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Adenosine receptors enhance the ATP-induced odontoblastic differentiation of human dental pulp cells

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ABSTRACT

Purinergic signaling regulates various biological processes through the activation of adenosine receptors (ARs) and P2 receptors. ATP induces the odontoblastic differentiation of human dental pulp cells (HDPCs) via P2 receptors. However, there is no information available about the roles of ARs in HDPC odontoblastic differentiation induced by ATP. Here, we found that HDPCs treated with ATP showed higher activity of *ADORA1* (A_1R), *ADORA2B* ($A_{2B}R$), and *ADORA3* (A_3R). Inhibition of A_1R and $A_{2B}R$ attenuated ATP-induced odontoblastic differentiation of HDPCs, whereas activation of the two receptors enhanced the odontoblastic differentiation induced by ATP. However, activation of ARs by adenosine did not induce the odontoblastic differentiation of HDPCs independently without induction of ATP. Our study indicates a positive role for ARs in ATP-induced odontoblastic differentiation of HDPCs, and demonstrates that ATP-induced odontoblastic differentiation of HDPCs may be due to the combined administration of ARs and P2 receptors. This study provides new insights into the molecular mechanisms of pulpal injury repair induced by ATP.

1. Introduction

Human dental pulp cells (HDPCs) have attracted the interest of researchers in the field of tissue regeneration because of their accessibility and abundance of stem/progenitor cells [1,2]. Recent studies have reported that HDPCs can differentiate not only into odontoblasts for dentin regeneration, but also into osteoblasts, which can repair bone defects under appropriate conditions [3,4]. The differentiation of mesenchymal cells in HDPCs into odontoblasts is induced by multiple cytokines such as four and a half LIM domains 2 (FHL2) [5], bone morphogenetic protein (BMP2) [6], and ID1 (a downstream target of BMP2 signaling) [7].

Purinergic signaling can regulate the proliferation [8], differentiation [9], and death [8] of different types of stem cells. As critical signaling molecules in this pathway, adenosine triphosphate (ATP) and its hydrolysates act through purinergic receptors, which are classified into P1 (adenosine receptors [ARs], e.g. A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R) and P2 (P2XR, e.g. $P2X_{1-7}R$; P2YR, e.g. $P2Y_{1,2,4,6,11-14}R$) receptors. P1 receptors are primarily activated by adenosine, whereas those in the P2 category are mainly regulated by purines such as ATP and adenosine diphosphate (ADP) [10]. Cutarelli et al. [11] were the first to report the biphasic effects of ATP on differentiation and mineralization in human osteoblasts, showing an increase in these processes in response to low concentrations (<100 μM), whereas high concentrations (>100 μM) led to a decrease in these processes, which they suggested was due to the combined activation of P2 receptors and ATP hydrolysis products (e.g. ADP, AMP, adenosine, the mineralization inhibitor PP_i, and the mineralization promoter inorganic phosphate (P_i]). In contrast, the osteogenic effects of ATP on human bone marrow mesenchymal stem cells was due to adenosine stimulation of the AR subtype, $A_{2B}R$ [12].

Extracellular ATP and downstream purinergic signaling have also been proposed to contribute to dental pulp tissue healing and dentin regeneration. Mechanical and thermal stimulation of external dentin can induce ATP release in dental pulp through pannexins [13]. Cold stimulation was also reported to induce ATP release from human odontoblast-like cells [14]. Our previous study demonstrated that high concentrations of ATP (800 μM) can induce odontoblastic differentiation of HDPCs, whereas low

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concentrations (<400 μM) promoted cell proliferation, and that P2 receptors and the ERK/MAPK signaling pathway were involved in this ATP-induced odontoblastic differentiation [15]. These investigations indicated positive roles for ATP and P2 receptors in dental pulp wound healing and dentin formation. However, the specific role of ARs in ATP-induced odontoblastic differentiation of HDPCs, and the effects of adenosine, the hydrolysate of ATP, on HDPC odontoblastic differentiation remain unknown.

All four AR subtypes were demonstrated to be expressed in dental pulp stem cells (DPSCs), and stimulation of A_1R might enhance the osteogenic differentiation of DPSCs, moreover, the progress of dentinogenesis is similar to that of osteogenesis, to some extent [16]. We conducted this study to identify the role of ARs in ATP-induced odontoblastic differentiation of HDPCs, and to determine whether the ARs activation by adenosine can induce HDPC odontoblastic differentiation independently, without the induction of ATP.

2. Methods

2.1. Cell culture

This study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology (IRB-2013055), and informed consent was obtained. HDPCs were isolated according to a previous report [15]. Cells were cultured in proliferation medium (PM) containing α-minimum essential medium (α-MEM) (Gibco, Grand Island, NY) with 10% fetal bovine serum (Corning Cellgro, NY), 1% L-glutamine (Sigma-Aldrich, St. Louis, MO), and 1% penicillin/streptomycin (Gibco, BRL). For ATP-induced odontoblastic differentiation, cells were cultured in standard PM containing additional 600 μM ATP (ATP medium). Every experiment was repeated three times with HDPCs at the third passage from five different donors (three males and two females; 19–29 years old).

2.2. Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA isolation and RT-PCR were performed as described in the earlier study [15]. *GAPDH* was measured as a reference. The primers used in this process are listed in Table 1.

2.3. Quantitative real-time PCR

Real-time PCR was performed using an ABI 7500 real-time PCR machine (Applied Biosystems, Foster City, CA) and SYBR Green chemistry, according to the manufacturer's protocol. Expression of *DMP1* and *DSPP* (both specific markers in odontoblastic differentiation) in HDPCs cultured in PM, ATP medium (presence or absence of selective inhibitors/agonists of A_1R, $A_{2B}R$, and A_3R), and adenosine medium (0, 0.1, 1, 10, 100, 200, 400, 600, 800, 1000 μM) was detected at 24 h, 48 h, and 7 days. These time points were chosen for mRNA analysis, as they are the critical windows in the differentiation of HDPCs into odontoblasts. Adenosine and the selective antagonists of A_1R (8-cyclopentyl-1,3-dipropylxanthine (DPCPX)), $A_{2B}R$ (MRS-1754), and A_3R (PSB-11 hydrochloride), as well as the selective agonists of A_1R (2-Chloro-N^6-cyclopentyladenosine (CCPA)), $A_{2B}R$ (NECA), and A_3R (IB-MECA) were all purchased from Abcam (Cambridge, MA).

2.4. Western blotting

Western blotting analysis of A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R in HDPCs cultured in ATP medium was performed at 14 days, as described in a previous study [16]. The protein expression of DMP1 and DSPP in cells cultured in ATP medium (presence or absence of selective inhibitor/agonist of A_1R, $A_{2B}R$, and A_3R) and adenosine medium was detected at 48 h and 7 days. We chose the 7-day time point for western blotting because DMP1 and DSPP protein levels showed a visible response to the odontoblastic differentiation around 7 days [17–19]. The primary antibodies used were anti-GAPDH, anti-DMP1, anti-DSPP (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA); anti-A_1R, anti-$A_{2A}R$, anti-$A_{2B}R$, anti-A_3R (1:1000, Abcam, Cambridge, MA); GAPDH was evaluated as an internal control.

2.5. Alizarin Red S staining and quantification

After 21 days of odontoblastic induction, mineralization was analyzed using Alizarin Red staining. The 21-day time point is frequently used in Alizarin Red S staining in osteo/odontogenic differentiation research, as mineralized nodules are obviously visible around 21 days [16]. For quantification of mineralization, the stained mineralized nodules were dissolved in 10% (w/v) cetylpyridinium chloride (Sigma-Aldrich, St. Louis, MO) for 1 h, and the concentration was measured at 562 nm using a spectrophotometer (BioTek Instruments, Winooski, VT).

2.6. Statistical analyses

Data were presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using Student's *t*-test and one-way ANOVA, and differences were considered significant at $P < 0.05$. Experimental data were analyzed using Prism 6 software (GraphPad, San Diego, CA).

Table 1
Primers for adenosine-receptor-related and odontoblast-related gene expression analysis.

Gene	Accession Number	Primer	Sequences (5′-3′)	Fragment Size (bp)
ADORA1 (RT-PCR and Real-time PCR)	NM_000674	Forward	CAAGATCCCTCTCCGGTACAA	109
		Reverse	GCCAAACATAGGGGTCAGTCC	
ADORA2A (RT-PCR and Real-time PCR)	NM_000675	Forward	CATGCTAGGTTGGAACAACTGC	185
		Reverse	AGATCCGCAAATAGACACCCA	
ADORA2B (RT-PCR and Real-time PCR)	NM_000676	Forward	CTGTCACATGCCAATTCAGTTG	134
		Reverse	GCCTGACCATTCCCACTCTTG	
ADORA3 (RT-PCR and Real-time PCR)	NM_000677	Forward	GTGCTGGTCATGCCTTTGG	100
		Reverse	CGTGGGTAAAGATAAGCAGTAGG	
DMP1 (Real-time PCR)	NM_001079911	Forward	GTGAGTGAGTCCAGGGGAGATAA	111
		Reverse	TTTTGAGTGGGAGAGTGTGTGC	
DSPP (Real-time PCR)	NM_014208	Forward	TGGAGCCACAAACAGAAGCAA	127
		Reverse	TCCAGCTACTTGAGGTCCATC	
GAPDH (RT-PCR and Real-time PCR)	NM_001256799	Forward	ATGGGGAAGGTGAAGGTCG	108
		Reverse	GGGGTCATTGATGGCAACAATA	