Regulation of Cbfa1 Expression by Total Flavonoids of *Herba Epimedii*

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Abstract. Core binding factor $\alpha 1$ (Cbfa1) is a member of the runt family of transcription factors, which appears to play a pivotal role in regulating the differentiation of osteoblastic precursors and the activity of mature osteoblasts. Total flavonoids of *Herba epimedii* (HEF) is a recognized bone anabolic agent, but there is lack of reports on the modulation of Cbfa1 expression by HEF. Here we investigated the effect of HEF on Cbfa1 expression in the bone of ovariectomized (OVX) rats. 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.

Key words: Total flavonoids of *Herba epimedii*, Core binding factor $\alpha 1$, Osteoporosis

(Endocrine Journal 53: 87–94, 2006)

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 an 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 total flavonoids of *Herba epimedii* (HEF). At present, HEF anabolic effects on bone have been verified in many studies. In experimental animals with osteoporosis, administration of HEF increases bone mass by stimulating *de novo* bone formation [1]. *In vitro*, this increase in bone mass is associated with an increased activity of existing osteoblasts and an increased differentiation of osteoblasts [2]. But the underlying molecular mechanisms accounting for these osteoblastic effects of HEF remain unclear.

The recent discovery of Cbfa1 (Runx2, OSF2, AML3,

Pebp2 α A) as 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 [4]. Targeted deletion of the Cbfa1 gene resulted in a complete lack of skeleton and absence of functional osteoblasts due to maturational arrest [5]. Specific expression of a dominant-negative Cbfa1 in mature osteoblasts resulted in severe reduction in bone formation and bone mass due to a decrease in osteoblast activity [6]. 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 [4–6].

Based on *in vitro* data and the phenotypes of mice with altered Cbfa1 expression, 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. In this context, it is reasonable to speculate that HEF anabolic effect on bone may be involved in the control of a master regulatory gene(s) whose expression and activity governs

Received: January 6, 2005

Accepted: October 24, 2005

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osteoblast function.

In the present study, our data demonstrated that the expression of Cbfa1 mRNA was enhanced by HEF in the bone of ovariectomized rats in a dose-dependent manner.

Materials and Methods

Materials

HEF is composed of icariin and icariside. Icariin is the main active constituent. Its molecular weight is 676.65 and its structural formula is shown in Fig. 1. HEF and icariin were purchased from Pharmaceutical Industry Research Institute, Shanghai, China. They were isolated from dried aerial parts of *Herba epimedii* by the method described previously [7]. 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, CHCl_3 and n-BuOH to obtain different fractions. Different fractions were combined and dis-

tilled. The crude extract was then purified twice with methanol. The final extract contained 63.41% total flavonoids (27.99% icariin) as determined by HPLC. For *in vitro* experiments, 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.

Nilestriol, a derivative of estriol, was purchased from Hualian Pharmaceutical Co., Ltd., Shanghai, China. Generally, it is used for hormone replacement therapy in perimenopausal women. Its molecular weight is 380.53, and its structural formula is also shown in Fig. 1.

Experimental design

In vitro experiments

Rat primary osteoblasts were isolated from newborn pups (<24 h old). Calvariae were removed and washed with PBS. The fibrous tissue surrounding the bone was gently scraped off, the calvariae were divided in two halves, and sutures were removed. The trimmed calvariae were treated with 0.25% trypsin (Amresco, USA) solution for 10 min at 37°C. Then calvariae were subjected to two 60-minute sequential collagenase (0.1%) (Sigma, USA) digestions in an oscillating 37°C water bath. Digestions were neutralized with α -MEM (Gibco, USA), pooled, and filtered through a sterile polypropylene mesh of 200–297 μ . The filtrate was centrifuged for 10 min at 1000 rpm, the supernatant was removed, and cells were resuspended in 3–5 ml α -MEM containing 10% fetal calf serum (FCS) (Sino-American Biotechnology Co, China). Cells were then diluted to 2×10^4 per ml and plated onto culture dishes (2 ml per culture dish). The next morning the medium was exchanged. Thereafter, cells were grown with a regular medium exchange every third day.

For the set of the cells treated with icariin, the cells were cultured in the presence of 1 ng, 10 ng or 100 ng/ml of icariin. Icariin treatment was conducted for 48 h using the cells at 80% confluency.

In vivo experiments

Three-month-old female SD rats were obtained from the Central Animal Department of Fudan University, Shanghai, China. The animals were housed in a controlled environment animal facility at 24 ~ 28°C with a

(A)

(B)

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

12-h light/dark cycle. During the experiment, the animals were given free access to food and distilled water. The Institutional Animal Care and Use Committee (IACUC) approved all animal procedures.

After 7 days of acclimation, the rats were divided into two groups: bilateral ovariectomy or sham operation. A week after surgery, the rats were divided into six groups ($n = 9$): (1) sham operated + vehicle (0.9% sodium chloride solution, Shanghai Worldbest Treeful Pharmaceuticals Co., Ltd., China); (2) ovariectomized (OVX) + vehicle; (3) OVX + nilestriol (0.1 mg/kg per week, gavage); (4) OVX + Low dose-HEF (L-HEF) (40 mg/kg per day, gavage); (5) OVX + Middle dose-HEF (M-HEF) (80 mg/kg per day, gavage); (6) OVX + High dose-HEF (H-HEF) (160 mg/kg per day, gavage). The rats received drug treatment for 12 weeks. Body weights were determined every week. After treatments, blood samples were collected by heart puncture, and the serum was centrifuged (3,000 rpm, 20 min at 4°C), aliquoted, and stored at -20°C until measurements were made. Skulls were dissected and stored in liquid nitrogen until measurements were made.

Measurement of bone mineral density (BMD)

BMD was measured in total body by dual-energy x-ray absorptiometry (DEXA) (Lunar, USA), which was calculated as quotient of the projected bone area (cm²) and bone mineral content (g). The machine was calibrated daily with a phantom provided by the manufacturer. The coefficient of variation (CV) of DEXA was less than 1.0%.

Serum osteocalcin (BGP) and estradiol (E₂)

Serum BGP and E₂ were measured by a radio-immunological kit (Chemclin Biotech Co. Ltd., Beijing, China).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was harvested from the cells with Trizol reagent (Gibco, USA) and isolated according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA by using Oligo(dT) primer and M-MLV reverse transcriptase (Promega, USA). Samples were incubated at 42°C for 1 h. Subsequently, PCR was performed with Taq DNA Polymerase

(Promega, USA); initial denaturation occurred at 94°C for 5 min, with final extension at 72°C for 7 min for each set of primers. The amplification was carried out using the following specific oligonucleotides:

Bone morphogenetic protein 2 (BMP-2):

5'-GGACTGCGGTCTCCTAAA-3' and 5'-GCTCAAACTCAACTCCGAC-3'

GAPDH:

5'-ACCACAGTCCATGCCATCAC-3' and 5'-ATGTCGTTGTCCCACCACCT-3'

GAPDH served as control. Conditions set for the investigated genes were: 94°C 30 sec, 57°C 45 sec, 72°C 1 min (32 cycle) for BMP-2; 94°C 30 sec, 56°C 45 sec, 72°C 1 min (32 cycle) for GAPDH. The PCR products were analyzed by electrophoresis on 2% agarose gels, stained with ethidium bromide. Finally, the gels were subjected to imaging and densitometric scanning of the resulting bands under UV light.

Real-time quantitative RT-PCR

RNA isolation and reverse transcription

The skull was dissected from each rat and stored in liquid nitrogen until used. Total RNA was isolated from the skull using RNeasy[®] total RNA isolation system (Promega, USA). Five µg of total RNA was reverse-transcribed by incubation with 1 µl of 5 µM oligo (dT) primer 65°C for 5 min; afterwards 2 µl of 10 mM dNTPs, 1 µl of 0.1 M DTT (Invitrogen, USA), 4 µl of reverse transcriptase buffer (Promega, USA), and 50 units of M-MLV reverse transcriptase (Promega, USA) were added in a total volume of 20 µl. The mixture was incubated at 42°C for 1 hr.

Determination of optimum primer and probe

Taqman PCR assays were performed on a Corbett Rotor-Gene 3000 real-time PCR system (Corbett Research, Australia). First optimum primer concentrations for different expressed sequenced tags (ESTs), which combined same concentrations of both forward and reverse primers with a constant probe concentration, were determined. Then optimum probe concentrations were determined.

Determination of amplification efficiency for target and reference

The optimal concentrations for primer and probe were used in reactions for determination of amplification efficiency. Duplicates of cDNA templates were

used for every run. To determine amplification efficiency of target and reference, a calibration curve was used. For this purpose, a stock solution of cDNA from which we prepared serial dilution was prepared. A calibration curve with 125, 25, 5, 1, 0.2 ng of cDNA was created for ESTs. Overall efficiencies (E) of PCR were calculated from the slopes of the standard curves according to the $E = 10^{[-1/\text{slope}]}$ for serial dilution in steps of 10 [log (10) scale]. $E = 2$ reflects a doubling of DNA in each PCR cycle over all dilution steps. Two regression lines were used to compare the significance of difference between two slopes of curve regression for target and reference, respectively. If there was no significant difference, relative quantification of Cbfa1 mRNA expression was calculated by $\Delta\Delta C_t$; otherwise, it was calculated by Rasmussen [8, 9].

Real-time quantitative RT-PCR

Due to valuable samples and more fragments requiring validation, the reaction setup was scaled down appropriately to 25 μ l. The thermal cycling conditions were at 95°C for 3 min, followed by 45 cycles of at 94°C for 5 sec and at 65°C for 40 sec. Primer and Taqman probe sets were designed from sequences in the Genebank database using Primer Express 2.0 software. The sequences are listed in Table 1.

Statistical analysis

Data were presented as the mean $\pm$ SEM. For statistical evaluation, data were compared using the ANOVA test and $P < 0.05$ was considered significant.

Results

Body weight was not influenced by HEF

Before treatments, body weights of six groups of rats did not differ (data not shown). After experiments, body weight of rats in OVX group was higher than that in sham-operated group ($P < 0.01$). Intake of HEF for 12 weeks did not affect the body weight ($P > 0.05$) (Fig. 2).

Increase of BMD by HEF

Marked bone loss occurred in the rats of OVX group, and this loss was prevented by treatment with HEF. Administration of nilestriol also prevented the bone loss in OVX rats.

Ovariectomy led to significant decrease of BMD of

Fig. 2. Final body weight in sham-operated rats (Sham); ovariectomized (OVX) rats treated with vehicle; OVX rats treated with L-HEF or M-HEF or H-HEF; OVX rats treated with nilestriol. Data are means $\pm$ SEM of nine rats. Different from OVX group: * $P < 0.05$, ** $P < 0.01$ by ANOVA. HEF, total flavonoids of *Herba epimedii*; OVX, ovariectomized; L-HEF, Low dose HEF; M-HEF, Middle dose HEF; H-HEF, High dose HEF.

Table 1. Primer and probe sequence (forward primer, reverse primer, Taqman probe)

Name	Gen Bank accession number	Sequence
Cbfa1	AF053950	Forward primer
		Reverse primer
		Probe
GAPDH	U75401	Forward primer
		Reverse primer
		Probe

Table 2. Effect of HEF on bone mineral density (BMD) in ovariectomized rats

Group	Bone mineral density (g/cm ²)
L-HEF	0.2920 ± 0.006874
M-HEF	0.2931 ± 0.006294
H-HEF	0.2975 ± 0.006626*
Nilestriol	0.2964 ± 0.006464*
OVX	0.2918 ± 0.002438
Sham	0.3030 ± 0.012294*

Data are means ± SEM of nine rats.

After 12 weeks, BMD was measured in total body by dual-energy x-ray absorptiometry, which was calculated as quotient of the projected bone area (cm²) and bone mineral content (g).

Different from OVX group: * $P < 0.05$ by ANOVA

HEF, total flavonoids of *Herba epimedii*; OVX, ovariectomized; L-HEF, Low dose HEF; M-HEF, Middle dose HEF; H-HEF, High dose HEF

total body compared to the sham-operated group ($P < 0.05$). However, HEF administration increased BMD of total body in a dose-dependent manner. The statistical significant increase of BMD could only be found in the group dosed with H-HEF concentration ($P < 0.05$). Nilestriol group demonstrated similar action on the BMD indicating the nilestriol was an effective positive control in the present study (Table 2).

Serum E₂ was not altered by HEF

As expected, the serum level of E₂ strikingly decreased in OVX group, indicating that the rats were estrogen deficient. Treatment with nilestriol slightly increased the serum level of E₂, but the value was not significant compared to the OVX group. Treatment with various concentrations of HEF did not alter the serum level of E₂ ($P > 0.05$) (Fig. 3).

Serum level of BGP was enhanced by HEF

The serum level of BGP in OVX group was higher than that in sham-operated group though no significance was observed ($P > 0.05$). After treatment with HEF, the serum level of BGP was increased. Moreover, high-dose HEF administration remarkably increased the serum level of BGP, compared with sham-operated and OVX groups ($P < 0.05$) (Fig. 4).

Fig. 3. Serum E₂ concentration in sham-operated rats (Sham); ovariectomized (OVX) rats treated with vehicle; OVX rats treated with L-HEF or M-HEF or H-HEF; OVX rats treated with nilestriol for 12 weeks. Data are means ± SEM of nine rats. Different from OVX group: * $P < 0.05$, ** $P < 0.01$ by ANOVA. HEF, total flavonoids of *Herba epimedii*; OVX, ovariectomized; L-HEF, Low dose HEF; M-HEF, Middle dose HEF; H-HEF, High dose HEF.

Fig. 4. Serum BGP concentration in sham-operated rats (Sham); ovariectomized (OVX) rats treated with vehicle; OVX rats treated with L-HEF or M-HEF or H-HEF; OVX rats treated with nilestriol for 12 weeks. Data are means ± SEM of nine rats. Different from OVX group: * $P < 0.05$; different from sham group: # $P < 0.05$ by ANOVA. HEF, total flavonoids of *Herba epimedii*; OVX, ovariectomized; L-HEF, Low dose HEF; M-HEF, Middle dose HEF; H-HEF, High dose HEF.

Effect of icariin on BMP-2 expression in primary osteoblasts

After incubation with icariin, expression of BMP-2 was increased in a dose-dependent manner ($P < 0.05$).

However, reduced BMP-2 expression was observed at higher icariin concentration (100 ng/ml) (Fig. 5).

Relative quantitative expression analysis

Real time amplification profiles for the target genes or ESTs fragments in bone tissues are shown in Fig. 6. After normalization to GAPDH, the values for fold changes were expressed relative to the calibrator with the lowest expression and displayed as relative expression. Genes that were not detected in any subject were excluded from statistical analysis. The expression level of Cbfa1 in sham-operated group was much higher than that in OVX group ($P<0.01$). However, the administration of H-HEF and nilestriol recovered expression of Cbfa1. Furthermore, expression of Cbfa1

in H-HEF group was even higher than that in sham-operated group ($P<0.01$) (Fig. 7).

Fig. 5. Effect of icariin on BMP-2 mRNA expression in rat primary osteoblasts. Primary osteoblasts were treated with 1 ng/ml, 10 ng/ml and 100 ng/ml icariin or control for 48 h. Total RNA was isolated and subjected to semi-quantitative RT-PCR analysis of BMP-2 mRNA expression under the conditions described in "Methods". Results were obtained from four independent experiments and expressed as means $\pm$ SEM. Different from control group: * $P<0.05$ by ANOVA. (A: 1 ng/ml; B: 10 ng/ml; C: 100 ng/ml; D: Control).

Fig. 6. Calibration curves constructed by measuring serial dilutions of rat bone tissue total RNA for relative quantification of target and reference genes expression. Ct values versus cDNA (reverse transcribed total RNA) concentration input were plotted to calculate the slope. The corresponding real time PCR efficiencies were calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ (Rasmussen, 2001). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Ct, threshold cycle.

Fig. 7. Relative quantification of Cbfa1 mRNA expression in sham-operated rats (Sham); ovariectomized (OVX) rats treated with vehicle; OVX rats treated with L-HEF or M-HEF or H-HEF; OVX rats treated with nilestriol for 12 weeks. Data are means $\pm$ SEM of nine rats. Different from OVX group: * $P<0.05$, ** $P<0.01$; different from sham group: # $P<0.01$ by ANOVA. HEF, total flavonoids of Herba Epimedii; OVX, ovariectomized; L-HEF, Low dose HEF; M-HEF, Middle dose HEF; H-HEF, High dose HEF.

Discussion

Estrogen has an important impact on bone. Loss of ovarian function may cause a 30% decrease of skeletal mass in women after menopause, due to their bone resorption increase and imbalance between the amounts of resorbed and formed bone at each remodeling site [10, 11]. Bone loss in OVX animals is similar to that in postmenopausal women. Therefore, OVX rat represents an appropriate model to study postmenopausal osteoporosis.

Indeed, decreased level of serum E_2 and BMD, increased serum BGP, urine NTx (data not listed) and body weight observed in OVX rats collectively suggested our animal model of osteoporosis by ovariectomy was successful.

Besides, treatment with nilestriol increased the serum level of E_2 , but the value was not significant from that in OVX group. As nilestriol derives from estril (E_3), the above result might be relevant to conversion of E_3 to E_2 .

We observed that the bone loss induced by ovariectomy was successfully prevented by the treatment with HEF, but HEF failed to improve the level of serum E_2 in OVX rats, indicating that HEF might contribute to its antiosteoporotic effect without increasing the level of serum E_2 . In addition, the present finding implies a possible involvement of a master regulatory gene(s) in this process.

Cbfa1 is a transcription factor, which is essential for osteoblast differentiation and bone formation. The expression of Cbfa1 mRNA in bone tissues was reduced by ovariectomy, which might be related to the lower level of serum E_2 in OVX rats [12–15]. Treatment with HEF prevented reduction of Cbfa1 mRNA expression from ovariectomy indicating that HEF may exert its action on bone metabolism by modulating Cbfa1. It seemed that this regulation on Cbfa1 was not relevant to the level of serum E_2 because the level of serum E_2 was not influenced by HEF. Our study demonstrated that icariin, the major constituent of HEF, could increase the expression of BMP-2 mRNA in primary osteoblasts. BMP-2 is a member of transforming growth factor- β family. It appears to be an effective inducer

for osteogenesis and upregulates the expression of Cbfa1 through Smads signal pathway [16]. Another study also showed that BMP-2 can enhance osteoblast differentiation by an increase in expression of Cbfa1 [17]. However, the pathway of regulation on Cbfa1 by HEF remains unknown and it needs further research.

BGP is a bone matrix protein secreted exclusively by differentiated osteoblasts and is believed to be one of the most reliable makers for bone formation. Earlier studies demonstrated that runt domain in Cbfa1 gene could bind to osteoblast-specific cis-acting element 2 (OSE2) in the promoter of the rat BGP gene, thereby Cbfa1 regulated the expression of BGP [18]. The expressions of these genes might be induced by Cbfa1, but a Cbfa1-specific antisense oligonucleotide could block Cbfa1-induced bone anabolic activity [6, 19]. Treatment with HEF increased the level of serum BGP in a dose-dependent manner, which was consistent with the regulation of HEF on Cbfa1 expression. It suggests that HEF exerts *in vivo* osteogenic effects via Cbfa1 transactivation, which then enhances expression of BGP. But an additional question of interest pertains to the reason(s) that expression of Cbfa1 was decreased in OVX group compared with sham-operated group, even though the level of serum BGP in OVX group was higher. Fujita *et al.* [20] found that ovariectomy could suppress expression of BGP, which was in accordance with the present finding. So the exact role of Cbfa1 on BGP expression in OVX animals is subject for further investigation.

In conclusion, our findings demonstrated that HEF had antiosteoporotic effects on ovariectomized rats. HEF could promote the mRNA expression of Cbfa1, which might partly contribute to its antiosteoporotic effect.

Acknowledgements

We would like to thank Zhousheng Xiao, Bogeng Song and Hong Ao for their excellent technical assistance during this study. This study was supported by the National Natural Sciences Foundation of China (30070375).

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