European Journal of Pharmacology 636 (2010) 28–35

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

Inhibition of osteoclastogenic differentiation by Ikarisoside A in RAW 264.7 cells via JNK and NF-κB signaling pathways

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ARTICLE INFO

Article history:
Received 4 July 2009
Received in revised form 22 February 2010
Accepted 14 March 2010
Available online 29 March 2010

Keywords:
Ikarisoside A
Osteoclast
Osteoclastogenesis
Bone
RAW 264.7
TRAP
RANKL

ABSTRACT

Osteoclasts are specialized bone-resorbing cells derived from multipotent myeloid progenitor cells. They play a crucial homeostatic role in skeletal modeling and remodeling and destroy bone in many pathologic conditions. Receptor activator of NF-κB ligand (RANKL) is essential to osteoclastogenesis. In this study, we investigated the effects of Ikarisoside A, isolated from *Epimedium koreanum* (Berberidaceae), on osteoclastogenesis in RANKL-treated murine monocyte/macrophage RAW 264.7 cells. The results indicate that Ikarisoside A is a potent inhibitor of osteoclastogenesis in RANKL-stimulated RAW 264.7 cells as well as in bone marrow-derived macrophages. The inhibitory effect of Ikarisoside A resulted in decrease of osteoclast-specific genes like matrix metalloproteinase 9 (MMP9), tartrate-resistant acid phosphatase (TRAP), receptor activator of NF-κB (RANK), and cathepsin K. Moreover, Ikarisoside A blocked the resorbing capacity of RAW 264.7 cells on calcium phosphate-coated plates. Ikarisoside A also has inhibitory effects on the RANKL-mediated activation of NF-κB, JNK, and Akt. Finally, Ikarisoside A clearly decreased the expression of c-Fos and nuclear factor of activated T cells c1 (NFATc1) as well as the transcriptional activity of NFATc1, the master regulator of osteoclast differentiation. The data indicate that Ikarisoside A has potential for use in treatment of diseases involving abnormal bone lysis such as osteoporosis, rheumatoid arthritis, and periodontal bone erosion.

1. Introduction

Bone remodeling is a physiological process that involves the resorption of bone by osteoclasts and the synthesis of bone matrix by osteoblasts (Karsenty and Wagner, 2002). Osteoclasts are known to be formed by the fusion of hematopoietic cells of the monocyte-macrophage lineage during the early stage of the differentiation process (Mohamed et al., 2007). Terminal differentiation in this lineage is characterized by acquisition of mature phenotypic markers such as expression of tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, matrix metalloproteinase 9 (MMP9), and cathepsin K, as well as morphological conversion into large multinucleated cells and the capability to form resorption lacunae on bone (Anusaksathien et al., 2001; Motyckova et al., 2001; Reddy et al., 1995).

The essential signaling molecules for osteoclast differentiation include RANKL (receptor activator of NF-κB ligand) and M-CSF (macrophage colony-stimulating factor) in bone marrow-derived macrophage precursor cells (Takayanagi et al., 2002). RANKL induces the signaling essential for precursor cells to differentiate into osteoclasts (Theill et al., 2002), whereas M-CSF, secreted by osteoblasts, provides the survival signal to these cells (Yoshida et al., 1990). Binding of RANKL to its receptor RANK activates TNF receptor-associated factor 6, which is linked to nuclear factor κB (NF-κB) and mitogen-activated protein kinases (Kobayashi et al., 2001; Lee et al., 2002; Chang et al., 2007). Active extracellular signal-regulated kinase (ERK) can directly phosphorylate c-Fos and active c-Jun-*N*-terminal kinase (JNK) phosphorylates c-Jun (Miyazaki et al., 2000). Thus, AP-1 transcription factor, a heterodimer composed of a Fos family member and a Jun family member, can be a target of ERK and JNK in response to RANKL stimulation of osteoclast precursor cells. In addition, RANKL induces the key transcription factor for osteoclastogenesis, nuclear factor of activated T cells c1 (NFATc1) (Takayanagi et al., 2002; Yamashita et al., 2007; Zhou et al., 2002).

As the bone and the immune system are so closely intermingled, all factors that regulate immune cells should be investigated for their effect on bone and vice versa. For this reason, treatment strategies for bone disease focus on the suppression of bone destruction and

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doi:10.1016/j.ejphar.2010.03.023

inflammation-associated bone loss. Bone-resorbing osteoclasts are important effector cells in inflammation-induced bone loss such as rheumatoid arthritis or periodontitis (Jimi et al., 2004; Mino et al., 1998). Inflammatory cytokines and prostaglandins up-regulate RANKL in osteoblasts, synovial fibroblasts, and activated T cells (Coon et al., 2007; Kotake et al., 1999). RANK–RANKL signaling was shown to be essential for osteoclast differentiation in inflammatory bone destruction (Anandarajah and Schwarz, 2006). In addition, many cytokines affected by inflammation, including the proinflammatory cytokines TNF-α and interleukin-1 (IL-1), may contribute to osteoclast differentiation and activation (Han et al., 2007).

Flavonoids among novel therapeutic agents are well known to especially suppress inflammation. Ikarisoside A is a natural flavonoid of the Ikarisoside family (Kuroda et al., 2000; Li et al., 1998, 1996). Data from a previous study of ours showed that Ikarisoside A isolated from *Epimedium koreanum* (Berberidaceae) exerted antioxidant potential and anti-inflammatory effects in LPS-stimulated bone marrow-derived macrophage precursor cells and RAW 264.7 cells (Choi et al., 2008a). Therefore, we examined the anti-osteoclastogenic effects and signaling pathways of Ikarisoside A with RANKL-stimulated macrophages. We demonstrate here for the first time that Ikarisoside A significantly suppresses RANKL-induced osteoclast differentiation by modulating osteoclast-specific genes, transcription factors, and signaling molecules.

2. Materials and methods

2.1. Materials

Cell culture medium, fetal bovine serum (FBS), and horse serum were obtained from Invitrogen (Gaithersburg, MD, USA). RANKL was obtained from PeproTech (Rocky Hill, NJ, USA). M-CSF was from R&D Systems (Minneapolis, MN, USA). Leukocyte Acid Phosphatase Assay Kit was obtained from Sigma (St. Louis, MO, USA). Commercially available OAAS kit for osteoclastic bone resorption assay was obtained from Oscotec (Choongnam, Korea). All other chemicals were purchased from Sigma and/or the same as described previously (Soh et al., 2000, 2003, 1998), unless otherwise indicated.

2.2. Isolation of Ikarisoside A

Ikarisoside A (Fig. 1A) from *E. koreanum* methanol extract was isolated by successive fractionation with ethyl acetate, *n*-butanol, chloroform, and hexane as Icariside II was purified (Choi et al., 2008b).

2.3. Isolation of bone marrow-derived macrophages and co-culture

Six-week-old ICR (Institute of Cancer Research) mice were purchased from Damool Science (Daejeon, Korea), bred and maintained in accordance with the guidelines of the Chonbuk National University Animal Ethics Commiittee. Cells were obtained from tibia and femur bone marrow and were cultured in α-MEM with 10% FBS containing 20 ng/ml macrophage colony-stimulating factor (M-CSF). After 3 days, the nonadherent cells were removed by washing and adherent cells were used as bone marrow-derived macrophages. For co-culture experiment, primary osteoblasts were prepared from the calvaria of 1 day-old mouse and seeded on 96-well plates (1×10^4 cells per well). After 1 day, bone marrow cells were added at 1×10^5 cells per well to the osteoblasts and cultured for 5 days in the presence of vitamin D3 (10^{-8} M) and prostaglandin E2 (10^{-6} M).

2.4. Cell culture and treatment

The murine monocyte/macrophage cell line RAW 264.7 was purchased from American Type Culture Collection (Manasas, VA, USA) and grown in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C. For

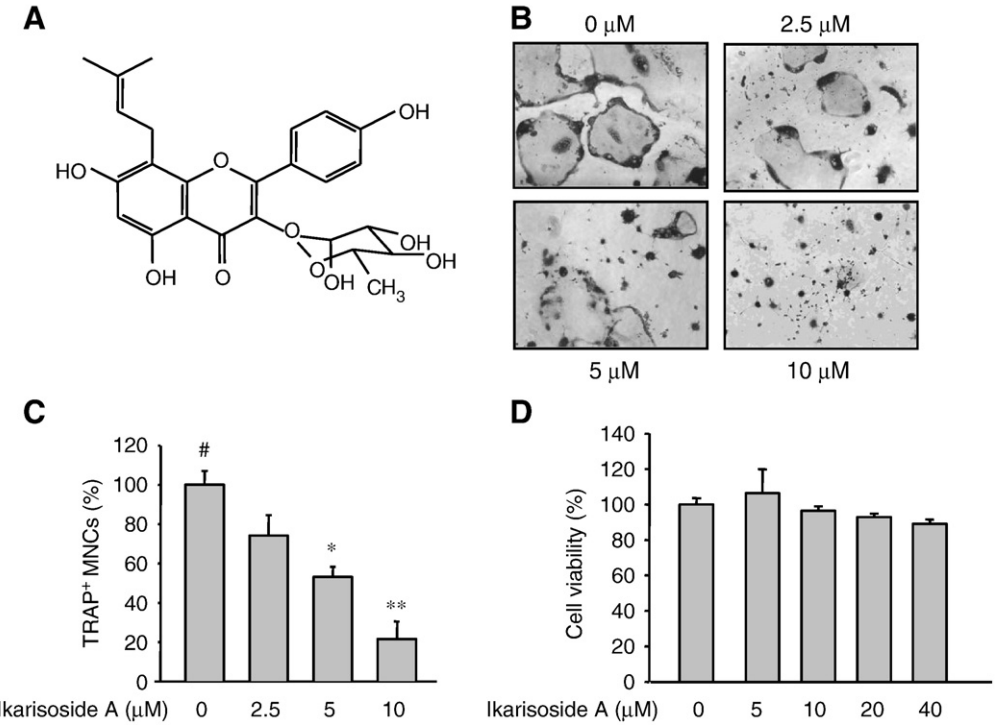

Fig. 1. Inhibitory effects on osteoclast differentiation in RANKL-stimulated RAW 264.7 cells. (A) Chemical structure of Ikarisoside A. (B) RAW 264.7 cells were cultured with the indicated concentration of Ikarisoside A in the presence of RANKL (50 ng/ml). After 6 days, cells were fixed and stained for TRAP. (C) TRAP-positive multinucleated cells ($TRAP^+$) were counted. (D) The effect of Ikarisoside A on cell viability was measured with MTT assay. The results are expressed as mean ± S.E.M. $^{**}P<0.01$, $^{*}P<0.05$ versus vehicle-treated cells (#).