Bone 101 (2017) 191–201

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

Calcium-sensing receptor-ERK signaling promotes odontoblastic differentiation of human dental pulp cells

Hiroyuki Mizumachi [a], Shinichiro Yoshida [b], Atsushi Tomokiyo [b], Daigaku Hasegawa [b], Sayuri Hamano [a], Asuka Yuda [a], Hideki Sugii [a], Suguru Serita [a], Hiromi Mitarai [a], Katsuaki Koori [b], Naohisa Wada [c], Hidefumi Maeda [a,b,*]

[a] Department of Endodontology and Operative Dentistry, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, Fukuoka, Japan
[b] Division of Endodontology, Kyushu University Hospital, Kyushu University, Fukuoka, Japan
[c] Division of General Dentistry, Kyushu University Hospital, Kyushu University, Fukuoka, Japan

ARTICLE INFO

Article history:
Received 17 November 2016
Revised 10 May 2017
Accepted 11 May 2017
Available online 12 May 2017

Keywords:
CaSR
L-VDCC
HDPC
Odontoblastic differentiation
ERK 1/2

ABSTRACT

Activation of the G protein-coupled calcium-sensing receptor (CaSR) has crucial roles in skeletal development and bone turnover. Our recent study has identified a role for activated CaSR in the osteogenic differentiation of human periodontal ligament stem cells. Furthermore, odontoblasts residing inside the tooth pulp chamber play a central role in dentin formation. However, it remains unclear how CaSR activation affects the odontoblastic differentiation of human dental pulp cells (HDPCs). We have investigated the odontoblastic differentiation of HDPCs exposed to elevated levels of extracellular calcium (Ca) and strontium (Sr), and the contribution of CaSR and the L-type voltage-dependent calcium channel (L-VDCC) to this process. Immunochemical staining of rat dental pulp tissue demonstrated that CaSR was expressed at high levels in the odontoblastic layer, moderate levels in the sublayer, and low levels in the central pulp tissue. Although normal HDPCs expressed low levels of CaSR, stimulation with Ca or Sr promoted both CaSR expression and odontoblastic differentiation of HDPCs along with increased expression of odontoblastic makers. These effects were inhibited by treatment with a CaSR antagonist, whereas treatment with an L-VDCC inhibitor had no effect. Additionally, knockdown of CaSR with siRNA suppressed odontoblastic differentiation of Ca- and Sr-treated HDPCs. ERK1/2 phosphorylation was observed in Ca- and Sr-treated HDPCs, whereas CaSR antagonist treatment or CaSR knockdown blocked ERK1/2 phosphorylation. Furthermore, inhibition of ERK1/2 suppressed mineralization of Ca- and Sr-treated HDPCs. These results suggest that elevated concentrations of extracellular Ca and Sr induce odontoblastic differentiation of HDPCs through CaSR activation and the ERK1/2 phosphorylation.

1. Introduction

Dentin is a hard tissue from which much of the tooth is formed. Dental pulp tissue is included within a chamber surrounded by dentin. This pulp tissue plays important roles in tooth nourishment, inhibiting bacterial invasion, and reacting to mechanical and chemical stimuli. To protect itself from exogenous stimuli, pulp tissue contains stem cells that differentiate into odontoblasts, ultimately leading to reparative dentin formation [1,2].

Dentin includes two major non-collagenous extracellular proteins produced by odontoblasts: dentin matrix protein 1 (DMP-1) and dentin sialophosphoprotein (DSPP). These proteins and their encoding genes are similar in sequence and structure [3]. DMP-1 modulates mineralization, and when implanted into exposed pulp tissue in vivo, promotes early mineralization during the wound healing process [4]. DSPP is a calcium binding protein involved in mammalian dentin calcification that is found predominately within predentin at low mineralization levels [5]. Loss of DSPP results in widened predentin, an irregular mineralization front, and hypomineralization, supporting its roles in dentin mineralization [6]. These findings implicate DSPP and DMP-1 as positive conditioners of hard tissue formation, with DSPP influencing dentin and DMP-1 regulating both bone and dentin [5].

Exposure of dental pulp tissue by caries or trauma is generally treated by application of calcium hydroxide-containing agents to the affected area to promote reparative dentin formation. However, the alkaline environment created by the hydroxide ion often induces necrosis of the pulp tissue that is in direct contact with these agents, with formation of porous including embedded cells reparative dentin adjacent to this necrotic tissue [7]. This porous dentin could become a route for bacterial infection,

* Corresponding author at: Department of Endodontology and Operative Dentistry, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail address: hide@dent.kyushu-u.ac.jp (H. Maeda).

http://dx.doi.org/10.1016/j.bone.2017.05.012
8756-3282/

leading to pulpitis or pulp necrosis. Additionally, physiological dentin with dentinal tubules sometimes forms just below or above imperfect dentin, depending on the type of agent used. While the precise mechanisms underlying formation of porous dentin tissue remain unclear, preservation of healthy pulp tissue requires that porous dentin be avoided.

Elevated concentrations of extracellular calcium induce the odontoblastic differentiation, proliferation, and migration of dental pulp cells [8]. Furthermore, a recent study reported that strontium could be also a candidate material for this purpose [9]. Both calcium and strontium are known as calcium-sensing receptor (CaSR) agonists [10,11].

CaSR is a class 3 G-protein coupled receptor (GPCR) that was first cloned from the bovine parathyroid gland [10], and is expressed on the cell membrane as a disulfide-linked constitutive homodimer [12,13]. CaSR is also expressed in a number of tissues not contributing to the regulation of extracellular calcium concentration [14,15], and is involved in systemic calcium and skeletal homeostasis. CaSR is a key modulator of the osteoblastic differentiation of rat mesenchymal stem cells [16]. Consistently, elevated concentrations of extracellular calcium activate CaSR to induce osteoblastic growth [17]. Additionally, CaSR-null mice exhibit osteomalacia [18,19]. Lastly, a high level of extracellular calcium is believed to control bone formation by stimulating osteoblastic proliferation, chemotaxis, differentiation, and mineralization [20,21]. Taken together, these reports indicate that CaSR signaling is essential for bone development. Consistently, our recent study demonstrated that exposure of periodontal ligament stem cells to high concentrations of calcium promoted increased proliferation and mineralization through activation of CaSR, but not L-type voltage-dependent calcium channels (L-VDCCs) [22].

CaSR signaling is mediated by several mitogen activated protein kinase (MAPK) families, with the specific MAPK pathway involved dependent on the cell type [23,24,25]. In osteoblastic cells, several MAPK cascades are activated by stimulation of CaSR with polycationic CaSR agonists such as gadolinium, neomycin, and spermine [26]. CaSR signaling in MC3T3 cells treated with strontium induces differentiation through ERK [27]. However, the signaling pathway used during CaSR-mediated odontoblastic differentiation of dental pulp cells has been unknown until now.

In the present study, we have examined the role of CaSR in the odontoblastic differentiation of human dental pulp cells treated with calcium and strontium. Furthermore, we have identified the signaling pathways associated with this process.

2. Materials and methods

2.1. Cell culture

Human dental pulp cells (HDPCs) were used as described in our previous studies [28,29]. Briefly, cells were isolated from the third molars of three different patients: a 25-year-old female (3U), a 24-year-old male (3R), and a 20-year-old female (3T) who visited Kyushu University Hospital for tooth extraction. HDPCs were maintained in α-minimum essential medium (α-MEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 50 μg/ml streptomycin and 50 U/ml penicillin (Gibco-BRL) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. HDPCs were cultured in 100-mm tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ, USA) and half of the medium in each dish was replenished every 2–3 days to prevent nutrient depletion. All procedures were performed in compliance with the Research Ethics Committee, Faculty of Dentistry, Kyushu University.

2.2. Immunohistochemical and immunofluorescent assays

Immunohistochemical analysis was performed as described in our recent study [22]. Briefly, 5-week-old male Sprague Dawley (SD) rats (Kyudo, Saga, Japan) were anesthetized by intra-peritoneal injection of 2 mg/kg midazolam (Sandoz Inc., Tokyo, Japan), 0.15 mg/kg medetomidine (Kyoritsu Seiyaku Co, Ltd. Tokyo, Japan), and 2.5 mg/kg butorphanol tartrate (Meiji Seika Pharma Co. Ltd., Tokyo, Japan). Next, animals were sacrificed by transcardial perfusion with 4% paraformaldehyde (PFA; Merck Millipore, Darmstadt, Germany) in phosphate-buffered saline (PBS). Maxillae were removed and decalcified in 10% ethylenediaminetetraacetic acid (Wako Pure Chemical Industries Ltd., Osaka, Japan) at 4 °C for 1 month before dehydration and embedding in paraffin. Following the preparation of 5 μm sections, tissues were blocked with 2% bovine serum albumin (BSA; Nacalai Tesque, Kyoto, Japan) in PBS at room temperature for 1 h. The sections were incubated overnight with mouse monoclonal anti-CaSR antibody (1:500 dilution; Abcam, Cambridge, UK) at 4 °C. Following three washes with PBS, sections were incubated with biotinylated anti-mouse secondary antibody (Nichirei Biosciences Inc., Tokyo, Japan), followed by avidin-peroxidase conjugation (Nichirei Biosciences Inc.). Diaminobenzidine (Nichirei Biosciences Inc.) was used to produce a color reaction. The tissues were imaged by using an inverted microscope (BX41; Olympus Medical, Tokyo, Japan).

Calcium chloride ($CaCl_2$; WAKO Pure Chemical Industries Ltd.) and strontium ranelate (SR; LKT Laboratories, Inc., St. Paul, MN, USA) were dissolved in distilled water. HDPCs (2×10^4 cells/dish) were seeded on 35 mm dishes (Becton Dickinson) and cultured in control medium (CM) and CM containing 2 mM $CaCl_2$ or 10 mM SR for 3 days. HDPCs were then fixed with 4% PFA including 0.5% dimethyl sulfoxide (DMSO; WAKO Pure Chemical Industries Ltd.) in PBS for 20 min at room temperature. Next, the cells were washed five times with PBS. After blocking by incubation with 2% BSA and 0.01% sodium azide in PBS for 1 h at 4 °C, cells were incubated overnight with mouse monoclonal anti-CaSR antibody (1:500 dilution) at 4 °C. Following three washes with PBS, cells were incubated with Alexa-Fluor-488-conjugated chicken anti-mouse antibody (1:200 dilution; Invitrogen, Carlsbad, CA) for 10 min at 4 °C, followed by avidin-peroxidase conjugate. Nuclei were stained with 4′,6-diamidino-2-phenylindole solution (DAPI; Nacalai Tesque). Finally, cells were imaged using a Biozero digital microscope (Keyence, Osaka, Japan).

2.3. Mineralization assay

The CaSR specific antagonist NPS2143 (NPS; Santa Cruz Biotechnology Inc., Dallas, TX, USA), calcium channel inhibitor Nifedipine (WAKO Pure Chemical Industries Ltd.; NIF), and ERK inhibitor U0126 (Merck Millipore) were dissolved in DMSO. HDPCs (2×10^4 cells/well) were seeded on 24-well plates (Becton Dickinson) in CM and CM containing 1, 2, or 4 mM $CaCl_2$, or 1, 5, or 10 mM SR. After 7 days of culture, calcified deposits were detected by von Kossa staining and Alizarin Red S staining according to our previous report [29]. Total RNA was extracted from cells cultured for 3 days using TRIzol Reagent (Invitrogen) for real time RT-PCR analysis to measure the expression levels of osteoblast-associated marker genes.

HDPCs were also seeded on 24-well plates in CM containing 0, 1, or 5 μM NPS, or 0 or 7.5 μM NPS. After 24 h, the media were changed to CM including 2 mM $CaCl_2$ with 0, 1, or 5 μM NPS, or 10 mM SR with 0 or 7.5 μM NPS. Additionally, HDPCs were cultured on 24-well plates in CM containing 5 or 7.5 μM NPS, or 10 μM NIF. After 24 h, the media were changed to CM including 2 mM $CaCl_2$, 2 mM $CaCl_2$ with 5 μM NPS, 2 mM $CaCl_2$ with 10 μM NIF, 10 mM SR, 10 mM SR with 7.5 μM NPS, or 10 mM SR with 10 μM NIF. As a control, cells were pretreated with CM for 24 h and the medium was changed to CM. After an additional 7 days of culture, calcified deposits were detected by von Kossa and Alizarin Red S staining. Total RNA was extracted from cells cultured for 4 days (pretreatment for 24 h and treatment for 3 days) using TRIzol Reagent prior to real time RT-PCR analysis to measure the expression levels of osteoblast-associated marker genes. After pretreatment for 24 h and treatment for 10 min, cells were rinsed three times with PBS and lysed