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Sappanone A inhibits RANKL-induced osteoclastogenesis in BMMs and prevents inflammation-mediated bone loss

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ABSTRACT

Receptor activator of nuclear factor-kB ligand (RANKL) is a key factor in the differentiation and activation of osteoclasts. Suppressing osteoclastogenesis is considered an effective therapeutic approach for bone-destructive diseases, such as osteoporosis and rheumatoid arthritis. Sappanone A (SPNA), a homoisoflavanone compound isolated from the heartwood of *Caesalpinia sappan*, has been reported to exert anti-inflammatory effects; however, the effects of SPNA on osteoclastogenesis have not been investigated. In the present study, we describe for the first time that SPNA inhibits RANKL-induced osteoclastogenesis in mouse bone marrow macrophages (BMMs) and suppresses inflammation-induced bone loss in a mouse model. SPNA inhibited the formation of osteoclasts from BMMs, osteoclast actin-ring formation, and bone resorption in a concentration-dependent manner. At the molecular level, SPNA significantly inhibited RANKL-induced activation of the AKT/glycogen synthase kinase-3β (GSK-3β) signaling pathway without affecting its activation of the mitogen-activated protein kinases (MAPKs) JNK, p38, and ERK. In addition, SPNA suppressed the induction of nuclear factor of activated T cells cytoplasmic 1 (NFATc1), which is a crucial transcription factor in osteoclast differentiation. As a result, SPNA decreased osteoclastogenesis-related marker gene expression, including *CtsK*, *TRAP*, *dendritic cell-specific transmembrane protein* (*DC-STAMP*), *MMP-9* and *osteoclast-associated receptor (OSCAR)*. In a mouse inflammatory bone loss model, SPNA significantly inhibited lipopolysaccharide (LPS)-induced bone loss by suppressing the number of osteoclasts. Taken together, these findings suggest that SPNA inhibits osteoclastogenesis and bone resorption by inhibiting the AKT/GSK-3β signaling pathway and may be a potential candidate compound for the prevention and/or treatment of inflammatory bone loss.

1. Introduction

Bone mass homeostasis is regulated by the coordinated action of osteoclast-mediated bone resorption and osteoblast-induced bone formation, a process termed remodeling [1]. Osteoclasts are multi-nucleated, specialized bone-resorbing cells that arise from hematopoietic progenitors of the monocyte/macrophage lineage [2]. Many pathological bone diseases, including postmenopausal osteoporosis, periodontitis, rheumatoid arthritis, and lytic bone metastasis, are characterized by progressive and excessive bone resorption [3,4,5]. Identification of agents that inhibit osteoclast differentiation and function could be a strategy for the development of therapeutic or preventive agents for osteoclast-related bone diseases.

Osteoclast differentiation is dependent on two cytokines, a tumor necrosis factor (TNF) family cytokine, receptor activator of nuclear factor-κ B (NF-κ B) ligand (RANKL), and macrophage-colony stimulating factor (M-CSF) [6,7,8]. M-CSF promotes the proliferation and survival of myeloid cells and induces expression of RANK, the receptor for the key inducer of osteoclastogenesis RANK ligand (RANKL) [7]. The binding of RANKL to the RANK receptor activates multiple downstream signaling pathways such as the NF-κB, phosphatidylinositol 3-kinase (PI3K)/AKT, and mitogen-activated protein kinases (MAPKs) pathways [9]. Activation of PI3K ultimately releases intracellular Ca^{2+} [10,11], which can activate the key central transcription factor for osteoclastogenesis, nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) [12]. As a result, a number of osteoclastic genes including tartrate-resistant acid phosphatase (TRAP), cathepsin K (CtsK), and matrix metalloproteinase 9 (MMP-9) are upregulated [13].

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Natural products are a great source of lead compounds for developing novel drugs [14]. Many active compounds derived from natural products have inhibitory effects on osteoclast differentiation and function, and so have potential therapeutic value for treating or preventing bone diseases characterized by excessive bone resorption [15]. Sappanone A (SPNA) is a homoisoflavanone compound from the heartwood of *Caesalpinia sappan* L. (Leguminosae). SPNA has various pharmacological activities such as anti-melanogenic and anti-inflammatory activities [16,17,18]. We have previously shown that SPNA inhibits LPS-induced inflammatory responses both *in vitro* and *in vivo via* the induction of HO-1 [17]. On the basis of the association between chronic inflammatory and bone diseases [19], we investigated the pharmacological effects of SPNA on RANKL-induced osteoclast differentiation and function in bone marrow-derived macrophages (BMMs) and on bone destruction in a lipopolysaccharide (LPS)-induced bone loss mouse model. We show here that SPNA inhibited RANKL-induced osteoclast differentiation at an early stage of osteoclastogenesis *in vitro* and protected mice from LPS-induced bone loss *in vivo*.

2. Materials and methods

2.1. Antibodies and reagents

Recombinant mouse M-CSF and RANKL were obtained from R & D systems, Inc. (Minneapolis, MN, USA). Antibodies against NFATc1, phospho-AKT, AKT, phospho-p38, p38, phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, p-GSK-3β and GSK-3β were from Cell Signaling Technology *(*Danvers, MA, *USA)*. Fluorescein isothiocyanate (FITC)-conjugated phalloidin was from Invitrogen (Invitrogen, Carlsbad, CA, USA). The Antibody against for CD11b was from Abcam (Cambridge, MA, USA) and antibodies for CtsK, c-Src, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sappanone A (SPNA, Fig. 1A) was isolated from the heartwood of *C. sappan* as previously described [17].

2.2. Isolation of BMMs and osteoclast differentiation

Bone marrow cells were isolated from the femurs and tibias of 6-week old male ICR mice and cultured in α-MEM (Hyclone, Logan, UT, USA) containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 ng/mL M-CSF (Prospec, East Brunswick, NJ, USA) for overnight in a humidified incubator with 5% CO_2 at 37 °C. Floating cells were collected and cultured for three days with 30 ng/mL M-CSF and cells adhering to the bottom of the culture dish were classified as BMMs. The adherent BMMs were washed with PBS, harvested by gently scraping with a cell scrapper and used for further experiments. For tartrate-resistant acid phosphatase (TRAP) staining, BMMs (5×10^4/well) were seeded on 96-well plates and cultured in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for seven days with or without SPNA, then fixed in 3.7% formalin for 15 min, permeabilized with 0.1% Triton X-100, and stained for TRAP. TRAP-positive multinucleated cells with more than five nuclei were counted as osteoclasts.

2.3. Cytotoxicity assay

Cytotoxicity was determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. BMMs were seeded into 96-well plates (1×10^4 cells/well) and then incubated with 30 ng/mL M-CSF in the presence of various concentrations of SPNA (0, 3, 10, and 30 μM). After 72 h of incubation, MTT (0.5 mg/mL) was added to each well for 3 h. At the end of the incubation, the insoluble formazan products were dissolved in dimethyl sulfoxide, and absorbance at 540 nm was determined.

2.4. Bone resorption assay

BMMs (5×10^4 cells/well) were seeded on Corning OsteoAssay Surface 96-well plates (Corning, NY, USA) with α-MEM containing 10% FBS, 1% penicillin and streptomycin, 30 ng/mL M-CSF, and 100 ng/mL RANKL together with various concentrations of SPNA (0, 3, 10, and 30 μM) and cultured in a humidified incubator with 5% CO_2 at 37 °C. After seven days, differentiated BMMs were washed with tap water and the surface of resorption pits were observed using an H550L Nikon Microscope and quantified using image J (NIH).

2.5. Actin ring formation and immunofluorescence

BMMs were seeded onto a cover glass and incubated with 100 ng/mL RANKL and 30 ng/mL M-CSF together with various concentrations of SPNA (0, 3, 10, and 30 μM) and cultured in a humidified incubator with 5% CO_2 at 37 °C. After seven days, BMMs were washed with PBS, and fixed with 4% paraformaldehyde for 15 min. After permeabilization with 0.1% Triton X-100, BMMs were blocked, stained with FITC-conjugated phalloidin for 10 min at room temperature, washed twice in PBS and mounted. Images were acquired using a Zeiss LSM510 META NLO inverted confocal laser scanning microscope (Zeiss, Jena, Germany; Korea Basic Science Institute Chuncheon Center) equipped with an external Argon, HeNe laser and HeNe laser II.

2.6. Western blot analysis

To prepare whole cell lysates, cells were lysed with lysis buffer [50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL pepstatin A, 10 μg/mL aprotinin, 2 mM benzamidine, 50 mM NaF, 5 mM sodium orthovanadate, and 150 mM NaCl]. Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Hybond-P membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked with 5% skim milk at room temperature for 1 h, and then incubated for 2 h with primary antibodies (1:1000 dilution). After washing, membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (1:2000 dilution). The signal was detected using an enhanced chemiluminescence system (Thermo Scientific Pierce, Rockford, IL, USA).

2.7. Real–time quantitative PCR

The cells were harvested and total RNA was isolated using RNeasy Mini Kits according to the manufacturer's instructions (Qiagen, Santa Clarita, CA, USA). One μg of total RNA was used to synthesize first stranded cDNA using an RT-PCR kit (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR (qPCR) amplification was carried out using TOPreal qPCR 2 × PreMIX (SYBR Green, Enzynomics. Daejeon, Korea) and Rotor-Gene Q real-time PCR cycler (Qiagen). The following primers were used. *MMP9*, 5′-TGG GCA AGC AGT ACT CTT CC-3′ (sense) and 5′-AAC AGG CTG TAC CCT TGG TC-3′ (antisense); *CtsK*, 5′-GAC ACC CAG TGG GAG CTA TG-3′ (sense) and 5′-AGA GGC CTC CAG GTT ATG GG-3′ (antisense); *TRAP*, 5′-ACT TGC GAC CAT TGT TAG CC-3′ (sense) and 5′-TTC GTT GAT GTC GCA CAG AG-3′ (antisense); *β-actin*, 5′-GGG AAA TCG TGC GTG ACA TCA AAG-3′ (sense) and 5′-AAC CGC TCC TTG CCA ATA GT-3′ (antisense). Primers for OSCAR and DC-STAMP were purchased from Origene (Rockville, MD, USA). The optimized real-time PCR conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, and 60 °C for 15 s, and 72 °C for 20 s. All reactions were performed in triplicate and β-Actin was used as the internal control. Quantification of relative gene expression was analyzed using the 2-ΔΔCt method.