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An 8-*O*-4′ norlignan exerts oestrogen-like actions in osteoblastic cells via rapid nongenomic ER signaling pathway

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ABSTRACT

Ethnopharmacological relevance: *Sambucus williamsii* Hance (SWH), which belongs to the *Caprifoliaceae* family distributed in various regions of China, Korea and Japan, has been used as a folk medicine for treatment of bone and joint diseases in China for thousands of years. In previous studies, SWH was shown to possess anti-osteoporosis, healing fracture, anti-inflammatory and analgesic activities. Our previous studies showed that SWH extract effectively suppressed ovariectomy-induced increase in bone turnover and improved bone mineral density and bone biomechanical strength in rats as well as in mice. An 8-*O*-4′ norlignan, (7*R*,8*S*)-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropyl)-2-methoxyphenoxy]-1,3-propanediol (PPD) was previously isolated and identified as the bioactive ingredient in SWH. The present study aimed to characterize the bone protective effects as well as its mechanism of actions in osteoblasts.

Materials and methods: Bone protective actions of PPD on different cells were determined by proliferation assay, alkaline phosphatase (ALP) activity assay, calcium deposition as well as real-time reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, estrogen receptor (ER) antagonist ICI182,780 and mitogen-activated protein kinase kinase (MEK) inhibitor U0126 blocking assays, competitive ER radioligand binding assay, ERE-dependent luciferase reporter assay and immunoblotting were used to determine if PPD activated ER and if the effects of PPD on osteoblastic functions were ER dependent.

Results: PPD exerted anabolic effects in osteoblasts and its effects were abolished by co-incubation with ICI182,780 or U0126. PPD induced mRNA expressions of Runx2, ALP, osteocalcin, and increased the ratio of osteoprotegerin/receptor activator of nuclear factor κB (OPG/RANKL). PPD failed to bind to either ERα or ERβ and did not activate ERE-luciferase activity via ER. PPD induced the phosphorylation of extracellular regulated kinases (ERK) and its effect was completely abolished by U0126. It also induced the phosphorylation of ERα at serine 118.

Conclusion: These data show that PPD is a bioactive compound in SWH that exerts oestrogen-like actions in osteoblast-like cells via ligand-independent, estrogen response element (ERE)-independent and mitogen-activated protein (MAP) Kinase-mediated rapid nongenomic ER signaling pathway.

1. Introduction

Recently, there is strong interest for the use of phytoestrogens, plant derived polyphenolic nonsteroidal compounds with oestrogen-like biological activity, for treatment of menopausal related diseases and postmenopausal osteoporosis (Albertazzi and Prudie, 2008; Bedell et al., 2014). Isoflavones and lignans are the two most studied types of phytoestrogen and their efficacies as a HRT substitute have been explored (Bedell et al., 2014). Lignans with the similar structures as oestradiol are widely found in oilseeds, cereals, legumes,

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fruit, vegetables and flaxseed. Some studies have reported the beneficial effects of lignans on bone both *in vivo* and *in vitro*. The total lignans extracted from *Eucommia ulmoides* Oliv. Barks effectively suppressed the loss of bone induced by ovariectomy (OVX), and promoted primary osteoblastic cell proliferation, differentiation and osteoprotegrin (OPG) expression (Zhang et al., 2014). Sesamin, the major lignan in sesame seeds, stimulated osteoblasts to mature and function by accelerating osteoblastogenic gene expression (Wanachewin et al., 2012). Lignans from the fruits and seeds of *Schisandra chinensis* (Turcz.) stimulated the proliferation and the activity of alkaline phosphatase in osteoblasts (Caichompoo et al., 2009). Although some lignans have been demonstrated for its anabolic effects on bone, two clinical studies indicated that lignan from flaxseed was not effective in protecting against bone loss in menopausal women (Dodin et al., 2005; Cornish et al., 2009). Indeed, reports on the effects of lignans on bone are relatively limited and that the lack of protective effects of lignans from flaxseed on bone could be due to the differences in chemical structures of lignans being used. Thus, in order to determine if the specific class of lignans is useful for management of postmenopausal osteoporosis, it is important to systematically evaluate it is bone protective and delineate the mechanism involved in mediating its actions.

An 8-*O*-4′ neolignan, namely (7*R*,8*S*)-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropyl)-2-methoxyphenoxy]-1,3-propanediol (PPD), was recently identified to be the bioactive ingredient from *Sambucus williamsii* Hance (SWH) through *in vivo* and *in vitro* bioactivity-guided fractionation (Yang et al., 2007; Xiao et al., 2011). SWH (*Caprifoliaceae*), as a folk medicine, has been used for treatment of bone and joint diseases in China for thousands of years (Zhong et al., 1985; Song, 2000; Zhao, 2010; Han et al., 2013). Our previous studies showed that SWH extract effectively suppressed ovariectomy-induced increase in bone turnover and improved bone mineral density (BMD) and biomechanical strength in rats (Xie et al., 2005) as well as in mice (Zhang et al., 2011). PPD has been isolated from many plants, such as Cultivar of *Helianthus annuus* (Macías et al., 2004), fruit of *Euterpe oleracea* (Chin et al., 2008) and *Tsuga dumosa* (Zhao et al., 2004). It is reported that PPD (both PPD and its isomer) could significantly induce alkaline phosphatase (ALP) activity in UMR 106 cell, and exhibited potent activities in hydroxyl radio scavenging assay (Fujimoto et al., 2004; Chin et al., 2008). However, it is unclear if PPD exerts oestrogen-like effects, and the signaling pathways that mediate the actions of PPD in osteoblastic cells are yet to be determined.

In the present study, we hypothesize that PPD is a phytoestrogen that exerts oestrogen-like activities in bone cells. The present study aims to study the effects of PPD on osteoblast proliferation and differentiation, calcium deposition and the expression of genes involved in osteoblast differentiation in UMR 106 cells, MC3T3-E1 cells and BMSC cells, and to determine if and how PPD activates estrogen receptor (ER) in UMR 106 cells. We hope this study will improve our understanding of the potential of using lignans as an alternative regimen for treatment of postmenopausal osteoporosis.

2. Materials and methods

2.1. Plant materials and compounds identification

The stems and branches of *S. williamsii* Hance (SWH) were collected in Shenyang, Liaoning Province in Northeast of China in April 2007 and authenticated according to a method listed in the Chinese Bencao (Song, 2000) with the help of Professor Zerong Jiang (Shenyang Pharmaceutical University, Shenyang, China). The identification of SWH was confirmed by analyzing its major ingredients pentacyclic triterpenoid, phenolic acid and derivatives and protein. A voucher specimen (HHXSWGZ-2007) was deposited in the Herbarium of the Institute of Traditional Chinese Medicine and Natural Products (Jinan University, Guangzhou, China).

(7*R*,8*S*)-1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropyl)-2-methoxyphenoxy]-1,3-propanediol (PPD) was isolated and elucidated from the bioactive fraction of *S. williamsii* Hance (SWH) as previously described (Xiao et al., 2011) by comparing its physical properties and infrared absorption (IR), nuclear magnetic resonance (NMR) and circular dichroism (CD) spectral data with literature values (Arnoldi and Merlini, 1985; Zhao et al., 2004). The purity of the PPD was more than 98%.

2.2. Cell culture

Rat osteoblast-like UMR 106 cells (ATCC no. CRL-1661) and murine pre-osteoblastic MC3T3-E1 cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and were routinely cultured as previously described (Xiao et al., 2014b). Bone marrow mesenchymal stem cells (BMSC) were harvested and cultured as previously described (Xiao et al., 2014b). PPD and 17β-oestradiol (E2) (Sigma-Aldrich, St Louis, MO, USA) were dissolved in ethanol to get 10^{-2} M stock, respectively. The stocks of PPD were diluted with ethanol to 10^{-9} to 10^{-4} M, finally, serially diluted with medium to achieve treatment concentration at 10^{-12} M to 10^{-7} M. The stock of E2 was diluted with ethanol to 10^{-5} M, and then diluted with medium to treatment concentration.

UMR-106 cells were seeded in 96-well plates at a cell density of 4×10^3 per well. After 48 h, medium was changed to pheol-red free DMEM (pf-DMEM) (Gibco, Grand Island, NY, USA) supplemented with 1% dextran-charcoal-stripped fetal bovine serum (sFBS) (Gibco, Grand Island, NY, USA) and incubated for 24 h. Then the cells were treated with PPD (10^{-12} to 10^{-7} M), 17β-oestradiol (E2, 10^{-8} M) or vehicle (0.1% ethanol) in the presence or absence of ICI 182,780 (10^{-6} M) (Tocris, Minneapoils, MN, USA) or U0126 (10^{-5} M) (Tocris, Bristol, UK) for 24 or 48 h. MC3T3-E1 cells were subjected to similar treatment in the presence of ascorbic acid (Sigma-Aldrich, St Louis, MO, USA) and β-glycerophosphate (Calbiochem, Darmstadt, GER). BMSC cells were also subjected to similar treatment in medium with dexamethasone (1×10^{-8} M) (J&K, Peking, China), ascorbic acid (50 μg/ml) and β-glycerol sodium phosphate (5×10^{-3} M) (Aladdin, Shanghai, China). The medium with drugs was changed per 2 days and the cells were incubated for 18 days.

2.3. Cell proliferation assay and alkaline phosphatase (ALP) activity assay

Upon treatment with PPD (10^{-12} M to 10^{-7} M), E2 (10^{-8} M) or vehicle (0.1% ethanol) in the presence or absence of ICI182,780 (10^{-6} M) (Tocris, Minneapoils, MN, USA) or U0126 (10^{-5} M) (Tocris, Bristol, UK) for 24 or 48 h, the proliferation and alkaline phosphatase activities were measured as described in our previous study (Xiao et al., 2014b).

2.4. von Kossa staining for deposits of calcium

Treated cells were fixed in 4% paraformaldehyde and incubated with 1% silver nitrate solution. Unreacted silver was removed with 5% sodium thiosulfate (Aladdin, Shanghai, China). Cells were then counterstained with nuclear fast red (Aladdin, Shanghai, China) for 5 min. The stained deposits of calcium were recorded under microscope.

2.5. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated and reverse transcribed using our previous method (Xiao et al., 2014b). The sequences of the PCR