Phytomedicine 18 (2011) 176–185

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Icariin inhibits osteoclast differentiation and bone resorption by suppression of MAPKs/NF-κB regulated HIF-1α and PGE_2 synthesis

Tsai-Pei Hsieh[a], Shiow-Yunn Sheu[a,**], Jui-Sheng Sun[b,c,*], Ming-Hong Chen[d]

[a] School of Pharmacy, College of Pharmacy, Taipei Medical University, Taiwan, ROC
[b] Graduate Institute of Clinical Medicine, Taipei Medical University, No. 250, Wusing St., Sinyi District, Taipei City 11031, Taiwan, ROC
[c] Department of Orthopedic Surgery, Hsin Chu General Hospital, Department of Health, Executive Yuan, Taiwan, ROC
[d] Institute of Biomedical Engineering, National Yang-Ming University, Taipei, Taiwan, ROC

ARTICLE INFO

Keywords:
Icariin
Epimedium pubescens
Osteoclast
LPS
Gene expression
HIF-1α
MAPKs
NF-κB

ABSTRACT

Icariin has been reported to enhance bone healing and treat osteoporosis. In this study, we examined the detail molecular mechanisms of icariin on lipopolysaccharide (LPS)-induced osteolysis. Our hypothesis is that icariin can inhibit osteoclast differentiation and bone resorption by suppressing MAPKs/NF-κB regulated HIF-1α and PGE_2 synthesis.

After treatment with icariin, the activity of osteoclasts differentiation maker, tatrate resistances acid phosphatease (TRAP), significantly decreased at the concentration of 10^{-8} M. Icariin (10^{-8} M) reduced the size of LPS-induced osteoclasts formation, and diminished their TRAP and acid phosphatease (ACP) activity without inhibition of cell viability. Icariin also inhibited LPS-induced bone resorption and interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) expression. The gene expression of osteoprotegerin (OPG) was up-regulated, while receptor activator of NF-κB ligand (RANKL) was down-regulated. Icariin also inhibited the synthesis of cyclo-oxygenase type-2 (COX-2) and prostaglandin E_2 (PGE_2). In addition, icariin had a dominant repression effect on LPS-induced hypoxia inducible factor-1α (HIF-1α) expression of osteoclasts. On osteoclasts, icariin suppresses LPS-mediated activation of the p38 and JNK; while on the osteoblasts, icariin reduced the LPS-induced activation of ERK1/2 and I-kappa-B-alpha (IκBα), but increased the activation of p38.

In conclusion, we demonstrated that icariin has an *in vitro* inhibitory effects on osteoclasts differentiation that can prevent inflammatory bone loss. Icariin inhibited LPS-induced osteoclastogenesis program by suppressing activation of the p38 and JNK pathway.

Introduction

The human bone is a highly dynamic organ that maintains its homeostasis through a delicate balance between the bone-forming osteoblasts (bone formation) and the bone-eroding osteoclasts (bone resorption). The dynamic balance between these two cells types results in bone remodeling. Increased osteoclast activity induces erosion of trabecular bone and fragile bones. Conversely, increased osteoblast activity increases bone density, which is associated with bone deformity and osteopetrosis (Boyle et al. 2003; Teitebaum 2000).

Osteoclasts are multinucleated giant cells that differentiated from cells of hematopoietic monocyte–macrophage linage under the presence of two critical factors: the receptor activator of NF-κB ligand (RANKL) and the macrophage/monocyte colony-stimulating factor (M-CSF). Both factors (the RANKL and M-CSF) are produced by osteoblasts or stromal cells (Boyle et al. 2003). Osteoprotegerin (OPG), a soluble decoy receptor for RANKL, produced by osteoblasts can inhibit osteoclasts formation by blocking RANKL binding to RANK (Hsu et al. 1999). RANKL represents the osteoblasts-derived factor required for osteoclasts formation, whereas OPG blocks these

Abbreviations: ACP, acid phosphatease; BMD, bone mineral density; BMMSCs, bone marrow-derived mesenchymal stem cells; BMP-2, bone morphogenetic protein-2; Cbfa1/Runx2, core binding factor A1/runt-related transcription factor 2; COX-2, cyclo-oxygenase type-2; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinase1/2; FBS, fetal bovine serum; HEF, flavonoids of Herba Epimedii; HIF-1α, hypoxia inducible factor-1α; ICR mice, Imprinting Control Region mice; IL-6, interleukin-6; IκBα, inhibitor of the nuclear transcription factor NF-κB; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; M-CSF, macrophage/monocyte colony-stimulating factor; MTT assay, 3-[4,5-dimethylthiazol]-2,5-diphenylterazolium bromide assay; NF-κB, nuclear factor of kappa light polypeptide gene enhancer in B-cells; NO, nitric oxide; OPG, osteoprotegerin; p38, p38 MAPK; PCR, polymerase chain reaction; PGE_2, prostaglandin E_2; RANKL, receptor activator of NF-κB ligand; TNF-α, tumor necrosis factor-α; TRAP, tatrate resistances acid phosphatease; α-MEM, α-minimum essential medium.

* Corresponding author at: No. 25, Lane 442, Sec. 1, Jingguo Rd., Hsinchu City 30059, Taiwan, ROC. Tel.: +886 2 27361661x3229/3 5326151; fax: +886 2 27390500.
** Corresponding author at: School of Pharmacy, College of Pharmacy, Taipei Medical University, No. 250, Wu-Shin Street, Taipei City 11031, Taiwan, ROC. Tel.: +886 2 27361661x6129; fax: +886 2 27361661x6129.
E-mail addresses: amel@tmu.edu.tw (S.-Y. Sheu), drjssun@tmu.edu.tw, drjssun@hch.gov.tw (J.-S. Sun).

0944-7113/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.phymed.2010.04.003

effects and prevents bone resorption in the various microenvironments (Teitebaum 2000). Many factors, such as lipopolysaccharide (LPS), 1,25-dihydroxyvitamin D_3 (Vit D_3), and pro-inflammatory cytokines, can increase osteoclasts formation via up-regulating the expression of RANKL and/or down-regulating OPG in osteoblasts or stromal cells (Lorenzo et al. 2008).

LPS, a bacteria-derived cell wall product, has long been recognized as a key factor implicated in the development of bone loss (Smith et al. 2006). LPS plays an important role in bone resorption by initiating a local host response that involves recruitment of inflammatory cells, production of prostanoids [such as prostaglandin E_2 (PGE_2)], synthesis of cytokines [such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)], and activation of osteoclasts formation and differentiation (Islam et al. 2007). LPS induces production of pro-inflammatory cytokines on osteoclasts through the NF-κB pathway and the three major mitogen activated protein kinases (MAPKs): extracellular signal regulated kinase 1/2 (ERK1/2), c-Jun-N-terminal kinase (JNK), and p38 (Hotokezaka et al. 2007; Kirkwood et al. 2007; Rogers et al. 2007). In macrophages, LPS induces hypoxia-inducible factor-1α (HIF-1α) protein accumulation under normoxic condition, and it has demonstrated that HIF-1α plays an essential role in the inflammatory response (Nishi et al. 2008; Tacchini et al. 2008). However, the biological effect of LPS on HIF-1α protein during inflammation of osteoclasts and/or osteoblasts has not been reported.

Many plant-derived natural products have been used in traditional medicine for the treatment of various diseases. Herba Epimedii is a traditional Chinese herbal medicine, which has been commonly used as tonic, aphrodisiac and anti-rheumatic in China for thousands of years. Its physical and functional characteristics have been thoroughly documented in the Chinese pharmacopoeia 2005. It was reported as an effective enhancer of bone healing (Qin et al. 2005) that could be prescribed for treating osteoporosis. It is known to increase the overall mineral content, therefore, to promote bone formation and to increase lumbar bone mineral density (BMD) (Zhang et al. 2009). Several compounds derived from natural products have been recently reported to possess inhibitory effects on osteoclast differentiation and function, leading to decreased bone loss *in vivo*. Zhang et al. reported that the flavonoids of Herba Epimedii (HEF) could concurrently improve osteogenic differentiation and inhibit the osteoclast differentiation of human bone marrow-derived MSCs (BM-MSCs) (Zhang et al. 2009).

Icariin ($C_{33}H_{40}O_{15}$; molecular weight: 676.67), the main active flavonoid glucoside isolated from *Epimedium pubescens*, was found to have a therapeutic effect on osteoporosis by ovariectomy rat models and postmenopausal women (Zhang et al. 2007). Further studies demonstrated that icariin also suppressed mice osteoclast differentiation (Huang et al. 2007), but the detailed molecular mechanisms underlying these effects remain unclear. Recently, we found that icariin might exert its osteogenic effects through induction of bone morphogenic protein-2 (BMP-2) and NO synthesis, subsequently regulate Core binding factor A1/runt-related transcription factor 2 (Cbfa1/Runx2), OPG, and RANKL genes expression (Hsieh et al. 2009). In the present study, we further examined the detailed molecular mechanisms of the effect of icariin on LPS-induced osteoysis by primary co-culture models obtained from adult female mice. Our hypothesis is that icariin can inhibit osteoclast differentiation and bone resorption by suppressing MAPKs/NF-κB regulated HIF-1α and PGE_2 synthesis.

Materials and methods

Chemicals and reagents

Icariin was obtained from Biotic Chemical Co., Ltd., Taipei, Taiwan. Icariin stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma Chemical, St. Louis, MO, USA) and stored at −20 °C. The final concentration of DMSO used in the culture was 0.01%. Lipopolysaccharide (LPS, Sigma Chemical, St. Louis, MO, USA) was obtained from Escherichia coli O26:B6.

Cell culture: osteoblasts and osteoclasts co-culture

This study was reviewed and received prior approval of the Taipei Medical University Animal Research Committee (Affidvait of Approval of Animal Use Protocol Taipei Medical University, LAC-96-0098). For the osteoclasts co-culture cell, 8-month old female Imprinting Control Region (ICR) mice were killed by CO_2 asphyxia, then the femur and tibia bones were dissected aseptically. The marrow cells were flushed out with α-Minimum Essential Medium (α-MEM, GibcoBRL; Grand Island, NY, USA). The bones (without marrow) were cut into pieces (less than 1 mm in diameter), digested with 0.2% collagenase for 1 h to harvest the mature osteoblast cells. For the osteoclast co-culture cells model, the cells were seeded into 6-wells plates at a density of osteoblasts 2×10^5 cells and bone marrow cells 1.4×10^7 cells/well in α-MEM supplemented with 10% fetal bovine serum (FBS, Sigma Chemical, St. Louis, MO, USA), antibiotics (100U/ml of penicillin G and streptomycin 100 μg/ml, GibcoBRL; Grand Island, NY, USA), 28 nM ascorbic acid 2-phosphate (Sigma Chemical, St. Louis, MO, USA), and 10 nM 1α,25$(OH)_2$Vit D_3 (Sigma Chemical, St. Louis, MO, USA) in a humidified atmosphere with 5% CO_2 and 95% air at 37 °C.

Osteoclasts validation and viability

Tartrate-resistant acid phosphatase (TRAP) stain

Cells were treated with different concentration of icariin in 90% α-MEM with 1% FBS, 28 nM ascorbic acid 2-phosphate, 10 nM 1α,25$(OH)_2$Vit D_3, and 1 μg/ml LPS with/or without icariin. After 6 days incubation, cells were fixed in 4% formaldehyde in PBS for 20 min, stained for TRAP activity using a commercialized kit (Sigma Chemical, St. Louis, MO, USA) according to the manufacturer's instruction. After incubation at room temperature for 1 h, cells were washed with distilled water for three times. Red color-TRAP-positive cells were observed and photographed for further analysis.

Acid phosphatase (ACP) and TRAP activity

TRAP is a maker of osteoclast differentiation, the effects of icariin on osteoclasts differentiation can be observed by the changes in TRAP activity. In this study, osteoclasts co-culture was treated with different concentration of icariin in α-MEM with 1% FBS, 28 nM ascorbic acid 2-phosphate, 10 nM 1α,25$(OH)_2$Vit D_3, and 1 μg/ml LPS with/or without icariin. The treatments were exchanged every 3 days. The ACP and TRAP assays for osteoclasts differentiation were performed in 3rd, 6th, and 9th day of culture. Briefly, cells were lysis by 0.2% Trion-X 100 in PBS, the collections was measured with a commercially available ACP and TRAP assays kit (Acid Phosphatase Liquicolor: Human Gesellschaft; Germany) by ELISA reader (Spectra max 340, molecular Devices; CA, USA) at wavelength of 405 nm. The measured ACP/TRAP activities were then normalized with their specific protein titer.

3-[4,5-Dimethylthiazol]-2,5-diphenylterazolium bromide assay (MTT assay)

Co-culture cells were treated with 10^{-8} M icariin in α-MEM with 1% FBS, 28 nM ascorbic acid 2-phosphate, 10 nM 1α,25$(OH)_2$Vit D_3, and 1 μg/ml LPS with/or without icariin. The MTT (Sigma Co., St. Louis, MO, USA) assay for cell viability was performed on 6th day of culture. During the experiment, the treatments (including medium and medication) were changed every 3 days and that fresh icariin was added at each medium change. The mitochondrial activity of