Icariin promotes osteoblastic differentiation in OVX mice via MAPK signaling pathway revealed by profiling

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Objective: Icariin (ICA), an extract from epimedium, has been reported to be effective in promoting bone formation. The objective of the study is to search for the molecular targets of ICA in bone mesenchymal stem cells (bMSCs) from the mice with ovariectomy (OVX)-induced osteoporosis.

Methods: Six-month-old Imprinting Control Region (ICR) mice that underwent OVX were treated with ICA. After three months, bone mass was evaluated by microcomputed tomography, morphometry and immunohistological detection. bMSCs were isolated from the femur and tibia to observe the self-renewal and differentiation capacities using colony-forming unit fibroblastic (CFU-F), colony-forming unit adipocyte (CFU-Adipo) and alkaline phosphatase (ALP) staining. In addition, microarray of bMSCs *ex vivo* was measured two weeks after ICA treatment and analyzed by heatmap and pathway analysis. The signaling pathway was further explored by western blot assay and inhibitors of p38 and ERK: SB203508 and PD98059.

Results: μ CT displayed a decrease in bone mass for three months after OVX. ICA treatment increased the trabecular thickness (Tb.Th), osteoblast number while decreased osteoclast number, elevating osteocalcin (OC) protein levels *in vivo* and facilitating the self-renewal and osteoblastic differentiation of bMSCs *ex vivo*. Microarray data indicated ICA rescued several gene expressions that were dysregulated by OVX. Pathway analysis revealed that the core genes acted by ICA were highly involved in MAPK signaling pathway. Further study demonstrated ICA suppressed ERK while stimulated p38 phosphorylation to promote osteoblastic differentiation *in vitro*.

Conclusion: ICA promotes osteoblastic differentiation of bMSCs in OVX mice. MAPK signaling pathway might be involved in the process.

Keywords: Icariin; MAPK; microarray; bone mesenchymal stem cell; osteoporosis.

Introduction

Icariin (ICA), a flavonoid glycoside as the principal component of Epimedium, has been reported to be effective in promoting osteogenesis. It increased bone mass density (BMD) and

biological strength partly through facilitating osteoblast proliferation and differentiation.^{1–5} The signaling molecules targeted by ICA in the process of pro-osteogenic differentiation are involved in a bone morphogenetic protein (BMP) and Runx2 signaling, estrogen receptor, TGF and Wnt pathway,

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and ERK, JNK activation.^{6–8} However, the molecular mechanism of ICA acting on bone mesenchymal stem cells (bMSCs), the progenitors of osteoblasts, remains unclear.

In the present study, we evaluated the protective effect of ICA in ovariectomy (OVX)-induced osteoporotic mice (three months after OVX surgery) by μ CT, morphometry and immunohistochemical detection *in vivo*. The function of bMSCs from OVX mice was tested by colony-forming unit fibroblastic (CFU-F), colony-forming unit adipocyte (CFU-Adipo) and ALP staining. In addition, microarray data from bMSCs of two weeks ICA treatment mice were analyzed by heatmap and pathway analysis, and verified using western blot with inhibitors of p38 and ERK: SB203508 and PD98059. Our study aims to search for the molecular targets of ICA in bMSCs from the mice with OVX-induced osteoporosis, and provide an alternative for the treatment of post-menopausal osteoporosis.

Materials and Methods

Preparation of drugs

ICA (purity > 98%, MW: 676.65) was purchased from You-si Biotech Co. (Shanghai, CN). The solutions of ICA were prepared in dimethylsulfoxide (DMSO, Sigma, USA) and corn oil (1:3) to give a final concentration of 10 mg/mL for experiment *in vivo*. About 10^{-5} M of ICA was used for *in vitro* study and the final concentration of DMSO was less than 0.1%.

The inhibitors of two proteins stored in DMSO, SB203580 and PD98059 were obtained from Beyotime Institute of Biotechnology (Jiangsu, CN), and diluted into 50, 100 μ M, respectively, with culture medium.

Animals grouping and treatments

Six-month-old female Imprinting Control Region (ICR) mice ($n = 60$) were provided by the Shanghai Laboratory Animal Center (SCXK 2007-0005, Science and Technology Commission of Shanghai Municipality; Shanghai Animal Ethics Committee approved for the experimental research on animals). The mice were randomly divided into sham, OVX and OVX + ICA (ICA) groups. These groups were subdivided into two weeks and three months groups ($n = 10$ per group). The mice in ICA groups were intraperitoneal injected with ICA at dose of 100 mg/(kg $\cdot$ d) twice every three days for two weeks or three months after the OVX surgery. About 10 one-month-old ICR mice were applied for *in vitro* experiment.

μ CT

Lumbar spine specimens were fixed in 4% paraformaldehyde for 24 h, washed for 2 h and examined. Six L_4 vertebrae in

each group were used for a three-dimensional (3D) model construction excluding adnexa, such as transverse and spinous processes. Analyses were performed using the μ CT 80 radiograph microtomograph (Scanco Medical AG, Switzerland), associated 3D Calc, cone reconstruction and AVG model building software (HP, Japan). A reconstruction of the bitmap dataset was obtained and employed to build the 3D model. Scores for the ratio of BMD, bone volume to tissue volume (BV/TV), the connectivity density of trabeculae (Conn.D.), the trabecular number (Tb.N), the trabecular thickness (Tb.Th) and the trabecular spaces (Tb.Sp) were directly obtained from the 3D model.

Histological and histomorphometric analyses

Lumbar spines from the mice were fixed in 4% paraformaldehyde for 24 h, decalcified in 10% EDTA for 4 weeks and embedded in paraffin wax. The sections were stained with hematoxylin and eosin or followed by trap staining procedure. A morphometric study was performed with an image auto-analysis system (Olympus BX50; Japan). L_4 were examined. The static parameters were the trabecular bone area (T.Ar) and the bone perimeter (B.Pm), which were utilized to calculate the parameters of bone formation: osteoblast activity (N.ob/T.Ar, N.ob/B.pm) or the parameters of bone resorption: osteoclast activity (N.oc/B.pm).⁹

Immunohistochemical and immunofluorescence analyses

Sections were pretreated and stained with osteocalcin (OC) (1:100) as previously described⁹ ($n = 6$). A morphometric study was performed with an image auto-analysis system (Olympus BX50; Japan). The data were quantified using a medical image management system (Cmias, CN).

bMSCs culture and treatment and CFU-F, CFU-Adipo assays

bMSCs were obtained from the bone marrow of the bilateral tibia and femur of ten mice per group in the OVX experiment to evaluate the self-renewal and differentiation capacity (three month group) or for microarray detection (two week group) or from 10 one-month-old mice for *in vitro* observation. The marrow cavity was flushed with α -MEM (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin–streptomycin (Gibco, USA). The cells were cultured in 10 cm dishes (two mice per dish). The number of CFU-F or CFU-Adipo was counted under a light inverted microscope on the 4th or 7th day post-surgery.

ALP assay

For the differentiation assay, bMSCs were cultured in a basal culture medium or osteogenic supplement (OS, Dexamethasone 10^{-8} M, β -Glycerophosphate 10 mM, L-ascorbic acid 2-phosphate 50 μ g/mL, Sigma-Aldrich, USA) plus various doses of ICA (10% FBS) for five days. The cells were fixed with 4% paraformaldehyde and stained with NBT-BCIP (Pierce, USA) for 30 min. Lyons blue represents the positive staining.

NimbleGen gene expression profiling

Total nine pieces of NimbleGen Gene Expression Profiling (No. PXH100525) containing 26991 genes were used (three pieces per group). Total RNAs were harvested using TRIzol (Invitrogen) and the RNeasy kit (Qiagen) according to manufacturer's instructions, including a DNase digestion step. After having passed RNA measurement on the Nano-drop ND-1000 and denaturing gel electrophoresis, the samples were amplified and labeled using a NimbleGen One-Color DNA Labeling Kit and hybridized in NimbleGen Hybridization System. After hybridization and washing, the processed slides were scanned with the Axon GenePix 4000B microarray scanner. Raw data were extracted by NimbleScan software (version 2.5). NimbleScan software's implementation of RMA offers quantile normalization and background correction. The gene summary files were imported into Agilent GeneSpring Software (version 11.0) for further analysis. Differentially expressed genes were identified through Fold-change and *T*-test screening. The profiling identified a subset of the total number of probes that are differentially expressed.

Western blotting

Whole cell extracts (50 μ g protein per lane, 15 min ICA treatment) were separated on an 8–12% SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Membranes were blocked with TBST containing 5% (W/V) defatted milk powder at room temperature (RT) for 2 h and then incubated for 1–2 h at RT with rabbit polyclonal p38, pp38, ERK and pERK antibody (1:1000, Cell signaling Technology Inc., USA) or mouse monoclonal β -actin antibody (1:4000, Sigma-Aldrich Co. LLC, USA).

Statistical analyses

The data are expressed as means $\pm$ SE, and statistical significance was calculated using One-Way ANOVA followed by a post-hoc LSD test (homogeneity of variance) or a Tukey's test (heterogeneity of variance) using SPSS software (SPSS Inc, Chicago, USA). The significance level was defined as $p < 0.05$. For microarray expression data analysis, the web-based DAVID DATABASE (<http://david.abcc.ncifcrf.gov/>) was used. Pathway analysis was achieved through functional analysis mapping of genes to KEGG pathways. The *p*-value (EASE-score, Fisher-*P*-value or Hypergeometric-*P*-value) denotes the significance of the pathway correlated to the conditions. Less the *p*-value, more significant is the pathway (The *p*-value cut-off is 0.05). Clustering analysis was performed by R software (version 3.2.3).

Results

ICA increases bone mass of OVX mice

Lumbar samples of mice were collected three months post-surgery. Quantitation of μ CT data indicated that BMD, BV/TV, Tb.N and Tb.Th values significantly decreased in the OVX group, while Tb.Sp values significantly increased. After ICA treatment for three months, the Tb.Th was observed to be significantly elevated ($P < 0.05$). The values for BV/TV, Tb.N and Tb.Sp were improved, although no statistical differences were observed (Table 1).

ICA increases osteoblast but decreases osteoclast activities, elevating the OC protein distribution in OVX mice

Histological examination indicated that Tb.N reduced at three months after OVX surgery. ICA partly inhibited this bone loss (Fig. 1(a)). The values of N.ob/T.Ar, N.ob/B.pm were decreased ($P < 0.01$), while N.oc/B.pm were increased in the OVX group ($P < 0.01$), indicating a decrease in osteoblast activity and an increase in osteoclast activity, which could be partly improved by ICA treatment ($P < 0.01$) (Figs. 1(b)–1(e)).

Table 1. Micro-CT data of L₄ vertebrae ($n = 6$ per group).

	BMD (1/cm)	BV/TV	Conn.D. (1/mm ³)	Tb.N (1/mm)	Tb.Th (mm)	Tb.Sp (mm)
Sham	2.98 $\pm$ 0.05	0.23 $\pm$ 0.07	90.23 $\pm$ 9.84	3.36 $\pm$ 0.48	0.06 $\pm$ 0.01	0.31 $\pm$ 0.04
OVX	2.86 $\pm$ 0.05**	0.09 $\pm$ 0.03**	81.19 $\pm$ 16.00	2.27 $\pm$ 0.35**	0.04 $\pm$ 0.00**	0.46 $\pm$ 0.08*
ICA	2.90 $\pm$ 0.06	0.13 $\pm$ 0.03	79.69 $\pm$ 41.62	2.79 $\pm$ 0.70	0.05 $\pm$ 0.00#	0.38 $\pm$ 0.10

Notes: * $p < 0.05$, ** $p < 0.01$ versus Sham, # $p < 0.05$ versus OVX.

Figure 1. (a) Representative images of H & E staining of lumbar vertebrae section indicated a loss in the number of trabecular bone at three-month post-surgery, while ICA reduced this damage. (b, d) Morphometry methods displayed a great decrease in N.Ob/T.Ar and N.Ob/B.pm induced by OVX, but these two indicators were partially increased by three-month treatment of ICA. (c, e) TRAP staining showed an increase in N.Oc/B.pm after OVX surgery, but ICA treatment reduced the index. The columns represent the means $\pm$ SE from six mice per group. ** $p < 0.01$ versus sham, ## $p < 0.01$ versus OVX.

OC, secreted by osteoblasts and accumulated in the extracellular matrix of bone, is considered to be one of the most sensitive markers associated with bone formation.^{10,11} The positive staining for OC was weakened by OVX surgery, but ICA significantly increased OC expression (Figs. 2(a) and 2(b)).

ICA promotes MSCs self-renewal and differentiation to osteoblast, but inhibits MSCs differentiation to adipocytes of OVX mice

To test the effect of ICA treatment *in vivo*, bMSCs were isolated and cultured for four and seven days *ex vivo*. The results of CFU-F and CFU-Adipo assays showed that ICA

Figure 2. (a) Immunostaining of lumbar vertebrae section with antibody against OC. Reduced positive staining (brown) was observed, especially on the surface of bone lacuna in the OVX group. In the ICA group, OC protein was increased. PBS instead of OC antibody was applied in negative section. (b) Quantification of (a). The columns represent the means $\pm$ SE from six mice per group. ** p < 0.01 versus sham, ## p < 0.01 versus OVX.

Figure 3. (a) Representative images and (b, c) quantification of CFU-F and CFU-Adipo, indicating a decrease in CFU-F on 4th day while an increase in CFU-Adipo on 7th day of OVX-bMSCs. ICA treatment reversed these two markers. (d) ALP assay showed OVX-inhibited osteoblastic differentiation of bMSCs, but the level of ALP-positive staining was increased after ICA treatment.

maintained CFU numbers of bMSCs, while decreased those of adipocytes differentiated from bMSCs (Figs. 3(a)–3(c)).

ALP assay was done to determine whether ICA treatment enhances osteoblastic differentiation of bMSCs. It showed that ICA dramatically increased ALP activity (Fig. 3(d)).

ICA rescues gene expression pattern and targets on MAPK signaling pathway in OVX mice

The bMSCs from Sham, OVX and ICA treated mice were collected for microarray profiling. Total 514 differential expressed genes (fold changes > 1.5, $p < 0.05$) among three groups were selected for hierarchical clustering analysis. Result shown as a heatmap indicated the similarity in gene expression among either samples or genes. The samples fell into two major classes shown by the two branches of the heatmap. Sham and ICA samples grouped together in one class while OVX samples were in the other. The results manifested that ICA reversed the gene expression toward the Sham mice. The samples from the same group cluster together, suggesting a reliability of our gene expression measurement (Fig. 4(a)). The genes reversed by ICA were shown in four kinds of tendencies in terms of gene expression pattern (Figs. 4(b)–4(e)).

According to the KEGG pathway analysis, MAPK signaling pathway has the –LgP score of nearly nine in different gene signaling pathways, the second highest among all signaling pathways (Fig. 4(f)). Among MAPK signaling pathways, the expressions of five genes including IL1, IL1R, PAK1/2, MST1/2 and GSK were regulated by ICA treatment in OVX mice according to the microarray data (Fig. 5(a), red). We then examined two typical proteins in MAPK pathways including p38, ERK1/2 using primary murine bMSCs. The results of Western blot demonstrated that ICA increased phosphorylation of p38 while decreased that of ERK1/2 (Fig. 5(b)).

Moreover, ALP staining became stronger when used the inhibitors of ERK1/2 protein: PD98059 while weaker used those of p38:SB203580 for three days. The combination of ICA strongly increased ALP positive staining compared to PD98059 treatment alone, but showed no significant changes when p38 MAP kinase was inhibited (Fig. 5(c)).

Discussion

Menopause can cause loss of bone mass due to low hormone levels. So far, an ideal medicine with fewer side effects is still required. *Epimedium* is one of the most widely prescribed herbs for treating osteoporosis in China. Under Chinese medical theory, *epimedium* may help to keep bone healthy through kidney boosting effects, because the kidney is responsible for nourishing bones.¹² As an active component of

epimedium, ICA was speculated to stimulate osteogenesis by regulating the differentiation of bMSCs to osteoblasts. It was tested using OVX-induced osteoporotic mice. The study presented that ICA promoted bone formation and stimulated differentiation of bMSCs towards osteoblastic lineage. The activation of p38 and attenuation of ERK1/2 may be involved in the process.

We have reported that OVX led to the reduction in BV/TV, Tb.Th and Tb.N values while increase in Tb.Sp value of rats three months post-surgery.¹³ The animal model was replicated in mice with the same periods in the present study. However, the results showed that ICA significantly increased Tb.Th while only improved tendency presented in other three indexes with no statistical significance. That would be caused by the existence of big intragroups difference due to the old age of mice, or the differences in both or either administration routes and doses. Others reported that ICA increased BMD in the treatment for OVX rats with 225 mg/kg per day for eight weeks,¹⁴ supporting that ICA may improve indexes of bone mass in the OVX condition. Moreover, the results of the histomorphometry analysis indicated an increase in the N. Ob while a decrease in the osteoclast number, suggesting that ICA exerted anabolic and anti-resorptive effects on trabecular bone of OVX mice that also observed in non-surgery mice.¹⁵ We found the increased level of OC secretion by ICA treatment, which is similar to others' reports of rats.^{16,17}

To further observe if ICA acts on bMSCs in *in vivo* system, CFU-F, CFU-Adipo and ALP assays were performed in *ex vivo* studies which are responsible for the evaluation of the direct function of bMSCs *in vivo*.¹⁸ We determined that ICA increased the number of CFU-F on the 4th day while reduced that of CFU-Adipo on the 7th day without any adipogenic supplement, and enhanced ALP positive staining without any osteogenic supplement, suggesting that ICA stimulated osteoblastic differentiation and inhibited the adipocyte formation in *in vivo* condition.

The possible molecular targets of ICA were further explored in the study using gene expression profiling analysis. Several genes expressions were reversed by ICA in OVX mice, and these genes focused on several signal pathways including metabolic pathway, MAPK signaling pathway, endocytosis, etc. Among these high-related pathways involved in the ICA action, we chose MAPK pathway to further determine if ICA exerted a pro-osteogenic effect partially through it, because MAPK pathway has been reported to play a role in osteoblastic differentiation and might be a target of ICA in other cell lineages. For instance, in a murine embryonic stem cells *in vitro* study, ICA-stimulated cardiomyocyte differentiation was abolished when the p38 pathway was inhibited, whereas ERK1/2 showed different response to ICA treatment.^{19–22} On osteoblasts, ICA reduced the lipopolysaccharide (LPS)-mediated activation of

Figure 4. (a) Heatmap and hierarchical cluster analysis (HCL) of 514 differential expressed genes of nine samples from three groups: Sham, OVX and ICA. Three replicates from each group cluster together, and nine samples fell into three major classes, indicating reliable quality of microarray data. (b-e) The genes reversed by ICA were shown in four kinds of tendencies in terms of gene expression pattern. (f) The differential gene signaling pathways ranked by -LgP score according to the KEGG analysis.

Figure 5. (a) MAPK signaling pathway in KEGG database. Five differential expressed genes by OVX that were regulated with ICA treatment (red). (b) Western blot analysis of total p38, ERK1/2 and their phosphorylation by 10⁻⁵ M of ICA treatment for 15 min. (c) ALP staining on the 3rd day after supplement of p38, ERK1/2 inhibitors: SB203580 and PD98059 with or without ICA treatment.

ERK1/2, but induced ERK activation on MC3T3-E1 cells while on osteoclasts, it suppressed that of p38.^{8,23} ERK inhibitor PD98059 significantly attenuated ICA-induced angiogenic events of endothelial cells.²⁴ p38 pathway were suppressed in LPS or hydrogen peroxide-induced neural injury response in neuronal cell lines by ICA.^{25,26} So, the effect of ICA on p38, ERK is not consistent according to the literature. In the present study, we found that ICA activated p38 MAPK while suppressed ERK1/2. The pro-osteogenic effect of ICA could be inhibited using p38 inhibitor SB203580, but that was enhanced by ERK1/2 inhibitor PD98059. It demonstrated the ICA-stimulated osteoblastic differentiation of bMSCs was partially through p38 activation and ERK1/2 suppression. The exact action of ICA on p38 MAPK and ERK1/2 might depend on cell types, cultural conditions, and up-stream molecular actions.

Taken together, ICA could be considered as a useful drug for menopausal osteoporotic therapy.

Conclusions

Our findings suggest that ICA exerts a pro-osteogenic effect in both OVX-induced osteoporotic mice and primary murine bMSCs *in vitro*. The screening genes expression is highly related to MAPK signaling pathway, and the molecular targets of ICA on bMSCs might be partial through activation of p38 MAPK and inactivation of ERK1/2.

Conflict of Interest

The authors have no conflict of interest to declare.

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