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Paeonol inhibits RANKL-induced osteoclastogenesis by inhibiting ERK, p38 and NF-κB pathway

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ABSTRACT

Numerous studies have indicated that inflammatory cytokines play a major role in osteoclastogenesis, leading to the bone resorption that is frequently associated with osteoporosis. Paeonol (2′-hydroxy-4′-methoxyacetophenone), the main active compound of the traditionally used Chinese herb *Paeonia lactiflora* Pallas, has anti-inflammatory activity. Here we found that paeonol markedly inhibited the receptor activator of nuclear factor kappa B ligand (RANKL) plus macrophage colony stimulating factor (M-CSF)-induced osteoclastic differentiation from bone marrow stromal cells and RAW264.7 macrophage cells. In addition, in an assay of osteoclast activity on substrate plates, paeonol significantly decreased the resorption activity of mature osteoclasts. Treatment of RAW264.7 macrophages with RANKL induced extracellular signal-regulated kinases (ERK), p38 and c-Jun N-terminal kinase (JNK) phosphorylation. However, RANKL-induced ERK, p38 but not JNK phosphorylation was attenuated by paeonol. Furthermore, RANKL-mediated increase of IκBα phosphorylation, p65 phosphorylation at Ser^{536}, κB-luciferase activity and NF-κB binding activity was inhibited by paeonol. In addition, paeonol also prevented the bone loss inducing by ovariectomy *in vivo*. Our data suggest that paeonol inhibits osteoclastogenesis from bone marrow stromal cells and macrophage cells via attenuated of RANKL-induced ERK, p38 and NF-κB activation, which in turn protect bone loss from ovariectomy.

1. Introduction

Bone is a complex tissue containing several cell types which are undergoing renewal and repair process termed “bone remodeling”. The two major cell types responsible for bone remodeling are osteoclasts, which resorb bone, and osteoblasts, which form new bone (Goltzman, 2002). Bone remodeling is regulated by several systemic hormones (including: parathyroid hormone, 1, 25-dihydroxyvitamin D_3, sex hormones and calcitonin), and local factors (including: nitric oxide, prostaglandins, growth factors and cytokines) (van't Hof and Ralston, 2001). Osteoporosis ensues from an imbalance bone resorption and bone formation with a net bone loss that may be induced by several conditions, such as hormonal imbalance, diseases, or medications (e.g. corticosteroids or anti-epileptic agents) (Goltzman, 2002). Current drugs used to treat osteoporosis include bisphosphonates, calcitonin and estrogen. These drugs are all bone resorption inhibitors, which maintain bone mass by inhibiting the function of osteoclasts (Rodan and Martin, 2002). Since the osteoclasts are responsible for bone resorption, therefore they are one of the main target for treatment of osteoporosis.

Osteoclasts are multinucleated cells formed by the fusion of mononuclear progenitors of the monocyte/macrophage family (Teitelbaum, 2000). In vitro maturation of macrophages into osteoclasts requires the presence of stromal cells or their osteoblast progeny (Udagawa et al., 1990). Extensive research in the last few years has indicated that these accessory cells express macrophage colony stimulating factor (M-CSF) and receptor for activation of NF-κB (RANK) ligand (RANKL) that are essential for osteoclastogenesis (Yao et al., 1998). Besides M-CSF and RANKL, several other inflammatory cytokines and osteotropic agents including tumor necrosis factor (TNF) and interleukin (IL)-1β have also been implicated in osteoclastogenesis, most likely through the osteoblastic modulation of RANKL, its decoy receptor osteoprotegerin, and

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M-CSF (Yao et al., 1998). RANKL, a member of the TNF superfamily (Darnay et al., 1999), interacts with the cell surface receptor RANK and in turn recruits TNFR associated factors (TRAF)1, 2, 3, 5, and 6 (Darnay et al., 1998). The receptor deletion analysis has shown that sequential recruitment of TRAF6 and NF-κB-inducing kinase by RANK leads to NF-κB activation, and recruitment of TRAF2 leads to c-Jun N-terminal kinase (JNK) activation (Lee et al., 1997; Darnay et al., 1999). RANKL is also known to activate NF-κB, JNK, and p38 and p44/p42 mitogen-activated protein kinase (MAPK) (Lee et al., 1997; Darnay et al., 1999), how this cytokine mediates osteoclastogenesis is not fully understood. Thus agents that can suppress RANKL signaling can suppress osteoclastogenesis-induced bone loss.

Paeonol (2′-hydroxy-4′-methoxyacetophenone) is the main active compound of the *Paeonia lactiflora* Pallas, a traditional Chinese herb used in Asia and Europe to improve blood flow; to down-regulate transcription factors NF-κB and AP-1 (Ishiguro et al., 2006; Nizamutdinova et al., 2007); to suppress the expression of cyclooxygenase-2, nitric oxide synthase, cell surface adhesion molecules, TNF-α and IL-1β (Chou, 2003; Nizamutdinova et al., 2007); and to inhibit the activity of extracellular signal-regulated kinase (ERK) and p38 (Nizamutdinova et al., 2007). This compound has antioxidant and anti-inflammatory activity and found to suppress tumor formation (Chung, 1999; Chou, 2003; Nizamutdinova et al., 2007; Xu et al., 2007). The effect of paeonol has been shown to suppress NF-κB activation by TNF-α (Nizamutdinova et al., 2007), and we examined the effect of paeonol on RANKL-induced NF-κB activation and on osteoclastogenesis in osteoclast precursor cells. We demonstrate that RANKL induces MAPK and NF-κB activation. Paeonol inhibits the RANKL-induced ERK, p38 and NF-κB activation in macrophages. On the other hand, paeonol did not affect the proliferation and differentiation of human osteoblasts. Therefore, our data provide evidence that paeonol may be an anti-resorption agent for treatment of osteoporosis.

2. Materials and methods

2.1. Materials

Paeonol was purchased from Wako Chemicals (Osaka, Japan). Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for phospho-IκBα, phosho-p38, phospho-ERK, phospho-JNK, p38, ERK, JNK, p65 and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p65 phosphorylated at Ser^{536} was purchased from Cell Signaling and Neuroscience. The osteoclast activity assay substrate (OAAS) was purchased from OCT USA, Inc. (Torrance, CA, USA). Recombinant human M-CSF, RANKL and BMP-2, TNF-α, IL-1β and IL-6 ELISA kit were purchased from R&D Systems (Minneapolis, MN, USA). The osteocalcin ELISA kit was purchased from Biosource Technology (Nivelles, Belgium, USA). The osteopontin ELISA kit was purchased from Assay Designs Inc. (Ann Arbor, MI, USA). An NF-κB TransAM kit was purchased from Active Motif (Carlsbad, CA). PD98059, SB203580, SP600125 and alendronate were purchased from Calbiochem (San Diego, CA, USA). The NF-κB inhibitor peptide (in a cell-permeable form) was purchased from BIOMOL (Butler Pike, PA). pSV-β-galactosidase vector, luciferase assay kit was purchased from Promega (Madison, MA, USA). The NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA, USA). The C-terminal telopeptides of type-I collagen ELISA kit was obtained from Cross Laps (Herlev, Denmark). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Bone marrow cells were prepared by removing from femurs of 6–8 week-old Sprague–Dawley rats and flushing the bone marrow cavity with α-MEM which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The non-adherent cells (hematopoietic cells) were collected after 24 h and used as osteoclast precursors. Cells were seeded at a density of 1×10^6 cells/well in 24-well plates in the presence of human recombinant soluble RANKL (50 ng/ml) and M-CSF (20 ng/ml) for 7 days. The culture medium was replaced every 3 days.

Murine RAW264.7 cells (a mouse macrophage cell line obtained from American Type Culture Collection) were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For differentiation of osteoclasts, RAW264.7 cells (2×10^4, in 24-well plate) were cultured in the presence of RANKL (50 ng/ml) and M-CSF (20 ng/ml) for 5 days. The culture medium was replaced every 3 days.

Mature osteoclasts were isolated as described previously (Tang et al., 2007a). Long bones were isolated from 6-day-old rabbits (body weight, 70–90 g). After removal of muscle and cartilage, the bones were minced in α-MEM. Cells were dissociated from bone fragments by gentle vortexing, then bone fragments were allowed to settle under normal gravity. The supernatant was removed and saved, and the mincing and sedimentation were repeated three more times. The supernatants were pooled and centrifuged for 5 min at 60 ×*g*. The cells were plated in OAAS plate, after overnight incubation, the adherent cells were washed three times with PBS and then treated with trypsin/EDTA for 10 min at 37 °C to remove contaminating cells. Additionally, treatment with 0.1% collagenase for 5 min at room temperature was performed to remove completely most of the stromal cells. The highly enriched osteoclasts (>90%) were washed three times in PBS.

The human osteoblast-like cell line MG-63 (CRL-1427) was purchased from American Type Culture Collection. Cells were cultured in α-MEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The conditionally immortalized human fetal osteoblastic cell line (hOB; CRL-11372) was maintained in a 1:1 mixture of DMEM/Ham's F-12 medium containing 10% FBS supplemented with Geneticin (300 μg/ml) and antibiotics at 37 °C.

2.3. Osteoclast differentiation assay

Osteoclast formation was measured by quantifying cells positively stained by tartrate-resistant acid phosphatase [TRAP (Acid Phosphatase Kit 387-A; Sigma-Aldrich, St. Louis, MO, USA)]. Briefly, the cells were fixed for 30 s and then stained with Naphthol AS-BI phosphate and a tartrate solution for 1 h at 37 °C, followed by counterstaining with a hematoxylin solution. Osteoclasts were determined to be TRAP-positive staining multinuclear (>3 nuclei) cells using light microscopy. The total number of TRAP-positive cells and the number of nuclei per TRAP-positive cell in each well were counted. The morphological features of osteoclasts were also photographed (Tang et al., 2007a).

2.4. mRNA analysis by reverse transcriptase-polymerase chain reaction (RT-PCR)

The method of RT-PCR was prepared as described previously (Tang et al., 2005). Total RNA was extracted from macrophage using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using oligo(dT) primer, then amplified for 33 cycles using two oligonucleotide primers:

Mouse TRAP: TGACAAGAGGTTCCAGGA and AGCCAGGACAGCTGAGTG
Mouse matrix metalloproteinases (MMP)-9: AGTTTGGTGTCGCG-GAGCAC and TACATGAGCGCTTCCGGCAC
Mouse GAPDH: AAGCCCATCACCATCTTCCAG and AGGGGCCATCCACAGTCTTCT

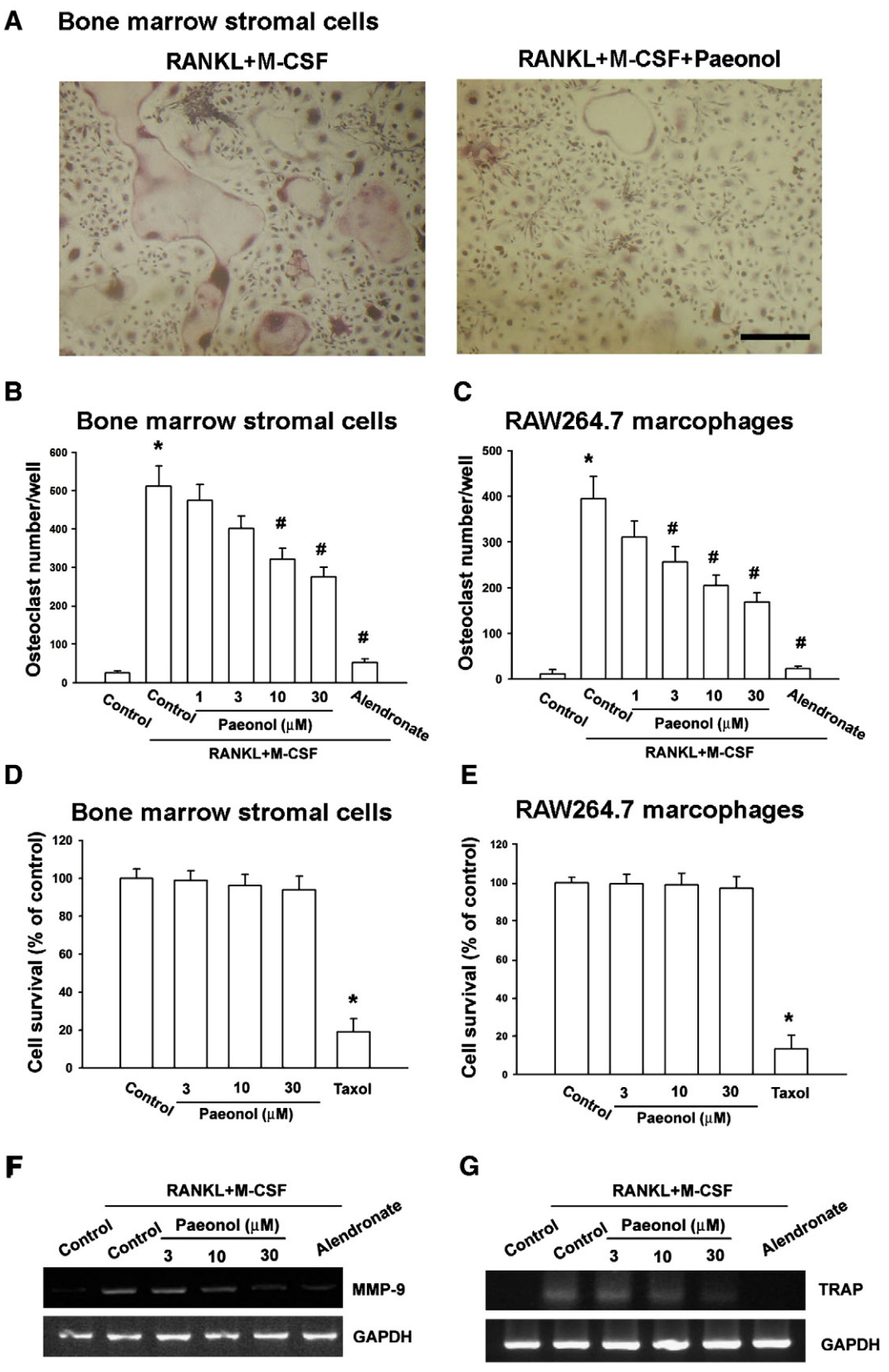


Fig. 1. Inhibition of osteoclast differentiation by paeonol. Osteoclast precursors isolated from long bones of adult male rats were plated on a 24-well plate at 1×10^6 cells/well and cultured in the presence of M-CSF (20 ng/ml) and RANKL (50 ng/ml) for 7 days. Following TRAP staining, the cells with more than 3 nuclei were counted (A). Bar = 100 μm. Compared with RANKL plus M-CSF, paeonol (30 μM) or alendronate (10 nM) treatment markedly inhibited the differentiation of osteoclast. The quantitative data are shown in B ($n=4$). RAW264.7 cells were seeded at 2×10^4 and incubated for 5 days with M-CSF (20 ng/ml) and RANKL (50 ng/ml) without or with paeonol. Treatment with paeonol inhibited osteoclastogenesis in a concentration-dependent manner (C). Bone marrow stromal cells or RAW264.7 cells were treated with paeonol (3–30 μM) or taxol (100 nM) for 2 days and cell viability was evaluated by the MTT assay. Note that paeonol did not affect the cell viability of bone marrow stromal cells and RAW264.7 cells ($n=4$) (D and E). RAW264.7 cells were treated with M-CSF (20 ng/ml) and RANKL (50 ng/ml) without or with paeonol (3–30 μM) or alendronate (10 nM) for 5 days. After incubation, TRAP (F) and MMP-9 (G) mRNAs were detected by RT-PCR. Results are expressed as the mean ± S.E.M. of four independent experiments. *: $p<0.05$ as compared with control group. #: $p<0.05$ as compared with RANKL–M-CSF-treated group.

Each PCR cycle was carried out for 30 s at 94 °C, 30 s at 55 °C, and 1 min at 68 °C. PCR products were then separated electrophoretically in a 2% agarose DNA gel and stained with ethidium bromide.

2.5. Cell viability

Cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay. After treatment with paeonol for 3 days, cultures were washed with PBS. MTT (0.5 mg/ml) was then added to each well and the mixture was incubated for 2 h at 37 °C. Culture medium was then replaced with equal volume of DMSO to dissolve formazan crystals. After shaking at room temperature for 10 min, absorbance of each well was determined at 550 nm using a microplate reader (Bio-Tek, Winooski, VT).

2.6. Assay of resorbing activity of osteoclast

The mature osteoclasts were prepared from rabbit long bones as described previously (Tang et al., 2007a). The cells were re-suspended in complete α-MEM medium and plated into a calcium phosphate apatite-coated OAAS plate. After 2-days' cultures, the remaining cells on the plate were lysed using 1 N NaOH. Five images per well were obtained using inverted microscope (200x), and the resorbed area was measured using an image analyzer.

2.7. Western blot analysis

The cellular lysates were prepared as described previously (Tang et al., 2006, 2007b). Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-mouse antibodies against IκBα, p-IκBα, p-p38, p-ERK, p-JNK, p38, ERK or JNK (1:1000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.8. Transfection and reporter gene assay

Cells were co-transfected with 0.8 μg κB-luciferase plasmid, 0.4 μg β-galactosidase expression vector. The cells were grown to 80% confluent in 12-well plates and were transfected on the following day by Lipofectamine 2000 (LF2000; Invitrogen, Carlsbad, CA). DNA and LF2000 were premixed for 20 min and then applied to the cells. DMEM containing 20% FBS was added 4 h later. After 24 h transfection, the cells were then incubated with the indicated agents. After further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector (Tang et al., 2007b).

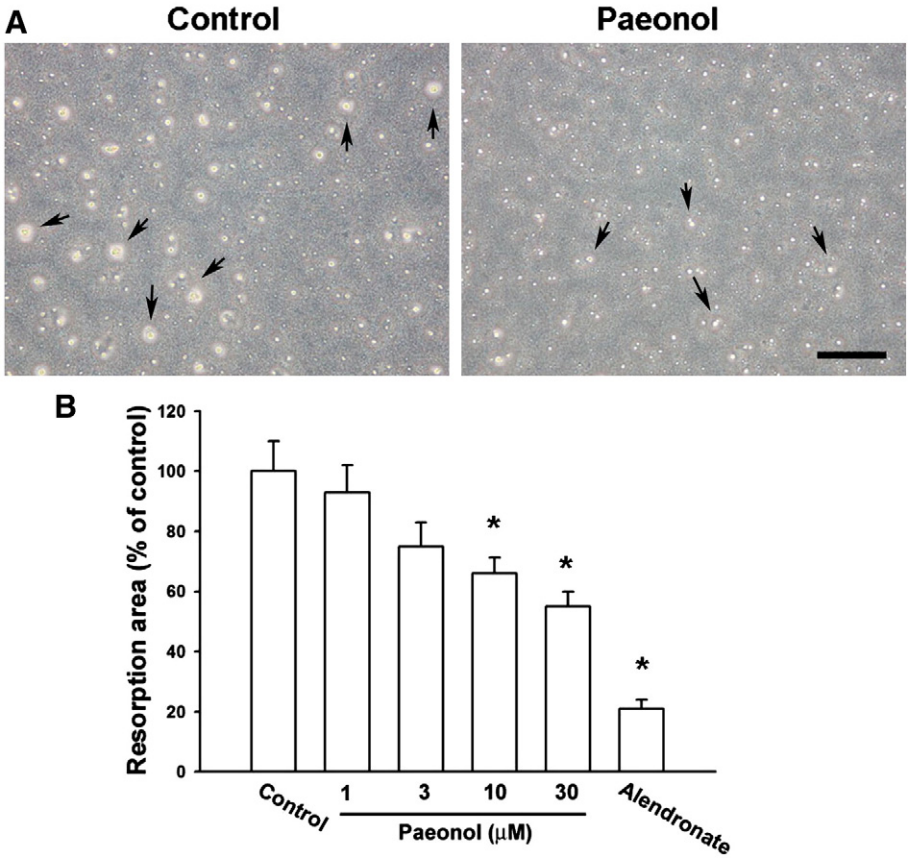


Fig. 2. Inhibition of bone resorption by paeonol. Mature osteoclasts isolated from rabbit long bones were cultured in a bone resorption well (OAAS). Osteoclasts were treated with various concentrations of paeonol for 2 days. Resorbed lacunae on the plates were photographed under an inverted microscope (X200). Note that as compared with control, paeonol (30 μM) or alendronate (10 nM) treatment significantly decreased the resorption activity of osteoclasts. Total resorption area per well was graphed and measured by image analyzer (C). Data are presented as mean ± S.E. ($n=3$). Bar = 100 μm. *, $p<0.05$ as compared with control.

2.9. Assay of cell proliferation

Osteoblasts (2×10^4 cells/well) were seeded on 24-well plates. Cells were incubated in serum-free medium for 24 h before the addition of paeonol. After incubation with paeonol for 48 h, BrdU incorporation was assayed according to the protocol of enzyme-linked immunosorbent assay chemiluminescence detection kit (Roche Molecular Biochemicals) using a luminescence counter (TopCount; Packard Instruments, Meriden, CT). The counts per second correlate directly to the amount of DNA synthesis and the number of proliferating cells.

2.10. Measurement of alkaline phosphatase (ALP) activity

Osteoblasts cultured in 24-well plates in the presence or absence of paeonol were harvested in 0.2% Nonidet P-40 and the cell suspension was disrupted by sonication. After centrifugation at 1500 ×*g* for 5 min, ALP activity in the supernatant was measured as described by Yang et al. (2005).

2.11. Assay of osteocalcin and osteopontin

Osteocalcin and osteopontin ELISA kits were used to detect osteocalcin and osteopontin levels, respectively. Briefly, cells were treated with various concentrations of paeonol for the 3 days. The culture medium was collected for measurement of osteocalcin and osteopontin, respectively. These samples were placed in 96-well microtiter plates coated with monoclonal detective antibodies and incubated for 2 h at room temperature. After removing unbound material by washing with washing buffer (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20), horseradish peroxidase-conjugated streptavidin was added to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution, with color intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm. Results are presented as the percentage of change of the activity compared with the untreated controls.

2.12. Ovariectomy-induced osteoporosis

Female ICR mice (4 week-old; 22–28 g) were used for this study. Mice were ovariectomized bilaterally under trichloroacetaldehyde (100 mg/kg) anesthesia and control mice were sham-operated (Sham) for comparison. Bone mineral density and bone mineral content were measured after treated with various concentrations of paeonol by orally feeding them every 2 days for 4 weeks. Bone mineral density and bone mineral content of full-body were determined by dual-energy X-ray absorptiometer (DEXA; XR-26; Norland, Fort Atkinson, WI) using a mode for small subjects as described previously (Tang et al., 2005, 2007a). All protocols complied with institutional guidelines and were approved by Animal Care Committee of China Medical University.

2.13. Statistics

The values given are means±S.E.M. The significance of difference between the experimental groups and control was assessed by Student's *t* test. The difference is significant if the *p* value is <0.05.

3. Results

3.1. Paeonol inhibits osteoclastogenesis from bone marrow stromal cells and macrophages

Osteoclasts are specialized monocyte/macrophage family members that differentiate from bone marrow hematopoietic precursors. Cultures of osteoclast precursors in the presence of RANKL (50 ng/ml) and M-CSF (20 ng/ml) for 7 days induced the formation of large mature osteoclasts

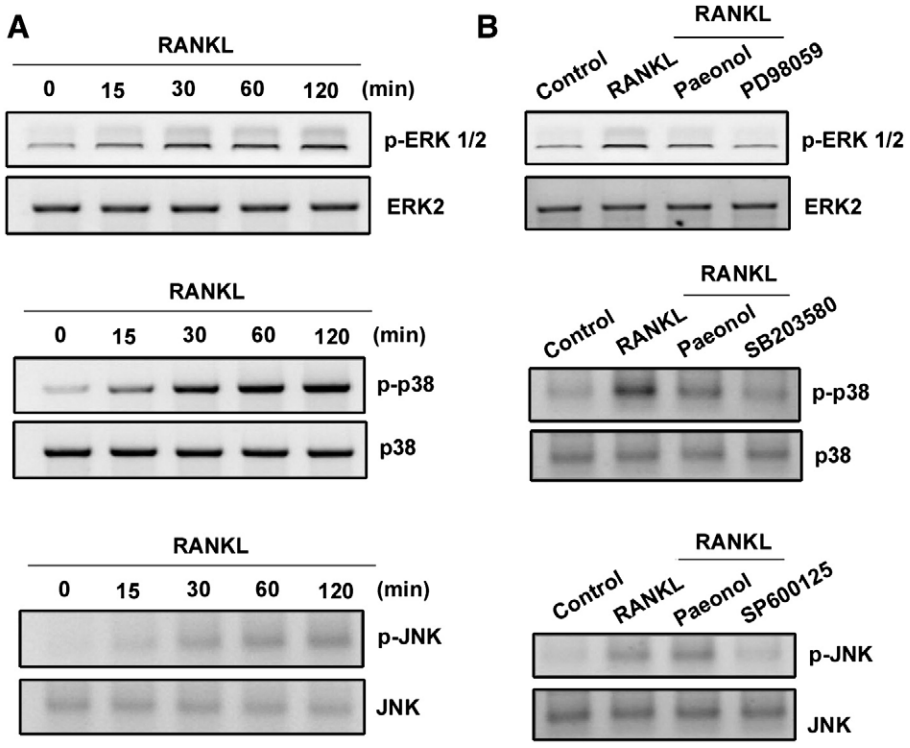


Fig. 3. Paeonol inhibits the RANKL-induced ERK and p38 phosphorylation. RAW264.7 cells were incubated with RANKL (50 ng/ml) for indicated time intervals (A), or pretreated with (30 μM), PD98059 (10 μM), SB203580 (10 μM) or SP600125 (10 μM) for 30 min followed by stimulation with RANKL (50 ng/ml) for 120 min (B), and the ERK, p38 and JNK phosphorylation was determined by immunoblotting using phospho-ERK, p38 and JNK specific antibody, respectively. Note that paeonol inhibits the RANKL-induced ERK and p38 but not JNK phosphorylation. Typical traces represent three experiments with similar results.

with multi-nuclei characterized by the acquisition of mature phenotypic markers, such as TRAP (Fig. 1A). Paeonol markedly inhibited the differentiation of osteoclast in a dose-dependent manner [alendronate (10 nM) was used for comparison] (Fig. 1A and B). The stimulating effect on osteoclast differentiation was also observed in murine RAW264.7 macrophages, where RANKL (50 ng/ml) and M-CSF (20 ng/ml) were able to cause osteoclast formation. Culturing for 5 days in RAW264.7 cells, paeonol dose-dependently inhibited the formation of TRAP-positive cells (Fig. 1C). In addition, treatment of bone marrow stromal cells or RAW264.7 macrophages for 3 days with paeonol (3–30 μM) did not affect cell viability, which was assessed by MTT assay [Taxol (100 nM) was used for positive control] (Fig. 1D and E). To the functional characterization, we examined whether TRAP and MMP-9 were expressed in the differentiated osteoclast. These two molecules have been widely used as specific markers of osteoclasts (Miyazaki et al., 2000). Dramatic levels of MMP-9 and TRAP mRNA, which were absent in undifferentiated RAW264.7 macrophages, were induced by RANKL and M-CSF using RT-PCR analysis (Fig. 1F and G). Treatment with paeonol (3–30 μM) inhibited the expression of these markers in RAW264.7 macrophages (Fig. 1F and G). These data suggest that paeonol inhibited osteoclastogenesis from bone marrow stromal cells and murine macrophages.

3.2. Effect of paeonol on the resorption activity of mature osteoclasts

To directly evaluate the effect of paeonol on the resorption activity of osteoclasts, mature osteoclasts were isolated from long bones of rabbit and cultured on an OAAS plate. Treatment with paeonol for 2 days significantly decreased the resorption activity of mature osteoclasts in a concentration-dependent manner [alendronate (10 nM) was used for comparison] (Fig. 2).

3.3. Paeonol inhibits the RANKL-induced ERK and p38 activation

Three members of mitogen-activated protein kinase (MAPK)s, ERK, p38 and JNK have been implicated in the mediation of RANKL-regulated osteoclastogenesis (Yasuda et al., 1998). To elucidate the signaling pathways of paeonol, we examined the activation of MAPKs in RAW264.7 cells treated with RANKL by immunoblotting. Stimulation by RANKL (50 ng/ml) markedly induced the phosphorylation of ERK, p38 and JNK (Fig. 3A). RANKL-induced increase in ERK and p38 but not JNK phosphorylation was inhibited by the pretreatment of cells for 30 min with paeonol (Fig. 3B). In addition, PD98059 (selective mitogen-activated protein kinase inhibitor), SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor) were used to confirm the involvement of ERK, p38 and JNK in RANKL-induced ERK, p38 and JNK phosphorylation (Fig. 3B).

3.4. Paeonol inhibits RANKL-induced NF-κB activation

In addition to MAPKs, activation of transcription factor NF-κB is also involved in osteoclast differentiation (Bossard et al., 1999). Activation of NF-κB by most agents requires phosphorylation of its inhibitory subunit IκBα. Stimulation of RAW264.7 cells with RANKL induced the IκBα phosphorylation in a time-dependent manner (Fig. 4A). In addition, treatment of cells with RANKL also increased the phosphorylation of p65 Ser^{536}. Pretreatment of cells with paeonol attenuated the RANKL-induced IκBα and p65 Ser^{536} phosphorylation (Fig. 4B). To further confirm the NF-κB activation involved in the action of paeonol, transient transfection was performed using the κB promoter-luciferase constructs. RAW264.7 cells incubated with RANKL (50 ng/ml) led to a 3.2-fold increase in κB promoter activity. The increase of κB promoter activity by RANKL was antagonized by paeonol (NF-κB inhibitor peptide was used for positive control) (Fig. 4C). In addition, paeonol also reduced the RANKL-increased NF-κB binding ability by using TransAM kits (Fig. 4D). These results suggest that ERK, p38 and NF-κB signaling pathways are necessary for paeonol-reduced osteoclastogenesis from macrophages. It has been reported that the NF-κB-derived formation of mediators such as cytokines including TNF-α, IL-1β and IL-6 is important in osteoclasts production and function (Udagawa et al., 1990). RANKL-induced TNF-α, IL-1β and IL-6 production was markedly reduced by paeonol in marcophages (Fig. 4E–G).

3.5. Paeonol did not affect the proliferation and differentiation of cultured osteoblasts

We next determined the effect of paeonol on the cell proliferation of MG-63 and hOB human osteoblast cells by BrdU assay. As shown in Fig. 5A, paeonol did not exhibit significant effects on cell growth at the concentrations used (10 or 30 μM) after 48 h of treatment in either cell line. Differentiated osteoblasts exhibit elevated ALP activity, which is correlated with high levels of enzyme expression (Tang et al., 2007a). We then investigated the effects of paeonol on the ALP activity of osteoblasts. Treatment of osteoblasts with BMP-2 (10 ng/ml) but not paeonol (10 or 30 μM) for 72 h significantly increased ALP activity (Fig. 5B). The effect of paeonol on the terminal differentiation of osteoblast cells was also assessed by determining the synthesis of osteocalcin and osteopontin. As shown in Fig. 5C and D, treatment of MG-63 and hOB cells with paeonol did not affect the expression of osteocalcin and osteopontin after 72 h of treatment (BMP-2 was used for positive control).

3.6. Inhibition of bone loss by paeonol in ovariectomized mice

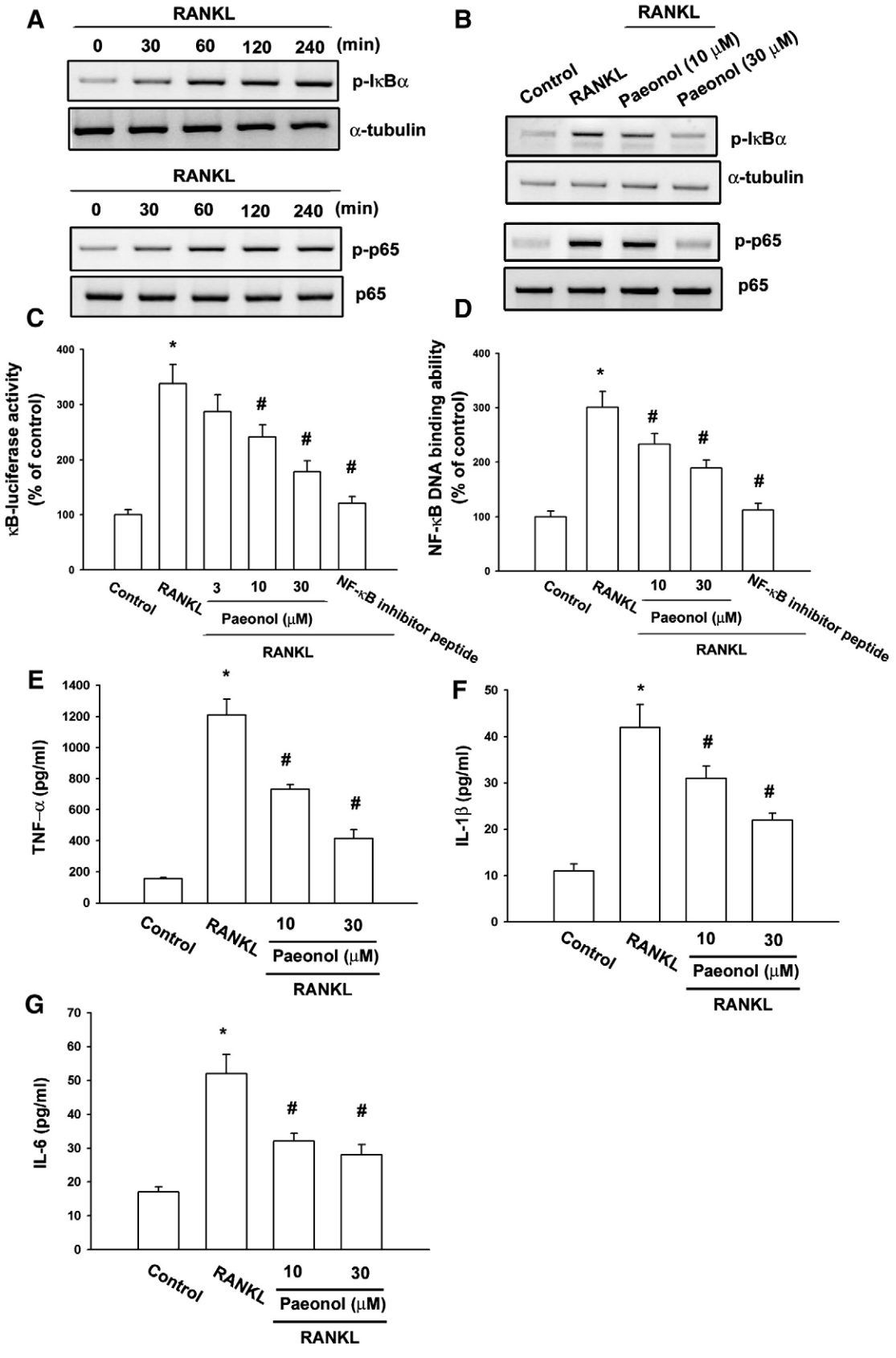
To examine the effect of paeonol on bone loss, female mice were induced osteoporosis by ovariectomy. Ovariectomized mice showed a decreased bone mineral density and bone mineral content of total body. Treatment with paeonol (10–50 mg/kg) or estradiol (0.1 mg/kg; estradiol was used for comparison) for 4 weeks inhibited the loss of bone mineral density and bone mineral content in a dose-dependent manner (Fig. 6A and B). Blood concentration of C-terminal telopeptide of collagen can reflect the osteoclastic activity. As shown in Fig. 6C, paeonol or estradiol also inhibited the increase of osteoclast activity by ovariectomy. In addition, treatment with paeonol (50 mg/kg) did not affect the bone mineral density, bone mineral content and osteoclast activity in normal mice (Fig. 6A–C). On the other hand, paeonol also inhibited the ovariectomized-induced TNF-α, IL-1β and IL-6 release (Fig. 6D–F).

4. Discussion

Paeonol has various biological activities such as anti-aggregatory, antioxidant, anxiolytic-like and anti-inflammatory functions (Hirai et al., 1983; Chou, 2003; Mi et al., 2005). Numerous studies have indicated that inflammatory cytokines play a major role in osteoclastogenesis, leading to the bone resorption that is frequently associated with osteoporosis. Our current study showed that paeonol inhibited osteoclastogenesis from bone marrow stromal cells and macrophages. The RANKL-induced ERK, p38 and NF-κB activation was attenuated by paeonol. Paeonol also prevented ovariectomized-induced bone loss *in vivo*.

To evaluate the effects of paeonol on the formation of osteoclasts, we used a bone marrow culture system from healthy rats and murine macrophages to generate osteoclasts with in vitro RANKL/M-CSF stimulation. We showed in this study for the first time that paeonol can inhibit the formation of osteoclast. To simply examine the effect of paeonol on resorption activity of osteoclasts, the mature osteoclasts were isolated from long bones of rabbit. Treatment with paeonol could concentration-dependently decrease the resorption activity of mature osteoclasts. Our results suggest that paeonol is able to suppress osteoclastic differentiation as well as the resorption activity of mature osteoclasts. We further demonstrated the suppression effects on the expression of MMP-9 and TRAP in differentiated osteoclast. Whether the other signaling pathways, e.g. Cathepsin K (Nishida and Gotoh, 1993), are involved in paeonol-inhibited bone resorption needs further investigation.

MAPK family members are proline-directed serine/threonine kinases that play important roles in cell growth, differentiation, and apoptosis (Hirai et al., 1983). Some external stimuli activate the phosphorylation of threonine and tyrosine (Ducy et al., 2000; Berg et al., 2003). MAPK family members are classified into the ERK, p38 and JNK groups, and it is widely accepted that peptide growth factors and phorbol esters preferentially activate ERK (Ducy et al., 2000). Furthermore, there are some studies that showed that RANKL–RANK



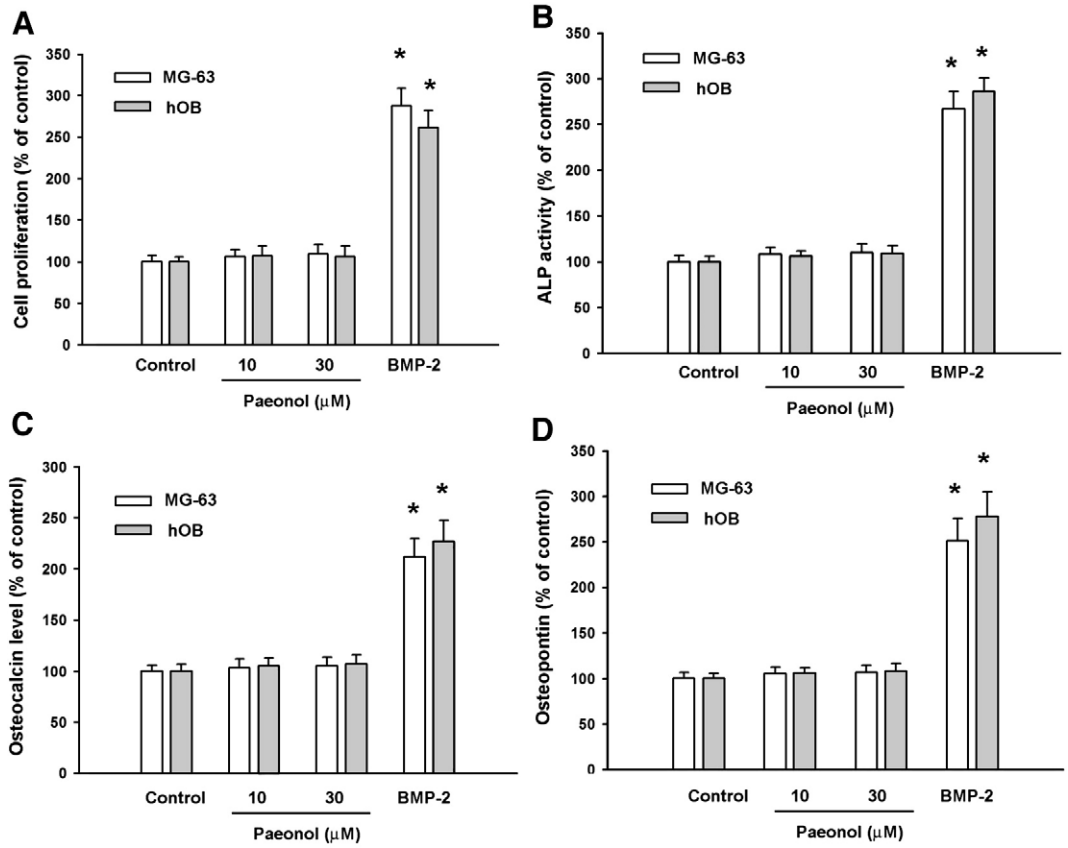


Fig. 5. Paeonol did not affect the cell proliferation and differentiation of osteoblasts. (A) MG-63 or hOB cells were treated with paeonol (10 or 30 μM) or BMP-2 (10 ng/ml) for 2 days and cell proliferation was evaluated by the BrdU assay as described in "Materials and method". Note that paeonol did not affect the cell proliferation of osteoblast. (B) MG-63 or hOB cells were treated with paeonol (10 or 30 μM) or BMP-2 (10 ng/ml) for 72 h. ALP activity was assessed using the commercial ALP kit. (C and D) Cells were treated with paeonol (10 or 30 μM) or BMP-2 (10 ng/ml) for 72 h. The amount of osteocalcin and osteopontin in culture medium was assessed by osteocalcin and osteopontin ELISA kit. Note that paeonol did not affect the differentiation of osteoblast cells. Whereas BMP-2 could stimulate the expression of ALP, osteocalcin and osteopontin by MG-63 or hOB cells. Data are presented as mean ± S.E. ($n=4$). *, $p<0.05$ as compared with control.

binding causes the phosphorylation of ERK, p38 and JNK, and that such phosphorylation leads to osteoclast differentiation (Yasuda et al., 1998; Ducy et al., 2000; Berg et al., 2003). In the present study, we used a homogenous clonal population of murine monocytic RAW 264.7 cells to clarify the effects of paeonol on the signaling pathways in osteoclast progenitor cells. We found that paeonol strongly inhibited the phosphorylation of ERK and p38 but not JNK in RAW 264.7 cells stimulated with RANKL. These findings suggest that the inhibitory effect of paeonol on osteoclast differentiation into mature osteoclasts may be responsible for the regulation of phosphorylation of ERK and p38. Further, when mouse bone marrow stromal cells were cultured for 7 days in the presence of both RANKL and M-CSF, paeonol significantly reduced osteoclast formation. Based on our findings, we speculated that this phenomenon was caused by the inhibitory effect of paeonol on RANK binding to RANKL and subsequent phosphorylation of ERK and p38 signal transduction.

NF-κB plays a critical role in the regulation of the cell cycle, cell adhesion, cytokine production, apoptosis, and other important cellular processes in macrophages. Osteoclast formation and their functions are mediated by RANKL-induced NF-κB activation (Bossard et al., 1999). Therefore, the inhibition of NF-κB activity might be an effective approach to target osteoclast activity as well as treatment of osteoporosis. In this study, we demonstrated that paeonol inhibited RANKL-induced NF-κB activation in RAW 264.7 cells by inhibiting the phosphorylation IκBα and p65. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that RANKL-increased NF-κB activity was inhibited by paeonol. These data support the hypothesis that paeonol inhibits NF-κB activity in macrophages, resulting in inhibition of osteoclast formation, and resorption activity.

Bone is a complex tissue composed of several cell types which are continuously undergoing a process of renewal and repair. When resorption and formation of bone are not coordinated and bone breakdown overrides bone building, osteoporosis results. Here we found that paeonol prevented the ovariectomized-induced bone loss. In addition, paeonol also antagonized the ovariectomized-induced osteoclast activity *in vivo*. Therefore, paeonol may be an anti-resorption agent for treatment of osteoporosis. Current drugs used to treat osteoporosis include bisphosphonates, calcitonin and estrogen. These are all anti-resorption medications, which maintain bone mass by inhibiting the function of osteoclasts (Goltzman, 2002). Their

Fig. 4. Paeonol inhibits RANKL-induced NF-κB activation. RAW264.7 cells were incubated with RANKL (50 ng/ml) for indicated time intervals (A), or pretreated with paeonol (10 or 30 μM) for 30 min followed by stimulation with RANKL (50 ng/ml) for 120 min (B), and IκBα phosphorylation and p65 phosphorylation at Ser^{536} were determined by immunoblotting using phospho-IκBα and phospho-p65 specific antibodies, respectively. (C) Cells were transfected with κB-luciferase expression vector and then pretreated with paeonol (3–30 μM) or NF-κB inhibitor peptide (10 μg/ml) for 30 min before incubation with RANKL (50 ng/ml) for 24 h. Luciferase activity was then assayed. Results are expressed as the mean ± S.E.M. ($n=4$). (D) Cells were pretreated with paeonol (3–30 μM) or NF-κB inhibitor peptide (10 μg/ml) for 30 min before incubation with RANKL (50 ng/ml) for 3 h. Nuclear fractions were extracted and assayed for κB binding activity by TransAM kits. (E–G) RAW264.7 cells were treated with RANKL (50 ng/ml) and paeonol (10 or 30 μM) for 2 days. Cell culture medium was then used to measure TNF-α, IL-1<0.05 as compared with RANKL-treated control group.

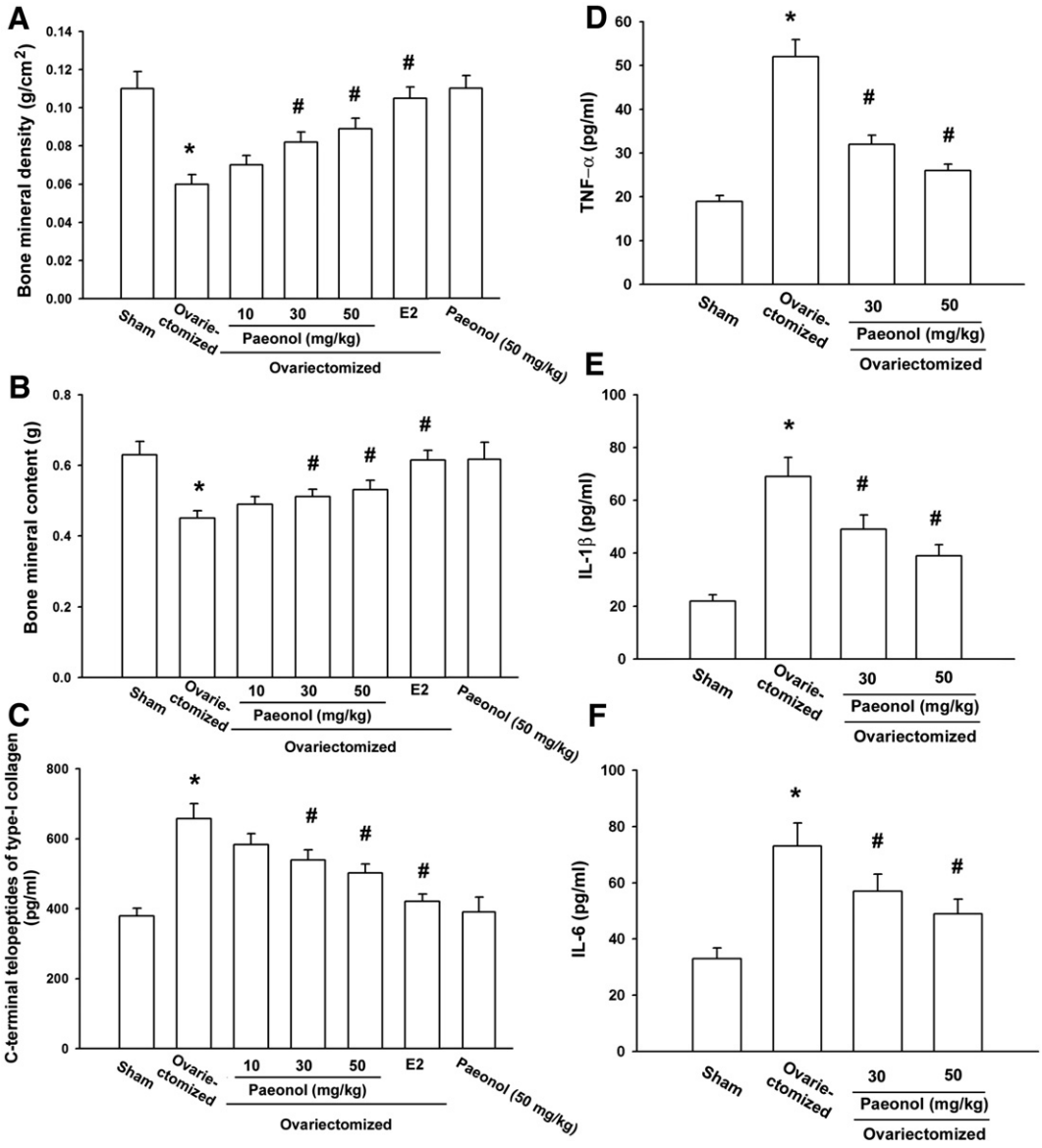


Fig. 6. Inhibition of ovariectomy induced a decrease of bone mineral density and bone mineral content by paeonol. Female ICR mice were given a sham operation or were ovariectomized. Mice that underwent ovariectomized were treated with the indicated concentrations of paeonol (10, 30, 50 mg/kg) or estradiol (0.1 mg/kg) by oral feeding. Total body bone mineral density (A), bone mineral content (B), serum C-terminal telopeptides of type-I collagen (C), TNF-α (D), IL-1β (E) and IL-6 (F) were determined 4 weeks after surgery. Data are presented as mean±S.E. (n=8–10 mice/group). *: $p<0.05$ as compared with sham. #: $p<0.05$ as compared with ovariectomized-group.

effects in increasing or recovering bone mass is relatively small, certainly no more than 2% per year (Rodan and Martin, 2002). It is desirable, therefore, to have satisfactory bone-building agents, such as teriparatide, that would stimulate new bone formation and correct the imbalance of trabecular microarchitecture that is characteristic of established osteoporosis (Cobb and Goldsmith, 1995; Julian et al., 1998). Since new bone formation is primarily a function of the osteoblast, agents that act by either increasing the proliferation of cells of the osteoblastic lineage or inducing differentiation of the osteoblasts can enhance bone formation (Cobb and Goldsmith, 1995; Julian et al., 1998). Unfortunately we found that paeonol did not affect the proliferation of two osteoblast cell lines. Compared with BMP-2, paeonol also did not affect the differentiation marker including ALP, osteocalcin and osteopontin of osteoblasts. These data suggested the anti-resorption effect but not bone formation activity of paeonol.

In conclusion, the present study demonstrated that paeonol inhibits the osteoclastogenesis from bone marrow stromal cells and macrophages. Paeonol also reduced resorption activity of mature osteoclasts. In addition, paeonol attenuated the RANKL-induced ERK, p38 and NF-κB activation. Therefore, paeonol may show beneficial effects in reducing the osteoclast formation and activity to promote bone health in osteoporosis therapy.

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References

Berg, C., Neumeyer, K., Kirkpatrick, P., 2003. Teriparatide. Nat. Rev. Drug Discov. 2, 257–258.

Bossard, M.J., Tomaszek, T.A., Levy, M.A., Ijames, C.F., Huddleston, M.J., Briand, J., Thompson, S., Halpert, S., Veber, D.F., Carr, S.A., Meek, T.D., Tew, D.G., 1999. Mechanism of inhibition of cathepsin K by potent, selective 1, 5-diacylcarbohydrazides: a new class of mechanism-based inhibitors of thiol proteases. Biochemistry 38, 15893–15902.

Chou, T.C., 2003. Anti-inflammatory and analgesic effects of paeonol in carrageenan-evoked thermal hyperalgesia. Br. J. Pharmacol. 139, 1146–1152.
Chung, J.G., 1999. Paeonol promotion of DNA adduct formation and arylamines *N*-acetyltransferase activity in human colon tumour cells. Food Chem. Toxicol. 37, 327–334.
Cobb, M.H., Goldsmith, E.J., 1995. How MAP kinases are regulated. J. Biol. Chem. 270, 14843–14846.
Darnay, B.G., Haridas, V., Ni, J., Moore, P.A., Aggarwal, B.B., 1998. Characterization of the intracellular domain of receptor activator of NF-kB (RANK): interaction with tumor necrosis factor receptor-associated factors and activation of NF-kB and c-Jun N-terminal kinase. J. Biol. Chem. 273, 20551–20555.
Darnay, B.G., Ni, J., Moore, P.A., Aggarwal, B.B., 1999. Activation of NF-kB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kB-inducing kinase: identification of a novel TRAF6 interaction motif. J. Biol. Chem. 274, 7724–7731.
Ducy, P., Schinke, T., Karsenty, G., 2000. The osteoblast: a sophisticated fibroblast under central surveillance. Science 289, 1501–1504.
Goltzman, D., 2002. Discoveries, drugs and skeletal disorders. Nat. Rev. Drug Discov. 1, 784–796.
Hirai, A., Terano, T., Hamazaki, T., Sajiki, J., Saito, H., Tahara, K., Tamura, Y., Kumagai, A., 1983. Studies on the mechanism of antiaggregatory effect of Moutan Cortex. Thromb. Res. 31, 29–40.
Ishiguro, K., Ando, T., Maeda, O., Hasegawa, M., Kadomatsu, K., Ohmiya, N., Niwa, Y., Xavier, R., Goto, H., 2006. Paeonol attenuates TNBS-induced colitis by inhibiting NF-kappaB and STAT1 transactivation. Toxicol. Appl. Pharmacol. 217, 35–42.
Julian, M., Quinn, M., Elliott, J., Gillespie, M.T., Martin, T.J., 1998. A combination of osteoclast differentiation factor and macrophage-colony stimulating factor is sufficient for both human and mouse osteoclast formation in vitro. Endocrinology 139, 4424–4427.
Lee, S.Y., Reichlin, A., Santana, A., Sokol, K.A., Nussenzweig, M.C., Choi, Y., 1997. TRAF2 is essential for JNK but not NF-kB activation and regulates lymphocyte proliferation and survival. Immunity 7, 703–713.
Mi, X.J., Chen, S.W., Wang, W.J., Wang, R., Zhang, Y.J., Li, W.J., Li, Y.L., 2005. Anxiolytic-like effect of paeonol in mice. Pharmacol. Biochem. Behav. 81, 683–687.
Miyazaki, T., Katagiri, H., Kanegae, Y., Takayanagi, H., Sawada, Y., Yamamoto, A., Pando, M.P., Asano, T., Verma, I.M., Oda, H., Nakamura, K., Tanaka, S., 2000. Reciprocal role of ERK and NF-kB pathways in survival and activation of osteoclasts. J. Cell Biol. 148, 333–342.
Nishida, E., Gotoh, Y., 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. Trends Biochem. Sci. 18, 128–131.
Nizamutdinova, I.T., Oh, H.M., Min, Y.N., Park, S.H., Lee, M.J., Kim, J.S., Yean, M.H., Kang, S.S., Kim, Y.S., Chang, K.C., Kim, H.J., 2007. Paeonol suppresses intercellular adhesion molecule-1 expression in tumor necrosis factor-alpha-stimulated human umbilical vein endothelial cells by blocking p38, ERK and nuclear factor-kappaB signaling pathways. Int. Immunopharmacol. 7, 343–350.
Rodan, G.A., Martin, T.J., 2002. Therapeutic approaches to bone diseases. Science 289, 1508–1514.
Tang, C.H., Yang, R.S., Fu, W.M., 2005. Prostaglandin E2 stimulates fibronectin expression through EP1 receptor, phospholipase C, protein kinase Calpha, and c-Src pathway in primary cultured rat osteoblasts. J. Biol. Chem. 280, 22907–22916.
Tang, C.H., Yang, R.S., Huang, T.H., Lu, D.Y., Chuang, W.J., Huang, T.F., Fu, W.M., 2006. Ultrasound stimulates cyclooxygenase-2 expression and increases bone formation through integrin, focal adhesion kinase, phosphatidylinositol 3-kinase, and Akt pathway in osteoblasts. Mol. Pharmacol. 69, 2047–2057.
Tang, C.H., Hsu, T.L., Lin, W.W., Lai, M.Z., Yang, R.S., Hsieh, S.L., Fu, W.M., 2007a. Attenuation of bone mass and increase of osteoclast formation in decoy receptor 3 transgenic mice. J. Biol. Chem. 282, 2346–2354.
Tang, C.H., Yang, R.S., Chen, Y.F., Fu, W.M., 2007b. Basic fibroblast growth factor stimulates fibronectin expression through phospholipase C gamma, protein kinase C alpha, c-Src, NF-kappaB, and p300 pathway in osteoblasts. J. Cell. Physiol. 211, 45–55.
Teitelbaum, S.L., 2000. Bone resorption by osteoclasts. Science 289, 1504–1505.
Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., Koga, T., Martin, T.J., Suda, T., 1990. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proc. Natl. Acad. Sci. U. S. A. 87, 7260–7264.
van't Hof, R.J., Ralston, S.H., 2001. Nitric oxide and bone. Immunology 103, 255–261.
Xu, S.P., Sun, G.P., Shen, Y.X., Wei, W., Peng, W.R., Wang, H., 2007. Antiproliferation and apoptosis induction of paeonol in HepG2 cells. World J. Gastroenterol. 13, 250–256.
Yang, R.S., Lin, W.L., Chen, Y.Z., Tang, C.H., Huang, T.H., Lu, B.Y., Fu, W.M., 2005. Regulation by ultrasound treatment on the integrin expression and differentiation of osteoblasts. Bone 36, 276–283.
Yao, G.Q., Sun, B., Hammond, E.E., Spencer, E.N., Horowitz, M.C., Insogna, K.L., Weir, E.C., 1998. The cell-surface form of colony-stimulating factor-1 is regulated by osteotropic agents and supports formation of multinucleated osteoclast-like cells. J. Biol. Chem. 273, 4119–4128.
Yasuda, H., Shima, N., Nakagawa, N., 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc. Natl. Acad. Sci. U. S. A. 95, 3597–3602.