Hyperoside suppresses osteoclasts differentiation and function through downregulating TRAF6/p38 MAPK signaling pathway

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ABSTRACT

Hyperoside (HP), as a natural product, can promote proliferation and differentiation of osteoblasts and presents a protective effect on ovariectomized (OVX) mice. However, the inhibitory effect of HP on osteoclasts (OCs) and the potential mechanism remain to be elucidated. In this study, it was found that HP could effectively inhibit the differentiation and bone resorption of OCs, and its intrinsic molecular mechanism was related to the inhibition of TRAF6/p38 MAPK signaling pathway. Therefore, HP could be a promising natural compound for lytic bone diseases.

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1. Introduction

Osteoporosis (OP), a disease of bone metabolism, is characterized by the promoted bone resorption by OCs and the insufficient of osteoblasts formation, resulting in loss of bone mass, deterioration of bone microstructure and increased risk of fracture. Under normal physiological conditions, osteoclast-mediated bone resorption and osteoblast-mediated bone formation are maintained in a balance state to keep the homeostasis of the skeletal system. Hence, the imbalance will lead to the occurrence of bone disease [1]. Overabsorption of OCs can lead to several lytic bone diseases, such as postmenopausal osteoporosis, inflammatory arthritis, and neoplastic bone metastasis, therefore, the strengthening of OCs activity is the main cause of most adults’ bone diseases [2]. The World Health Organization reports [3] that more than 1.02 billion people are suffering from OP worldwide, and the number is expected to rise to 1.36 billion by 2030, so the prevention and treatment of OP are vital to the improvement of citizens’ health.

OPG/RANKL/RANK signaling pathway is a relatively mature theory of OCs differentiation at present [4]. Osteoprotegerin (OPG) can act as a pseudo-receptor of receptor activator of NF-κB ligand (RANKL) in the process of bone transformation. After binding to RANKL, OPG can block the RANKL/RANK signaling pathway, thus inhibiting the differentiation and maturation of OCs [5]. When RANKL is combined with receptor activator of NF-κB (RANK), RANK initiates the transduction of intracellular signaling by recruiting docking proteins such as tumor necrosis factor receptor-related factors (TRAFs) [6]. TRAF6 is critical for OCs differentiation, then, the mitogen-activated protein kinase (MAPK) signaling pathway, nuclear factor-κB (NF-κB) signaling pathway and anti-apoptotic serine/threonine kinase (Akt/PKB) signaling pathway are activated [7]. The MAPK signaling pathway mainly includes three signaling pathways: c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38. The p38 signaling pathway can up-regulate the expression of c-fos and promote its combination with nuclear factor of activated T-cell cytoplasmic 1 (NFATc1), which is one of the major downstream targets of RANK signaling [8]. NFATc1 activates and induces the expression of OCs specific genes through self-amplification and interaction with other transcription factors, which encodes proteins related to OCs differentiation, fusion and function [9], such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK) and dendritic cell-specific transmembrane protein (DC-STAMP). In this article, the TRAF6/p38 MAPK signaling pathway was mainly studied.

At present, some drugs with anti-osteoporosis effect are widely used in clinic, such as calcitonin, bisphosphonates, vitamin D and hormones (estrogen and parathyroid hormone), but the side effects of these drugs cannot be ignored. A number of Chinese herbal medicines and their natural products have been found to exhibit favorable anti-osteoporosis properties with minor side effects, which are more advantageous than chemical drugs and hormonal drugs [10]. HP (Figure 1), a flavonol glycoside compound, is widely found in various plants, such as the fruits and whole plants of Hypericaceae, Campanulaceae and Ericaceae. It was found that HP could protect mice from OP caused by ovariectomy [11], reduce the apoptosis and autophagy rate of MC3T3-E1 [12], and attenuate inflammation in C57BL/6J mice with acute
liver injury (ALI), and reduce the expression of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in lipopolysaccharide (LPS)-induced RAW264.7 cells [13]. But, the effect of HP on the differentiation and function of OCs and its potential molecular mechanism have not been reported. In this article, the cell model of RAW264.7 induced by RANKL was utilized, combined with TRAP staining, determination of TRAP activity and bone resorption activity assay, to study the effect of HP on OCs differentiation and bone resorption activity, meanwhile, the molecular mechanism of HP on OCs was explored through TRAF6/p38 MAPK signaling pathway.

2. Results and discussion

2.1. HP inhibited the osteoclastogenesis induced by RANKL

Before studying the effect of HP on the differentiation of RAW264.7 cells into OCs, the effect of HP on the cell survival rate of RAW264.7 cells was observed. In Figure 2B, it was shown that HP, below 100 μM, had no significant effect on cell survival rate. Hence, HP below 100 μM can be considered less toxic to RAW264.7 cells. In this study, 10, 25, and 50 μM HP were chosen to study the effect on the osteoclastogenesis of RAW264.7 cells. RAW264.7 cells were continuously induced by RANKL for 5 d. After staining, it was found that some of RAW264.7 cells differentiated into large, multinucleated cells (usually more than 3), which were regarded as TRAP positive OCs. As can be seen in Figure 2A, OCs in the negative control were most strongly generated, with a large number and volume. With the increase of HP concentration, the number of TRAP positive cells decreased. The statistical results of TRAP positive cells were shown in Figure 2C. In addition, HP suppressed the TRAP activity of RANKL-induced OCs in a dose-dependent manner, as shown in Figure 2D. Compared with the negative control, HP significantly inhibited osteoclastogenesis, and 50 μM HP exhibited the best inhibitory effect.

2.2. HP inhibited the bone resorption function of OCs

Under the induction of RANKL, RAW264.7 cells differentiated into OCs. OCs adsorbed on the bone slices to perform the function of bone resorption and the resorption pits on the bone surface were obviously observed through the staining of

Figure 1. Chemical structure of HP.
toluidine blue. OCs are the only cells *in vivo* to perform the function of bone resorption, and the area of bone pits can reflect and evaluate the bone resorption capacity of OCs. It could be seen from Figure 3A that the bone resorption area of the negative control was the largest. With the increase of HP concentration, the bone resorption area gradually decreased. The statistical results of the area of bone resorption pits were shown in Figure 3B. The result indicated that HP had an inhibitory effect on the bone resorption function of OCs.

### 2.3. Inhibitory effect of HP on actin ring structure of mature OCs

The bone resorption function of OCs depends on dynamic regulation of the actin cytoskeleton. Actin ring structure is a characteristic cytoskeletal feature of functional OCs. Therefore, the actin ring structure of mature OCs affected by HP would be examined. The F-actin was arranged into a ring-like structure (actin ring) at the cell periphery in mature OCs [14]. In Figure 4A, B, the treatment of mature OCs with HP caused both shrinkage of OCs and disruption of actin ring structure in a dose-dependent manner, and the number of intact actin rings gradually decreased with the increase of HP concentration.

### 2.4. HP inhibited the mRNAs expression levels of osteoclast-related genes

In order to further demonstrate the role of HP in the differentiation of OCs, the mRNAs expression levels of osteoclast-related genes were studied by real-time PCR. RAW264.7 cells induced by RANKL were treated with different concentration of HP. As shown in Figure 5, compared with the negative control, the mRNAs expression...
levels of TRAF6, c-fos, NFATc1, CTSK, and TRAP exhibited a downward trend. TRAF6 can initiate the p38 MAPK signaling pathway, and up-regulate the expression levels of c-fos and NFATc1, ultimately leading to the fusion and maturation of OCs. TRAP and CTSK are related to the executive function of OCs. The results explained that HP inhibited OCs differentiation by regulating the mRNAs expression levels of these genes.

### 2.5. HP inhibited the proteins expression levels of osteoclast-related signaling pathway

To examine the molecular mechanism of the anti-osteoclastogenic action of HP, RAW264.7 cells induced by RANKL were treated with different concentration of HP. Western blot results were shown in Figure 6A and B. Compared with the negative control, the proteins expression levels of TRAF6, c-fos, NFATc1, TRAP, CTSK, and the phosphorylation level of p38 MAPK decreased. These proteins are related to OCs
differentiation in the upstream and downstream of the TRAF6/p38 MAPK signaling pathway. The results suggested that HP exerted an anti-osteoclastogenic effect by regulating the TRAF6/p38 MAPK signaling pathway.

2.6. Discussion

HP, as a kind of flavonoids, is found in many Chinese herbal medicines. In recent years, HP has also been studied in anti-osteoporosis. HP can protect MC3T3-E1 from oxidative stress via inhibiting the MAPK signaling pathway and oxidative damage of the cells [15], and reduce the apoptosis and autophagy rate of MC3T3-E1. In addition, HP also has a good anti-osteoporosis performance in OVX mice through NF-κB signaling pathway.

In this research, the effect of HP on RANKL induced OCs from RAW264.7 cells was studied, including TRAP-positive cell counting and TRAP activity test. TRAP, as a landmark phosphatase in OCs, can reflect the inhibitory ability of HP on osteoclastogenesis. As shown in Figure 2A and C, compared with the negative control, HP groups (10, 25, and 50 μM) gradually decreased the number of TRAP positive cells. In Figure 2D, TRAP activity in HP groups significantly decreased in a concentration dependent-manner. As shown in Figure 3A and B, compared with the negative control, HP groups reduced the area of bone resorption pits significantly. The above results indicated that HP could suppress the differentiation and bone resorption capacity of OCs.

The assembly of F-actin to form an organized ring is necessary for osteoporotic function. Mature OCs will form a fold boundary rich in F-actin structure on the bone surface, separating the absorption cavity from the extracellular space. The sealing area forms a large circular F-actin band and contains densely podosomes as an adhesion structure. In mature OCs, the podosomes will dynamically arrange into a

Figure 5. HP suppressed the mRNAs expression levels of osteoclast-related pathway genes. RAW264.7 cells were cultured with or without HP (10, 25, and 50 μM) or E2 (10⁻² μM) in an osteoclast-inducing media for 5 d. HP down-regulated the mRNAs expression levels of TRAF6, c-fos, NFATc1, CTSK and TRAP. All values represent mean ± SD. N = 3. *p < 0.05 and **p < 0.01 versus the negative control.
specialized near-membrane ring to migrate and absorb bone. These osteoclast-specific structures are essential for OCs to perform their functions [16, 17]. As shown in Figure 4(A,B), compared with the negative control, HP groups caused both the shrinkage of OCs and disruption of actin ring structure, and the number of intact actin rings gradually decreased in a dose-dependent manner.

In the signaling pathway of RANKL/RANK/TRAF6, NFATc1 plays a crucial role. The function of NFATc1 depends on three stages: initiation, amplification and targeting [18]. The initiation and amplification of NFATc1 mainly rely on its upstream signal regulation, such as the combination of RANKL and RANK. TRAF6 mediates the up-expression of signal molecules such as NF-κB, c-Jun, and p38 after its recruitment, and activates the downstream transcription process of NFATc1 [19]. It is reported [20] that the combination of c-fos, activated by p38, and NFATc1 can induce an increased expression level of NFATc1. NFATc1 affects the differentiation, fusion and function of OCs through the induction and expansion of target cell mRNA levels. While OCs precursor cells fused and OCs perform functions, the proteins encoded by the downstream target gene of NFATc1 play an important role, such as TRAP, CTSK, DC-STAMP, and tonoplast proton pump subunit (atp6v0d2) [21, 22]. Among them, CTSK can denature bone collagen [23], TRAP can dephosphorylate the bone matrix phosphate osteopontin and the bone sialoglycoprotein [24, 25].

Collectively, HP inhibited the expression level of TRAF6, and attenuated the phosphorylation of p38, so that the expressions of c-fos and NFATc1 were downregulated, and ultimately, the differentiation and bone resorption capacity of OCs were inhibited. As shown in Figures 5 and 6, HP had a certain inhibitory effect on the expression levels of genes and proteins related to the TRAF6/p38 MAPK signaling pathway.
3. Experimental

3.1. Materials, reagents, and antibodies

Hyperoside, purity > 98%, was purchased from Chengdu Pusi Biotechnology Co., Ltd (Chengdu, China). Estradiol (E2), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and TritanX-100 were purchased from Beijing Solarbio Biological Technology Co., Ltd (Beijing, China). Mouse recombinant RANKL protein was obtained from R&D Systems (Minneapolis, MN, USA). Fetal bovine serum was obtained from Zhejiang Tianhang Biotechnology Co., Ltd (Zhejiang, China). Leukocyte acid phosphatase (trap) kit and toluidine blue were purchased from Sigma System (Saint Louis, USA). Tartaric-resistant phosphatase (StrACP) test kit was obtained from Nanjing Jiancheng Institute of Bioengineering (Nanjing, China). The bone slices were purchased from Orthopedic Hospital of Lanzhou Military Region (Gansu, China). Phalloidin-tetramethylrhodamine conjugate was purchased from Shanghai Absin Company (Shanghai, China). BCA Total Protein Quantitative Kit was obtained from Beyotime (Shanghai, China). RT EasyTM and Real Time PCR Easy TM-SYBR Green I kit were purchased from FOREGENE (Chengdu, China). Total RNA Kit I was obtained from Omega BIO-TEK (Vermont, USA). Rabbit anti-mouse β-actin, c-fos, NFATc1 primary antibodies were purchased from Cell Signaling Technology (Danvers, USA). Rabbit anti-mouse CTSK, TRAP, TRAF6 primary antibodies were obtained from Abcam (Cambridge, UK). Goat anti-rabbit secondary antibody was obtained from Shanghai Absin Company (Shanghai, China).

3.2. Cell culture

RAW264.7 cells were cultured in 10% FBS medium (DMEM high glucose medium plus 10% FBS, 100 μg/ml penicillin streptomycin and 2 mM glutamine). In this study, cells were incubated in a CO₂ incubator (Thermo Fisher Scientific, Waltham, USA) at constant high humidity, 37 °C, and 5% CO₂ atmosphere.

3.3. Cytotoxicity assay

The cells were seeded into 96-well plates, which were randomly divided into experimental groups and control group. In the experimental groups, different concentrations of HP (0.01, 0.1, 1, 10, 100, and 1000 μM) were added to the 10% FBS medium. After 3 d, MTT (5 mg/ml; Solarbio, Beijing, China) was added to all wells, the plates were incubated for 4 h, and the absorbance value was measured at 490 nm using a multifunctional microplate reader (Thermo Fisher Scientific, Waltham, USA). Cell survival rate was calculated relative to the control group using the following formula: (experimental group OD – zeroing OD)/(control group OD – zeroing OD) ×100%.

3.4. Experiment grouping

RAW264.7 cells were divided into experimental groups: 10% FBS medium contains different concentrations of HP (10, 25, and 50 μM) and RANKL (30 ng/ml), the
negative control: 10% FBS medium contained RANKL (30 ng/ml), and the positive control: 10% FBS medium contained E2 (10^{-2} \mu M) and RANKL (30 ng/ml).

### 3.5. TRAP staining

RAW264.7 cells were inoculated with or without HP (10, 25, and 50 \mu M) or E2 (10^{-2} \mu M) in the osteoclast-inducing media for 5 d. Cultured cells were fixed with 10% formaldehyde, and stained according to Leukocyte acid phosphatase (trap) kit (Sigma System, Saint Louis, USA). The TRAP-positive cells (usually more than 3 nuclei) were counted under an inverted fluorescence microscope (Leica-microsystems, Hessen, Germany).

### 3.6. Determination of TRAP activity

RAW264.7 cells were seeded in 48-well plates, cultured with or without HP (10, 25, and 50 \mu M) or E2 (10^{-2} \mu M) in an osteoclast-inducing media for 5 d. The cells were rinsed with PBS after cell induction. About 1% TritanX-100 (Solarbio, Beijing, China) was added to the cells, then, the cells were put in an incubator at 37°C for 5 min. The obtained lysis solution was detected in accordance with the tartaric-resistant phosphatase (StrACP) test kit (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China).

### 3.7. Bone resorption activity assay

The bone slices (Orthopedic Hospital of Lanzhou Military Region, Gansu, China) were cut into appropriate sizes (0.5 × 0.5 cm) and placed into a 48-well plate. RAW264.7 cells were cultured with or without HP (10, 25, and 50 \mu M) or E2 (10^{-2} \mu M) in an osteoclast-inducing media for 7 d. The bone slices were firstly fixed with 10% formaldehyde, then, sonicated with 0.25 M ammonia water. 80%, 85%, 90%, and 100% ethanol were used for gradient elution, and finally the bone slices were stained with 0.1% toluidine blue (Sigma system, Saint Louis, USA). The bone slices were observed and photographed by a microscope, and the area of the bone resorption pits was calculated.

### 3.8. Actin ring formation assay

RAW264.7 cells were seeded in 96-well plates, inoculated with or without HP (10, 25, and 50 \mu M) or E2 (10^{-2} \mu M) in an osteoclast-inducing media for 5 d. Cells were rinsed with PBS, and fixed in 10% formaldehyde for 30 minutes. Then, 0.1% Triton X-100 was added into the fixed cells for 5 minutes to increase the permeability of cells. The fixed cells were rinsed for three times, then incubated with phalloidin-tetramethylrhodamine conjugate (1 \mu g/ml) for 30 min. F-actin stained with phalloidin-tetramethylrhodamine conjugate was photographed using a fluorescence microscope at a wavelength of 546 nm.
3.9. Real-time PCR analysis

RAW264.7 cells were cultured in six-well plates with or without HP (10, 25 and 50 μM) or E2 (10^{-2} μM) in an osteoclast-inducing media for 5 d. The induced cells were subjected to RNA extraction according to Total RNA Kit I (Omega BIO-TEK, Vermont, USA). Reverse transcription conditions and PCR reaction conditions were in accordance with RT EasyTM and Real Time PCR Easy TM-SYBR Green I kit (FOREGENE, Chengdu, China) respectively. The PCR primers were designed as follows: TRAF6, 5'-AGTATGAGTGTCCCCATCTGCT-3' and 5'-TTTACCGTCAGGGAAAGAAT-3'; c-fos, 5'-GGGTTGACCGTTCTTATTATCCT-3' and 5'-AGCCGCTGGAGAAGGAG-3'; NFATc1, 5'-GCCTCGAACCCTATCGAGTG-3' and 5'-TGCCCTCTTCGCTTC-3'; TRAP, 5'-CTCGACGTCGTTTTCTATCC-3' and 5'-ATGGTCACGCGATTC-3'; b-actin, 5'-CGGAGATCTGACGACGTC-3' and 5'-ATGGTCACGCGATTC-3'. Using b-actin as an internal reference, the mRNAs expression levels of osteoclast-related genes were calculated by the 2^-ΔΔct method.

3.10. Western blot analysis

Total protein was extracted using RIPA lysis buffer (containing 1% protease inhibitor). The total protein concentration was determined according to the BCA Total Protein Quantitative Kit (Beyotime, Shanghai, China). The sample proteins (20 μg) were resolved on 8% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), and then the protein was transferred to polyvinylidene difluoride membrane (Millipore, Massachusetts, USA). About 5% skimmed milk powder in TBS-Tween (25 mM Tris–HCl, pH 7.4; 150 mM NaCl; and 0.1% Tween 20) was used for blocking for 1 h. After incubating the primary antibody (rabbit anti-mouse) overnight at 4°C, the secondary antibody (goat anti-rabbit) was used to incubate membranes for 1 h. Protein signals were detected using the Immobilon Western HRP Substrate Peroxide Solution (Burlington, MA, USA) and the V3 Western Blotting System (Bio-Rad, State of California, USA). The band intensities of each protein were compared with b-actin, then, the ratio was compared with the negative control to obtain the relative quantification.

3.11. Statistical analysis

All experiments were conducted three times. The experimental data were expressed as mean ± standard deviation (SD) and compared by the two-tailed Student’s t-test or one-way analysis of variance (ANOVA) with Tukey’s multiple comparison post-hoc test. p < 0.05 was considered statistically significant.

4. Conclusions

In summary, this study demonstrated that HP had an inhibitory effect on the differentiation and function of OCs in the cell model of RAW264.7 induced by RANKL, and the underlying molecular mechanism was associated with the inhibition of
TRAF6/p38 MAPK signaling pathway. These results suggested that HP could be a promising natural compound for lytic bone diseases.

**Disclosure statement**

The authors have no conflicting interests.

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