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Gentiopicroside inhibits RANKL-induced osteoclastogenesis by regulating NF-κB and JNK signaling pathways

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ABSTRACT

Gentiopicroside, a main active component from the traditional Chinese herb medicine *Gentiana manshurica Kitag*, has been shown to possess anti-arthritis effect. However, the molecular mechanism of gentiopicroside on the osteoclast formation remains unclear. The present study was designed to investigate the effects and mechanisms of gentiopicroside on receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis. The results showed that pre-treatment with gentiopicroside significantly inhibited RANKL-induced osteoclast formation from mouse bone marrow macrophages (BMMs). In addition, we observed that gentiopicroside efficiently suppressed osteoclastogenesis-related marker genes expression in RANKL-stimulated BMMs. Mechanistically, gentiopicroside suppressed RANKL-induced the activation of JNK and NF-κB signaling pathways in BMMs. Taken together, the present study demonstrated that gentiopicroside inhibits RANKL-induced osteoclastogenesis through the inactivation of JNK and NF-κB signaling pathways. Thus, gentiopicroside may be a promising agent for the treatment of osteoporosis.

1. Introduction

Osteoporosis is the most common bone remodeling disease that affects about millions of people in the world. It results from the imbalance of bone resorption and formation [1]. Although current drugs used to treat osteoporosis include bisphosphonates, calcitonin and estrogen, the effect of these drugs is limited [2]. Thus, there is a requirement of safe and efficacious compounds that can inhibit bone loss.

Osteoclasts, bone-specialized multinucleated cells, which are derived from monocyte-macrophage lineage cells, play a pivotal role in osteoporosis [3]. Receptor activator of nuclear factor-κB ligand (RANKL) is a member of the tumor necrosis factor superfamily and induces differentiation of osteoclast precursors into osteoclasts [4]. RANKL binding to RANK could activate different transcription factors such as NF-κB, AP-1 and nuclear factor of activated T-cells (NFATc1), which are necessary for the regulation of osteoclast differentiation-related genes, consequently resulting in osteoclast formation and bone resorption [5,6]. Thus, targeting RANKL signaling pathway has been a promising strategy for the treatment of osteoporosis.

Gentiopicroside, a main active component from the traditional Chinese herb medicine *Gentiana manshurica Kitag*, has been shown to possess diverse biological properties, including antinociceptive, anti-inflammatory and hepato-protective activities [7–9]. It was reported that gentiopicroside significantly suppressed IL-1β-induced inflammation response in rat articular chondrocyte [10]. However, the molecular mechanism of gentiopicroside on the osteoclast formation remains unclear. The present study was designed to investigate the effects and mechanisms of gentiopicroside on RANKL-induced osteoclastogenesis. Our results indicated that gentiopicroside inhibits RANKL-induced osteoclastogenesis through the inactivation of JNK and NF-κB signaling pathways.

2. Materials and methods

2.1. Animals, reagents and antibodies

C57BL/6 mice (5-week-old) were purchased from the School of Basic Medicine, Nanjing Medical University (Nanjing, China). All

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reagent-grade chemicals, gentiopicroside (purity ≥ 98%), macrophage colony-stimulating factor (M-CSF) and RANKL were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), the BCA Protein Assay Kit and the commercial kit for the detection of osteoclast formation were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). The Cell Counting Kit-8 (CCK-8) was purchased froom Dojindo (Kumamoto, Japan). Penicillin and streptomycin were purchased from Gibco (Rockville, MD, USA). Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore Life Sciences (Billerica, MA, USA). Antibodies against NFATc1, cathepsin K, c-Src, p-JNK, JNK, p-p65, p65, IκBα, GAPDH, and horseradish peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were from Sigma-Aldrich.

2.2. Cell culture

The protocol and procedure of the experiment were approved by the Institutional Animal Care and Use Committee of The Third Clinical Medical College, Nanjing Medical University (China). Mouse bone marrow cells were obtained from femurs and tibias of 5-week-old C57BL/6 mice and cultured in DMEM containing 10% FBS, 100 units/ml penicillin-streptomycin at 37 °C with 5% CO_2 in a humidified incubator. Non-adherent bone marrow cells were transferred to 10-cm Petri dishes and cultured in the presence of M-CSF (30 ng/ml) for 3 days. Adherent cells were used as bone marrow macrophages (BMMs), as osteoclast precursors.

2.3. Osteoclast differentiation assay

The stock solution was prepared by dissolving 1.0 mg of gentiopicroside into 1.0 mL of 100% methanol to a final concentration of 1.0 mg/ml, and stored at −20 °C before use. Osteoclast formation was detected using a TRAP staining kit (Invitrogen) according to the manufacturer's instructions. Briefly, BMMs at a density of 1×10^4 cells/well were placed in a 96-well plate and then treated with recombinant-soluble RANKL (100 ng/ml) and M-CSF (30 ng/ml) in the absence or presence of various concentrations of gentiopicroside (12.5, 25 and 50 μM) at 37 °C. After 4 days, the cells were washed with PBS and fixed with 10% formaldehyde for 5 min, and then stained with naphthol AS-Mx phosphate and tartrate solution for 1 h at 37 °C. TRAP-positive multinucleated cells (≥3 nuclei) were counted using a light microscopy. The controls were exposed to the vehicle.

2.4. Bone resorption assay

BMMs at a density of 1×10^4 cells/well were plated onto BioCoat Osteologic™ slides and incubated 50 μM gentiopicroside in the presence of RANKL (100 ng/ml) for 5 days. The slides were washed with 6% sodium hypochlorite solution to remove the cells, and the resorption pits were photographed and analyzed using Image-Pro Plus version 4.0 (Media Cybernetics, Silver Spring, MD, USA).

2.5. Cell viability assay

Cell viability was evaluated using the CCK-8 assay. In brief, BMMs (1×10^4 cells/well) were placed in a 96-well plate and cultured with various concentrations of gentiopicroside (12.5, 25 and 50 μM) for 48 h in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml). Then, 10 μL of the CCK-8 solution was added to each well and the mixture was incubated for 4 h at 37 °C. The absorbance was evaluated at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.6. Western blot

After treatment, BMMs were harvested after washing with ice-cold PBS and then lysed with RIPA lysis buffer (200 mM Tris–HCl (pH 7.5), 1.5 M NaCl, 10 mM EDTA, 25 mM sodium pyrophosphate, 10 mM glycerolphosphate, 10 mM sodium orythovanadate, 50 mM NaF, 1 mM PMSF, in combination with protein inhibitor cocktail). The cell lysates were incubated in an ice box for 30 min and then centrifuged at 8000 rpm for 10 min, and the protein concentration was quantified by the BCA Kit. 25 μg of protein from each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. Non-specific binding was blocked with 5% non-fat milk at room temperature for 1 h and then incubated with primaries antibodies against NFATc1 (1:2,000), cathepsin K (1:2,500), c-Src (1:2,000), p-JNK (1:1,500), JNK (1:1,500), p-p65 (1:2,500), p65 (1:3,000), IκBα (1:2,500) and GAPDH (1:3,000) at 4 °C overnight. After washing with PBS containing 0.5% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:3,000) for 1 h at room temperature. Immunocomplexes were visualized using the enhanced ECL detection system (Pierce, Rockford, IL, USA).

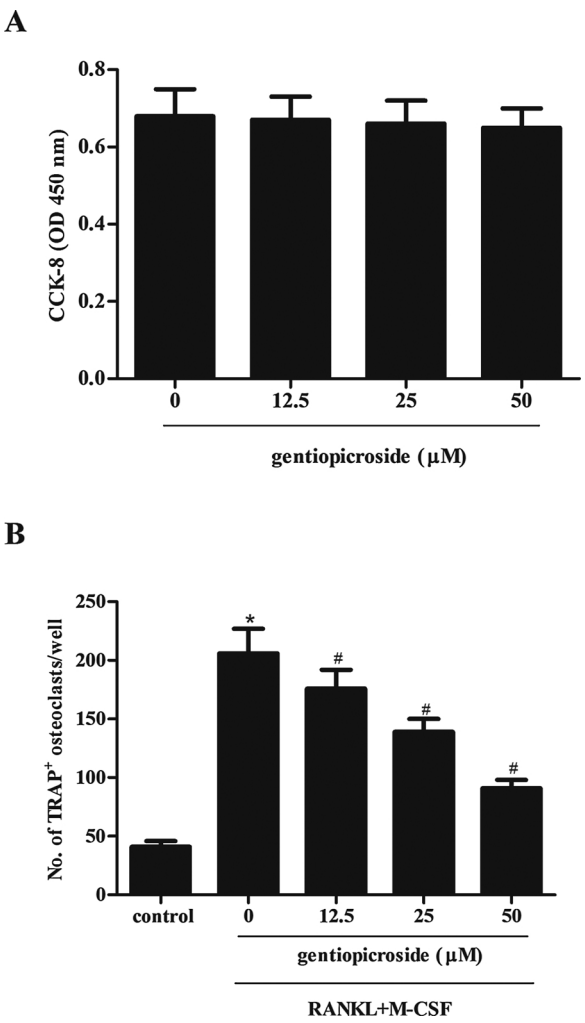

Fig. 1. Gentiopicroside dose-dependently inhibits RANKL-induced osteoclast formation from mouse BMMs. A, BMMs were cultured with different concentrations of gentiopicroside (12.5, 25 and 50 μM). Cell viability was evaluated using the CCK-8 assay. B, BMMs were cultured with RANKL (100 ng/ml) and M-CSF (30 ng/ml) in the absence or presence of various concentrations of gentiopicroside (12.5, 25 and 50 μM) for 4 days. Osteoclast formation was measured using a TRAP staining kit. All values are expressed as means ± SD (n = 3). *$P < .05$ versus untreated control, #$P < .05$ versus RANKL + M-CSF group.

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