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STIM1 and TRPV4 regulate fluid flow-induced calcium oscillation at early and late stages of osteoclast differentiation

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ABSTRACT

Bone resorption is mainly mediated by osteoclasts (OCs), whose formation and function are regulated by intracellular Ca^{2+} oscillation. Our previous studies demonstrated that fluid shear stress (FSS) lead to Ca^{2+} oscillation through mechanosensitive cation-selective channels. However, the specific channels responsible for this FSS-induced Ca^{2+} oscillation remain unknown. In the present study, we examined the expression of several Ca^{2+} channels in OCs, including STIM1, ORAI1, TRPV1, TRPV4, TRPV5, and TRPV6, by western blotting and reverse transcription-polymerase chain reaction. The results showed that STIM1 was highly expressed in early stage OCs, while TRPV4 was highly expressed in late stage OCs. We observed intracellular Ca^{2+} responses in OCs that were mechanically stimulated by FSS. When we blocked STIM1-dependent store-operated Ca^{2+} entry or inhibited TRPV4 using siRNA or drug inhibition, FSS-induced Ca^{2+} oscillations were almost undetectable in early and late stage OCs, respectively. These results indicate that STIM1 and TRPV4 act as mechanical transduction channels for OCs during the early and late differentiation stages, respectively, suggesting that these calcium channel could serve as markers of osteoclastogenesis or bone resorption.

1. Introduction

Osteoclasts (OCs) originate from hematopoietic stem cells, and their differentiation is initiated by the formation of monocyte precursors in response to macrophage-colony stimulating factor (M-CSF), which subsequently fuse into multinucleated OCs in response to M-CSF and receptor activator of nuclear factor-κB ligand (RANKL) [1]. OCs are unique cells with the ability to resorb bone, and their dysfunction can lead to osteoporosis or osteosclerosis. It has long been known that the architecture of bone is continuously adapting to the mechanical load it bears during daily activities [2]. The cavities within bone are fully filled with interstatial fluid, which may be driven to flow on OCs locating on the bone surface and further to produce fluid shear stress (FSS) on OCs. The process of bone remodeling is mainly composed of osteoblast-dominated bone formation and OC-dependent bone resorption. Despite several studies regarding the mechanotransduction pathways of osteoblasts, little is known about these pathways in OCs [3].

Binding of RANKL to its receptor, RANK, on OC membranes induces dynamic fluctuation of the intracellular calcium concentration ($[Ca^{2+}]_i$), called calcium oscillation [4]. Calcium oscillation activates downstream regulators of osteoclastogenesis, such as calcineurin, nuclear factor of activated T cells (NFATc1), NF-κB, c-Fos, and β-catenin [5]. Inhibition of RANKL-induced calcium oscillation reduces OC precursor fusion and resorption ability. Similar to excitable cells, Ca^{2+}-release-activated Ca^{2+} (CRAC) channels or store-operated Ca^{2+} entry (SOCE) activity are also major means of Ca^{2+} influx in non-excitable hematopoietic cells [6,7] and OCs [8]. The increase in $[Ca^{2+}]_i$ mainly derives from Ca^{2+} release from intracellular stores such as the endoplasmic reticulum (ER) through inositol trisphosphate receptors on the ER membrane, and from extracellular calcium influx. The type 1A transmembrane protein stromal interacting molecule 1 (STIM1) on the ER membrane [9] and the plasma membrane Ca^{2+} channel ORAI CRAC modulator 1 (ORAI1) [10] have been identified as the molecular mediators of SOCE activity. Some studies have shown that SOCE through the STIM1-ORAI1 pathway plays important roles in OC formation and function [11], and blocking the SOCE in OCs with 3,4-

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Table 1
Mouse primer sequences for quantitative real-time −PCR.

Gene name	Gene ID	Full gene name	Forward	Reverse	NCBI reference sequence
Orai1	109305	calcium release-activated calcium modulator 1	CATAAGACGGACCGGCAGTT	GCCCGGTGTTAGAGAATGGT	NP_780632.1
GAPDH	14433	glyceraldehyde-3-phosphate dehydrogenase	CCGGGAAACTGTGGCGTGATGG	AGGTGGAGGAGTGGGTGTCGCTGTT	NP_001276655.1
STIM1	20866	Stim1 stromal interaction molecule 1 [Mus musculus (house mouse)]	GAATTGACAAGCCCCTGTGT	ATGACTTCCATGCCTTCCAC	NM_009287.4
TRPV1	193034	transient receptor potential cation channel, subfamily V,member 1	GACTTCAAGGCTGTCTTCATCATCC	CATGCACTTCAGGAAACT	NM_001001445.2
TRPV4	63873	transient receptor potential cation channel, subfamily V, member 4	TCTTCACCCTCACCGCCTACT	TCCACTGTGGTCCGGTAAG	NM_022017.3
TRPV5	194352	transient receptor potential cation channel, subfamily V, member 5	CGTTGGTTCTTACGGGTTGAAC	GTTTGGAGAACCACAGAGCCTCTA	NM_001007572.2
TRPV6	64177	transient receptor potential cation channel, subfamily V, member 6	ATCCGCCGCTATGCACA	AGTTTTTCTCCTGAATCTTTTTCCA	NM_022413.4

dichloropropioanilide can treat arthritis-induced bone erosion [12].

Other important Ca^{2+}-influx channels in OCs include transient receptor potential (TRP) family members. TRP channels are expressed in almost every cell and tissue type, and are involved in diverse homeostatic functions. It has been demonstrated that RANKL can activate the expression of TRPV2 [13], TRPV4 [14], and TRPV5 [15], which promotes extracellular Ca^{2+} influx in OCs. Among these channels, TRPV4 mediates Ca^{2+} influx during the late stage of OCs differentiation [16] and Ca^{2+} influx through TRPV5 is involved in the process of estrogen-inhibited osteoclastogenesis and bone resorption [17]. In addition, in vitro findings suggest that the TRPV2 channel [13] is likely one of the Ca^{2+} entry pathways that contributes to $[Ca^{2+}]_i$ oscillations [13,18].

Our previous studies have demonstrated that fluid shear stress (FSS) could induce $[Ca^{2+}]_i$ oscillation in OCs, which had significantly different profiles at the early and late differentiation stages, i.e., stronger $[Ca^{2+}]_i$ fluctuations in monocytes or small OCs with 2–5 nuclei after 4 days of induction than in large OCs after 8 days of induction [19]. In addition, mechanosensitive cation-selective channels (MSCCs), phospholipase C (PLC), and the ER constitute the major signaling pathway for mechanical stimulation-induced calcium response in OCs [20]. It was concluded that extracellular Ca^{2+} influx leads to cytosolic Ca^{2+} oscillation because FSS could induce only one Ca^{2+} responsive peak when the extracellular Ca^{2+} was removed. In the present study, we further investigated the specific mechanosensitive ion channels mediating FSS −induced $[Ca^{2+}]_i$ oscillation. The results demonstrate that SOCE through the STIM1 pathway produced $[Ca^{2+}]_i$ oscillation at the early differentiation stage of OCs, while TRPV4 regulated the Ca^{2+} response at the late stage.

2. Materials and methods

2.1. Cell culture

RAW264.7 macrophage cells were purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, high glucose) containing 10% fetal bovine serum, 100 units/mL of penicillin, and 100 units/mL of streptomycin. The cells were cultured with 30 ng/mL M-CSF and 50 ng/mL RANKL for 4 or 8 days for differentiation into OCs. To identify OC formation, the cells were stained with tartrate-resistant acid phosphatase (TRAP) and Hoechst.

Briefly, for TRAP staining, the cells were fixed with citrate-acetone solution for 30 s at room temperature (26–28 °C) and then immersed in a reaction solution containing fast garnet GBC, naphthol AS-BI phosphate, acetate, and tartrate, and incubated for 1 h at 37 °C. Subsequently, the cells were counterstained for 2 min with hematoxylin and observed under a microscope. OCs with active acid phosphatase displayed purple to dark red cytoplasmic granules.

For Hoechst staining, the cells were first fixed with 3.7% formaldehyde for 5 min, rinsed three times with phosphate-buffered saline (PBS), and then stained with 0.2 mM Hoechst for 10 min at room temperature. The number of nuclei was counted in each OC in the fluorescent images.

2.2. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

To quantify the mRNA expression of STIM1, ORAI1, TRPV1, TRPV4, TRPV5, TRPV6, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), total RNA was extracted from RAW264.7 cells after 4 or 8 days of induction using TRIzol reagent (Takara). PCR primers were purchased from AuGCT (Beijing, China). The isolated total RNA was reverse transcribed and then used in two-step quantitative RT-PCR according to the manufacturer's instructions. Briefly, total RNA aliquots (1 μg) were reverse transcribed at 25 °C for 10 min, 42 °C for 60 min, and 70 °C for 15 min using a Reverse Transcription System (Promega, USA). Reaction mixture aliquots (cDNA, 1 μL) were used as templates for RT-PCR using qPCR Master Mix (Promega, USA). PCR cycling conditions were 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s, then the final melting curve program. Relative differences in the amounts of various messenger RNAs were calculated using the comparative cycle threshold method. The primer sets for STIM1, ORAI1, TRPV1, TRPV4, TRPV5, TRPV6, and GAPDH are listed in Table 1.

2.3. RNA interference

Short hairpin RNA constructs against STIM1, and TRPV4 were generated using the psiHIV-mH1 vector, which was purchased from GeneCopoeia. The target small interfering RNA (siRNA) sequence for STIM1 was: 5′- GGUGUCUAUCGUU-3′ (sense) and 5′-AACGAUAGACA CCAC-3′ (anti-sense). The siRNA target sequence for TRPV4 was: 5′-CCGUGUCCUUCUACAUCAATT-3′ (sense), and 5′-UUGAUGUAGAA GGACACGGTT-3′ (anti-sense). The sequence of small interference RNA (siRNA) specifically targeting mRNA of TRPV6 is 5′-AACTTGAG CAG CTTGCTCAGAGCCT-3′.

Cells were transfected with 50 nM of siRNA against STIM1, TRPV4, TRPV6 or corresponding scrambled negative controls siRNA (Abnova, USA) using Lipofectamine 3000 (Invitrogen, USA) for 48 h according to the manufacturer's instructions.

2.4. Measurement of FSS-induced $[Ca^{2+}]_i$

The FSS-induced cytosolic Ca^{2+} response in OCs was measured and analyzed as described in our previous studies [19,20]. Briefly, after 4 or 8 days of differentiation, the cytosolic Ca^{2+} in RAW264.7 cells was stained with 5 μM Fluo-4 AM in DMSO and 0.02% Pluronic F-127 for 2 h in culture medium at room temperature (26–28 °C). The cells were