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

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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⁺/Ca²⁺ 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.

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-kB 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+/Ca2+ 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+/Ca2+ exchangers, which are expressed in osteoclasts, are important in Ca2+ 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% CO2 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 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, ×40; 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, ×40; 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 OD260nm (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'-ATTCTTGAATGGGCCAGGT-3' and reverse: 5'-CTGGCTCAACACCTCAACC-3' for β-actin, forward: 5'-GATGCCAGGCAGAACAGGTTT-3' and reverse: 5'-CATACACCGGGGTAGTTGGCGA-3' for TRAP, forward: 5'-GAGAAGAGACTCCCGAGAC-3' and reverse: 5'-TCCAGGTATTGGGCAAGATTT-3' for CTK, forward: 5'-CTGGACAGCCACAGCATCATAGAG-3' and reverse: 5'-CTCGCCGGCAGTCTCTTCAAG-3' for MMP-9, forward: 5'-CAGGAGAGGCGATTAGCA-3' and reverse: 5'-GGTACCTTCCCTGGTTCGAT-3' for Rank, forward: 5'-CTGCTGGTAAAGGGAGCTC-3' and reverse: 5'-CCAAGGAGCCAGAACATCTTCAAG-3' and reverse: 5'-CCAAGGAGCCAGAACATCTTCAAG-3'.
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.
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
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 osteoclast 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 IkB. These findings indicated that amiloride may have a potential indication in the treatment of bone loss-related diseases.

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References