (serotonin receptor 1A) responsiveness to agonist [23]. In MLO-Y4 osteocytes, flow activated rapid release of ATP through the hemichannel, which can feed back on P2Y receptors on the cell
Flow also caused release of PGE2, but this was not entirely through hemichannel but based on upstream factors such as ATP [17]. Therefore, it is possible that ATP played the role in changing the serotonin receptor responsiveness following OFF exposure. In which case, OFF caused rapid release of ATP, which feeds back on MLO-Y4 cells through P2Y receptors to decrease the responsiveness of serotonin receptors. This then caused decreased response of serotonin-induced PGE2 release.
Chapter 3: Experimental Approaches

This project evaluates the effect of mechanical stimulation on the chemical sensitivity of osteocytes in MLO-Y4 osteocytes in vitro. An extensive literature research was conducted initially on osteocyte response to various chemical stimuli. I identified serotonin as the chemical to study, and intracellular calcium and PGE₂ release as the responses to measure.

3.1 Serotonin Confirmation Study

My first task was to confirm the literature result that serotonin can induce 3-fold PGE₂ increase in MLO-Y4 osteocytes (Figure 2-4). Cells seeded on collagen-coated dishes were incubated with 100μM serotonin media for one hour.

3.2 Investigate OFF’s Effect on Serotonin-Induced Calcium Response

To study the first objective, MLO-Y4 cells seeded on collagen-coated glass slides were pre-incubated with Fura-2AM dye and then assembled in small parallel plate flow chamber [68]. After 30 minutes of rest on the imaging stage, the cells were exposed to 3 minutes of no flow (static) or OFF precondition. Then, fluorescence images were taken continuously for 1 minute at both 340nm and 380nm at baseline condition. It is followed by 3 minutes of imaging with the addition of serotonin. OFF precondition was achieved by OFF at 1 Pa and 1 Hz for 3 minutes, which are based on previous studies on osteocytes [5, 69]. Serotonin (100μM, Sigma) in working media (phenol red-free α-MEM with 1% FBS, 1% CS, 1% PS) was slowly perfused using a syringe pump (Cole Parmer, USA) and the flow rate was minimized to reduce the shear stress generated by the perfusion to not stimulate the cells.
by the process. Chart 3-1 shows the scenarios of serotonin-induced calcium without or with 3 minutes of OFF precondition.

**Chart 3-1:** Serotonin-induced calcium with or without 3 minutes of OFF precondition.

### 3.3 Calcium Pathway Blocking Approach

For objective 2, the goal was to investigate the mechanism of how OFF can change the calcium response to serotonin in osteocytes. Since OFF can induce calcium response in osteocytes [69] and in my study I showed that serotonin can induce calcium response as well, I speculate that they share similar pathways to induce calcium response, and hence leading to shared activation of specific calcium signaling events. I used four different inhibitors to block different parts of calcium pathway in osteocytes. Thapsigargin (1μM, Sigma) was used to inhibit ca\(^{2+}\) ATPase pump and cause intracellular calcium store depletion in the endoplasmic reticulum [15]. Calcium free media was used to eliminate extracellular calcium and blocking its influx upon activation. Gadolinium (Gd\(^{3+}\), 10μM, Sigma) inhibits mechanosensitive cation channel and voltage operated L-type calcium channel [70]. Cadmium chloride (Cd\(^{2+}\), 100μM, Sigma) is used to block calcium channels on the cell surface [67]. All inhibitors were applied by pre-treating the cells with inhibitor for 30 minutes prior to placing into flow chamber and the concentrations based on previous findings. The procedure is illustrated in Chart 3-2.
3.4 Investigate OFF’s Effect on serotonin-induced PGE₂ release

To study the fourth objective, experimental approach in Chart 3-3 was used. Flow precondition was achieved by 2 hours of OFF at 1 Pa and 1 Hz. The flow parameters were chosen based on previous studies on osteocytes to significantly alter the cell’s signalling response [5]. Immediately after OFF precondition, the slides were extracted from flow chambers and placed in 100 mm tissue culture dishes where serotonin was administrated at 100μM in α-MEM with reduced serum (0.2% FBS, 0.2% CS, 1% PS) for 2 hours. A total of 10ml of media was added to each dish to ensure covering of the slide.

3.5 ATP inhibition

To investigate the role of ATP in the mechanism of OFF changing serotonin-induced calcium and PGE₂, I used suramin, which is a non-selective P2 receptor inhibitor and has been used to inhibit P2 receptors in MLO-Y4 cells to prevent ATP autocrine/paracrine effects [14]. The cells were pre-treated with 100uM suramin for 30 minutes prior assembling into the flow chambers and followed procedures in Chart 3-1 and Chart 3-2 to test the involvement of ATP with P2 receptor inhibited.
Chart 3-3: Experimental procedure for combined chemical and mechanical stimulation

3.6 Cell Culture

Murine long bone osteocyte Y4 (MLO-Y4) cells (gift of Dr. Lynda Bonewald, University of Missouri - Kansas City, MO, USA) were cultured on type I rat tail collagen (BD Laboratory)-coated 100-mm tissue culture dishes in α-MEM (GIBCO) supplemented with 2.5% (v/v) Fetal Bovine Serum (FBS, Hyclone), 2.5% Calf Serum (CS, Hyclone), and 1% Penicillin/Streptomycin (P/S, GIBCO). Cells were maintained at 37 °C and 5% CO₂ in a humidified incubator (Thermo Scientific). Cell subculture was performed when the cells reached 70% confluence. Cells were seeded on collagen-coated small glass slides (75mm×26mm×1mm) for calcium imaging and on large glass slides (75mm×38mm×1mm) for PGE₂ experiment at 150,000 cells per slide 48 hours prior to experiment to ensure 80% confluence at the time of experiment. For PGE₂ studies, the cells were media starved of nutrients (using 0.05% PBS and 0.05% PS) 12 hours prior to flow experiment to synchronize the cell cycles. For serotonin confirmation study, cells were seeded on collagen coated 6-well
tissue culture dishes at 75,000 cells/well 48 hours prior to experiment to ensure 80% confluence at the time of experiment.

3.7 Real-Time Calcium Imaging

Cells were incubated at room temperature with Fura-2 AM (5 μM) (Invitrogen), a ratiometric fluorescent calcium indicator, for 1 hour prior to imaging with fluorescent microscope (Nikon) equipped for computer image acquisition (PTI, USA). Temporal profiles were determined for approximately 40 cells per field. Each cell was classified as responding or not responding: a cell response was defined as a transient increase in the fura-2 340 nm/380 nm ratio of at least 2 times the maximum oscillation recorded during the baseline period [69]. Responsiveness was characterized by the fraction of responding cells. The calcium data from the software (EasyRatioPro, PTI) was quantified using Matlab by percentage of cells responding and magnitude of response.

3.8 Oscillatory Fluid Flow

Oscillatory fluid flow is a common mechanical stimulation used to model physiological loading on bone experienced by osteocytes. In brief, the laminar oscillatory fluid flow was driven by an electro-mechanical loading device (Mechanical & Industrial Engineering, University of Toronto) mounted with Hamilton glass syringe in series with rigid walled tubing and parallel plate flow chamber as described before [68]. For PGE₂ studies, MLO-Y4 cells seeded on collagen-coated glass slides were mounted in the large parallel plate flow chambers (chamber size: 38mm × 10mm × 0.254mm) and exposed to a total of 2 hours of OFF with peak sinusoidal wall shear stress of 1 Pa at 1 Hz incubated at 37°C and 5% CO₂. Control slides were incubated in the parallel flow chamber, and were not subjected to
OFF (static cells). Fresh culture media was used as flow media during the experiment. For calcium studies, OFF of 1 Pa at 1 Hz for 3 minutes was used to precondition or to stimulate the cells.

3.9 PGE\(_2\) Release

Immediately following experiments, conditioned media from the chamber as well as the inlet and outlet tubing (approximately 10ml in total) were collected and centrifuged at 12,000 g. Supernatant PGE\(_2\) levels were measured using Prostaglandin E2 EIA Kit (sensitivity: 50% B/B0: 50 pg/ml; 80% B/B0: 15 pg/ml) (Cayman Chemical, USA). PGE\(_2\) levels of each experimental group were normalized to cell number.

3.10 Statistics

Student's t-test was used for two sample comparisons. ANOVA were used to compare observations from more than two groups, followed by Tukey and Dunnet post-hoc tests. Confidence level of 95% (*p < 0.05) was considered statistically significant for all statistical analyses. Data were reported as mean value ± standard error (SE).
Chapter 4: Results

4.1 Serotonin Treatment Induces PGE\textsubscript{2} Release in Osteocytes

I confirmed literature result [24] and demonstrated that one hour of serotonin treatment increased PGE\textsubscript{2} release by 3-fold in MLO-Y4 cells (Figure 4-1).

![Figure 4-1](image)

*Figure 4-1*: Normalized PGE\textsubscript{2} concentration for chemical treatment in 6-well for 1 hour on MLO-Y4 cells, n=6.

4.2 OFF Decreases Osteocyte Response to Serotonin in Triggering Calcium Mobilization

The application of 100μM serotonin induced rapid calcium response in osteocytes (Figure 4-2B). However, when the cells were mechanically preconditioned by OFF (3min, 1Pa, 1Hz), the cells (Figure 4-2C) displayed reduced calcium response to serotonin (Figure 4-2D, Figure 4-21E). The percentage of cells responding to serotonin decreased from 73% in the non-preconditioned case to 19% in the OFF preconditioned case (Figure 4-2D), while the average magnitude of the cells with response was also reduced (Figure 4-2E).
Figure 4-2: Serotonin-induced calcium response (340nm/380nm intensity ratio) of MLO-Y4 cells to A) control (media with no serotonin) B) serotonin C) serotonin after precondition of
3min OFF. Each line represents the intracellular calcium levels of a single cell within the field of view D) percentage response and E) magnitude of response between (B) and (C), n>4.

4.3 OFF Regulates Serotonin-Induced Intracellular Calcium Response by Utilizing Same Calcium Pathways

Calcium inhibition studies were conducted to investigate the calcium pathways induced by serotonin and OFF, using the procedure outlined in Chart 3-2. A sample of calcium profile for each inhibition condition is shown in Appendix G (OFF-induced calcium response) and Appendix H (Serotonin-induced calcium response). All inhibitors significantly blocked calcium response to both stimuli with the only exception of gadolinium, which did not block serotonin-induced calcium. This suggests that both intracellular and extracellular calcium are involved in OFF- and serotonin-induced calcium response, and the mechanosensitive channel is not involved in serotonin-induced calcium (Figure 4-3A). In contrast, the magnitudes of responding cells were generally not affected by the calcium pathway inhibitions (Figure 4-3B). The only difference was seen in the thapsigargin-inhibited groups where the magnitude of cell response was greatly reduced by nearly 2-fold.

4.4 OFF Regulation of Serotonin-Induced Intracellular Calcium Response Does Not Depend on ATP

Since ATP is a fast acting molecule, ATP may also act through P2Y receptors to affect calcium, and it has been reported recently that ATP inhibition reduces osteocyte’s calcium response to OFF [14]. Therefore, I investigated whether ATP plays a similar role in serotonin-induced calcium response in osteocytes with or without OFF-precondition. By
adding an extra 30 minutes of suramin inhibition and following procedures outlined in Chart 3-1 and Chart 3-2, I obtained calcium profiles shown in Appendix I. My result (Figure 4-4A Left Column) is consistent with previous result [14] that suramin reduced osteocyte response to OFF. However, ATP inhibition by suramin did not affect serotonin-induced calcium response with or without OFF precondition (Figure 4-4A Middle and Right Columns).

Figure 4-3: Calcium inhibition results for serotonin-induced (red) and OFF-induced (blue) calcium on MLO-Y4 cells, showing A) percentage of responding cells B) magnitude of responding cells. n>3 for all groups. *p<0.05 vs. no inhibition control.
This suggests that serotonin-induced calcium response does not depend on ATP signaling through its receptors, and that OFF regulating serotonin-induced calcium response also does not depend on ATP signaling through the P2 receptors. The magnitudes of the responses (Figure 4-4B) were not affected by ATP inhibition.

![Figure 4-4](image)

**Figure 4-4**: ATP inhibition by suramin on calcium response induced by OFF alone, serotonin alone, or serotonin with 3 minutes of OFF precondition, showing A) % response and B) magnitude of response. n>3 for all groups.
4.5 OFF Decreases Osteocyte Response to Serotonin in Releasing PGE$_2$

Following procedure illustrated in Chart 3-3, I obtained the 4 conditions for the combined mechanical and chemical stimulation on osteocyte PGE$_2$ release. Without mechanical stimulation, serotonin treatment (100uM, 2 hours) increased osteocyte PGE$_2$ release by approximately 3-fold (Figure 4-5A, NN vs. NS). However, after MLO-Y4 osteocytes have been mechanically stimulated for 2 hours at 1Pa and 1Hz, the cells were no longer affected by serotonin treatment in PGE$_2$ release (Figure 4-5A, FN vs. FS). Although the baseline PGE$_2$ release was increased by OFF precondition (Figure 4-5A, FN), serotonin treatment (100uM, 2 hours) did not cause further increase in PGE$_2$ release.

**Figure 4-5:** A) PGE$_2$ concentration for combined chemical and mechanical treatment on MLO-Y4 cells according to procedure outlined in Chart 3-3, n=4 for all groups. B) Effect of ATP receptor inhibition using suramin on PGE$_2$ release for combined chemical and mechanical treatment on MLO-Y4 cells. Experiment procedure was similar to (A) except that
all groups were pre-incubated in suramin for 30 minutes. NN = no flow, no serotonin. NS = no flow, with serotonin. FN = with flow, no serotonin. FS = with flow, with serotonin. n=8 for all groups.

4.6 OFF Regulation of Serotonin-Induced PGE$_2$ Release Depends on ATP Signaling Through P2 Receptors

To test whether ATP plays a role in OFF modifying osteocyte sensitivity to serotonin in releasing PGE$_2$, the P2 purinergic receptor blocker suramin was used, which inhibits both P2X and P2Y receptors on the cell surface and abolishes subsequent signaling by ATP through the receptors. With the suramin precondition, serotonin alone still stimulated PGE$_2$ release by approximately 3-fold, from 118 pg/ml per 20,000 cells (Figure 4-5B NN) to 385 pg/ml per 20,000 cells (Figure 4-5B NS). In the case with the cells being mechanically preconditioned by OFF (2 hours, 1 Pa, 1 Hz), the serotonin treatment (100uM, 2 hours) caused a significant increase in PGE$_2$ release in osteocytes (Figure 4-5B FN vs. FS). Although the increase of approximately 1.6-fold (Figure 4-5B FN: 199, FS: 327) was lower than that induced by serotonin alone, this was significantly different than the non ATP inhibited case where the sensitivity to serotonin was completely abolished (Figure 4-5A FN vs. FS). This data suggests that ATP plays a part in OFF regulation of serotonin-induced PGE$_2$ release in osteocytes, although it does not explain the full effect. Another effect of suramin was the reduction in the overall PGE$_2$ release by approximately 2-fold compared to non-suramin treated case (Figure 4-5A all groups vs. Figure 4-5B all groups).
Chapter 5: Discussion

The present study explored the effect of mechanical stimulation on chemo-sensitivity of osteocytes. I first showed that serotonin can induce rapid calcium response in MLO-Y4 osteocytes, and that 3 minutes precondition of mechanical stimulation by OFF significantly reduced the response (Figure 4-2). I also demonstrated that MLO-Y4 osteocytes respond to exogenous serotonin by increasing the release of PGE$_2$, which is consistent with previous findings [24]. Furthermore, I found that when the cells were preconditioned with 2 hours of OFF, serotonin treatment no longer had any effect on PGE$_2$ release (Figure 4-5A). Together, these data suggest that mechanically pre-treating the osteocytes by OFF can reduce their sensitivity towards serotonin in triggering intracellular calcium mobilization and release PGE$_2$.

To investigate the mechanism underlying OFF reducing calcium response to serotonin, I have looked at the difference in calcium pathway between OFF and serotonin stimulation of calcium. I have found that both intracellular calcium and extracellular calcium were involved in both types of stimulation (Figure 4-3). The only exception is that gadolinium did not block serotonin-induced calcium response. Gadolinium is an inhibitor for the mechanosensitive cation channel and voltage operated L-type calcium channel. This makes sense for serotonin as it functions mainly through the specific serotonin receptors [63]; and serotonin receptors have been linked to the IP3 pathway [71]. Therefore, it is possible for serotonin to induce intracellular calcium directly through receptor activation leading to IP3 release, and does not involve the mechanosensitive or voltage operated calcium channels. My result is consistent with literature where extracellular calcium has been shown to be involved in serotonin-induced calcium response in rat sensory neurons [67]. For OFF-induced calcium...
response, recent studies have reported both intracellular and extracellular involvement [13-15, 69, 72-74]. Overall, since both intracellular and extracellular calcium were important in serotonin and OFF induced calcium response, I speculated that precondition by OFF activated calcium pathways, causing calcium pathway components to be pre-occupied when the same components were subsequently activated by serotonin, resulting in reduced response due to cell recovery. A recent mathematical model has been developed for the calcium response to fluid shear stress [50], where ATP precondition reduced shear stress-induced intracellular calcium response in bone cells. It was believed that precondition with extracellular ATP caused messenger molecules (e.g. IP3) to be kept at a higher level than the un-pretreated case, therefore making shear stress after precondition to only induce less augmenting of these messages, resulting in lower intracellular calcium response. Likewise, I speculate that precondition by OFF would cause messenger molecules like IP3 to be up-regulated and pre-occupied, so serotonin would be able to induce less calcium response than in the un-pretreated case. Indeed, it has been shown that osteoblast cells responded with more calcium response if OFF was applied with rest periods inserted rather than continuous loading [4], suggesting that cells need time to recover for calcium response.

ATP was selected as a candidate molecule to study in the mechanism of OFF affecting serotonin-induced effects because it has been shown that ATP was rapidly released following OFF exposure [17] and can act on P2 receptors to induce calcium response and decrease the sensitivity of serotonin receptors [23]. Therefore, it is possible that ATP could potentially be involved in changing the sensitivity of osteocytes to serotonin in triggering calcium or releasing PGE2. Suramin was used as it effectively blocks purinergic P2 receptors in MLO-Y4 osteocytes [14]. I first investigated whether ATP plays a role in OFF regulating
serotonin-induced calcium response. I found ATP to be involved in OFF-induced calcium response (Figure 4-4A Left Column); this is consistent with literature [14]. ATP was not involved in serotonin-induced calcium regardless of OFF precondition (Figure 4-4 Middle and Right Columns). This suggests that OFF-induced calcium response depend on ATP whereas serotonin-induced calcium response does not. The role of ATP in OFF-induced calcium response has been recently reported, as OFF causes rapid release of ATP [17], which can in turn cause calcium response. ATP also has the ability to travel to neighbouring cells to stimulate multiple responses in osteocytes and osteoblasts [15]. Since I did not find serotonin-induced calcium response to be affected by ATP, it suggests that serotonin does not trigger ATP release. It is likely for serotonin-induced calcium response to be mediated by serotonin receptors, but the specific receptor subtype has not been confirmed in MLO-Y4 osteocytes [24]. The ATP inhibition studies also demonstrate that ATP is not involved in OFF modulation of serotonin-induced calcium. In this regard, the OFF precondition up-regulated message molecules in the cytosol (e.g. IP3, ATP), but serotonin stimulation of calcium response does not depend on ATP, so no effect of ATP blocking was observed. Overall, I found the main difference between OFF and serotonin stimulation of calcium response to be the involvement of mechanosensitive cation channels and ATP signaling in OFF. Based on the evidences from literature and results from this study, I have summarized and proposed the mechanism for OFF regulation of serotonin-induced calcium response (Figure 5-1).

I have also investigated whether ATP is involved in OFF regulation of serotonin-induced PGE₂ release. By using the P2 receptor blocker suramin, I found a partial restoration of the OFF abolishment on serotonin-induced PGE₂ release (Figure 4-5B). With both OFF
precondition and ATP receptor inhibition, serotonin had a relatively small but significant increase (1.6-fold) in PGE2 release compared to just OFF precondition without ATP inhibition where PGE2 release was not affected by serotonin. Although ATP does not explain the full effect of OFF abolishing serotonin-induced PGE2 release, it does however suggest the involvement of ATP via acting on the P2 receptors. One possible implication is that ATP released by OFF activated subsequent pathways that decreased PGE2 release by serotonin. One such candidate molecule is Arachidonic Acid (AA), which is required for the synthesis of PGE2. It has been shown that both OFF [75] and serotonin [76] can regulate AA, therefore serving as a common pathway in osteocyte PGE2 release linked to serotonin, OFF and ATP. A proposed mechanism of osteocyte PGE2 release in response to OFF and serotonin is shown in Figure 5-2.

Another interesting observation was that ATP inhibition by suramin reduced overall PGE2 release by approximately half in all cases of OFF and serotonin stimulation (Figure 4-5A vs. Figure 4-5B). Various studies have shown ATP acting on P2 receptors to affect downstream PGE2 release induced by both OFF [17, 18, 49] and serotonin [23, 77]. Therefore, my result is consistent with the fact that ATP is involved in OFF regulation of PGE2 release and possibly on serotonin induced PGE2 release as well. The exact mechanism of OFF modulation of osteocyte sensitivity to serotonin is still unclear and certainly more complex than just ATP.
In retrospect, selective serotonin reuptake inhibitors (SSRIs) have been used clinically to treat depression; however, it is associated with catabolic effects on bone health [63]. It has recently been shown that serotonin synthesized in the gut can travel through the bloodstream to reach bone cells, where serotonin can reduce osteoblast activity [62]. Therefore, this is one potential mechanism mediating the deleterious effects of SSRI on bone. However, in my study, I have shown that mechanical stimulation can reduce the osteocyte...
Figure 5-2: Proposed mechanism of osteocyte PGE$_2$ release upon stimulation by OFF and serotonin. Question marks indicate unidentified yet possible mechanisms. COX2 = Cyclooxygenase 2, AA = Arachidonic acid.

sensitivity towards serotonin. If the same relationship is true for osteoblasts, it can be speculated that under mechanical loading conditions, osteoblasts would also be less responsive to the harmful effects of serotonin. Therefore, doing more exercise to keep osteoblasts under mechanical loading can be a strategy to mitigate the deleterious effects of serotonin on bone when treating with SSRI.
Chapter 6: Future Work

6.1 Explore More Chemicals on Osteocyte Effect Modulation by Mechanical Stimulation

In this preliminary study on mechanical regulation on osteocyte chemo-sensitivity, I looked at only one specific chemical stimulation, serotonin. However, osteocytes are sensitive to a variety of chemical stimulations as listed in Chapter 2, Section 2.6. In future, more chemical stimulations sensitive to the osteocytes can be studied, specifically focusing on their effects modulation by mechanical stimulation. It would be interesting to see whether the same relationship for OFF and serotonin hold for other chemical stimulations.

6.2 Study Additional Effects of Serotonin on Osteocytes

In this study, I only studied two effects of osteocyte induced by serotonin: intracellular calcium mobilization and PGE₂ release. These effects were found to be regulated by mechanical loading by OFF. This is because of the lack of knowledge of the effects of serotonin on osteocytes. However, osteocytes are known to be very responsive to OFF (as shown in Chapter 2, Section 2.5), by secreting a variety of cytokines and alter their gene expressions. Potentially, osteocytes have the capability to respond to serotonin in ways other than intracellular calcium and PGE₂ release. These additional effects that serotonin might have on osteocytes can be identified and explored on their sensitivity change by OFF. This would help characterize the degree of modulation by OFF on osteocyte sensitivity towards serotonin.
6.3 Explore Calcium in OFF Regulation of Serotonin-Induced PGE$_2$

Response

I have considered the idea that calcium mobilization is involved in OFF regulation of serotonin-induced PGE$_2$ release. I speculate that OFF could modify PGE$_2$ by changing calcium response. Previous literature have shown that flow-induced PGE$_2$ in rat calvarial osteoblasts [78] and osteocytes [75] were decreased by calcium inhibition. However, studies have also shown that flow-induced PGE$_2$ can occur without intracellular calcium store [69]. And more importantly, PGE$_2$ can be inhibited without noticeable change in calcium signal in osteocytes [69, 79]. Therefore, the relationship between calcium and PGE$_2$ show mixed results, and it is unclear whether calcium can affect the PGE$_2$ response in osteocytes. This remains an interesting area to explore in future studies.

6.4 Improve calcium inhibition study for serotonin

In this study, I only explored 4 inhibitors of calcium pathway. Although a number of calcium pathways have been reported in osteocytes in response to OFF [14], the same is not true for serotonin. Serotonin-induced calcium response in osteocytes has never been studied before, and this study is the first to show the relationship. In future studies, more inhibitors can be used to pinpoint the exact calcium pathway for osteocyte calcium response to serotonin. Inhibitors that block IP$_3$, IP$_3$ receptor or specific serotonin receptors could be used.
6.5 Study the Reverse: Effect of Chemical Stimulation on Osteocyte Mechano-Sensitivity

Osteocytes experience both chemical and mechanical stimulation in the bone. This study looked at one side of the problem, how mechanical stimulation can affect the osteocyte sensitivity towards chemical stimulation. The other side of the problem is also interesting and perfectly valid to study. That is, how can the precondition of osteocytes using chemical stimulation affect their sensitivity towards mechanical stimulation? Due to the similarities in the pathways found in this study, I speculate the trend to be the same as in this study. One can take this even further by proposing to study both stimulations at the same time. Based on the results from this study, I do not speculate additive effects. The resulting response for the combined treatment may be between the response levels for each individual treatment.
Chapter 7: Conclusion

This is the first study to investigate the effect of mechanical stimulation on the chemo-sensitivity of osteocytes. I first showed that serotonin can induce rapid calcium response in MLO-Y4 osteocytes, and that 3 minutes precondition of mechanical stimulation by OFF significantly reduced the response (Figure 4-2). I also demonstrated that MLO-Y4 osteocytes respond to exogenous serotonin by increasing the release of PGE2 (Figure 4-5A), which is consistent with previous findings [24]. Furthermore, I found that when the cells were preconditioned with 2 hours of OFF, serotonin treatment no longer had any effect on PGE2 release (Figure 4-5A). Together, these data suggest that mechanically pre-treating the osteocytes by OFF can reduce their sensitivity towards serotonin in triggering intracellular calcium mobilization and release PGE2.

I have investigated the mechanism of OFF affecting serotonin-induced calcium response by using a number of calcium inhibitors and looking at the calcium response to OFF and to serotonin. I have shown that both OFF- and serotonin-induced calcium depend on intracellular and extracellular calcium. The similarity in their calcium pathways can be used to explain the OFF-induced calcium sensitivity effects, and suggests that precondition by OFF already activated calcium pathways and when serotonin was trying to activate the same calcium pathways, the response was lower due to cell recovery. I have also determined that serotonin-induced calcium response does not depend on ATP signaling through its receptors, and that OFF regulating serotonin-induced calcium response also does not depend on ATP signaling through the P2 receptors. Overall, I found the main difference between OFF and serotonin stimulation of calcium response to be the involvement of mechanosensitive cation channels and ATP receptors in OFF.
I have also identified ATP as a likely target molecule involved in OFF affecting serotonin-induced PGE$_2$ release. In this study, I showed that ATP plays a part in OFF regulation of serotonin-induced PGE$_2$ release in osteocytes, although it does not explain the full effect. I also found the ATP inhibition to reduce the overall PGE$_2$ release by 2-fold when stimulated by OFF or serotonin. This is consistent with literature and suggests that ATP is involved in OFF regulation of PGE$_2$ release and possibly on serotonin induced PGE$_2$ release as well.

Mechanical stimulation and chemical stimulation has not been studied together on osteocytes, which are mechanosensitive and respond to a large number of cytokines. Serotonin has recently been found to be involved in bone metabolism and its current cellular mechanisms are under debate. We hope this study will expand our knowledge on the effect of serotonin on osteocytes and shed light on combinational therapies using exercise and chemical-based strategy in treating osteoporosis and other musculoskeletal diseases.
References


33. Gray, H., *Anatomy of the Human Body*, Fig. 77 available online at http://www.bartleby.com/107/illus77.html1918.


Appendices

Appendix A: Parallel Plate Flow Chamber System

The parallel plate flow chambers shown below consist of a chamber and a cover, each piece contains ten screw holes for fixing. The glass slide with cells seeded on one side is placed inside the chamber, layered with a gasket, and covered with the cover. To ensure optimal performance, media is filled in the chamber before assembling the slide to ensure no bubbles are trapped inside the flow chamber system. The existence of bubbles in the flow chamber is problematic because it changes the fluid flow induced shear stress applied to the cells.

The fully assembled chamber is then attached to a Hamilton glass syringe via PVC tubing (VWR). The syringe is mounted on the electro-linear actuator (Machine Shop, Mechanical & Industrial Engineering, University of Toronto) to drive fluid in oscillatory motion.
The small parallel plate flow chamber is theoretically similar to the large flow chamber but with a few structural differences. There is no cover and instead the glass slide (also smaller) serves as the cover with the side without cells facing out. Rather than placing the glass slide into the chamber the gasket is inserted between the flow chamber and the gasket. Holes are punched in the gasket exterior region to allowing sealing by vacuum. The gasket interior is cut out to allow fluid to traverse. The setup for small flow chamber is shown below [1].

Fluid in oscillatory motion flows between the inlet and outlet to apply shear stress to the cells in the chamber. A cross-section of the flow chamber is depicted below to illustrate the
shear stress force with chamber dimensions. Two types of flow chambers are used in this study, the interior dimensions of flow chambers are listed below:

- Small flow chamber for calcium imaging experiment: 38mm (w) ×10mm × 0.254mm (Δ)
- Large flow chamber for PGE2 release experiment: 75mm (w) × 34mm × 0.28mm (Δ)

The reason why two types of flow chambers are used is because of the distinctive advantages for each type. The large flow chamber can be assembled inside the hood to keep the samples sterile. This is ideal for experiments requiring long duration, in which bacterial infection could become an issue if the chamber is not kept in sterile environment. The small flow chamber, however, is more ideal for calcium analysis. It does not have the cover like in the large flow chamber and instead uses the glass slide. This makes imaging through the slide possible, as most microscope objectives have a relatively short working distance. Although the small flow chamber is not assembled inside the hood, this is not a problem for the purpose of calcium imaging, as the experiments are very short.

Appendix B: Assembling the Flow Chambers

The large flow chamber is assembled by using screws.

The procedure for assembling small flow chambers is trickier and uses vacuum and grease:

**Step 1:**

Carefully grease the outer edge of the inner platform of the flow chamber.

**Step 2:**

Carefully place the gasket onto the flow chamber and press down on the areas that are greased to make a tight seal.

**Step 3:**
Use the syringe to push media into inlet and outlet tubing, allowing a small bolus of media to come up onto the flow chamber and limit the bolus to the flow area only. Make sure that there are no bubbles in the inlet and outlets (scraping a 10\(\mu\)l pipette tip in the inlet and outlet slots on the flow chamber can be effective in getting rid of bubbles).

**Step 4:**

Connect the outlet tubing end into a 20ml syringe filled with medium. Get rid of the bubble in the end. (This will prevent the cell being exposed to air if leakage is a problem)

**Step 5:**

Carefully (use tweezers) to lift the glass slide containing cells off the tissue culture dish, then grab edges with hands (be careful not to touch surface of the slide). Flip over so that cells face down, and quickly place on the flow chamber and push very hard until the sound of vacuum is stopped. Ensure that the slide is properly placed. Wipe off excess fluid on the bottom of the slide.

**Step 6:**

Flip the chamber over (so the slide is on the bottom) and secure to microscope stage for calcium imaging.
Appendix C: Flow Rate Calculations

Calculation for Linear Actuator Syringe Displacement

Shear stress:
\[ \tau = \frac{Q_{\text{max}}}{k w} \]
\[ Q_{\text{max}} = \frac{\tau h w}{\mu} \quad (1) \]

- \( h \) = height between slide and flow chamber, aka \( \Delta \) (mm)
- \( w \) = width of flow chamber (mm)
- \( \mu \) = viscosity (Pa.s)
  \( \approx 1 \times 10^{-2} \) Pa.s (at room temp)
- \( \tau \) = shear stress (1-3 Pa)

Displacement:
\[ d = d_0 \sin(\omega t) \]
\[ d_0 = \text{max. displacement} \]
\[ \omega = \text{angle speed} \quad \text{for 1-Hz: } \omega = 2\pi, \ s^{-1} \]

Velocity:
\[ u_{\text{max}} = \frac{\partial d}{\partial t}|_{\text{max}} \]
\[ u_{\text{max}} = d_0 \frac{\partial}{\partial t} \left[ \omega t \right] |_{\text{max}} \quad (3) \]

- \( u_{\text{max}} \) = sub (2) in (3)
- \( u_{\text{max}} = \omega d_0 \cos(\omega t) \) |_{\text{max}}
- \( u_{\text{max}} = 2\pi d_0 \) |_{\text{max}}

\[ Q_{\text{max}} = u_{\text{max}} A \quad (5) \]
- \( A \) = cross-sectional area of syringe = \( \frac{\pi}{4} D^2 \)
- \( D \) = inner diameter of syringe

Now, sub (4) into (5):
\[ Q_{\text{max}} = (2\pi d_0) \left( \frac{\tau h w}{4\mu} \right) \]
\[ Q_{\text{max}} = \frac{\pi^2}{2} d_0 D^2 \quad (6) \]

Finally, sub (6) into (1):
\[ Q_{\text{max}} = \frac{\tau h w}{3\pi^2 \mu} \]
\[ d_0 = \frac{\tau h w}{3\pi^2 \mu} D^2 \quad \Rightarrow \text{solve for } d_0 \]

*** NB: This is the distance from 0 to max. distance travelled by the plunger.

To get total syringe displacement, multiply by 2!

\[ d_{\text{plunger}} = \frac{2\pi h w}{3\pi^2 \mu D^2} \]

* this solution is valid for both small and large flow chambers

\[ d_{\text{plunger}} = 6.356(\tau h w) \]
- \( d_{\text{plunger}} \) = full distance travelled by plunger (mm)
- \( h \) = height between slide and flow chamber, aka \( \Delta \) (mm)
- \( w \) = width of flow chamber (mm)
- \( \tau \) = shear stress (Pa)
Calculation for Linear Actuator Syringe Displacement

Sample Calculations:

**Large Flow Chamber**

\[ \tau = 1 \text{ Pa} \]

\[ h = 0.28 \text{ mm} \]

\[ w = 34 \text{ mm} \]

\[ d_{\text{plunger}} = 6.356 \times (1 \text{ Pa}) \times (0.28 \text{ mm})^2 \times (34 \text{ mm}) = 16.9 \text{ mm} \approx 17 \text{ mm} \]

<table>
<thead>
<tr>
<th>Shear stress ((\tau, \text{ Pa}))</th>
<th>Displacement ((d_{\text{plunger}}, \text{ mm}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
</tr>
</tbody>
</table>

**Small Flow Chamber**

\[ \tau = 1 \text{ Pa} \]

\[ h = 0.32 \text{ mm} \] [using newest gasket, McMaster cat. # 87315K73]

\[ w = 10 \text{ mm} \]

\[ d_{\text{plunger}} = 6.356 \times (1 \text{ Pa}) \times (0.32 \text{ mm})^2 \times (10 \text{ mm}) = 6.5 \text{ mm} \]

<table>
<thead>
<tr>
<th>Shear stress ((\tau, \text{ Pa}))</th>
<th>Displacement ((d_{\text{plunger}}, \text{ mm}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.5</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>19.5</td>
</tr>
</tbody>
</table>
Appendix D: Protocol for Real-Time Intracellular Calcium Imaging on MLO-Y4 Cells

Materials

- Fura-2 AM (Invitrogen)
- DMSO, anhydrous
- Hamilton glass syringe (500 μl) with integrated needle
- 15 ml centrifuge tube
- Working media (MEM-alpha without phenol red + 1% calf serum + 1% FBS + 1% P/S)
- MLO-Y4 cells on glass slide

Procedure

* Fura-2 AM dye needs to be freshly diluted just before the experiment, and is valid for 24 hrs at room temperature and 1 month at -20°C (sealed with parafilm and wrapped in Al foil)

Diluting Fura-2 AM dye

- Wash Hamilton syringe with DMSO twice
- Thaw Fura-2 AM dye for 2-3 min (stored in -20°C freezer)
- Transfer 50 μl DMSO\(^1\) to the tube containing Fura-2 dye - the color of the solution should change
- Vortex solution for 1 min
- Disassemble syringe to air dry
- Dilute the Fura-2 solution in the 5 ml of working media in a 15ml centrifuge tube -> 10 μM final Fura-2 concentration
- Vortex for 1 min

Loading cells with Fura-2 AM dye

- Discard culturing media from the dish
- Wash slide with PBS twice; discard all PBS at the end of the wash
- Vortex Fura-2 solution for 1 min
- Apply enough to cover the slide (~ 1 ml)
- Incubate at room temperature for 30 min - 1 hr (1hr for optimal loading)

\(^1\) Make sure the DMSO container is tightly capped and wrapped with parafilm, replace DMSO every 3 months
• NB: loading at high temperature (i.e. 37°C) is more physiologically relevant, but it may cause dye particle leakage and non-homogeneous loading of fura, if that occurs, try loading at reduced temperatures and longer durations, such as 33°C and 31°C or RT.

• Discard Fura-2 solution into chemical waste bottle
• Wash slide with PBS twice, discard
• Wet slide with working media
• load slide onto flow chamber
• Let the cells sit for 30 min on microscope stage (at room temperature or 37°C) before treatment and imaging

Power on the systems

• computer
• turn on monochrometer and Xenon lamp; adjust current to ~0.70 Volts (replace bulb when current above 14 Watts)
  • NB: do not turn on/off the lamp frequently; allow to cool for at least 15 min before turning on again
• Open physical shutter on the microscope and change to correct lens (S Fluor 20x) and filter (Ca)
• camera (connect power, wait for green light, then connect USB to computer)

Setup EasyRatioPro software

• Click on Fura2 button, save the data on D drive in your own folder
• Camera -> Load -> Grabber -> OK
• Change camera properties “clocking mode” to “alternate normal”
• Click on “R” (red) on 340, 380 and ratio
• Select channels 340, 380 and ratio
• Adjust the exposure time to ~100ms
• Adjust binning (use 8x for OFF-induced calcium, use 1x for maximum resolution image)
• Click on “record” button
• Adjust the brightness by clicking “A” or sliding the small triangles
• Check the intensity, should be around 500 – 1000 (horizontal axis on histogram)
• If intensity falls below the range, try turning up the right knob on monochrometer
• Choose an imaging field of view
• Click on “stop” to close the shutter (avoid prolonged exposure to minimize photobleaching)
Draw ROI using the oval tool around a sample of cells (~10-20)
  • NB: the more ROI’s selected, the more RAM you will be using, so the camera imaging will be slower and may crash the software
  • If want to store image, check “authoring tools -> stream to tiff”

Record real-time calcium experiment

  • Click “>” (green) play button
  • To insert event (ie. flow on), click on “J” button to add label to the journal events
  • Click “stop” and save the session

Data Analysis

  • Open the session in EasyRatioPro
  • Delete the current ROIs by removing all cells from 340, 380 and ratio channels
  • Select channels 340, 380 and ratio
  • Redraw ROIs on more cells (~40) using polygon tool
  • Click on “play”
  • Click on “Bypass image manipulation” and hide Graph Window (Ctrl+Alt+2) to speed up the calculation
  • Each EasyRatioPro programs utilizes 1 core of CPU, so you can do multiple files at same time
  • In Graph Window, click File -> Save Record As -> change type Felix Text File -> *.txt
  • Open the file in excel, find the event column (empty except for event rows), record the rows for events, remove the columns, save in another .txt file
  • Open the new .txt file in Matlab, run testcodes.m to obtain % of cell response and magnitudes of responses

For chemical induction of calcium response in MLO-Y4 cells (i.e. control/proof-of-concept):

  • Lets cells sit for 30 min on microscope stage prior to imaging
  • Start baseline imaging (~3 min)
  • Add 20mM to 1M KCl to cells and continue imaging for several minutes
  • Alternatively, add 5uM ionomycin to cells
    • NB: adding ionomycin will cause irreversible Ca mobilization (open up the Ca channel), so you will not be able to use the cells after

Background subtraction
• Add “BGSubtract” to channel
• Find out-of-focus background image
• Click on “snap background”

*Need to subtract BG first and then calculate ROI
Appendix E: Matlab Code for Analyzing Calcium Data Outputted from EasyRatioPro

The calcium analysis uses 3 Matlab functions:

1. testcodes.m (main driver of the analysis)
2. readcadata.m (for handling file import)
3. peakdet.m (for detecting the maximum and minimum peaks within each calcium response for a single cell)

The Matlab codes for all three files are shown below.

1) testcodes.m

% Instructions -----------------------------------------------
% The file reading function in MatLab doesn't like the event marker columns
% in the exported calcium file. So you need to open the exported txt in Excel, delete the columns with the event marker, save as a new txt file (tab delimited). Then proceed with the script.

% Things to remember (or write down) while looking at the data in Excel:
% 1) the column number at which the ratio data starts
% 2) the row number of your event marker (e.g. flow start)

% This script outputs a table of results with the headings:
% Baseline amp | If 1st peak 4x (1 or 0) | Multiple 2x peaks (1 or 0) |
% Fold increase of 1st peak | Fold increase of subsequent peaks (each column represent a peak)

% required file: peakdet.m, readcadata.m

% Chao Liu, 1.0 (Explicitly not copyrighted).
% Johnny Zhang, 1.1 - Changes
% -added color, removed linfit, and norm plot
% -changed threshold for 1st peak to 2x
% -skip plotting of first line
% -declared first peak threshold as constant
% -output to .csv file
% ---------------------------------------------------------------

clear;

% Need inputs here ---------------------------------------------

% Name of your text file goes here
filename = '../../../Calcium Raw/20120427_OFF_inhib/5_OFF_thaps_79';
% Enter the row number of the events
flowon = 79;
% logics
ANALYSE_PEAK_TO_TROUGH = 1;
WRITE_FILE_MODE = 1;

% End of inputs حرية

readcadata([filename '.txt']);
% mydata=dlmread('fix.txt','	',4,0);
mydata = data;

% Enter the column number that the ratio data start at (the time column)
% ratiostart = 81;
ratiostart = size(mydata,2)/3*2+1;

% Start of baseline in terms of row#, not seconds (to compensate for the
% transient spikes at the beginning of each recording)
basestart = 20;
baseend = 50;
if ratiostart < baseend
    baseend = ratiostart;
end

% Detection threshold (# of times increase over baseline) for the
% first peak
FIRST_PEAK_THRES = 2;
%  clearfix

% output from EasyRatio Pro arranges the time series in odd numbered
% columns, the ratio series in even numbered columns
totalsample = max(size(mydata(1,1:end)));
k = 0;
% output = 0;
output = zeros((totalsample-ratiostart+1)/2,4);
hold on;

for samplenum=ratiostart:2:totalsample
    x=mydata(1:end, samplenum);
y=mydata(1:end, samplenum+1);

    % linear fit
    % P1 = polyfit(x,y,1);
    % ylinfit = polyval(P1,x);

    % baseline drift compensation
    ymean = mean(y(basestart:baseend));
    % ynew = ymean+(y - ylinfit);
    ynew = y-mean;
% change unit of time axis to seconds (used to be msec)
xsec = x/1000;

% get the amplitude of baseline
baselineamp = max(ynew(basestart:baseend)) - min(ynew(basestart:baseend));

% get the peaks during flow
% [MAXTAB, MINTAB] = peakdet(ynew, 0.015, xsec);
% [MAXTAB, MINTAB] = peakdet(ynew, baselineamp);

% output a table of results with the headings:
% Baseline amp | If 1st peak 4x (1 or 0) | Multiple 2x peaks (1 or 0) |
% Fold increase of 1st peak | Fold increase of subsequent peaks (each column represent a peak)
k = k+1;
output(k,1) = baselineamp;
firstpeak = 1;

% make sure there is at least 1 max
if isempty(MAXTAB) == 0
    sizeMAXTAB = size(MAXTAB);
    j = 0;
    % *** amplitude of peaks calculated from mean, baseline fit
    for i = 1:sizeMAXTAB(1,1) % if 1st peak is 2x or more
        if (MAXTAB(i,1) > flowon)
            % determine corresponding local min
            if (ANALYSE_PEAK_TO_TROUGH)
                if ( (size(MINTAB,1)>=i) && (MINTAB(i,1)<MAXTAB(i,1)) )
                    peaksize = MAXTAB(i,2) - MINTAB(i,2);
                elseif ( i>1 && size(MINTAB,1)>1 )
                    peaksize = MAXTAB(i,2) - MINTAB(i-1,2);
                else
                    peaksize = MAXTAB(i,2);
                end
            else
                peaksize = MAXTAB(i,2);
            end
            if (peaksize > (baselineamp * FIRST_PEAK_THRES))
                j = j+1; % inc number of peaks
                if (output(k,2) == 0)
                    % output true/false of 1st peak
                    output(k,2) = 1;
                    % fold increase of 1st peak
                    output(k,4) = peaksize/baselineamp;
                end
                output(k,3) = j;
            end
        end
    end
end

% plot for each ROI

64
rndclr=[rand,rand,rand];
plotsize = size(xsec,1);
plot(xsec(2:plotsize),ynew(2:plotsize), 'DisplayName','xsec vs.
ynew', 'XDataSource','xsec', 'YDataSource', 'ynew', 'color', rndclr); figure(gcf)
end
hold off;

if min(size(output)) > 1
  percentresp = sum(output(:,2))/size(output(:,2),1) * 100;
  count=0;
  for i = 1:size(output(:,3))
    if (output(i,3) > 1)
      count=count+1;
    end
  end
  percentmulti = count/size(output(:,3),1) * 100;
  multi_index = find(output(:,3));
  multis = output(multi_index,3);
  meanmulti = mean(multis);
  stdmulti = std(multis);
  percentresp = sum(output(:,2))/size(output(:,2),1) * 100;
  count=0;
  for i = 1:size(output(:,3))
    if (output(i,3) > 1)
      count=count+1;
    end
  end
  percentmulti = count/size(output(:,3),1) * 100;
  multi_index = find(output(:,3));
  multis = output(multi_index,3);
  meanmulti = mean(multis);
  stdmulti = std(multis);

  mag_index = find(output(:,4));
  mags = output(mag_index,4);
  meanmag = mean(mags);
  stdmag = std(mags);
end

% number of cells analyzed
numcells = size(output,1);

% output to .csv file
if (WRITE_FILE_MODE)
  write_a = {'Numcells', numcells;
  'Percentresp', percentresp;
  'Percentmulti', percentmulti;
  'Mean_mag', meanmag;
  'Std_mag', stdmag;
  'Mean_multi', meanmulti;
  'Std_multi', stdmulti};
  write_b = mags;
  write_c = multis;
xlswrite([filename '_new.xls'], write_a, 'Sheet1', 'A1');
  if (percentresp > 0)
    xlswrite([filename '_new.xls'], write_b, 'Sheet1', ['A' 
      num2str(length(write_a)+1)]);
    xlswrite([filename '_new.xls'], write_c, 'Sheet1', ['B' 
      num2str(length(write_a)+1)]);
  end
end

% save figure
saveas(gcf(),filename,'fig');
% close;

% echo to the main window
output
numcells
percentresp
meanmag
stdmag
percentmulti
meanmulti
stdmulti

2) readcadata.m

function readcadata(fileToRead1)
% Imports data from the specified file
% FILETOREAD1: tofile read

% Auto-generated by MATLAB on 21-Jun-2011 00:04:37
DELIMITER = '\t';
HEADERLINES = 4;

% Import the file
newData1 = importdata(fileToRead1, DELIMITER, HEADERLINES);

% Create new variables in the base workspace from those fields.
vars = fieldnames(newData1);
for i = 1:length(vars)
    assignin('base', vars{i}, newData1.(vars{i}));
end

3) peakdet.m

function [maxtab, mintab]=peakdet(v, delta, x)
%PEAKDET Detect peaks in a vector
%   [MAXTAB, MINTAB] = PEAKDET(V, DELTA) finds the local
% maxima and minima ("peaks") in the vector V.
% MAXTAB and MINTAB consists of two columns. Column 1
% contains indices in V, and column 2 the found values.
% With [MAXTAB, MINTAB] = PEAKDET(V, DELTA, X) the indices
% in MAXTAB and MINTAB are replaced with the corresponding
% X-values.
% A point is considered a maximum peak if it has the maximal
% value, and was preceded (to the left) by a value lower by
% DELTA.
maxtab = []; 
mintab = [];

v = v(:); % Just in case this wasn't a proper vector

if nargin < 3
    x = (1:length(v))';
else
    x = x(:);
    if length(v)~= length(x)
        error('Input vectors v and x must have same length');
    end
end

if (length(delta(:)))>1
    error('Input argument DELTA must be a scalar');
end

if delta <= 0
    error('Input argument DELTA must be positive');
end

mn = Inf; mx = -Inf;
mnpos = NaN; mxpos = NaN;

lookformax = 1;

for i=1:length(v)
    this = v(i);
    if this > mx, mx = this; mxpos = x(i); end
    if this < mn, mn = this; mnpos = x(i); end

    if lookformax
        if this < mx-delta
            maxtab = [maxtab ; mxpos mx];
            mn = this; mnpos = x(i);
            lookformax = 0;
        end
    else
        if this > mn+delta
            mintab = [mintab ; mnpos mn];
            mx = this; mxpos = x(i);
            lookformax = 1;
        end
    end
end
Appendix F: Using Matlab Code to Analyze Calcium Data

The software used for calcium imaging is EasyRatioPro from Photon Technology International (PTI). After recording the calcium oscillations, the software displays the output as shown below. Each line represents the time course of ratio intensity of a region within the field of view. The regions are manually drawn in the software prior to imaging to encapsulate the cells of interest being monitored. The intensity represents the ratio of intensities measured at 340nm to 380nm. Intensity measured at 340nm corresponds to Fura-2 that is found to calcium, and 380nm intensity corresponds to Fura-2 that is not bound to calcium, therefore, the ratio represents the intracellular calcium concentration.

In order analyze and quantify the data, a text version of the intensities for each cell can be exported. This file is first opened in Microsoft Excel to remove the Event Columns, which represent the “Serotonin” at time 60000 ms. This step is necessary for the operation of Matlab code as Matlab can only import data field that contains only numbers, thus the text “Serotonin” is invalid.
After the exported text file has been modified, the Matlab code testcodes.m can be run to generate quantitative data for calcium analysis. A few inputs should be modified for each file before the analysis.

- Filename - specifies the directory and name of the text file to be analyzed
- Flowon - a number describing the onset of stimulation application, corresponding to the row number of the event in original exported text file from EasyRatioPro.

After these inputs have been modified, the code does the following things:

1. Calls ‘readcadata.m’ to import the text file into Matlab.
2. Find the baseline oscillation amplitude.
3. Determines the columns of data representing the ratio values.
4. For easy ratio value (corresponding to each cell), normalize the intensity values to the average baseline value, and calls ‘peakdet.m’ to find the local minimum and maximum values.
5. For each peak detected after the stimulation is applied, record if the peak is greater than 2 times the baseline amplitude.
6. Plot the data
7. Export the results (including % of cells responding, and magnitude of response) into a .xls file with the same name, and display in Matlab

In the end, the matlab output looks like the following:
Notice the main difference is that the cells are normalized to the baseline, so all intensities start at ~0 and overlaps. The transient increase in calcium also shows much clearer to the viewer. The x-axis is modified to display in seconds rather than ms, which is default for the imaging software. Numcells is the total number of cells analyzed. %Resp is the percentage of cells with response. %Multi is the percentage of cells with more than one response. Mean_mag and Std_mag are the mean and standard deviation for the magnitudes of cells.
with response. Mean_multi and Std_multi are the mean and standard deviation for the magnitudes of cells with multiple responses.
Appendix G: Calcium Profiles of OFF-Induced Calcium Response with Calcium Inhibition

Representative calcium temporal profiles shown for OFF-induced calcium response in MLO-Y4 osteocytes, with various inhibitors used in the study.
Cadmium Inhibition

340nm/380nm Intensity

Time (seconds)
Appendix H: Calcium Profiles of Serotonin-Induced Calcium Response with Calcium Inhibition

Representative calcium temporal profiles shown for serotonin-induced calcium response in MLO-Y4 osteocytes, with various inhibitors used in the study.
Cadmium Inhibition

340nm/380nm Intensity

Serotonin

Time (seconds)
Appendix I: Calcium Profiles of Calcium Responses in MLO-Y4 Cells with ATP Inhibition

Representative calcium temporal profiles shown for ATP inhibition using suramin, on OFF and serotonin induced calcium response in MLO-Y4 osteocytes.

A) OFF-induced calcium response, with or without suramin inhibition:

B) Serotonin-induced calcium response, with or without suramin inhibition:
C) Serotonin-induced calcium response on osteocytes that have been preconditioned by 3 minutes of OFF, with or without suramin inhibition:
Appendix J: Protocol for Cell Culture of MLO-Y4 Cells

Materials:

- Complete medium:
  - 94% α-MEM (GIBCO Cat: 12571, Lot: 1403808)
  - 2.5% FBS (Hyclone Cat: SH30396.03, Lot: KPF21344),
  - 2.5% CS (Hyclone Cat: SH30072.03, Lot: AQE23766),
  - 1% PS (Cat: 15140-122, Lot: 1402758)
- Collagen coated culture dish
- trypan blue
- hemocytometer
- PBS
- Trypsin-EDTA
- Pipette aid
- 1000 pipettor
- 50ml pipette
- 25ml pipette
- tube rack

Procedure:

1. Prepare water bath to 37°C.
2. Heat complete media, Trypsin-EDTA and PBS to 37°C.
3. Prepare hood: alcohol spray clean. Place materials inside hood:
   - Pipette aid, 1000 pipettor, 50 ml tube, pipettes, tube rack
4. Aspirate old medium from dishes.
5. Rinse cell layer twice with 25 ml of PBS solution to remove dead cells and debris
   (using 25 ml pipette). **Gently add PBS. Pipette the PBS onto a wall of the dish, not
   onto the cells, to avoid dislodging loosely adherent cells.**
6. Add 3 ml of Trypsin-EDTA solution to flask dish. Return cells to incubator for 3 min.
   Observe cells under an inverted microscope. If cell layer is not yet dispersed, tap
   sides of dish while gently shaking and observe again.
7. Add 7 ml (to bring total volume up to 10 ml) of complete growth medium. **Pipette
   vigorously to break cell clumps. Check on the inverted scope to make sure that
   you have a single cell suspension.**
8. Pipette cells into a 50 ml falcon tube.
9. Transfer 1mL of cells/medium into a microfuge tube.
10. Place 50 ml falcon tube of cell suspension into centrifuge (remembering to balance
    the weight) and centrifuge for 10 minutes at 1000 rpm.
11. Count cells with hemocytometer or Vi-Cell cell counter

**Hemocytometer:**
- Add 20 µL of cells/medium from the 1ml microfuge tube to a well.
- Add 20 µL of trypan blue to the same well.
- Use pipettor to mix cells/medium in the well. **Pipette vigorously to break cell clumps.**
- Add 10 µl of cells/medium to fill the hemocytometer cover slip.
- Total number of cells = count (average per unit) * 10,000 (hemacytometer factor) *2 (dilution factor) * 10ml
- When done with the hemocytometer, rinse with water, spray alcohol, then dry with Kimwipe.

**Vi-Cell cell count:** (~ 2min/sample, need to sign up on the log book by the machine)
- Get cell count vials from JED’s lab
- Load each vial with one sample, > 0.5ml, >50k/ml
- Load cell count vials in Vi-Cell machine
- Log in: youlab, password: utyou325
- Log in sample: position #, sample ID, cell type (PAVIC: 5-50 um, or MLO-Y4), uncheck “save image”; check “Export to Excel”
- Check sample log in info: Autosample queue
- Start Queue
- Cell count result: upper right panel
- Record: viability, viable cells/ml

12. Determine the split ratio: total number of cells / 300,000 – if 300,000 cells per dish; (when calculating total number of cells, remember to multiply total volume, since the cell counter gives cells/ml).
13. As soon as centrifuge has finished, remove supernatant (being careful not to disturb cell pellet).
14. Immediately dilute remaining cells with 10 ml of complete medium (10 ml pipette), **breaking up cell pellet and mixing well.**
15. Add complete medium to the 50ml tube such that each new dish will receive 10 ml cell/medium solution and **pipette vigorously to break cell clumps.**
16. Pipette the remaining cell solution into new dishes: 10 ml each dish. **Rotate and shake the dish well to be sure the cells are dispersed evenly over the surface.**
17. Mark the dish with cell info. (cell name, person’s name, date, passage no. cell density).
18. Observe cells under microscope, then return to incubator.
19. Clean hood and dispose of biohazard waste by adding bleach and letting sit 3 min or until colour changes to yellowish-white.
Appendix K: Protocol for Collagen Coating

Materials:

- Rat tail collagen, 3.5 mg/ml
- Acetic Acid: Sigma 537020, 99%. MW 60.05.
- Gloves: Fisherbrand Non-latex examination gloves (36-099-31XX) or Vinyl exam gloves (11-394-120X). (Both latex and Nitrile gloves offer poor protection against acetic acid)
- Graduated cylinder (500ml)
- Sterilization filter
- 500ml sterilized bottle (x2)
- 100mm tissue cultural dishes (x20)
- 100ml sterilized bottle
- Pipette aid
- 25ml transfer pipettes
- 10ml transfer pipettes

Methods:

1. Place a few 50 ml pipettes in the 4 °C refrigerator for later use.
2. To prepare 0.02N acetic acid solution, do the calculation; dilute the concentrated acetic acid with deionized water. (If 1.00N stock solution is used, to prepare 500ml of solution, 490ml of deionized water is needed and 10ml of stock solution is to be mixed with the water.)
3. Prepare the hood. Spray and place materials inside hood. (from this point on, all work are to be done in the laminar hood)
4. Use 0.2µm filter to sterilize the 0.02N acetic acid solution.
5. To prepare 0.15 mg/ml collagen solutions in 0.02 N acetic acid, do the calculation. The stock collagen density is usually around 3.5mg/ml. (To prepare 210ml of final solution, 9ml collagen and 201ml of 0.02N acetic acid solution are needed.)
6. Transfer 201ml of 0.02N acetic acid solution to a new bottle.
7. Add 9ml of collagen using the chilled pipette to avoid the collagen stick on the inside wall of the pipette. Pipette the solution up and down to rinse the residual collagen in the pipette.
8. Add 5ml of the collagen solution to each of the tissue cultural dishes; make sure the entire bottom of the dish is covered by the solution. If coating slides, add 1ml of collagen solution to each slide, make sure the entire surface of the slide is covered by the solution and no collagen solution spills out of the slide into the dish.
9. Leave the lids open, in the laminar hood, at room temperature for 45 minutes.
10. Remove the collagen solution in the dishes and store them in a 100ml bottle for reuse. It can be reused up to 5 times maximum. Store the solution in 4 °C refrigerator.

11. Leave dishes or slides open for another 45min-1hour to allow collagen solution to dry.

12. Close the lids for the dishes, place them sealed sterile bags, and store in 4 °C refrigerator.

13. Clear the hood.