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Pulsed electromagnetic field inhibits RANKL-dependent osteoclastic differentiation in RAW264.7 cells through the Ca^{2+}-calcineurin-NFATc1 signaling pathway

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ABSTRACT

Pulsed electromagnetic field (PEMF) has been reported to improve bone healing in osteoporosis patients. However, the precise mechanism has remained largely unknown. This study aimed to investigate the effects of PEMF on nuclear factor κB ligand (RANKL)-dependent osteoclastic differentiation and the Ca^{2+}-calcineurin-NFATc1 signaling pathway in RAW264.7 cells in vitro. Treating RAW264.7 cells with RANKL for 4 days induced osteoclastic differentiation in vitro, and the formation of multinucleated osteoclasts, bone resorption-pit formation, tartrate-resistant acid phosphatase (TRAP) activity and the protein levels of cathepsin K, TRAP, Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) and matrix metalloproteinase 9 (MMP-9) were significantly decreased. The mRNA levels of specific genes related to osteoclastogenesis (*TRAP, NFATc1, CTSK* and *MMP-9*) were also reduced. Moreover, the oscillations of intracellular Ca^{2+} in RANKL-dependent RAW264.7 cells were suppressed by PEMF, as well as by inhibitors of membrane and store-operated Ca^{2+} channels. Meanwhile, calcineurin activity was increased, although its protein level was not changed. PEMF increased phospho-NFATc1 in the cytosol while suppressing the nuclear translocation of NFATc1, thus inhibiting osteoclastic differentiation by suppressing the Ca^{2+}-calcineurin-NFATc1 signaling pathway. Although many questions remain unresolved, to our knowledge, this is the first report demonstrating that PEMF is beneficial against RANKL-dependent osteoclastic differentiation in RAW264.7 cells in vitro via inhibiting the Ca^{2+}-calcineurin-NFATc1 signaling pathway.

1. Introduction

Osteoporosis is a severe, chronic skeletal disease characterized by decreased bone mass and impaired microarchitecture [1]. The potential mechanism of osteoporosis may be excess osteoclastic activity, leading to an imbalance of bone remodeling processes mediated by osteoclasts and osteoblasts [2]. The effect of pharmacological intervention is barely satisfactory and accompanied by undesirable side effects [3]. Abundant evidence has substantiated that pulsed electromagnetic field (PEMF) can increase bone mineral density (BMD) in patients [4] and prevent bone loss in ovariectomized (OVX) rats [5]. In spite of the beneficial biological effects of PEMF, the precise mechanisms have not been fully understood.

Osteoclasts are derived from monocyte/macrophage cell lineage [6]. In osteoclast differentiation, receptor activator of nuclear factor κB (RANK), RANK ligand (RANKL) and osteoprotegrin (OPG) have been considered the vital mediators [7]. Activation of signaling pathways in the RANK/RANKL/OPG system leads to complex responses that ultimately generate mature multinucleated osteoclasts. Several publications have noted that PEMF with different frequencies and intensities could suppress osteoclastic differentiation via modulating pathways in this signaling system [8,9]; however, further studies addressing the mechanisms are still needed.

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Papers in recent years have claimed that the Ca^{2+}-calcineurin-NFATc1 signaling pathway is one of the major mechanisms in this signaling system [10]. Evidence has shown that activation of Ca^{2+}-calcineurin-NFATc1 signaling pathway can accelerate osteoclastic differentiation through mechanisms including increasing intracellular Ca^{2+} oscillations and translocating osteoclastogenic transcription factors [11–14]. Nevertheless, few studies have investigated the results of PEMF on the Ca^{2+}-calcineurin-NFATc1 signaling pathway. To our knowledge, this is the first report to determine the contribution of PEMF to the Ca^{2+}-calcineurin-NFATc1 signaling pathway in vitro. We selected experimental parameters of PEMF based on our previous study [15]. Here, we focused on PEMF may regulate osteoclastic differentiation by modulating the Ca^{2+}-calcineurin-NFATc1 signaling pathway.

2. Materials and methods

2.1. Osteoclastogenesis

For osteoclastic differentiation experiments, RAW264.7 cells were cultured in alpha-minimum essential media (α-MEM, Thermo Scientific, USA) containing 10% FBS and 1% penicillin/streptomycin solution in the presence of 50 ng/ml of RANKL (Peprotech, Rocky Hill, USA) for 4 days. The culture medium was refreshed every other day.

2.2. PEMF stimulation

RAW264.7 cells were exposed to a sinusoidal 50 Hz PEMF with a magnetic flux density of 1 mT [15]. The cells were divided into four groups: Control, RANKL (50 ng/ml), RANKL (50 ng/ml) + PEMF and PEMF. The electromagnetic exposure was performed in a humidified incubator at 37 °C under 5% CO_2 for 3 h per day for all the groups. Because the coils produced heat when generating PEMF, groups without PEMF stimulation were placed in another cell incubator containing the solenoid and properly energized so as not to generate a PEMF but eventually to produce the same heat.

2.3. Cell viability assay

The cell viability was quantified using a Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay according to the manufacturer's instructions. At the indicated time points, the floating RAW264.7 cells were removed, and 100 μl of fresh medium containing 1/10 (v/v) CCK-8 reagent was added to each well before incubation for 4 h at 37 °C. The number of viable cells in each well was measured at an absorbance wavelength of 450 nm.

2.4. The tartrate-resistant acid phosphatase (TRAP) staining and TRAP activity assay

TRAP staining (Sigma-Aldrich, USA) was performed to confirm the formation of mature osteoclasts which containing three of more nuclei. After induction, cells were fixed and stained for TRAP according to instructions. Then TRAP-positive multinucleated cells (≥3 nuclei/cell) were counted under a light microscope. TRAP activity was measured in terms of the absorbance at a wavelength of 540 nm using a microplate reader.

2.5. Resorption pit assay

The pit formation assay was conducted using covered bovine bone slices (Third Military Medical University, Chongqing, China) in a 48-well plate. At day 7 of the induction, cells were removed by 10% sodium hypochlorite solution for 5 min at room temperature, and then the bovine bone slices were stained with 1% toluidine blue for 30 min. The slices were then washed with pure water and observed under a light microscope and the resorbed pit areas were identified using Image J software.

2.6. Real-time quantitative reverse transcription-PCR

Total RNA was extracted using Trizol according to the manufacturer's instructions. The first-strand cDNA was synthesis from 1 μg of total RNA using All-in-one™ first-strand cDNA synthesis kits (GeneCopoeia, USA) and oligo dT primers. The primers for *NFATc1, MMP-9, CTSK, TRAP* and *GAPDH* were designed and purchased from Shanghai Biological Engineering.

qPCR was performed using an ABI 7500 Real-time PCR system. The comparative threshold cycle (Ct) method was used to calculate mRNA expression of genes specific for osteoclastic differentiation, and the Ct values of the calibrator and the samples of interest were verified by normalizing to *GAPDH*. The comparative Ct method is known as the $2^{-\Delta\Delta Ct}$ method and was relatively quantified by comparison with values of a calibrator, which was cDNA extracted from RAW264.7 cells without any PEMF exposure.

2.7. Western blots

RAW264.7 cells were prepared with various combinations of PEMF and other treatments, and cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer containing 10 mM Tris, pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% deoxycholic acid. The samples (50 μg protein/well) were resolved using SDS–PAGE (6 and 10% gels), and proteins were transferred to nitrocellulose membranes. The membrane was blocked in 5% Bovine Serum Albumin and incubated with antibodies against p-NFATc1 (1:1000, CST, USA), n-NFATc1 (1:1000, CST, USA), calcineurin (1:1000, CST, USA), CTSK (1:1000, Proteintech, USA), TRAP (1:1000, Proteintech, USA), MMP-9 (1:1000, Proteintech, USA), β-actin (1:1000) and Lamin A/C (1:4000) overnight at 4 °C. This procedure was followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:4000) for 1 h. Chemiluminescence was detected using an ECL system. Blots against β-actin and Lamin A/C served as loading controls.

2.8. Calcineurin enzymatic assay

Calcineurin activity was measured from RAW264.7 cell extracts using a colorimetric Calcineurin Cellular Activity Assay Kit (Enzo Life Sciences, USA). Calcineurin activity was determined as the difference between total phosphatase activities minus the phosphatase activity in the presence of 10 mM EGTA that blocks calcineurin activity. Data were determined at 620 nm using a plate reader.

2.9. Measurement of intracellular Ca^{2+}

RAW264.7 cells cultured in confocal dishes and activated for 24 h with RANKL were first loaded with 10 μM Fluo 4-AM in physiological salt solution (final concentration of DMSO, 0.1%) at room temperature for 30 min protected from light, then washed twice in Hanks' balanced salt solution (HBSS) and incubated with nifedipine or SKF-96365 for 30 min at room temperature. The PEMF-treated cells were kept within a water bath apparatus connected to a confocal laser-scanning microscope as described previously [15]. Fluorescence was measured every 0.6 s for 2 min. Image analysis was performed using Zeiss LCS software, and fluorescence of every cell in each field was measured [16].

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