Effects of Adenosine Triphosphate on Proliferation and Odontoblastic Differentiation of Human Dental Pulp Cells

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Abstract

Introduction: Adenosine 5′-triphosphate (ATP) is a potent signaling molecule that regulates diverse biological activities in cells. Its effects on human dental pulp cells (HDPCs) remain unknown. This study aimed to examine the effects of ATP on proliferation and differentiation of HDPCs. **Methods:** Reverse transcription polymerase chain reaction was performed to explore the mRNA expression of P2 receptor subtypes. Cell Counting Kit-8 test and flow cytometry analysis were used to examine the effects of ATP on proliferation and cell cycle of HDPCs. The effects of ATP on differentiation of HDPCs were examined by using alizarin red S staining, energy-dispersive x-ray analysis, Western blot analysis, and real-time polymerase chain reaction. **Results:** The purinoceptors P2X3, P2X4, P2X5, P2X7, and all P2Y receptor subtypes were confirmed to present in HDPCs. ATP enhanced HDPC proliferation at 10 μmol/L concentration. However, it inhibited cell proliferation by arresting the cell cycle in G_0G_1 phase ($P < .05$ versus control) and induced odontoblastic differentiation, ERK/MAPK activation, and dentin matrix protein 1 (DMP1) and dentin sialophosphoprotein (DSPP) mRNA transcriptions at 800 μmol/L concentration. Suramin, an ATP receptor antagonist, inhibited ERK/MAPK activation and HDPC odontoblastic differentiation ($P < .05$ versus control). **Conclusions:** Extracellular ATP activates P2 receptors and downstream signaling events that induce HDPC odontogenic differentiation. Thus, ATP may promote dental pulp tissue healing and repair through P2 signaling. Results provide new insights into the molecular regulation of pulpal wound healing. *(J Endod 2016;42:1483–1489)*

Key Words

Dentin matrix protein 1, dentin sialophosphoprotein, dentinogenesis, ERK pathway, odontoblast, purinergic P2 receptors

Significance

These results indicate that ATP may promote dental pulp tissue healing and repair through P2 signaling and provide new insights into the therapy of pulpal wound.

Dental pulp cells (DPCs) possess multipotent differentiation potential and retain the ability to form dentin throughout life if stimulated. Many investigators are interested in DPCs because of their characteristics. High molecular weight hyaluronan could promote the early mineralization of DPCs mediated via CD44 (1). Rosiglitazone inhibits proliferation in human dental pulp cells (HDPCs) but induces osteopontin gene expression (2). Dental pulp stem cells have been used for pulp regeneration therapy, and the efficacy of this therapy could be assessed by magnetic resonance imaging (3).

When dental pulp is confronted by microbes, trauma, or chemicals, a host of inflammatory cytokines are released (4). Simultaneously, underlying progenitor pulp cells are stimulated to differentiate to odontoblasts, and dentin matrix proteins are secreted as part of reparative dentinogenesis to resist the external stimuli (5, 6). Odontoblasts secrete several collagenous and non-collagenous proteins, such as type-1 collagen, osteopontin, dentin matrix protein 1 (DMP1), and dentin sialophosphoprotein (DSPP), which are special markers for the odontoblast/osteoblast-like differentiations of HDPCs (7, 8). However, the molecular control mechanisms underlying the effects of the inductive signal on cell growth and odontoblastic differentiation remain unclear (9, 10).

Adenosine 5′-triphosphate (ATP) is present in the cytoplasm of mammalian cells at concentrations between 2 and 5 mmol/L (11). ATP is released via exocytosis, transporters, and lysosomes, and this process is under control (12). Highly regulated ATP release occurs in response to various stimuli via membrane channels, including connexin hemichannels (13) and pannexins (14). ATP release from cells increases in response to different external stimuli, such as hypoxia (15), mechanical stress (16, 17), fluid flow (18), and shockwaves (19). The concentrations of ATP release vary during cell damage and under active conditions. Entire ATP content of cytoplasm is released on cell destruction (20), compared with only 1–200 mmol/L in active conditions (21). ATP can participate in both short-term and long-term purinergic signaling; short-term purinergic signaling is involved in neurotransmission, muscle contraction, endocrine secretion, vasodilation, acute inflammation, and chemoattraction, whereas long-term purinergic signaling is involved in cell migration, proliferation, differentiation, and hyp-

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oxia (22, 23). The role of ATP in wound healing was also demonstrated through its recruitment of immune cells to the site of injury and its promotion of angiogenesis (24, 25). Possible correlation has been indicated between ATP signaling disorders and the pathogenesis of some diseases such as glaucoma, retinal detachment, and diabetic retinopathy (26, 27).

Extracellular ATP acts by binding to the purinergic P2 receptors. Purinergic P2 receptors have 2 subtypes, the ionotropic receptor P2X ($P2X_{1-7}$) and the G-protein–coupled receptor P2Y ($P2Y_{1,2,4,6,11-14}$) (28, 29).

Purinergic signaling may play a critical role in regulating the differentiation of mesenchymal stem cells to adipocytes or osteoblasts (30, 31). Shockwaves (0.18 mJ/mm^2 for 100 impulses generated at a generator voltage of 8.5 kV and a capacitance of 0.3 μF) could induce osteogenic differentiation of human mesenchymal stem cells through ATP signaling (19). Endogenous ATP released by osteoblasts might also act as a key inhibitor of tissue calcification because mineralization increased in osteoblast cultures when osteoblasts were treated with apyrase (which sequentially hydrolyzes extracellular ATP to adenosine diphosphate to adenosine monophosphate + 2 P(i)) (32), ATP release inhibitors, and P2 receptor antagonists.

Another well-known regulator of osteogenesis is ERK, which is activated in response to growth factors to stimulate osteoblastic differentiation and to mediate osteogenic gene expression (33, 34). Although an increasing amount of evidence suggests that purinergic signaling is also involved in osteogenic differentiation of progenitor cells, its relationship to the ERK signaling pathway is unknown.

Liu et al (14) have detected ATP release via pannexins in dental pulp, which is induced by mechanical or thermal stimulation of external dentin. This study supports a plausible role for ATP signaling in dentin hypersensitivity and dental pain, but the definite effects of ATP on dental pulp remain unclear.

Therefore, the present study had been designed to examine the presence of P2 receptors and the effects of ATP on the proliferation and odontoblastic differentiation of HDPCs. The role of ERK signaling in ATP-mediated odontoblastic differentiation was also investigated.

Materials and Methods

Culture of HDPCs

Human dental pulps were obtained from normal third molars extracted from healthy human volunteers (19–29 years old). The study was approved by the Ethics Committee of the authors' institution (IRB-2013055). HDPCs were isolated from teeth (35), as described in the Supplemental Material, and cultured in α-minimum essential medium (α-MEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mmol/L L-glutamine (Sigma-Aldrich, St Louis, MO), and 1% penicillin/streptomycin (Gibco, BRL) at 37°C in 5% CO_2. Five cell lines from 5 teeth of 5 different individuals (3 men and 2 women) were named cell lines 1–5 and used in the following experiments.

Reverse-Transcription Polymerase Chain Reaction

Reverse-transcription polymerase chain reaction (RT-PCR) was used to explore the mRNA expression of P2 receptor subtypes in HDPCs. Total RNA was extracted from the HDPCs via TRIzol reagent (Invitrogen, Carlsbad, CA), and the experimental details are described in the Supplemental Material. The primer sets used are listed in Table 1.

Quantitation of Cell Proliferation

Cell proliferation was measured by using the Cell Counting Kit-8 (CCK-8; Dojin, Tokyo, Japan). Cells (5×10^3) were plated in 100 μL growth medium in a 96-well plate. After overnight incubation, cells were treated with α-MEM containing 0.1–1000 μmol/L ATP, 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1% penicillin/streptomycin for 72 hours to determine the study of dosage effect. The medium was not changed for 72 hours. The detailed method was applied according to the manufacturer's instructions, as described in the Supplemental Material.

Flow Cytometry Analysis

HDPCs treated with ATP at 10 and 800 μmol/L concentrations and untreated controls were subjected to a flow cytometry system (Beckman Coulter, Fullerton, CA), as described in the Supplemental Material. Cell cycle distributions were analyzed in percentages of cells in G_0G_1, S, and G_2M phases.

Alizarin Red S Staining

The HDPCs were seeded into 12-well plates (4×10^4 cells/well). After overnight incubation in the absence of osteogenic medium, the cells were treated with α-MEM supplemented with ATP at final concentrations of 10 and 800 μmol/L, 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1% penicillin/streptomycin. As positive control, HDPCs were treated with osteogenic medium (10^{-7} mol/L dexamethasone, 10 nmol/L L-ascorbic acid phosphate magnesium salt n-hydrate, and 10 mmol/L sodium β-glycerophosphate in the growth medium). Qualitative and quantitative analyses were performed after 21 days of continuous culture. ATP treatment and osteogenic media were separately changed every 3 days for 21 days, as described in the Supplemental Material.

Energy-dispersive X-ray Microanalysis for Mineral Deposition

To analyze the mineral composition of the crystals, energy-dispersive x-ray (EDX) analysis was performed by using a transmission electron microscope (Hitachi S-4800, Tokyo, Japan) equipped with an energy-dispersive spectrometer (D76181; Siemens AG, Karlsruhe, Germany). Energy-dispersive x-ray spectroscopy (EDS) was performed for elemental analysis of minerals after sample preparations, as described in the Supplemental Material.

Western Blot Analysis

HDPCs were seeded in 60-mm dishes at a density of 1×10^6 cells/well. For time-course experiments, HDPCs were treated with 800 μmol/L ATP for 0, 1, 5, 10, 20, 30, and 60 minutes. To determine the intracellular signaling pathway, HDPCs were treated with suramin (100 μmol/L) for 60 minutes and U0126 (20 μmol/L) for 30 minutes before 800 μmol/L ATP treatment. Cells were collected 10 minutes after ATP exposure. Western blot analyses were performed as described in the Supplemental Material.

Real-time PCR

Real-time PCR was performed by using ABI 7500 (Applied Biosystems, Foster City, CA) and SYBR Green chemistry, as described in the Supplemental Material. The primer sets used are listed in Table 1. Relative expressions of the target genes were calculated by using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Data were presented as mean ± standard error of the mean. Statistical analysis was performed by using one-way analysis of variance

TABLE 1. Primers for P2 Receptor–related and Odontoblast-related Gene Expression Analysis

Gene	Accession number	Primer	Sequences (5′-3′)	Fragment size (base pairs)
P2X1 (RT-PCR)	NM_002558	Forward	ATGGTGCTGGTGCGTAATAAG	217
		Reverse	GGAAGACGTAGTCAGCCACA	
P2X2 (RT-PCR)	NM_174873	Forward	CTGCCTCGTCAGGCTACAAC	142
		Reverse	GTGGGAATCAGGCTGAACTTC	
P2X3 (RT-PCR)	NM_002559	Forward	AGTCGGTGGTTGTGAAGAGC	104
		Reverse	AGCCTTCTCGTGCAAGAAAAC	
P2X4 (RT-PCR)	NM_002560	Forward	TGGCGGATTATGTGATACCAGC	112
		Reverse	GTCGCATCTGGAATCTCGGG	
P2X5 (RT-PCR)	NM_175080	Forward	CTGTCGCTGTTCGACTACAAG	112
		Reverse	CCCATACGACCAGGTACGC	
P2X6 (RT-PCR)	NM_005446	Forward	TCAACTTCTCTAAGTCCAATGCC	88
		Reverse	CAGTAGGGGCTGAATTGTGGT	
P2X7 (RT-PCR)	NM_002562	Forward	TGTGTCCCGAGTATCCCACC	203
		Reverse	GGCACTGTTCAAGAGAGCAG	
P2Y1 (RT-PCR)	NM_002563	Forward	GGGATGCCATGTGTAAACTGC	159
		Reverse	CGCTGATACAGATCGCATTCTT	
P2Y2 (RT-PCR)	NM_176072	Forward	CCGCTTCAACGAGGACTTCAA	211
		Reverse	GCGGGCGTAGTAATAGACCA	
P2Y4 (RT-PCR)	NM_002565	Forward	GGAGCTGGACTGTTGGTTTGA	105
		Reverse	CATAGGGTTGGGGCGTTAAGG	
P2Y6 (RT-PCR)	NM_176797	Forward	GTGTCTACCGCGAGAACTTCA	159
		Reverse	CCAGAGCAAGGTTTAGGGTGTA	
P2Y11 (RT-PCR)	NM_002566	Forward	GCTGCCGACGACAAACTCA	67
		Reverse	GGAACTCAACCACCAGTATGG	
P2Y12 (RT-PCR)	NM_176876	Forward	TTTGTGTGTCAAGTTACCTCCG	101
		Reverse	CTGGTGGTCTTCTGGTAGCG	
P2Y13 (RT-PCR)	NM_176894	Forward	ATCGTGCTGTTAGGGCTCATA	153
		Reverse	CAAGATCGTATTTGGCAGGGAG	
P2Y14 (RT-PCR)	NM_001081455	Forward	TACGTGCCCAGCTCTAAGAGT	99
		Reverse	GTCACCAAGGATCTTGAAAGGAA	
Glyceraldehyde-3-phosphate dehydrogenase (RT-PCR and real-time PCR)	NM_001256799	Forward	ATGGGGAAGGTGAAGGTCG	108
		Reverse	GGGGTCATTGATGGCAACAATA	
DMP1 (real-time PCR)	NM_001079911	Forward	GTGAGTGAGTCCAGGGGAGATAA	111
		Reverse	TTTTGAGTGGGAGAGTGTGTGC	
DSPP (real-time PCR)	NM_014208	Forward	TGGAGCCACAAACAGAAGCAA	127
		Reverse	TCCAGCTACTTGAGGTCCATC	

followed by Bonferroni post hoc test. Differences were considered significant at $P < .05$.

Results

Expression of Purinoceptor mRNA in HDPCs

The HDPCs exhibited mRNA expression of purinergic receptors (P2X3, P2X4, P2X5, P2X7, and all P2Y subtypes) after cells reached confluence (Fig. 1*A*). These subtypes had varying magnitudes of expression. The expressions of P2X5, P2Y1, P2Y2, P2Y6, and P2Y13 were much greater than other subtypes among P2X and P2Y receptors.

Effect of ATP on HDPC Proliferation

To test the effects of extracellular ATP on cell proliferation, primary cultures of HDPCs were exposed to various ATP concentrations for 72 hours. As shown in Figure 1*B*, lower ATP concentrations (0.1–100 μmol/L) increased cell proliferation in a concentration-dependent manner. A significant effect was observed at 1 μmol/L ($n = 5$, $P < .05$ versus control), and peak effect was observed at 10 μmol/L ATP. In contrast, the proliferative capacity of HDPCs was inhibited at higher ATP concentrations ($\geq$400 μmol/L). At 800 μmol/L ATP, HDPC numbers were significantly decreased compared with those in the controls (Fig. 1*B*; $n = 5$, $P < .05$).

Effects of ATP on Cell Cycle Progression

To investigate the ATP-stimulated regulation of cell cycle progression, cells were synchronized in G_0G_1 by serum deprivation for 24 hours and then treated with ATP. A higher percentage of cells in S phase (16.9% versus 4.8%) and a lower percentage in G_0G_1 phase (58.3% versus 73.2%) were detected in 10 μmol/L ATP-treated cells compared with untreated cells (Fig. 1*C*; $n = 5$, $P < .05$ versus control). In 800 μmol/L ATP-treated group, most cells remained in the G_0G_1 phase (87%). No sub-G_1 peaks were present in either 10 μmol/L or 800 μmol/L ATP-treated groups. Combined with the CCK-8 test, the flow cytometry results suggested that high ATP concentrations inhibit the growth of HDPCs through G_1 phase cell cycle arrest.

ATP-induced Mineralization in HDPC Cultures

With alizarin red S staining, the extracellular matrix calcium deposits were clearly visible at 21 days for the 800 μmol/L ATP-treated and the positive control groups (Fig. 2*A*). However, no mineral deposit was found in the 10 μmol/L ATP-treated group. Quantitative measurement of alizarin red S staining was also consistent with the image patterns (Fig. 2*B*; $n = 5$, $P < .05$).

Figure 2*C* shows the mineral elements in the ATP-treated cultures analyzed by EDS. The Ca/P ratio of the mineral deposits induced by 800 μmol/L ATP treatment was 1.62 (standard deviation = 0.123),

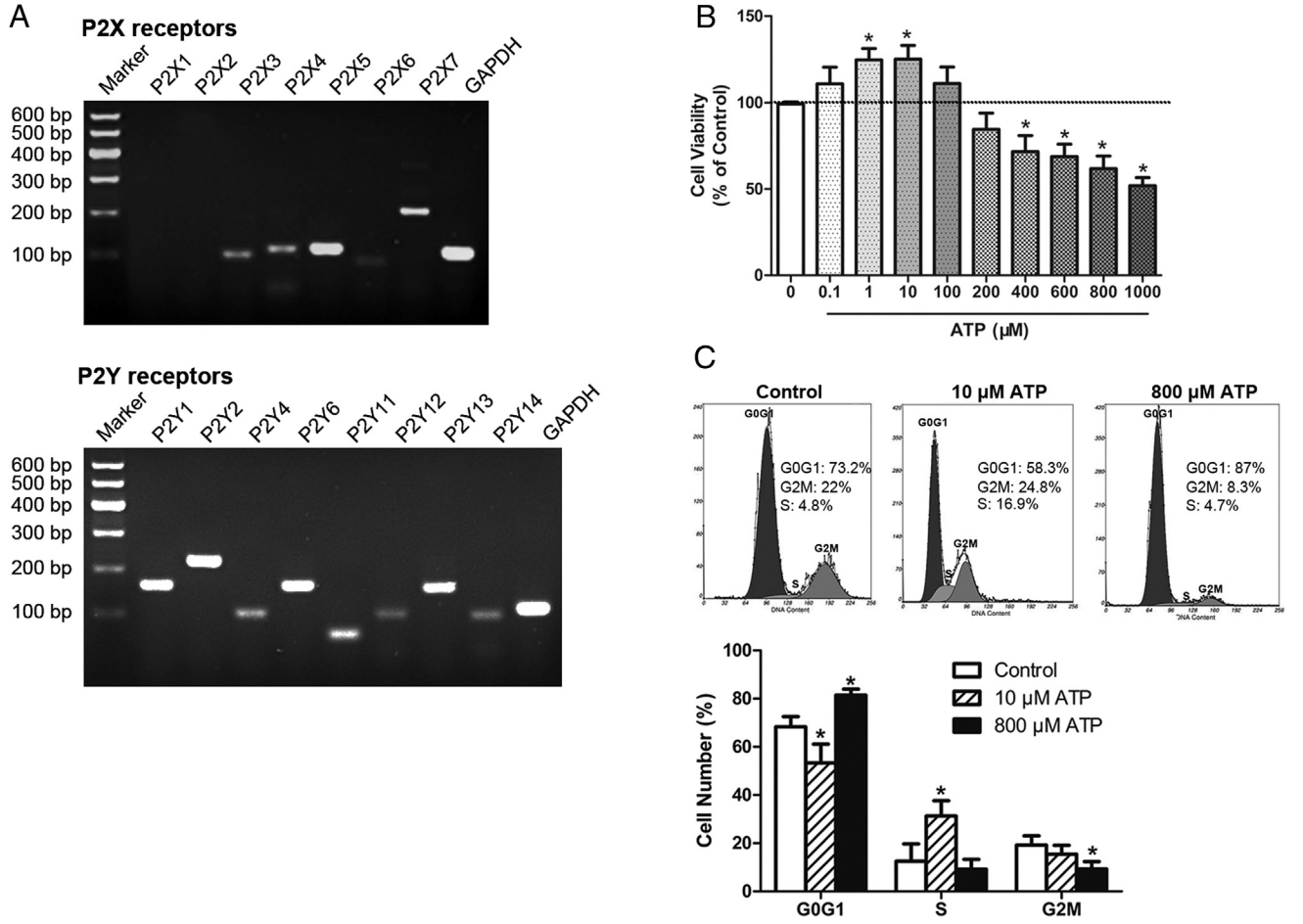

Figure 1. P2 receptor mRNA expression and effect of ATP on cell proliferation. (*A*) mRNA expression of P2X and P2Y receptors in HDPCs. Total RNA extracted from HDPCs was reverse-transcribed into cDNA. The first lane is the DNA marker ladder. The relative amount of PCR product for each purinergic receptor subtype expressed in HDPCs was determined by RT-PCR. The number of PCR cycles for all P2 receptors was 35. (*B*) Cell proliferation was stimulated by ATP at a series of concentrations (0, 0.1, 1, 10, 100, 200, 400, 600, 800, and 1000 μmol/L) for 3 days to study the dosage effect ($n = 5$, $^*P < .05$ versus control). (*C*) Effect of ATP on cell cycle progression by flow cytometry. Representative flow cytometry graphs in cells with 0, 10, and 800 μmol/L ATP for 3 days (up). Mean values of cell cycle distribution in different groups for 3 days ($n = 5$, $^*P < .05$ versus control). The indicated $n = 5$ represents the 5 cell lines from 5 different teeth used to produce the data in this figure. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

compared with 1.77 (standard deviation = 0.095) for the positive control group (Fig. 2*D*).

Effects of ATP on ERK Activation

ATP treatments resulted in the activation of ERK/MAPK pathway. As shown in Figure 3*A*, ATP activated p-ERK in HDPCs in a time-dependent manner. The p-ERK/ERK ratio, an indication of ERK phosphorylation, peaked at 10 minutes after treatment with ATP and was 2.2 times greater than that at the basal level ($n = 3$, $P < .05$).

For HDPCs untreated or pretreated with 100 μmol/L suramin, a P2 purinoceptor unselective antagonist, the ERK activity was greatly reduced compared with HDPCs subjected to ATP treatment alone (Fig. 3*B*).

As shown in Figure 3*C*, U0126, a selective ERK inhibitor, significantly inhibited the ATP-induced activation of ERK phosphorylation. The combined treatment with U0126 and ATP attenuated ERK activity by 67% compared with the level treated with ATP alone.

ATP Upregulation of DMP1 and DSPP Gene Expression in HDPCs

To examine the reparative responses of HDPCs to stress stimuli, cells were treated with ATP for 24 or 48 hours. HDPCs exhibited early expression of odontoblast-related mRNA, namely DMP1 and DSPP (Fig. 4). This response was time-dependent, and the maximum expressions of DMP1 and DSPP mRNA were produced with ATP at 24 hours. The DMP1 and DSPP mRNA expressions were greatly reduced by cotreatment with suramin or U0126 compared with ATP treatment alone, further suggesting the involvement of ERK pathway in ATP-mediated odontoblastic differentiation.

Discussion

ATP, a stress signaling molecule, could affect proliferation and osteogenic differentiation of HDPCs. A majority of the purinergic receptors, including P2X3, P2X4, P2X5, P2X7, and all P2Y subtypes, are present in HDPCs. ATP promoted proliferation of HDPCs at lower concentrations but inhibited cell proliferation by arresting cells in the G_0G_1 phase at higher concentrations. ATP enhanced osteogenic differentiation and mineral deposition at 800 μmol/L but not at 10 μmol/L concentrations, which suggested that ATP affected the proliferation and differentiation of HDPCs in a dose-dependent manner.

The effect of extracellular ATP on cell proliferation has been reported in various types of cells (36–38). Some studies reported that ATP promoted cell growth and proliferation (38), whereas others

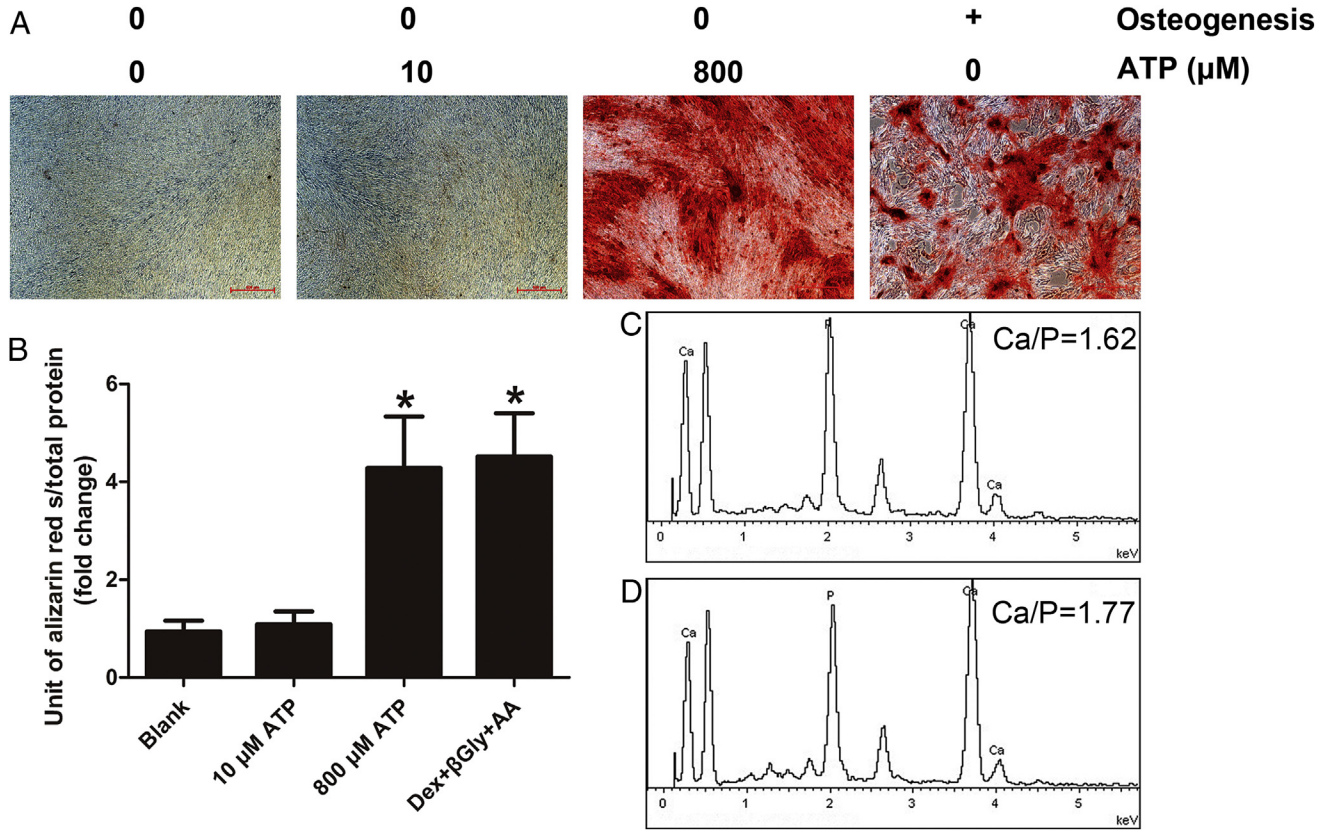

Figure 2. Osteogenic potential of ATP to HDPCs *in vitro*. (*A*) Alizarin red staining was positive in the 800 μmol/L ATP-treated group and osteogenic media group at 21 days. The scale bar represents 500 μm. (*B*) The mineralization nodes in the cultures were quantified by dissolving alizarin red staining in 10% (w/v) cetylpyridinium chloride and measuring absorbance. The mineralization activities were remarkably observed in the 800 μmol/L ATP-treated and osteogenic media groups, and no significant differences were found between each group ($n = 5$, $^*P < .05$ versus blank control). EDX analysis of Ca/P ratios for cultured HDPCs for 21 days. Similar special peaks for Ca and P were found in 800 μmol/L ATP (*C*) and osteogenesis (*D*) treatments. Osteogenic medium (10^{-7} mol/L dexamethasone, 10 nmol/L L-ascorbic acid phosphate magnesium salt n-hydrate, and 10 mmol/L sodium β-glycerophosphate in growth medium) was used as the positive control. The indicated $n = 5$ represents the 5 cell lines from 5 different teeth used to produce the data in this figure.

have shown that it inhibited cell proliferation and differentiation (37). The concentrations of extracellular ATP vary from nmol/L to μmol/L levels (39). The effects of ATP on cell proliferation and differentiation in human dental pulp differed significantly at high and low concentrations. In the range 0.1–1000 μmol/L, ATP increased proliferation of pulp cells between 1 and 10 μmol/L but inhibited cell proliferation at concentrations greater than 400 μmol/L (Fig. 1). Because the cell numbers at higher ATP concentrations (≥400 μmol/L) were lower than those in the control group, we further tested to see whether the reduction in cell numbers was a result of ATP-induced apoptosis. We found no sub-G_1 peaks and apoptosis at 800 μmol/L ATP by using flow cytometry, but we found a much greater percentage (87%) of HDPCs in the G_0G_1 phase than that (58%) with ATP concentration of 10 μmol/L. ATP at 800 μmol/L inhibited cell proliferation not by inducing apoptosis but by arresting the cells in the G_0G_1 phase. The effect of ATP on cell proliferation is consistent with that reported for human cardiac fibroblasts (36) and mouse retinal cells (38).

In this study, mineralization was not affected when HDPCs were treated with ATP at 10 μmol/L, which is a concentration that is closer to the physiological range. However, ATP at 800 μmol/L induced calcified nodule formation in the absence of other osteogenic agents. Compared with the positive control group, the Ca/P ratio in the ATP-treated group was slightly lower (1.62 versus 1.77), but it was similar to the theoretical value of Ca/P in stoichiometrically pure hydroxyapatite (1.67). ATP might inhibit or promote bone formation with variations in ATP concentrations in the μmol/L range (40). The balance between pyrophosphate and orthophosphate is a critical determinant of osteogenesis and calcification (41). Phosphate release after ATP hydrolysis is essential for the formation of mineralized nodules. ATP at 800 μmol/L, but not at 10 μmol/L, may generate adequate quantities of phosphates necessary for mineralization in the absence of osteogenic media.

Although the exact role of ATP in osteogenesis remains to be clarified, the ERK signaling pathway is likely involved in ATP-mediated osteogenic differentiation of HDPCs and the subsequent formation of mineralized nodules. Phosphates from ATP hydrolysis enhanced mineral deposition by osteoblasts, and ATP promoted calcification by affecting Ca^{2+} transporting or accumulation; phosphorylation of ERK is involved in the osteogenic differentiation of osteoblasts and dental pulp cells (33, 42). In the present study, ATP significantly enhanced ERK phosphorylation in a time-dependent manner. After treatment with ATP at 800 μmol/L, ERK phosphorylation increased immediately and peaked at 10 minutes, and this could be prevented either by a P2 receptor antagonist or by an ERK inhibitor (Fig. 3). ATP-mediated osteogenesis may be linked to the ERK/MAPK signaling pathway. This observation is consistent with the effect of bisphosphonates on osteoblast-like cells (43). Bisphosphonates, which are commonly used for treating osteoporosis, may induce ATP release and then act via P2Y receptors to activate ERK (43).

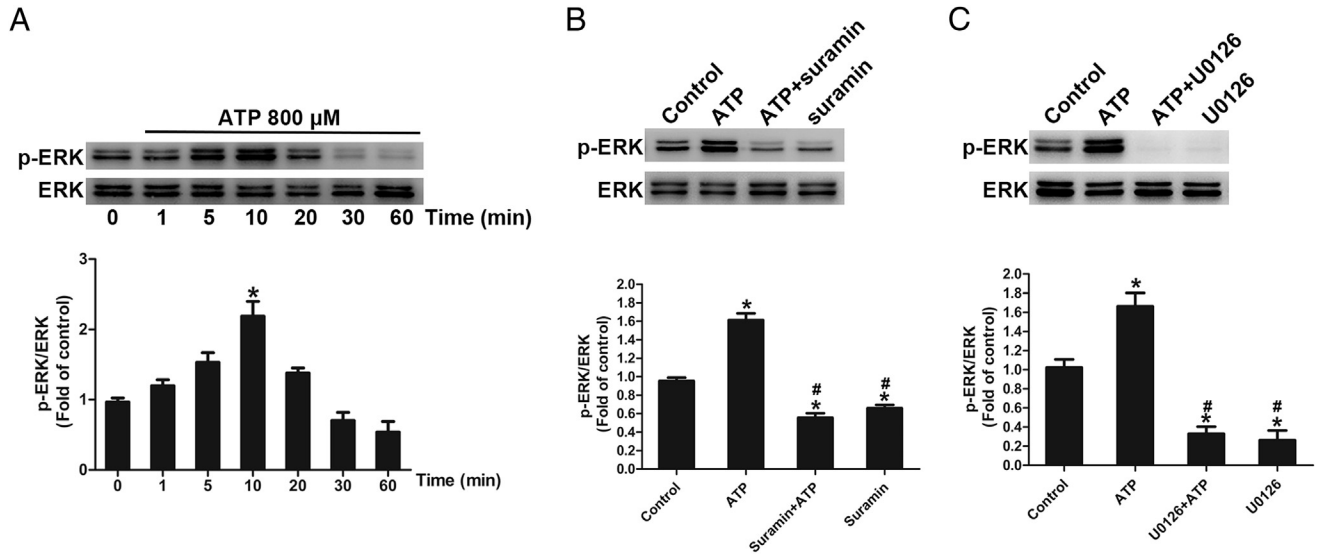

Figure 3. Western blot of p-ERK. (*A*) Time course of ATP on ERK activation in HDPCs. HDPCs were treated with 800 μmol/L ATP for 0, 1, 5, 10, 20, 30, or 60 minutes. ERK level was detected by Western blot analysis. Data were shown as the relative ratio to basal levels. Values of 3 different patients were presented as mean ± standard error of the mean. Differences were considered significant at $P < .05$. *$P < .05$ versus control. The effect of suramin (*B*) or U0126 (*C*) on ATP-induced ERK activation in HDPCs. HDPCs were pretreated with suramin (100 μmol/L) for 60 minutes or U0126 (20 μmol/L) for 30 minutes as well as treated with 800 μmol/L ATP. Cells were collected 10 minutes after ATP exposure for Western blot analysis. Error bars represent standard error of the mean. *$P < .05$ versus control; #$P < .05$ versus ATP. There were 3 cell lines ($n = 3$), named cell lines 1, 3, and 5, from 3 different teeth used to produce the data in this figure.

DMP1 and DSPP are important markers of odontoblastic differentiation of HDPCs. In this present study, DMP1 and DSPP were upregulated in ATP-treated HDPCs at mRNA level during early stages of treatment. The highly selective ERK antagonist U0126 abolished the ATP-induced upregulation of DMP1 and DSPP mRNAs, thereby providing further evidence that the ERK signaling pathway plays an important role in ATP-mediated odontoblastic differentiation of HDPCs.

Although numerous studies have shown that ATP affects wound healing, osteoblast differentiation, and bone metabolism (31, 44), it is unclear which subtypes of P2 receptors are involved in these biological processes. Further studies are needed to elucidate which P2 receptors participate in the osteogenic differentiation of HDPCs. We demonstrated that P2X3, P2X4, P2X5, P2X7, and all P2Y subtypes were expressed in HDPCs, with P2X5, P2Y1, P2Y2, P2Y6, and P2Y13 showing particularly higher levels than other P2 subtypes. The widespread presence of P2 receptor subtypes suggests that ATP may affect the functions of HDPCs broadly. The expression levels of different P2 receptors may also change with the stages of cell differentiation (30, 45). P2X receptors and ecto-ATPase may mediate the transmission of pain in dental pulp (14, 46, 47). Extracellular ATP has been considered as a universal "danger signal" and may activate multiple signaling pathways and elicit complex protective and reparative responses after tissue injury (48, 49). ATP release in low concentrations at early stage of dental pulp injury may activate the pain transmission pathway and enhance proliferation of dental pulp progenitor cells, and in high concentrations at later stages of injury it may activate osteogenic signaling pathways and enhance reparative mineralization in dental pulp.

In summary, this study demonstrated that multiple P2 receptors are expressed in HDPCs. ATP promotes cell proliferation at low concentrations but inhibits cell proliferation by arresting cells at G_0G_1 stage of the cell cycle and enhances odontoblastic differentiation and mineralization at high concentrations. A novel extracellular ATP signaling pathway that triggers the DMP1 and DSPP gene expressions in HDPCs

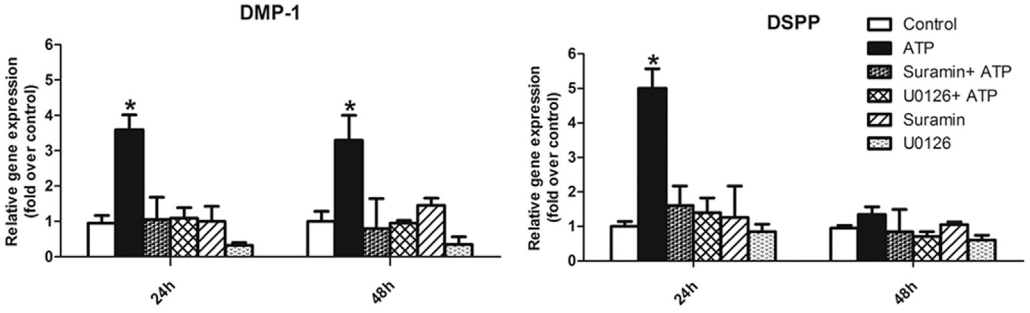

Figure 4. Effects of suramin and U0126 on ATP-induced and odontoblast-related mRNA expression in HDPCs. HDPCs were treated with 800 μmol/L ATP in the presence or absence of suramin (100 μmol/L) or U0126 (20 μmol/L). The mRNA expression levels of DMP1 and DSPP were detected by real-time PCR analysis. Data were expressed as mean ± standard error of the mean ($n = 3$, *$P < .05$). The indicated $n = 3$ represents 3 cell lines (1, 3, and 5) from 3 different teeth used to produce the data in this figure.

was demonstrated. The ERK/MAPK signaling pathway was shown to be involved in ATP-mediated odontoblastic differentiation. These findings provided insights into the molecular regulation of pulpal wound healing and the roles of ATP signaling in the reparative mechanism of dental pulp injury.

Acknowledgments

Wei Wang and Xiaosong Yi contributed equally to this study and should be considered co-first authors. Yanfang Ren and Qiufei Xie contributed equally to this study.

The authors deny any conflicts of interest related to this study.

Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jendodon.com (http://dx.doi.org/10.1016/j.joen.2016.07.013).

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