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Oleanolic acid acetate inhibits osteoclast differentiation by downregulating PLCγ2–Ca^{2+}-NFATc1 signaling, and suppresses bone loss in mice

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ABSTRACT

Owing to their potential pharmacological activities in human disease, natural plant-derived compounds have recently become the focus of increased research interest. In this study, we first isolated oleanolic acid acetate (OAA), a triterpenoid compound, from *Vigna angularis* (azuki bean) to discover anti-bone resorptive agents. Many studies have identified and described the various medicinal effects of *V. angularis* extract. However, the pharmacological effect of OAA-derived *V. angularis* extract, particularly the effect on osteoclastogenesis, is not known. Therefore, we investigated the effect and mechanism of OAA in receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis. OAA inhibited RANKL-induced osteoclast differentiation in bone marrow macrophages (BMMs) without any evidence of cytotoxicity. Interestingly, OAA significantly inhibited Btk phosphorylation, phospholipase Cγ2 (PLCγ2) phosphorylation, calcium ion (Ca^{2+}) oscillation, and nuclear factor of activated T cell c1 (NFATc1) expression in RANKL-stimulated BMMs, but did not affect RANKL-induced mitogen-activated protein kinase. OAA also inhibited the bone-resorbing activity of mature osteoclasts. Furthermore, mice treated with OAA demonstrated marked attenuation of lipopolysaccharide-induced bone erosion based on micro-computed tomography and histologic analysis of femurs. Taken together, the results suggested that OAA inhibited RANKL-mediated osteoclastogenesis *via* PLCγ2-Ca^{2+}-NFATc1 signaling *in vitro* and suppressed inflammatory bone loss *in vivo*.

Introduction

Bone diseases, caused by bone loss and impaired bone quality, are a major health problem in the aging population and are associated with high morbidity and mortality. Bone homeostasis is dynamically regulated through the coordinated action of osteoclast-mediated bone resorption and osteoblast-induced bone formation [1]. Enhanced bone resorption by osteoclasts, which is not fully compensated by bone formation, is a critical mechanism in pathological bone diseases such as osteoporosis, rheumatoid arthritis, and periodontal disease [2,3].

Osteoclasts, which are multinucleated giant cells, originate from hematopoietic mononuclear precursors of the monocyte/macrophage lineage under the control of two main cytokines: receptor activator of nuclear factor-κB (NFκB) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [4,5]. The M-CSF receptor transmits survival signals to osteoclasts, and RANKL mediates osteoclastogenesis through binding to its receptor RANK on osteoclast precursors [4,5]. It initiates the recruitment of tumor necrosis factor receptor-associated factor 6 (TRAF6) and induces mitogen-activated protein kinases (MAPKs), including p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinases 1/2 (ERK 1/2) [5,6]. RANKL–RANK signaling also activates calcium ion (Ca^{2+}) signaling through the activation of phospholipase Cγ (PLCγ) [7,8]. PLCγ regulates protein kinase C (PKC) activation, intracellular Ca^{2+} levels, and nuclear factor of activated T cell c1

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(NFATc1) expression in hematopoietic systems in response to the stimulation of immune receptors [7,9,10]. PLCγ cleaves membrane phospholipid phosphatidylinositol-4,5-biphosphate (PIP2) into the second messenger molecules inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 directly increases intracellular Ca^{2+} levels by inducing the release of endoplasmic reticulum calcium stores, while DAG activates PKC at the plasma membrane [7]. During osteoclastogenesis, the increased Ca^{2+} level induces NFATc1 dephosphorylation and NFATc1 translocation into the nucleus. NFATc1 induces the expression of target genes by binding to NFAT-binding sites in the promoter region of genes such as tartrate-resistant acid phosphatase (*TRAP*) and osteoclast-associated receptor (*OSCAR*), which are important for osteoclast differentiation or function [6,11,12]. Recently, we reported that calcium signaling is important in metabolic bone diseases through the study of vesicle-associated membrane protein B (VapB), which regulates osteoclastogenesis *via* PLCγ2–Ca^{2+}-NFAT signaling [13].

Many compounds derived from natural products have pharmacological applications and have therapeutic value for treating or preventing several bone diseases characterized by excessive bone resorption [14–17]. To discover new compounds that can act as anti-resorptive agents, we screened 50 natural compounds using analysis of TRAP staining in the condition of RANKL-induced osteoclast differentiation, and found that oleanolic acid acetate (OAA) has inhibitory effects on osteoclast differentiation. OAA is a triterpenoid compound isolated from *Vigna angularis* (azuki bean). Many studies have identified and described the various medicinal effects of *V. angularis* extract, such as its tumor-suppressive [18], renal-protective [19,20], antidiabetic [20–22], and anti-inflammatory effects [23]. However, the pharmacological effect of OAA derived from *V. angularis* remains unknown; in particular, the effect of OAA on osteoclast differentiation and pathological bone destruction has not yet been well defined.

Therefore, we investigated the effects of OAA on osteoclast differentiation and function, the RANKL-induced signaling pathway, and bone destruction in a mice model of induced bone erosion.

Materials and methods

Preparation of V. angularis extract and purification of OAA

V. angularis was obtained from a herbal medicine store (Jeonbuk, Korea). The plant material (10 kg) was dried, ground to a fine powder, and extracted with 95% ethanol at 70 °C. The extracts were filtered through a 0.45-mm filter and concentrated under reduced pressure to yield the ethanol extracts, which were further extracted with ethyl acetate. The organic layer was applied to a silica gel column (230–400 mesh, 1 kg, Merck, Dannstad, Germany) and eluted with a hexane–ethyl acetate solvent system (100:1, 80:1, 60:1, 40:1, 20:1, 10:1, 1:1; each 1 L, v/v) to yield 5 fractions (H1–H5) according to thin-layer chromatography profiles. Lastly, the OAA was obtained by the recrystallization of H-3 in methyl alcohol. The OAA was prepared as a 20 mM stock in dimethyl sulfoxide (DMSO) and stored at −20 °C. The OAA was added to cell culture medium such that the DMSO comprised <0.1% of the volume of the culture medium.

Mice and reagents

Male, 5-week-old ICR mice were purchased from Damul Science (Daejeon, Korea). The mice were kept in controlled temperature (22–24 °C) and humidity (55–60%) with 12 h light/dark cycles. All experiments in this study were performed in accordance with the animal experiment guidelines of the Institute Committee of Wonkwang University. Recombinant soluble human M-CSF and human RANKL were obtained from PeproTech EC Ltd. (London, UK). Anti-JNK, anti-phospho-JNK, anti-ERK 1/2, anti-phospho-ERK 1/2, anti-p38, and anti-phospho-p38 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-phospho-Btk, anti-Btk, anti-c-Fos and anti-NFATc1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal β-actin antibody was obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), α-minimum essential medium (α-MEM), and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). All other chemicals were of analytical grade or complied with the standards required for cell culture experiments.

Mouse bone marrow macrophage preparation and osteoclast differentiation

Bone marrow cells (BMCs) were obtained from 5-week-old male ICR mice by flushing the femurs and tibias with α-MEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). To obtain bone marrow macrophages (BMMs), the BMCs were cultured in α-MEM supplemented with 10% FBS and M-CSF (10 ng/mL) for 1 day on culture dishes. Non-adherent cells were transferred to 10 cm Petri dishes and further cultured in the presence of M-CSF (30 ng/mL) for 3 days. Adherent cells were used as BMMs, as osteoclast precursors, after the non-adherent cells were removed. To generate osteoclasts from the BMMs culture system, BMMs (3.5×10^4 cells/well) were cultured in complete medium containing M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 4 days in a 48-well plate with or without OAA. The cells were fixed in 3.7% formalin for 10 min, permeabilized with 0.1% Triton X-100, and then stained with TRAP (Sigma). TRAP-positive multinucleated cells with more than three nuclei were counted as osteoclasts.

Cell viability assay

BMMs were seeded in 96-well plates (1×10^4 cells/well) and cultured overnight, and then treated with M-CSF (30 ng/mL) and various concentrations of OAA. After 3 days, 50 μL XTT reagent (sodium 3′-[1-(phenyl-aminocarbonyl)-3,4-tetrazolum]-bis(4-methoxy-6-nitro), benzenesulfonic acid hydrate, and N-methyl dibenzopyrazine methyl sulfate) was added to each well, and then incubated for 4 h. The optical density of each well was then measured at 450 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Quantitative real-time RT-PCR

Total RNA was isolated with QIAzol reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Equal amounts of total RNA were reverse-transcribed to cDNA using SuperScript II Reverse Transcriptase (Invitrogen, San Diego, CA, USA). Real-time RT-PCR was conducted using a Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer Co., Daejeon, Korea) in a 20 μL reaction mixture containing 10 μL SYBR Green Premix (Bioneer Co.), 10 pmol forward primer, 10 pmol reverse primer, and 1 μg cDNA. The primers used to detect the genes of interest were *c-Fos*, forward 5′-GGTGAAGACCGTGT CAGGAG-3′ and reverse 5′-TATTCCGTTCCCTTCGGATT-3′; *NFATc1*, forward 5′-GAGTACACCTTCCAGCACCTT-3′ and reverse 5′-TATGATGT CGGGGAAAGAGA-3′; *OSCAR*, forward 5′-GGAATGGTCCTCATCTGCTT-3′ and reverse 5′-GGAATGGTCCTCATCTGCTT-3′; *TRAP*, forward 5′-TCATGGGTGGTGCTGCT-3′ and reverse 5′-GCCCACAGCCACAAATCT-3′; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), forward 5′-TCAAGAAGGTGGTGAAGCAG-3′ and reverse 5′-AGTGGGAGTTGC TGTTGAAGT-3′. The mouse *GAPDH* gene was used as the internal control. The amplification parameters consisted of initial denaturation at 95 °C for 5 min and 40 cycles of 3-step PCR (denaturation at 95 °C for 1 min, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min). The fluorescence resulting from the incorporation of SYBR Green 1 dye into the double-stranded DNA produced during the PCR and the emission data were quantified using the threshold cycle (C_t) value. The data were normalized to *GAPDH* and presented as the mean fold change as compared to controls.