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Licochalcone A inhibits the formation and bone resorptive activity of osteoclasts

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Abstract

Licochalcone A on the formation and bone resorptive activity of osteoclasts up to 5 μM significantly inhibited the receptor activator of nuclear factor κB (NF-κB) ligand (RANKL)-induced activity of tartrate-resistant acid phosphatase (TRAP) activity and formation of osteoclasts without any effect on cell viability. Interestingly, licochalcone A was shown to inhibit the RANKL-induced activation of extracellular signal-regulated kinase, translocation of NF-κB into nucleus and mRNA expression of Fra-2. Licochalcone A also inhibited the bone resorptive activity of mature osteoclasts and the expression of bone resorption-related genes. Inhibitory effects of licochalcone A on the formation and bone resorptive activity of mouse bone marrow macrophage-derived osteoclasts were also observed. In conclusion, licochalcone A has the potential to inhibit the formation of osteoclasts as well as the bone resorptive activity of mature osteoclasts.

Keywords: Licochalcone A; Osteoclast formation; Bone resorption; ERK; NF-κB; Fra-2

1. Introduction

Bone homeostasis during remodeling is maintained by osteoclastic bone resorption and osteoblastic bone formation (Parfitt, 1987). Generally, an imbalance in bone remodeling due to increased bone resorption over bone formation leads to bone disorders such as osteoporosis (Boyle et al., 2003). Osteoclasts, multinucleated cells, are differentiated from hematopoietic cells of the monocyte/macrophage family in response to osteoclastogenic factors, especially RANKL, and mature osteoclasts have the ability to resorb mineralized bone (Teitelbaum, 2000).

Recently, beneficial effects of natural products and their derivatives on the skeleton were reported by their influencing the process of bone remodeling, in particular inhibiting bone resorption (Putnam et al., 2007). For example, soybean isoflavones, a subclass of flavonoids, mainly represented by genistein and daidzein, have received considerable attention for their potential role in preventing postmenopausal bone loss (Morabito et al., 2002), and their actions probably result in a decrease in osteoclast differentiation (Yamagishi et al., 2001; Rassi et al., 2002). During screening of natural compounds for their potential to inhibit osteoclast differentiation, we identified licochalcone A (Fig. 1) as a compound inhibiting RANKL-induced tartrate-resistant acid phosphatase (TRAP) activity in mouse monocyte/macrophage RAW264.7 cells. Licochalcone A is a flavonoid derived from licorice, which is one of the most frequently used plants in traditional

Abbreviations: AP, activator protein; ATP6v0d2, v-ATPase V0 subunit d2; BMM, bone marrow-derived macrophage; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκBα, inhibitor of κB-α; LDH, lactate dehydrogenase; MAP kinases, mitogen-activated protein kinases; M-CSF, macrophage colony stimulating factor; MMP, matrix metalloproteinase; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; RANKL, receptor activator of nuclear factor-κB ligand; TNFR, tumor necrosis factor receptor; TRAF, tumor necrosis factor receptor-associated factor; TRAP, tartrate-resistant acid phosphatase.

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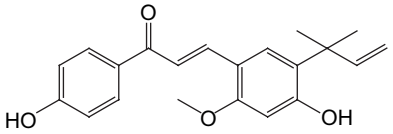

Fig. 1. Structure of licochalcone A.

Oriental medicine (Shibata, 2000). Licochalcone A has anti-inflammatory activity (Shibata et al., 1991; Kolbe et al., 2006), anti-parasitic activity (Chen et al., 1993; Mi-Ichi et al., 2005), anti-cancer activity (Rafi et al., 2000; Fu et al., 2004), anti-bacterial activity (Tsukiyama et al., 2002) and anti-browning and depigmenting activity (Fu et al., 2005), but its effect on bone metabolism has not yet been studied.

Therefore, we investigated the effect of licochalcone A on the formation and bone resorptive activity of osteoclasts. To elucidate the action mechanism of licochalcone A in the processes of osteoclast differentiation and bone resorption, we also examined the effect of licochalcone A on the activation of mitogen-activated protein (MAP) kinases and transcription factors, such as NF-κB, activator protein (AP)-1 and nuclear factor of activated T cells (NFAT) c1, known to play a critical role in the induction of osteoclast-specific genes and the activation of mature osteoclasts to resorb mineralized bone (Boyle et al., 2003; Lee and Kim, 2003). The effect of licochalcone A on the expression levels of osteoclast-specific genes has also been examined.

2. Materials and methods

2.1. Cell culture and induction of multinucleated osteoclasts

Osteoclast generation was achieved using either mouse monocyte/macrophage RAW264.7 cells or the primary culture of mouse bone marrow-derived macrophages (BMMs). RAW264.7 cells have been shown to retain the capacity to differentiate into osteoclast-like cells in the presence of RANKL (Hsu et al., 1999). RAW264.7 cells were purchased from the American Type Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, UT) supplemented with 10% fetal bovine serum (FBS, HyClone), 100 U/ml of penicillin and 100 mg/ml streptomycin, with a change of medium every 3 days in humidified atmosphere of 5% CO_2 in air at 37 °C. To differentiate into osteoclasts, RAW264.7 cells were suspended in α-minimal essential medium (α-MEM, HyClone) supplemented with 10% FBS and 100 ng/ml RANKL (R&D Systems Inc., MN) and plated in a 96-well plate at 1×10^3 cells/well. After 3–4 days, multinucleated osteoclasts were observed. To generate BMMs-derived osteoclasts, monocytes were isolated from femur and tibiae of BALB/c mice (Central Lab. Animal Inc., Korea), seeded and cultured in α-MEM with 10% FBS and 10 ng/ml macrophage colony stimulating factor (M-CSF; R&D Systems) for 1 day. Suspended cells at this stage were considered M-CSF-dependent BMMs and used as osteoclast precursors. Induction of their differentiation into osteoclasts was done by culturing the cells plated into a 96-well plate at 3×10^5 cells/well in α-MEM with 10% FBS, 100 ng/ml RANKL and 30 ng/ml M-CSF. Multinucleated osteoclasts were observed on differentiation day 6.

2.2. Cell viability assay

RAW264.7 cells were suspended in α-MEM with 10% FBS and 100 ng/ml RANKL, and plated in 96-well plates 1×10^3 cells/well. After 24 h, serially diluted licochalcone A (Calbiochem, Germany) was treated and incubated for 1 or 3 days. Cell viability was then evaluated by Cell Counting Kit-8 (Dojindo Molecular Technologies, ML, USA) according to the manufacturer's protocol. The results are presented as mean of measured absorbance (at 450 nm) ± 1 standard deviation, experiments being performed in triplicate. Absorbance was measured using a Wallac EnVision microplate reader (PerkinElmer, Finland).

2.3. TRAP staining and activity assay

Multinucleated osteoclasts were fixed with 10% formalin for 10 min and ethanol/acetone (1:1) for 1 min, and stained with Leukocyte Acid Phosphatase Kit 387-A (Sigma, MO, USA). The images of TRAP-positive multinucleated cells were taken under a microscope with DP Controller (Olympus Optical, Japan). For measuring TRAP activity, multinucleated osteoclasts were fixed with 10% formalin for 10 min and 95% ethanol for 1 min, followed by 100 μl citrate buffer (50 mM, pH 4.6) containing 10 mM sodium tartrate, and 5 mM *p*-nitrophenyl phosphate (Sigma) was added to the dried cell-containing wells. After incubation for 1 h, the enzyme reaction mixtures in the wells were transferred into new plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at 410 nm and TRAP activity was presented as a percentage of control. The experiment was performed in triplicate and differences were considered significant when $p < 0.01$.

2.4. Isolation of total RNA

Total RNA was isolated with TRIzol reagent (Life Technologies, MD, USA) according to the manufacturer's protocol. The concentration of total RNA was calculated from the absorbance at 260 and 280 nm with a BioPhotometer (Eppendorf AG, Germany).

2.5. Primer design and real-time quantitative PCR (QPCR)

Primers were chosen with an on-line primer design program (Rozen and Skaletsky, 2000; see Table 1). First-strand cDNA was synthesized with 2 μg total RNA, 1 μM of oligo-dT_{18} primer and 10 units of RNase inhibitor RNasin (Promega, WI, USA) using an Omniscript RT kit (Qiagen, CA, USA), according to the manufacturer's protocol. The SYBR green-based QPCR was performed using the Stratagene