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Inhibitory activity of linarin on osteoclastogenesis through receptor activator of nuclear factor κB ligand-induced NF-κB pathway

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ABSTRACT

Linarin, a natural flavonoid glycoside widely found in plants, has been reported to possess anti-inflammation, neuroprotection and osteogenic properties. However, its impact on osteoclast remains unclear. In the present study, the effects of linarin on osteoclastogenesis and its underlying molecular mechanisms of action were investigated. Using the culture systems of osteoclasts derived from bone marrow macrophages (BMMs), we found that linarin dose-dependently inhibited osteoclasts formation and bone resorptive activity. The Cell Counting Kit-8 test displayed that the viability of cells was not influenced by linarin at doses up to 10 μg/mL. In addition, linarin downregulated osteoclast-related genes expression, including nuclear factor of activated T cells cytoplasmic 1 (NFATc1), tartrate resistant acid phosphatase (TRAP), osteoclast-associated receptor (OSCAR) and c-Fos, as shown by quantitative real time polymerase chain reaction (RT-qPCR). Western blot analysis further showed that linarin inhibited receptor activator of nuclear factor κB ligand (RANKL)-induced nuclear factor kappa B (NF-κB) p65 and NFATc1 activity. The present findings show that linarin exerted a potent inhibitory effect on osteoclastogenesis through RANKL-induced NF-κB signaling pathway. In conclusion, the results suggest that linarin has anti-osteoclastic effects and may serve as potential modulatory agents for the prevention and treatment of bone loss-associated diseases.

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1. Introduction

Osteoclasts, the multinuclear giant cells derived from the monocyte-macrophage lineage of hematopoietic stem cells, have been shown to play a crucial physiological function in continuous and dynamic bone remodeling [1]. Enhanced formation and/or excessive activation of osteoclasts can lead to osteolytic lesions, such as osteoporosis, Paget's disease, rheumatoid arthritis, multiple myeloma, metastatic bone tumours and aseptic loosening of prostheses [2–4]. Therefore, Osteoclasts become the main targets for the treatment of such diseases due to excessive bone resorption [5]. Currently, there are various drugs available to inhibit osteoclast function in clinical [6]. However, long-term use of these drugs may have severe side effects. For instance, the prolonged use of bisphosphonate can cause oesophageal cancer [7], osteonecrosis of the jaw [8] and atypical bone fracture [9], while estrogen can increase the risk of breast cancer and heart disease [10]. Therefore, searching for natural compounds or other synthetic substances with preventing bone loss and fewer undesirable side effects is an alternative strategy for the treatment of osteolytic diseases.

Linarin, a natural flavonoid glycoside widely found in plants such as *Flos chrysanthemi indici, Buddleja officinalis* and *Mentha arvensis*, has been shown to possess various pharmacological effects, including analgesic, antipyretic [11], anti-inflammatory [12], neuroprotective [13], cardioprotective [14] and antioxidative effects [15]. Linarin has also been reported to exert osteogenic properties by promoting osteoblastic differentiation or/and protecting osteoblast dysfunction [16,17]. However, the effect of linarin on osteoclasts remains unclear. In the present study, we have performed a series of experiments *in vitro* investigating the effect of linarin on osteoclastogenesis and bone resorption and the involvement of the RANKL-induced NF-κB signaling pathway. Our results suggest that this compound may be a new therapeutic option for treatment of osteolytic bone diseases.

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2. Materials and methods

2.1. Media and reagents

Linarin (Purity>98%) was obtained from Shanghai Yuanye Bio-Technology Co. Ltd (Shanghai, China), and dissolved in Dimethyl sulfoxide (DMSO). Alpha Modified Minimal Essential Medium (α-MEM), Dulbecco's modified eagle's media (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, were purchased from TRACE (Sydney, Australia). Receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) were purchased from R&D Systems (Minneapolis, MN, USA). The Cell Counting Kit-8 (CCK-8) was bought from Dojindo Molecular Technologies (Tokyo, Japan). Antibodies against NFATc1, p65, p-p65and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). Tartrate resistant acid phosphatase (TRAP) enzymatic activity was detected using the Leukocyte acid phosphatase staining kit (Sigma, St. Louis, MO, USA).

2.2. Cell culture

Bone marrow macrophages (BMMs) were isolated from the long bone of 8-week-old C57BL/6 mice. The animal protocols and procedures in this study were approved by the Medical Ethics Committee of Soochow University. RAW264.7 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). RAW264.7 cells and BMMs were cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin-streptomycin (complete α-MEM) at 37 °C in a humidified atmosphere with 5% carbon dioxide. To differentiate macrophages into osteoclast precursors, primary BMMs were grown in complete α-MEM with the addition of macrophage-colony stimulating factor (M-CSF, 10 ng/mL) for 3 days.

2.3. Osteoclast formation and differentiation inhibition assay

BMMs were seeded at a density of 1×10^5 cells/well in 24-well plate and cultured in the presence of complete α-MEM medium containing M-CSF(30 ng/mL), RANKL (50 ng/mL) and different concentrations of linarin (0, 0.1, 1, and 10 μg/mL). The medium was changed every two days. After 5 days, the cultured cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Fixed cells were then dyed with TRAP staining kit according to the manufacturer's instructions. Cells that stain positive for TRAP contain red granular substances in their cytoplasm. TRAP-positive cells containing 3 or more nuclei, which were identified as osteoclasts, were imaged and counted using an inverted microscope (Nikon Corporation, Tokyo, Japan).

2.4. Cytotoxicity assay

BMMs were seeded at a density of 2×10^4 cells/well in 96-well plate overnight, followed by incubation with complete α-MEM medium, M-CSF (30 ng/mL) and different concentration of linarin (0, 0.1, 1, 10, 15, 20, and 25 μg/mL). After 48 h incubation, 10 μl CCK-8 reagent was added to each well and cultured in darkness at 37 °C for 2 h. The value of optical density was then measured at 450 nm using a microplate reader (Biotek, Winooski, VT, USA). All tests were performed in triplicate.

2.5. Osteo assay

2×10^5 cells/well BMMs were plated into 24-well Osteo Assay Plates (Corning, NY, USA) and treated with complete α-MEM medium, M-CSF (30 ng/mL), RANKL (50 ng/mL) and different concentration of linarin (0, 0.1, 1, and 10 μg/mL) at 37 °C with 5% carbon dioxide. After 6 days, the cells were removed completely by cell dissociation buffer. The area of the bone resorption region per well was then observed under an optical microscope.

2.6. RNA extraction and real time-quantitative polymerase chain reaction (RT-qPCR)

BMMs were seeded at a density of 1×10^5 cells/well in 24-well plate and cultured in the presence of complete α-MEM medium containing M-CSF(30 ng/mL), RANKL (50 ng/mL) and different concentrations of linarin (0, 0.1, 1, and 10 μg/mL) for 5 days. Total RNA was isolated from the cells using Trizol regent (Sigma, St. Louis, MO, USA). cDNA was synthesized from 1 μg of total RNA using reverse transcriptase with oligo-dT primer (Promega, Mandison, WI, USA). qPCR was performed on a ViiA™ 7 Real-time PCR machine (Applied Biosystems, Paisley, UK) using SYBR Premix Ex Taq kits (Takara Bio, Inc.). The comparative $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of each target gene. GAPDH was used as the housekeeping gene and all reactions were run in triplicate. The target primer sequences are listed in Table 1.

2.7. Western blot assays

RAW264.7 cells (2×10^6 cells/well) were seeded in 6-well plates. After the cells fusion, some wells were pretreated with different concentration of linarin (0, 0.1, 1 and 10 μg/mL) for 4 h and then stimulated with RANKL (50 ng/mL) for 15 min, some wells were only incubated with 10 μg/mL linarin for 4 h and then added with RANKL (50 ng/mL) for different time intervals (0, 5, 15, 30 and 60 min). Subsequently, the cells were washed three times with cold phosphate-buffered saline (PBS) and lysed using radioimmunoprecipitation assay buffer for 30 min at 4 °C. The whole cell protein was obtained by centrifugation to pellet the cell debris. Protein samples were separated using sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis and transferred onto nitrocellulose membranes; these were then blocked with 5% skim milk for 1 h and incubated at 4 °C overnight with 1:1000 dilutions of target primary antibodies. After three washes, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Protein bands were then detected with an enhanced chemiluminescence (ECL) system (Sigma, St. Louis, MO, USA). β-actin was used as the internal control. The experiments were replicated three times.

Table 1
Specific primers used for RT-qPCR.

Gene	Forward	Reverse
GAPDH	5′-ACCCAGAAGACTGTGGATGG-3′	5′- CACATTGGGGGTAGGAACAC-3′
NFATc1	5′- CCGTTGCTTCCAGAAAATAACA-3′	5′- TGTGGGATGTGAACTCGGAA-3′
TRAP	5′-CTGGAGTGCACGATGCCAGCGACA-3′	5′- TCCGTGCTCGGCGATGGACCAGA-3′
c-Fos	5′- CCAGTCAAGAGCATCAGCAA-3′	5′-AAGTAGTGCAGCCCGGAGTA-3′
Oscar	5′-CTGCTGGTAACGGATCAGCTCCCCAGA-3′	5′-CCAAGGAGCCAGAACCTTCGAAACT-3′

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