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Oleanolic acid exerts bone protective effects in ovariectomized mice by inhibiting osteoclastogenesis

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ABSTRACT

Postmenopausal osteoporosis (POP) is quite prevalent and many new drugs are under development to obtain better therapeutic outcomes. Oleanolic acid (OA) has been reported to prevent bone loss in ovariectomized (OVX) rats by stimulating osteoblastogenesis. One previous study has demonstrated that acetate of OA suppressed lipopolysaccharides (LPS)-induced bone loss in mice. However, the role of OA in the receptor activator of nuclear factor kappa-B ligand (RANKL)-mediated osteoclastogenesis is still not elucidated. Here we show that OA dose-dependently inhibits RANKL-mediated osteoclastogenesis and the formation of functional osteoclasts without impairing the viability and osteoclastic potential in bone marrow macrophages (BMMs). Moreover, OA administration attenuates bone loss in OVX mice by inhibiting osteoclast's densities. Mechanistically, OA does not affect RANKL-induced activation of the NF-κB, JNK, p38, ERK and Akt pathways, but inhibits the expression of the nuclear factor of activated T-cells c1(NFATc1) and c-Fos. Moreover, OA significantly suppresses the expression of RANKL-activated osteoclast genes encoding matrix metalloproteinase 9 (*MMP9*), Cathepsin K(*Ctsk*), tartrate-resistant acid phosphatase (*TRAP*) and carbonic anhydrase II (*Car2*). This work has elucidated the molecular mechanism of OA in RANKL-mediated osteoclastogenesis and revealed the promising potential of OA to be further developed as a new drug to prevent and treat POP.

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1. Introduction

The homeostasis of bone metabolism is maintained by the tight coupling between bone-forming osteoblasts and bone-resorbing osteoclasts throughout the lifetime.[1] Imbalance between bone formation and bone resorption, in favor of bone resorption, leads to the development of postmenopausal osteoporosis (POP).[2–4] POP, a major public health problem, is characterized by low bone mass and structural deterioration of the skeletal system.[5] Therefore, the effective inhibition on bone resorption has long been recognized as an important therapeutic strategy for osteoporosis in the clinic. Currently, there are several anti-resorptive drugs (agents capable of inhibiting osteoclast formation and/or function) on the market: estrogen,[6] selective estrogen receptor modulators (SERMs),[7] bisphosphonates,[8] and calcitonin.[9] However, these drugs often have potential to cause side effects and the curative effects are far from ideal in the management of POP.

Active principles or ingredients from natural herbs, such as icariin,[10] osthole,[11] and psoralen,[12] have been found to exert an

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anabolic effect on osteoporotic bone by increasing bone formation. Moreover, several active ingredients were also reported to inhibit osteoclast differentiation.[13,14]Oleanolic acid (OA), one of active ingredients from Nvzhenzi (*Fructus Ligustri Lucidi*), and its derivatives possess the effects of hepatoprotection, anti-inflammation, and anticancer.[15] Several previous studies revealed that OA exerted bone protective effects in ovariectomized (OVX) rats by stimulating osteoblastogensis.[16,17] However, although numerous studies have shown that glycosides or derivatives of OA negatively regulated osteoclastogenesis *in vitro* [18–20], the regulatory role and molecular mechanism of OA in osteoclastogenesis are not fully understood. Notably, one previous study demonstrated that acetate of OA (OAA), which is structurally very similar to OA, inhibits osteoclast differentiation from BMMs in male mice by suppressing the PLCγ2-Ca^{2+}–NFATc1 and prevents lipopolysaccharides (LPS)-induced bone loss.[21] Taken together, these findings indicated that OA directly inhibits osteoclastogenesis and additional studies are necessary to further investigate the regulatory role and molecular mechanism of OA inosteoclastogenesis.

Abnormal osteoclast formation has been implicated in the pathogenesis of POP.[3] Osteoclasts differentiate from mononuclear cells of the monocyte/macrophage lineage when stimulated by the macrophage colony stimulating factor (M-CSF) and the receptor activator of nuclear factor kappa-B ligand (RANKL).[22] It is well established that M-CSF stimulates proliferation and survival of bone marrow macrophages (BMMs, osteoclast precursors). In contrast, RANKL primarily promotes osteoclast differentiation by activating numerous signaling pathways, such as NF-κB, JNK, ERK, p38 and Akt.[22,23] In addition, RANKL also up-regulates the expression of NFATc1 and c-Fos, two master transcription factors for osteoclastogenesis.[24,25] Consequently, RANKL-induced expression of NFATc1 and c-Fos leads to the expression of the genes encoding matrix metalloproteinase 9 (*MMP9*), cathepsin K (*Ctsk*), tartrate-resistant acid phosphatase (*TRAP*) and carbonic anhydrase II (*Car2*)[26,27] in osteoclastogenesis.

In the present study, we investigated the effect of OA on osteoclastogenesis using both *in vitro* and *in vivo* approaches, and examined the molecular mechanism by which OA modulates osteoclastogenesis. This work has established the role of OA and provided new insights into the molecular mechanism underlying the inhibitory effects of OA on osteoclastogenesis. Importantly, our findings further support the notion that OA has potential to be a novel and effective new drug for POP.

2. Materials and methods

2.1. Reagents

OA (purity>99%; Supplemental Fig. 1.) were purchased from Yousi Company (Shanghai, China) and dimethyl sulphoxide (DMSO) were purchased from Sigma–Aldrich (D2438; St. Louis, MO,USA). Stock solutions (2×10^4 μM) of OA were prepared in DMSO monthly and stored at 4 °C. Recombinant purified glutathione S-transferase-RANKL and Mouse M-CSF was prepared as described previously.[28] Antibodies were purchased from Cell Signaling Technology Inc (Beverly, MA, USA): IκBα, phosphor-IκBα, p44/42ERK, phospho-44/42ERK,JNK, phospho-JNK, p38, phospho-p38. Antibodies against NFATc1, and c-Fos were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animals

C57BL/6 mice for *in vitro* analysis were purchased from Harlan Industries (Indianapolis, IN,USA) and were maintained in the animal facility at University of Alabama at Birmingham (UAB). For *in vivo* analysis, C57BL/6 mice (SLAC Laboratory Animal Co. Ltd., Shanghai, China) were maintained in animal center of Longhua hospital with light: dark (12 h:12 h). Ovariectomized (OVX) mice were created[29] and divided into three groups (8 mice) at 7 d after ovariectomy: sham operated mice (Sham), OVX mice treated with saline (OVX) and OVX mice treated with OA (10 mg/kg) intraperitoneally (i.p.) every 2 ds for 3 months. The experiments involving mice was performed in accordance with the approval of Institutional Animal Care and Use Committee of the Shanghai University of TCM (Animal Protocol Approval No.: SZY201603007).

2.3. Micro-CT analysis

Lumbar spine specimens were fixed in 4% paraformaldehyde for 24 h, washed for 2 h, and examined. Six L4 vertebrae in each group were described for a 3-dimensional (3D) model without exhibiting adnexa, such as transverse and spinous processes. Analyses were performed using the μCT 80 radiograph microtomography, associated 3DCalc cone reconstruction, μCT Ray V3.4 A model building software (Scanco Medical AG, Bruttisellen, Switzerland). Scores for the bone mineral density (BMD), the ratio of bone volume over tissue volume (BV/TV), the trabecular number (Tb.N), the trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) were directly obtained.

2.4. TRAP staining

TRAP staining was performed on paraffin sections (5 μm) of L4 vertebrae. Briefly, the sections were deparaffinized, and dehydrated with 0.02 g/ml Napthol-Ether solution and incubated with the diluted Napthol-Ether solution at 37 °C for 30 min. Then, a 0.04 g/ml sodium nitrite solution and a 0.05 g/ml pararosaniline dye/2 N hydrochloric acid were mixed and added to the basic stock solution for additional 15 min. After counter staining with 0.5% fast green and dehydration, the sections were mounted and observed using light microscope (Olympus BX50, Tokyo, Japan) and were analyzed. TRAP activity was quantified by calculating the ratio of the number of TRAP-positive osteoclast.

2.5. Immunohistochemistry analysis

Left tibia sections (5 μm) were deparaffinized, rehydrated with phosphate buffer saline (PBS, pH 7.4), treated with aqueous 3% H_2O_2 for 10 min, and antigen was retrieved with 0.1% trypsin (M/V) in 0.1% $CaCl_2$ at 37 °C for 10 min. The sections were blocked with 5%

Table 1
Names and sequences of primers used for polymerase chain reaction analysis.

Gene	Sequence
ß-actin	F: 5′–CCTGTACGCCAACACAGTGC-3′
	R:5′- ATACTCCTGCTTGCTGATCC-3′
MMP9	F: 5′- CTTCTTCTCTGGACGTCAAATG -3′
	R:5′-CATTTTGGAAACTCACACGCC -3′
Car2	F:5′- AGAGAACTGGCACAAGGACTT -3′
	R 5′- CCTCCTTTCAGCACTGCATTGT -3′
Ctsk	F:5′- GATGCTTACCCATATGTGGGC -3′
	R:5′- CATATCCTTTGTTTCCCCAGC -3′
TRAP	F:5′- GCCAAGATGGATTCATGGGTGG -3′
	R:5′- CAGAGACATGATGAAGTCAGCG-3′
NFATc1	F:5′- TGTTCTTCCTCCCGATGTCT -3′
	R:5′- CCCGTTGCTTCCAGAAAATA -3′
c-Fos	F:5′- TGTTCTTCCTCCCGATGTCT -3′
	R:5′-GGATTTGACTGGAGGTCTGC -3′
GAPDH	F:5′ACATCATCCCTGCATCCACTG-3′
	F:5′TCATTGAGAGCAATGCCAGC-3′

Car2, carbonic anhydrase II;*Ctsk*, cathepsin K;*Trap*, tartrate-resistant acid phosphatase; *Mmp9*, matrix metalloproteinase 9; *NFATc1*, nuclear factor of activated T-cells c1; *c-Fos*, FBJ osteosarcoma oncogene; F, forward; R, reverse.

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