Amiloride inhibits osteoclastogenesis by suppressing nuclear factor-κB and mitogen-activated protein kinase activity in receptor activator of nuclear factor-κB-induced RAW264.7 cells

XIANGDONG WANG[1*], YUANLI ZHU[2*], SHOUCHAO ZHENG[3], CHAOCHAO NI[4], LIBO ZHAO[1], CHANGYU LIU[5], ANMIN CHEN[1] and JUN XIAO[1]

Departments of [1]Orthopedics and [2]Pathology, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, Hubei 430030; [3]Department of Spine and Joints, Cangzhou Hospital of Integrated Traditional Chinese and Western Medicine, Hebei Medical University, Cangzhou, Hebei 061001; [4]Department of Endocrinology, Tongji Hospital; [5]Department of Orthopedics, Liyuan Hospital, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, Hubei 430030, P.R. China

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Abstract. Amiloride is widely used in clinical practice as a diuretic and is known to interact with the epithelial sodium channel and acid-sensing ion channel proteins, as well as Na^+/H^+ antiporters and Na^+/Ca^{2+} exchangers. The aim of the present study was to examine the effects of amiloride on receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis and to elucidate the underlying mechanisms in the RAW264.7 murine macrophage cell line. The number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells were counted and the bone resorption area was estimated. In addition the expression levels of nuclear factor of activated T cells, cytoplasmic 1 (*NFATc1)* mRNA and osteoclast-specific genes, including TRAP, matrix metalloproteinase 9, cathepsin K and osteoclast-associated receptor, were examined using reverse transcription-quantitative polymerase chain reaction. The nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways were also investigated using western blotting. The results showed that amiloride significantly reduced the number of TRAP-positive multinucleated cells as well as the bone resorption area. Amiloride also downregulated the expression of *NFATc1* mRNA and inhibited the expression of osteoclast-specific genes. A possible underlying mechanism may be that amiloride suppresses the degradation of the inhibitor of NF-κB and blocks the activation of c-Jun N-terminal kinase, extracellular signal-regulated kinase and p38, thus implicating the NF-κB and MAPK pathway is this process. In conclusion, the current data suggest that amiloride is a strong inhibitor of osteoclast differentiation, indicating a novel indication for amiloride in the treatment of bone-loss-related diseases.

Correspondence to: Mr. Jun Xiao or Mr. Anmin Chen, Department of Orthopedics, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan, Hubei 430030, P.R. China
E-mail: xiaojun301@sina.com
E-mail: amchen@tjh.tjmu.edu.cn

*Contributed equally

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Introduction

Osteoclasts are large multinucleated cells that are derived from a monocyte-macrophage lineage and have been shown to possess a number of essential physiological functions for development, in particular, in the dynamic remodeling of bone (1). Excessive bone resorption by osteoclasts is observed in certain lytic bone diseases, including osteoporosis, hypercalcemia and rheumatoid arthritis, as well as tumor metastases in the bone, periodontitis and Paget's disease (2,3). Patients with lytic bone diseases are at a higher risk of sustaining fractures. Therefore, lytic bone diseases are an increasingly serious social and economic issue, due to the high medical costs associated with hospitalization (4). Therefore, inhibiting osteoclast formation may represent a treatment option for disease involving excessive bone resorption (5).

Receptor activator nuclear factor-κB ligand (RANKL) is the primary cytokine responsible for the differentiation of osteoclast precursors into osteoclasts *in vitro* and *in vivo* (6). The binding of RANKL to its receptor, RANK, leads to activation of components of the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 (7). There is accumulating evidence that nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), which acts as the principal regulator of osteoclastogenesis, is upregulated by RANKL in osteoclast precursors

through mechanisms that depend on NF-κB and MAPK (8). NFATc1 is known to directly regulate a number of osteoclast-related marker genes, including tartrate-resistant acid phosphatase (TRAP), matrix metalloproteinase 9 (MMP9), cathepsin K (CTSK) and osteoclast-associated receptor (OSCAR) (8-10). Therefore, suppression of RANKL signaling may be beneficial in the treatment of osteoclast-related diseases (5).

Amiloride is a small molecule diuretic, which was discovered in 1965 and is used for the long-term treatment of hypertension in combination with other diuretics (11,12). This drug is known to interact with the epithelial sodium channel (ENaC) and acid-sensing ion channel proteins (ASICs), as well as Na^+/H^+ antiporters and Na^+/Ca^{2+} exchangers (13-15). Previous studies have shown that isolated human monocytes express ASIC1, 2, and 3, and that this expression persists following induction of osteoclast differentiation (16). Additionally, ASIC1a is involved in the induction of osteoclastic genes, known to be direct transcriptional targets of NFATc1, during osteoclastogenesis (17). The Na^+/H^+ antiporter may also be involved in the recovery of intracellular pH during osteoclast activation (18). Furthermore, Na^+/Ca^{2+} exchangers, which are expressed in osteoclasts, are important in Ca^{2+} transportation and regulation during bone resorption (19). Therefore, since amiloride inhibits these channels, it was hypothesized that amiloride may also suppress osteoclast differentiation. The present study examined the effects of amiloride on RANKL-induced osteoclastogenesis in a murine RAW264.7 macrophage cell line, which is a classic model of osteoclast precursors that differentiates into TRAP-positive multinuclear osteoclasts following treatment with RANKL (20).

Materials and methods

Cell culture. The RAW264.7 murine macrophage cell line was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Gibco Life Technologies) was used for the routine subculture of RAW 264.7 cells. Cells were maintained in a humidified incubator (Shanghai Laboratory Instrument Works Co., Ltd, Shanghai, China) with 95% air/5% CO_2 at 37˚C and were subcultured every two days.

Proliferation assays. Cell viability was assessed using a Cell Counting kit-8 (CCK-8, Boster Biological Technology Ltd., Wuhan, China). RAW264.7 cells were seeded at 3,000 cells per well in 96-well plates. Following incubation for 24 h, cells were treated with 0, 10, 50 or 100 μM amiloride (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich). Medium (DMEM supplemented with FBS and 1% penicillin-streptomycin) with fresh amiloride was exchanged daily. After 24, 72 or 120 h, 100 μl of medium from each well (containing 10% CCK-8) was added and incubated in darkness at 37˚C for 2 h. The plate was then read using a spectrophotometer (ELx808 Absorbance Microplate Reader, BioTek Instruments, Inc. Winooski, VT, USA) at 450 nm. The number of surviving cells was quantified by measuring the absorbance at this wavelength.

TRAP staining. RAW264.7 cells were treated with RANKL (50 ng/ml, Peprotech, Rocky Hill, NJ, USA) and different concentrations of amiloride, and media were refreshed daily over the course of five days. Cells were washed twice with phosphate-buffered saline (Boster Biological Technology, Ltd), fixed with 4% formaldehyde (Boster Biological Technology, Ltd) for 15 min and then subjected to TRAP staining (Sigma-Aldrich) for 60 min, according to the manufacturer's instructions. Cells were considered osteoclasts if they were stained dark red and contained ≥3 nuclei when viewed under a light microscope (magnification, x40; Nikon TE2000-S, Nikon Corporation, Tokyo, Japan). For each sample three fields of vision were examined.

Pit-formation assays. The bone resorption function of osteoclasts derived from RAW264.7 cells induced by RANKL was analyzed using Osteo Assay plates (Corning Inc., Corning, NY, USA). Briefly, RAW264.7 cells (6,000 cells/well) were plated on Osteo Assay plates and treated with amiloride (0, 10, 50 or 100 μM) in the presence RANKL (50 ng/ml) for 7 days. Subsequently, the cells were removed completely. Relative resorption area per well was observed under a light microscope (magnification, x40; Nikon TE2000-S, Nikon Corporation, Tokyo, Japan) and measured by ImageJ 1.47 software (National Institutes of Health, Bethesda, MD, USA). For each sample three fields of vision were examined.

Reverse transcription-quantitative polymerase chain reaction. Total RNA was extracted from RAW264.7 cells using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and integrity of the extracted RNA were analyzed by measurement of the $OD_{260/280}$ (Eppendorf 22331, Eppendorf, Hamburg, Germany). The purified RNA was converted into cDNA using a First Strand cDNA Synthesis kit (TransGen Biotech Co., Ltd., Beijing, China). The resulting cDNAs were subjected to qPCR. The total volume of the reaction was 20 μl, which consisted of 10 μl Top Green qPCR SuperMix (TransGen Biotech Co., Ltd.), 8 μl RNase-free water, 1 μl primer solution and 1 μl cDNA. qPCR was performed on a Bio-Rad Q5 instrument (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: Denaturation at 94˚C for 30 sec, followed by 40 cycles of denaturation at 94˚C for 5 sec and annealing at 60˚C for 30 sec. The primers used in the amplification were as follows: Forward:5'-ATTTCT GAATGGCCCAGGT-3' and reverse: 5'-CTGCCTCAACAC CTCAACC-3' for β-actin, forward: 5'-GATGCCAGCGAC AAGAGGTT-3' and reverse: 5'-CATACCAGGGGATGT TGCGAA-3' for TRAP, forward: 5'-GAAGAAGACTCA CCAGAAGCAG-3' and reverse: 5'-TCCAGGTTATGGGCA GAGATT-3' for CTSK, forward: 5'-CTGGACAGCCAGACA CTAAAG-3' and reverse: 5'-CTCGCGGCAAGTCTTCAG AG-3' for MMP-9, forward: 5'-CAGGAGAGGCATTATGAG CA-3' and reverse: 5'-GGTACTTTCCTGGTTCGCAT-3' for RANK, forward: 5'-CTGCTGGTAACGGATCAGCTCCCC AGA-3' and reverse: 5'-CCAAGGAGCCAGAACCTTCGA

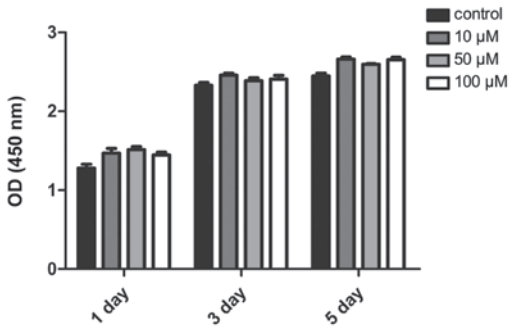

Figure 1. The cytotoxicity of amiloride in RAW264.7 cells. RAW264.7 cells were treated with amiloride (0, 10, 50 or 100 μM) for 1, 3 or 5 days. Cytotoxicity was determined by a CCK-8 assays. Data are expressed as the mean ±standard deviation of three independent experiments. *P<0.05 vs. control, $^{\#}$P<0.05 vs. 10 μM amiloride and $^{\Delta}$P<0.05 vs. 50 μM amiloride. OD, optical density, CCK-8, Cell Counting kit -8.

AACT-3' for OSCAR and forward: 5'-GGTAACTCTGTC TTTCTAACCTTAAGCTC-3' and reverse: 5'-GTGATGACC CCAGCATGCACCAGTCACAG-3' for NFATc1. β-Actin was included as housekeeping gene. The comparative $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of each gene.

Western blotting. Serum-starved RAW264.7 cells were pretreated with 100 μM amiloride for 12 h and then stimulated with RANKL (50 ng/ml) for the appropriate times (0, 5, 15, 30 and 60 min). Subsequently, cells were lysed in a radioimmunoprecipitation assay lysis buffer containing inhibitors of protease and a phosphorylase (Protease Inhibitor Cocktail and Phosphatase Inhibitor cocktail; Boster Biological Technology Ltd.). The lysates were centrifuged at 14,000 x g for 20 min, and the supernatants were collected. Protein concentrations of the supernatants were determined using a bicinchoninic acid Protein Assay kit (Boster Biological Technology Ltd.), standardized with bovine serum albumin (Gibco Life Technologies). Cellular proteins (30 μg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Nonspecific interactions were blocked with 5% bovine serum albumin for 2 h and probed with the following primary antibodies: Rabbit polyclonal anti-α-Tubulin (1:1,000; #2148), rabbit monoclonal anti-inhibitor of κB (IκB; 1:1,000; #4812), rabbit monoclonal anti-JNK (1:1,000; #9258), rabbit monoclonal anti-phosphorylated (p-) JNK (1:1,000; #4671), rabbit monoclonal anti-ERK (1:1,000; #4348), rabbit monoclonal anti-p-ERK (1:1,000; #4094), rabbit monoclonal anti-p38 (1:1,000; #14451) and rabbit monoclonal anti-p-p38 (1:1,000; #4511), which were all purchased from Cell Signaling Technology (Danvers, MA, USA). Membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, 1:200, BA1003; Boster Biological Technology Ltd.) and immunoreactivity was detected using enhanced chemiluminescence reagents (Pierce Biotechnology, Inc., Rockford, IL, USA).

Statistical analyses. Statistically significant differences between groups were determined by one-way analysis of variance using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation of ≥3 independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of amiloride on cell viability. In order to determine whether the effects of amiloride on osteoclastogenesis were due to the potential toxicity of this drug, the cytotoxicity of amiloride was examined using a CCK-8 assay in RAW264.7 cells. Cytotoxicity was very low in cells treated with all concentrations of amiloride (10, 50 or 100 μM) for 1, 3 or 5 days (Fig. 1), suggesting that the effects of amiloride on osteoclast differentiation are not mediated by cytotoxicity of this compound.

Effects of amiloride on osteoclast differentiation in RANKL-induced RAW264.7 cells. In order to examine the effects of amiloride on osteoclast differentiation, the formation of osteoclast-like cells induced by RANKL (50 ng/ml) stimulation in RAW264.7 cells was examined in the presence of various concentrations of amiloride by counting the number of TRAP-positive multinucleated cells. The following doses of amiloride were selected for subsequent experiments: 0, 10, 50 and 100 μM. As shown in Fig. 2, TRAP-positive osteoclasts with multiple nuclei were formed within 5 days in response to RANKL stimulation, and this response was inhibited by amiloride in a concentration-dependent manner.

Effects of amiloride on bone resorption in RANKL-induced RAW264.7 cells. Osteo Assay plates were used to determine whether amiloride treatment affected the bone resorption function of osteoclasts. RAW264.7 cells were plated onto bone slices and stimulated with RANKL (50 ng/ml) for 7 days in the presence of amiloride (0, 10, 50 or 100 μM). As shown in Fig. 3, numerous resorption pits were formed on the bone slices. Amiloride decreased the area of the bone resorption pits in a concentration-dependent manner.

Effects of amiloride on the expression of osteoclast differentiation marker genes in RANKL-induced RAW264.7 cells. Changes in the expression of osteoclast differentiation genes in response to amiloride treatment were examined using RT-qPCR analysis of osteoclasts. The expression of osteoclast differentiation markers, including TRAP, MMP9, CTSK and OSCAR, have previously been shown to be detectable following the formation of mature osteoclasts (8-10). As shown in Fig. 4, the expression of these genes decreased in response to amiloride treatment, in a concentration-dependent manner. The expression of the *NFATc1* gene, which acts as the master switch in osteoclastogenesis and regulates the expression of osteoclastogenic genes (8), was downregulated by amiloride in a concentration-dependent manner. A downregulation of 80% was observed following treatment with 100 μM amiloride.

Effects of amiloride on the phosphorylation of NF-κB and MAPK family proteins in RANKL-induced RAW264.7 cells. Activation of the NF-κB and MAPK is important in osteoclastogenesis (21). In order to evaluate the effects of 12 h amiloride treatment on these signaling pathways, following RANKL

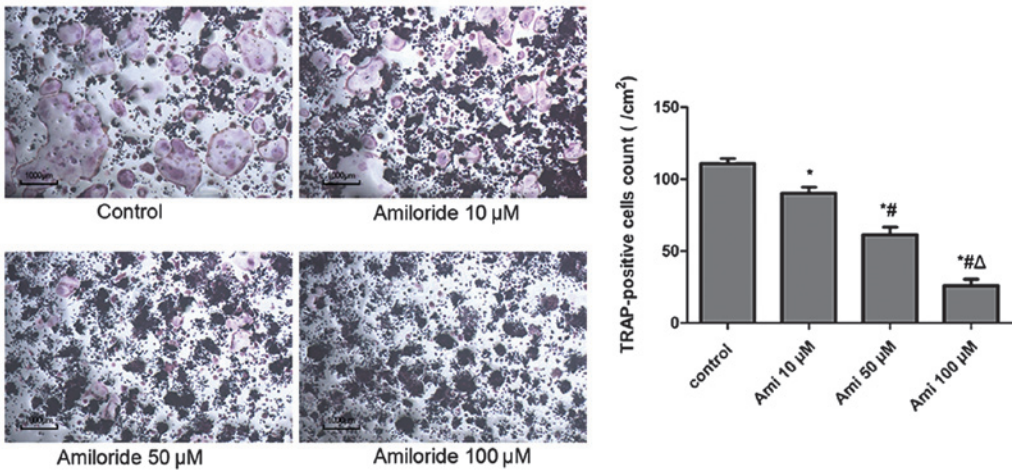

Figure 2. Amiloride inhibited the formation of RANKL-stimulated TRAP-positive multinucleated RAW264.7 cells in a concentration-dependent manner. RAW264.7 cells were cultured with RANKL (50 ng/ml) and varying concentrations of amiloride for five days. The number of TRAP-positive multinucleated cells was counted in each well (x100, scale bar=1,000 μm). Data are expressed as the mean ±standard deviation of three independent experiments with triplicate cultures. *P<0.05 vs. control, $^{\#}$P<0.05 vs. 10 μM amiloride and $^{\Delta}$P<0.05 vs. 50 μM amiloride. RANKL, receptor activator of nuclear factor-κB; TRAP, tartrate-resistant acid phosphatase.

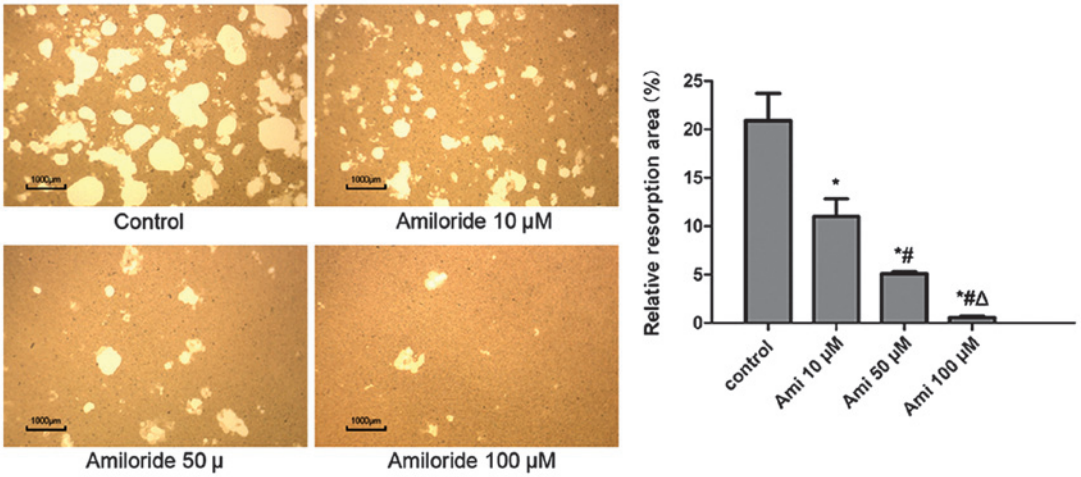

Figure 3. Amiloride inhibited RANKL-induced bone resorption in RAW264.7 cells. RAW264.7 cells were cultured on Osteo Assay plates and stimulated with 50 ng/ml RANKL in the presence different concentrations of amiloride. Following seven days of culture, the relative resorption area per well was measured using an image analyzer and was observed under a microscope (x100, scale bar=1000 μm). Data are expressed as the mean ±standard deviation of three independent experiments. *P<0.05 vs. control, $^{\#}$P<0.05 vs. 10 μM amiloride and $^{\Delta}$P<0.05 vs. 50 μM amiloride. RANKL, receptor activator of nuclear factor-κB; Ami, amiloride.

stimulation in RAW264.7 cells, the phosphorylation of p38, JNK and ERK, and the degradation of IκB was evaluated using western blot analysis. The results demonstrated that amiloride significantly inhibited the RANKL-induced activation of JNK, ERK and p38, and the degradation of IκB (Fig. 5). These results indicate that amiloride inhibits RANKL-induced activation of the NF-κB and MAPK pathways in osteoclasts.

Discussion

In the present study, the effects of amiloride on RANKL-induced osteoclast differentiation and its function in murine RAW264.7 cells were investigated, and the signaling mechanisms associated with this process were examined. The results demonstrated that amiloride markedly suppressed RANKL-induced osteoclast differentiation and resorption, and that these effects were not due to the cytotoxicity of the drug. It was also shown that amiloride inhibited the expression of *NFATc1* and other osteoclast-related genes through RANKL-induced inhibition of the NF-κB and MAPK signaling pathways.

Activation of the NF-κB pathway is essential for RANKL-induced osteoclast differentiation (22). NF-κB is localized in the cytoplasm, in association with a number of inhibitory IκB proteins, including IκBα, IκBβ and IκBε, of which IκBα is the most abundant (23). The results of the present indicated that amiloride inhibits the degradation of IκBα, suggesting that suppression of the NF-κB pathway is a mechanism underlying the anti-osteoclastogenic effect of amiloride.

Three major MAPKs have been identified in mammalian cells: JNK, ERK and p38. These proteins are activated by RANKL stimulation and have been shown to be involved in osteoclastogenesis (21,24). Moreover, inhibitors of JNK, ERK and p38 have been shown to inhibit RANKL-induced osteoclastogenesis (24-26). In the current study, it was shown

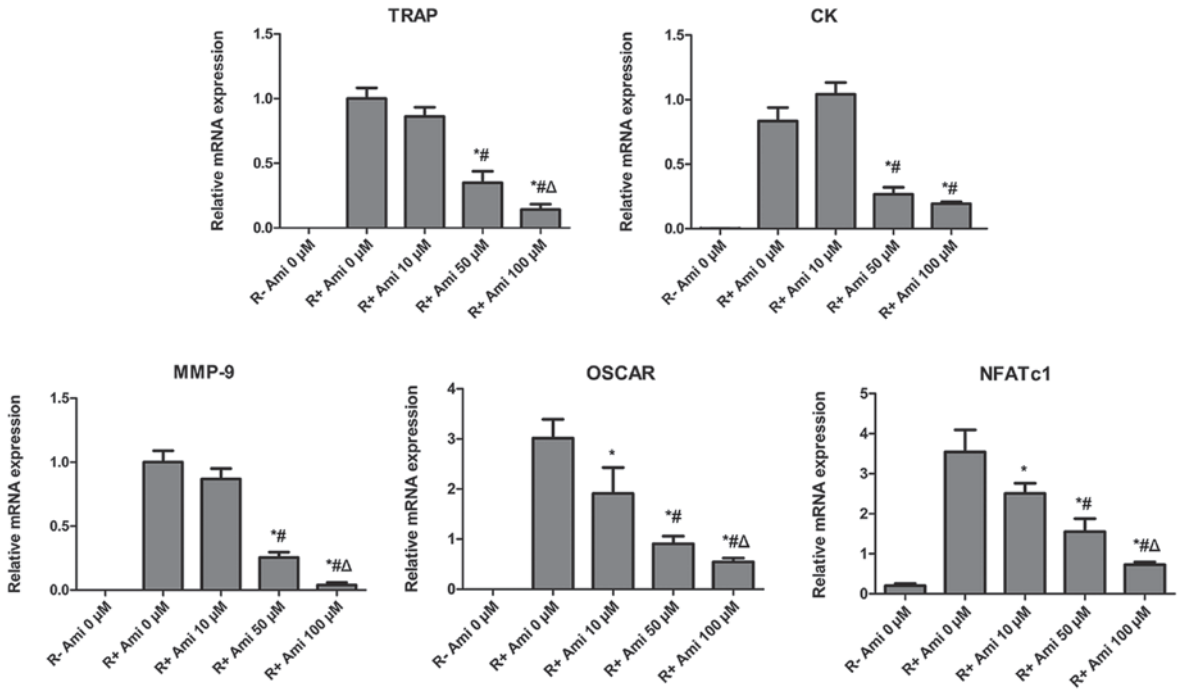

Figure 4. Amiloride downregulated osteoclast-specific gene expression in RANKL-induced RAW264.7 cells. RAW264.7 cells were cultured with RANKL in the presence or absence of amiloride for five days. The relative mRNA expression levels of *TRAP*, *CTSK*, *MMP9*, *OSCAR*, and *NFATc1* genes were determined by quantitative polymerase chain reaction, following normalization to β-actin mRNA expression. *P<0.05 vs. control, #P<0.05 vs. 10 μM amiloride and ΔP<0.05 vs. 50 μM amiloride. RANKL, receptor activator of nuclear factor-κB; Ami, amiloride; TRAP, tartrate-resistant acid phosphatase; CTSK, cathepsin K; MMP-9, matrix metalloproteinase-9; OSCAR, osteoclast-associated receptor; NFATc1, nuclear factor of activated T cells cytoplasmic 1.

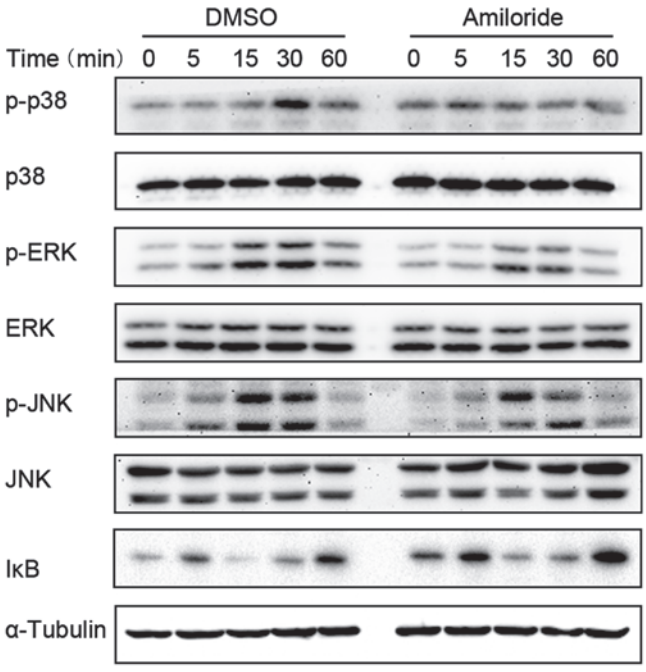

Figure 5. Amiloride downregulated RANKL-induced IκB degradation and MAPK phosphorylation in RAW264.7 cells. Cells were pretreated with amiloride (100 μM) or vehicle (DMSO, equal final concentrations) for 12 h and then stimulated with RANKL (100 ng/ml) for the indicated times. Whole cell lysates were extracted and subjected to western blotting analysis with antibodies targeting p-p38, p38, p-ERK, ERK, p-JNK, JNK, and IκB. α-Tubulin served as a reference protein. RANKL, receptor activator of nuclear factor-κB; MAPK, mitogen-activated protein kinase; p-p38, phosho-p38; ERK, extracellular signal-regulated kinase; p-ERK, phospho-ERK; JNK, c-Jun N-terminal kinase; p-JNK, phospho-JNK; DMSO, dimethyl sulfoxide.

that amiloride markedly inhibited the phosphorylation of JNK, ERK and p38, thereby contributing to the inhibition of RANKL-induced osteoclast differentiation.

It was also demonstrated that amiloride downregulates RANKL-induced expression of *NFATc1* mRNA in RAW264.7 cells. NFATc1 has been shown to be the predominant regulator

of osteoclastogenesis, and overexpression of the *NFATc1* gene is associated with the efficient induction of mature osteoclasts. The expression of this gene is known to be dependent on NF-κB and MAPKs (8,27). NFATc1 also regulates the expression of a number of osteoclast-specific genes, including *TRAP*, *CTSK*, *MMP9* and *OSCAR*, which, in the present study, were shown to be inhibited by amiloride in a concentration-dependent manner. Therefore, the inhibition of RANKL-induced *NFATc1* expression by amiloride is likely to be involved in the inhibition of osteoclastogenesis. Further investigation is required in order to confirm the mechanisms involved in osteoclastogenesis inhibition. In addition, the efficacy of amiloride in treating excessive bone resorption should be evaluated *in vivo*.

In conclusion, the results of the present study demonstrated that amiloride markedly inhibited osteoclastogenesis *in vitro* without exerting cytotoxic effects. It also reduced the RANKL-induced expression of osteoclastic marker genes and suppressed the expression of *NFATc1*, the principal regulator of osteoclastogenesis. In addition, amiloride attenuated RANKL-induced JNK, ERK and p38 activation, and the degradation of IκB. These findings indicated that amiloride may have a potential indication in the treatment of bone loss-related diseases.

Acknowledgements

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