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Isofraxidin Inhibits Receptor Activator of Nuclear Factor-κB Ligand–Induced Osteoclastogenesis in Bone Marrow–Derived Macrophages Isolated from Sprague–Dawley Rats by Regulating NF-κB/NFATc1 and Akt/NFATc1 Signaling Pathways

Wei Wang[1] **and Bo Wang**[1]

Abstract

Osteoporosis is a common bone disease that is characterized by decreased bone mass and fragility fractures. Isofraxidin is a hydroxy coumarin with several biological and pharmacological activities including an anti-osteoarthritis effect. However, the role of isofraxidin in osteoporosis has not yet been investigated. In the present study, we used receptor activator of nuclear factor-κB ligand (RANKL) to induce osteoclast formation in primary bone marrow macrophages (BMMs). Our results showed that RANKL treatment significantly increased tartrate-resistant acid phosphatase (TRAP) activity, as well as the expression of osteoclastogenesis-related markers including MMP-9, c-Src, and cathepsin K at both mRNA and protein levels; however, these effects were inhibited by isofraxidin in BMMs. In addition, luciferase reporter assay demonstrated that isofraxidin treatment suppressed the RANKL-induced an increase in nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) transcriptional activity. Besides, the decreased expression level of IκBα and increased levels of p-p65, p-IκBα, and p-Akt in RANKL-induced BMMs were attenuated by isofraxidin. Moreover, NFATc1 overexpression rescued the anti-osteoclastogenic effect of isofraxidin with increased expression levels of MMP-9, c-Src, and cathepsin K. Taken together, these findings indicated that isofraxidin inhibited RANKL-induced osteoclast formation in BMMs via inhibiting the activation of NF-κB/NFATc1 and Akt/NFATc1 signaling pathways. Thus, isofraxidin might be a therapeutic agent for the treatment of osteoporosis.

Keywords

osteoporosis, isofraxidin, osteoclast formation, RANKL, NFATc1

Introduction

Osteoporosis is a common bone disease that is characterized by decreased bone mass, microarchitectural deterioration, and fragility fractures[1]. Osteoporosis is widespread in older women and men, particularly in postmenopausal women[2]. In recent years, increasing researchers have devoted to exploring the pathogenesis of osteoporosis[3]. Osteoblasts are specialized, terminally differentiated products of mesenchymal stem cells, which are essential for normal bone health and mediate bone resorption[4]. Previous studies have shown that osteoblasts play a very important role in the progression of osteoporosis via influencing bone anabolic function[5]. Receptor activator of nuclear factor-κB ligand (RANKL) is necessary for osteoclast formation and function through the signaling receptor, RANK. The RANKL/RANK signaling activates nuclear factor of activated T-cells cytoplasmic 1

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(NFATc1), the master regulator of osteoclastogenesis, to induce osteoclastogenic gene expression[6,7]. Accumulating data indicate that inhibition of RANKL-mediated osteoclastogenesis may serve as a therapeutic strategy for osteoporosis.

Isofraxidin (7-hydroxy-6, 8-dimethoxy coumarin) is a hydroxy coumarin that has been proven to possess several biological and pharmacological activities, such as anti-inflammatory, antioxidant, anticancer, cardioprotective, and neuroprotective effects[8–11]. Considering the multiple activities, isofraxidin can be used as a potent multitarget agent for some diseases, e.g. cancer, neurodegenerative diseases, and heart diseases. Shen et al. reported that isofraxidin inhibits proliferation and induces apoptosis through blockage of Akt pathway in human colorectal cancer cells[9]. Bai et al. reported that isofraxidin protects against Aβ(25-35)-induced atrophies of axons and dendrites[10]. A study by Chen et al. showed that isofraxidin alleviates myocardial infarction via NLRP3 inflammasome inhibition[11]. Additionally, isofraxidin also has an anti-osteoarthritis effect. Isofraxidin inhibits interleukin (IL)-1β-induced inflammatory response in human osteoarthritis chondrocytes via the regulation of NF-κB signaling[12]. Isofraxidin prevents osteoarthritis development via targeting the toll-like receptor 4/myeloid differentiation protein-2 axis[13]. However, the role of isofraxidin in osteoclast differentiation has not yet been investigated. In the present study, we examined the effect of isofraxidin on RANKL-induced osteoclast formation in bone marrow macrophages (BMMs).

Materials and Methods

Cell Culture

Primary BMMs were isolated from Sprague–Dawley rats (aged 6 to 8 weeks) as previously described[14]. Briefly, bone marrow cells were harvested from the femurs and tibiae of Sprague–Dawley rats and cultured with a-MEM medium, which was supplemented with 10 ng/ml recombinant rat macrophage-colony stimulating factor (Biorbyt, Cambridge, UK), 10% fetal bovine serum, and 100 U/ml penicillin/100 mg/ml streptomycin for 24 h. Then the adherent cells (considered as BMMs) were collected and classified as osteoclast precursor cells. All animal experiments were approved by the Institution Animal Care and Use Committee of the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

Osteoclast Differentiation Assay

To generate osteoclasts, the primary BMMs (1×10^3 cells/well) were cultured in the presence of 100 ng/ml of RANKL (R&D Systems, Minneapolis, MN, USA). After 4 days, cells were collected for the preparation of cell lysates with 0.05% Triton X-100 at 4°C. Then the TRAP activity was measured using an acid phosphatase assay kit (BioVision, Milpitas, CA, USA). Finally, the absorbance at 405 nm was determined using a microplate reader (Bio-Tek, Winooski, VT, USA).

Cell Viability Assay

BMMs (5×10^4 cells) were seeded into 96-well plates and incubated for 16 h in media as mentioned in Cell Culture section. Various concentrations (0, 6.25, 12.5, and 25 μM) of isofraxidin (Sigma-Aldrich, St. Louis, MO, USA) were added to cells and maintained for 24 h at 37°C in a 95% air and 5% CO_2 atmosphere incubator. After that, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT) was added and incubated for 2 h. Finally, BMMs were treated with 200 μl of dimethyl sulfoxide and the absorbance was quantified at 490 nm wavelength using a microplate reader (Bio-Tek).

Lactate Dehydrogenase Assay

The supernatant of culture medium after different treatments was collected for the detection of lactate dehydrogenase (LDH) content using an LDH Cytotoxicity Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Absorption at 490 nm was measured using a microplate reader (Bio-Tek).

Luciferase Reporter Assay

To examine the NFATc1 activation, BMMs were transfected with luciferase reporter construct containing NFATc1-binding promoter element. The BMMs (1×10^4 cells/well) were then plated in 96-well plates and pretreated with isofraxidin for 30 min and then stimulated with 100 ng/ml of RANKL for 8 h. At the end of the culture period, cell lysates were prepared, and the luciferase activity was measured using a luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Quantitative Real-time Polymerase Chain Reaction

Total RNA was isolated from BMMs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for the quantitative real-time polymerase chain reaction (qRT-PCR). The single-stranded cDNA was synthesized using 1 μg of total RNA with SuperScript II reverse transcriptase (TaKaRa Biotechnology, Otsu, Japan). RT-PCR was then performed using SYBR® Premix Ex Taq™ II (TaKaRa Biotechnology) and the results were analyzed using an ABI 7500 Sequencing Detection System (Applied Biosystems, Foster City, CA, USA). Primers were designed against the following mouse sequences: MMP-9 (forward: 5′-AGT TTG GTG TCG CGG AGC AC-3′, reverse: 5′-TAC ATG AGC GCT TCC GGC AC-3′), c-Src (forward: 5′-CCA GGC TGA GGA GTG GTA CT-3′, reverse: 5′-CAGCTTGCGGATCTTGTAGT-3′), Cathepsin K (forward: 5′-GGC CAA CTC AAG AAG AAA AC-3′, reverse: 5′-GTG CTT GCT TCC CTT CTG G-3′), NFATc1 (forward: 5′-TGC TCC TCC TCC TGC TGC TC-3′, reverse: 5′-CGT CTT CCA CCT CCA CGT CG-3′), and β-actin (forward: 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′, reverse: 5′-CTA GAA GCA TTT GCG GTG GAC GAT G-3′). Data were analyzed by the $2^{-\Delta\Delta CT}$ method, and all mRNA levels were normalized to the level of β-actin.

Western Blot

Whole cell lysates were prepared from BMMs using radioimmunoprecipitation assay buffer (Beyotime Biotechnology, Shanghai, China) containing protease and phosphatase inhibitors (Beyotime Biotechnology). Samples of whole cell extracts were normalized to determine protein concentration using the BCA method and then separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gels were then subjected to western blot analysis with primary antibodies against MMP-9 (Abcam, Cambridge, MA, USA), c-Src (Abcam), cathepsin K (Abcam), p-p65 (Invitrogen), p-IκBα (Invitrogen), IκBα (Invitrogen), p-Akt (Cell Signaling Technology, Boston, MA, USA), Akt (Cell Signaling Technology), NFATc1 (Invitrogen), or β-actin (Abcam) and horseradish peroxidase–conjugated secondary antibody (Abcam). Thereafter, the bands were developed using the enhanced chemiluminescence system (Cell Signaling Technology). The membranes were scanned and quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Cell Transfection

A total of 5×10^5 BMMs were seeded into a six-well plate and incubated until the cells reached 80% to 90% confluence. The cells were transfected with the appropriate construct (pcDNA3.1-NFATc1 or pcDNA3.1) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Statistical Analysis

All data in the present study were analyzed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA) and expressed as the mean ± standard error of the mean. The results are representative of three independent experiments. One-way analysis of variance followed by Dunnett's multiple comparison test was used to perform for the comparison of differences among multiple groups. In all cases, $P < 0.05$ suggested the significant differences.

Results

Effect of Isofraxidin on Cell Viability

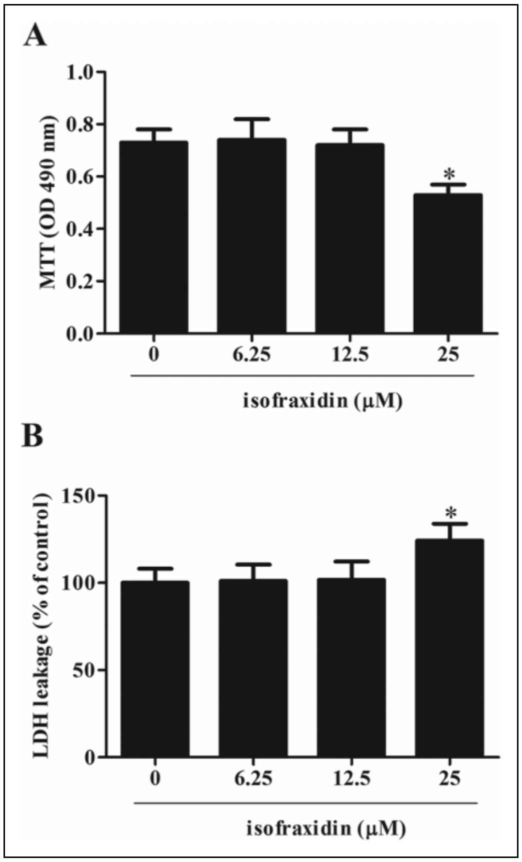

Figure 1. Evaluation of the cytotoxicity effect of isofraxidin on BMMs. BMMs were incubated with various concentrations (0, 6.25, 12.5, and 25 μM) of isofraxidin for 24 h. (A) MTT assay was performed to evaluate cell viability. (B) LDH release assay was carried out to assess the LDH content. $^{*}P < 0.05$ versus control BMMs. BMM: bone marrow macrophage; LDH: lactate dehydrogenase; MTT: 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium.

BMMs were incubated with various concentrations (0, 6.25, 12.5, and 25 μM) of isofraxidin followed by MTT and LDH release assays. The results revealed that treatment with 25 μM isofraxidin caused the decrease in cell viability; however, isofraxidin at concentrations of 6.25 and 12.5 μM did not affect the cell viability of BMMs (Fig. 1A). Besides, the LDH contents in the isofraxidin (6.25 and 12.5 μM)-treated BMMs exhibited no significant difference with control BMMs (Fig. 1B). Thus, the dosages of 6.25 and 12.5 μM were chosen for further experiments.

Isofraxidin Inhibits Osteoclast Formation in BMMs

In order to evaluate the effects of isofraxidin on RANKL-induced osteoclast formation in BMMs, the BMMs were treated with various doses of isofraxidin in the presence of RANKL (100 ng/ml). TRAP activity was determined to evaluate the osteoclast formation of BMMs. The results illustrated that the TRAP activity was significantly increased in RANKL-induced BMMs compared with control group. However, the increased TRAP activity was inhibited by isofraxidin treatment (Fig. 2).

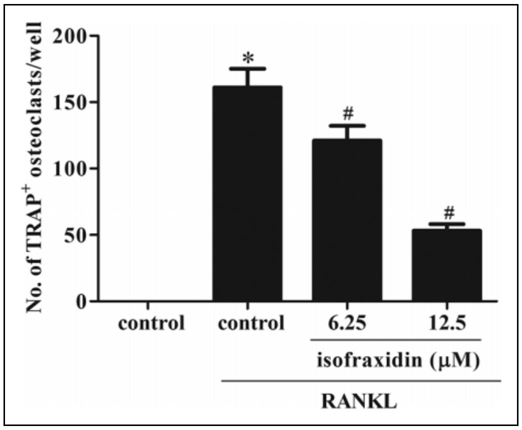

Figure 2. Isofraxidin inhibits RANKL-induced osteoclast formation in BMMs. BMMs were treated with various doses of isofraxidin (0, 6.25, and 12.5 μM) in the presence or absence of RANKL (100 ng/ml). TRAP activity was determined to evaluate the osteoclast formation of BMMs. *$P < 0.05$ versus control BMMs. $^{\#}P < 0.05$ versus RANKL-induced BMMs. BMM: bone marrow macrophage; RANKL: receptor activator of nuclear factor-κB ligand.

Isofraxidin Inhibits Osteoclastogenesis-related Markers Expression in BMMs

To further explore the role of isofraxidin in osteoclast differentiation, we analyzed the mRNA and protein levels of osteoclastogenesis-related markers including MMP-9, c-Src, and cathepsin K. The mRNA levels of MMP-9, c-Src, and cathepsin K were markedly increased in RANKL-induced BMMs, while isofraxidin exhibited inhibitory effects on the mRNA levels of MMP-9, c-Src, and cathepsin K (Fig. 3A). Consistently, the RANKL-induced protein levels of MMP-9, c-Src, and cathepsin K were suppressed by isofraxidin (Fig. 3B–E).

Isofraxidin Inhibits RANKL-induced NFATc1 Activation in BMMs

To explore the NFATc1 activation in the effect of isofraxidin on RANKL-induced BMMs, luciferase reporter assay was performed. As shown in Fig. 4A, isofraxidin inhibited the RANKL-induced NFATc1 activation in BMMs. In addition, the expression level of NFATc1 was determined using

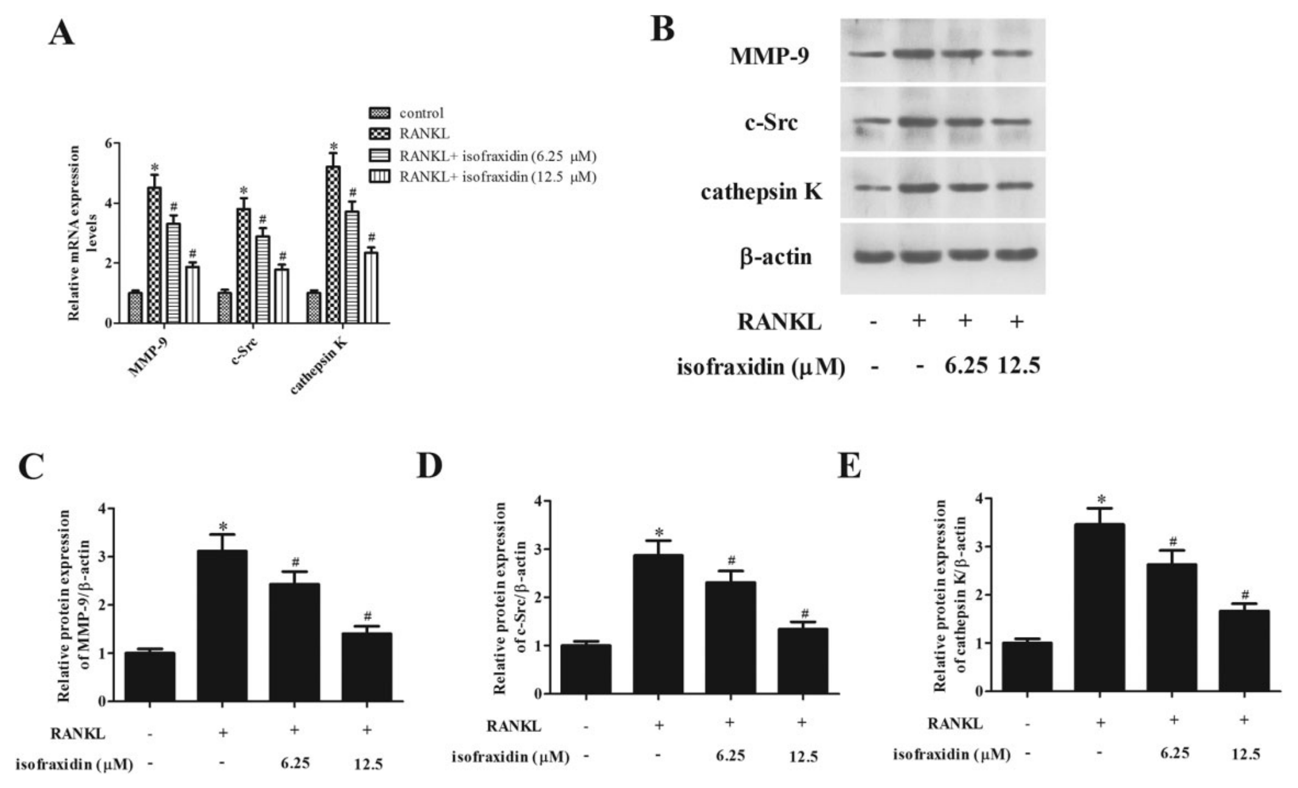

Figure 3. Isofraxidin inhibits the expressions of osteoclastogenesis-related markers in RANKL-induced BMMs. BMMs were treated with various doses of isofraxidin (0, 6.25, and 12.5 μM) in the presence or absence of RANKL (100 ng/ml). (A) The mRNA levels of MMP-9, c-Src, and cathepsin K were determined by qRT-PCR. (B) The protein levels of MMP-9, c-Src, and cathepsin K were determined by western blot analysis. (C–E) Quantification analysis of MMP-9, c-Src, and cathepsin K. *$P < 0.05$ versus control BMMs. $^{\#}P < 0.05$ versus RANKL-induced BMMs. BMM: bone marrow macrophage; qRT-PCR: quantitative real-time polymerase chain reaction; RANKL: receptor activator of nuclear factor-κB ligand.

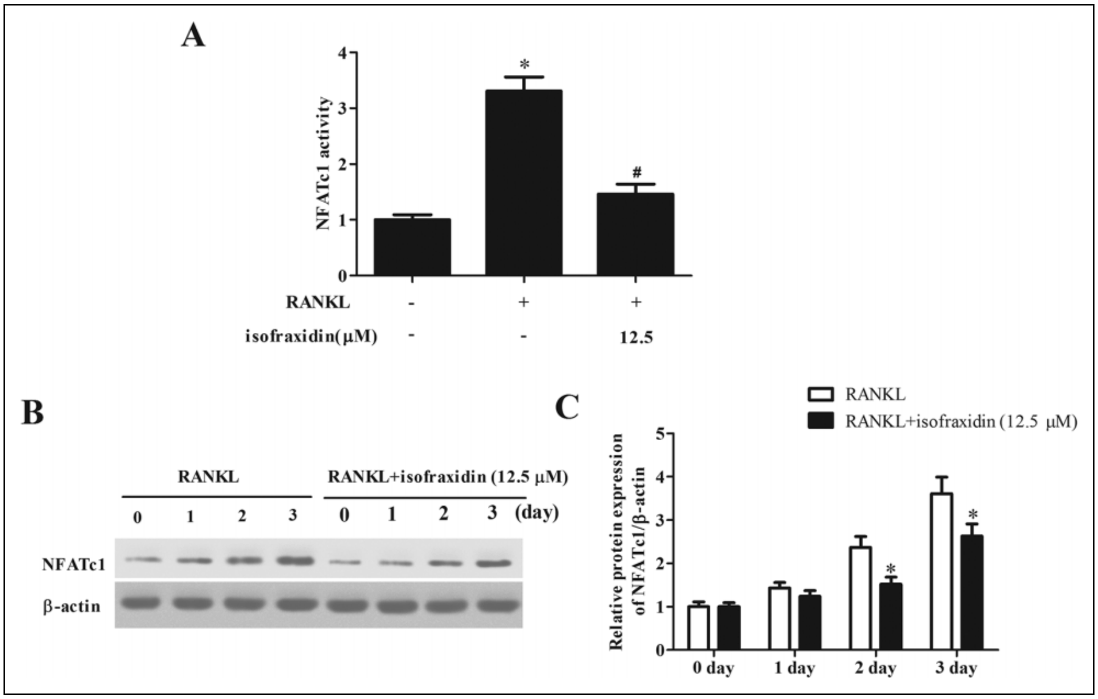

Figure 4. Isofraxidin inhibits RANKL-induced NFATc1 activation in BMMs. BMMs were treated with isofraxidin (12.5 μM) in the presence or absence of RANKL (100 ng/ml). (A) Luciferase reporter assay was performed to explore the NFATc1 activation. (B) The protein level of NFATc1 was determined by western blot analysis. (C) Quantification analysis of NFATc1. $^{*}P < 0.05$ versus control BMMs. $^{\#}P < 0.05$ versus RANKL-induced BMMs. BMM: bone marrow macrophage; NFATc1: nuclear factor of activated T-cells cytoplasmic 1; RANKL: receptor activator of nuclear factor-κB ligand.

western blot. The results showed that the protein level of NFATc1 was increased by the treatment with RANKL. However, treatment with isofraxidin significantly suppressed the NFATc1 expression at protein level (Fig. 4B, C).

Isofraxidin Inhibits RANKL-induced Activation of NF-κB Pathway in BMMs

To further explore the molecular mechanisms underlying the inhibitory effects of isofraxidin on RANKL-induced osteoclast formation, we examined the effects of isofraxidin on NF-κB pathway activation in RANKL-induced BMMs. The results of western blot analysis showed that decreased expression levels of IκBα and increased level of p-IκBα and p-p65 were found in RANKL-induced BMMs. However, treatment with isofraxidin inhibited the RANKL-induced activation of NF-κB pathway in BMMs (Fig. 5A–D).

Isofraxidin Inhibits RANKL-induced Activation of Akt Pathway in BMMs

It has been previously reported that the PI3K/Akt signaling pathway enhances NFATc1 expression and regulate osteoclast differentiation[15]. We next elucidated the role of Akt pathway in the effect of isofraxidin on RANKL-induced osteoclast differentiation. RANKL caused significant increase in the expression level of p-Akt, which was attenuated by isofraxidin. The results implied that isofraxidin inhibited the activation of Akt pathway in RANKL-induced BMMs (Fig. 6).

NFATc1 Overexpression Rescued the Anti-osteoclastogenic Effect of Isofraxidin

Subsequently, the NFATc1-overexpressing BMMs was established to further confirm the role of NFATc1 in osteoclastogenesis. Results from qRT-PCR and western blot showed that the mRNA and protein levels of NFATc1 were increased in pcDNA3.1-NFATc1 transfected BMMs (Fig. 7A, B). Moreover, we observed that the inhibitory effects of isofraxidin on mRNA and protein levels of MMP-9, c-Src, and cathepsin K were attenuated by NFATc1 overexpression, suggesting that NFATc1 rescued the anti-osteoclastogenic effect of isofraxidin (Fig. 7C–F).

Discussion

Osteoporosis is caused by the imbalance between bone formation of osteoblasts and bone resorption of osteoclasts[16]. Osteoclasts are bone-resorbing cells that are derived from hematopoietic precursor cells and require macrophage-colony stimulating factor and RANKL for their survival, proliferation, differentiation, and activation[17]. The binding of RANKL to its receptor RANK triggers osteoclast

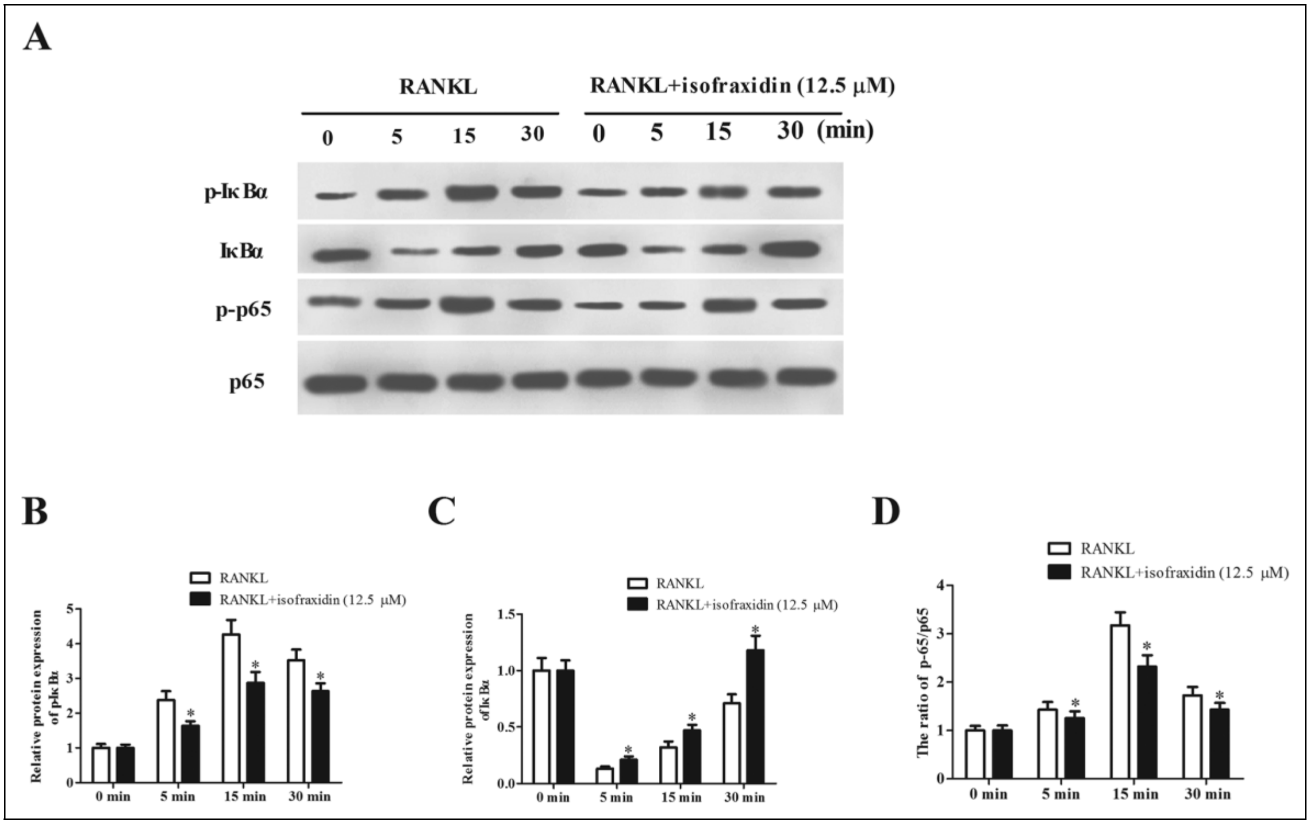

Figure 5. Isofraxidin inhibits RANKL-induced NF-κB activation in BMMs. BMMs were treated with isofraxidin (12.5 μM) in the presence or absence of RANKL (100 ng/ml) for different times. (A) The protein levels of p-IκBα, IκBα, p-p65, and p65 were determined by western blot analysis. (B–D) Quantification analysis of p-IκBα, IκBα, and p-p65. *$P < 0.05$ versus control BMMs. #$P < 0.05$ versus RANKL-induced BMMs. BMM: bone marrow macrophage; RANKL: receptor activator of nuclear factor-κB ligand.

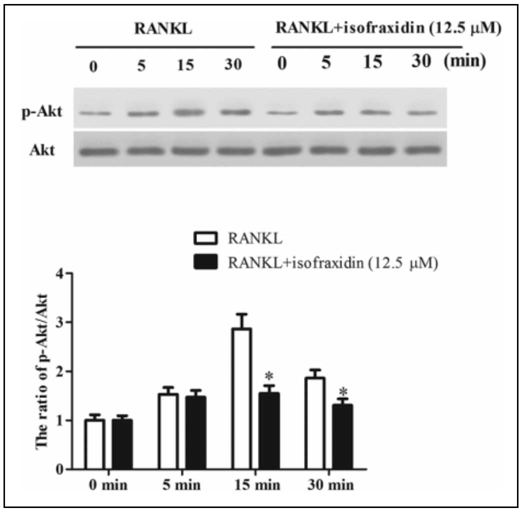

Figure 6. Isofraxidin inhibits RANKL-induced Akt activation in BMMs. BMMs were treated with isofraxidin (12.5 μM) in the presence or absence of RANKL (100 ng/ml) for different times. The protein levels of p-Akt and Akt were determined by western blot analysis. *$P < 0.05$ versus control BMMs. #$P < 0.05$ versus RANKL-induced BMMs. BMM: bone marrow macrophage; RANKL: receptor activator of nuclear factor-κB ligand.

precursors to differentiate into osteoclasts, which is crucial for the development of osteoporosis[17]. Targeting RANKL signaling pathway appears to be an efficient and relevant approach for identifying potential new drug for preventing osteoporosis and other bone-related diseases[18,19]. In the current study, we found that RANKL caused significant increase in TRAP activity, as well as the expression levels of osteoclastogenesis-related markers including MMP-9, c-Src, and cathepsin K, indicating that RANKL induced the osteoclast formation in BMMs. However, treatment with isofraxidin inhibited RANKL-induced osteoclast formation in BMMs.

NFATc1 is a broadly expressed member of NFATc family, which has five members (NFATc1 through NFATc5)[20]. It has been demonstrated that NFATc1 plays a pivotal role in osteoclast activation via upregulation of various genes in a series processes, such as osteoclast adhesion, migration, acidification, and degradation of inorganic and organic bone matrix[21]. Moreover, NFATc1 regulates many osteoclast-specific genes, such as cathepsin K, TRAP, calcitonin receptor, and osteoclast-associated receptor, in cooperation with other transcription factors[22]. NFATc1 acts as a therapeutic target for the treatment of osteoporosis. Therefore, we

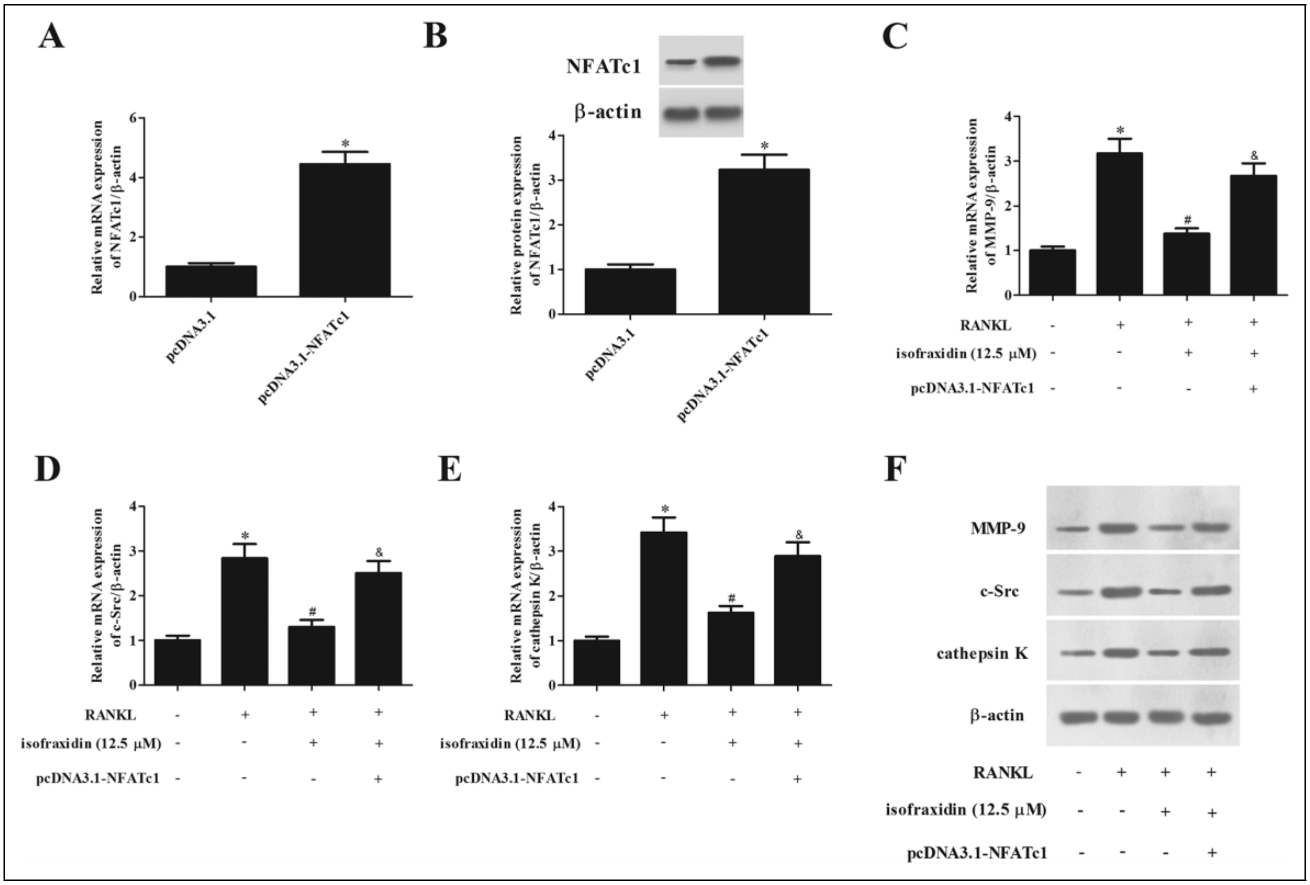

Figure 7. NFATc1 overexpression rescued the anti-osteoclastogenic effect of isofraxidin. (A and B) BMMs were transfected with pcDNA3.1-NFATc1 or pcDNA3.1, followed by the qRT-PCR and western blot analysis. Transfected BMMs were cultured with RANKL (100 ng/ml) in the presence of isofraxidin (12.5 μM). (C–E) The mRNA levels of MMP-9, c-Src, and cathepsin K were determined by qRT-PCR. (F) The protein expression levels of MMP-9, c-Src, and cathepsin K were detected by western blot. $^{*}P < 0.05$ versus control BMMs. $^{\#}P < 0.05$ versus RANKL-induced BMMs. $^{\&}P < 0.05$ versus RANKL + isofraxidin. BMM: bone marrow macrophage; NFATc1: nuclear factor of activated T-cells cytoplasmic 1; qRT-PCR: quantitative real-time polymerase chain reaction; RANKL: receptor activator of nuclear factor-κB ligand.

evaluated the effect of isofraxidin on NFATc1 activation. The results showed that isofraxidin prevented the RANKL-induced NFATc1 activation in BMMs, as evidenced by decreased NFATc1 transcriptional activity and NFATc1 expression. Moreover, overexpression of NFATc1 rescued the anti-osteoclastogenic effect of isofraxidin.

The RANKL/RANK transduces the activation of various downstream signaling pathways including NF-κB, JNK, p38 MAPK, extracellular signal-related kinase, and Akt to induce the expression of osteoclastogenesis-related genes such as c-Fos, TRAP, NFATc1, and osteoclast-associated receptor[23]. NF-κB is a crucial transcription factor that controls the expression of numerous genes involved in cell proliferation, apoptosis, and inflammation[24,25]. NF-κB also plays an important role in RANKL-induced osteoclastogenesis[26,27]. Stimulation of RANKL leads to the activation of the kinase of IκB and IκBα phosphorylation. Subsequently, the dissociative p65 subunit of NF-κB translocates to the nucleus and then initiates the transcription of target genes including NFATc1[26]. Previous studies have proven that isofraxidin has the ability to regulate the NF-κB signaling pathway. Isofraxidin suppresses IL-1β-induced IκBα degradation and NF-κB activation in human osteoarthritis chondrocytes[10]. Isofraxidin may have a therapeutic effect against LPS-induced inflammatory disease through regulation of NF-κB signal[28]. Isofraxidin alleviates IL-1β-induced inflammation in human nucleus pulposus cells via inhibiting the NF-κB activation[29]. Our results proved that expression level of IκBα was decreased, while expression level of p-IκBα and p-p65 was increased in RANKL-induced BMMs. The RANKL-induced changes in the expression levels of IκBα and p-p65 were attenuated by isofraxidin, indicating that RANKL-induced the activation of NF-κB in BMMs was prevented by isofraxidin.

In addition to NF-κB, PI3K/Akt is an important signaling that mediates various cellular functions, including mitogenesis, survival, motility, and differentiation[30,31]. It has been evidenced that Akt induces osteoclasts survival and differentiation

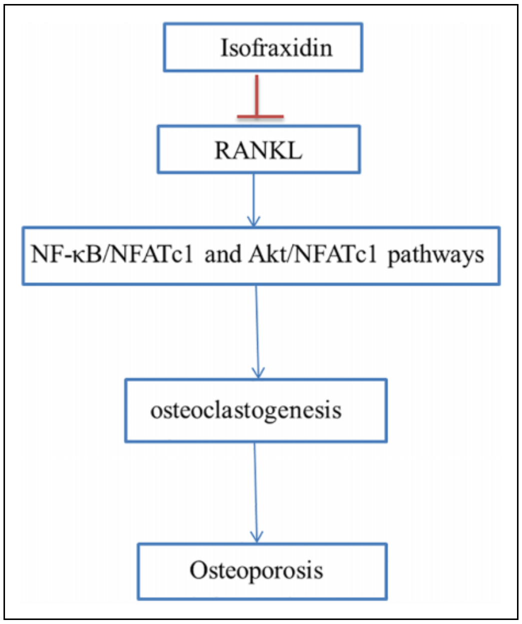

Figure 8. A brief schematic diagram of isofraxidin inhibits osteoclastogenesis in BMMs by regulating NF-κB/NFATc1 and Akt/NFATc1 signaling pathways. BMM: bone marrow macrophage; NFATc1: nuclear factor of activated T-cells cytoplasmic 1.

through regulating the GSK-3β/NFATc1 signaling cascade. Inhibition of the PI3K/Akt by LY294002 reduces the formation of osteoclasts and attenuates the expression of NFATc1, suggesting that PI3K/Akt/NFATc1 signaling axis is necessary for RANKL-induced osteoclastogenesis[32]. Isofraxidin was found to exhibit inhibitory effect on the activation of Akt signaling. For instance, isofraxidin inhibits proliferation and induces apoptosis of human colorectal cancer cells via blockage of Akt pathway[9]. Isofraxidin ameliorates influenza A virus–induced severe lung damage and lethal infection via regulating PI3K/Akt and MAPK pathways[33]. Isofraxidin inhibits the PI3K/Akt signaling pathway by reducing the expression level of p-Akt to hinder the development of lung cancer cells[34]. Our results proved that RANKL induced the activation of Akt in BMMs, which was prevented by isofraxidin. These findings illustrated that the anti-osteoclastogenic effect of isofraxidin might be mediated by the NF-κB/NFATc1 and Akt/NFATc1 signaling pathways (Fig. 8).

In this study, we demonstrated that isofraxidin inhibited RANKL-induced osteoclast formation in BMMs. The anti-osteoclastogenic effect of isofraxidin might be mediated by the NF-κB/NFATc1 and Akt/NFATc1 signaling pathways. Thus, isofraxidin might be a therapeutic agent for the treatment of osteoporosis.

Ethical Approval

This study was approved by our institutional review board.

Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology of Ethics Committee's (Approval Number: 200143) approved protocols.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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