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Arctigenin suppresses receptor activator of nuclear factor κB ligand (RANKL)-mediated osteoclast differentiation in bone marrow-derived macrophages

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ABSTRACT

Osteoclasts, multinucleated bone-resorbing cells, are closely associated with bone diseases such as rheumatoid arthritis and osteoporosis. Osteoclasts are derived from hematopoietic precursor cells, and their differentiation is mediated by two cytokines, including macrophage colony stimulating factor and receptor activator of nuclear factor κB ligand (RANKL). Previous studies have shown that arctigenin exhibits an anti-inflammatory effect. However, the effect of arctigenin on osteoclast differentiation is yet to be elucidated. In this study, we found that arctigenin inhibited RANKL-mediated osteoclast differentiation in bone marrow macrophages in a dose-dependent manner and suppressed RANKL-mediated bone resorption. Additionally, the expression of typical marker proteins, such as NFATc1, c-Fos, TRAF6, c-Src, and cathepsin K, were significantly inhibited. Arctigenin inhibited the phosphorylation of Erk1/2, but not p38 and JNK, in a dose-dependent manner. Arctigenin also dramatically suppressed immunoreceptor tyrosine-based activation motif-mediated costimulatory signaling molecules, including Syk and PLCγ2, and Gab2. Notably, arctigenin inhibited the activation of Syk through RANKL stimulation. Furthermore, arctigenin prevented osteoclast differentiation in the calvarial bone of mice following stimulation with lipopolysaccharide. Our results show that arctigenin inhibits osteoclast differentiation in vitro and in vivo. Therefore, arctigenin may be useful for treating rheumatoid arthritis and osteoporosis.

1. Introduction

Bone remodeling depends on maintaining a balance between bone formation by osteoblasts and bone resorption by osteoclasts (Takayanagi, 2005a, 2005b). Thus, bone-forming osteoblasts and bone-resorbing osteoclasts play essential roles in bone homeostasis (Rahman et al., 2006). Excessive resorption by osteoclasts may lead to pathological destruction such as osteoporosis and rheumatoid arthritis (Boyle et al., 2003). Osteoclasts are large multinucleated cells derived from hematopoietic precursor cells of the monocyte/macrophage lineage (Rodan and Martin, 2000). The in vivo activity of osteoclasts is supported by bone marrow stromal cells or osteoblasts through cell–cell interactions (Teitelbaum, 2000a, 2000b). Osteoclast numbers must be regulated to sustain physiological remodeling and inhibit excess bone resorption leading to pathological bone destruction (Del Fattore et al., 2008).

Osteoblasts and stromal cells express macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL). These cytokines are essential for osteoclastogenesis (Teitelbaum, 2000a, 2000b). M-CSF maintains proliferation and survival and upregulates RANK expression in osteoclast precursor cells. RANKL is a member of the tumor necrosis factor (TNF) family and interacts with the receptor RANK, which recruits adaptor molecules such as TNF receptor-associated factors (TRAFs) (Asagiri and Takayanagi, 2007), and is involved in the downstream activation of the mitogen-activated protein (MAP) kinases, nuclear factor κB (NF-κB), and activator protein-1 (AP-1) (Walsh et al., 2006). RANKL plays a role in bone metabolism by mediating osteoclast formation and activation and calcium homeostasis (Dempsey et al., 2003; Feng, 2005). Among the TRAFs, TRAF6 is the most important adaptor molecule for RANKL-mediated signaling because it activates various kinases via its multiple domains (Bharti and Aggarwal, 2004; Lamothe et al., 2007; Wada et al., 2006).

Arctigenin is a lignan found in some plants, including *Arctium lappa* L. (Compositae) and *Saussurea medusa* (Asteraceae) (Sun et al., 1992; Takasaki et al., 2000). Traditionally, extracts from these plants have been used as therapeutic agents to treat inflammation-associated diseases such as throat swelling and syphilis. It has also

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been reported that arctigenin exhibits antioxidant, antitumor, and anti-inflammatory activities (Awale et al., 2006; Matsumoto et al., 2006; Zhao et al., 2009). Recently, we reported that a combination of herbal extracts from Trachelospermi caulis and Moutan cortex radicis significantly reduced the number of osteoclasts in the proximal tibia of CIA mice; several components, including arctigenin, have been identified from the mixture (Kim et al., 2011). To examine the anti-osteoclastogenic effect of the compound from the herbal mixture, we evaluated the effect of arctigenin on the differentiation of osteoclasts and examined the mechanism of action. In this study, we showed that arctigenin had inhibitory effects on RANKL-mediated osteoclast differentiation, bone resorption, and lipopolysaccharide (LPS)-induced osteoclast differentiation in mice, likely through inhibition of NF-κB and Syk kinase.

2. Materials and methods

2.1. Reagents

The following reagents were obtained: arctigenin was purchased from Tocris Bioscience (Bristol, UK); recombinant M-CSF and RANKL were from Peprotech, Inc. (Rocky Hill, NJ, USA); transcription factor probes against nuclear factor κB (NF-κB) were from Panomics (Fremont, CA, USA); antibodies against TRAF6, NFATc1, c-Fos, and cathepsin K were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibody against c-Src was from Millipore (London, UK); and antibodies against the phosphorylated forms of Syk (Tyr525/526), ERK1/2 (Thr202/Tyr204), p38 (Thr180/Tyr182), JNK (Thr183/Tyr185), PLCγ2 (Tyr1217), and Gab2 (Tyr452) were from Cell Signaling Technology (Beverly, MA, USA). Cell culture media and other culture reagents were from Gibco-BRL (Grand Island, NY, USA).

2.2. Measurement of cytotoxicity

BMMs (1×10^4 cells/well) were plated on 96-well plates in 30 ng/ml of M-CSF for 24 h. Next, cells were treated with the indicated concentrations of arctigenin (0.3–3 μM) in the presence of 100 ng/ml of RANKL and 30 ng/ml of M-CSF. Cell viability was determined every 24 h for 4 days by using a cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's protocol. Briefly, CCK-8 solution (at a 1:10 ratio of CCK-8 to media) was added to each well of the plate, and the plates were incubated for 2 h in a CO_2 incubator at 37 °C. The color generated by living cells was measured at 450 nm. Arctigenin was dissolved in dimethyl sulfoxide to make a 100-mM stock solution, and the stock was diluted to various concentrations of arctigenin (0.3–3 μM) for the experiments. The corresponding concentration (0.003%) of dimethyl sulfoxide for the 3-μM arctigenin solution did not show cytotoxic effects.

2.3. Cell culture and osteoclast differentiation

BALB/c mice (aged 6 weeks) were purchased from Orient (Seongnam, Korea) and housed in a pathogen-free animal facility at Konkuk University (Seoul, Korea). Bone marrow cells (BMCs) were obtained from the tibiae and femurs of 6-week-old BALB/c mice by flushing the marrow space with α-minimal essential medium (α-MEM). After removing red blood cells, cells were plated on 100-mm culture dishes, and cultured in α-MEM containing 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/ml penicillin–streptomycin with 30 ng/ml M-CSF. After 3 days, adherent cells were used as bone marrow macrophages (BMMs). BMMs (1×10^4 cells/well) were cultured with 30 ng/ml M-CSF and 100 ng/ml RANKL for 4 days with or without arctigenin. The animal study was performed in accordance with the institutional guidelines. The protocol was approved by the Institutional Animal Care and Use Committee at Konkuk University.

2.4. Tartrate-resistant acid phosphatase (TRAP) activity and TRAP staining

Four days after stimulating the BMMs with M-CSF and RANKL, the cells were fixed with 4% paraformaldehyde for 5 min. Fixed cells were permeabilized using 0.1% Triton X-100 for 5 min and were stained using the Leukocyte Acid Phosphatase Kit (Sigma, St. Louis, MO, USA). Images of TRAP-positive cells were captured under a microscope using a DP Controller (Olympus Optical, Tokyo, Japan). TRAP-positive multinucleated cells with more than 3 nuclei were counted as osteoclasts. To measure TRAP activity, permeabilized cells were treated with 100 μL of citrate buffer (50 mM, pH 5.2) containing 10 mM sodium tartrate and 5 mM *p*-nitrophenylphosphate. After incubating for 5 min at room temperature, the mixtures were transferred to new plates containing 50 μL of 0.1 N NaOH, and absorbance was measured at 405 nm.

2.5. Bone resorption assay

BMMs (1×10^4 cells/well) were plated on Osteologic discs (BD Biosciences, Bedford, MA, USA), which are coated with calcium phosphate film. Cells were cultured for 5 days in supplemented α-MEM with 30 ng/ml M-CSF and 100 ng/ml RANKL with or without 3 μM arctigenin. Cells were removed using 6% NaOCl and the areas of resorption pits were measured using Multi Gauge V3.1 software (Fujifilm, Tokyo, Japan).

2.6. Cell stimulation and immunoblotting analysis

BMMs (4×10^5 cells/well) were starved in serum-free α-MEM for 6 h. Cells were stimulated through the addition of 100 ng/ml of RANKL for 7 min with or without arctigenin pretreatment for 30 min, and then washed twice with ice-cold phosphate buffered saline (PBS). Cells were lysed in 50 μL ice-cold lysis buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 60 mM octyl β-glucoside, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenylphosphate, 0.7 μg/ml pepstatin, and a protease-inhibitor cocktail tablet). Lysates were kept on ice for 30 min and then centrifuged at $13{,}000\times g$ for 10 min at 4 °C. Supernatants were denatured by boiling at 95 °C for 5 min in 2× Laemmli buffer. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride membranes by using standard protocols. After blocking the membranes in TBS-T buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 0.05% Tween-20) containing 5% skim milk powder or bovine serum albumin, the membranes were incubated individually with specific antibodies. The immunoreactive proteins were detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemoluminescence according to the manufacturer's protocols (Amersham Biosciences, Piscataway, NJ, USA).

2.7. Electrophoretic mobility shift assay

BMMs (2×10^6 cells/well) were starved in serum-free α-MEM for 6 h. Cells were stimulated by adding 100 ng/ml of RANKL for 7 min with or without arctigenin pretreatment for 30 min. Nuclear extracts were prepared using the Nuclear Extraction Kit (Panomics). An electrophoretic mobility shift assay (EMSA) was performed using the EMSA "Gel-Shift" kit (Panomics) according to the manufacturer's protocol. Briefly, nuclear extracts were incubated with a biotin-labeled oligonucleotide harboring the consensus binding sequence for NF-κB (5′-AGTTGAGGGGACTTTCCC-AGGC-3′) for 30 min at 15 °C, and the mixtures were separated on a 6.0% non-denaturing polyacrylamide gel and then blotted onto a Biodyne B membrane (Pall Corporation, Ann Arbor, MI, USA) for 30 min at 300 mA. Band shifts were detected using HRP-conjugated streptavidin.

2.8. Immunoprecipitation and in vitro kinase assay

BMMs (2×10^6 cells/well) were starved in serum-free α-MEM for 6 h. Cells were stimulated by addition of 100 ng/ml of RANKL for indicated times with or without arctigenin pretreatment for 30 min, then washed twice with ice-cold PBS, and lysed using ice-cold lysis buffer. Lysates were incubated on ice for 30 min and then centrifuged at $15{,}000\times g$ for 15 min at 4 °C. The supernatant fraction was "precleared" by addition of 50 μL protein A-agarose. An equal amount of protein was used for immunoprecipitation. Syk was immunoprecipitated by overnight incubation (at 4 °C with gentle rocking) with a specific antibody and, subsequently, protein A-agarose. The agarose was washed 5 times using a washing buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 2.5 mM nitrophenylphosphate, 0.7 μg/ml pepstatin, and a protease-inhibitor cocktail tablet). The immunoprecipitates were assayed for kinase activity using an ELISA-based Universal Tyrosine Kinase Assay Kit (Gen Way, San Diego, CA, USA) according to the manufacturer's instructions.

2.9. LPS-induced osteoclast formation in mouse calvaria

Mice (BALB/c mice, male, 5 weeks old) were obtained from Orient (Seongnam, Korea). After a 1-week acclimation, 10 mg/kg LPS or PBS vehicle was injected subcutaneously over the mice calvariae under light anesthesia, daily for 7 days. Arctigenin (10 mg/kg) was orally administered daily for 7 days, 30 min before LPS injection. Mice were killed at 7 days after the first injection of LPS. Whole calvariae were removed, fixed in 4% paraformaldehyde, and stained for TRAP using a leukocyte acid phosphatase kit (Sigma). For histological analysis, calvarial tissues were fixed in 4% paraformaldehyde in PBS for 3 days, decalcified in 10% EDTA in 4% paraformaldehyde for 30 days at 4 °C, and then embedded in paraffin. Serial 5-μm paraffin sections were stained for TRAP by using the leukocyte acid phosphatase kit and counterstained using hematoxylin.

2.10. Statistical analysis

The data are presented as the mean ± S.E.M. from 3 or more independent experiments. Statistical analysis was performed using one-way analysis of variance and the Dunnett test. All statistical calculations ($^*P<0.05$ and $^{**}P<0.01$) were performed using the software SigmaStat (Systat Software, Inc., Point Richmond, CA, USA).

3. Results

3.1. Effect of arctigenin on RANKL-mediated osteoclast differentiation from mouse BMMs and bone resorption

Differentiation of osteoclast has been observed in RANKL-stimulated bone marrow monocyte/macrophage lineage cells in the presence of M-CSF (Asagiri and Takayanagi, 2007; Yoshida et al., 1990). Previously, we identified arctigenin (Fig. 1A) from herbal extracts of Trachelospermi caulis and Moutan cortex radicis that reduced the number of osteoclasts in the proximal tibia of collagen-induced arthritis mice (Kim et al., 2011). Therefore, we initially determined whether arctigenin has an effect on osteoclast differentiation. Cells were cultured in the presence of RANKL (100 ng/ml) and M-CSF (30 ng/ml) for 4 days with or without arctigenin (0.3–3 μM) to induce formation of TRAP-positive multinucleated osteoclasts. Arctigenin significantly inhibited the differentiation of osteoclasts and formation of peripheral actin belt in a dose-dependent manner (Fig. 1B and C). Similarly, arctigenin decreased RANKL-induced TRAP activity of osteoclasts in a dose-dependent manner (Fig. 1D). In the dose range, arctigenin did not show a cytotoxic effect (Fig. 1E).

Bone resorption is the process by which osteoclasts break down bone and release minerals. Excessive osteoclastic bone resorption causes bone diseases such as rheumatoid arthritis and osteoporosis (Rodan and Martin, 2000). We investigated the effect of arctigenin on RANKL-mediated bone resorptive activity during osteoclastogenesis in BMMs. BMMs were cultured on Osteologic discs (BD Biosciences) for 5 days with 3 μM arctigenin in the presence of 100 ng/ml RANKL and 30 ng/ml M-CSF. Arctigenin dramatically attenuated RANKL-mediated resorption pit formation (Fig. 1F), most likely due to inhibited osteoclast formation (Fig. 1B and C). The formation of the resorption pit was significantly suppressed by 89 ± 0.2% at 3 μM arctigenin (Fig. 1G).

3.2. Effect of arctigenin on the expression of RANKL-induced osteoclast marker proteins

Typical osteoclast marker proteins such as TRAF6, NFATc1, and c-Fos are known to regulate RANKL-mediated osteoclast differentiation from BMMs (Walsh et al., 2006). c-Src and cathepsin K are also expressed in mature osteoclasts and play an essential role in the process of bone resorption (Wada et al., 2006). In this study, we investigated the effect of arctigenin on the expression of osteoclast-specific genes during RANKL-mediated osteoclastogenesis. RANKL stimulation substantially enhanced expression levels of NFATc1, c-Fos, c-Src, TRAF6, and cathepsin K in BMMs (Fig. 2). Arctigenin (3 μM) significantly suppressed the expression of the markers at most time points (Fig. 2A–E). These data strongly suggest that arctigenin inhibits RANKL-induced osteoclast differentiation and bone resorption by suppressing the expression of NFATc1, c-Fos, TRAF6, c-Src, and cathepsin K.

3.3. Effect of arctigenin on RANKL-induced signaling pathway in BMMs

Binding of RANKL to RANK triggers various intracellular signaling cascades that control lineage commitment and activation of osteoclasts (Wada et al., 2006). RANKL stimulation triggers the formation of the RANK-TRAF6 complex, which leads to the activation of NF-κB, Akt, and MAP kinases (Takayanagi, 2007). We evaluated the effect of arctigenin on the activation of MAP kinases by RANKL in BMMs. Arctigenin substantially inhibited RANKL-induced phosphorylation of Erk1/2 in a dose-dependent manner. However, the phosphorylations of p38 and JNK were not suppressed by arctigenin (Fig. 3A and B).

Immunoreceptor tyrosine-based activation motif (ITAM)-mediated costimulatory signals are critical for RANKL-stimulated induction of osteoclast differentiation. Phosphorylation of ITAMs of DAP12/FcRγ by RANKL induces the recruitment and activation of Syk, leading to the activation of PLCγ and calcium signaling, which is crucial for NFATc1 induction in BMMs (Koga et al., 2004). Gab2 is also a crucial molecule of RANK signaling and osteoclastogenesis (Wada et al., 2005). Therefore, we examined the effect of arctigenin on the activation of Syk, PLCγ, and Gab2 by RANKL in BMMs. RANKL-induced activation of Syk, PLCγ, and Gab2 was dramatically suppressed by arctigenin in a dose-dependent manner (Fig. 3C and D).

Next, we determined whether the DNA binding activity of NF-κB, an essential factor for osteoclast differentiation (Wada et al., 2006), was inhibited by arctigenin. Arctigenin significantly suppressed RANKL-stimulated NF-κB binding to DNA in BMMs (Fig. 3E and F).

3.4. Effect of arctigenin on Syk activation

Syk kinase is a pivotal kinase that stimulates ITAM-mediated signaling cascades during RANKL-mediated osteoclast formation in BMMs (Mócsai et al., 2004). We next examined the effect of arctigenin on RANKL-mediated Syk activation in BMMs. The kinase activity of Syk was stimulated by RANKL in a time-dependent manner in BMMs and it significantly inhibited the RANKL-mediated activation of Syk (Fig. 4).

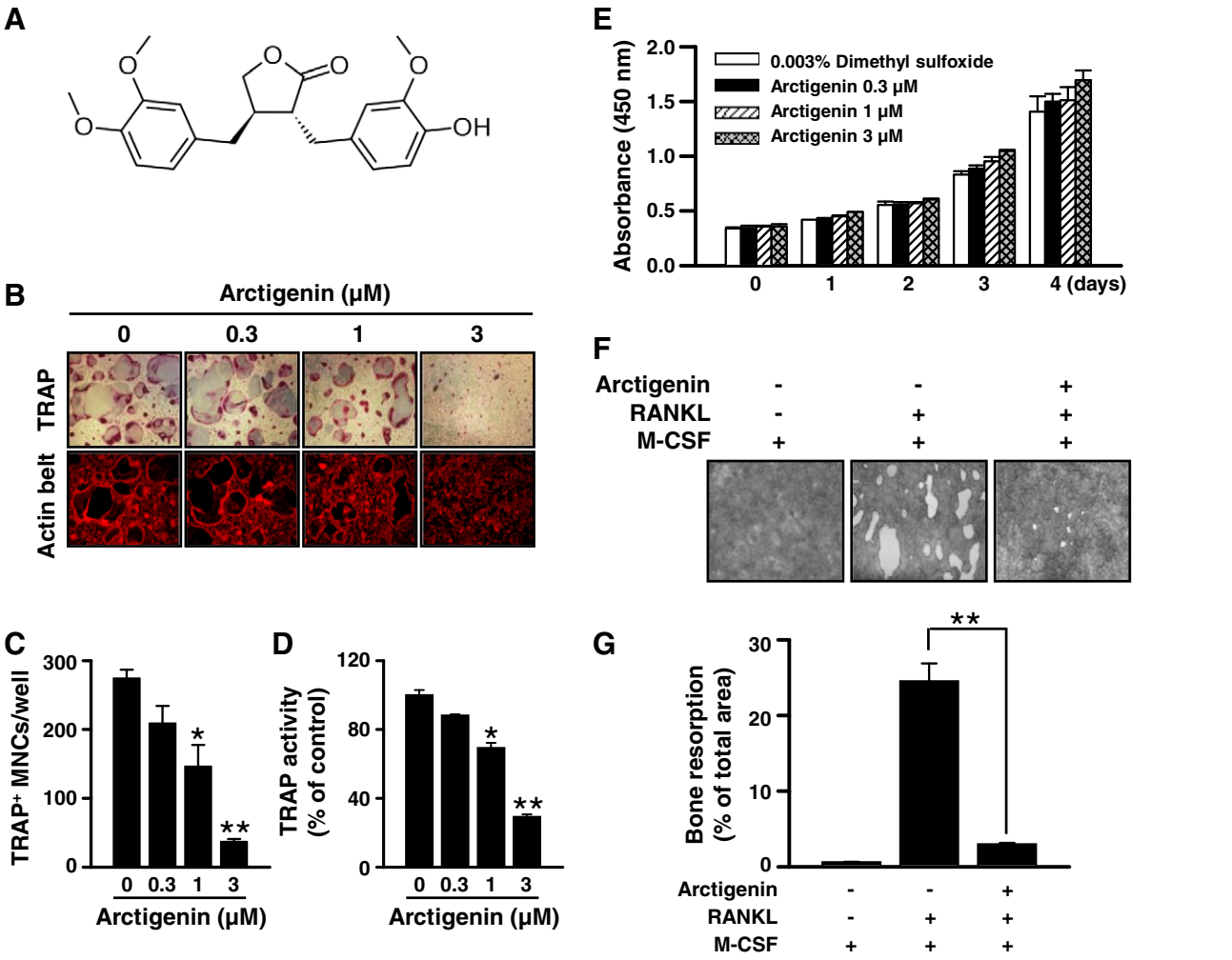

Fig. 1. Effect of arctigenin on RANKL-mediated osteoclast differentiation and bone resorption in BMMs. Mouse BMMs (1×10^4 cells/well in 96-well culture plates) were cultured in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 4 days with or without arctigenin (0.3, 1, and 3 μM) as indicated. TRAP-positive cells that contain more than 3 nuclei were counted as osteoclasts after staining for TRAP. (A) Chemical structure of arctigenin. (B) Representative images are shown from 3 independent experiments: cell images for TRAP staining (upper panel); actin ring staining (lower panel). The treatment with 0 μM arctigenin contains 0.003% dimethyl sulfoxide. (C) The number of TRAP-positive osteoclasts (C) and TRAP activity were analyzed. (E) Cell viability was determined by using a cell counting kit-8 as described in "Materials and methods". For bone resorption assay, mouse BMMs (1×10^4 cells/well) were cultured on Osteologic discs for 5 days in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) with or without arctigenin (3 μM). (F) Representative images of resorption pit formation are shown. (G) Total resorption pit area was analyzed as described in "Materials and methods". (C–D, E, and G) Results are expressed as the mean ± S.E.M. from 3 independent experiments. *P<0.05, and **P<0.01, compared with the values without arctigenin (0).

3.5. In vivo effect of arctigenin on LPS-induced osteoclast differentiation in mice

We evaluated whether arctigenin exerts any effect on osteoclast differentiation in mice. LPS is a pathological factor involved in bone destruction (Ozaki et al., 2009), and it stimulates the activity and formation of osteoclasts (Soysa et al., 2011). The mechanism of LPS-induced bone loss includes, at least partially, indirect stimulation of osteoclasts by RANKL expressed on osteoblasts or stromal cells (Suda et al., 2004; Zou and Bar-Shavit, 2002). As expected, arctigenin suppressed osteoclast differentiation by LPS on the whole surface of the calvaria and sutures (Fig. 5A). Furthermore, the number of osteoclasts in mice calvariae was significantly inhibited by arctigenin (Fig. 5B). These results suggest that arctigenin has an inhibitory effect on LPS-induced osteoclast differentiation in mice.

4. Discussion

Arctigenin is known to have anti-inflammatory activity, likely through the inhibition of production of TNF-α and nitric oxide (Cho et al., 1999; Zhao et al., 2009). However, the effect of arctigenin on osteoclast differentiation is unknown. Our results showed that arctigenin suppresses RANKL-mediated osteoclast differentiation by inhibiting Syk activation in BMMs.

Bone homeostasis is primarily regulated by 2 types of cells, including bone-forming osteoblasts and bone-resorbing osteoclasts (Takayanagi, 2009). Two cytokines, M-CSF and RANKL are essential for osteoclast formation from osteoclast precursors. M-CSF is essential for the survival and proliferation of osteoclast precursor cells (Takayanagi, 2010) and the induction of RANK expression in BMMs (Miyamoto et al., 2000). In RANKL-induced signaling, the cytoplasmic domain of RANK recruits adaptor molecules, such as the TRAF6, to initiate a signaling cascade (Lamothe et al., 2007). TRAF6 is also involved in the activation of transcription factors such as NF-κB, NFATc1, and c-Fos, which play significant roles in osteoclast differentiation (Feng, 2005). Particularly, TRAF6 is critical for RANKL-induced activation of NF-κB (Asagiri and Takayanagi, 2007). In addition to RANKL-induced activation of TRAF6, ITAM-mediated costimulatory signals lead to the induction of NFATc1, a dominant transcription factor that is also crucial for osteoclast differentiation (Gohda et al., 2005). NFATc1 expression is also regulated by the TRAF6-NF-κB pathways during osteoclast differentiation (Asagiri and Takayanagi, 2007). In this study, arctigenin inhibited RANKL-

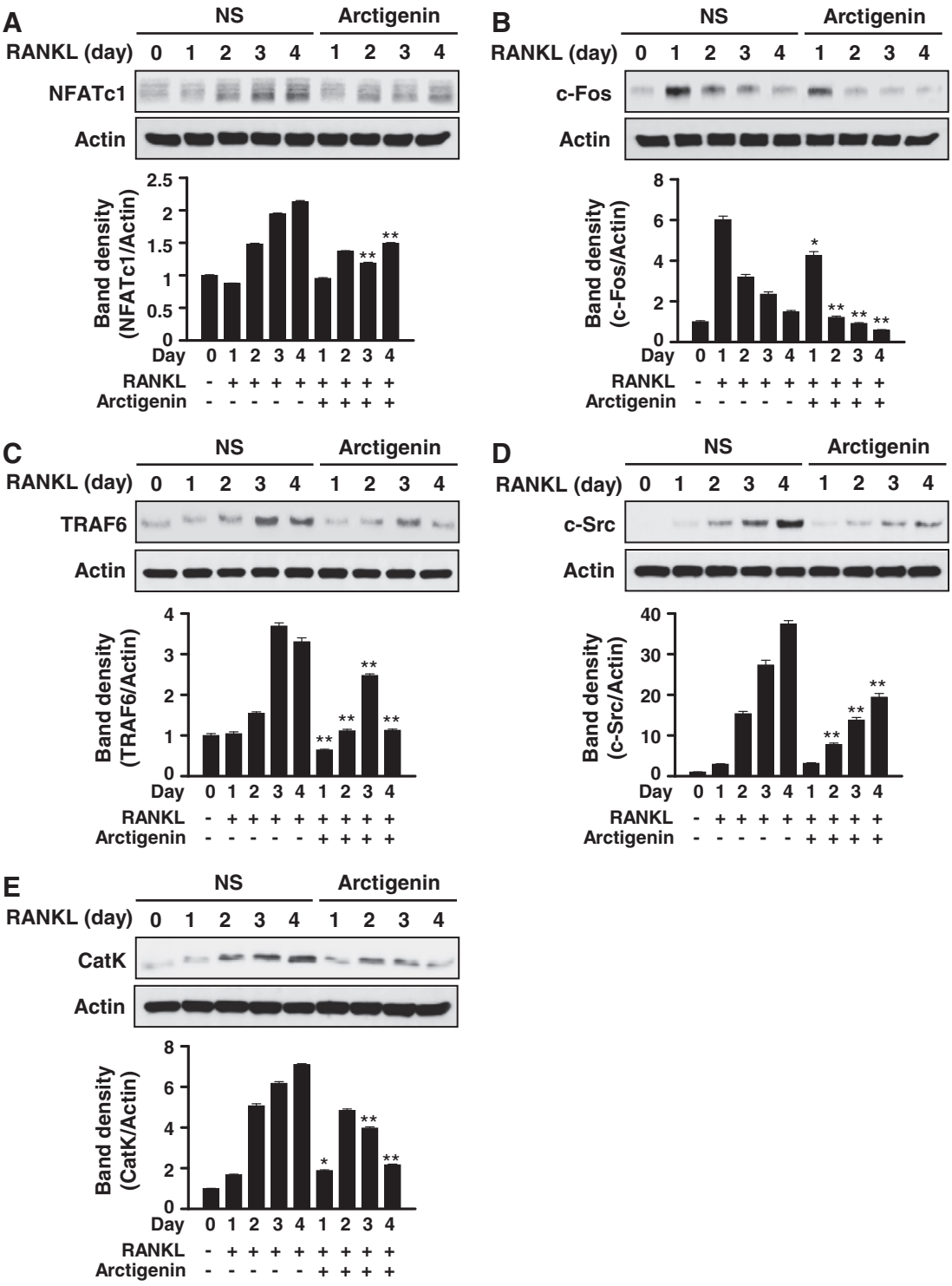

Fig. 2. Effect of arctigenin on the expression of RANKL-induced osteoclast marker proteins. Mouse BMMs (4×10^5 cells/well) were incubated in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml) for the indicated number of days with or without 3 μM arctigenin. Expression levels of NFATc1 (A), c-Fos (B), TRAF6 (C), c-Src (D), and cathepsin K (E) were examined using western blot analysis. The graphs indicate relative band density. Results are expressed as the mean ± S.E.M. from 3 independent experiments, and statistical significance was analyzed between corresponding values in the presence or absence of arctigenin. *$P<0.05$ and **$P<0.01$.

mediated activation of NF-κB (Fig. 3E). Furthermore, arctigenin suppressed RANKL-mediated expression of NFATc1 and c-Fos (Fig. 2A and B). These results suggest that arctigenin inhibits RANKL-stimulated differentiation of osteoclasts by downregulating NFATc1, c-Fos, and NF-κB in BMMs.

In addition to TRAF6-dependent signaling cascades, ITAM-dependent costimulatory signals of the adaptor proteins FcRγ and DAP12 are also essential for the stimulation of osteoclastogenesis (Koga et al., 2004). Phosphorylation of ITAMs results in the recruitment of Syk and leads to the activation of PLCγ and calcium mobilization (Shinohara et al., 2008), which are crucial for the induction of NFATc1 for osteoclast differentiation. Therefore, Syk activity is critical for activating the NFATc1 pathway (Mócsai et al., 2004; Teitelbaum, 2000a, 2000b; Vines et al., 2001). In this study, arctigenin inhibited the phosphorylation of Syk and the signaling molecule PLCγ, which is critical for the activation of calcium signals (Fig. 3C). Furthermore,

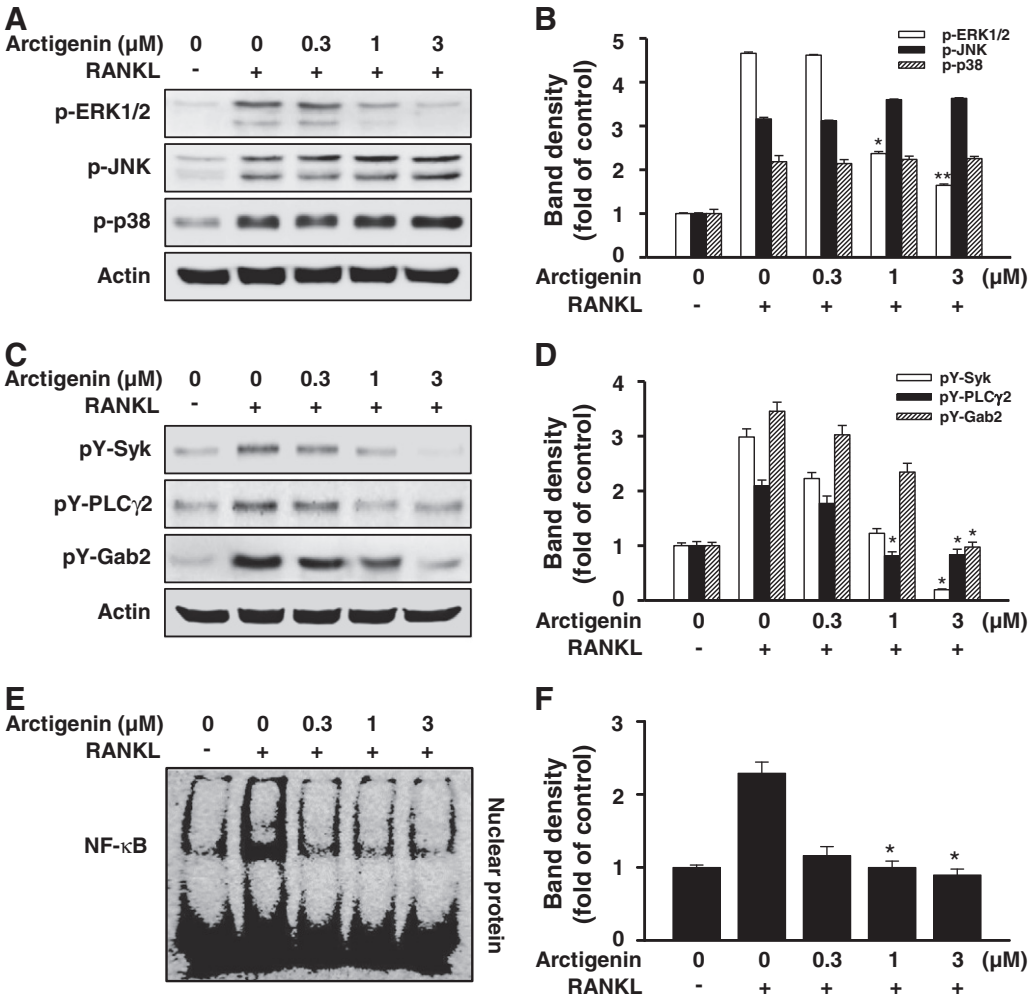

Fig. 3. Effect of arctigenin on RANKL-stimulated signaling molecules in BMMs. BMMs (4×10^5 cells/well) starved in serum-free α-MEM for 6 h. Cells were stimulated using 100 ng/ml of RANKL for 7 min with or without pretreatment with arctigenin for 30 min. Whole cell lysates were analyzed by immunoblotting analysis using specific antibodies. (A) Representative images for phosphorylated Erk1/2, JNK, and p38 are shown. (C) Representative images for phosphorylated Syk, PLCγ2, and Gab2 are shown. (E) DNA binding activity of NF-κB was assessed using an electrophoretic mobility shift assay as described in "Materials and methods." (B, D, F) The graphs indicate relative band densities for panels A, C, and E, respectively. Results are expressed as the mean ± S.E.M. from 3 independent experiments. *P<0.05 and **P<0.01, compared with the values from the RANKL-stimulated group without arctigenin.

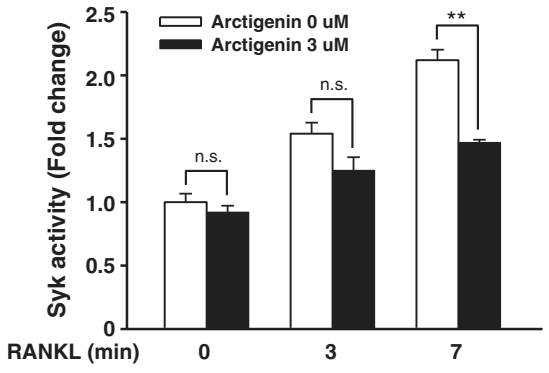

Fig. 4. Effect of arctigenin on RANKL-mediated activation of Syk in BMMs. Mouse BMMs (2×10^6 cells/well) were stimulated using RANKL (100 ng/ml) for 3 or 7 min, with or without pretreatment with arctigenin for 30 min. Cell lysates were prepared and subjected to immunoprecipitation with Syk antibody. Immunoprecipitates were assayed for Syk activity by using an ELISA-based Universal Tyrosine Kinase Assay Kit. Values indicate the relative kinase activity. Results are expressed as the mean ± S.E.M. from 3 independent experiments. **P<0.01.

arctigenin suppressed RANKL-mediated Syk activation (Fig. 4). The results strongly suggest that arctigenin inhibits RANKL-mediated osteoclast differentiation by inhibiting Syk activation.

The molecular scaffold protein Gab2 is also critical for RANK signaling and osteoclast differentiation (Wada et al., 2005). Stimulation of Gab2 by RANKL leads to the activation of JNK, Akt, and NF-κB in BMMs. Arctigenin inhibited the phosphorylation of Gab2 in a dose-dependent manner (Fig. 3C), indicating that arctigenin also suppresses RANKL-mediated osteoclast differentiation by inhibiting Gab2. However, it is still unclear whether Gab2 is a downstream molecule of Syk.

Among the MAP kinases, JNK and p38 are activated by RANKL stimulation through the RANK-TRAF6 signaling pathway during osteoclast differentiation (Negishi-Koga and Takayanagi, 2009). However, it is still unclear how Erk1/2 is activated by RANKL in cells. Although the critical targets of these MAP kinases have not been clarified, they are at least partially responsible for the formation of the AP-1 complex, an important transcription factor for osteoclast differentiation, through JNK-mediated phosphorylation of c-Jun (Negishi-Koga and Takayanagi, 2009). In our results, the activation of Erk1/2, not JNK and p38, was inhibited by arctigenin; this carefully suggested that

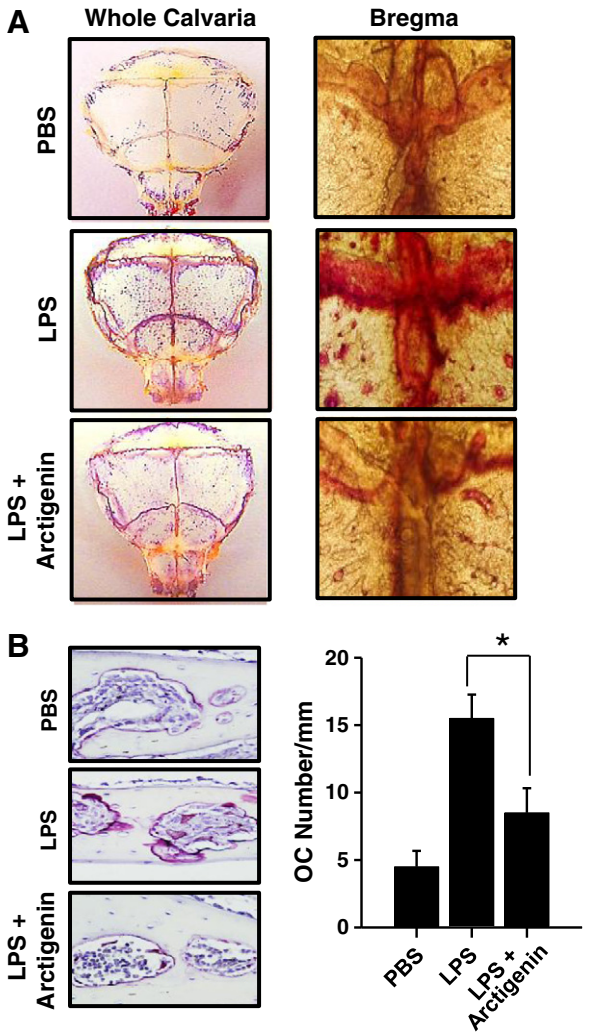

Fig. 5. In vivo effect of arctigenin on LPS-induced osteoclast differentiation. BALB/c mice were subcutaneously injected over the calvaria with LPS (10 mg/kg), daily for 7 days. Arctigenin (10 mg/kg) was orally administered daily for 7 days, 30 min before LPS injection. Mice were killed at 7 days after the first LPS injection. Whole calvariae were removed and stained for TRAP. (A) Representative images of TRAP staining for whole calvaria and bregma. (B) Representative images for TRAP-stained histological sections of calvariae. All images were obtained from 3 independent experiments with five mice each group.

arctigenin primarily suppresses the ITAM-mediated costimulatory signaling pathway, including Syk, rather than the RANK-TRAF-6 pathway. However, the implications of Erk1/2 suppression by arctigenin remain unclear.

LPS is a membrane component of gram-negative bacteria that stimulates osteoclast formation by acting on osteoblasts and pre-osteoclasts (Sakuma et al., 2000; Suda et al., 2004; Ueda et al., 1998). LPS is known to stimulate osteoclast differentiation by inducing RANKL expression in osteoblasts (Suda et al., 2004). In this study, arctigenin inhibited LPS-stimulated osteoclast formation in mice calvariae (Fig. 5), indicating that arctigenin can inhibit osteoclast formation in vivo.

Osteoprotegerin (OPG), a decoy receptor for RANKL expressed on pre-osteoblasts/osteoblasts, is a critical negative regulator for the differentiation of osteoclasts. OPG binding to RANKL suppresses osteoclast differentiation. Therefore, the expression ratio of OPG/RANKL in pre-osteoblasts/osteoblasts is an important factor in determining osteoclastogenesis (Teitelbaum, 2000a, 2000b; Yasuda et al., 1998). Further studies are necessary to determine the effect of arctigenin on OPG expression in pre- osteoblasts/osteoblasts during LPS-stimulated osteoclastogenesis in mice.

In summary, we have shown that arctigenin suppresses RANKL-stimulated osteoclast differentiation by inhibiting the activation of Syk and Gab2. Therefore, our results strongly suggest that arctigenin may be useful for treating bone-related diseases, including rheumatoid arthritis and osteoporosis.

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