

Crocin inhibits RANKL-induced osteoclastogenesis by regulating JNK and NF- κ B signaling pathways

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Abstract. Receptor activator of nuclear factor- κ B ligand (RANKL), a member of the tumor necrosis factor receptor-ligand family, is a crucial factor involved in osteoclast differentiation. Crocin, a pharmacologically active component of *Crocus sativus* L., has been reported to attenuate ovariectomy-induced osteoporosis in rats. However, the molecular mechanism underlying the effect of crocin on osteoclast formation remains to be determined. The present study aimed to investigate the effect of crocin on RANKL-induced osteoclastogenesis and its underlying molecular mechanism. Results demonstrated that crocin decreased osteoclastogenesis in bone marrow-derived macrophages (BMMs). In addition, the expression levels of osteoclast marker proteins were downregulated by crocin. Mechanistically, crocin inhibited RANKL-induced activation of nuclear factor- κ B (NF- κ B) by suppressing inhibitor of κ B α degradation and preventing NF- κ B p65 subunit nuclear translocation, and by activating c-Jun N-terminal kinase (JNK) in BMMs. In summary, the results of the present study suggested that crocin down-regulates osteoclast differentiation via inhibition of JNK and NF- κ B signaling pathways. Thus, crocin may be a potential therapeutic agent for the treatment of osteoclast-associated diseases, including osteoporosis.

Introduction

Osteoporosis is a skeletal disorder characterized by reduced bone mass and micro-architectural deterioration of bone tissue (1). It is estimated that, in the United States, ~50% of women aged >50 years may sustain an osteoporotic fracture during their lifetime (2). Despite the high incidence rate of osteoporosis, the effect of therapeutic agents, including bisphosphonates, calcitonin and estrogen in recovering bone

mass is limited. Osteoclasts are unique bone-resorbing cells derived from cells of the monocyte-macrophage lineage (3). Numerous factors are involved in osteoclast differentiation (4-6). Receptor activator of nuclear factor- κ B ligand (RANKL) is a membrane protein of the tumor necrosis factor (TNF) family, and increasing evidence indicates that RANKL serves a critical role in osteoclast differentiation (6-8). It has been reported that RANKL may activate various downstream signaling pathways, including the nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling pathways. Activation of these pathways may upregulate expression of osteoclast-specific genes, including those encoding tartrate-resistant acid phosphatase (TRAP) and enzymes involved in cell fusion (9). Thus, inhibiting RANKL signaling may suppress osteoclastogenesis.

Crocus sativus L. (saffron) belongs to the Iridaceae family and has been utilized widely as a culinary spice, an anodyne, or a tranquilizer in traditional Chinese medicine. Crocin is a pharmacologically active component of *Crocus sativus* L. Various studies have demonstrated that crocin possesses a wide range of pharmacological effects, including anti-arthritis, anti-inflammatory, anti-oxidant and anti-tumor properties (10-13). Ding *et al* (14) demonstrated that crocin inhibited interleukin (IL)-1 β -induced activation of the NF- κ B signaling pathway by suppressing the degradation of inhibitor of κ B α (I κ B α) in rabbit chondrocytes. In addition, crocin has been demonstrated to restore cartilage and reduce bone deterioration, inflammation and oxidative damage (10). A recent study reported that administration of crocin may prevent ovariectomy-induced osteoporosis in rats (15). However, the molecular mechanism underlying the effect of crocin on osteoclast formation remains to be investigated. The present study aimed to evaluate the effect of crocin on RANKL-induced osteoclastogenesis and determine its underlying molecular mechanism.

Materials and methods

Animals, reagents and antibodies. A total of 6 female C57BL/6 mice (age, 8-12 weeks old) were obtained from the Animal Breeding Center of Shaanxi Provincial People's Hospital (Xi'an, China) and maintained at a constant temperature (21 \pm 2°C) and humidity in a holding facility under a 12 h light/dark cycle, with free access to food and water. Animal experiments were approved by the Institutional Animal Care and Use

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Committee of Shaanxi Provincial People's Hospital (Shaanxi, China). All reagent-grade chemicals, including crocin (purity >95%), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, recombinant macrophage-colony stimulating factor (M-CSF), RANKL, α -minimum essential medium (α -MEM), TRAP Staining kit, Cell Counting kit-8 (CCK8) and Bicinchoninic Acid (BCA) Protein assay kit were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Radioimmunoprecipitation assay (RIPA) lysis buffer was purchased from Beyotime Institute of Biotechnology (Haimen, China). Polyvinylidene difluoride (PVDF) membranes were purchased from EMD Millipore (Billerica, MA, USA). Rabbit anti-mouse antibodies against nuclear factor of activated T cells cytoplasmic 1 (NFATc1; 1:2,500; cat. no. sc-13033), c-Fos (1:3,000; cat. no. sc-253), cathepsin K (1:1,500; cat. no. sc-30056), I κ B α (1:3,000; cat. no. sc-371), phosphorylated (p)-p65 (1:3,000; cat. no. sc-33020), p-c-Jun N-terminal kinase (JNK; 1:2,500; cat. no. sc-135642), JNK (1:3,000; cat. no. sc-572) and GAPDH (1:3,000; cat. no. sc-25778) were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA). A goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (1:3,000; cat. no. A16104) and the Enhanced Chemiluminescence (ECL) reagent were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Cell culture. Primary bone marrow-derived macrophages (BMMs) were prepared by removing bone marrow from the femora and tibiae of C57BL/6 mice, and flushing the bone marrow cavity with α -MEM supplemented with 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. The following day, all non-adherent cells were collected and considered to be osteoclast precursors. Mice were sacrificed intraperitoneally with sodium pentobarbital (50 mg/kg; Sigma-Aldrich; Merck Millipore).

Osteoclast differentiation assay. During differentiation, BMMs were treated with 0, 10, 20 or 40 μ M crocin at room temperature, and then cultured in α -MEM supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin in the presence of 50 ng/ml M-CSF and 50 ng/ml RANKL for 4 days. The control group was BMMs exposed to medium alone at room temperature. Subsequently, osteoclast formation was measured using a TRAP Staining kit according to the manufacturer's protocol. Briefly, cells were fixed with 10% formaldehyde in PBS for 3 min and stained with Naphthol AS-BI phosphate and a tartrate solution for 1 h at 37°C, prior to counterstaining with hematoxylin. Osteoclasts were considered to be TRAP-positive if multinuclear staining (>3 nuclei) was observed via light microscopy. The total number of TRAP-positive cells and the number of nuclei per TRAP-positive cell in each well were counted (data not shown).

Cell viability assay. Cell viability was measured with CCK8 according to the manufacturer's protocol. Briefly, BMMs at a density of 1×10^4 cells/well were treated with 10, 20 or 40 μ M crocin for 3 days in 96-well plates. Subsequently, 10 μ l CCK8 solution was added to each well for 2 h. The absorbance in each well was measured at a wavelength of 490 nm using a microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA).

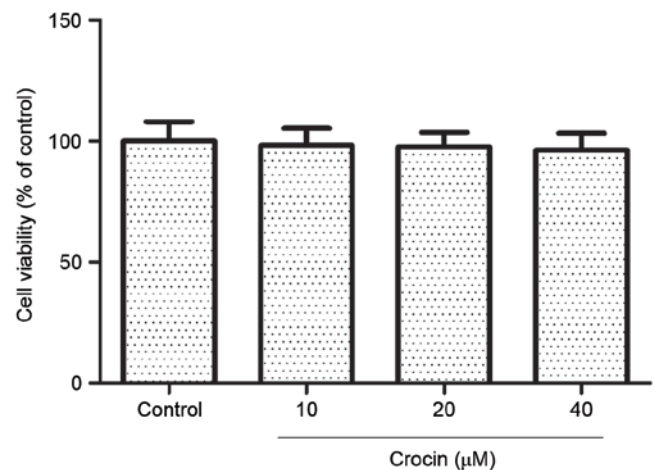


Figure 1. Effect of crocin on cell viability. Bone marrow-derived macrophages were treated with 0, 10, 20 or 40 μ M crocin for 72 h, control group was BMMs exposed to medium alone. Cell viability was measured using the CCK8 assay. Data are expressed as the mean \pm standard error. All experiments were repeated at least three times.

Western blotting. Total protein was extracted from BMMs by using RIPA lysis buffer according to the manufacturer's protocol. Lysates were sonicated for 5 sec on ice and centrifuged at 6,000 \times g for 5 min at 4°C. Supernatants were collected and the protein concentration was determined using a BCA assay kit. Equal quantities of protein (40 μ g per lane) were loaded onto 10% gels and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, prior to transfer onto PVDF membranes. Membranes were blocked with 10% fat-free milk in TBS (20 mmol/l Tris, 0.15 mol/l NaCl; pH 7.0) containing 0.1% Tween-20 (TBST) for 1 h at room temperature prior to probing with specific antibodies against NFATc1, c-Fos, cathepsin K, p-JNK, JNK, I κ B α , p-p65 and GAPDH at 4°C overnight. Following washing with TBST, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Proteins were visualized using an ECL reagent and images were captured using the ImageQuant™ LAS 4000 imager (Fujifilm, Tokyo, Japan). Densitometry was performed using Gel-Pro Analyzer software version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data was analyzed using SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA), and are presented as the mean \pm standard error of triplicate. Significant differences were analysed using a Student's t-test or one-way analysis of variance followed by Tukey's Honest Significant Difference post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of crocin on cell viability. BMMs were treated with 10, 20 or 40 μ M crocin for 72 h, and cell viability was assessed using a CCK8 assay. As presented in Fig. 1, crocin treatment had no cytotoxic effect on cells compared with the control group.

Crocin inhibits osteoclast formation in BMMs. Subsequently, the effect of crocin on RANKL-induced osteoclast

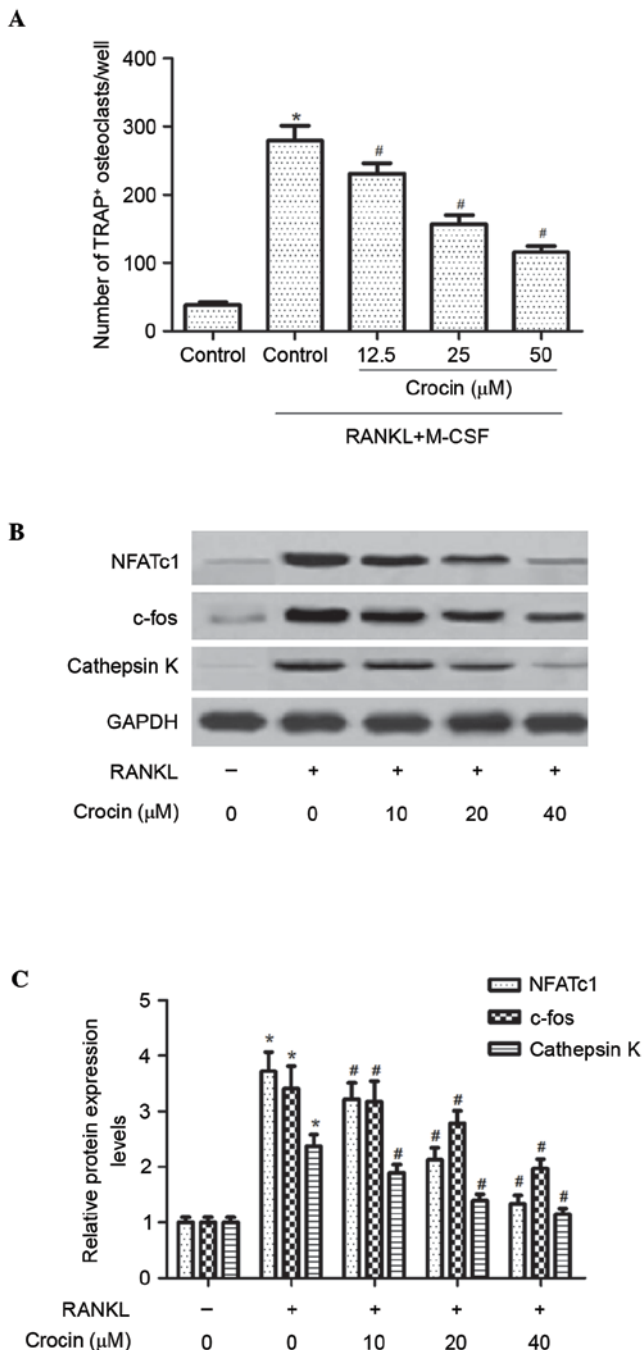


Figure 2. Crocin inhibits osteoclast formation in BMMs. (A) BMMs were cultured with crocin in the presence of RANKL and M-CSF for 4 days. Subsequently, cells were fixed and stained with TRAP. Multinucleated osteoclasts were counted. * $P < 0.05$ vs. control; # $P < 0.05$ vs. RANKL + M-CSF only. (B) Western blotting was performed to assess the protein expression levels of NFATc1, c-Fos and cathepsin K. (C) NFATc1, c-Fos and cathepsin K protein expression levels were quantified by densitometry. * $P < 0.05$ vs. control; # $P < 0.05$ vs. RANKL. Data are expressed as the mean \pm standard error. All experiments were repeated at least three times. BMMs, bone marrow-derived macrophages; RANKL, receptor activator of nuclear factor- κ B; M-CSF, macrophage-colony stimulating factor; NFATc1, nuclear factor of activated T cells cytoplasmic 1; TRAP, tartrate resistant acid phosphatase.

differentiation from BMMs was investigated. As presented in Fig. 2A, the number of TRAP-positive multinuclear osteoclasts was significantly increased following RANKL stimulation ($P < 0.05$). However, crocin significantly inhibited

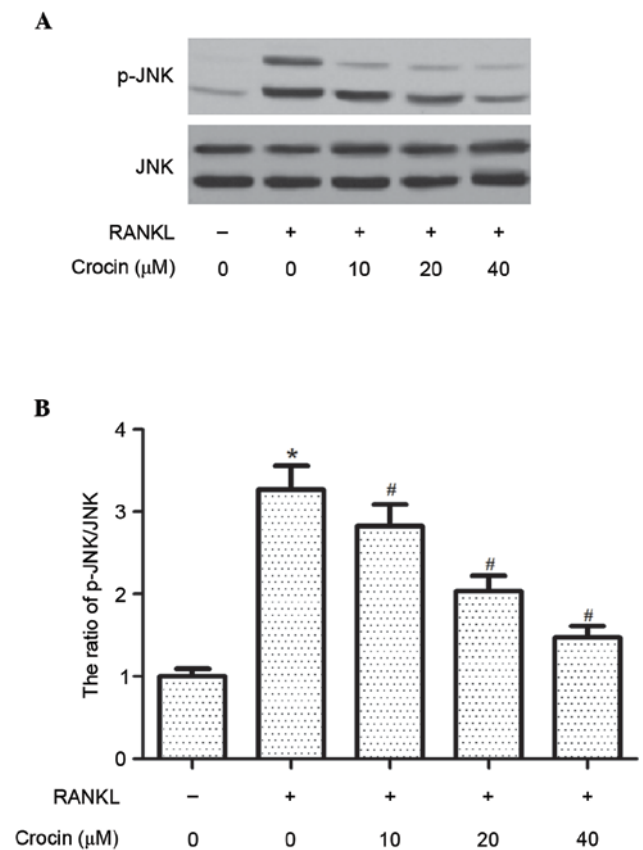


Figure 3. Crocin inhibits RANKL-induced activation of JNK in BMMs. (A) BMMs were pre-incubated for 30 min with the indicated concentrations of crocin and subsequently activated for 1 h with RANKL. JNK phosphorylation was determined by western blotting. (B) Quantification of the ratio of p-JNK/JNK protein expression levels by densitometry. Data are expressed as the mean \pm standard error. All experiments were repeated at least three times. * $P < 0.05$ vs. control; # $P < 0.05$ vs. RANKL. RANKL, receptor activator of nuclear factor- κ B; JNK, c-Jun N-terminal kinase; BMMs, bone marrow-derived macrophages; p, phosphorylated.

the formation of TRAP-positive multinuclear osteoclasts in a dose-dependent manner ($P < 0.05$).

Furthermore, the effect of crocin on the expression of osteoclast-associated proteins, including NFATc1, c-Fos and cathepsin K, was determined. Western blotting demonstrated that the protein expression levels of NFATc1, c-Fos and cathepsin K were increased following RANKL stimulation; crocin treatment significantly inhibited the expression levels of these proteins in BMMs compared with RANKL treatment alone ($P < 0.05$), in a dose-dependent manner (Fig. 2B and C).

Crocin inhibits RANKL-induced activation of JNK in BMMs.

To investigate the molecular mechanisms underlying the inhibitory effect of crocin on RANKL-induced osteoclast formation, the effect of crocin on JNK activation in RANKL-induced BMMs was examined. Western blotting demonstrated that the protein expression levels of p-JNK were significantly increased following RANKL stimulation ($P < 0.05$), whereas crocin significantly inhibited the RANKL-induced phosphorylation of JNK in BMMs ($P < 0.05$; Fig. 3).

Crocin inhibits RANKL-induced NF- κ B activation in BMMs. RANKL-induced NF- κ B activation is essential for osteoclast differentiation and function. Thus, the effect of crocin on RANKL-induced NF- κ B activation in BMMs was assessed. I κ B α protein expression levels were decreased following 1 h of RANKL stimulation ($P < 0.05$), whereas crocin significantly suppressed the RANKL-induced degradation of I κ B α in BMMs ($P < 0.05$; Fig. 4). In addition, crocin inhibited the RANKL-stimulated phosphorylation of NF- κ B p65 in BMMs ($P < 0.05$; Fig. 4).

Discussion

The present study revealed that crocin inhibited the osteoclastogenesis of BMMs, as the expression of osteoclast marker proteins was downregulated by crocin. Mechanistically, crocin inhibited RANKL-induced activation of NF- κ B by suppressing I κ B α degradation and preventing NF- κ B p65 nuclear translocation, and activating JNK in BMMs.

To evaluate the effects of crocin on the formation of osteoclasts, a bone marrow culture system was utilised to induce the formation of osteoclasts in the presence of RANKL/M-CSF *in vitro*. A previous study has reported that M-CSF induces the proliferation of osteoclast precursor cells and their differentiation into the osteoclast lineage, whereas RANKL induces subsequent differentiation of osteoclast precursors into osteoclasts (16). Consistent with these results, the present study revealed that RANKL stimulation induced the formation of osteoclasts; however, crocin treatment inhibited the RANKL-induced formation of osteoclasts from osteoclast precursors, as the number of TRAP-positive multinuclear cells from BMMs was reduced following treatment with crocin. These results suggested that crocin suppresses osteoclast differentiation by acting directly on osteoclast precursors.

NFATc1, a key transcriptional factor downstream of c-Fos, regulates the expression of TRAP, calcitonin receptor, and cathepsin K during osteoclast differentiation (17). Takayanagi *et al* (18) reported that NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts following RANKL stimulation, and that ectopic expression of NFATc1 causes precursor cells to undergo differentiation. c-Fos serves an essential role in the osteoclastic differentiation of precursor cells generated by M-CSF and RANKL. c-Fos-deficient mice exhibit a severe osteoporotic phenotype due to the absence of osteoclast differentiation (19). The present study demonstrated that crocin suppressed the expression of osteoclast marker proteins in BMMs. These results suggested that suppression of RANKL-induced activation of NFATc1 and c-Fos by crocin is associated with the inhibition of osteoclastogenesis.

Previous studies have revealed that RANKL stimulates JNK, leading to the activation of the transcription factor c-Jun (20,21). Blockade of this signalling pathway by treatment with a JNK inhibitor, SP600125, suppressed RANKL-induced osteoclast differentiation (22). Furthermore, suppression of c-Jun using small interfering RNA significantly inhibited RANKL-induced osteoclast differentiation (23). The present study demonstrated that crocin greatly inhibited RANKL-induced phosphorylation of JNK in BMMs, which suggested that the inhibitory effect of crocin on the

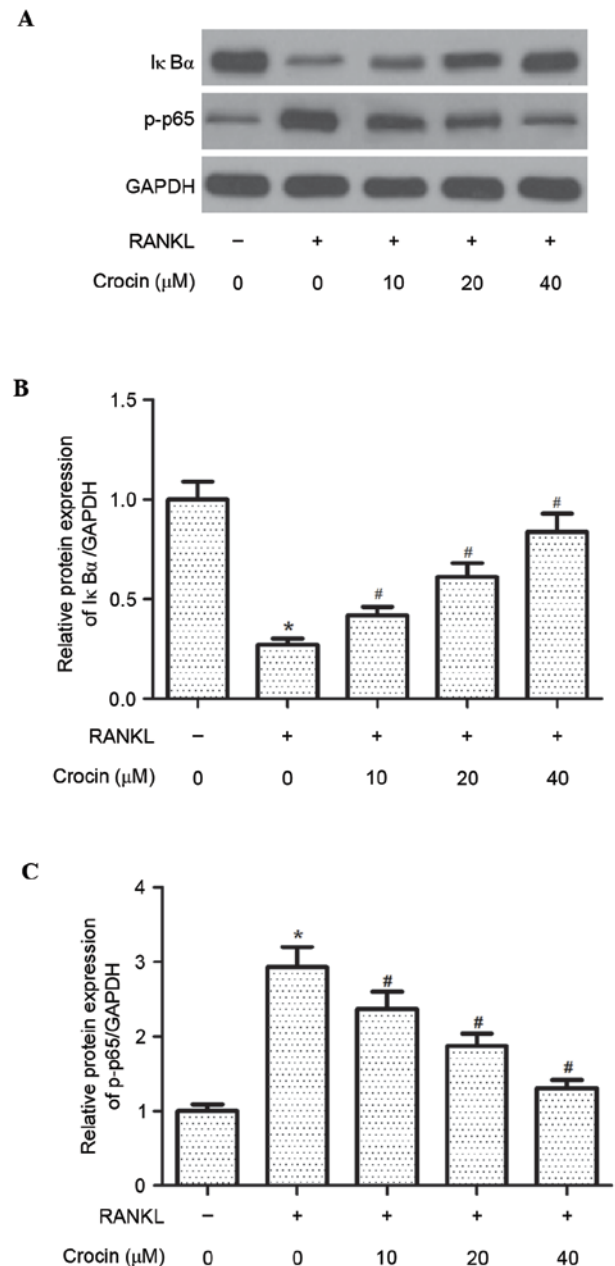


Figure 4. Crocin inhibited RANKL-induced NF- κ B activation in BMMs. (A) BMMs were pre-incubated for 30 min with the indicated concentrations of crocin and subsequently activated for 1 h with RANKL. I κ B α and p-p65 protein expression levels were determined by western blotting. (B) I κ B α and (C) p-p65 protein expression levels were quantified by densitometry. Data are expressed as the mean \pm standard error. All experiments were repeated at least three times. * $P < 0.05$ vs. control; # $P < 0.05$ vs. RANKL. RANKL, receptor activator of nuclear factor- κ B; NF- κ B, nuclear factor- κ B; BMMs, bone marrow-derived macrophages; I κ B α , inhibitor of κ B; p, phosphorylated.

differentiation of osteoclast precursors into mature osteoclasts may be mediated by the regulation of JNK phosphorylation.

The NF- κ B signaling pathway serves an important role in osteoclast formation (24-26). It has been reported that NF- κ B knockout mice exhibit severe osteopetrosis and failed to stimulate osteoclastogenesis (27). Genetic inactivation of I κ B kinase (IKK) α or IKK β is sufficient to inhibit osteoclastogenesis (28). Furthermore, osteoclast formation

has been demonstrated to be mediated by RANKL-induced NF- κ B activation. For example, one study demonstrated that RANKL induces TNF- α production primarily via the activation of NF- κ B in RAW264.7 cells (29). Therefore, inhibition of NF- κ B activity may be an effective strategy for the treatment of osteoporosis. The present study revealed that crocin greatly suppressed the RANKL-induced degradation of I κ B α and phosphorylation of NF- κ B p65 in BMMs. This data supported the hypothesis that crocin inhibits NF- κ B activity in BMMs, resulting in inhibition of osteoclast formation.

In conclusion, the results of the present study suggested that crocin suppresses osteoclast differentiation via the JNK and NF- κ B signaling pathways. Thus, crocin may be a potential therapeutic agent for the treatment of osteoclast-associated diseases, including osteoporosis.

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