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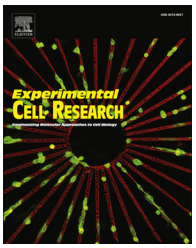

Research Article

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.

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Alkaline phosphatase activity (ALP)

The ALP activity of cell lysates was colorimetrically determined using a commercially available kit (Wiener Lab., Rosario, Argentina); sodium phenylphosphate was used as a substrate in this assay; ALP in the presence of methyl propanol amine (pH 10) releases phenol. The phenol released was combined with a color-generating reagent solution of 4-amino-antipyrine and ferrocyanide, and quantified at 520 nM. The ALP activity in primary osteoblasts was measured after 0, 4, 7 and 10 days of treatment. Briefly, cell layers were washed and cells gathered together in 200 μl PBS using a scraper, followed by sonication at 4 °C and centrifugation at 500*g*. The supernatant was collected and stored at 4 °C until assaying at pH 10, as indicated above. A blank (B) and standard (S) (200 IU/l phenol) were also processed. Optical density of the samples (D) was measured and ALP activity was calculated as follows: ALP (IU/l) = 200 IU/l × (D−B)/(S−B).

Alizarin red staining

Calvarial osteoblasts seeded in 12-well plates were cultured and treated as above for 0, 4, 7, 10, 14, 17, 20, and 22 days. The cells were then fixed with 2% glutaraldehyde in PBS (pH 7.4) at room temperature for 10 min, washed three times with PBS, and then incubated with 2% alizarin red (pH 4.2) for 30 min at 37 °C. The cells were washed thoroughly with deionized water, and microscopic photographs were then taken by a Nikon Eclipse TE 300 microscope coupled to a Nikon Digital Sight DS U2 camera. Images were analyzed with the software Image J (NIH, Bethesda, MD). For illustration purposes, images were processed using CorelPhotoshop with identical scale parameters. After alizarin red staining, the samples were incubated with 0.1 M NaOH, and O.D. was measured by spectrophotometer at 540 nm. Results were expressed in arbitrary units taking the control value obtained at day 0 as 1.

Real-time quantitative polymerase chain reaction (RQ-PCR)

Total RNA was isolated using Quick-RNA™ Miniprep. The amount of RNA was measured by spectrophotometry. Reverse transcription of total RNA was performed using the High-Capacity cDNA Reverse Transcription Kit. Quantitative PCR was done by SYBR Green real-time PCR methods. PCR was performed according to manufacturer's instructions, with denaturation cycles at 95 °C for 3 s and annealing/extend cycles at 60 °C for 30 s. The relative mRNA expression was calculated using the comparative threshold method (Ct-method) with GAPDH for normalization. The average value of expression level of different genes in control cells was defined as 1. All experimental conditions were processed in triplicate.

RQ-PCR primers were specifically designed to amplify the following cDNAs (Table 1).

Statistical analysis

Statistical significance of data was evaluated using Student's *t*-test, and probability values below 0.05 ($p<0.05$) were considered significant. Quantitative data were expressed as means ± standard deviation (SD) from the indicated set of experiments.

Results

ATP and UTP stimulate rat calvarial osteoblast differentiation

Alkaline phosphatase (ALP) is an early differentiation marker for the osteoblast. Treatment of osteoblasts with 10 μM ATP stimulates 33–39% ALP activity at days 7 and 9, what suggests that purinergic signaling pathway plays a positive role in modulation of osteoblast maturation [18]. In the present work, we use several nucleotides to evaluate the possible purinergic receptor subtypes involved in the osteoblast differentiation. Osteoblasts were incubated in a serum deprived- medium and, 18 h later, treated with 10 or 100 μM ATP, ATPγS (a nonhydrolyzable analogue of ATP), UTP, ADP, ADPβS (a nonhydrolyzable analogue of ADP) or UDP in osteogenic medium. Subsequently, the ALP activity of cell lysates was determined colorimetrically as it is described in Materials and Methods section. In an initial series of experiments, we investigated the time course of the treatment with 10 μM ATP or UTP in osteoblast differentiation. Cells incubated in osteogenic medium under control conditions showed noticeable increases in APL activity from day 4 onwards, APL activity peaked on day 7, and it remained elevated until day 14 (Fig. 1A). Cell treatment with 10 and 100 μM ATP, ATPγS or UTP stimulated ~35% ALP activity over the control on day 7 of the treatment (Fig. 1A and B) whereas 10 and 100 μM ADP, ADPβS and UDP did not show any effect neither during the 7 days of treatment (Fig. 1B), nor at the other times studied (data not shown). Although the response to 100 μM UDP seems to be higher than controls and comparable to that of 100 μM UTP, this change was not statistically significant. Because ATPγS is a weak agonist at rat $P2Y_4$ receptor, this result suggests that signaling pathway modulated by $P2Y_2$ receptor subtypes (responsive to ATP, ATPγS and UTP) might be involved in the osteoblast differentiation induced by ATP [31].

ATP and UTP up-regulate ALP, BMP-4, -2, -5 and BSP gene expression in osteoblasts

To determine whether $P2Y_2$ receptor stimulation modifies the expression levels of genes related with osteoblast differentiation and function, cells were treated with 10 and 100 μM ATPγS or UTP in osteogenic medium for different times, and then ALP, BMP-4, -2, -5 and BSP mRNA levels were quantified by RQ-PCR analysis as it is described in the Materials and methods section. Osteoblast stimulation with 10 and 100 μM ATPγS or UTP increased the ALP mRNA levels around 30% over the control, reaching a peak on day 4 (Fig. 2A). Furthermore, BMP-4 mRNA levels increased ~2.5 folds with respect to the controls after cell treatment with 100 μM ATPγS or UTP, showing a peak on day 7 (Fig. 2B); however, cell treatment with 10 μM ATPγS did not affect the BMP-4 mRNA levels in the studied times. Because of this, we decided to perform the BMP-2, -5 and BSP gene expression quantification by RQ-PCR in presence or absence of 100 μM ATPγS or UTP. Osteoblasts stimulation with 100 μM ATPγS or UTP increased, in a time-dependent manner, the BMP-2 and BMP-5 mRNA levels over the control (Fig. 3A and B). The effect on BMP-2 was evident from day 7, and remained elevated until day 9 of the treatment. BMP-5 mRNA levels were robustly increased between day 3 and 9 showing a peak on day 7 of ~2 and 2.5 folds over the control for ATP and UTP,

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Table 1 – Primers for ALP: alkaline phosphatase, BSP: bone sialo-protein, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, rBMP: rat bone morphogenetic protein.

Target	Forward	Reverse
ALP	5′ AGAACTACATCCCCCACG 3′	5′ CAGGCACAGTGGTCAAGGT 3′
BSP	5′ TCCCCACCAGCCAGAAAG 3′	5′ TCGTTGCCTATTTGTTCGTATTCTT 3′
GAPDH	5′ GGCAAGTTCAACGGCACAGT 3′	5′ TGGTGAAGACGCCAGTAGACTC 3′
rBMP-2	5′ GGCCACGACGGTAAAGGA 3′	5′ GCTTCCGCTGTTTGTGTTTG 3′
rBMP-4	5′ CCAAGCGTAGTCCCAAGCAT 3′	5′ CGACGGCAGTTCTTATTCTTCTTC 3′
rBMP-5	5′ TCCCTTTGATGGCGTTGGT 3′	5′ AATCTGCCGGTCAGAAGCAA 3′

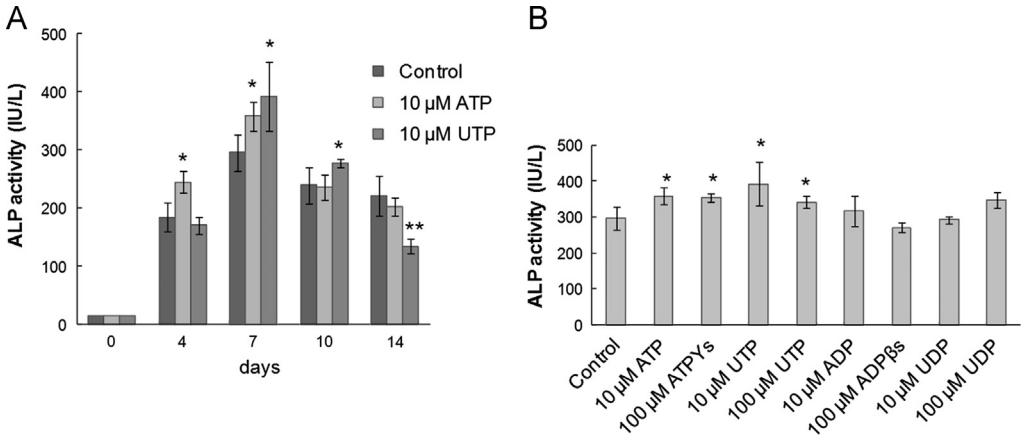

Fig. 1 – Effect of extracellular nucleotides on alkaline phosphatase (ALP) activity in osteoblasts. (A) Cells were treated with 10 μM ATP, UTP or vehicle (control) for indicated times in osteogenic medium. (B) Cells were treated with 10 and 100 μM ATP, ATPγS, UTP, ADP, ADPβS, UDP or vehicle (control) for 7 days in osteogenic medium. In both cases, ALP activity was measured as described in Materials and Methods. Values (IU/L) are the average ± SD of three independent experiments performed in triplicate. *$p < 0.05$, **$p < 0.02$ versus control.

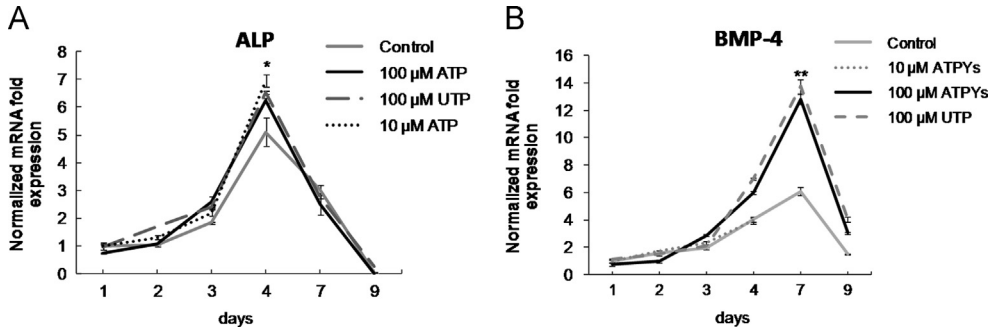

Fig. 2 – ATP and UTP increase ALP and BMP-4 mRNA levels in osteoblasts. The cells were treated with ATPγS, UTP, at the indicated concentration, or vehicle (control) in osteogenic medium for different times (1–9 days), and total cellular RNA was extracted. Then, quantification of (A) ALP and (B) BMP-4 mRNA level by real-time PCR analysis was performed as indicated in the Materials and methods section. The expression levels of target genes were standardized by GAPDH level in each sample and normalized with respect to day 1 of control. Results are shown as average value ± SD of data from at least three separate experiments, each sample processed in triplicate. *$p < 0.05$, **$p < 0.02$ versus control.

respectively. Moreover, BSP mRNA levels increased ~2 folds because of the cell exposure to 100 μM ATPγS or UTP (Fig. 3C), showing a peak on day 7 and remaining high with respect to controls in the studied times. These results show ALP, BMPs and BSP genes as possible targets of purinergic signaling pathway involved in osteoblast differentiation.

Involvement of PI3K/AKT in the ATP-induced up-regulation of BMP-4 and ALP gene expression in osteoblasts

PI3K inhibitor Ly294002 abolishes both cell proliferation and ALP activity induced by ATP in rat primary osteoblasts, suggesting that PI3K/AKT pathway activation is necessary for osteoblast proliferation

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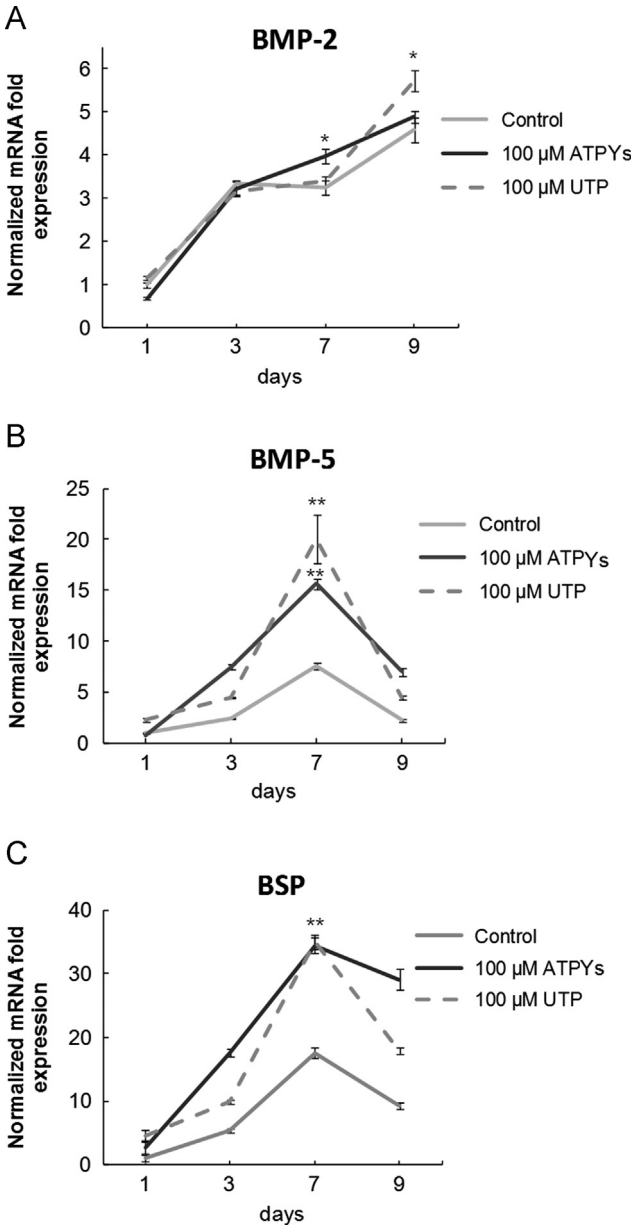

Fig. 3 – ATP and UTP increase BMP-2, BMP-5 and BSP mRNA levels in osteoblasts. The cells were treated with 100 μM ATPγS, UTP or vehicle (control) in osteogenic medium for different times (1–9 days), and total cellular RNA was extracted. The quantification of (A) BMP-2 (B) BMP-5 and (C) BSP mRNA level by real-time PCR analysis was performed as indicated in Materials and Methods. The expression levels of target genes were standardized by GAPDH level in each sample and normalized with respect to day 1 of control. Results are shown as average value ± SD of data from at least two separate experiments, each sample processed in triplicate. *$p<0.05$, **$p<0.02$ versus control.

and differentiation [18]. Now, we show that both ALP activity induced by 100 μM ATP and UTP are suppressed by Ly294002 (Fig. 4A). Additionally, we studied the PI3K/AKT involvement in osteoblast differentiation by measurement of ALP and BMP-4 mRNA levels under ATP treatment by RQ-PCR. To determine if ALP activity inhibition by Ly294002 (Fig. 4A) was due to down-regulation of ALP

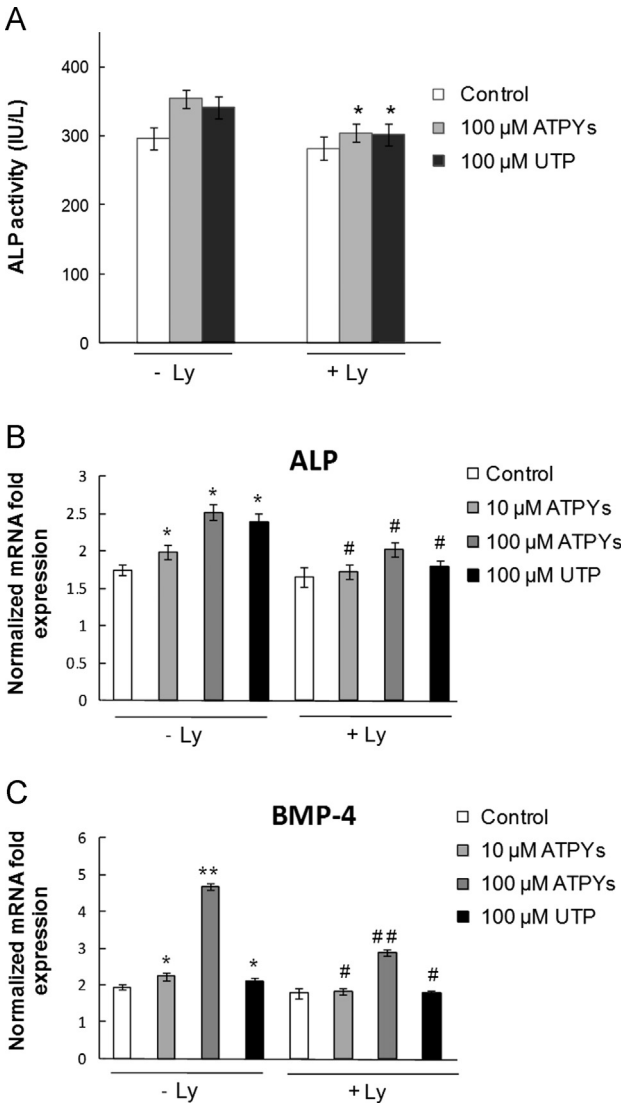

Fig. 4 – Involvement of PI3K/AKT on ATP and UTP effects on ALP activity and ALP and BMP-4 mRNA levels in osteoblasts. (A) Osteoblasts were treated with 100 μM ATPγS, UTP or vehicle (Control) for 7 days in osteogenic medium in the absence (–Ly) or presence of 10 μM LY294002 (+Ly), a PI3K inhibitor. Next, ALP activity was measured as indicated in Materials and Methods. The values (IU/L) shown in the figure are the average ± SD of three independent experiments performed in triplicate. *$p<0.05$ versus cell group without Ly treatment. For the quantification of (B) ALP and (C) BMP-4 mRNA level, the cells were treated with ATPγS, UTP, at the concentration indicated, or vehicle (control) in absence (–Ly) or presence of 10 μM Ly294002 (+Ly) in osteogenic medium for three days; then, the total cellular RNA was extracted. Real-time PCR analysis was performed as described in Materials and Methods. The expression levels of target genes were standardized by GAPDH level in each sample and normalized with respect to day 1 of control. Results are shown as the average value ± SD of data from at least three separate experiments, each sample processed in triplicate. *$p<0.05$, **$p<0.02$ versus control. # $p<0.05$, # # $p<0.02$ versus each cell group without Ly treatment.

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mRNA levels caused by PI3K inhibition, we made RQ-PCR in cell treated with ATP or UTP in presence or absence of the PI3K inhibitor. The Ly294002 treatment suppressed the increment induced by 10 μM ATP and 100 μM UTP on ALP mRNA levels, and a statistically significant inhibition for 100 μM ATP-induced up-regulation of ALP mRNA was observed (Fig. 4B). In parallel, the Ly294002 treatment inhibited the 10 and 100 μM ATP- and UTP-induced up-regulation of BMP-4 mRNA levels in a statistically significant manner (Fig. 4C).

ATP and UTP enhance mineralization by osteoblasts

The typical morphology of actively secreting mature osteoblasts is cuboidal [32]. We observed that treatment of osteoblasts cultured in osteogenic medium with 100 μM ATP or UTP for several days, induced changes in cell morphology with respect to control. After examining the cells with a phase contrast microscope, the treated group with ATP and UTP, but not those treatment with ADP or UDP, showed more cells with cuboidal and irregular polygonal shapes than in the control group from day 7 of treatment (Fig. 5A). *In vitro* studies have shown that ATP and UTP, at concentrations ≥1 μM, strongly inhibit bone mineralization by cultured primary osteoblasts [33,13]. However, as BMP-2, BMP-4 and BMP-5 are inducers of bone formation, we decided to evaluate the effect of osteoblasts treatment with different nucleotides at 100 μM on bone mineralization, as indicated in the Materials and methods section. Calvarial osteoblasts reach a mature stage after long incubation times (~15 day) in osteogenic medium, and mineralization nodules can be visualized around day 20, or even later. For this reason, we studied the effect of nucleotides until day 22 of treatment in osteogenic medium. Bone mineralization nodules contain large amount of calcium that can be easily detected by alizarin red staining. When osteoblasts were cultured in osteogenic medium, deposited accumulations of calcium began to appear around day 17 and were easily detected at day 22 (the calcium depositions appeared in red color after alizarin red staining in Fig. 5B). Cell treatment with 100 μM ATP or UTP significantly enhanced the formation of calcium depositions when compared with control at day 22 (1.9 ± 0.2 and 2.3 ± 0.3 versus 1.3 ± 0.1 arbitrary units, respectively) (Fig. 5B and C).

Discussion

In the present study, we show for the first time the up-regulation of the ALP, BMP-4, BMP-2, BMP-5 and BSP gene expression, and the stimulation of bone mineralization by cultured rat osteoblasts under ATP or UTP treatment in an osteogenic medium. In addition, we demonstrate the participation of PI3K/AKT pathway in the stimulation of osteoblast differentiation induced by these purinergic agonists. Rat osteoblasts express mRNA and protein for all P2 receptor subtypes, except $P2Y_{11}$, and that P2 receptor pattern expression changes during osteoblast differentiation *in vitro* [32,33]. A detailed study conducted by Wildman et al. on the pharmacological properties of rat $P2Y_2$ and $P2Y_4$ receptors expressed in oocytes demonstrates that both receptors exhibit equipotent responses to ATP and UTP but not to ATPγS. They found that ATPγS was a full agonist at $P2Y_2$ receptors and a weak agonist at $P2Y_4$, suggesting that ATPγS may also help to distinguish between $P2Y_2$ and $P2Y_4$ subtypes [31]. Considering this information, the $P2Y_2$ receptor subtype, responsive to ATP, ATPγS and UTP, would be involved in the purinergic action observed in our study, because the other nucleotides used were unable to stimulate osteoblast differentiation, as it was demonstrated by the measurement of ALP activity. The fact that $P2Y_6$ receptor agonist UDP (100 μM) seemed to have an effect on ALP activity, although resulted statistically not significant, did not ruled out the involvement of $P2Y_6$ receptor subtype. $P2X_7$ receptor acting through a pathway involving lysophosphatidic acid promotes osteogenesis [34]. As we have used a concentration of 100 μM ATP, which can also stimulate P2X receptors, we cannot exclude the contribution of P2X receptors subtypes such as $P2X_7$ in the ATP osteogenic action observed here. As, to our knowledge, a P2X activation by UTP has not been reported yet, we underestimated this possibility. The contribution of each purinergic receptor subtype in modulating the osteoblast differentiation and osteogenic protein expression will be assessed in future studies by using specific purinergic receptor inhibitors and siRNA. Our results are in disagreement with the ones which suggest that ATP and UTP, at concentrations ≥1 μM, strongly inhibit bone mineralization by cultured rat primary osteoblasts [35,13]. We found two main differences between these studies and the present work. First, they used Sprague Dawley whereas we used Wistar rats. Second, we did not used dexamethasone in the osteogenic medium during osteoblast differentiation. We believe that the latter is the main reason for such discrepancy because dexamethasone down-regulates Runx-2, a transcription factor required for osteoblasts differentiation [36]. Moreover, in mesenchymal progenitor cells, dexamethasone inhibites Wnt/β-catenin, a signaling pathway involved in osteoblast differentiation [37]. In addition, glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength [38].

Different stages during osteoblast maturation in osteogenic medium have been reported. In the first stage (day 4), ALP activity, an osteoblast differentiation marker began to increase. On the other hand, the Quarles et al.'s classification postulated that the second stage of osteoblast differentiation is a transformation period through which premature osteoblasts turn into mature osteoblasts (around day 7) [39]. In agreement with this, in our osteogenic culture conditions, ALP activity began to increase at day 4, and reached a peak on day 7. Moreover, the ALP gene expression changes occurred prior to the increase in ALP activity, showing a temporal correlation. Thus, the appearance of differentiation markers in control conditions, as ALP, BSP and the osteogenic proteins BMP-2, BMP-4 and BMP-5 suggests that our culture conditions appropriately promoted differentiation and maturation of osteoblasts. Although the nucleotide treatment did not seem to affect the temporal pattern of differentiation marker expression because the peak was reached at the same time, the robust increase observed in ALP, BSP and osteogenic protein expression levels suggests that $P2Y_2$ purinergic pathway may improve osteoblasts maturation and function. Supporting this speculation, the conditions treated with ATP or UTP showed, from day 7 onwards, the presence of more cuboidal and irregular polygonal cell shapes than in the control group. These results agree with studies reported by Cooper et al. [40], which show that typical morphology of the active secreting mature osteoblasts is cuboidal.

The most relevant finding of the present study is the stimulation by ATP or UTP of the BMP-2, BMP-4 and BMP-5 gene expression in osteoblasts. Mechanical strain might promote osteoblasts differentiation through BMPs/Smad signaling pathway [30] and promotes ATP release from cells [7–9]. BMP-2, 4, 5, 6, and 7, have strong osteogenic capacity. Addition of BMP-2 to culture medium or a short-term expression of the BMP-2 is necessary and sufficient

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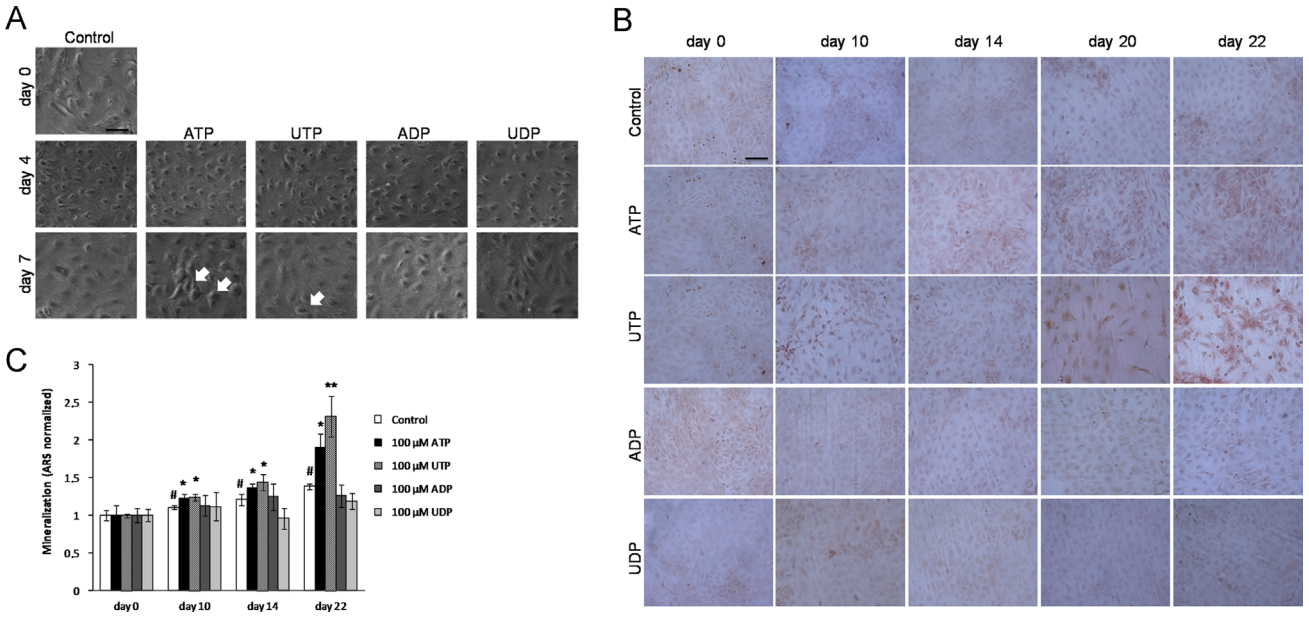

Fig. 5 – Stimulation of bone nodule mineralization by ATP and UTP. Osteoblasts were cultured in osteogenic medium for several days in the presence or absence of 100 μM of each indicated nucleotide before being photographed or stained with alizarin red. The microphotographs in (A) show cells by phase contrast microscopy. The typical mature "cuboidal-shape" osteoblasts were marked with white arrows. The 50 μM scale bar can be seen. The images in (B) show the alizarin red-stained cells cultured in different conditions and times of treatments. Osteoblasts treated with 100 μM ATP or UTP exhibit typical mature osteoblast morphology from day 7 of treatment with detectable discrete mineralization from day 10. The data shown are representative of two independent experiments. Scale bar: 120 μM. (C) Quantification of mineral matrix deposition after alizarin red staining was performed as indicated in Materials and Methods. Each value is the average ± SD of two independent experiments performed in triplicate. *$p<0.05$, **$p<0.02$ versus control condition and #$p<0.05$ versus control day 0.

to irreversibly induce bone formation [41,42]. Genetic analysis of the roles of BMP-2 and BMP-4 showed that the loss of both BMP-2 and BMP-4 produced a severe impairment of osteogenesis [43]. Mice lacking ability to produce BMP-2 in their limb bones have spontaneous fractures that are not consolidated suggesting that BMP-2 activity is required for the initiation of fracture healing [23].

Our results also support the involvement of PI3K/AKT pathway in the modulation of osteoblasts differentiation induced by ATP because both ALP activity and gene expression, as well as BMP-4 gene expression, were reduced by the specific PI3K inhibitor Ly294002. Consistent with this, we previously demonstrated stimulation of PI3K/AKT pathway by ATP and UTP in an osteoblastic cell line and in MCF-7cell [17,18]. In addition, modulation of PI3K/AKT signaling pathway by ATP was shown in several systems [44–46].

Several lines of evidence suggest a crucial role for PI3K/AKT signaling pathway in osteoblasts differentiation and bone formation [47–50]. Besides, a concerted action between AKT and BMP-2 mediating osteoblast differentiation from mesenchymal stromal cells has been observed [50,51]. Published data reveal that PI3K signaling and its downstream effector AKT interact mainly with osteoblast transcription factors at the level of gene transcription [52,53].

On the other hand, extracellular ATP and UTP activate the transcription factor Runx2, which controls osteoblast differentiation by upregulating the expression of several critical osteoblastic genes including osteopontin, BSP and osteocalcin [54–56]. In accordance with this, we report here an increase in the BSP expression in osteoblasts treated with ATP or UTP. Besides, BSP is primarily expressed by mature osteoblasts and may play a role in mineralization [57,58]. This protein can capture calcium ions while it is conserving polyglutamate regions, which exhibit hydroxyapatite crystal nucleation potential [59]. Finally, we observed an increment in the calcium depositions by osteoblasts under 100 μM ATP or UTP treatment in the third stage (days 14 and 22).

Mechanical strain of these cells promotes ATP release [7–9]. It has also been associated with increased ALP activity and activation of the BMPs/Smad pathway in osteoblasts [30]. Nevertheless, to our knowledge, the involvement of extracellular nucleotides in modulation of BMPs gene expression has not been reported until the present study.

To summarize, our results suggest that PI3K/AKT signaling pathway modulated by $P2Y_2$ receptor subtype might be involved in the osteoblast differentiation induced by ATP or UTP. We demonstrate a novel target for extracellular ATP and UTP signaling pathway that triggers the BMPs and BSP gene expression in osteoblasts. Our findings indicate that extracellular nucleotides can play a pivotal role in regulating osteogenesis, showing ALP, BMPs and BSP as possible target genes of purinergic signaling pathway. Our results would help to set up new pharmacological strategies for the intervention in bone loss pathologies.

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