Osteocytes' Expression of P2Y2 Negatively Regulates the Anabolic Response to Exercise

Joseph Gardinier[1,2,3], Amit Chougule[1,2,3], Nickolas Vinokurov[4], Devin Mendez[4], Chunbin Zhang[2]
[1]Henry Ford Health + Michigan State University Health Sciences, Detroit, MI, [2]Dept of Orthopedic Surgery, Henry Ford Health System, Detroit, MI
[3]Dept Physiology, College of Human Medicine, Michigan State University, East Lansing, MI, [4]School of Medicine, Wayne State University, Detroit, MI
Email of Presenting Author: jgardin2@hfhs.org

Disclosures: Joseph Gardinier (N), Amit Chougule (N), Devin Mendez (N), Chunbin Zhang (N)

INTRODUCTION: Osteocytes sensation and response to dynamic loading plays a crucial role in maintaining bone mass. Loading of the skeleton produces interstitial fluid flow that activates an array of cell-signaling pathways. For example, in osteocytes initial influx of calcium through mechano-sensitive cation channels induces purinergic signaling through the release of adenosine triphosphate (ATP) [1]. The subsequent activation of the P2X7 receptor is known to mediate the down-stream induction of bone formation and overall anabolic response to loading [2-3]. In contrast, less is known regarding the role of P2Y receptors. To date we have demonstrated in osteoblasts that P2Y2 activation has the potential to down-regulate mechanosensitivity by increasing actin-stress fiber formation (ASFF) [4]. Therefore, the purpose of this study is to examine P2Y2 function in osteocytes' for response to loading. The central hypothesis states that P2Y2 activation in response to loading produces a negative feedback, limiting the anabolic potential of dynamic loading.

METHODS: The P2ry2 gene was edited in MLO-Y4 cells using CRISPR-Cas9 to produce a knockout cell-line (KO). Wild-type (WT) cells were generated by transfecting MLO-Y4 cells with a non-targeting control vector that does not recognize or edit the mouse genome. Both KO and WT clones were then exposed to oscillatory fluid flow (OFF) with a peak shear stress of 15 dynes/cm2 at 1 Hz using a parallel plate flow. Changes in protein expression, secreted factors, and cytoskeleton organization were evaluated after 15, 30 and 60 minutes OFF. Conditional knockout mice targeting osteocytes' P2ry2 expression ($P2ry2^{cKO}$) were generated by crossing $Dmp1\text{-}Cre^{ERT2}$ with P2ry2-floxed mice. Littermates that did not express Cre were used as wild-type controls (WT). Both WT and $P2ry2^{cKO}$ mice were subjected to treadmill exercise (15m/min on a 5% incline) for 30 and 60 minutes each day. Mice were exercised 5 days each week for 5 weeks. Tibia samples were isolated for histomorphometry, micro-CT analysis and mechanical testing. Statistical differences between WT and KO cell lines were identified using a student t-test with a p-value < 0.05 considered statistically significant. Animal procedures were conducted under Institutional Animal Care Use Committee (IACUC) approval at the Henry Ford Hospital.

RESULTS SECTION: The response to OFF in KO cells was characterized by a lack of RhoA activation (Fig 1A) and actin cytoskeleton organization when compared to WT controls. KO cells also exhibited a greater increase in ATP (Fig 2B) release at the onset of OFF when compared to WT controls. The degree of extracellular ATP under fluid flow conditions was also sustained longer by KO cells, while extracellular levels of ATP significantly decreased from 30 minutes of OFF to 60 minutes. The KO cells also displayed a greater increase in ERK1/2 phosphorylation under fluid flow when compared to WT cells (Fig 1C). To understand how P2Y2 may be limiting ERK1/2 phosphorylation under fluid flow, we pre-treated WT cells with 1mM ATP for 1 hour before subjecting them to OFF for 15 minutes. Compared to non-treated controls, pre-treating WT cells with ATP required greater shear stress to induce an increase in ERK phosphorylation (Fig 1D). Conversely, pre-treating KO cells with ATP did not affect the level of ERK1/2 phosphorylation at each magnitude of shear stress. We then subjected $P2ry2^{cKO}$ mice to varying durations of treadmill exercise. In WT mice, 30 minutes of treadmill exercise each day for 5 weeks significantly increased the degree of periosteal mineralizing surface (Ps.MS/BS) when compared to sedentary controls (Fig 1E). A similar increase in Ps.MS/Bs was observed in $P2ry2^{cKO}$ mice subjected to the same exercise regimen. However, increasing the duration of treadmill exercise to 60 minutes significantly increased Ps.MS/BS in $P2ry2^{cKO}$ mice only, while WT controls failed to exhibit any additional gains in Ps.MS/BS (Fig 1E).

DISCUSSION: Based on our in-vitro findings, P2Y2 plays a critical role in regulating RhoA activation and actin stress fiber formation in response to loading. The increase in actin stress fiber formation through P2Y2 appears to shift the degree of strain needed to induce ERK1/2 phosphorylation. As a result, the loss in P2Y2 expression allowed a greater degree of ATP release and ERK1/2 activation under loading. In-vivo analysis of $P2ry2^{cKO}$ mice targeting osteocytes' P2Y2 expression confirmed a greater degree of periosteal bone formation under prolonged exercise regimens. Altogether, these findings suggest that P2Y2 activation produces a negative feedback loop that then limits the degree of bone formation under physiological loading.

SIGNIFICANCE/CLINICAL RELEVANCE: Overall this study presents a novel negative feedback mechanism that regulates the anabolic response to loading. Targeting the P2Y2 signaling mechanism has the potential to enhance the anabolic efficacy of loading and prevent bone loss.

REFERENCES: [1] Jacobs et al., Ann Rev Biomed Engineering, 2010, 12:369-400; [2] Ke et al., Mole Endo, 2003, 17(7):1356-67; [3] Li et al., J Bio Chem, 2005, 280(52):42952-9; [4] Gardiner et al., Am J Phys Cell Phys, 2014, 306(11):C1058-67.

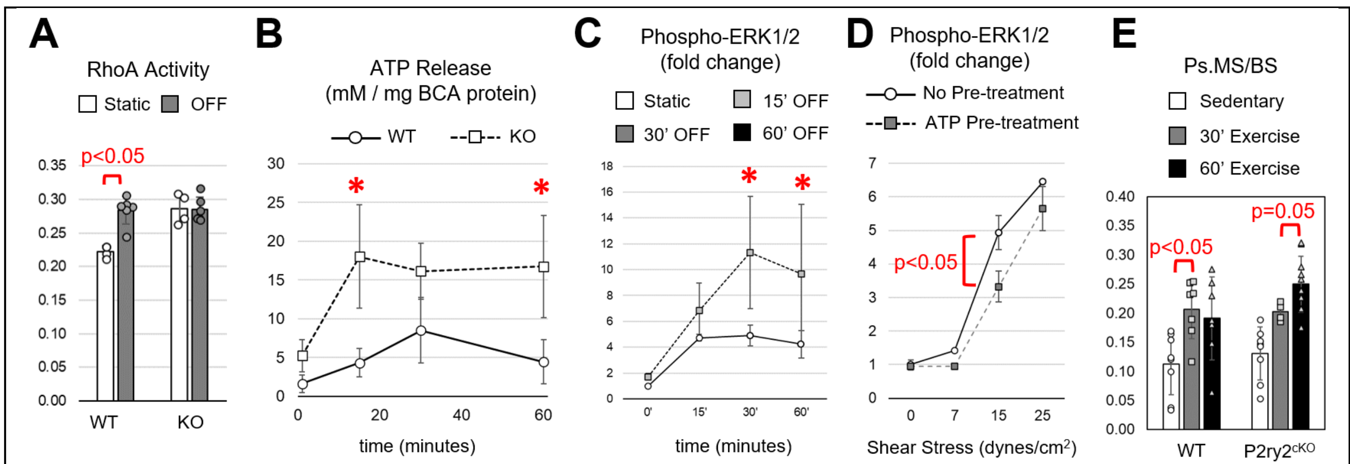

Figure 1: A) Protein samples were collected to measure RhoA activation in WT and KO cells after 30 minutes of OFF (mean ± stdev., n=5). Extracellular ATP **(B)** and ERK1/2 phosphorylation **(C)** were measured at 15, 30, and 60 minutes of OFF using a luciferase assay and western blot analysis respectively (mean ± stdev., n=4). Significant difference compared to WT control denoted by '*'. **D)** ERK1/2 phosphorylation was measured based on western blot analysis of WT cells pretreated with 1mM of ATP (1 hour) and then exposed to different magnitudes of shear stress (mean ± stdev., n=4). **D)** Periosteal mineralizing surface (Ps.MS/BS) was measured in the tibia of WT and $P2ry2^{cKO}$ mice that were subjected to 30 or 60 minutes of exercise 5 out of the week for 5-weeks (mean ± stdev., n= 7).