Equol promotes rat osteoblast proliferation and differentiation through activating estrogen receptor

J. Wang¹², J. Xu¹, B. Wang¹², F.R. Shu¹², K. Chen¹² and M.T. Mi¹²

¹Department of Nutrition and Food Hygiene, College of Military Preventive Medicine, Third Military Medical University, Chongqing, China
²Chongqing Key Laboratory of Nutrition and Food Safety, Chongqing Medical Nutrition Research Center, Chongqing, China
³Department of Nutrition, Xinqiao Hospital, Third Military Medical University, Chongqing, China
⁴Department of Endocrinology, Xinqiao Hospital, Third Military Medical University, Chongqing, China

Corresponding author: M.T. Mi
E-mail: mimantian@126.com

Received July 1, 2013
Accepted December 3, 2013
Published July 4, 2014
DOI http://dx.doi.org/10.4238/2014.July.4.21

ABSTRACT. Phytoestrogens have been suggested as alternative treatment for postmenopausal osteoporosis. Equol, a metabolite of daidzein, has been shown to inhibit bone loss in ovariectomized mice and rats. However, whether or not equol influences the formation of bone has not yet been investigated. Therefore, we investigated the effect of equol on the proliferation and differentiation of rat primary osteoblasts and explored the involved mechanisms. Different equol concentrations significantly promoted the proliferation of osteoblasts after 48- and 72-h incubations. The alkaline phosphatase (ALP) activity also increased significantly in all of the equol and 17β-estradiol (E₂) groups, except for the lowest (0.01 µM) equol group. Equol also significantly elevated the
osteocalcin levels. The effects of equol on osteoblast proliferation, ALP activity, and osteocalcin levels were blocked by the estrogen receptor (ER) antagonist ICI182780. After a 24-h incubation, the expression of protein kinase C alpha (PKCα) in osteoblasts was significantly increased by equol. In conclusion, our study demonstrated that equol could promote the proliferation and differentiation of rat osteoblasts through activating the ER-PKCα-related signaling pathway, suggesting that equol could promote bone formation. These results suggest that equol could be a potential alternative agent for the management of postmenopausal osteoporosis.

**Key words:** Osteoblast; Osteoblast proliferation; Equol; Postmenopausal osteoporosis; Osteoblast differentiation

**INTRODUCTION**

Estrogen deficiency is recognized as the main reason for the occurrence of osteoporosis in postmenopausal women (Riggs et al., 2002). Due to the side effects of hormonal replacement therapy, phytoestrogens, which are plant-derived substances with estrogenic activity, have been suggested as alternatives for the treatment of postmenopausal osteoporosis without displaying cancer-related side effects (Zavatti et al., 2013).

Among the phytoestrogens, isoflavones have been shown to be capable of increasing the bone mineral density in ovariectomized animals and postmenopausal women (Occhiuto et al., 2007; Shedd-Wise et al., 2011; Chang et al., 2013). Equol, a metabolite of daidzein, which is a major isoflavone of soybean, is more biologically active than any other isoflavone aglycone (Yuan et al., 2007; Setchell and Clerici, 2010). Equol was shown to inhibit bone loss in ovariectomized mice and rats (Fujioka et al., 2004; Ohtomo et al., 2008; Kolios et al., 2009). In an *in vitro* study, equol significantly inhibited the osteoclast formation induced by 1α, 25(OH)2D3 (Ohtomo et al., 2008). However, whether or not equol might influence the formation of bone has not yet been investigated. Therefore, we aimed to study the effect of equol on the proliferation and differentiation of rat primary osteoblasts and to explore the involved mechanisms.

**MATERIAL AND METHODS**

**Reagents**

Alpha minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT, USA). Equol, 17β-estradiol (E2), ICI182780, vitamin C, and sodium β-glycerophosphate (β-GP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). P-nitrophenol phosphate and the alkaline phosphatase (ALP) and hydroxyproline assay kits were purchased from the Nanjing Jiancheng Biological Engineering Institute.

**Cell culture**

Primary osteoblasts were isolated from neonatal calvaria of 1-day-old Wistar rats as
Equol promotes osteoblast proliferation and differentiation

described previously (Hu et al., 2012). Briefly, calvaria of neonatal rats were cut into small pieces and cultured for 5 days on collagen gel prepared in α-MEM containing 10% FBS. Pre-osteoblasts grown from the calvarium were collected by treatment of collagen gel cultures with collagenase, and were then further cultured in α-MEM with 10% FBS. The medium was changed three times a week.

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay

Primary rat osteoblasts were seeded on 96-well plates at a 1 x 10^4 cells/well density in phenol-red-free α-MEM supplemented with 10% charcoal-stripped FBS 24 h before treatment with 0.01-1 µM equol, 0.01-1 µM E_2, or 0.01-1 µM equol/E_2 combined with 1 µM ICI182780. Twenty-four or forty-eight hours later, 10 µL 5 mg/mL MTT solution was added to each well. The plates were incubated at 37°C for 4 h, and then the supernatant was discarded and 100 µL dimethyl sulfoxide was added to each well and mixed thoroughly before taking measurements in a microplate reader.

Measurement of ALP activity

Primary rat osteoblasts were seeded onto 24-well plates at a 1 x 10^5 cells/well density in phenol-red-free α-MEM supplemented with 10% charcoal-stripped FBS. After 24 h, they were subsequently cultured for 7 or 14 days in differentiation medium consisting of 50 mg/L ascorbic acid and 10 mM β-GP. The cultures on 24-well plates were rinsed with phosphate-buffered saline. The cells were sonicated in 0.1 M Tris buffer, pH 7.2, containing 0.1% Triton-X-100. The ALP activity was measured with an ALP detection kit according to manufacturer instructions.

Statistical analysis

Data are reported as means ± SD. Statistical analysis was performed using ANOVA followed by Fisher protected least significant differences. P < 0.05 was considered to be statistically significant.

RESULTS

Identification of primary osteoblasts by ALP staining

The morphology of the cultured osteoblast cells was observed under a light microscope (Figure 1A). There were many shapes of osteoblasts observed, including stellate-shaped, short spindle-shaped, and fusiform-shaped cells. Osteoblast staining showed that there were many ALP-positive dark brown or black particles in the cytoplasm of the cells (Figure 1B), suggesting that the isolated cells were indeed osteoblasts.

Effect of equol on the proliferation of primary osteoblasts

To investigate the effect of equol on the proliferation of cultured osteoblasts, the MTT
assay was performed. The results showed that all of the equol concentrations tested promoted the proliferation of osteoblasts after 48- and 72-h incubations, which was consistent with the effect of E₂ (Figure 2). This effect was blocked by the estrogen receptor (ER) antagonist ICI182780.

Figure 1. Identification of isolated rat osteoblasts. A. Light microscope observation of the osteoblasts (40X). B. ALP staining of the isolated osteoblasts (100X).

Effect of equol on the ALP activity of primary osteoblasts

To further study the effect of equol on the differentiation of cultured osteoblasts, we determined the ALP activity of the osteoblasts. After 48- and 72-h incubations with different equol or E₂ concentrations, the ALP activity increased significantly in all of the equol and E₂ groups except for the 0.01 μM equol group (Figure 3). When co-incubated with ICI182780, the effects of equol and E₂ on the ALP activity were abolished.

Figure 2. Effect of equol on the proliferation of primary osteoblasts. Osteoblasts were seeded on 96-well plates. After 24 h, the cells were treated with equol (0.01-1 μM), E₂ (0.01-1 μM) or equol/E₂ (0.01-1 μM) combined with ICI182780 (1 μM) for 48 or 72 h, then MTT assay was performed. *P < 0.05, **P < 0.01, compared with the control group; #P < 0.05, ##P < 0.01 compared with equol or E₂ groups at the same concentration.

Effect of equol on the ALP activity of primary osteoblasts

To further study the effect of equol on the differentiation of cultured osteoblasts, we determined the ALP activity of the osteoblasts. After 48- and 72-h incubations with different equol or E₂ concentrations, the ALP activity increased significantly in all of the equol and E₂ groups except for the 0.01 μM equol group (Figure 3). When co-incubated with ICI182780, the effects of equol and E₂ on the ALP activity were abolished.
Effect of equol on the osteocalcin level in primary osteoblasts

Next, the osteocalcin levels in osteoblasts were examined. The results showed that the osteocalcin levels in the equol- or E2-treated osteoblasts were increased significantly (Figure 4). Equol showed a weak effect on the regulation of osteocalcin levels at the same concentration. The effects of both equol and E2 on osteocalcin levels were blocked by ICI182780.

Figure 3. Effect of equol on the ALP activity of primary osteoblasts. Osteoblasts were seeded on 12-well plates. After 24 h, the cells were treated with equol (0.01-1 μM), E2 (0.01-1 μM) or equol/E2 (0.01-1 μM) combined with ICI182780 (1 μM) for 48 or 72 h, then ALP activity was determined as described above. *P < 0.05, **P < 0.01, compared with the control group; #P < 0.05, ##P < 0.01 compared with equol or E2 groups at the same concentration.

Figure 4. Effect of equol on the osteocalcin in primary osteoblasts. Osteoblasts were seeded on 12-well plates. After 24 h, the cells were treated with equol (0.01-1 μM), E2 (0.01-1 μM) or equol/E2 (0.01-1 μM) combined with ICI182780 (1 μM) for 48 or 72 h, then the osteocalcin levels were determined. *P < 0.05, **P < 0.01, compared with the control group; #P < 0.05, ##P < 0.01 compared with equol or E2 groups at the same concentration.
Equol upregulated the expression of protein kinase C alpha (PKCα)

Finally, we determined the expression of PKCα after a 24-h incubation with equol or E₂. As shown in Figure 5, the expression of PKCα was significantly increased in both equol- and E₂-treated cells.

**DISCUSSION**

In the present study, our results showed that equol could promote the proliferation, and increase the ALP activity and osteocalcin levels of primary osteoblasts, and that these effects were blocked by the ER antagonist ICI182780. In addition, equol elevated the expression of PKCα, suggesting that the effects of equol are mediated by a PKC-related pathway.

Bone is one of the most important target organs of estrogen. Estrogen deficiency has been recognized as one of the most important causes of osteoporosis, particularly in menopausal women (Corina et al., 2012; Tyagi et al., 2012). Hormone therapy is widely used for the management and prevention of postmenopausal osteoporosis in older women; however, the occurrence of undesired side effects has limited the use of hormone therapy (Bakhireva et al., 2010). Previous studies have shown that phytoestrogens are a suitable alternative for the treatment of postmenopausal osteoporosis (Al-Anazi et al., 2011; Zavatti et al., 2013). Phytoestrogens could exert weak estrogen-like effects without the side effects of estrogen (Dang and Lowik, 2005). Soy isoflavone, which is a type of phytoestrogen with a similar molecular structure to estrogen, could combine with the ER (Tang et al., 2008). In previous studies, soy isoflavones and their metabolites, soy isoflavone aglycones, were shown to be capable of reducing bone loss in ovariectomized rats, suggesting that soy isoflavone and its metabolites may be active substances in the prevention of osteoporosis in postmenopausal women (Mathey et al., 2007; Shiguemoto et al., 2007). However, the outcomes of soy isoflavone treatment for the prevention of menopausal bone loss are conflicting. Although many researchers have shown that daily supplementation with soy isoflavones could prevent postmenopausal osteoporosis and improve bone strength, thus decreasing the risk of fracture in menopausal women (Wong et al., 2009; Taku et al., 2010, 2011), other studies have indicated that the daily administration of soy isoflavones could not prevent bone loss in menopausal women (Arjmandi et al., 2005; Levis et al., 2011).
Equol promotes osteoblast proliferation and differentiation

Equol is a metabolic end-product of soy isoflavones with high stability and a slow excretion speed. In addition, it has stronger physiological activity compared to its precursor soy isoflavone aglycone. In the current study, we showed that equol could promote the proliferation of primary cultured osteoblasts. We also evaluated the effects of equol on osteoblast differentiation by measuring two osteoblast differentiation markers, ALP and osteocalcin. The results showed that equol enhanced ALP activity and increased the osteocalcin content. The stimulatory effect of equol on osteoblast proliferation, ALP activity, and osteocalcin content was blocked by the ER antagonist ICI182780, suggesting that equol acts as an ER agonist on osteoblasts. However, it was previously found that only 30 to 50% of individuals produce equol, and the metabolic ability of equol differs among different races (Setchell et al., 2003; Jou et al., 2008). Therefore, the controversial results of soy isoflavones in the management of postmenopausal osteoporosis may be due to differences in the production and metabolism of equol in different populations.

PKC signaling mediates various cellular functions, such as cell proliferation and differentiation, and the signaling of various osteogenic regulators, such as parathyroid hormone and estrogen (Migliaccio et al., 1998; Capiati et al., 1999; Lampasso et al., 2002; Longo et al., 2006). Inhibition of PKCα activity in osteoblasts results in a marked decrease in mitogen-activated protein kinase activity, which is known to play an important role in the proliferation of cells, leading to a significant decrease in proliferation (Lampasso et al., 2002); on the other hand, activation of PKCα promotes human osteoblast proliferation (Villa et al., 2003; Hsieh et al., 2010; Liang et al., 2012). In rat and human osteoblast-like cell lines, PKC signaling can induce the transcription of osteocalcin (Boguslawski et al., 2000). However, the function of PKCα in osteoblast differentiation is controversial. PKCα is necessary for basic fibroblast growth factor-induced bone formation (Tang et al., 2004), whereas in MC3T3-E1 cells, overexpression of PKCα decreases ALP activity and attenuates osteoblastic differentiation (Nakura et al., 2011). In this study, we showed that both equol and E, upregulated the expression of PKCα, suggesting that the effect of equol on osteoblast proliferation, ALP activity, and osteocalcin content may be mediated by PKCα-related signaling.

In conclusion, our study demonstrated that equol may promote the proliferation and differentiation of rat osteoblasts through activating the ER-PKCα-related signaling pathway, suggesting that equol may promote bone formation. These results suggest that equol could be a potential alternative agent for the management of postmenopausal osteoporosis. However, since this is only an in vitro study, further studies should be performed to validate the anti-postmenopausal osteoporosis effects of equol in vivo.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (#81273058) and the Special Funds of Chongqing Key Laboratory for Independent Research Projects (#2011).

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES

Equol promotes osteoblast proliferation and differentiation

Maturitas 70: 333-338.