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Original Article

Dopamine receptor expression on primary osteoblasts and bone marrow mesenchymal stem cells of rats

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Abstract: Bone metabolism and the underlying mechanisms are of great interest, particularly in the context of clinical bone tissue repair and tissue engineering. Neurotransmitters play an important role in bone metabolism. Dopamine is an important neurotransmitter with several critical functions in the central nervous system, and is likely involved in bone metabolism. However, the expression of dopamine receptors in the bone marrow stem cells and osteoblasts has not been extensively studied to date. In this study, we determined whether two types of dopamine receptors, dopamine receptor 1 (D1R) and dopamine receptor 2 (D2R), were expressed on osteoblasts and bone marrow mesenchymal stem cells (BMMSCs) in rats, by using polymerase chain reaction, western blot, and immunofluorescence. Immunohistochemistry was used to confirm the expression of D1R and D2R in the mandible of the rats. The results showed that D1R and D2R are expressed on rat osteoblasts and BMMSCs. Both receptors were distributed in the dental pulp, periodontal tissue, and bone tissue. Our results suggested that dopamine could affect the biological behavior of osteoblasts and BMMSCs by directly binding to its receptors. D1R and D2R could therefore directly modulate bone metabolism.

Keywords: Dopamine, dopamine receptor, osteoblast, bone marrow mesenchymal stem cell, mandible, rats

Introduction

Leptin is a polypeptide hormone that exerts gonadal functions by binding to specific receptors in the hypothalamus. The discovery of leptin-mediated bone mass modulation via the sympathetic nervous system suggested that neurotransmitters play a role in bone formation [1, 2]. Dopamine, a catecholamine neurotransmitter widely expressed in the central nervous system (CNS) and some peripheral areas, regulates cognitive and behavioral functions in the CNS, including voluntary movement, memory, attention, learning, and reward [3]. Several effects of dopamine and its receptors in the modulation of bone modeling have been reported. Long-term treatment with D2R antagonist induced bone loss in female rats [4]. The absence of dopamine transporter caused lower bone mass in mice [5]. In clinical trials, patients with Schizophrenia undergoing treatment with D2R antagonist showed lower bone mineral density [6]. On the other hand, the risk of osteoporosis was higher in patients with Parkinson's disease. These were related to dopamine and its receptors [7, 8]. Based on these data, we hypothesized that dopamine could be involved in mediating bone metabolism.

Dopamine exerts its physiological actions by activating specific seven-transmembrane G-protein-coupled dopamine receptors. Five such receptors, D1R, D2R, D3R, D4R, and D5R, have been discovered. These receptors have been divided into the D1-like and D2-like subfamilies, based on pharmacology and the ability to modulate cyclic adenosine monophosphate (cAMP) concentration. D1R and D5R are included in the D1-like family and tend to increase the concentration of cAMP. The D2-like family, which includes D2R, D3R, and D4R, inhibits the production of cAMP by inhibiting adenylate cyclase [3, 9]. Dopamine receptors are widely expressed in the CNS and peripheral tissues [9], such as the renal system [10] and coronary vessels [11].

Table 1. Primers used for PCR

Gene	Sequence (5'-3')
D1R*	5'-CAGTCCATGCCAAGAATTGCC-3'
	5'-AATCGATGCAGAATGGCTGGG-3'
D2R	5'-CACCACGGCCTACATAGCAA-3'
	5'-GGCGTGCCCATTCTTCTCT-3'
GAPDH	5'-ATGCTCTACCCACGGCAAG-3'
	5'-GGAAGATCCTGATCCCTTTC-3'

*D1R gene reference was obtained from Zhou et al [29].

Hanami et al detected all five dopamine receptors in human osteoclasts. Further, the number of osteoclasts decreased after culture with D2-like receptor agonist but not with D1-like receptor agonist [12]. Lee et al reported that mouse MC3T3-E1 osteoblastic cells expressed all the dopamine receptors, based on RT-PCR and western blot data [13]. However, previous studies showed that only D1R, D3R, and D5R were expressed in MC3T3-E1 cells [14]. Thus, the expression pattern of dopamine receptors in rats remains unclear. In this study, we aimed to determine whether rat primary osteoblasts and BMMSCs expressed D1R and D2R, by using polymerase chain reaction (PCR), western blot, and immunofluorescence. We also studied the distribution of dopamine receptors in the rat mandible by immunohistochemistry. The results could improve our understanding of the mechanisms by which dopamine modulates bone metabolism.

Materials and mthods

Cell culture

Primary Sprague-Dawley (SD) rat calvarial osteoblasts were obtained and cultured as reported previously [15]. Briefly, the fibrous layers of the periosteum from the parietal and frontal bones were isolated and cleaned with 0.1M phosphate-buffered saline (1×PBS). The isolated material was digested by treating with 0.2% collagenase I (Sigma, USA) for 10 min twice and 3 times for 15 min at 37°C. The reaction mixtures were centrifuged for 6 min at 1500 rpm. Cells were resuspended in Dulbecco's minimum essential medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin, and cultured at 37°C in a 5% CO_2 humidified atmosphere.

Rat BMMSCs (Cyagen Bioscience Inc., USA) were cultured in α-Minimal Essential Media (α-MEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin, and cultured at 37°C in a 5% CO_2 humidified atmosphere. Culture medium was replaced every 2-3 days.

Polymerase chain reaction (PCR)

RNA was isolated from osteoblasts and BMMSCs by using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The isolated RNA was reverse-transcribed to cDNA by using the PrimeScript RT Reagent Kit (TaKaRa Bio Inc. Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a normalizing control. PCR amplification was performed using the following cycling settings: 29 cycles of 94°C for 40 s, 55°C for 45 s, and 72°C for 40 s, and finally 70°C for 5 min. After amplification, electrophoresis was conducted in 1% agarose gel stained with GelRedTM Nucleic Acid Gel Stain (Biotium, Inc., CA, USA), and the bands were visualized and photographed using the Vilber Lourmat Fusion FX5 system (Vilber Lourmat, Marne-la-Vallée, France). The primers used are listed in **Table 1**.

Western blot

We used PC12 as the positive control and beta actin as a loading control. The processing of cells, including PC12, osteoblasts, and BMMSCs, was conducted as reported previously [16]. Cells lysis was performed in radio immunoprecipitation assay buffer (50 mmol/L Tris-HCL: pH 8.0, 5 mmol/L EDTA, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride and phosphatase & protease inhibitor cocktail). The mixture was centrifuged at 14,000 rpm, and the cell lysate (supernatant) was collected and loaded onto 12% NuPAGE SDS-PAGE Gel (Invitrogen, Carlsbad, CA, USA). The resolved bands were transferred to PVDF membranes, blocked in 5% skimmed milk for 30 min, and incubated overnight at 4°C with primary antibodies against D1R and D2R (ratio 1:200; Abcam, Cambridge, MA, USA). The membrane was washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Milipore, Billerica, MA, USA) at 37°C for 1 h. The bands were detected using chemiluminesce-

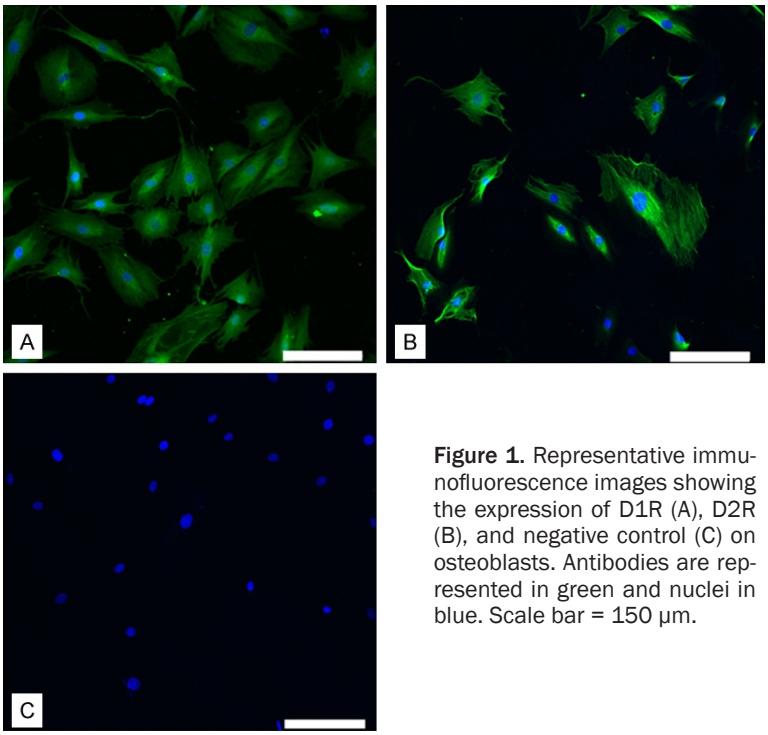

Figure 1. Representative immunofluorescence images showing the expression of D1R (A), D2R (B), and negative control (C) on osteoblasts. Antibodies are represented in green and nuclei in blue. Scale bar = 150 μm.

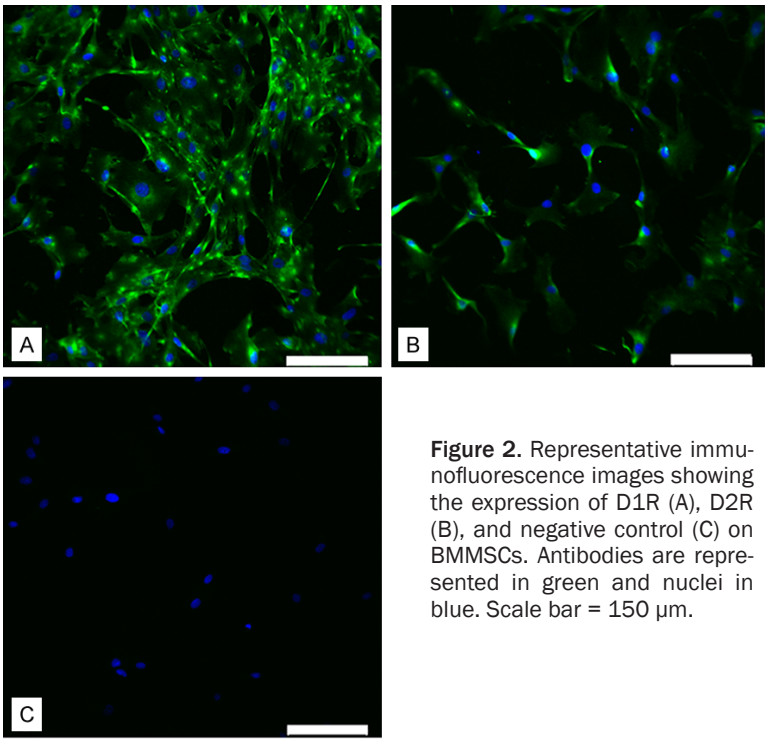

Figure 2. Representative immunofluorescence images showing the expression of D1R (A), D2R (B), and negative control (C) on BMMSCs. Antibodies are represented in green and nuclei in blue. Scale bar = 150 μm.

nce reagent and visualized by the EasySee western blot kit (Transgen, China).

Immunofluorescence

Immunofluorescence studies were performed according to previously described protocols [12]. The osteoblasts and BMMSCs were soaked in paraformaldehyde for 20 min. The cells were incubated with 20% goat serum and then immunostained overnight at 4°C with antibodies against D1R and D2R (1:500; Abcam, Cambridge, MA, USA) after serially washing in PBS. Cells incubated in PBS were used as negative control. Subsequently, the cells were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Milipore, Billerica, MA, USA). The fluorescence signal was observed and photographed with a fluorescent microscope (NIKON, ECLIPSE E600).

Tissue preparation

After the Sprague-Dawley (SD) rats were sacrificed by cervical dislocation, the mandibles were removed and fixed in 4% paraformaldehyde. After 1 month, the tissues were immersed in a 10% EDTA solution for demineralization, until the dental hard tissue could be punctured with a fine needle without obvious resistance. After rinsing under running water, the tissue samples were dehydrated in an ethanol gradient series (50%-60%-80%-90%-95), and sequentially immersed in 100% ethanol for 3 h twice, normal butanol for 20 h, and trichloromethane for 5 h. Finally, the tissue was subjected to wax immersion for 24 h twice to obtain a wax-embedded block. The block was cut into 5-μm sagittal serial sections parallel to the long axis of the teeth and mounted on glass slides. The sections were then subjected to immunohistochemistry.

Immunohistochemistry

Slide-mounted tissue sections were heated for 30 min at 58°C, deparaffinized in xylene, and

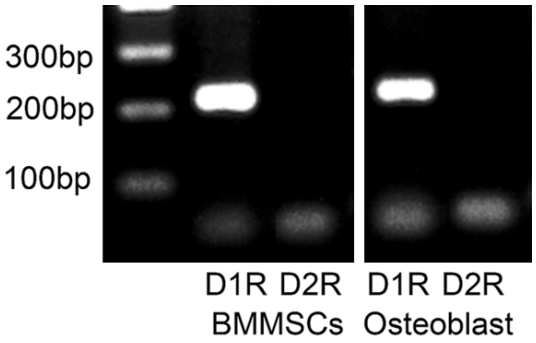

Figure 3. D1R and D2R gene expression in osteoblasts and BMMSCs.

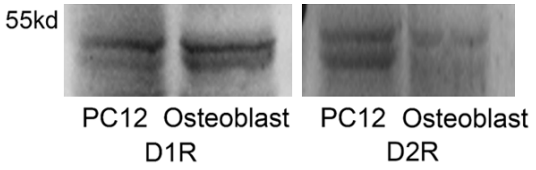

Figure 4. Western blot analysis of D1R and D2R expression in osteoblasts. PC12 was used as a positive control. Beta actin was used as a loading control.

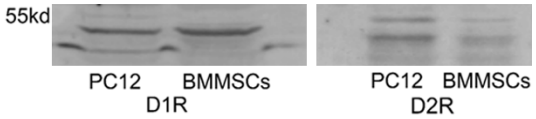

Figure 5. Western blot analysis of D1R and D2R expression in bone marrow mesenchymal stem cells. PC12 was used as a positive control. Beta actin was used as a loading control.

rehydrated in an ethanol gradient series. After blocking endogenous peroxidase with 3% H_2O_2 for 10 min at room temperature, the sections were incubated in 1 mg/ml trypsin at 37°C for 10 min for antigen retrieval. 10% Goat Serum was used for blocking at room temperature, and the sections were incubated with rabbit anti-dopamine receptor D1R polyclonal antibody (1:400; Abcam, Cambridge, MA, USA) or rabbit anti-dopamine receptor D2R polyclonal antibody (1:400; Abcam, Cambridge, MA, USA) at 4°C overnight in a humid atmosphere. One of the sections was incubated in PBS as a negative control. After washing in PBS, the sections were incubated in the presence of peroxidase-conjugated goat anti-rabbit IgG (H+L; 1:400; Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h to visualize DR subtypes. Images were captured using a digital microscopic system (Olympus BX51/DP72, Tokyo, Japan).

Results

Immunofluorescence analysis of dopamine receptors

We used immunofluorescence to determine whether D1R and D2R were expressed in osteoblasts and BMMSCs. As shown in **Figures 1** and **2** significant D1R and D2R expression was observed in both cell types. Higher D1R expression was observed in BMMSCs, while similar levels of D1R and D2R were observed in osteoblasts.

D1R and D2R gene expression in osteoblasts and BMMSCs

D1R and D2R gene expression in osteoblasts and BMMSCs was confirmed by PCR. As shown in **Figure 3**, gel electrophoresis of the D1R and D2R PCR products from BMMSCs and osteoblasts yielded identical DNA bands.

Western blot analysis of dopamine receptor expression in osteoblasts and BMMSCs

Figures 4 and **5** show representative results of western blot analysis of D1R and D2R. PC12 was used as the positive control. D1R and D2R were clearly detected in osteoblasts and BMMSCs.

Distribution of D1R and D2R in rat mandible

We studied the distribution of D1R and D2R in the rat mandible by immunohistochemistry. As shown in **Figure 6**, immunohistochemical staining of D1R and D2R was observed in the dental pulp, periodontal tissue, and bone tissue in the rat mandible. Only some cells in the bone were stained, and were distributed irregularly. Additionally, D1R and D2R were observed on the cell membrane and cytoplasm.

Discussion

Neurotransmitter-mediated bone mass regulation was first reported in 2000 and 2002 [1, 2], after which evidence of neurotransmitter involvement in bone metabolism accumulated rapidly. Dopamine, an important neurotransmitter involved in severe central nervous system (CNS) diseases such as Parkinson's disease, is associated with a high risk of osteoporosis [17], and is likely involved in bone metabolism. Increased bone mineral density was observed in

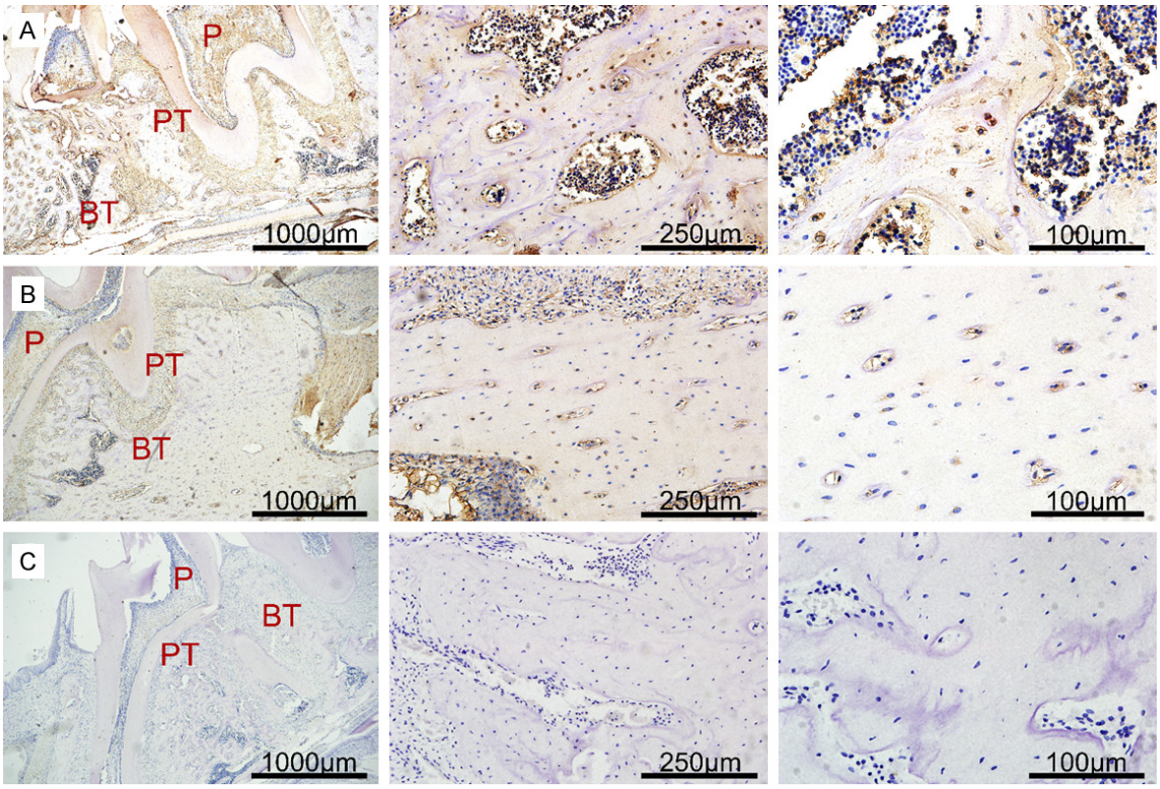

Figure 6. Immunohistochemistry showing the distribution of D1R (A), D2R (B), and negative control (C) in the rat mandible. Brown lines and dots represent positive D1R and D2R binding to their receptors. P: Dental Pulp; PT: Periodontal Tissue; BT: Bone Tissue.

patients with metabolism disorders and osteopenia who were treated with dopamine receptor agonists [18, 19]. Further, dopamine antagonist treatment could decrease bone mineral density [4]. However, the mechanism by which dopamine regulates bone metabolism remains unclear.

Dopamine could mediate different physiological actions by binding to specific receptors. The structures of dopamine receptors of the same subfamily are similar; the transmembrane domains of D1R and D5R exhibit 80% homology. Similarly, D3R and D4R show 75% and 53% homology, respectively, with D2R [3]. Dopamine receptors have been detected in a variety of cells, and have different functions [20-22]. Immune cells such as B-cells, T-cells, neutrophils, and natural killer cells express dopamine receptors, and the various functions of activated T-cells could be affected by the binding of dopamine to its receptors [20, 21]. In the gastrointestinal system, dopamine receptors were localized on the pepsin C-positive chief cells, and regulated pepsin secretion [22]. All five types of dopamine receptors have been detected in osteoblasts [13] and osteoclasts [12]. Dopamine-mediated reduction of RANKL-dependent osteoclast formation and differentiation was mimicked by treatment with a D2R agonist [12, 23]. D1R-like receptor antagonists could decrease osteoclast differentiation and bone destruction in a collagen-induced arthritis mouse model [24].

Our results confirmed D1R and D2R expression in rat osteoblasts and BMMSCs. D1R and D2R were expressed on the cell membrane and cytoplasm, suggesting that exogenous dopamine could affect the behavior of osteoblasts and BMMSCs by directly binding to D1R and D2R. In the mandible of rats, D1R and D2R were widely expressed in the dental pulp, periodontal tissue, and bone tissue, possibly in mesenchymal stem cells (MSCs). MSCs have been successfully isolated from premolar periodontal ligaments and dental pulp [25] by digesting in collagenase and dispase [26] or sorting by fluorescence-activated analysis [27], and could differentiate into bone, cartilage, and

adipose cells under specific conditions [26]. The possibility of other cell types expressing D1R and D2R in dental pulp and periodontal tissue, however, could not be excluded, and requires further study. In our study, the expression of D2R appeared lower than that of D1R, although published data suggests that D2R is pivotal in bone remodeling [23]. Based on our results, we assumed that D1R and D2R are expressed throughout bone remodeling, and may be involved in the regulation of osteoblastic induction of BMMSCs.

Our results on D1R and D2R expression seem to be in broad agreement with clinical and experimental evidence of dopamine receptor modulation of bone metabolism [4, 12, 13, 18, 23, 24, 28]. Expression of the remaining members of the dopamine receptor subfamilies, with homologous transmembrane domains and similar functions [3], in osteoblasts, BMMSCs, and the rat mandible could not be excluded in our study. Furthermore, the functional effects of different receptors and the underlying molecular mechanisms require further study.

In conclusion, we found that D1R and D2R were expressed on osteoblasts and BMMSCs, and widely distributed in the rat mandible dental pulp, periodontal tissue, and bone tissue. Dopamine could affect the biological behavior of osteoblasts and BMMSCs by directly binding to these receptors.

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Disclosure of conflict of interest

None.

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