·论著·

Role of MAPK signal pathyway in the regulation of Cbfa1 protein expression in osteoblast by Icariin

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Abstract: **Objective** To investigate the effect of icariin (ICA) on mitogen-activated protein kinase (MAPK) signal pathway in rat osteoblasts cultured *in vitro* and the role of MAPK signal pathway in the icariin-promoting expression of core binding factor-1 (Cbfa1) in osteoblasts, and to elucidate the signal mechanism of icariin on osteoblasts. **Methods** Calvarial osteoblasts were obtained from newborn (< 24 h) SD rats by trypsin-collagenase digestion method. After 5 min, 10 min, 30 min, 60 min of treatment with icariin (10 ng/mL) or estrodial (E_2) (10^{-8} mol/L), total protein was isolated from osteoblasts and proteins of ERK, p-ERK, P38 and p-P38 were detected by western-blot analysis. Then calvarial osteoblasts were cultured in the medium containing icariin (10 ng/mL), estrodial (10^{-8} mol/L) with or without u0126, SB203580 for 24 h respectively, nucleus protein was isolated from osteoblasts and protein of Cbfa1 was detected by western-blot analysis. **Results** 1. The protein of p-ERK in calvarial osteoblasts increased at 30 min and lasted for 60 min ($P < 0.05$, contrast to the blank group) when treating osteoblasts with icariin(10 ng/mL); the protein of p-P38 in calvarial osteoblasts increased at 5 min and was at the peak at 30 min, and lasted for 60 min ($P < 0.05$, contrast to the blank group) when treating osteoblasts with icariin(10 ng/mL). 2. The protein of p-ERK in calvarial osteoblasts increased at 30 min ($P < 0.05$, contrast to the blank group) when treating osteoblasts with estrodial (10^{-8} mol/L); the protein of p-P38 in calvarial osteoblasts increased at 10 min and continuously increased to 30 min($P < 0.05$, contrast to the blank group) when treating osteoblasts with estrodial (10^{-8} mol/L). 3. Both icariin and estrodial could promote the expression of Cbfa1 protein ($P < 0.05$, contrast to the blank group) and this effect could be weakened by SB203580 or u0126. **Conclusion** Icariin and estrodial can activate ERK/MAPK and P38/MAPK signal pathways and promote the expression of Cbfa1 protein in osteoblasts respectively. ERK/MAPK and P38/MAPK signal pathway are involved in the processes of icariin-promoting and estrodial-promoting Cbfa1 expression.

Key words: Mitogen-activated protein kinase; Core binding factor-1; Icariin; Osteoblasts

MAPK信号通路在淫羊藿甙促进成骨细胞Cbfa1蛋白表达中的作用 张秀珍,宋利格,王博 同济大学附属同济医院内分泌科,上海 200065

摘要：**目的** 观察淫羊藿甙对体外培养大鼠成骨细胞丝裂原活化蛋白激酶(mitogen-activated protein kinase,MAPK)信号通路的影响以及MAPK信号通路在淫羊藿甙促成骨细胞核心结合因子α1(core binding factor-1,Cbfa1)蛋白表达中的作用,以探讨淫羊藿甙对成骨细胞作用的信号传导机制。**方法** 用酶消化法分离24 h内新生SD大鼠颅盖骨成骨细胞,进行原代培养。在培养液中加入淫羊藿甙(10 ng/mL),作用于成骨细胞5 min、10 min、30 min、60 min,抽提总蛋白,用Western blot法检测细胞中ERK、p-ERK、P38和p-P38蛋白的表达。用雌二醇(10^{-8} mol/L)作用于成骨细胞5 min、10 min、30 min,同上法检测细胞中ERK、p-ERK、P38和p-P38蛋白的表达。用淫羊藿甙(10 ng/mL)、雌二醇(10^{-8} mol/L)和u0126或SB203580单独或共同干预成骨细胞24 h,抽提核蛋白,用Western blot法检测Cbfa1蛋白的表达。**结果** ①淫羊藿甙作用于成骨细胞,30 min时可促进ERK蛋白的磷酸化,并可持续至60 min,和

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空白组比较,差异有显著性($P < 0.05$)淫羊藿甙作用于成骨细胞,5 min 时即可促进 P38 蛋白的磷酸化,在 30 min 时达高峰,并可持续至 60 min,和空白组比较,差异有显著性($P < 0.05$)。②雌二醇作用于成骨细胞,在 30 min 时可促进 ERK 蛋白的磷酸化,和空白组比较,差异有显著性($P < 0.05$)雌二醇作用于成骨细胞,在 10 min 时可促进 P38 蛋白的磷酸化,并可持续至 30 min,和空白组比较,差异有显著性($P < 0.05$)。③淫羊藿甙和雌二醇均能促进成骨细胞中 Cbfa1 蛋白的表达,和空白组相比,差异有显著性($P < 0.05$)。u0126 和 SB203580 可以抑制淫羊藿甙和雌二醇促进 Cbfa1 蛋白表达的作用。结论 ①淫羊藿甙和雌二醇均可以激活成骨细胞中 ERK/MAPK 和 P38/MAPK 信号通路;②淫羊藿甙和雌二醇均能促进成骨细胞中核转录因子 Cbfa1 的表达,并且 ERK/MAPK 和 P38/MAPK 信号通路参与了此过程。

关键词:丝裂原活化蛋白激酶;核心结合因子 α1;淫羊藿甙;成骨细胞

HERBA epimedii (which also goes by the names Yin Yang Huo and Horny Goat Weed) is a traditional botanical medicine used in China, Japan and Korea. As a herb, it has a history of traditional use for disorders of the kidneys, joints, liver, back and knees. Pharmacological studies have shown its potential activity against osteoporosis[1-3] and its main constituent is the total flavonoids of Herba epimedii (HEF), in which icariin is the main active constituent. At present, the anabolic effects of icariin on bone have been verified in many studies. In vitro, icariin enhances significantly the proliferation and differentiation of osteoblasts, which may be mediated by increasing the expression of BMP-2 mRNA[4-6]. Icariin could also significantly promote the expression of OPN mRNA and type I collagen in rat osteoblasts *in vitro*[7].

The recent discovery of Cbfa1 (Runx2, OSF2, AML3, Pebp2-A), a master regulatory gene in the osteoblast, is of great importance. Cbfa1 is a member of the runt family of transcription factors, whose expression is required for the differentiation of osteoblastic precursors and modulating the activity of mature osteoblasts[8]. Additionally, several *in vitro* studies have demonstrated that Cbfa1 is a major regulator of the osteoblast phenotype and is necessary for osteoblast-specific expression of genes such as osteocalcin, type I collagen, osteopontin, and bone sialoprotein[8-10]. Based on *in vitro* data, it seems that the expression and regulation of Cbfa1 activity in osteoblastic lineage cells might be important in determining bone formation induced by anabolic agents.

Our previous animal experiment has revealed that HEF could increase the expression of Cbfa1 mRNA in the bone of ovariectomized rats in a dose-dependent manner. Furthermore, the high dose HEF (160 mg/kg) administered for 12 weeks *in vivo* stimulated osteocalcin expression. These findings suggest that Cbfa1 is required for mediating the anabolic effects of HEF[11]. In vitro, icariin could exert its potent osteogenic effect through induction of Cbfa1 mRNA expression, production of BMP-4 and activation of BMPsignaling[12].

Evidence have proven that the proliferation and differentiation of osteoblasts were associated with MAPK signaling pathways, in which ERK and P38 sub-pathways are involved. ERKs are not only essential for the growth and differentiation of osteoblasts but also are important for adhesion, spreading, migration, and integrin expression of osteoblasts[13], and simultaneously ERKs also play an important role in driving the ECM-induced osteogenic differentiation of hMSC[14]. Treated C2C12 cells by BMP-2, results indicated that P38 and ERK cascades played a crucial role in the differentiation of osteoblasts[15]. Furthermore, distinct MAPK pathways seemed to independently modulate the proliferation and differentiation of osteoblastic cells, with ERK playing an essential role in cell replication and whereas P38 being involved in the regulation of ALPexpression during osteoblastic cell differentiation[16]. Stimulation of MAPK by transfecting a constitutively active form of MEK1, MEK(SP) into MC3T3-E1 preosteoblast cells increased endogenous OCN mRNA, while a dominant negative mutant, MEK(DN), was inhibitory. MEK(SP) also stimulated activity of a 147-base pair minimal OCN promoter, and this stimulation required an intact copy of OSE2, the DNA binding site for Cbfa1. A purified His-tagged Cbfa1 fusion protein was directly phosphorylated by activated recombinant MAPK *in vitro*. This study demonstrated that Cbfa1 was controlled by MAPKs and suggested that this pathway had an important role in the

control of osteoblast-specific gene expression[17].

Based on these background informations, it is reasonable for us to speculate whether MAPKs are involved in the expression of Cbfa1 protein in osteoblasts by icariin. In the present study, our data demonstrated that MAPK signal pathyway could be activated by icariin and was required for the expression of Cbfa1 protein in osteoblasts by icariin.

1 Materials and Methods

1.1 Reagents and antibodies

Icariin: Its molecular weight is 676.65 and its structural formula is shown in Fig. 1. Icariin was purchased from Pharmaceutical Industry Research Institute, Shanghai, China. It was isolated from dried aerial parts of Herba epimedii by the method described previously[18]. Dried aerial parts of Epimedii herba was extracted 3 times with ethanol, yielding an ethanol extract upon removal of the solvent in vacuo. The ethanol extract was then suspended in water and partitioned successively with n-hexane, CHCl3 and n-BuOH to obtain different fractions. Then the n-BuOH fraction was spread out using silica gel column chromatography to isolate icariin, which was purified through repeated recrystallization from MeOH. This resulted in an amorphous yellow powder that consisted of 91% icariin as verified by HPLC.

Estradiol: Its molecular weight is 272.38 and its structural formula is also shown in Fig. 1. Estradiol was purchased from Huamei Bio-engineering Company, Luoyang, China. It is a kind of hormone which can protect women from Postmenopausal Osteoporosis.

Antibodies: Anti-Erk1/2, anti-Phospho-ERK1/2, anti-P38 MAPKinase, anti-Phospho-P38 MAPKinase and anti-glyceraldehyde-3H-phosphate Dehydrogease (GAPDH) were purchased from Kangchen Bio-technology Company, Shanghai, China. Anti-Cbfa1 was purchased from Santa Cruz Biotechnology, Inc.

1.2 Experimental design

1.2.1 Cell culture: Rat primary osteoblasts were isolated from newborn SD rats (< 24 h old). Calvariae were removed and washed with PBS. The fibrous tissue surrounding the bone was scraped off softly, the calvariae were divided into two halves, and sutures were removed. The trimmed calvariae were treated with 0.25% trypsin

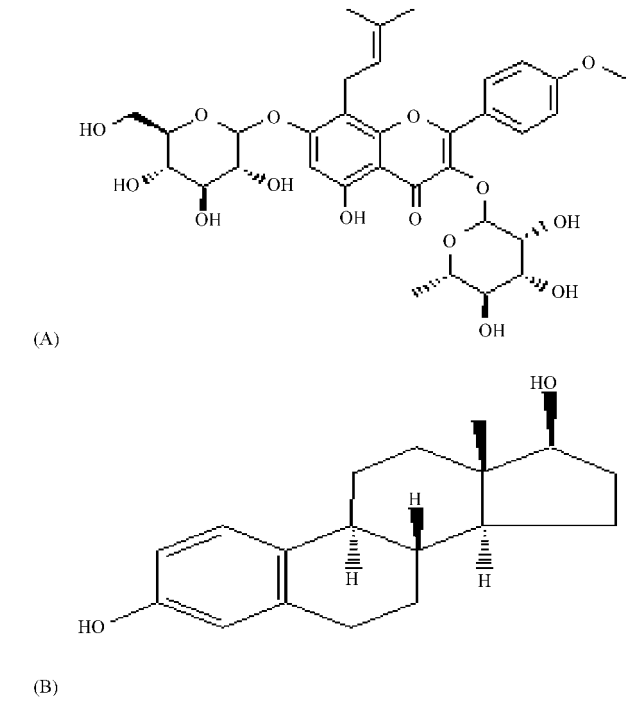

Fig.1 Structures of icariin (A) and estrodial (B).

(Amresco, USA) solution for 10 min at 37℃. Then calvariae were subjected to two 60-minute sequential collagenase (0.1%) (Sigma, USA) digestions in an oscillating 37℃ water bath. Digestions were neutralized with DMEM (Gibco, USA), pooled, and filtered through a sterile polypropylene mesh of 200 ~ 297 μ. The filtrate was centrifuged for 10 min at 1000 rpm, and the supernatant was removed, and then cells were resuspended in 3 ~ 5 ml DMEM containing 10% fetal calf serum (FCS) (Sino-American Biotechnology Co, China). Cells were then diluted to 2×10^4 per ml and plated into flasks. The next morning the medium was exchanged. Thereafter, cells were cultured in a regular medium, exchanged every two days. The second generation cells were used in our experiment. Before treated with reagents, the cells were cultured in non-serum medium for 24 h.

1.2.2 Treat with reagents: To examine the proteins of p-ERK, p-P38, ERK and P38, the cells were treated by icariin (10 ng/mL) for 5 min, 10 min, 30 min and 60 min, and by estrodial (10^{-8} mol/L) for 5 min, 10 min, 30 min. To examine the protein of Cbfal, the cells were treated by icariin (10 ng/mL) or estrodial (10^{-8} mol/L) for 24 h. Before treated by icariin and estrodial, some cells were pre-incubated by U0126 or SB203580.

1.2.3 Western-blot analysis

Proteins of MAPKs

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 aprotinin, 2 mM EDTA and 1 mM vanadate. After 15 min on ice, the supernatants were collected by centrifugation and total cellular protein amount was determined by the Micro BCA Protein Assay Reagent Kit (Kangchen Bio-tech Company, Shanghai, China). 50 μg of cellular protein extract was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were immersed for 3 h in a blocking solution consisting of TTBS (Tris 20 mM, pH 7.4, NaCl 137 mM, 0.1% Tween 20) and 5% skim milk, and washed twice with TTBS for 15 min. For detection, the membranes were incubated overnight with primary antibody (1∶100 in 1% milk, 1% BSA, 0.05% Tween-20 inTBS for GAPDH, p-ERK, ERK, P38, p-P38) at 4℃. After incubation, the membranes were washed three times for 10 min, in TBS with 0.05% Tween-20 (TTBS), followed by incubation with HRP-conjugated appropriate secondary antibody(1∶1000 in 1% milk, 1% BSA in TTBS) for 60 min at room temperature. Finally, the membranes were washed repeatedly with TTBS and TBS and were developed with the KC (tm) chemiluminescence detection kit (Kangchen Bio-tech Company, Shanghai, China).

Protein of Cbfal

Each culture was rinsed with PBS twice and then the nuclear protein was extracted with Nuclear Protein Extract Kit (Santa Cruz Biotechnology, Inc. USA). 50 μg of nuclear protein extracts were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were immersed for 3 h in a blocking solution consisting of TTBS (Tris 20 mM, pH 7.4, NaCl 137 mM, 0.1% Tween 20) and 5% skim milk, and washed twice with TTBS for 15 min. For detection, the membranes were incubated overnight with primary antibody (1∶100 in 1% milk, 1% BSA, 0.05% Tween-20 inTBS for Cbfal) at 4℃. After incubation, the membranes were washed three times for 10 min, in TBS with 0.05% Tween-20 (TTBS), followed by incubation with HRP-conjugated appropriate secondary antibody(1∶1000 in 1% milk, 1% BSA in TTBS) for 60 min at room temperature. Finally, the membranes were washed repeatedly with TTBS and TBS and were developed with the KC™ chemiluminescence detection kit (Kangchen Bio-tech Company, Shanghai, China).

1.3 Statistical analysis

Data were presented as the mean ± SD. For statistical evaluation, data were compared using the ANOVA test and $P < 0.05$ was considered significant. Every data were repeated 5 times.

2 Results

2.1 ERK and P38 MAPK signal pathway in osteoblasts was activated by icariin

Osteoblasts were treated with icariin for 5, 10, 30 and 60 min. The stimulation of ERK phosphorylation was increased within 30 min after icariin treatment, last to 60 min ($P < 0.01$). But total ERK protein in osteoblasts was not increased with 60 min ($P > 0.05$). The stimulation of P38 phosphorylation was increased within 5 min after icariin treatment, and continued to increase with 30 min and then last to 60 min ($P < 0.01$). But total P38 protein in osteoblasts was not increased with 60 min ($P > 0.05$). This result suggests that the stimulatory effect of icariin on ERK and P38 phosphorylation is an early event in the cellular action of these molecules (Table.1) (Fig.2).

Table **1** Effect of icariin on the expression of ERK, p-ERK, P38 and p-P38 proteins in osteoblasts

Group	ERK/GAPDH Ratio	p-ERK/GAPDH Ratio	P38/GAPDH Ratio	p-P38/GAPDH Ratio
I0	1.154 ± 0.317	0.400 ± 0.257	0.640 ± 0.276	0.054 ± 0.046
I5	1.342 ± 0.371	0.618 ± 0.255	0.699 ± 0.316	0.256 ± 0.166*
I10	1.356 ± 0.481	0.664 ± 0.253	0.653 ± 0.213	0.380 ± 0.122**
I30	1.377 ± 0.536	0.879 ± 0.168**	0.674 ± 0.290	0.426 ± 0.079**#
I60	1.210 ± 0.444	1.096 ± 0.207**	0.699 ± 0.307	0.553 ± 0.128**##

Data are means ± SD; $n = 5$

ERK, p-ERK, P38 and p-P38 proteins in osteoblasts treated by icariin for different time; I0: blank group; I5: treated by icariin for 5min; I10: treated by icariin for 10 min; I30: treated by icariin for 30 min;

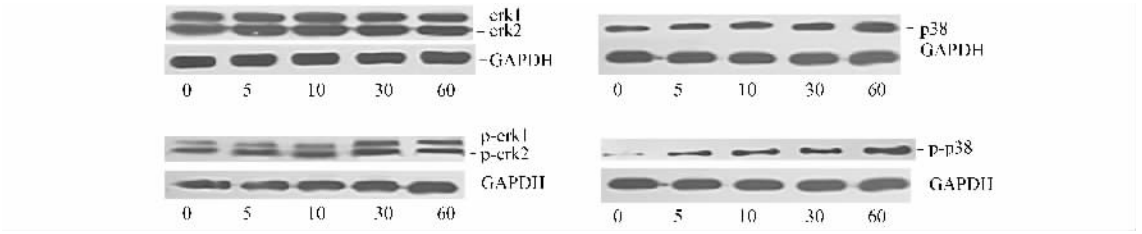

Fig.2 ERK, p-ERK, proteins in osteoblasts treated by icariin for different time; 0: blank group; 5: treated by icariin for 5min; 10: treated by icariin for 10 min; 30: treated by icariin for 30 min; 60: treated by icariin for 60 min.

I60: treated by icariin for 60 min. About ERK proteins, no difference occurs between each group. About p-ERK proteins, different from I0 group: $^{**}P < 0.01$ by ANOVA. About P38 protein, no difference occurs between each group. About p-P38 protein, different from E0 group: $^{*}P < 0.05$, $^{**}P < 0.01$ by ANOVA; different from E5 group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ by ANOVA.

2.2 ERK and P38 MAPK signal pathway in osteoblasts was activated by estrodial

Osteoblasts were treated with estrodial for 5, 10 and 30 min. The stimulation of ERK phosphorylation was increased within 30 min after estrodial treatment ($P < 0.05$). But total ERK protein in osteoblasts was not increased with 60 min ($P > 0.05$). The stimulation of P38 phosphorylation was increased at 10 min ($P < 0.01$) and last to 30 min ($P < 0.01$). But total P38 protein in osteoblasts was not increased with 60 min ($P > 0.05$). This result suggests that the stimulatory effect of estrodial on ERK and P38 phosphorylation is an early event in the cellular action of these molecules (Table.2) (Fig.3).

Table **2** Effect of estrodial on the expression of ERK, p-ERK, P38 and p-P38 proteins in osteoblasts

Group	ERK/GAPDH Ratio	p-ERK/GAPDH Ratio	P38/GAPDH Ratio	p-P38/GAPDH Ratio
E0	1.291 ± 0.389	0.708 ± 0.420	0.621 ± 0.167	0.090 ± 0.113
E5	1.334 ± 0.560	0.979 ± 0.347	0.679 ± 0.225	0.228 ± 0.152
E10	1.608 ± 0.713	1.131 ± 0.350	0.719 ± 0.246	0.468 ± 0.185**
E30	1.621 ± 0.785	1.308 ± 0.247*	0.691 ± 0.208	0.588 ± 0.268**

Data are means ± SD; $n = 5$.

ERK, p-ERK, P38 and p-P38 proteins in osteoblasts treated by estrodial for different time; E0: blank group; E5: treated by estrodial for 5 min; E10: treated by estrodial for 10 min; E30: treated by estrodial for 30 min. Data are means ± SD; $n = 5$. About ERK proteins, no difference occurs between each group. About p-ERK proteins, different from E0 group: $^{*}P < 0.05$ by ANOVA. About P38 proteins, no difference occurs between each group. About p-P38 proteins, different from E0 group: $^{**}P < 0.01$ by ANOVA.

2.3 Protein of Cbfa1 in osteoblasts was increased by icariin and estrodial, and this process involves the activation of ERK and P38

Our previous animal experiment show HEF, in which icariin is the main active constituent, can increase the expression of Cbfa1 mRNA in the bone of ovariectomized rats[11]. Now osteoblasts were treated with icariin for 24 h. After treatment with icariin, protein of Cbfal in calvarial osteoblasts was increased at 24 h. After treatment with estrodial, protein of Cbfa1 in calvarial osteoblasts was also increased at 24 h. Since tigogenin and resveratrol, two kind of chinese medicines, can stimulate the expression of Cbfa1 protein in osteoblasts through coupling to ERK and P38 activation[34,35], we speculate ERK and P38 signal pathways are involved in the expression of Cbfa1 protein in osteoblasts. Presently, this experiment also show icariin can stimulate ERK and

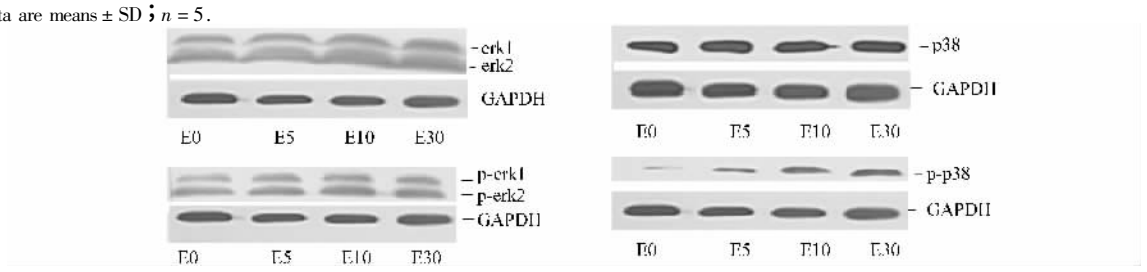

Fig.3 ERK, p-ERK, P38 and p-P38 proteins in osteoblasts treated by icariin for different time; E0: blank group; E5: treated by estrodial for 5 min; E10: treated by estrodial for 10 min; E30: treated by estrodial for 30 min.

P38 phosphorylation and increase Cbfa1 protein in osteoblasts. When osteoblasts were incubated with icariin in the presence of U0126 or SB203580, the stimulatory effect of icariin on Cbfa1 protein was markedly reduced. Taken together, these results suggest that ERK and P38 are both required for the expression of Cbfa1 protein. Incubating osteoblasts with icariin in the presence of U0126, the stimulatory effect of estrodial on Cbfa1 protein was markedly reduced. This result suggests that ERK is required for the increase of Cbfa1 protein by estrodial (Table.3) (Fig.4).

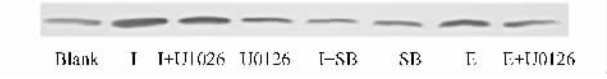

Fig.4 Cbfa1 proteins in osteoblasts treated by icariin, estrodial and/or U0126, SB203580; Blank: blank group; I: treated by icariin for 24 h; I + U0126: treated by icariin and U0126 for 24 h; E: treated by estrodial for 24 h; E + U0126: treated by estrodial and U0126 for 24 h; U0126: treated by U0126 for 24 h. I + SB: treated by icariin and SB203580 for 24 h; SB: treated by SB203580 for 24 h. Data are means ± SD; $n = 5$. Different from Blank group: $^{**}P < 0.01$ by ANOVA. Different from the corresponding group which was not treated by U0126: $^{\#}P < 0.05$ by ANOVA. Different from I group: $^{\#\#}P < 0.01$ by ANOVA.

Table **3** Effect of icariin, estrodial and/or U0126 on the expression of Cbfa1 protein in osteoblasts

Group	Cbfa1 gray value
Blank	1028.800 ± 397.699
I	2370.600 ± 429.276**
E	2544.400 ± 884.298**
I + U0126	1448.400 ± 574.604$^{\#}$
E + U0126	1280.800 ± 642.536$^{\#}$
U0126	1352.800 ± 811.835
I + SB	1171.600 ± 499.155$^{\#\#}$
SB	1106.200 ± 415.783

Data are means ± SD; $n = 5$.

Cbfa1 proteins in osteoblasts treated by icariin, estrodial and/or U0126, SB203580; Blank: blank group; I: treated by icariin for 24 h; E: treated by estrodial for24 h; I + U0126: treated by icariin and U0126 for 24 h; E + U0126: treated by estrodial and U0126 for 24 h; U0126: treated by U0126 for 24 h. I + SB: treated by icariin and SB203580 for 24 h; SB: treated by SB203580 for 24 h. Different from Blank group: $^{**}P < 0.01$ by ANOVA. Different from the corresponding group which was not treated by U0126: $^{\#}P < 0.05$ by ANOVA. Different from I group: $^{\#\#}P < 0.01$ by ANOVA.

3 Discussion

Osteoporosis is the most frequent bone remodeling disease, is defined by a low bone mass and a high risk of fractures, and is a major health problem for elderly women. For several years, estrogen replacement therapy (ERT) has been used to prevent osteoporosis in postmenopausal women[19]. However, studies have also reported that estrogen can lead to a higher incidence of breast carcinoma, endometrial cancer, and cardiovascular disease[20]. Plants derived medicines display less adverse effects and have been a part of traditional healthcare in China for thousands of years. Many of these have been shown to possess antiosteoporotic activities[21].

The theory of traditional Chinese medicine thinks that the kidney governs bones. It means that the development of the bones depends on the functions of the kidney. Many kinds of chinese medicines have the effects of invigorating kidney and strengthening bones. Icariin, as one kind of these chinese medicines, is belong to flavonols and flavonoids and has long been used for the treatment of osteoporosis. Icariin increased significantly osteoblast proliferation and ALPactivity, and inhibited the osteoclast TRAPactivity[22]. Icariin also caused an increase of bone mineral densities in ovariectomized rats and increased the expression of Cbfa1 mRNA in bone[11]. But the cellular signal pathway involved in these effects of icariin is unclear.

In this study, we clearly show that icariin and estrodial can rapidly (within minutes) activate the MAPK pathway and after treated by icariin, the phosphorylated ERK and P38 in osteoblasts began to increase almost at the same time. This phenomenon indicates icariin and estrodial may activate MAPK through the same signal pathway. Estrogen receptors exist as two subtypes ERa and ERb and osteoblasts express both receptors[23]. The interaction between flavonols and flavonoids with ERa and ERb is well documented[24,25]. Kuiper et al., who studied the estrogenic potency of several phytoestrogens, showed that kaempferol, and to a lesser extent quercetin, have the capacity to bind both ER subtypes[24]. Icariin can be thus considered to have the so-called "phytoestrogenic properties" and can rapidly (within minutes) activate the MAPK pathway through interacting

with ERs.

Cbfa1 is a transcription factor, which is essential for osteoblast differentiation and bone formation. Treating osteoblasts with icariin can promote the expression Cbfa1 protein, which indicates that icariin may exert its action on bone metabolism by modulating Cbfa1. The increase in Cbfa1 protein by icariin can be prevented by the ERK inhibitor U0126 or the P38 inhibitor SB203580, which demonstrates an involvement of the ERK pathway and P38 pathway in the stimulatory effect of icariin on Cbfa1. This result is in accordance with a report of Xiao G et al.[17] who showed that a dominant negative mutant, MEK (DN), markedly inhibits Cbfa1 expression in MC3T3-E1 preosteoblast cells. Runx2 can be phosphorylated and activated by the mitogen-activated protein kinase (MAPK) pathway. This pathway can be stimulated by a variety of signals including those initiated by extracellular matrix (ECM), osteogenic growth factors like bone morphogenic proteins (BMPs) and fibroblast growth factor-2 (FGF-2), mechanical loading and hormones such as parathyroid hormone (PTH)[26]. It is of note that Migliaccio et al., studying the mitogenic effect of estradiol, observed in an other experimental system (MCF-7 human cancer cells), that estradiol rapidly (within minutes) activates the MAPK pathway, an effect which is prevented by the anti-estrogen ICI 182780[27]. These authors also pointed at a rapid interaction of the estradiol-receptor complex with the tyrosine kinase Src. Src can phosphorylate Shc, an adaptor protein which in turn can associate with the Grb2/Sos complex, leading to p21ras activation[27], and therefore to a stimulation of the ERK pathway. A similar mechanism has also been reported in osteoblasts[28,29] as well as in osteoclasts[30]. According to this point of view, icariin, by its ability to bind the ERs, could secondarily activate the ERK pathway, an event required for the induction of Cbfa1 as suggested by the effect of U0126 or SB203580.

Because of the rapidity with which MAPkinase activation occurs, a pending question is that of the "identity" of the concerned ER (s). Are they the "classical" ERs, or the "membrane" ERs, the existence of which being supported by growing lines of evidence? Indeed, several studies have suggested that ERs located in the plasma membrane could be involved in the activation of the ERK pathway. In this regard, it could be conceived that icariin, as estradiol, could rapidly activate the MAPkinase pathway via their interaction with a subtype of ERs located in the plasma membrane. The mechanism whereby a rapid effect on ERK and P38 pathway, which takes place within minutes, induces a stimulatory effect on Cbfa1 remains to be specified. MAPkinase activation via a non genomic action of ER can lead to downstream modulation of several transcription factor such as ELK1, AP-1[31], CREB and C/EBPb[26]. Runx2 activity is enhanced by protein-protein interactions as are seen with PTH-induced Runx2/AP-1 and BMP-mediated Runx2/Smads interactions. Mechanisms for interaction with Runx2 are complex including binding of distinct components such as AP-1 factors and Smads proteins to separate DNA regions in target gene promoters and direct physical interactions between Runx2 and AP-1/Smad factors. In this regard, the fact that natural flavonols can reproduce the non genomic effects of estradiol may have some importance in the regulation of bone metabolism.

In conclusion, this study demonstrates that icariin can rapidly activate ERK and P38 in osteoblasts, which is possibly mediated by ER dependent pathway and also shows that icariin have a stimulatory effect on the expression of Cbfa1 protein in osteoblasts, in which MAPK signal pathyway is involved. These results with those previously reports[32,33] suggest that flavonols are substances which can both decrease osteoclastic activity and stimulate osteoblastic activity. Some other experiments also suggest that tigogenin and resveratrol[34,35], two kind of chinese medicines, stimulate the expression of Cbfa1 protein in osteoblasts through an ER-dependent mechanism and coupling to ERK and P38 activation. Such chinese medicines may represent new pharmacological tools for the treatment of osteoporosis.

References

[1] Xie F, Wu CF, Lai WP, et al. The osteoprotective effect of Herba epimedii (HEP) extract *in vivo* and *in vitro*. Evid Based Complement Alternat Med, 2005, 2(3): 353-361.

[2] Meng FH, Li YB, Xiong ZL, et al. Osteoblastic proliferative activity of Epimedium brevicornum Maxim. Phytomedicine, 2005, 12(3): 189-193.

[3] Qin L, Zhang G, Hung WY, et al. Phytoestrogen-rich herb formula

"XLGB" prevents OVX-induced deterioration of musculoskeletal tissues at the hip in old rats. J Bone Miner Metab, 2005, 23 Suppl: 55-61.

[4] Yin XX, Chen ZQ, Dang GT, et al. Effects of Epimedium pubescens icariine on proliferation and differentiation of human osteoblasts. Zhongguo Zhong Yao Za Zhi, 2005, 30(4): 289-291.

[5] Yin XX, Chen ZQ, Liu ZJ, et al. Icariine stimulates proliferation and differentiation of human osteoblasts by increasing production of bone morphogenetic protein 2. Chin Med J (Engl), 2007, 120(3): 204-210.

[6] Zhang DW, Cheng Y, Wang NL, et al. Effects of total flavonoids and flavonol glycosides from Epimedium koreanum Nakai on the proliferation and differentiation of primary osteoblasts. Phytomedicine, 2008, 15(1-2): 55-61.

[7] Xiao Q, Chen A, Guo F. Effects of Icariin on expression of OPN mRNA and type I collagen in rat osteoblasts *in vitro*. J Huazhong Univ Sci Technolog Med Sci, 2005, 25(6): 690-692.

[8] Ducy P, Zhang R, Geoffroy V, et al. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell, 1997, 89(5): 747-754.

[9] Komori T, Yagi H, Nomura S, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell, 1997, 89(5): 755-764.

[10] Ducy P, Starbuck M, Priemel M, et al. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev, 1999, 13(8): 1025-1036.

[11] Qian G, Zhang X, Lu L, et al. Regulation of Cbfa1 expression by total flavonoids of Herba epimedii. Endocr J, 2006, 53(1): 87-94.

[12] Zhao J, Ohba S, Shinkai M, et al. Icariin induces osteogenic differentiation *in vitro* in a BMP- and Runx2-dependent manner. Biochem Biophys Res Commun, 2008, 369(2): 444-448.

[13] Lai CF, Chaudhary L, Fausto A, et al. Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. J Biol Chem, 2001, 276(17): 14443 -14450.

[14] Salasznyk RM, Klees RF, Hughlock MK, et al. ERK signaling pathways regulate the osteogenic differentiation of human mesenchymal stem cells on collagen I and vitronectin. Cell Commun Adhes, 2004, 11(5-6): 137-153.

[15] Gallea S, Lallemand F, Atfi A, et al. Activation of mitogen-activated protein kinase cascades is involved in regulation of bone morphogenetic protein-2-induced osteoblast differentiation in pluripotent C2C12 cells. Bone, 2001, 28(5): 491-498.

[16] Suzuki A, Guicheux J, Palmer G, et al. Evidence for a Role of P38 MAPKinase in Expression of Alkaline Phosphatase During Osteoblastic Cell Differentiation. Bone, 2002, 30(1): 91-98.

[17] Xiao G, Jiang D, Thomas P, et al. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. J Biol Chem, 2000, 275(6): 4453-4459.

[18] Xin ZC, Kim EK, Lin CS, et al. Effects of icariin on cGMP-specific PDE5 and cAMP-specific PDE4 activities. Asian J Androl, 2003, 5(1): 15-18.

[19] Turner RT, Riggs BL, Spelsberg TC. Skeletal effects of estrogen. Endocrine Reviews, 1994, 15(3): 275-300.

[20] Orija IB, Mehta A. Hormone replacement therapy: current controversies. Clin Endocrinol (Oxf), 2003, 59(5): 657.

[21] Cassidy A. Dietary phytoestrogen and bone health. Journal of the British Menopause Society, 2003, 9(1): 17-21.

[22] Qin L, Han T, Zhang Q, et al. Antiosteoporotic chemical constituents from Er-Xian Decoction, a traditional Chinese herbal formula. J Ethnopharmacol, 2008, 118(2): 271-279.

[23] Arts J, Kuiper GG, Janssen JM, et al. Differential expression of estrogen receptors alpha and beta mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology, 1997, 138(11): 5067-5070.

[24] Kuiper GG, Lemmen JG, Carlsson B, et al. Interaction of estrogenic chemicals and phytoestrogens with receptor beta. Endocrinology, 1998, 139(10): 4252-4263.

[25] Harris HA, Bapat AR, Gonder DS, et al. The ligand binding profiles of estrogen receptors alpha and beta are species dependent. Steroids, 2002, 67(5): 379-384.

[26] Franceschi RT, Xiao G. Regulation of the osteoblast-specific transcription factor, Runx2: responsiveness to multiple signal transduction pathways. J Cell Biochem, 2003, 88(3): 446-454.

[27] Migliaccio A, Di Domenico M, Castoria G, et al. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J, 1996, 15(6): 1292-1300.

[28] Endoh H, Sasaki H, Maruyama K, et al. Rapid activation of MAPkinase by estrogen in the bone cell line. Biochem Biophys Res Commun, 1997, 235(1): 99-102.

[29] Kousteni S, Bellido T, Plotkin LI, et al. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell, 2001, 104(5): 719-730.

[30] Brubaker KD, Gay CV. Estrogen stimulates protein tyrosine phosphorylation and Src kinase activity in avian osteoclasts. J Cell Biochem, 1999, 76(2): 206-216.

[31] Kushner PJ, Agard DA, Greene GL, et al. Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol, 2000, 74(5): 311-317.

[32] Wattel A, Kamel S, Mentaverri R, et al. Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on *in vitro* osteoclastic bone resorption. Biochem Pharmacol, 2003, 65(1): 34-42.

[33] Miyake M, Arai N, Ushio S, et al. Promoting effect of kaempferol on the differentiation and mineralization of murine pre-osteoblastic cell line MC3T3-E1. Biosci Biotechnol Biochem, 2003, 67(6): 1199-1205.

[34] Zhou H, Yang X, Wang N, et al. Tigogenin inhibits adipocytic differentiation and induces osteoblastic differentiation in mouse bone marrow stromal cells. Mol Cell Endocrinol, 2007, 270(1-2): 17-22.

[35] Dai Z, Li Y, Quarles LD, et al. Resveratrol enhances proliferation and osteoblastic differentiation in human mesenchymal stem cells via ER-dependent ERK1/2 activation. Phytomedicine, 2007, 14(12): 806-814.

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