Icariin induces osteoblast proliferation, differentiation and mineralization through estrogen receptor-mediated ERK and JNK signal activation

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Abstract

Icariin (C33H40O15), the main active flavonoid glucoside isolated from Herba epimedii (HEF), is used in Chinese traditional medicine for treatment of kidney, joints, and liver disorders. In ovariectomized rats, icariin has been shown to prevent ovariectomy-induced bone loss and restored femoral strength (Hsieh et al., 2010; Nian et al., 2009; Mok et al., 2010). It could completely correct the decrease in the concentration of estrogen in serum and also could partly restore the decreased weight of uterus in ovariectomized rats (Nian et al., 2009; Chen et al., 2005). Recently, a 24-month randomized double-blind placebo-controlled clinical trial showed that HEF extracts (containing a daily dose of 60 mg icariin, 15 mg daidzein, and 3 mg genistein) prevent bone loss in postmenopausal women (Zhang et al., 2007). Although accumulative evidence have indicated anabolic effects of icariin on osteoblasts and bone formation, the detailed molecular mechanisms of icariin remained largely unknown.

The mitogen-activated protein kinases (MAPKs) are the family of secondary messengers that convey signals from the cell surface to the nucleus in response to a wide range of stimuli, including hormones, chemicals and stress. (Yang et al., 2013). Three major

1. Introduction

Osteoporosis, as one of the most common bone disorders, is characterized by a systemic decrease in bone density and microarchitectural integrity that increases likelihood of fragility fractures. Current treatments for osteoporosis are dominated by drugs that inhibit bone resorption although they also suppress bone formation that may contribute to pathogenesis of osteonecrosis (Bone, 2012). To restore the extensive bone loss, there is a great need for anabolic treatments that induce osteoblasts to build new bone. In addition to the currently approved parathyroid hormone or novel anabolic drugs i.e. antagonists of calcium sensing receptor or Wnt signaling inhibitor (Zaidi et al., 2007; Rachner et al., 2011), isoflavonoids are promising preventive treatment for osteoporosis in clinical (Ma et al., 2008).
families of MAPKs are extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun N terminal kinase (JNK) and each of these consists of their own subfamilies. MAPKs are activated via phosphorylation of tyrosine and threonine residues by upstream kinases or receptors. Activated MAPKs subsequently phosphorylate their specific substrates at serine and/or threonine residues to either positively or negatively regulate substrate and thus the entire signaling cascade activity (Yang et al., 2013). This complex control of signaling cascades modulate gene expressions to regulate mitosis, proliferation, differentiation, and apoptosis (Johnson and Lapadat, 2002). Numerous bone-active agents, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and estrogen, have been shown to induce ERK signaling in osteoblasts (Chau et al., 2009; Fulzele et al., 2007).

Cytokines activate p38 and JNK in osteoblasts (Pantouli et al., 2005; Matsuguchi et al., 2009), and subsequently promote osteogenic lineage specification of osteo-chondroprogenitor cells, osteoblast proliferation and differentiation (Matsushita et al., 2009; Wang et al., 2007).

Given the anabolic effects of Icariin and the roles of MAPK signaling in osteoblasts, here, we investigated the effect of Icariin on the proliferation, differentiation and mineralization of MC3T3-E1 cells. Furthermore, whether Icariin could affect MAPK activation was explored.

2. Materials and methods

2.1. Materials

MC3T3-E1 osteoblastic cell subclone 4 line was purchased from American Type Culture Collection (ATCC). α-modified minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Beijing, China). Icariin (Fig. 1) was obtained from Jiangsu Provincial Institute of Traditional Chinese Medicine, and purity was 98%. RT-PCR and Real-time PCR kits were purchased from Invitrogen (Beijing, China). Alizarin red S was purchased from Genmed Scientific Technology (Shanghai, China). Alizarin red S was purchased from Genmed Scientific Technology (Shanghai, China). ICI182780, Nilutamide, UO126 was purchased from Cell Signaling Technology (Shanghai, China). ICI182780, Nilutamide, UO126 was purchased from Cell Signaling Technology (Shanghai, China). ICI182780, Nilutamide, UO126 was purchased from Cell Signaling Technology (Shanghai, China). ICI182780, Nilutamide, UO126 was purchased from Cell Signaling Technology (Shanghai, China). ICI182780, Nilutamide, UO126 was purchased from Cell Signaling Technology (Shanghai, China). ICI182780, Nilutamide, UO126 was purchased from Cell Signaling Technology (Shanghai, China). ICI182780, Nilutamide, UO126 was purchased from Cell Signaling Technology (Shanghai, China).

2.2. Cell proliferation assay

Cell proliferation activity was assessed by 5-bromo-2-deoxyuridine (BrdU) incorporation using Cell Proliferation ELISA, BrdU kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. Cells were seeded in a 96-well plate at a density of 1 x 104 cells per well in 100 μl of culture medium. After 1 day, culture media were replaced with α-MEM containing 2.5% FBS and kanamycin, and then the cells were treated with Icariin for various time at 37 °C. BrdU labelling solution (100 mM) was added at 10 μl per well, and the cells were incubated for an additional 4 h at 37 °C. After this, the labelling media were removed, the cells were fixed and DNA was denatured with FixDenat solution. The cells were incubated with peroxidase-conjugated anti-BrdU antibody for 1.5 h at room temperature. The cells were then washed three times with PBS, followed by the addition of substrate solution (tetraethyl-benzidine) at 100 μl per well. After 15 min incubation, 1 M H2SO4 was added at 25 μl per well to stop the peroxidase reaction, and then absorbance of wells was measured at 450 nm using Multiskan FC (Thermo Fisher Scientific, Waltham, MA). In some experiments, cells were treated with UO126, SB203580, or SP600125 for 1 h before and during exposure to Icariin.

2.3. Water-soluble tetrazolium (WST) assay

Cell number was assessed as dehydrogenase activity by using Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). In this assay, WST-8 is reduced to water-soluble formazan outside the cells. Cells were seeded in a 96-well plate at a density of 10,000 cells per well in 100 μl of culture medium. After 1 day, culture media were replaced with α-MEM containing 2.5% FBS and kanamycin, and then the cells were treated with Icariin for various time. CCK-8 solution was added at 10 μl per well, and the cells were incubated for an additional 1.5 h at 37 °C. Then absorbance of wells was measured at 450 nm using Multiskan FC (Thermo Fisher Scientific).

2.4. MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the effects of Icariin on MC3T3-E1 cell viability. Cells were seeded on a 96-well plate (1 x 105 cells/ml) for 24 h prior to treatment with various doses of Icariin. After 24, 48 and 72 h of Icariin treatment, cells were washed with PBS, then 20 μl of 5 mg/ml MTT was added to each well and incubated for 4 h at 37 °C. The precipitated formazan was subsequently dissolved in dimethyl sulfoxide. The absorbance (ABS) in each well was then recorded at 570 nm using a microplate reader (KHB, Shanghai, China), and cell viability (%) was calculated against untreated cells as per following:

\[
\% \text{Cell viability} = \frac{\text{ABS(without cells)} - \text{ABS(treatment, without cells)}}{\text{ABS(control, with cells)} - \text{ABS(control, without cells)}} \times 100\%
\]

2.5. Osteoblast apoptosis assay

To determine the effect of Icariin on MC3T3-E1 cell apoptosis, MC3T3-E1 cells were seeded on a 96-well plate (1 x 105 cells/ml) and treated with Icariin in the absence of serum for 72 h. MC3T3-E1 cells were harvested and suspended in binding buffer containing annexin V-PE (BD Pharmingen) incubated for 20 min at room temperature in the dark. The cells were then analyzed by flow cytometry. Propidium iodide positive cells were excluded from analysis. In situ apoptotic cells were detected using the TUNEL assay (Apoptag Plus Fluorescein In Situ Apoptosis Detection Kit; Chemicon, Millipore) according to the manufacturer’s instructions. Cells with positive ApopTag labeling were counted and expressed as a percentage of total cell number.
2.6. Caspase-3 activity assay

MC3T3-E1 cells were treated with Icariin in the absence of serum and harvested at 72 h. Caspase activity was assayed by a fluorometric kit purchased from R&D Systems. In brief, the cells were washed with ice-cold PBS and lysed in a cell lysis buffer provided in the kit. The caspase-3 (DEVD-pNA) substrate was added to the cell lysates in a 96-well plate, and the plates were then incubated for 1 h. The release of pNA was measured at 405 nm in a microplate reader.

2.7. MC3T3-E1 cell differentiation and mineralization assay

MC3T3-E1 cells were plated at a density of 1 × 105 cells/ml in 12-well plates and cultured in α-MEM containing 10% FBS, 2 mM l-glutamine, and a mixture of 100 U/l penicillin and 100 mg/l streptomycin, at 37 °C in a humidified atmosphere of 95% air and 5% CO2. After cells reached confluence, the medium was replaced with α-MEM containing 5 mM β-glycerophosphate and 400 uM ascorbic acid to facilitate in vitro mineralization. Cells were then treated with several doses of Icariin as indicated in Section 3. The culture media, which included Icariin, were replaced every 3 days. For differentiation activity measurement, culture media and cell culture media, which included Icariin, were replaced every 3 days. For differentiation activity measurement, culture media and cell monolayers were harvested at 7 days after confluence. For analysis of mineralized nodule formation, cells were fixed at 14 days and stained with Alizarin red S as described below. In some experiments, cells were treated with UO126, SB203580, SP600125, ICI182780 or Nilutamide for 1 h before and during exposure to Icariin.

2.8. Alizarin red S staining

To determine bone nodule formation, MC3T3-E1 cells were washed twice with PBS then fixed with 4% paraformaldehyde in PBS, pH 7.4. The fixed MC3T3-E1 cells were stained with 0.1% Alizarin red S solution for 60 min, then washed twice with double distilled water, and then taken pictures with Nikon Camera. For the quantification of staining density, Alizarin red S staining was released from the cell matrix by incubation with 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for 20 min. The Alizarin red S concentration was determined by measuring the absorbance at 562 nm (Bio-Tek, Model ELX 808, CA, USA). This experiment was repeated for three times and data from representative experiments was shown.

2.9. Reverse transcription and real-time PCR analysis

Total RNAs were prepared as described previously with TRizol reagent and the first strand cDNA was synthesized with 500 ng of total RNA, 50 μM of oligoT primer, 100 μM of random 6 mers, 0.5 μl of prime script RT enzyme mix, and 2 μl 5’ primerscript buffer (DRR037A, TaKaRa Biotechnology (Dalian) Co., Ltd.). The SYBR green-based quantitative PCR amplification was then performed using Rotor Gene Real-time PCR system and the aim genes amplification was performed using a reaction mixture (25 μl) containing 100 ng of cDNA, 12.5 μl of 2 × SYBR Premix Ex Taq (DRR041A, TaKaRa Biotechnology (Dalian) Co., Ltd.) and 0.3 μM primers. The PCR reaction consisted of three segments. The first segment at 95 °C for 10 min was for the activation of the polymerase and the second one corresponded to 3-step cycling (40 cycles) at 94 °C for 40 s (denaturation), 60 °C for 40 s (annealing), and 72 °C for 1 min (extension). The third segment was for the generation of PCR product temperature dissociation curves (also called ‘melting curves’) at 95 °C for 1 min, 55 °C for 30 s, 95 °C for 30 s. All reactions were run in triplicate and analyzed by the 2-ΔΔCT method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control gene. The primers generated were based on the published mouse sequences (Table 1).

2.10. Western blot analysis

Each culture was rinsed with PBS twice and solubilized with radio-immuno precipitation assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate) containing 50 mg/ml leupeptin, 10 mg/ml aprotonin, 2 mM EDTA and 1 mM vanadate. After 15 min on ice, the supernatants were collected by centrifugation. Protein concentration was determined with the Micro BCA Protein Assay Reagent Kit (Kangchen Bio-tech Company, Shanghai, China). 48 μg of cellular protein extract was subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred onto PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was immersed in blocking solution consisting of TBST (Tris 20 mM, pH 7.4, NaCl 137 mM, 0.1% Tween 20) and 5% skim milk, and washed twice with TBST for 15 min. For detection, the membrane was incubated overnight with primary antibody at 4 °C. After incubation, the membrane was washed three times for 10 min, in TBST with 0.05% Tween-20, followed by incubation with HRP-conjugated appropriate secondary antibody (1:1000 in 1% milk, 1% BSA in TBST) for 60 min at room temperature. Finally, the membrane was washed repeatedly with TBST and developed with enhanced chemiluminescence (ECL) kit (GE Healthcare, Beijing, China).

2.11. Statistical analysis

All results are expressed as means ± S.D. Means were statistically compared by ANOVA followed by Student’s t-test. A value of P < 0.05 was considered to be significant.

3. Results

3.1. Icariin promotes osteoblast proliferation and reduces osteoblast apoptosis in a dose-dependent manner

Osteoblasts undergo three stages of maturation, namely proliferation, differentiation, and mineralization. We first estimated the effect of Icariin on osteoblast proliferation by BrdU incorporation and formazan formation. MC3T3-E1 osteoblastic cells were cultured in a 96-well plate and then treated with various doses of Icariin (0.1–100 nM) for 72 h. As shown in Fig. 2A and B, compared with control group, MC3T3-E1 treated with Icariin showed significant increase in DNA synthesis and cell number. We next examined the gene expression changes of genes involved in cell cycle regulation by real-time PCR after Icariin treatment. Icariin treatment dramatically elevated the mRNA levels of cyclin E, and showed a modestly stimulatory effect on PCNA expression, whereas Icariin significantly decreased the expression of Cdkn2b mRNA in MC3T3-E1 cells (Fig. 2C). These data demonstrated that Icariin promoted osteoblast growth, at least in part by enhancing cell cycle activator, cyclin E.
expression and reducing the expression of Cdkn2b, a cell cycle inhibitor.

We further determined whether Icariin affect MC3T3-E1 cell survival. By MTT cell viability assay, we found that Icariin treatment significantly increased osteoblast viability as compared to untreated control group in a dose-dependent manner (Fig. 2D). The maximal cell viability was observed at the 10 nM dose of Icariin. Furthermore, the effect of Icariin on MC3T3-E1 cell apoptosis in the serum-deprived culture condition was also investigated. As expected, Icariin could effectively inhibit serum starve-induced osteoblast apoptosis (Fig. 2E). Moreover, the caspase-3 activity was also significantly suppressed after Icariin treatment (Fig. 2F). Taken together, these results suggest that Icariin treatment could increase metabolically active cells by promoted osteoblastic proliferation and reduced apoptosis.

3.2. Icariin promotes osteoblast differentiation and mineralization

Next we examined the effect of Icariin on osteoblast differentiation. First we examined the effect of Icariin treatment on gene expression of common genetic markers of osteoblast differentiation. Collagen I (Col I) is an early marker of pre-osteoblast lineage which progressively expresses alkaline phosphatase (ALP) during maturation stage and osteopontin (OPN) during mineralization phase. Icariin treatment (0.1–100 nM) dramatically and significantly increased Col I and ALP mRNA expression in a dose-dependent manner until 10 nM concentration whereas OPN expression was marginally but significantly increased from 1 nM concentration onward (Fig. 3A). These data suggest that Icariin treatment mainly influenced the early stages of osteoblast differentiation.

Therefore, we next determined if Icariin treatment could promote osteoblast mineralization. To exclude the effect of Icariin on osteoblast mineralization was due to the increase of cell number, MC3T3-E1 cells were firstly cultured in osteogenic differentiation medium for 7 days, and then treated with various doses of Icariin for 7 days. MC3T3-E1 cells were stained with Alizarin red S at 14 days for the identification of mineralized nodules. As shown in Fig. 3B, all doses of Icariin significantly increased the staining density of Alizarin red S in visible observation and quantification by spectrophotometer, as compared to untreated control in which there were almost no mineralized nodules. An increase of nodule formation at lower doses of Icariin (0.1 nM) was about 50%, and higher doses of Icariin (10, 100 and 1000 nM) further increased nodule formation (3–4 fold), compared to the control cells. Moreover, we further determined the effect of Icariin on cell proliferation during mineralization by BrdU incorporation assay. As shown in Fig. 3C, although Icariin showed marginally enhancement on cell proliferation, but had no significant effect, suggesting that the potential increase in mineralized colonies did not result from increase cell number. Taken together, these data suggest that Icariin also could promote osteoblast differentiation and mineralization.

3.3. Icariin activates ERK and JNK MAPK pathways in osteoblast

As described in introduction, numerous growth factors, hormones, and cytokines have been shown to activate ERK, JNK, or p38 MAPK in osteoblasts to induce cell proliferation and differentiation. Therefore, we examined activation statue of MAPK signaling pathways in MC3T3-E1 cells after Icariin treatment by western blot analysis. As shown in Fig. 4A, 10 nM Icariin treatment
induced phosphorylation and activation of ERK and JNK whereas it had no effect on p38 MAPK activation. ERK and JNK were activated within 5 min after Icariin treatment and reached peak activation at 15 min. To further exclude the possibility that p38 unactivation by Icariin was result from anti-p-p38 inactivation, BMP-2, a well-known stimulator for p38 activation in osteoclasts (Guicheux et al., 2003), was used to treat MC3T3-E1 cells as a postive control. The result showed that in MC3T3-E1 cells, p38 activation was observed after BMP-2 treatment, suggesting that anti-p-p38 can detect p38 activation. p38 inhibitor SB203580 (10 μM) also could suppress BMP-2-induced activation of p38 (Fig. 1). Thus, these data further demonstrated that p38 signaling was not involved in Icariin-induced intracellular signaling.

Next we examined if the proliferative effect of Icariin was mediated via MAPK signaling. Icariin treatment-induced MC3T3-E1 proliferation was attenuated to control level in the presence of ERK inhibitor U0126 (10 μM) and JNK inhibitor SP600125 (10 μM) by Brdu incorporation assay. However, p38 inhibitor SB203580 (10 μM) showed no effect on Icariin-induced proliferation of MC3T3-E1 (Fig. 1). The stimulatory and inhibitory effects of Icariin on cyclin E1 (Fig. 4C) and Cdkn2b (Fig. 4E) gene expression respectively were also significantly reversed by treatment with U0126 and SP600125, whereas SB203580 also showed no effect. The effect of Icariin on PCNA gene expression was not significantly altered by either of the inhibitors of MAPKs (Fig. 4D). These observations strongly suggest that Icariin promotes proliferation of MC3T3-E1 cell at least in part via the activation of ERK and JNK MAPK signaling pathways.

3.4. Estrogen receptor signaling is involved in Icariin-mediated anabolic effects

As described in introduction section, in ovariectomized rats and postmenopausal women, Icariin has been shown to prevent bone loss induced by lack of estrogen. Moreover, Icariin has been shown to increase the circulating levels of estradiol in ovariectomized rats. Therefore, we hypothesized that Icariin might signal through estrogen receptor by acting as a phytoestrogen or cooperate with estrogen receptor signaling. To address this question, we examined the effect of the estrogen receptor inhibitor on Icariin-induced MC3T3-E1 differentiation. We observed that the stimulatory effects of Icariin on Col I, ALP and OPN mRNA expressions were significantly attenuated in the presence of estrogen receptor signaling inhibitor ICI182780 but not with androgen receptor signaling inhibitor Nilutamide (Fig. 5A and C). Moreover, the effect was mediated partially via MAPK signaling since the ERK and JNK activation by Icariin was reduced in the presence of ICI182780 (Fig. 5D). We further found that the enhancement of MC3T3-E1 mineralization by Icariin was also attenuated by ICI182780 treatment but not Nilutamide, suggesting that estrogen receptor signaling was involved in Icariin-mediated the anabolic actions (Fig. 5E).
4. Discussion

Icariin, a major active constituent in Epimedium, has been shown to prevent osteoporosis in late postmenopausal women (Zhang et al., 2007). This effect of Icariin on osteoblasts is likely due to its anabolic action, including induction and promotion of osteoblastic differentiation (Xiao et al., 2005; Hsieh et al., 2011). The signaling mechanisms of Icariin action remain elusive. In this study, we found that Icariin promoted osteoblast proliferation and differentiation at least via ERK and JNK MAPK signaling pathways. Our data also revealed that Icariin might act through estrogen receptor or cooperation with estrogen receptor signaling.

It was demonstrated that activation of ERK and JNK signaling pathways are major promoters of osteoblast proliferation. Numerous factors have been reported to promote osteoblast proliferation and survival through ERK, including insulin (Yang et al., 2010), glucocorticoid (Horsch et al., 2007; Wu et al., 2011), hyperbaric oxygen (Hsieh et al., 2010) and fibroblast growth factor-6 (FGF-6) (Bosetti et al., 2010). In this study, we examined the effects of Icariin on osteoblast proliferation and survival and the expression of genes (cyclin E, PCNA, and Cdkn2b) involved in cell cycle by real-time PCR. PCNA is synthesized in early G1 and S phase of the cycle, and serves as an excellent marker of proliferating cells (Strzalka and Ziemienowicz, 2011). Cyclin E-Cdk2 complex is essential to drive the G1/S transition whereas CDKN2B inhibits cell cycle progression through G1 phase (Aleem et al., 2005). Our data also revealed that Icariin might activate AP-1 transcription factors. Among numerous transcription factors activated by MAPK signaling, well recognized factors include c-fos, c-jun, AP-1, CREB and NFAT. Many of these transcription factors are directly involved in cellular proliferation in numerous cell types. Previous study has shown that Icariin treatment did not increase CREB gene expression in hypothalamicus (Pan et al., 2010). However, in cardiomyocytes Icariin treatment resulted in NF-κB and AP-1 activation in p38- and ERK-dependent manner (Wo et al., 2008). NF-κB is a cytoplasmic protein which is normally present in inactive form bound to its inhibitory subunit IκB. In cardiomyocytes, Icariin rapidly induced IκB phosphorylation by p38 and ERK activation and its subsequent degradation, resulting in translocation of released NF-κB into the nucleus (Wo et al., 2008). Furthermore, the activated p38 and ERK also up-regulated c-fos and c-jun expressions, subunits of AP-1 transcription factor. The various members of the AP-1 complex are differentially expressed during osteoblast differentiation and maturation (Marie, 2008). It has known that some factors such as parathyroid hormone, transforming growth factor β (TGF-β), and 1,25-dihydroxy vitamin D, could induce AP-1 and then regulate osteoblast proliferation, differentiation, and apoptosis (Matsumoto et al., 2012). Fos and Jun are highly expressed early during osteoblast differentiation and their expression levels decline with extracellular matrix production and mineralization (Marie, 2008). Given the stimulatory effects of Icariin on osteoblast proliferation and early genes involved in osteoblast differentiation i.e. Col I and ALP, we speculate that Icariin activate AP-1 transcription

![Fig. 4. Effect of Icariin on MAPK signaling in MC3T3-E1 osteoblastic cells. (A) Phosphorylation of MAPKs in MC3T3-E1 osteoblastic cells was determined by Western-blot after treated with 10^{-7} M Icariin for various time. (B) The proliferation of MC3T3-E1 osteoblastic cells was detected by BrdU incorporation assay after treatment with 10^{-7} M Icariin and/or the inhibitors of MAPKs, including U0126 (10 μM), SB203580 (10 μM) and SP600125 (10 μM) for 72 h. (C) The levels of mRNAs encoding Cyclin E, PCNA and Cdkn2b in MC3T3-E1 osteoblastic cells were determined by real-time PCR after treatment with 10^{-7} M Icariin and/or the inhibitors of MAPKs for 72 h. SB: SB203580, SP: SP600125. Each value is the mean ± S.D.; * P < 0.05, ** P < 0.01, versus the control group; # P < 0.05, ## P < 0.01, versus Icariin group. Each figure is representative data from three to five independent experiments.](image-url)
factor by up-regulating Fos and Jun protein expressions via activation of ERK and JNK signaling. However, the precise downstream pathways or target transcription factors of activated ERK and JNK signaling in osteoblasts in response to Icariin need further study. Previous studies have shown that Icariin treatment can prevent bone loss due to lack of estrogen in ovariectomized rats and postmenopausal women. Therefore, we examined whether Icariin can affect estrogen receptor signaling. We found that the effects of Icariin on gene induction of ALP, Col I, and bone nodule formation in osteoblasts were partly attenuated by the estrogen receptor signaling inhibitor ICI182780 but not by Nilutamide, inhibitor of androgen receptor signaling. These data suggest that the effect of Icariin on osteoblasts was mediated, at least partially by estrogen receptor signaling. Typically, the majority of estrogen receptor α and β are found in the cytoplasm and nucleus, although a small amounts are expressed on cell membrane (Lorenzo, 2003). The cytoplasmic estrogen–estrogen receptor complex translocates to nucleus and binds to estrogen response element (ERE) located in the target gene promoters, and stimulates gene transcription (Marino et al., 2012). Other transcription factors in nucleus, that include NF-κB, AP-1 and SP-1, interact with the ligand-receptor complex to influence gene transcription (Marino et al., 2012). Such nuclear canonical pathway mediates the long term actions of estrogen. Alternatively, estrogen can induce rapid effects through membrane-bound estrogen receptors. Both estrogen receptor α and β as well as androgen receptor can accumulate in caveolae of cell membrane. Upon binding to their respective ligands, these receptors then activate ERK, JNK and p38 MAPK signaling pathways in osteoblasts to activate AP-1 and CREB transcription factors to induce gene expressions responsible for osteoblast survival and differentiation (Jensen et al., 2010; Wagner, 2010). Indeed, here, we found that estrogen receptor antagonist ICI182780 could significantly reduce Icariin-induced ERK and JNK phosphorylation and activation. Thus, we speculated that Icariin behaved as a phytoestrogen that signals through estrogen receptor α or β, which was further supported by the fact that only estrogen receptor, but not androgen receptor antagonist could attenuate Col I, ALP and OPN gene expression induced by Icariin treatment. In conclusion, our data showed that Icariin promoted osteoblast proliferation, differentiation and mineralization by activating ERK and JNK MAPK pathways. We also demonstrated that Icariin act as a phytoestrogen to activate estrogen receptor signaling pathway or cooperate with estrogen–estrogen receptor complex in nucleus. These findings provide a new insight into the effect of Icariin on osteoblast proliferation and differentiation and may ultimately contribute to the future treatment of osteoporosis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2013.05.039.

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