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Research Article

ATP and UTP stimulate bone morphogenetic protein-2,-4 and -5 gene expression and mineralization by rat primary osteoblasts involving PI3K/AKT pathway

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ABSTRACT

The modulation of purinergic receptors plays an important role in the regulation of bone formation by the osteoblast. On the other hand, bone morphogenetic proteins (BMPs), members of the transforming growth factor-β superfamily, regulate the differentiation of osteoprogenitor bone cells and stimulate bone formation. In this study, we investigate the effects of several nucleotides on osteoblast differentiation and function, and their relation with the gene expression of osteogenic proteins BMP-2, BMP-4 and BMP-5 as well as of differentiation markers alkaline phosphatase (ALP) and bone sialoprotein (BSP). Our results indicate that 100 μM ATP, ATPγS and UTP, but not ADP, ADPβS or UDP, promote ALP activity in rat primary osteoblasts, showing a peak about day 7 of the treatment. ATP, ATPγS and UTP also increase the mRNA levels of ALP, BMP-2, BMP-4, BMP-5 and BSP. Both the ALP activity and ALP and BMP-4 mRNA increments induced by ATP and UTP are inhibited by Ly294002, a PI3K inhibitor, suggesting the involvement of PI3K/AKT signaling pathway in purinergic modulation of osteoblast differentiation. Furthermore, bone mineralization enhance 1 and 1.5 fold after culturing osteoblasts in the presence of 100 μM ATP or UTP, respectively, but not of ADP or UDP for 22 days. This information suggests that $P2Y_2$ receptors (responsive to ATP, ATPγS and UTP) enhance osteoblast differentiation involving PI3K/AKT signaling pathway activation and gene expression induction of ALP, BMP-2, BMP-4, BMP-5 and BSP. Our findings state a novel molecular mechanism that involves specific gene expression activation of osteoblast function by the purinoreceptors, which would be of help in setting up new pharmacological strategies for the intervention in bone loss pathologies.

Abbreviations: ATP, adenosine-5′-triphosphate; ATPγS, adenosine-5′-*O*-(thiotriphosphate); ADP, adenosine-5′-diphosphate); ADPβS, adenosine-5′-*O*-(2-thiodiphosphate); BMPs, bone morphogenetic proteins; UTP, uridine-5′-triphosphate; ALP, alkaline phosphatase; PI-PLC, phosphatidylinositol-specific phospholipase C; IP3, inositol trisphosphate; DAG, diacylglycerol; PI3K, phosphoinositide 3-kinase; AKT, serine/threonine kinase AKT (also known as protein kinase B or PKB); PKC, protein kinase C; TGF-β, transforming growth factor β; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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Introduction

Dynamic mechanical loading increases the bone density and strength. It also promotes proliferation, differentiation and activity of the osteoblasts, the bone forming cells, by acting at the gene expression level [1–3]. Although many experiments have been performed to study the mechanotransduction machinery in several cell types, the underlying molecular mechanisms that convert mechanical stimuli into cellular responses are not still totally understood. ATP can be released from cells via several physiological mechanisms or by cell exposure to different stress types, such as mechanical strain [4–9]. Purine and pyrimidine extracellular nucleotides are chemical messengers capable of acting on the plasma membrane receptors once released from cells in response to mechanical stimulation [10–12]. Extracellular nucleotides can regulate bone metabolism through activation of plasma membrane receptors [13,14]. Nucleotide receptors of the P2 family are subdivided into P2X ($P2X_{1-7}$) ionotropic and P2Y ($P2Y_{1,2,4,6,11-14}$) metabotropic sub-classes [14,15]. The different pharmacological response to nucleotides helps for the classification and identification of purinergic receptors in several cell types and tissues. Within the purinergic receptor family, ATP is the agonist of all P2 receptors. The P2X receptors subfamily responds only to ATP, whereas the P2Y subfamily responds not only to ATP but also to some other adenine and uridine nucleotides. Thus, $P2Y_{1,11,12,13}$ receptors respond to ATP and ADP but not to UTP; rat $P2Y_2$ and $P2Y_4$ receptors are activated by ATP and UTP but not by ADP; $P2Y_6$ responds to UDP, and $P2Y_{14}$ is activated by UDP-glucose. Because ATP can be hydrolyzed to ADP, AMP and adenosine by extracellular ectoenzymes, the effects observed upon ATP addition might result from the activation of several purinergic receptor subtypes. The use of nonhydrolyzable analogues of ATP and ADP such as ATPγS and ADPβS, respectively, helps to reduce these effects [14,15]. $P2Y_{1,\ 2,\ 4,\ 6,\ \text{and}\ 11}$ receptors couple to Gq/11 to activate phosphatidylinositol-specific phospholipase C (PI-PLC), with the consequent rise in inositol trisphosphate (IP3), diacylglycerol (DAG) and intracellular calcium [14,16]. ATP stimulation of $P2Y_{2/4}$ receptors activates the PI3K/AKT pathway through PLC/IP3/Ca^{2+}, PKC, and Src in MCF-7 cells as well as CaM in an osteoblastic cell line [17,18]. Activation of PI3K/AKT signaling pathway by ATP stimulates cell proliferation and alkaline phosphatase activity in rat primary calvarial osteoblasts, suggesting that purinergic signaling could play a positive role in modulation of the osteoblast maturation [18].

On the other hand, bone morphogenetic proteins (BMPs), functional growth factors belonging to the transforming growth factor β (TGF-β) superfamily, are potent osteoblastic differentiation factors which play a pivotal regulatory role in bone formation [19,20]. Particularly, BMP-2 and 4 are crucial when determining the dorsal-ventral axis in early embryogenesis [21,22]. Moreover, BMP-2 is a central and essential regulator during post natal bone formation and fracture healing [23,24]. Different stress types such as mechanical strain, hypoxia and ultrasound, among others, stimulate the BMPs expression and bone formation [25–29]. Besides, the mechanical strain increases ALP activity and activates the BMPs/Smad pathway in the osteoblasts [30]. As mentioned above, ATP can be released from cells after its exposure to different stress types, such as mechanical strain. However, the extracellular nucleotide action on BMPs expression has not been reported yet.

The aim of this study is to determine the effect of several nucleotides on osteoblast differentiation and maturation by evaluating the involvement of PI3K/AKT pathway and the role of extracellular nucleotides on the gene expression of osteogenic proteins BMPs.

In this work, we obtain evidences which indicate, for the first time, that ATP and UTP stimulate osteoblast differentiation and BMP-2, BMP-4 and BMP-5 gene expression. We also demonstrate the participation of the PI3K/AKT signaling pathway in osteoblasts differentiation and in BMP-4 gene expression.

Materials and methods

Materials

ATP, ATPγS, UTP, ADPβS, UDP, alizarin red and α-MEM were from Sigma Chemical Co. (St. Louis, MO, USA). Ly294002 was from Calbiochem (San Diego, CA, USA). Fetal bovine serum (FBS) was from Natocor (Córdoba, Argentina). Quick-RNA™ Miniprep for RNA isolation and the mix KAPPA SYBR® FAST qPCR Kit, for *Real Time Quantitative-PCR* (RQ-PCR), were from Biosystems S.A (Buenos Aires, Argentina). High-capacity cDNA Reverse Transcription Kit and primers for RQ-PCR were from Invitrogen S.A (Buenos Aires, Argentina). All other reagents used were of analytical grade.

Osteoblast isolation

Calvarial osteoblasts were obtained from 5-day-old neonatal rats which were sacrificed by fast decapitation. All procedures were carried out in conformity with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Briefly, calvarias were incubated in PBS containing 4 mM EDTA at 37 °C for two 10-min periods, and the supernatants were discarded. Subsequently, calvarias were rinsed in PBS and submitted to digestion in PBS containing 200 U/ml collagenase for four 15-min periods. Cells released during the first digestion were discarded, and those released during the subsequent digestions were spun down, collected and combined after centrifugation during 10 min at 1500 rpm. Then, cells were cultured at 37 °C in α-MEM supplemented with 15% FBS, 1% penicillin and streptomycin under humidified air (5.5% CO_2). After 24 h, the medium was replaced by α-MEM supplemented with 10% FBS, 1% penicillin and streptomycin, and the cells were cultured until ~80% of confluence (2–3 days). Then, the cells were frozen in liquid nitrogen until their use.

Osteoblast culture and treatment

Cells were cultured at the concentration of 5×10^3 cells/cm^2 for 2 days in α-MEM supplemented with 10% FBS, in a humidified atmosphere (5.5% CO_2) at 37 °C. Then, cells were starved in a 1% FBS medium for 18–21 h before starting treatment. Treatments were performed by replacing the medium for osteogenic medium (α-MEM supplemented with 1% FBS, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid) containing the indicated agonist amounts (ATP, ATPγS, UTP, ADP, ADPβS, UDP or the vehicle used in control conditions), in the presence or absence of 10 μM Ly294002. The osteogenic medium with agonists and/or Ly294002 was renewed every 2–3 days.