The inhibitory effect and the molecular mechanism of glabridin on RANKL-induced osteoclastogenesis in RAW264.7 cells

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Abstract. Osteoblastic bone formation and osteoclastic bone resorption are in balance to maintain a constant, homeostatically controlled amount of bone. Excessive bone resorption by osteoclasts is involved in the pathogenesis of bone-related disorders. In the present study, we evaluated the inhibitory effects of glabridin, a flavonoid purified from licorice root, on the receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation and its molecular mechanisms in murine osteoclast progenitor RAW264.7 cells. Glabridin significantly inhibited RANKL-induced tartrate-resistant acid phosphatase (TRAP) activity, the formation of multinucleated osteoclasts and resorption-pit formation. In mechanistic studies of the anti-osteoclastogenic potential of glabridin, we found that glabridin inhibited RANKL-induced expression of c-Fos and subsequent expression of NFATc1, which is a master regulator of osteoclastogenesis. Interestingly, glabridin inhibited the RANKL-induced expression of signaling molecules (TRAF6, GAB2, ERK2, JNK1 and MKK7) and osteoclast survival-related signaling pathways such as c-Src, PI3K and Akt2. Glabridin also inhibited the bone resorptive activity of mature osteoclasts by inhibiting osteoclast-associated genes (cathepsin K, MMP-9, CAII, TCIRG1, OSTM1 and CLCN7). Taken together, our data suggest that glabridin holds great promise for use in preventing osteoclastogenesis by inhibiting RANKL-induced activation of signaling molecules and subsequent transcription factors in osteoclast precursors and these findings may be useful for evaluating treatment options in bone-destructive diseases.

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Introduction

Bone homeostasis is balanced by the regulation of bone-forming osteoblasts and bone-resorbing osteoclasts. Excessive bone resorption due to increased osteoclast formation and activity is involved in the pathogenesis of several bone diseases, such as osteoporosis, hypercalcemia, rheumatoid arthritis, tumor metastasis into bone, periodontitis and Paget's disease (1). Osteoclasts are useful targets for the development of anti-resorptive drugs for bone-reducing diseases. Osteoclast differentiation takes place through multiple steps such as differentiation, fusion, and activation of mature osteoclasts by cell-to-cell contact with osteoblast lineages that express factors regulating osteoclast differentiation (2). Receptor activator of nuclear factor-κB (RANK), which belongs to the tumor necrosis factor superfamily, is present in osteoclasts. This factor promotes osteoclastogenensis when it binds to the RANKL, which is produced by the osteoblast and stromal cells (3). RANK and its ligand RANKL are key molecules regulating the differentiation and bone-resorbing capability of osteoclasts (4). The RANK-RANKL interaction triggers the activation of cytoplasmic TNF receptor associated factor-6 (TRAF6), which subsequently induces the activation of mitogen-activated protein (MAP) kinases, non-receptor tyrosine kinase c-Src (c-Src), phosphatidylinositol 3-kinase (PI3K), Akt, and transcription factors including activator protein (AP)-1 and nuclear factor of activated T cells c1 (NFATc1) (5). In addition, the molecular adaptor Grb-2-associated binder 2 (Gab2) is associated with RANK and mediates RANK-induced activation of Akt and c-jun N-terminal kinase (JNK) (6). In line with the induction and activation of the transcription factors for osteoclast differentiation, genetic studies have shown that deficiency in the c-Fos transcription factor, a major component of AP-1, or NFATc1 inhibits osteoclastogenesis (7,8). A large number of genes have been shown to play a crucial role in osteoclast differentiation and function. Deletion

of several genes including c-Src, TRAP, and cathepsin K have been shown to hinder osteoclast activity (9-11). There are also defects related to the differentiation of osteoclasts at earlier stages, where c-Src is uniquely required for cell spreading (12).

Mature osteoclasts secrete hydrogen ions and proteinases such as cathepsin K and matrix metalloproteinase (MMP)-9 from ruffled border, which dissolve the inorganic and organic components of bone, respectively. Hydrogen ions are produced via carbonic anhydrase II (CAII) in the cytoplasm and are secreted extracellularly by H^+-ATPases (13). The vacuolar (v)-type H^+-ATPase is the central driving force for acid secretion. One of the membrane subunits of this enzyme, T-cell, immune regulator 1 (TCIRG1), is essential for insertion of the osteoclastic H^+-ATPase into the osteoclast's extracellular membrane (14). In osteoclast, osteopetrosis-associated transmembrane protein 1 (OSTM1) and chloride channel 7 (CLCN7) colocalize with the a3 subunit of the v-type H^+-ATPase in the ruffled border (15). CLCN7 is a chloride channel that acts with the vacuolar H^+-ATPase to acidify the resorption space.

Given the association between osteoclastic bone-resorption and bone disorders, inhibition of osteoclastogenesis and functional activity of mature osteoclasts have been considered effective therapeutic strategies to inhibit bone disorders that are associated with excessive bone resorption. An increasing amount of evidence suggests that dietary flavonoids may provide desirable bone health benefits by restoration of the metabolic balance of bone formation and resorption (16,17). During screening of naturally occurring antiresorptive agents, we found that glabridin inhibited osteoclast differentiation. Glabridin is an isoflavan compound and is one of the major active flavonoids in licorice (18). Haraguchi *et al* (19) and Choi (20) reported that glabridin is effective in protecting mitochondrial function against oxidative stresses in osteoblast. In addition, it was shown that glabridin accumulation in macrophages was associated with reduced cell-mediated oxidation of low-density lipoproteins and decreased activation of the NADPH oxidase system (21). Kang *et al* (22) demonstrated that glabridin inhibited nitric oxide production by blocking NF-κB/Rel activation, and protected mice against LPS-induced sepsis. Moreover, Choi (23) reported that the enhanced osteoblast function by glabridin may prevent osteoporosis and inflammatory bone diseases. However, no study has evaluated the effects of glabridin on osteoclast differentiation and bone resorption. In this study, we first examined the effect of glabridin on osteoclastogenesis and gene expression to investigate the probable molecular mechanism of the anti-osteoclastogenic effect of glabridin in osteoclast progenitor RAW264.7 cells.

Materials and methods

Osteoclast differentiation of RAW264.7 cells. The RAW264.7 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin) and 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical, St. Louis, MO, USA) and maintained at 37˚C in 5% CO_2 humidified air. The cells were seeded at a density of $2x10^4$ cells/well in 24-well plates. After 24 h, the media were replaced and the cells were cultured for an additional 3 days in DMEM containing 100 ng/ml RANKL (Sigma Chemical) and different concentrations of glabridin (ChromaDex, Inc., Irvine, CA, USA) for 3 days with differentiation medium (DMEM containing 100 ng/ml RANKL). Glabridin was dissolved in dimethylsulfoxide (DMSO) and then diluted with the medium [final DMSO concentration ≤0.05% (v/v)].

TRAP activity measurement and TRAP-positive multinucleated cells. At the end of culture, the medium was removed and the cells were fixed with cold 10% formalin. The cells were washed three times with distilled water. A TRAP staining kit (Kamiya Biomedical Co., Seattle, WA, USA) was used to confirm the generation of multinucleated osteoclasts (MNC) and TRAP activity according to the instruction manual. Images of TRAP-positive cells were captured using a microscope. TRAP-positive cells appeared dark red, and TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. To preclude the possibility that the attenuation in TRAP activity was due to cytotoxicity, cell viability was simultaneously measured using the MTT assay.

Resorption pit assay. The resorptive function of mature MNC derived from RANKL-differentiated RAW264.7 cells was analyzed on BD Biocoat Osteologic Multitest Slide (BD Biosciences, San Jose, CA, USA). After 6 days of culture, the cell culture plates were treated with 6% sodium hypochlorite for 5 min. The plates were washed with deionized water, then dried and inaged. The resorption area was observed under a light microscope and analyzed.

RNA extraction. Total-RNA was isolated from cells using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA). After isolation, RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). cDNAs were synthesized with a Transcriptor first strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) and stored at -70˚C until further use. All procedures were performed according to the manufacturer's instructions.

Real-time RT-PCR. Real-time PCR was performed to verify the differential expression of selected genes using a Roche LightCycler 480 system (Roche Diagnostics GmbH) and the Taqman method using the Roche Universal ProbeLibrary (UPL) kit. Relative gene expression was determined by employing the comparative CT method. All reactions were carried out in a total reaction volume of 20 μl, which contained 10.0 μl of 2X UPL master mix, 1.0 μl of 5' primer (10 pmol/μl), 1.0 μl of 3' primer (10 pmol/ml), 0.2 μl of UPL probe, 1.0 μl of cDNA and 6.8 μl of sterile water. The thermal cycling conditions for PCR were an initial denaturation for 10 min at 95˚C, followed by 40 cycles of 94˚C for 10 sec and 60˚C for 30 sec. The primers summarized in Table I were designed by the Roche ProbeFinder assay tool. For the RT-PCR analysis, duplicate PCRs were carried out for each cDNA. Negative controls (except templates) were included in the PCR reaction to ensure specific amplification. LightCycler 480 software version 1.2 (Roche) was used for analysis of the quantitative PCR. The values obtained from each sample were

Table I. Primer sequences used in this study.

Genes	Sequence
Akt2	5'-CGA CCC AAC ACC TTT GTC A-3'
	5'-GAT AGC CCG CAT CCA CTC T-3'
CAII	5'-GGG GAT ACA GCA AGC ACA AC-3'
	5'-GAC TGC CGG TCT CCA TTG-3'
Cathepsin K	5'-CGA AAA GAG CCT AGC GAA CA-3'
	5'-TGG GTA GCA GCA GAA ACT TG-3'
c-Fos	5'-CAG CCT TTC CTA CTA CCA TTC C-3'
	5'-ACA GAT CTG CGC AAA AGT CC-3'
CLCN7	5'-TCG GAC AGA TGA ACA ACG TG-3'
	5'-GGT GTG AGG AGG ATC GAC TT-3'
ERK2	5'-GGA TTG AAG TTG AAC AGG CTC T-3'
	5'-GAA TGG CGC TTC AGC AAT-3'
GAB2	5'-AGA TCT GCG GCT TCA ATC AG-3'
	5'-GAC TGG CTG AAG AAA GGT TCC-3'
HPRT	5'-TCC TCC TCA GAC CGC TTT T-3'
	5'-CCT GGT TCA TCA TCG CTA ATC-3'
JNK1	5'-GAA GCT CTC CAG CAC CCA TA-3'
	5'-TAA CTG CTT GTC CGG GAT CT-3'
MKK7	5'-GGT GCT CAC CAA AGT CCT ACA-3'
	5'-TTT GGT CTC TTC CTG TGA TCT TTA-3'
MMP9	5'-ACG ACA TAG ACG GCA TCC A-3'
	5'-GCT GTG GTT CAG TTG TGG TG-3'
NFATc1	5'-TCC AAA GTC ATT TTC GTG GA-3'
	5'-CTT TGC TTC CAT CTC CCA GA-3'
OSTM1	5'-GGT CTC TGA GTT TTT CAA CAG CA-3'
	5'-CCT CAC CAT TGT TTG TTA GGC-3'
PI3K	5'-CCA GAC AGT GTT TTT GTA AGA GGA-3'
	5'-TCC ATG CCC TAT GCG ACT-3'
Src	5'-CTT CGG AGA GGT GTG GAT G-3'
	5'-GTG CCT GGG TTC AGA GTT TT-3'
TCIRG1	5'-CCA TAT CCC TTT GGC ATT GA-3'
	5'-GAG AAA GCT CAG GTG GTT CG-3'
Traf6	5'-TTG CAC ATT CAG TGT TTT TGG-3'
	5'-TGC AAG TGT CGT GCC AAG-3'

normalized to HPRT (hypoxanthine guanine phosphoribosyl transferase) expression. Levels of each gene expression in all experimental groups were compared to the expression levels of the control group.

Enzyme-linked immunosorbent assay (ELISA). The amounts of MMP-9 in the culture medium were determined using a MMP-9 immunoassay kit (R&D System, USA). c-Src was measured using PathScan® Total Src Sandwich ELISA Kit (Cell Signaling Technology, Danvers, MA, USA), which detects endogenous levels of total Src protein.

Statistical analysis. Results are expressed as mean ± SEM of at least three independent experiments. The data were analyzed by one-way analysis of variance (ANOVA) followed by the post-hoc Dunnett's t-test for multiple comparisons. A P-value <0.05 was considered significant.

Results

Glabridin inhibits the RANKL-induced osteoclastogenesis of RAW264.7 cells. To investigate the effects of glabridin on osteoclast differentiation, we first evaluated its effects on RANKL-induced TRAP activity and formation of TRAP-positive multinucleated osteoclasts using RAW264.7 cells. Glabridin significantly reduced the RANKL-induced TRAP activity in RAW264.7 cells at the concentrations of 2 and 5 μM (Fig. 1A). Moreover, glabridin (2 or 5 μM) significantly inhibited RANKL-induced formation of TRAP-positive multinucleated osteoclasts (Fig. 1B and E). This inhibition of osteoclastogenesis was not due to cytotoxicity since the MTT analysis demonstrated that glabridin did not affect cell viability at the concentrations used (Fig. 1C). To evaluate the effects of glabridin on the bone-resorbing activity of mature osteoclasts, osteoclasts were cultured on BD Biocoat Osteologic Multitest slides in the presence of glabridin, and resorption-pit formation was assessed. Glabridin (2 or 5 μM) markedly suppressed the resorption-pit formation induced by RANKL (Fig. 1D and F). These findings indicate that glabridin inhibits the bone-resorbing activity of mature osteoclasts.

Glabridin inhibits the RANKL-induced TRAF6 and GAB2 expression in RAW264.7 cells. The binding of RANKL to its receptor RANK results in the recruitment of adaptor molecules such as TRAF6 and GAB2, which activates MAPKs and transcription factors involved in osteoclastogenesis. To define the molecular mechanism by which glabridin inhibits osteoclast differentiation, we examined the effects of glabridin on the RANKL-induced expression of the TRAF6 and GAB2 genes. The RANKL-induced expression of TRAF6 and GAB2 was significantly inhibited by 5 μM glabridin (Fig. 2).

Glabridin inhibits the RANKL-induced ERK2, JNK1 and MKK7 expression in RAW264.7 cells. RANKL induces the activation of MAPKs (ERK, JNK and p38) and ERK and JNK participate in c-Fos and c-Jun activation in osteoclast precursors (24). Therefore, we determined whether MAPKs were involved in the inhibition of osteoclastogenesis by glabridin. RANKL strongly activated ERK2 and JNK1 gene expression and this induction was inhibited by glabridin at a concentration of 5 μM (Fig. 3A and B). MKK7 is the major activator of JNK in osteoclast precursors in response to RANKL. The presence of glabridin resulted in prominent reductions in RANKL-induced MKK7 expression at concentrations of 2 or 5 μM, suggesting that JNK1 may be inhibited through glabridin-induced reduction of MKK7 expression (Fig. 3C).

Glabridin inhibits the RANKL-induced expression of c-Fos and NFATc1 genes in RAW264.7 cells. Since c-Fos and NFATc1 have been shown to play a critical role in osteoclast differentiation, the effect of glabridin on the expression of c-Fos and NFATc1 genes was also evaluated. c-Fos and NFATc1 were highly expressed by RANKL treatment, but glabridin significantly inhibited RANKL-induced expression of those genes at a concentration of 5 μM (Fig. 4).

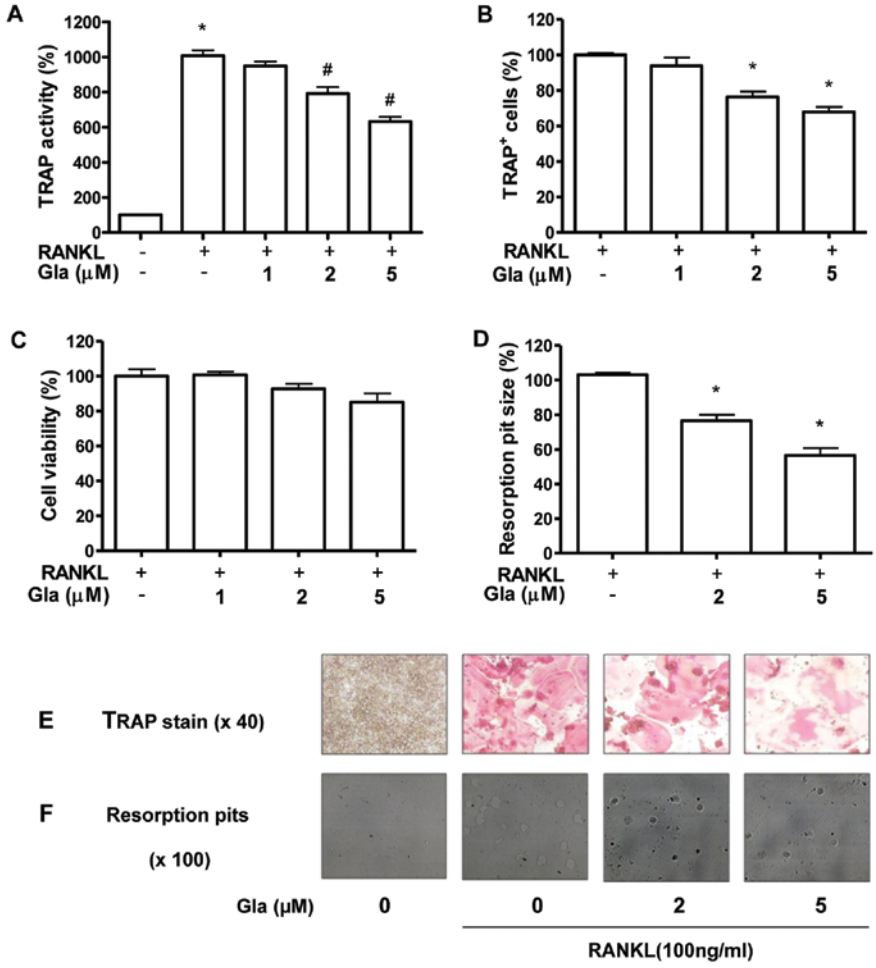

Figure 1. Inhibition of the RANKL-induced osteoclastogenesis in RAW264.7 cells by glabridin. (A) Effects of glabridin on RANKL-induced TRAP activity, (B) RANKL-induced formation of TRAP-positive multinucleated osteoclasts, (C) cell viability in RAW264.7 cells, and (D) osteoclastic resorption-pit formation. (E) Photograph of TRAP staining and (F) resorption pits. Data were expressed as a percentage of control. *P<0.05 vs. control. #P<0.05; RANKL vs. glabridin (Gla).

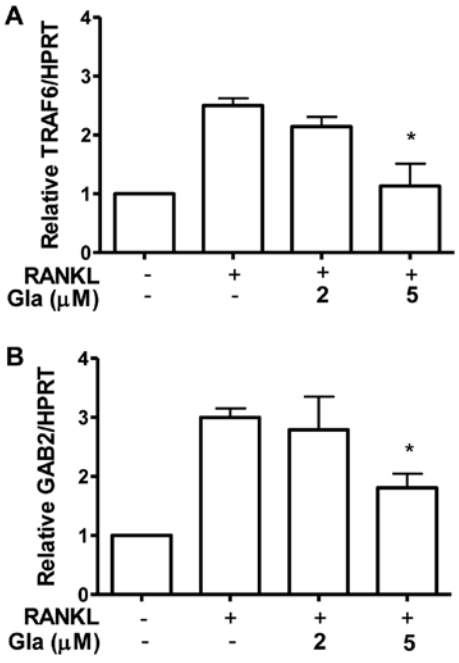

Figure 2. Suppression of the RANKL-induced expression of TRAF6 and GAB2 by glabridin. Gene expression of (A) TRAF6 and (B) GAB2 was detected by real-time QPCR. *P<0.05 compared with the results of the RANKL-treated cells without glabridin (Gla).

Glabridin inhibits the RANKL-induced expression of c-Src, PI3K, and Akt2 in RAW264.7 cells. c-Src, PI3K, and Akt2 are known to be involved in osteoclast survival and differentiation (25). The inhibitory effect of glabridin on RANKL-induced gene expression of c-Src, which is required for functional osteoclasts, was further evaluated by real-time PCR. Gene expression of Src was markedly increased in cells cultured with RANKL (Fig. 5A). However, glabridin (2 or 5 μM) decreased the gene expression of c-Src induced by RANKL. The inhibitory effect of glabridin on RANKL-induced protein production of c-Src was also evaluated by ELISA. Protein levels of c-Src were markedly increased in cells cultured with RANKL (Fig. 5B). However, glabridin (2 or 5 μM) decreased the protein levels of c-Src induced by RANKL. Glabridin also inhibited the increase in expression of PI3K and Akt2 induced by RANKL at a concentration of 5 μM (Fig. 5C and D).

Glabridin inhibits the RANKL-induced expression of cathepsin K and MMP-9 in RAW264.7 cells. To elucidate the mechanism of action of glabridin during the process of bone resorption, the effects of glabridin on the gene expression of cathepsin K and MMP-9 were evaluated by real-time PCR using RAW264.7 cells with or without glabridin in the presence of RANKL. We found that the expression of cathepsin K

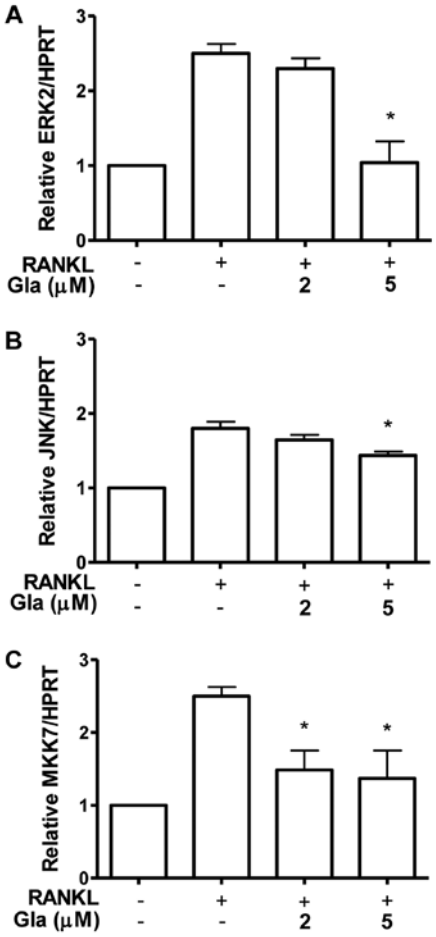

Figure 3. Suppression of the RANKL-induced expression of ERK2, JNK1, and MKK7 by glabridin. Gene expression of (A) ERK2, (B) JNK1 and (C) MKK7 was detected by real-time QPCR. *P<0.05 compared with the results of the RANKL-treated cells without glabridin (Gla).

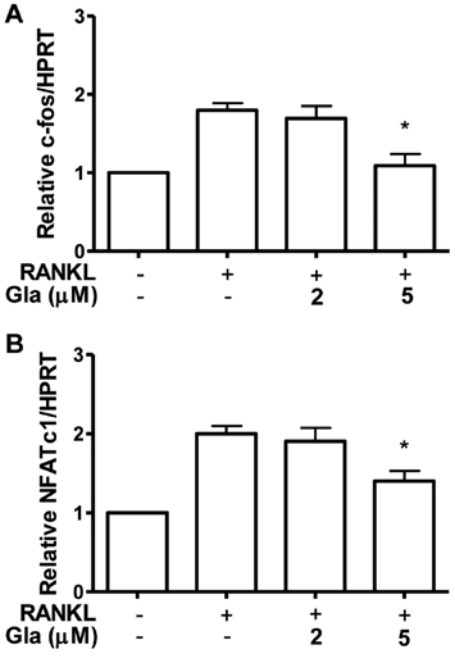

Figure 4. Suppression of the RANKL-induced expression of the transcription factors, c-Fos and NFATc1 by glabridin. Gene expression of (A) c-Fos and (B) NFATc1 was detected by real-time QPCR. *P<0.05 compared with the results of the RANKL-treated cells without glabridin (Gla).

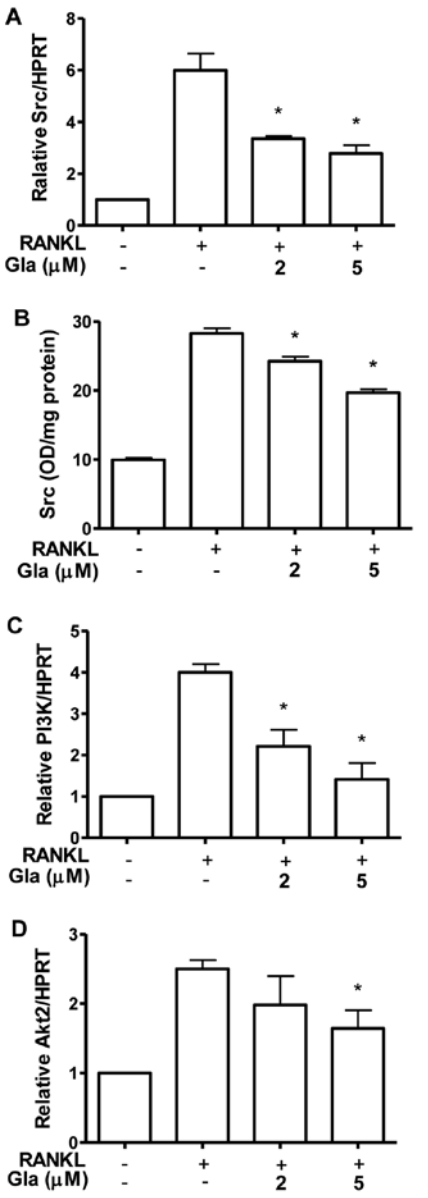

Figure 5. Suppression of the RANKL-induced expression of c-Src, PI3K, and Akt2 by glabridin. Gene expression of (A) c-Src, (C) PI3K and (D) Akt2 was detected by real-time QPCR. The protein level of (B) c-Src was detected by ELISA kit. *P<0.05 compared with the results of the RANKL-treated cells without glabridin (Gla).

was down-regulated with the treatment of glabridin at a concentration of 5 μM (Fig. 6A). We next examined whether or not glabridin could also reduce MMP-9. MMP-9 gene expression was markedly increased in cells treated with RANKL relative to untreated cells. Meanwhile, the addition of glabridin (2 or 5 μM) decreased the induction of MMP-9 by RANKL (Fig. 6B). The inhibitory effect of glabridin on RANKL-induced protein production of MMP-9 was evaluated by ELISA. Protein levels of MMP-9 were markedly increased in cells cultured with RANKL (Fig. 6C). However, glabridin (2 or 5 μM) decreased the protein levels of MMP-9 induced by RANKL.

Glabridin inhibits RANKL-induced gene expression of TCIRG1, OSTM1, CLCN7 and carbonic anhydrase II in RAW264.7

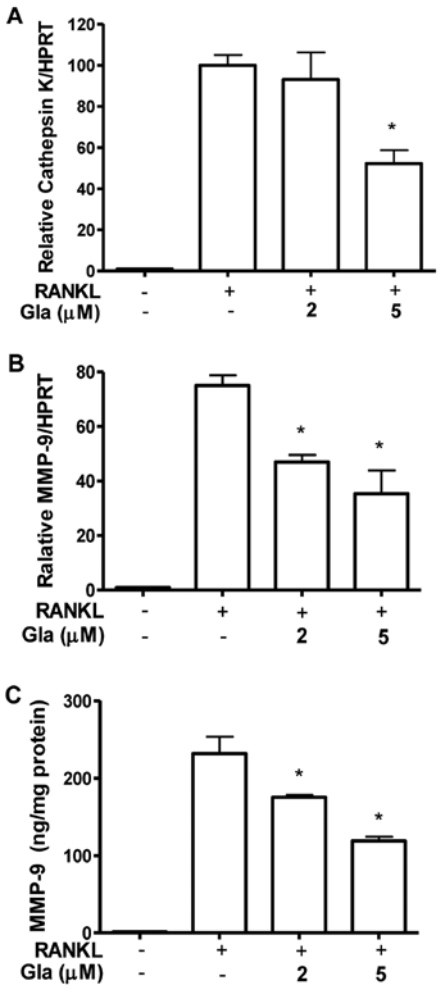

Figure 6. Suppression of the RANKL-induced expression of cathepsin K and MMP-9 by glabridin. Gene expression of (A) cathepsin K and (B) MMP-9 was detected by real-time QPCR. The protein level of (C) MMP-9 was detected by ELISA kit. *P<0.05 compared with the results of the RANKL-treated cells without glabridin (Gla).

cells. Mature osteoclasts were also characterized by high expression of a series of osteoclast markers such as TCIRG1, OSTM1, CLCN7 and CAII. The inhibitory effect of glabridin on RANKL-induced mRNA expression levels of these genes was further evaluated by real-time QPCR. The expression of osteoclast-associated genes including TCIRG1, OSTM1, CLCN7 and CAII was dramatically induced by RANKL, but the presence of glabridin (5 μM) significantly inhibited the expression of these genes (Fig. 7).

Discussion

Recent reports have indicated that dietary flavonoids provide health benefits by restoring the metabolic balance of bone formation and resorption, suggesting that consumption of certain flavonoids may contribute to bone health (16,17). Glabridin is an isoflavan compound and is one of the major active flavonoids in licorice. It has been reported that glabridin protects antimycin A-induced cytotoxicity and increases the function of bone-forming osteoblastic cells (20,23); however, little is known about the effects of glabridin on osteoclast differentiation and bone resorption. We first found that glabridin inhibited RANKL-induced osteoclastogenesis in RAW264.7 cells, which is a homogeneous clonal population of murine monocytic cells. We also evaluated its molecular mechanism of action during osteoclast differentiation and bone resorption. RANKL is known to be expressed in activated T cells as well as osteoblastic cells. RANK, which is a receptor for RANKL, is the sole signaling receptor for RANKL in inducing the development and activation of osteoclast cells.

The binding of RANKL to RANK results in the recruitment of TRAF6, which activates PI3K, Akt, MAP kinases and transcription factors such as AP-1 and NFATc1 (25). Glabridin suppressed the RANKL-induced mRNA expressions of c-Fos and NFATc1, which are important transcription factors for osteoclastogenesis (5,26). NFATc1 regulates the expressions of a number of osteoclast-specific genes including TRAP, Src, and cathepsin K, which are modulators of osteoclast fusion, activation and function (27). These reports support our data, demonstrating that glabridin suppresses osteoclastogenesis by inhibiting the expressions of these genes. An AP-1 complex containing c-Fos is required for the auto-amplification of NFATc1, enabling a robust induction of NFATc1. RANKL activates AP-1 partly through an induction of its critical component, c-Fos (28). Thus, we suggest that the inhibition of RANKL-induced c-Fos activation by glabridin is a relevant factor in the suppression of downstream NFATc1 signaling pathways. In addition, when considering that the induction and activation of c-Fos can be regulated by ERK (29), the induction of AP-1 in the osteoclast differentiation could result from the activation of ERK. ERK has been reported to play an important role in cell survival, and may also be involved in osteoclast differentiation (29). Our results showed that glabridin significantly inhibited RANKL-induced expression of MAPKs, especially ERK2 and JNK1. Therefore, a reasonable explanation for this anti-osteoclastogenesis activity may be that glabridin suppresses c-Fos expression via inhibition of upstream MAPKs activation.

Kim *et al* (30) previously reported that severe osteopetrosis and defective osteoclast formation occurred in TRAF6-deficient mice because of abrogated RANK signaling. Furthermore, it was reported that activation of JNK requires the phosphorylation and recruitment of the Grb2-binding adapter protein, GAB2, to RANK (6). GAB2 is rapidly phosphorylated upon RANK stimulation, associates with the cytoplasmic tail of RANK and controls RANK-mediated activation of JNK and Akt, which mediate essential signaling pathways for osteoclastogenesis (31). In the present study, we showed that glabridin-induced anti-osteoclastogenesis occurred at least in part through inhibition of TRAF6 and GAB2. This suggests that the assembly of a multiprotein complex including the RANK, TRAF6 and GAB2 may be facilitated by osteoclastogenic signals, and that glabridin may inhibit this multiprotein complex formation and consequently reduce RANK downstream signaling possibly through TRAF6 and GAB2. In addition, glabridin inhibited mitogen activated protein kinase kinase (MKK) 7 expression induced by RANKL. MKK7 is the major activator of JNK in osteoclast precursors in response to RANKL. JNK is known to activate AP-1 and to be activated by the phosphorylation of Thr and Tyr residues via MKK4 and/or MKK7.

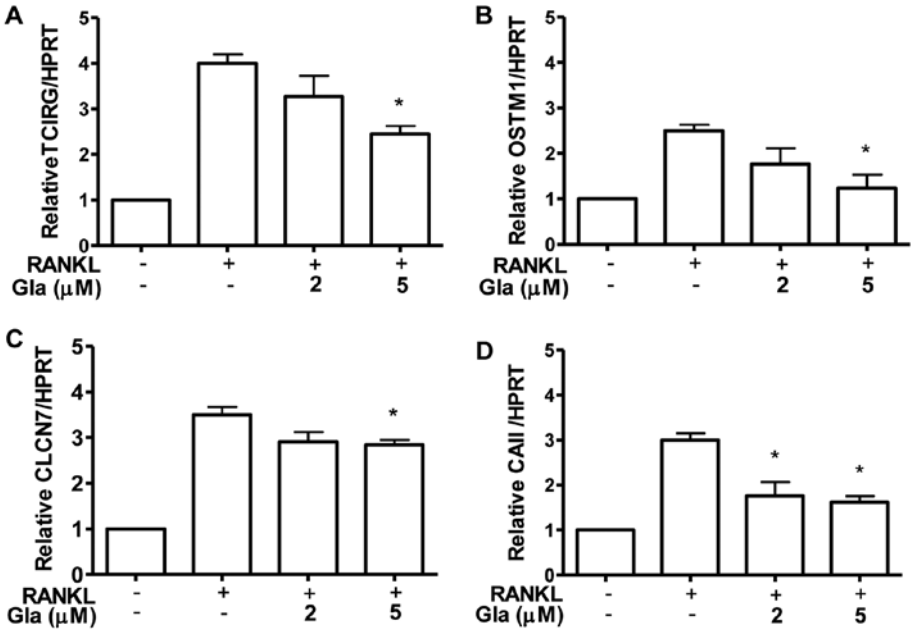

Figure 7. Glabridin inhibits RANKL-induced expression of TCIRG1, OSTM1, CLCN7 and carbonic anhydrase II (CAII) in RAW264.7 cells. Gene expression of (A) TCIRG1, (B) OSTM1, (C) CLCN7 and (D) CAII was detected by real-time QPCR. *P<0.05 compared with the results of the RANKL-treated cells without glabridin (Gla).

In the present study, the expression of other osteoclast associated genes, Src, PI3K and Akt2 were shown to be decreased by treatment with glabridin. Our results support the notion that glabridin could inhibit osteoclastogenesis by inhibiting RANKL-induced activation of osteoclast survival-related signaling molecules such as Src, PI3K and Akt2. In addition, Src has been reported to be required for osteoclastic bone resorption (32). c-Src-catalyzed phosphorylation of Cbl on Tyr-731 has been reported to create a binding site for the regulatory p85 subunit of PI3K (33), and induce its activation and recruitment to the cell membrane. PI3K in turn is involved in the reorganization of the cytoskeleton, which results in cell spreading and migration of several cell types including osteoclasts. PI3K/Akt-signaling plays a critical role in cell growth and differentiation. Activated PI3K transfers phosphate to the D3 position of the inositol ring present in the phosphatidylinositol 4,5-bisphosphate (PIP2) to produce the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3) and PIP3 recruits Akt to the plasma membrane. PI3K-dependent Akt kinase targets association of CBP with Smad 1/5 to regulate the transcription of CSF-1 in osteoblastic cells, which is necessary for osteoclast differentiation. Moreover, it is well known that Src-dependent Akt activation is important for RANKL-induced osteoclast survival (34).

Mature osteoclasts are also characterized by high expression of a series of osteoclast markers, of which TRAP, MMP-9, cathepsin K, carbonic anhydrase II, TCIRG1, CLCN7, and OSTM1 are the most prominent (35). In this study, we showed that glabridin inhibited osteoclast differentiation and also inhibited the expression of all of the above osteoclast specific genes, which play important roles in bone resorption. Moreover, the bone pit formation assay revealed the inhibitory effect of glabridin on bone resorption, suggesting that these effects could result from the ability of glabridin to inhibit the RANKL-induced expression of bone resorption-related genes. CAII is involved in the extracellular acidification caused by osteoclasts. CAII generates H^+ and $HCO3^-$ by the hydration of CO_2, and the H^+ protons are transported through the apical ruffled border of the osteoclasts to the resorption zone by vacuolar H^+-ATPase (36). This results in the secretion of HCl into the resorptive microenvironment, producing an acidic milieu (37). This condition first mobilizes bone minerals and subsequently, the demineralized organic component of the bone is degraded by a lysosomal protease, cathepsin K, and MMP-9 (36,38). Of the several proteolytic enzymes that degrade the bone matrix, cathepsin K and MMP-9 are highly expressed in osteoclasts. These genes are efficient collagenases and can cleave both collagen types I and II (39).

The vacuolar proton pump (VPP) is a multisubunit complex of more than 10 subunits, among which the a3 subunit was shown to be specific for osteoclasts (40). The TCIRG1 gene encodes the a3 isoform of the v-ATPase a subunit, which plays a critical role in the resorption activity of the osteoclast. In addition, it should be noted that the chloride channel may also be involved in the process to electrically shunt the VPP. In osteoclasts, CLCN7 is localized in the lysosomes and the ruffled border (41). The main role for CLCN7 is in enzyme dissolution of the inorganic phase of bone matrix (42), where it provides the chloride conductance required for efficient proton pumping by the vacuolar-ATPase (43), and thus acid efflux from osteoclastic cells. OSTM1 is a putative single-pass type I membrane protein with a heavily glycosylated extracellular domain. This gene encodes a protein that may be involved in the degradation of G proteins via the ubiquitin-dependent proteasome pathway. It is expressed in lysosomes and the ruffled border of osteoclasts (15). It is known that OSTM1 functions as a stabilizing β subunit for CLCN7 (15). The OSTM1 protein forms a complex with CLCN7 and as such has been proposed to be a β-subunit of active CLCN7 (15). Based on the above findings, it appears that glabridin may play an

important role in bone resorption inhibiting TCIRG1, CLCN7 and OSTM1 genes.

In summary, the present study demonstrated that glabridin could inhibit osteoclastogenesis, and that its inhibitory activity could result from its ability to block RANKL-induced activation of signaling molecules (TRAF6, GAB2, ERK2, JNK1 and MKK7), which regulate the expression of osteoclast-associated genes (cathepsin K, MMP-9, CAII, TCIRG1, OSTM1 and CLCN7) by modulating the activation and expression of transcriptional factors such as c-Fos and NFATc1. Glabridin could also inhibit the bone resorptive activity of mature osteoclasts by inhibiting osteoclast survival-related signaling pathways, such as Src, PI3K and Akt. Further studies need to be carried out in order to determine its precise mechanism and biological efficacy in *ex vivo* or *in vivo* models.

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