Phytomedicine xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Phloretin promotes osteoclast apoptosis in murine macrophages and inhibits estrogen deficiency-induced osteoporosis in mice

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ARTICLE INFO

Article history:
Received 25 August 2013
Received in revised form 12 February 2014
Accepted 2 April 2014

Keywords:
Phloretin
Osteoclast apoptosis
Estrogen deficiency
Osteoporosis
Ovariectomy

ABSTRACT

Bone-remodeling imbalance induced by increased osteoclast formation and bone resorption is known to cause skeletal diseases such as osteoporosis. The reduction of estrogen levels at menopause is one of the strongest risk factors developing postmenopausal osteoporosis. This study investigated osteoprotective effects of the dihydrochalcone phloretin found in apple tree leaves on bone loss in ovariectomized (OVX) C57BL/6 female mice as a model for postmenopausal osteoporosis. OVX demoted bone mineral density (BMD) of mouse femurs, reduced serum 17β-estradiol level and enhanced serum receptor activator of NF-κB ligand (RANKL)/osteoprotegerin ratio with uterine atrophy. Oral administration of 10 mg/kg phloretin to OVX mice for 8 weeks improved such effects, compared to sham-operated mice. Phloretin attenuated TRAP activity and cellular expression of β3 integrin and carbonic anhydrase II augmented in femoral bone tissues of OVX mice. This study further examined that osteogenic activity of phloretin in RANKL-differentiated Raw 264.7 macrophages into mature osteoclasts. Phloretin at 1–20 μM stimulated Smac expression and capase-3 activation concurrently with nuclear fragmentation of multi-nucleated osteoclasts, indicating that this compound promoted osteoclast apoptosis. Consistently, phloretin enhanced bcl-2 induction but diminished bax expression. Furthermore, phloretin activated ASK-1-diverged JNK and p38 MAPK signaling pathways in mature osteoclasts, whereas it dose-dependently inhibited the RANKL-stimulated activation of ERK. Therefore, phloretin manipulated ASK-1-MAPK signal transduction leading to transcription of apoptotic genes. Phloretin was effective in preventing estrogen deficiency-induced osteoclastogenic resorption.

Introduction

Osteoclasts specialized for bone resorption and osteoblasts responsible for bone formation play a key role in bone turnover (Proff and Römer 2009). There are multiple targets within osteoclasts for pharmacologic intervention to prevent bone loss. During osteoclast formation, receptor activator of nuclear factor (NF)-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) promote differentiation and modulate osteoclast survival (Blair and Zaidi 2006; Cicek et al. 2011; Xing et al. 2005). Several drugs act by targeting specific pathways within the osteoclastic cells (Rejnmark and Mosekilde 2011). Synthetic bisphosphonates for the antiresorptive treatment predominantly inhibit osteoclasts attachment to bone matrix and induce apoptosis of mature osteoclasts (Dominguez et al. 2011; Rejnmark and Mosekilde 2011). Accordingly, potential agents targeting osteoclast apoptosis may display favorable effects in combating resorptive bone diseases.

The reduction of estrogen levels at menopause is one of the strongest risk factors for developing postmenopausal osteoporosis (Fraser et al. 2011; Henriksen et al. 2011). Postmenopausal osteoporosis is characterized by low bone mass and microarchitectural deterioration of bone tissue (Kanis 2002). Osteoporosis treatments include assuring calcium and vitamin D as well as prescription of medications such as bisphosphonates (Akesson 2003; Beard 2012; Dawson-Hughes and Bischoff-Ferrari 2007). Potential first-line pharmacological therapies for postmenopausal osteoporosis include agents improving bone strength and reducing osteoporotic

Abbreviations: CA, carbonic anhydrase; RANKL, receptor activator of nuclear factor-κB ligand; Smac, second mitochondrial-derived activator of caspase; TRAP, tartrate-resistant acid phosphatase.

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http://dx.doi.org/10.1016/j.phymed.2014.04.002
0944-7113/

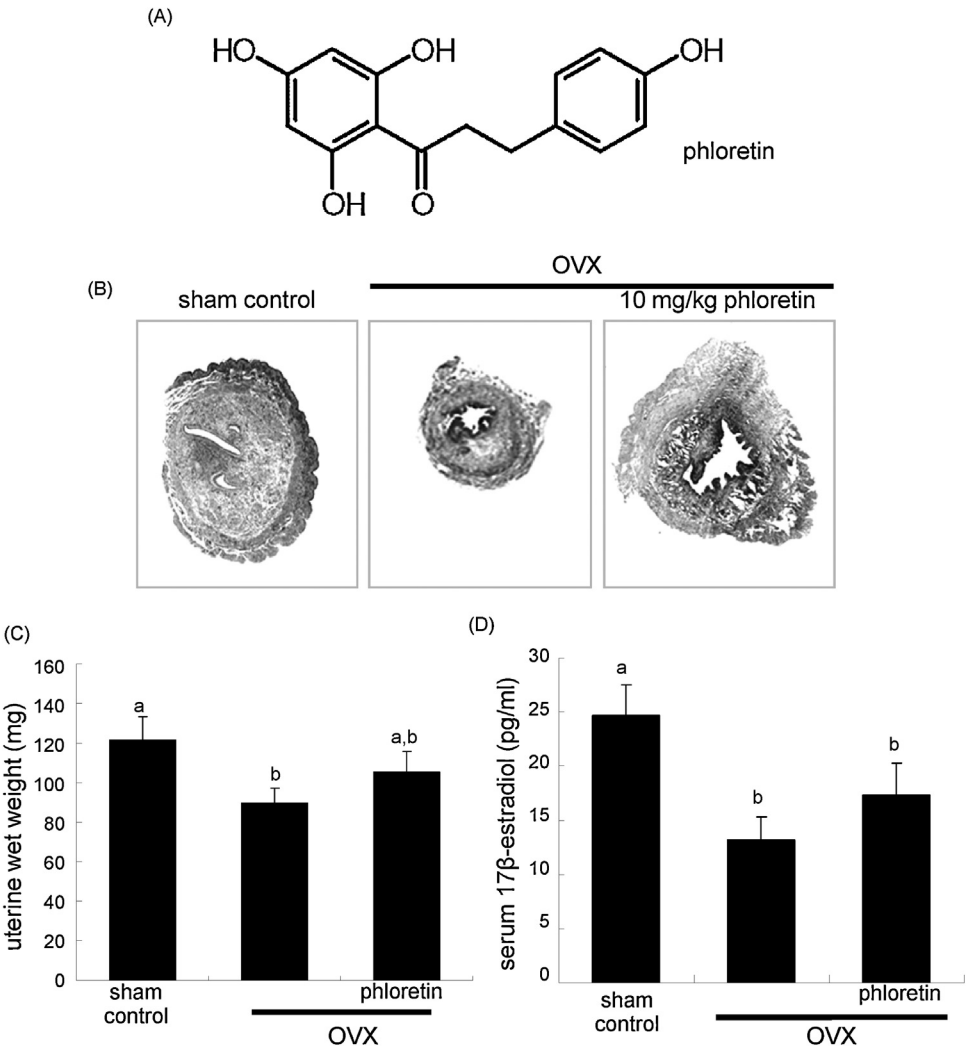

Fig. 1. Chemical structure of phloretin (A), uterus transverse section (B), wet weight of uterine tissues (C) and serum 17β-estradiol level (D) of OVX mice treated with 10 mg/kg phloretin daily for 8 weeks. Cross-sectional images of the uterine horn were obtained by staining with H&E and visualized under light microscopy. Magnification: 40-fold. Serum 17β-estradiol level was determined by using assay kits. Values in bar graphs (means ± SEM, $n = 9$) not sharing a letter are different at $p < 0.05$.

fracture (Josse et al. 2013). Hormone replacement therapy may help prevent adverse postmenopausal changes in bones (Eriksen 2012; Tao et al. 2011). However, its long-term use is controversial.

Concerns pertaining to the risk of long-term hormone therapy have prompted an increase in the use of natural alternatives (Bedell et al. 2014). Phytoestrogens mainly belong to naturally occurring nonsteroidal compounds that have estrogen-like biological activity primarily through binding to estrogen receptors (Al-Anazi et al. 2011; Turner et al. 2007). Numerous studies have focused on revealing osteoprotective mechanism(s) of phytoestrogens. The isoflavone formononetin prevents bone loss by reversing estrogen deficiency-induced detrimental bone biomechanical features (Kaczmarczyk-Sedlak et al. 2013). Several polyphenols including green tea (−)-epigallocatechin gallate (EGCG) prevent bone loss (Hagiwara et al. 2011). This polyphenol inhibits osteoclast formation and bone resorption (Kamon et al. 2009). Our previous study revealed that phloretin had an antiosteoclastogenic activity by suppressing RANKL-induced osteoclast differentiation (Kim et al. 2012). However, their action mechanisms for manipulating bone-specific remodeling process remain unclear under in vivo conditions.

Phloretin (Fig. 1A) is a natural dihydrochalcone present in apple peels and displays anti-oxidative and anti-inflammatory activity (Jung et al. 2009; Yang et al. 2011). Orally consumed phlorizin is mainly converted into phloretin by hydrolytic enzymes in the small intestine, and inhibits glucose absorption by the small intestine and renal glucose reabsorption as a glucose transporter 2 inhibitor (Idris and Donnelly 2009). This study investigated that phloretin would help to antagonize osteoporosis due to estrogen deficiency. This study evaluated the effects of phloretin on tartrate-resistant acid phosphatase (TRAP) activity and osteoclastogenic marker induction in femoral bone tissues of ovariectomized (OVX) mice. Also this study examined whether phloretin promoted osteoclast loss in femoral bones of OVX mice and apoptosis of mature osteoclasts differentiated from Raw 264.7 macrophages. The sequential molecular events and signaling of osteoclast apoptosis induced by phloretin were elucidated.

Materials and methods

Materials

Fetal bovine serum (FBS), penicillin–streptomycin, trypsin–EDTA were purchased from Lonza (Walkersville, MD). Minimum Essential Medium Alpha Medium (α-MEM), Dulbecco's

Please cite this article in press as: Lee, E.-J., et al., Phloretin promotes osteoclast apoptosis in murine macrophages and inhibits estrogen deficiency-induced osteoporosis in mice. Phytomedicine (2014), http://dx.doi.org/10.1016/j.phymed.2014.04.002