Inquiro, the UAB undergraduate science research journal

Volume 2008 | Number 2 Article 21

2008

Extracellular ATP Activates Multiple Calcium Signaling Pathways in Osteoblasts

Shweta Patel

Leah Strickland

Kaiyu Yuan

Min-Kyung Choo

Majd Zayzafoon

Extracellular ATP Activates Multiple Calcium Signaling Pathways in Osteoblasts

Shweta Patel, Leah Strickland, Kaiyu Yuan, Min-Kyung Choo, Majd Zayzafoon

Abstract

Mechanical stimulation of osteoblasts (bone forming cells) increases extracellular release of ATP and leads to an increase in intracellular calcium $[Ca^{+2}]_i$. This ultimately activates downstream targets such as Calmodulin Kinase (CaMK) and leads to an increase in osteoblast proliferation and bone formation. The role of CaMKII in regulating osteoblast proliferation in response to ATP is unclear. The purpose of this study is to determine the specific molecular mechanisms that are involved in the osteoblasts response to extracellular ATP treatment. To examine the effects of extracellular ATP on osteoblast proliferation, we treated MC3T3-E1 (murine clonal osteogenic cell line) with (100 uM) ATP for 24 hours. Here we show that ATP treatment caused an 86.7% increase in osteoblast proliferation. Furthermore, ATP treatment resulted in a 40% increase in the protein levels of Cyclin D1, which is known to be a critical mediator of cell growth and proliferation. Mechanistically, we demonstrate that ATP treatment (10 minutes) decreases the activation of CaMKII (75.4%) and increases the phosphorylation of ATF-2 and CREB (95.9%, 94.4%, respectively). The overexpression of CaMKII in MC3T3-E1 osteoblasts resulted in a 98.2% decrease in the ATF-2 activation and a 76.9% increase in the phosphorylation of CREB in response to ATP treatment. These results describe a novel signaling pathway in response to ATP that involves CaMKII, ATF-2, and CREB.

Introduction

Osteoblast cells are bone-forming cells that are essential for maintaining balance in the dynamic nature of bone formation[1]. Bone remodeling involves two main characteristics: resorbtion of bone by osteoclasts and formation of bone by osteoblasts[1]. In this study, the main focus is osteoblast proliferation and its response to extracellular ATP.

Mechanical stimulation, such as fluid shear stress or strain, of osteoblast cells is known to cause an increase in extracellular release of ATP[2]. This extracellular release of ATP plays an important role in bone remodeling because of its involvement in calcium signaling pathways[3]. The oscillations of intracellular calcium levels caused by the extracellular ATP in response to mechanical loading influence the activation of downstream targets involved in gene expression, osteoblast proliferation, and bone formation[4].

Moreover, in regards to the calcium signaling pathway, extracellular ATP is known to bind to two types of purinergic receptors, including the P2X ligand-gated ion channels and the P2Y G-protein coupled receptors[2]. There are seven known P2X subtypes and eight known P2Y subtypes[5]. The $P2Y_2$ receptor specifically could function as "switches" for bone formation[5]. For P2X receptors, ATP is the agonist that binds to the receptor to open the gate and allow extracellular calcium entry into the cell[1]. In respect to P2Y receptors, ATP activates the G-protein to activate phospholipase C, which produces inositol triphosphate[1]. The inositol triphosphate then allows the release of intracellular calcium stores from the endoplasmic reticulum and mitochondria[6].

In addition to the regulation of intracellular calcium through the purinergic receptors and its ability to activate downstream proteins, Ca^{+2}/CaM-dependent protein kinase II also regulates intracellular calcium and the phosphorylation of downstream molecules[7]. First, four calcium ions bind to calmodulin, a calcium binding protein, which induces conformational change[1]. Then, the activated calmodulin binds to calmodulin Kinase II (CaMKII), allowing the autophosphorylation of CaMKII[1]. Because of its ability to autophosphorylate, CaMKII is able to remain activated even after intracellular calcium levels decrease[1]. CaMKII is essential in bone growth because it is a multimeric serine/theronine kinase that plays an important role in calcium signaling pathway as a transducer, leading to activation of downstream proteins and osteoblast proliferation and differentiation[8].

Some of the downstream proteins include the following that were studied in this experiment: cyclin D1, ATF-2 and CREB. Activating Transcription factor (ATF) and cAMP response element binding (CREB) are a family of stress-responsive transcription factors[9]. As part of the Mitogen-Activated Protein Kinase (MAPK) pathway, p38 kinase and/or c-Jun N-terminal kinase (JNK) phosphorylates ATF-2, which ultimately activates various gene targets such as c*yclin D1*[9]. The protein Cyclin D1 is an important mediator of cell growth and proliferation, regulated by ATF-2[9]. Therefore, the activation of this calcium signaling pathway by extracellular ATP can eventually result in osteoblast proliferation and bone growth.

Method

Cell Culture

MC3T3-E1 GFP and MC3T3-E1 overexpressed CaMKII osteoblast cells were grown in α-MEM media containing phenol red (10% BSA, 1% P/S) for 72 hours. Cells were seeded at density of 100,000 cells/well. Media was changed to serum-free media 24 hours before treatment to starve the cells.

Cell Count
MC3T3-E1 (density of 30,000 cells/well) osteoblasts were grown in α-MEM media containing phenol red (10% BSA, 1% P/S) for 24 hours. Media was changed to serum-free media 24 hours before treatment. ATP (100uM) was added to cells for 24 hours, and cell count was conducted on these cells as well as control cells.

Intracellular Calcium Measurement
MC3T3-E1 parent cells were grown in α-MEM media containing phenol red (10% BSA, 1% P/S) for 24 hours on sterile coverslips. Cells were then washed with media containing no phenol red to remove all traces of phenol red containing media. Cells were loaded with Fluo-4 AM dye (10uM) and placed in incubator for 45 minutes. Then the coverslips were placed in new culture dish and washed with media containing no phenol red to remove any non-specific dye associations. Once the coverslip was placed under the microscope, a baseline was established. After the first ten frames, 10uM ATP was added.

Cell Treatment: Osteoblast Response to ATP Treatment Studies
MC3T3-E1 GFP and MC3T3-E1 overexpressed CaMKII osteoblast cells were treated with ATP (100uM) for 10 minutes. Prior to treatment, cells were starved for 24 hours in serum-free media. To stop the treatment, the media was removed from the wells. Protein was extracted using lysis buffer with protease inhibitor and phosphatase inhibitor.

Western Blot Analysis and Densitometries
Protein extracted from cells was separated using 10% SDS-PAGE. Proteins were transferred to immobilon P-PVDF membrane. Any non-specific binding was blocked with blocking solution. The following primary antibodies were probed for: Cyclin D1, p-ATF2, p-CREB, and p-CaMKII. To quantify the levels of protein expression, densitometry was conducted using the ImageJ program.

Immunohistochemistry
After treating MC3T3-E1 osteoblast cells (cell density of 25,000 cells/well) with ATP for 10 minutes, cells were fixed with 4% formaldehyde. Then cells were treated with Triton X-100. Cells were blocked in 5% BSA for 1 hour, and then primary antibody p-ATF2 was added (1:50 dilution) overnight in a humidity chamber at 4°C. After incubating the biotin-conjugated secondary antibody (1:750 dilution) for 30 minutes, a 1:20 dilution of avidin biotin enzyme reagent was added for 30 minutes. Cells for control and ATP treated cells were stained brown with peroxidase substrate (DAB) for 8 minutes and 30 seconds and pictures of slides were taken (200X).

Results
Extracellular ATP treatment increases osteoblast proliferation
Extracellular ATP treatment for 24 hours on MC3T3-E1 osteoblast cells caused an 86.7% increase in proliferation when compared to the control.

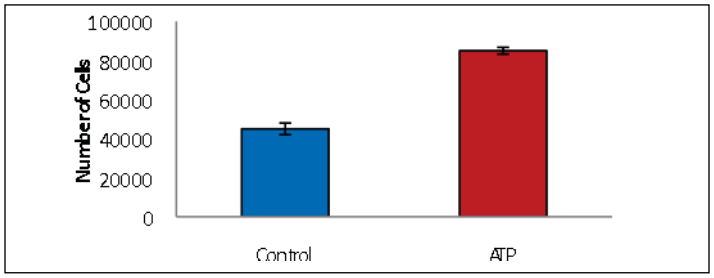
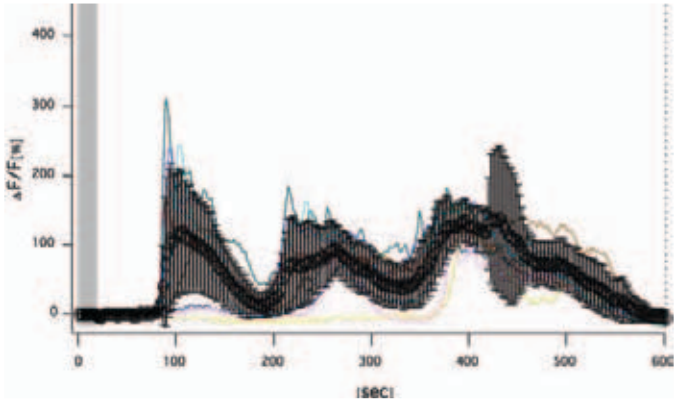


Figure 1: **Extracellular ATP treatment increases osteoblast proliferation.** MC3T3-E1 cells were treated with 100uM ATP for 24 hours. Cells were then counted. Values represent the mean ± SD of ATP treated cells compared to control. The study was performed once in triplicate.

Extracellular ATP increases intracellular calcium
Calcium imaging shows an increase in intracellular calcium when stimulated with 10uM ATP. Imaging also shows variations in relative levels of intracellular calcium release from one cell to another. Based on the ΔF/F graph, calcium release follows an oscillatory pattern, resulting in the rise and fall of calcium levels within the cell.



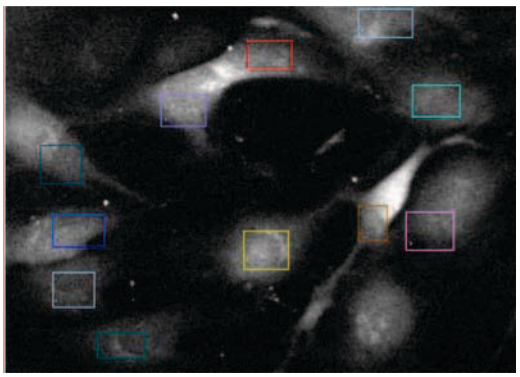


Figure 2: **Extracellular ATP increases intracellular calcium.** MC3T3-E1 osteoblasts were grown on coverslips and treated with 10uM ATP. All traces of media containing phenol red were removed before loading the dye. Fluo-4 AM (10uM)

dye was used for calcium imaging. Values represent the mean ± SD of ATP treated cells. The study was performed two times.

Extracellular ATP increases Cyclin D1 protein levels
Extracellular ATP treatment in MC3T3-E1 osteoblast cells led to a 40% increase in cyclin D1 protein levels compared to the control.

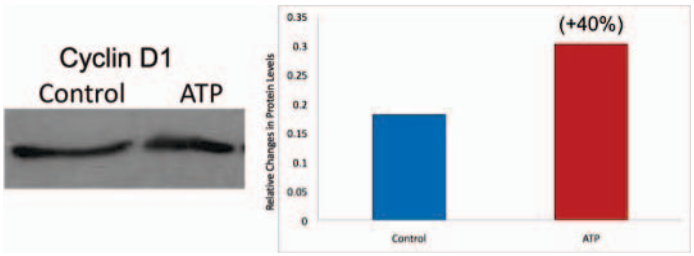
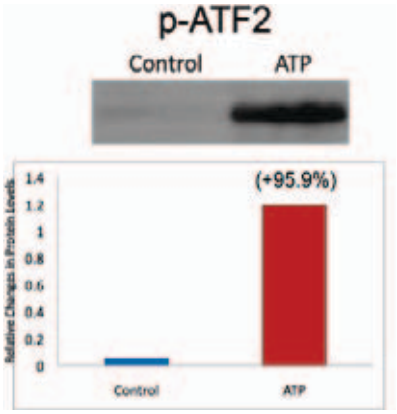
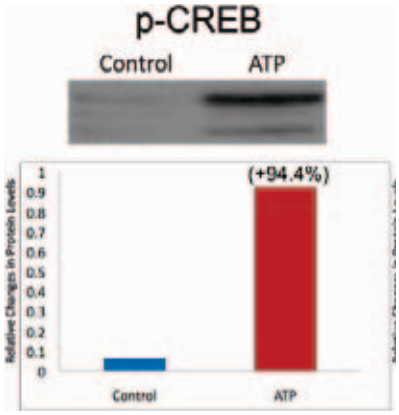


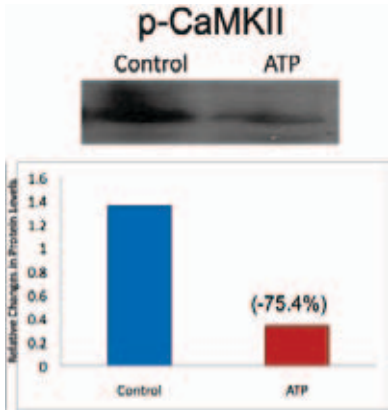
Figure 3: **Extracellular ATP increases Cyclin D1 protein levels.** MC3T3-E1 osteoblast cells were cultured in serum-free media for 24 hours before treatment. Cells were treated with ATP (100uM) for 10 minutes. Proteins were extracted and separated by SDS-PAGE. Immunoblots were developed using antibody against Cyclin D1. The ImageJ program was used to quantify changes in protein levels. The study was performed once.

Extracellular ATP increases the activation of calcium downstream molecules
Extracellular ATP treatment (10 minutes) in MC3T3-E1 osteoblast cells increased the phosphorylation of ATF-2 95.9% and increased the phosphorylation of CREB 94.4%. However, the same treatment caused a 75.4% decrease in the activation of CaMKII.

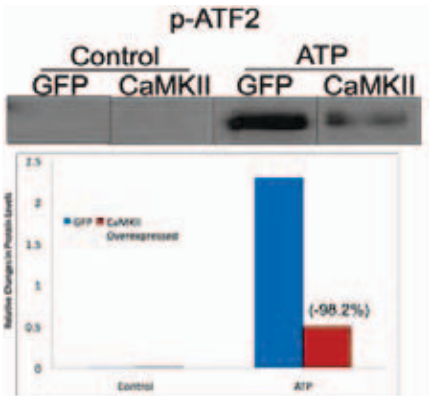
Figure 4: (next column) **Extracellular ATP increases the activation of calcium downstream molecules.** MC3T3-E1 osteoblast cells were cultured in serum-free media for 24 hours before treatment. Cells were treated with ATP (100uM) for 10 minutes. Proteins were extracted and separated by SDS-PAGE. Immunoblots were developed using antibodies against p-ATF2, p-CREB, and p-CaMKII. The ImageJ program was used to quantify changes in protein levels. The study was performed once.







ATF2 activation in response to extracellular ATP is CaMKII-dependent
In overexpressed CaMKII in MC3T3-E1 osteoblast cells, the activation of ATF-2 decreased (98.2%) and increased activation of CREB (76.9%).



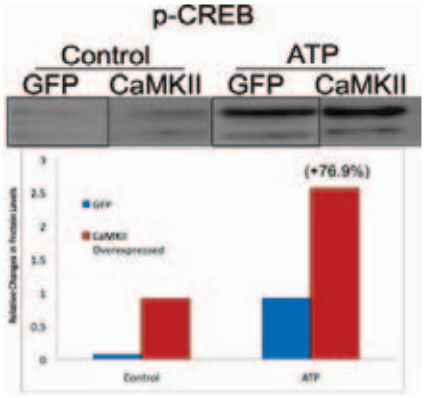


Figure 5: (bottom of page 41) **ATF2 activation in response to extracellular ATP is CaMKII-dependent.** Overexpressed CaMKII in MC3T3-E1 osteoblast cells were cultured in serum-free media for 24 hours before treatment. Cells were treated with ATP (100uM) for 10 minutes. Proteins were extracted and separated by SDS-PAGE. Immunoblots were developed using antibodies against p-ATF2 and p-CREB. The ImageJ program was used to quantify changes in protein levels. The study was performed once.

Extracellular ATP increases nuclear translocation of p-ATF2
Immunohistochemistry was done on MC3T3-E1 osteoblast cells, looking for translocation of p-ATF2. Looking at the image of the control sample, the protein p-ATF2 is mainly located in the cytoplasm and surrounds the nucleus. In regards to the ATP treated osteoblast cells, nuclear translocation is observed because dark staining is visible in the nucleus as well as slight staining in the cytoplasm.

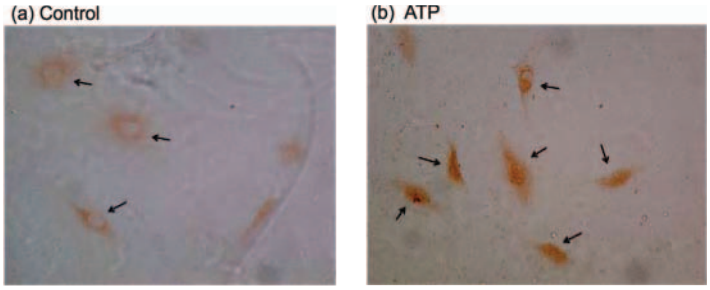


Figure 6: **Extracellular ATP increases nuclear translocation of p-ATF2.** MC3T3-E1 osteoblast cells were cultured on glass coverslips (25,000 cells/well) and changed to serum-free media 24 hours before treatment. Cells were treated with 100uM ATP (bottom panel) for 10 minutes and fixed with 4% formaldehyde. Primary antibody p-ATF2 was added (1:50 dilution). Cells were stained (brown), indicated by arrows. Photographs were taken with Nikon microscope (200X). The study was performed once. (a) Control (b) 100uM ATP

Discussion
The cell count experiment showed an increase in the number of osteoblast cells, demonstrating the direct affects of ATP on osteoblast proliferation. Therefore, when extracellular ATP is released as a result of mechanical stimulation, bone growth is also affected. The calcium imaging study showed that intracellular calcium levels increase in a cyclic pattern caused by the stimulation of extracellular ATP. This cyclic or wave pattern further suggests that extracellular calcium levels are strictly regulated. This regulation could occur through the purinergic receptors (P2X and P2Y) and through the activation of calmodulin and CaMK.

Based on the immunoblots and relative changes in protein levels, the phosphorylation of ATF2 seems to be CaMKII-dependent because in MC3T3-E1 osteoblast cells, p-ATF2 was upregulated, and in overexpressed CaMKII MC3T3-E1 osteoblast cells, p-ATF2 was downregulated. With p-CREB, an upregulation was seen when CaMKII was increased and decreased, meaning other factors might be controlling the phosphorylation of CREB in addition to CaMKII. To check the translocation of p-ATF2, immunohistochemistry was done on osteoblast cells. Based on the images, nuclear translocation is observed, meaning extracellular ATP treatment has the ability to influence the activation of ATF2 through the calcium signaling pathway. The data suggests this novel signaling pathway involving CaMKII and ATF2.

For future studies, more downstream proteins can be tested to determine if extracellular ATP influences their activation. Also, to determine if a specific purinergic receptor, P2X or P2Y, regulates the phosphorylation of downstream targets, a study can be done that inhibits one or more of these receptors. Another study that can be performed involves the nuclear factor of activated T cells (NFAT), a transcription factor that is regulated by calcium levels[10]. Specifically, the affects of extracellular ATP on the activation of NFAT can be tested.

Conclusion
ATP stimulates osteoblast differentiation through a novel signaling pathway that involves CaMKII, ATF2, and CREB.

References
(1) Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. 2002. Molecular Biology of the Cell. 4th Ed. 843-870, 1304-1308.

(2) Liu D, Genetos DC, Shao Y, Geist DJ, Li J, Ke HZ, Turner CH, Duncan RL. 2008. Activation of extracellular-signal regulated kinase (ERK1/2) by fluid shear is Ca^{2+} - and ATP-dependent in MC3T3-E1 osteoblasts. Bone 42:644-652.

(3) Qi J, Chi L, Faber J, Koller B, Banes AJ. 2007. ATP reduces gel compaction in osteoblast-populated collagen gels. J Appl Physiol 102:1152-1160.

(4) Genetos DC, Geist DJ, Liu D, Donahue HJ, Duncan RL. 2005. Fluid Shear-Induced ATP Secretion Mediates Prostaglandin Release in MC3T3-E1 Osteoblasts. J Bone Miner Res 20:41-49.

(5) Orriss IR, Knight GE, Ranasinghe S, Burnstock G, Arnett TR. 2006. Osteoblast responses to nucleotides increase during differentiation. Bone 39:300-309.

(6) Henriksen Zanne, Hiken JF, Steinberg TH, Jorgensen NR. 2006. The predominant mechanism of intercellular calcium wave propagation changes during long-term culture of human osteoblast-like cells. Cell Calcium 39:435-444.

(7) Akimova OA, Grygorczyk A, Bundey RA, Bourcier N, Gekle M, Insel PA, Orlov SN. 2006. Transient Activation and Delayed Inhibition of Na^+, K^+, Cl^-, Cotransport in ATP-treated C11-MDCK Cells Involve Distinct P2Y Receptor Subtypes and Signaling Mechanisms. J Biol Chem 281:31317-31325.

(8) Zayafoon M. 2006. Calcium/Calmodulin Signaling Controls Osteoblast Growth and Differentiation. J Cell Biochem 97:56-70.

(9) Vlahopoulos SA, Logotheti S, Mikas D, Giarika A, Gorgoulis V, Zoumpourlis V. 2008. The role of ATF-2 in oncogenesis. BioEssays 30:314-327.

(10) Tomida T, Hiroses K, Takizawa A, Shibasaki F, Iino M. 2003. NFAT functions as working memory of Ca^{2+} signals in decoding Ca^{2+} oscillation. The EMBO J 22:3825-3832.