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Comparative proteomic and metabolomic analysis reveal the antiosteoporotic molecular mechanism of icariin from *Epimedium brevicornu* maxim

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ABSTRACT

Ethnopharmacological relevance: Icariin, a principal flavonoid glycoside of *Epimedium brevicornu* Maxim, has been widely proved to possess antiosteoporotic activity with promoting bone formation and decreasing bone resorption. However, the involving mechanisms remain unclear.
Aim of the study: To clear a global insight of signal pathways involved in anti-osteoporotic mechanism of icariin at proteins and metabolites level by integrating the proteomics and NMR metabonomics, in a systems biology approach.
Material and methods: Mice were divided into sham, OVX model and icariin-treated OVX group, after 90 days treatment, difference gel electrophoresis combined with MALDI-TOF/TOF proteomics analysis on bone femur and serum metabolomics were carried out for monitor intracellular processes and elucidate anti-osteoporotic mechanism of icariin. Osteoblast and osteoclast were applied to evaluate the potential signal pathways.
Results: Twenty three proteins in bone femur, and 8 metabolites in serum, were significantly altered and identified, involving in bone remodeling, energy metabolism, cytoskeleton, lipid metabolism, MAPK signaling, Ca^{2+} signaling et, al. Furthermore, animal experiment show icariin could enhance the BMD and BMC, decrease CTX-I level in ovariectomized mice. The mitochondrial membrane potential and the intracellular ATP levels were increased significantly, and the cytoskeleton were improved in icariin-treatment osteoblast and osteoclast. Icariin also increased mRNA expression of Runx2 and osterix of OB, decreased CTR and CAII mRNA expression and protein expression of P38 and JNK. However, icariin did not reveal any inhibition of the collagenolytic activity of cathepsin K, mRNA expression of MMP-9 and protein expression of ERK in osteoclast.
Conclusion: we consider icariin as multi-targeting compounds for treating with osteoporosis, involve initiating osteoblastogenesis, inhibiting adipogenesis, and preventing osteoclast differentiation.

1. Introduction

Bone metabolism, which is normally maintained by osteoclast-mediated bone resorption and osteoblast-mediated bone formation, is a lifelong dynamic remodeling process (Rachner et al., 2011). Osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B ligand (RANKL) are central regulators of osteoclasts (OC) differentiation for activation of RANK in the osteoclast precursors. Osteoclasts adhere to bone surface through αvβ integrin, while creating F-actin sealing zone to resorb bone matrix. Osteoclasts are capable of generating an acidic microenvironment necessary for bone resorption. Carbonic anhydrase II generates H^+ and HCO_3^- by the hydration of CO_2, and the hydrogen ions are transported through the apical ruffled border of the osteoclasts to the resorption zone by a vacuolar H^+ adenosine triphosphatase (H^+ V-ATPase). The H^+ V-ATPase also provides the proton source for extracellular acidification by exchange of bicarbonate to chloride by HCO_3/ Cl^- exchanger (Laitala and Väänänen, 1993; Teitelbaum, 2000). Intracellular vesicles are fused to bone facing plasma membrane inside actin ring, release acid into the resorption space through ruffled border membrane, and starts

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dissolution of apatite crystals, exposing its organic matrix, with subsequent degradation by acidic proteases, such as cathepsin K (CatK), matrix metalloprotein 9 (MMP-9), and matrix metalloprotein 13 (MMP-13) (Teitelbaum, 2000). Meanwhile, osteoblasts secrete new bone matrixs (layers of oriented collagen fibers), and form dense crosslinked ropes. An essential event in osteoblast (OB) differentiation is the activation of runt-related transcription factor 2 (Runx2), a master switch can positively regulate mesenchymal stem cells (MSCs) to immature osteoblast (IO) and regulates expression of osterix (OSX). OSX, which are regulated by the Ca^{2+}-calcineurin pathway, interact with the nuclear factor for activated T cells 2 (NFAT2) to control the transcription of target genes, such as collagen 1 and osteocalcin (Nakashima et al., 2002). Bone morphogenetic proteins (BMP) in skeletal tissue belong to transforming growth factor-β (TGF-β) superfamily, play an important role in fracture healing. BMP and TGF-β signaling are via binding to specific receptors and activation of Smad effector proteins, such as Smad-1, −4 and −5, which also can interact with Runx2 and cooperates in controlling BMP signaling, ultimately stimulating bone formation (Feng and Derynck, 2005). The underlying mechanism of osteoporosis is an imbalance between bone resorption and bone formation during bone remodeling, characterized as excessive bone resorption, and inadequate formation of new bone.

Epimedii Folium (Chinese name as Yinyanghuo), the leaf of *Epimedium brevicornu* Maxim, *E. sagittatum* (Sieb. et Zucc.) Maxim, *E. pubescens* Maxim, *E. wushanense* T. S. Ying and *E. koreanum* Nakai (Chinese Pharmacopoeia Commission, 2015), has been clinically used for more than 2000 years. This herb, firstly recorded in the Shennong's Herbal Atlas (Shennong Bencao Jing, written around 221BCE to 220CE), then following recored in Chinese medicine books, was characterized as tonifying kidney, strengthening tendons and bones, treating impotence and rheumatoid arthritis. In recent decades, *Epimedii* Folium is frequently applied as the principal ingredients in many new Chinese formulas treating osteoporosis clinically in China (Wang et al., 2016; Zhang et al., 2016), and in experiment study, such as Er-xian Decoction(Xue et al., 2012a), Xianlingubao (Wang et al., 2015). Icariin (ICA, pubchem CID: 5,318,997), the most abundant active component of *Epimedii* Folium, possess anti-osteoporosis, anti-oxidation, anti-tumor, anti-aging, anti-depression and anti-atherosclerosis activity in pharmacological studies (Jia et al., 2012; Kang et al., 2012; Mao et al., 2000; Pan et al., 2013; Yang et al., 2013; Zhang et al., 2010). As a bone protective agent, ICA modulate bone remodeling by stimulating osteoblastic cell proliferation and differentiation through regulating the signaling of BMP, TGF-β, and insulin-like growth factors (IGFs), Wnt and Runx2/Osterix (Ma et al., 2011; Ma et al., 2013), and mediate bone resorption by inhibition of osteoclastic differentiation and bone resorption enzymes, such as CatK (Sun et al., 2013), MMP-9 (Zhang et al., 2015) and CAII. In our previous study, the bone protective effects of ICA has been demonstrated by the enhancement in bone microstructure of ovariectomized (OVX) rats, inhibition of osteoclasts differenation mainly through OPG/RANKL signal and MAPK signal. ICA also can reduce the number and activity of osteoclasts, show significantly inhibitory effects on tartrate-resistant acid phosphatase (TRAP) activity, CatK activity and the formation of F-actin ring (Nian et al., 2009; Xue et al., 2012a, 2012b). However, the mechanism involved in the bone protective effects of ICA is still not clear.

Currently, there is a great deal of scientific interest and debate concerning the possible advantages that transcriptomic, proteomic and metabonomic technologies ('Omics') might have over traditional biomarkers. Up to now, there is only the combined transcriptomic and proteomic approaches were applied to evalue antiosteoporostic of ICA in zebrafish larvae (Li et al., 2011). The detailed proteomic on bone tissue and serum metabonomic data in animal are still lacking. We propose modulating protein expression and serum metabolites may reveal more insights on bone protective effects of ICA. A comparative proteomic approach was used for identifying the proteins with altered expression levels in ICA-treated ovarictomized mice (classic osteoporostic model). A ^{1}H nuclear magnetic resonance spectroscopy (^{1}HNMR) -based metabonomics method was applied to obtain a systematic view of the serum metabolites. Based on proteomic and metabonomic findings, a hypothetical mechanism for antiosteoporotic activity of ICA were put forward. Furthermore, the effects of ICA on osteoblast and osteoclast were analyzed to confirm the implication of proteomic and metabonmic results. The global insights of effects of ICA in protein and metabolite level will be given in our study and the possible signal pathway involved in antiosteoporotic of ICA will be clarified.

2. Materials and methods

2.1. Reagents

ICA, purity 98%, was purchased from ChemFaces (CFN99554, China). Reagents used in this study included α-Modified minimum essential medium (α-MEM) and fetal bovine serum (FBS) (Gibco, US); trypsin and coomassie brilliant blue G-250 (Sigma, US); recombinant Rat macrophage colony-stimulating factor (M-CSF, 400–28) and RANKL (400-30) (Peprotech EC, US); β-actin (sc81178) antibody (Santa Cruz, US); *anti*-phospho-JNK (9251), anti-phospho-ERK (3371), *anti*-phospho-p38 (9211), anti-αvβ integrin (ab78289), *anti*-RhoGTPase (9968), anti-BMP-2 (ab14933), and anti-TGF-β1(3711) obtained from Cell Signaling Technology, Beverly, MA and abcam company. PrimeScriptTM RT Reagent Kit (Perfect Real Time DRR037S) and SYBR Premix Ex Taq™ (Perfect Real Time DRR041A) were purchased from Bao Biotech (China). NucBuster Protein Extraction kit was purchased from Merck (Novagen, Germany). ATP assay kit was from Beyotime Institute of Biotechnology, Shanghai, China; JC-1, pararosaniline, α-Cyano-4-hydroxycinnamic acid, hoechst 33258 and rhodamine-conjugated phalloidin were purchased from Sigma, USA. The Tris-HCl, glycerol, sodium dodecyl sulfate, diolamine, potassium sodium tartrate, disodium, potassium ferricyanide, sodium thiosulfate, 4-nitrophenylphosphate, Triton X-100 and 4-nitrophenol were of domestic AR grade.

2.2. Animal experimental protocol

Thirty female ICR mice, 3 months of age, were purchased from SLACOM Experimental Animal Company (Shanghai, China) and acclimated to conditions for 1 week before the experiment. The experimental animals were housed in an air-conditioned room with 12 h/12 h light-dark illumination cycles at constant temperature (25 ± 2 °C) and humidity (50 ± 10%). Food and drinking water were supplied ad libitum. Ten mice were sham-operated as control group, and the remaining mice were bilaterally ovariectomized and randomly divided into two groups with 10 per group, and respectively as model control and 40 mg/kg daily ICA-treated group. Mice received treatments starting from one day after surgery for 12 weeks. Success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of uterine horns. At the end of the treatment, blood samples of mice were withdrawn form heart and centrifuged to collect serum for metabolomic study. The tibia was cleaned off adhering soft tissues, stored in 75% ethanol for a week to analysis bone mineral density (BMD). The BMD were measured at 3 mm from the proximal epiphysis of right tibia with a peripheral quantitative computed tomography (pQCT) densitometry (StratecTM XCT Research SA, Germany). Serum carboxy-terminal