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Upregulation of osteoblastic differentiation marker mRNA expression in osteoblast-like UMR106 cells by puerarin and phytoestrogens from *Pueraria mirifica*

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ABSTRACT

Phytoestrogens have attracted attention for their potential in the prevention of postmenopausal osteoporosis. Recently, phytoestrogen-rich herb *Pueraria mirifica* has been demonstrated to possess an osteogenic effect on bone in ovariectomized rats, but its underlying cellular mechanism was not known. Here, we investigated the effects of *P. mirifica* extract and its major isoflavone compound, puerarin, on cell viability, cell proliferation and the expression of differentiation markers in rat osteoblast-like UMR106 cells. After exposure to 17β-estradiol (E2), genistein, *P. mirifica* extract and puerarin, proliferation but not viability of UMR106 cells was markedly decreased. Quantitative real-time PCR revealed that *P. mirifica* extract and puerarin significantly increased the mRNA expression of alkaline phosphatase (ALP) and osteoprotegerin, but not Runx2, osterix or osteocalcin. Puerarin also decreased the mRNA expression of receptor activator of nuclear factor-κB ligand, an osteoclastogenic factor, suggesting that it could induce bone gain by enhancing osteoblast differentiation and suppressing osteoclast function. Furthermore, after an exposure to high affinity estrogen receptor (ER) antagonist (ICI182780), the E2-, genistein-, *P. mirifica* extract- and puerarin-induced upregulation of ALP expressions were completely abolished. It could be concluded that *P. mirifica* extract and puerarin induced osteoblast differentiation rather than osteoblast proliferation in an ER-dependent manner. The present findings, therefore, corroborated the potential benefit of *P. mirifica* extract and puerarin in the prevention and treatment of postmenopausal osteoporosis.

Introduction

Osteoporosis, a metabolic bone disease characterized by low bone density and microarchitectural deterioration, progressively compromises bone strength, predisposing patients to the vertebral fractures, fragility fractures of the neck of the femur, and Colles fracture of the wrist (Cole et al. 2008; Lane 2006). The economic burden of osteoporosis is markedly increased paralleling the expansion of aging world population (Lane 2006). Approximately 9 million patients with osteoporotic fractures were reported worldwide in 2000, and the incidence has been predicted to reach 6.26 million by 2050 (Cole et al. 2008). Therefore, development of effective preventions, early interventions and treatments is needed to mitigate clinical complications of osteoporosis and the accompanying economic impact.

Since 17β-estradiol (E2) is an important hormone for maintaining bone mass, postmenopausal women with estrogen deficiency thus are prone to develop osteoporosis (Lane 2006). Although estrogen replacement therapy is an effective regimen for postmenopausal bone loss, long-term exposure to synthetic estrogens markedly increases the prevalence of malignant neoplasia in several tissues, such as mammary gland, endometrium, and ovary (Manolagas et al. 2002). Phytoestrogens—estrogen-like compounds of plant origin—are capable of binding to estrogen receptor (ER) with minimal cancer risk (Tham et al. 1998), and have become a widely accepted alternative treatment of choice (Setchell and Lydeking-Olsen 2003; Tham et al. 1998). Recently, the tuberous root extract of phytoestrogen-rich herb *Pueraria mirifica* has

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been shown to effectively prevent osteoporosis in ovariectomized rats by increasing bone mineral density (BMD) and bone mineral content (BMC) (Urasopon et al. 2008). However, the exact anti-osteoporotic mechanism of *P. mirifica* extract and its major constituent, puerarin, in osteoblasts (bone-forming cells) remained elusive.

P. mirifica belongs to the family Leguminosae endemic to Thailand (Malaivijitnond 2012; Chansakaow et al. 2000). Its tuberous root contains a number of isoflavones, such as puerarin, daidzin, genistin, daidzein and genistein (Cherdshewasart et al. 2007a,b; Cherdshewasart and Sriwatcharakul 2007). Some isoflavone derivatives are phytoestrogens that exert osteogenic effects on bone like estrogen (Urasopon et al. 2008; Marini et al. 2007; Morabito et al. 2002). For instance, a randomized double-blind placebo-controlled study in postmenopausal women showed that genistein administration significantly increased BMD and the circulating levels of bone formation markers, e.g., bone-specific alkaline phosphatase (ALP), osteocalcin and insulin-like growth factor (IGF)-1 (Marini et al. 2007; Morabito et al. 2002). It was shown that genistein exerted its action by binding to α- and β-isoforms of ER (Kuiper et al. 1998). Since the chemical structures and actions of isoflavones resemble those of E2, it is possible that *P. mirifica* extract and its major isoflavone compound, puerarin, may also induce positive osteogenic effect on osteoblasts.

It was noteworthy that although Thai *P. mirifica* contained some isoflavones similar to that found in Chinese and Korean *Pueraria lobata* (Cherdshewasart et al. 2007b), Kim et al. (2003) showed that Puerariae radix from different geographical regions exhibited different constituents and estrogenic activity as determined by uterotrophic assay. Specifically, the estrogenic activity of *P. mirifica* extract was stronger than that of *P. thunbergiana* (*lobata*). The *P. lobata* extract also contained kudzusapogenols and soyasaponin, both of which were not reported in *P. mirifica* extract (Malaivijitnond 2012). Up till now, the effect of *P. mirifica* extract on osteoblasts has never been investigated.

Although it was possible that *P. mirifica* extract prevented bone loss by promoting bone formation, whether it affected proliferation or differentiation stage of osteoblasts was not known. Under normal conditions, to induce bone formation, stromal cells or osteoprogenitor cells undergo proliferation, followed by differentiation with a slowdown of proliferation (Zaidi 2007; Owen et al. 1990). When osteoblasts differentiate, they sequentially express different markers specific to each stage of maturation, i.e., *runt*-related transcription factor 2 (Runx2), osterix, ALP, and osteocalcin in this order (Komori 2006; Stein et al. 2004). Moreover, differentiated osteoblasts also express the receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG), both of which are commonly used as markers for the assessment of osteoblast-regulated osteoclast function and bone resorption (Lin et al. 2007; Abdallah et al. 2005).

Therefore, the objectives of the present study were (i) to investigate the effect of *P. mirifica* extract and its major isoflavone, puerarin, on proliferation and viability of osteoblasts, (ii) to determine the direct effect of *P. mirifica* extract and puerarin on the expression of differentiation markers in osteoblasts, and (iii) to investigate whether ER mediated the osteoregulatory actions of *P. mirifica* extract and puerarin.

Materials and methods

Preparation of P. mirifica extract and high-performance liquid chromatography (HPLC)

The tuberous roots of *P. mirifica* cultivar SARDI 190 (lot no. 0070317) were purchased from Dr. Sompoch Tubcharoen, Kasetsart University, Kamphaeng Saen Campus, Thailand, and was authenticated as *P. mirifica* by comparing with the voucher specimens (nos. BCU010250 and BCU011045) kept at the Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand. The specimens were washed, sliced, dried in a hot-air oven at 70 °C, and ground. One gram of tuberous powder was extracted twice by mixing with 4 ml of 70% ethanol (Urasopon et al. 2008). The supernatants collected from two extractions were mixed together and dried for 4 h in a centrifugal concentrator at room temperature. Dry sample was kept at 4 °C until used in cell culture, and some portion was used for HPLC analysis.

The concentrations of isoflavones in *P. mirifica* extract (i.e., puerarin, daidzin, genistin, daidzein and genistein) were determined by HPLC, as described previously (Malaivijitnond et al. 2004). Briefly, 10 μl of extracted solution was injected through a sensory guard column [model Hyperclone ODS(C18); Phenomenex, Torrance, CA, USA] into a HPLC system (model Agilent 1000; Agilent, Waldbronn, Germany) equipped with a reverse phase Symmetry C18 column (250 mm × 4.6 mm, 5 μm; Phenomenex). Mobile phase consisted of 0.1% vol/vol phosphoric acid and acetonitrile with gradient elution (flow rate 1 ml/min). Ultraviolet detection was performed at a wavelength corresponding to the most intense absorption maximum at 255 nm. The isoflavone concentrations in each sample were analyzed in duplicate by comparing the retention times, and the amounts were quantified using the peak area of the standard curves. The standard compounds of daidzin, genistin, daidzein, and genistein (catalog nos. 30408, 48756, D7802 and G6649, respectively) were purchased from Sigma (St. Louis, MO, USA), and puerarin (catalog no. P5555) was purchased from Fluka (Buchs, Switzerland).

Cell culture

Rat osteoblast-like UMR106 cells [American Type Culture Collection (ATCC) no. CRL-1661] were cultured in 100-mm petri-dish with Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; PAA, Pasching, Austria), and 100 U/ml penicillin-streptomycin (Gibco, Grand Island, NY, USA). Cells were maintained at 37 °C in 5% CO_2 incubator, and subcultured according to the ATCC's protocol.

Experimental design

Prior to determination of osteoblast proliferation and differentiation marker expression, UMR106 cells were first investigated for the mRNA expression of ER-α and ER-β by quantitative real-time PCR (qRT-PCR). To investigate the time-dependent effect of E2 on osteoblast mRNA expression, UMR106 cells were incubated with 10 nmol/l E2 (Sigma) for 24, 48 and 72 h before determining the mRNA expression of ALP, an osteoblast differentiation marker. Thus, the incubating time (i.e., 48 or 72 h) was used in the subsequent experiments. To determine the dose-dependent effects of *P. mirifica* extract and related isoflavones on osteoblasts, confluent UMR106 cells were incubated for 48 or 72 h with vehicle [0.3% vol/vol dimethyl sulfoxide (DMSO); control group], or various concentrations of genistein (positive control; 0.1, 10 and 1000 nmol/l), *P. mirifica* extract (1, 10 and 100 μg/ml) or puerarin (major component of *P. mirifica* extract; 0.1, 10 and 1000 nmol/l). Thereafter, cell proliferation and viability were determined by 5-bromo-2′-deoxyuridine (BrdU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, respectively, while the mRNA expressions of osteoblast differentiation markers (i.e., Runx2, osterix, ALP, osteocalcin, RANKL and OPG) were quantified by qRT-PCR. Finally, to demonstrate whether *P. mirifica* extract exerted its osteogenic effect through ER in the same manner