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Acid-sensing ion channel 1a is involved in acid-induced osteoclastogenesis by regulating activation of the transcription factor NFATc1

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ABSTRACT

It has been known that osteoclastogenesis is induced by extracellular acidosis-evoked the rise of intracellular calcium ($[Ca^{2+}]i$), which regulate activation of the transcription factor nuclear factor of activated T cells c1 (NFATc1). However, the acid-sensing ion channels (ASICs) involved remain largely unknown. Here, we show that ASIC1a, ASIC1b, ASIC2a, and ASIC3 are expressed in rat osteoclasts, and only ASIC1a is highly upregulated in response to acidosis. Both the ASIC1a-specific blocker PcTX1 and specific siRNA significantly reduce this increase in acid-induced $[Ca^{2+}]i$ and acid-induced nuclear translocation of NFATc1, and inhibit acid-induced osteoclast differentiation and bone resorption. These findings show that ASIC1a-mediated calcium entry plays a critical role in osteoclastogenesis by regulating activation of the NFATc1.

1. Introduction

Bone homeostasis is strictly regulated by control of both bone resorption by osteoclasts and bone formation by osteoblasts [1]. Most adult skeletal diseases, such as osteoporosis and Paget's disease, are mostly or partially due to excess osteoclastic activity [2]. Osteoclasts are bone-resorbing cells that are specialized to break down and absorb bone tissue [3]. At present it is widely believed that two key regulators produced by bone marrow mesenchymal cells are essential for osteoclastogenesis: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) [4]. In addition to M-CSF and RANKL, Recent studies have demonstrated that extracellular acidosis is a potent modulator of the resorptive activity of mature osteoclasts [5]. Further study found that extracellular acidification enhances osteoclast differentiation by regulating the acid-induced rise in $[Ca^{2+}]i$ [6]. Although many acid-sensitive proteins such as ovarian cancer G protein-coupled receptor 1 (OGR1) and transient receptor potential (TRP) vanilloid-1 (TRPV1) ion channels involved in the activation of osteoclasts in extracellular acidosis have been identified [7–9], many more components are yet to be discovered.

ASICs, a novel class of ligand-gated cation channels, belong to the degenerin/epithelial Na^+ channel (DEG/ENaC) superfamily [8]. They are activated by a fall in the extracellular pH and are cation-selective. Recently, many studies have demonstrated that ASICs are expressed not only in nervous system but also in non-neuronal cells such as dendritic cells, chondrocytes, osteoblasts, osteoclasts, nucleus pulposus cells, carotid body glomus cells, and synoviocytes, suggesting more diverse roles for ASICs in physiological and pathogenic processes [10,11]. To date, at least six subunits of ASICs have been identified: 1a, 1b, 2a, 2b, 3 and 4. Of these, ASIC1a is the subunit that is reported to be permeable to Ca^{2+}, as it differs from other ASIC subunits [12]. Recent studies have indicated that ASIC1a is involved in synaptic plasticity, learning, memory, articular chondrocyte injury, and ischemic brain injury [13,14].

The increase in $[Ca^{2+}]i$ signaling is a critical regulator of osteoclastogenesis, by inducing nuclear factor of activated T cells c1 (NFATc1)-dependent gene transcription, the master regulator of osteoclastogenesis [15]. However, the way in which the $[Ca^{2+}]i$ increase is regulated during osteoclastogenesis in extracellular acidosis remains largely unknown. Previous work has shown that ASICs are expressed at high levels in bone cells [16]. However it is uncertain whether ASIC1a is expressed in rat osteoclasts, and whether it directly mediates Ca^{2+} influx and increased $[Ca^{2+}]i$ in extracellular acidosis. It is also uncertain whether ASIC1a contributes to osteoclastogenesis through an acidosis-evoked increase in $[Ca^{2+}]i$. Therefore, the aim of our current study was to establish the expression of ASIC1a genes in osteoclasts and identify their role

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in regulating osteoclastogenesis through acidosis-evoked calcium–NFATc1 signaling pathways.

2. Materials and methods

2.1. Isolation of osteoclasts

Bone marrow-derived macrophages (BMMs) were obtained as osteoclast precursors using the following procedures. BMMs were prepared by flushing out the bone marrow cavity of rats using α-MEM, which was supplemented with 10% fetal calf serum containing 30 ng/ml M-CSF. The non-adherent cells were collected after 24 h and used as osteoclast precursors. Cells were seeded at a density of 1×10^6 cells/well in the presence of RANKL (50 ng/ml) and M-CSF (30 ng/ml) for 5–6 days. The culture medium was replaced every 3 days. Samples were then stained for TRAP using the Leukocyte Acid Phosphatase Staining Kit (Sigma–Aldrich, St Louis, MO) according to the manufacturer's instructions. Multinuclear cells containing three or more nuclei that stained positive for TRAP were counted by light microscopy. TRAP-positive multinucleated cells containing three or more nuclei were considered to be osteoclasts.

2.2. Real-time PCR and RT-PCR analysis

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed using 0.1 μl of cDNA with SYBR Green dye detection of amplification products (Applied Biosystems, Foster City, CA) on a GeneAmp 5700 Sequence Detection System. To prepare culture media of different extracellular pH values, including pH 6.0, 6.5, 7.0, and 7.5, the pH buffering of cell culture medium was carried out as previously described [17]. In all acid-induced experiments, blockers of voltage-gated Ca^{2+} channels (5 μM nimodipine and 3 μM ω-conotoxin MVIIC), glutamate receptors (10 μM MK-801), TRPV1 (2 μM capsazepine), and OGR1 (100 μM, $CuCl_2$) were added to extracellular solutions to inhibit possible secondary activation of these channels. Gene expression levels were normalized to GAPDH and were calculated using the ΔΔCt method. The primers used are listed in Table S1. The RT-PCR product of ASIC1a was electrophoresed on 1.2% agarose gels and stained with ethidium bromide.

2.3. Western blot

Whole cells were lysed in lysis buffer, supplemented with protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). Protein concentration was determined for each sample using the BCA-200 Protein Assay Kit (Pierce, Rockford, IL). The lysates were resolved by denaturing 12% SDS–PAGE and transferred to Immobilon polyvinyldifluoride membranes. The membranes were probed with primary antibody against ASIC1a (Alpha Diagnostic International, San Antonio, TX) and NFATc1 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. After three washes, the blots were subsequently incubated with a secondary antibody for 1 h. The blots were developed using the Western Blue® stabilized substrate for alkaline phosphatase.

2.4. Immunofluorescent cell staining

Cells were permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS), and then the cells were washed in PBS containing 5% FBS and labeled with primary anti-ASIC1a antibody for 2 h. After washing with PBS, the primary antibody was detected with Texas red conjugated secondary antibodies (Molecular Probes).

For NFATc1 immunostaining, osteoclasts were switched to different pH levels for 1 h or mixed with the ASIC1a-specific blocker, PcTX1 (100 ng/ml, Alomone Labs, Ltd., Jerusalem, Israel). Cells were then washed with PBS and incubated with an anti-NFATc1 antibody. After washing, the cells were incubated with Alexa Fluor-488-conjugated secondary antibody (Molecular Probes). Cells were washed again with PBS and stained with DAPI (Sigma–Aldrich) for 15 min to identify multinucleated cells. Cells were washed and imaged using a laser scanning confocal microscope.

2.5. Fluorescence imaging of $[Ca^{2+}]i$

$[Ca^{2+}]i$ of isolated rat osteoclasts was monitored using fluorescence imaging [6]. Cells on glass coverslips were washed three times with D-Hanks' solutions and incubated with 4 μM fura-2 and 0.02% acetoxymethyl (Biotium, Hayward, CA) for 40 min, followed by three washes and additional incubation in normal Hanks' solutions for 30 min. Coverslips were washed and placed in a chamber mounted on the stage of an inverted microscope (Nikon) and superfused at room temperature with extracellular solution containing, which contained in mM: NaCl, 140; KCl, 5.4; glucose, 15; $MgCl_2$, 1; $CaCl_2$, 1.3; HEPES, 20; pH 7.5 using NaOH. Fura-2 was excited at 340 and 380 nm, and emission was measured at 510 nm. Digitized images were analyzed on a computer controlled by Leica-sp5 LAS AF software. Images (340:380 ratio) were analyzed by averaging pixel ratio values in circumscribed regions of cells in the field of view. The values were exported from Axon Imaging Workbench software to SigmaPlot for additional analysis and plotting.

2.6. siRNA transfection

The cDNA sequence of rat ASIC1a was obtained from GenBank (U94403). To stably knock down ASIC1a expression, we used lentivirus packing siRNA expression vector (synthesized by GenePharma, Shanghai, China) to infect cells. A negative control RNAi (NC-RNAi) was purchased from Genepharma. Target osteoclasts were infected with lentivirus for 24–48 h according to manufacturer's instruction. The RNAi oligonucleotides sequence used to knock down ASIC1a expression is in Table S2.

2.7. Osteoclast differentiation and resorption assays

Osteoclast differentiation and resorption were measured as described previously [6]. The cells were seeded into tissue culture dishes, and large multinucleated cells were counted in a standard central area (3.14 cm^2) using phase-contrast microscopy. The cells were then treated by incubation in different acid pH values for 18 h in α-MEM. Osteoclasts were then fixed in 4% paraformaldehyde, washed twice with PBS, and stained with TRAP to distinguish osteoclasts. To measure resorption, osteoclasts were cultured and treated as described above. The cells were scraped, counted, and re-plated onto dentine slices at 1×10^4 cells/well. Osteoclasts were incubated with different acid pH values for 18 h, then removed by sonication and the dentine was stained with toluidine blue to identify resorption pits. The resorption area was quantified with ImageJ software.

2.8. Statistical analyses

Data were expressed as the mean ± standard error of the mean. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) post hoc test and the Student's *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. ASICs are expressed in osteoclasts

To examine whether ASICs are involved in acid-induced osteoclast differentiation in rat bone marrow cells, we first measured the mRNA levels of ASICs in response to acidosis. BMMs were stimulated for 5 days with 50 ng/ml RANKL and 30 ng/ml M-CSF. Then the cells were stimulated at pH 6.0, 6.5, 7.0, and 7.5 for 18 h. Total cellular RNA was extracted from the induced cells and used for real-time RT-PCR. This analysis revealed that ASIC1a mRNA expression was highly upregulated in response to acidosis in rat osteoclasts, whereas the expression of other ASIC subtypes including ASIC1b, ASIC2a, and ASIC3 was unchanged in response to acidosis, however, no expression of either ASIC2b or ASIC4 was detected in rat osteoclasts (Fig. 1A). As shown in Fig. 1A, the expression of ASIC1a mRNA in response to acidosis was pH dependent, and the maximum response appeared at pH 6.0. This indicates that ASIC1a is pH responsive in rat osteoclasts (Fig. 1A).

We next examined the expression of mRNA encoding ASIC1a in osteoclasts. RT-PCR analysis showed clear expression of ASIC1a in rat osteoclasts (Fig. 1B), which was consistent with data from a positive control. Western blot analysis revealed that ASIC1a protein (approximately 80 kDa) was expressed at readily detectable levels in rat osteoclasts (Fig. 1C). To study the subcellular localization of ASIC1a by immunofluorescence, osteoclasts were fixed and co-stained for ASIC1a and indicated markers. As depicted in Fig. 1D, ASIC1a was found in dot-like structures dispersed throughout the cytoplasm, while an intense signal on the plasma membrane was also evident. Negative controls without anti-ASIC1a gave no signal (Fig. 1E). These data indicate the presence of ASIC1a in rat osteoclasts, providing a means to sense and respond to differences in extracellular pH.

3.2. Increased $[Ca^{2+}]i$ induced by acidic stimulation via ASIC1a in osteoclasts

Previous studies reported that acidosis also leads to the rise of $[Ca^{2+}]i$ in osteoclasts [7]. Using Ca^{2+}-imaging experiments, we determined whether ASIC1a in osteoclasts is Ca^{2+} permeable. From the Ca^{2+}-imaging (Fig. 2A), the application of extracellular acid (pH 6.0) caused transient rise of $[Ca^{2+}]i$ in osteoclasts (Fig. 2A and E). As an additional test of the involvement of ASIC1a, we applied the PcTX1 (100 ng/ml) and found that this response was diminished (Fig. 2B and E). To directly examine the involvement of ASIC1a in acid-induced changes in $[Ca^{2+}]i$, we knocked down the expression of ASIC1a using siRNA in BMMs. We made three ASIC1a siRNA constructs and found that siRNA#3 can effectively lower the cellular level of ASIC1a (Fig. S1A and 1B). Strikingly, knock-down of ASIC1a expression suppressed the acid-induced rise of Ca^{2+} in osteoclasts derived from BMMs (Fig. 2C and E). However, we did not observe any changes in NC-RNAi group (Fig. 2D and E). These data confirm an essential role for ASIC1a in mediating acid-induced $[Ca^{2+}]i$ in osteoclasts.

3.3. Acid-induced $[Ca^{2+}]i$ increase via ASIC1a is responsible for osteoclastogenesis

It has been established that the acid-induced rise of $[Ca^{2+}]i$ contributes to regulation of osteoclast differentiation [6]. To determine whether Ca^{2+} entry plays a role in acid-induced osteoclastogenesis, we treated cells with extracellular solution at pH 6.0, 6.5, 7.0 and 7.5 for 18 h (Fig. 3A). As shown in Fig. 3A and B, acidic pH-induced osteoclast differentiation was estimated using TRAP staining at 18 h. A significant increase was detected at pH 6.0, 6.5, 7.0, and

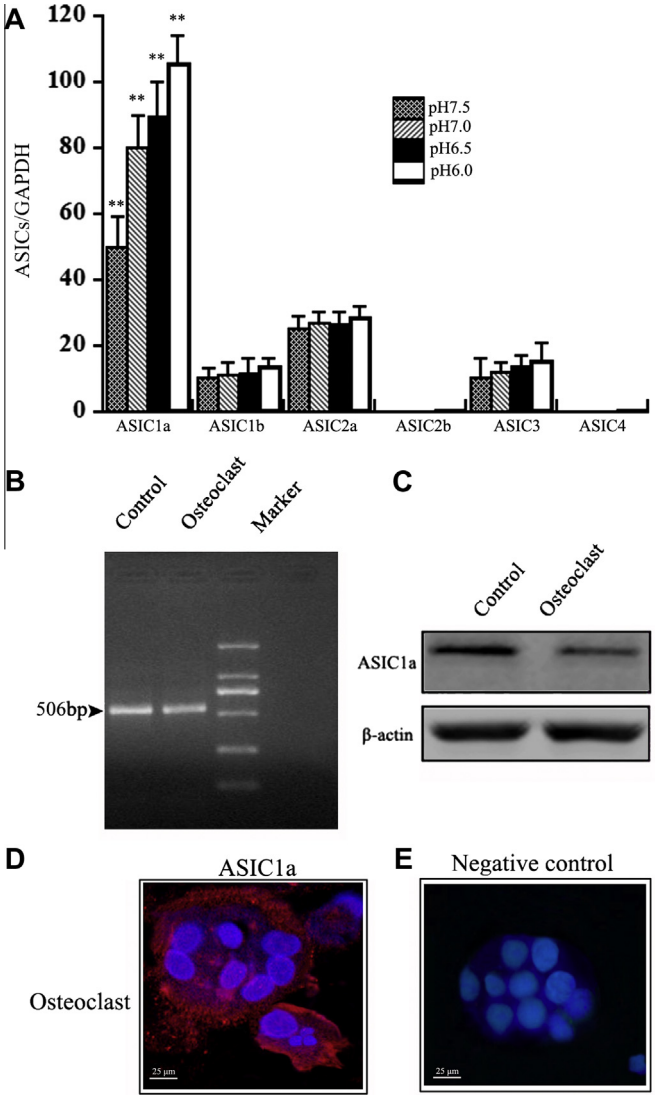

Fig. 1. Detection of ASICs in rat osteoclasts. (A) Real-time RT-PCR analysis of ASIC expression in osteoclasts incubated at different pH values for 18 h. Data were normalized to levels of β-actin. $^{**}P < 0.01$ compared with pH 7.5. (B) RT-PCR analysis of mRNA isolated from cultured osteoclasts revealed the presence of mRNA for ASIC1a. (C) Western blot of ASIC1a protein from cultured osteoclasts. A specific signal was detected at a molecular mass of approximately 80 kDa in the cultured rat osteoclasts, which corresponded to the expected molecular weight of recombinant ASIC1a protein. (D) Subcellular localization of ASIC1a protein in osteoclasts. A specific signal was detected in the the cytoplasm and plasma membrane of most osteoclasts (red). Nuclei were stained with DAPI (blue). Immunofluorescent double staining was observed in treated osteoclasts, and showed only nuclear staining. (E) No positive staining was observed in osteoclasts when non-immune IgG was used. Bar is 25 μm.

increasing extracellular acidification resulted in a dose-dependent increase in the osteoclast differentiation compare to control at pH 7.5 (Fig. 2B). To determine whether activation of ASIC1a is involved in acid-induced osteoclast differentiation, we tested the effect of PcTX1 and ASIC1a-specific RNAi, but not NC-RNAi, on osteoclast differentiation. As shown in Fig. 3B and C, the effect of acid incubation for 18 h on osteoclast differentiation was reduced by 100 ng/ml PcTX1 and by ASIC1a-specific RNAi.

It has been shown that the calcineurin inhibitor cyclosporine A and the Ca^{2+} chelator BAPTA potently suppress the RANKL-induced differentiation of osteoclasts through inhibition of NFATc1 nuclear translocation, suggesting that activation of the calcium–NFATc1 signaling pathway is crucial for osteoclast differentiation [18]. Thus, we next examined whether acid-induced $[Ca^{2+}]i$ elevation via acti-

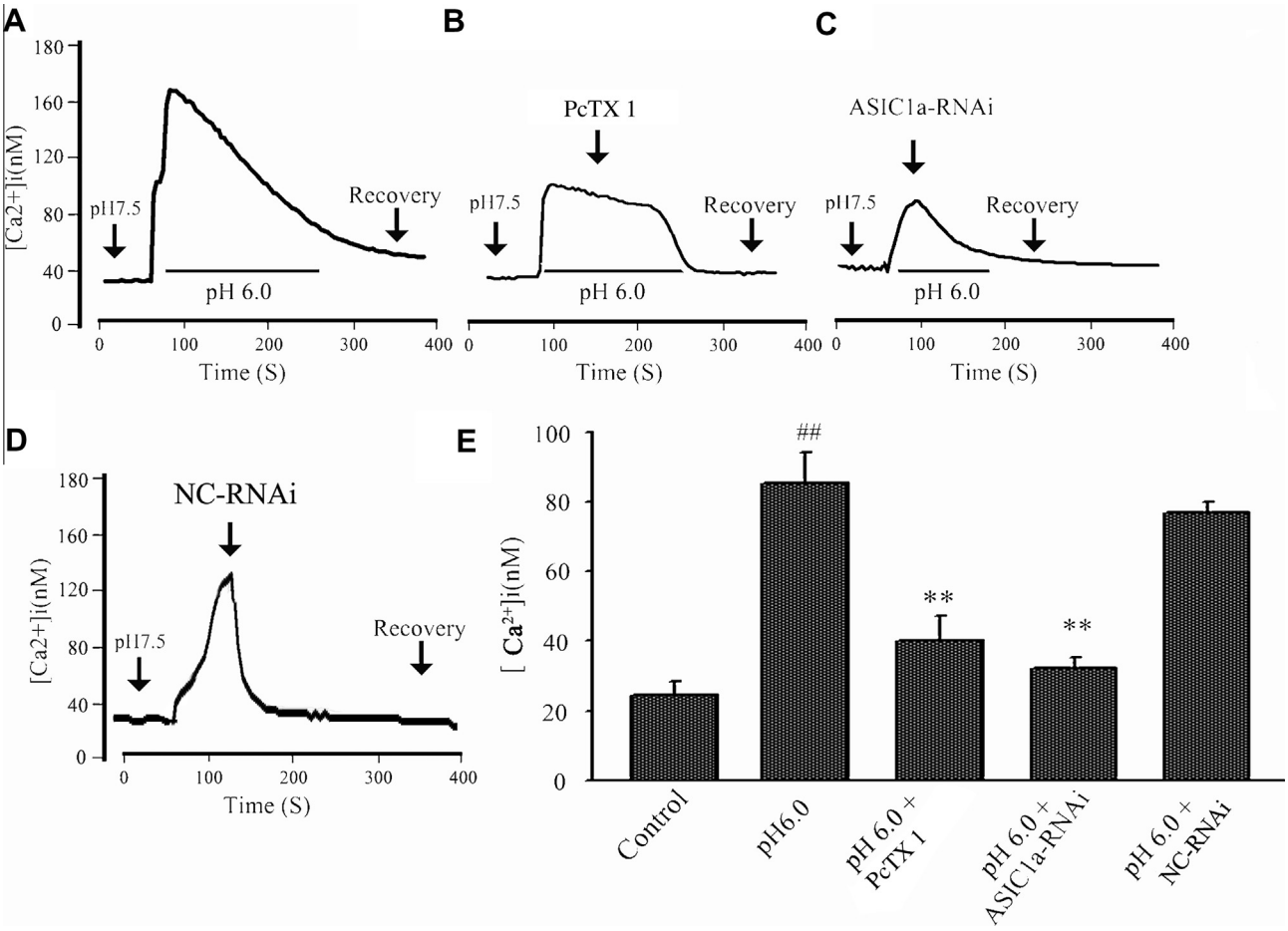

Fig. 2. Acid-induced elevation of $[Ca^{2+}]i$ level. (A) Acid-induced elevation of $[Ca^{2+}]i$ in the cultured rat osteoclasts. (B) Acid-induced elevation of $[Ca^{2+}]i$ in the cultured rat osteoclasts treated with PcTX1. (C) Acid-induced elevation of $[Ca^{2+}]i$ in the cultured rat osteoclasts treated with ASIC1a-specific RNAi. (D) Acid-induced elevation of $[Ca^{2+}]i$ in the cultured rat osteoclasts treated with NC-RNAi. (E) The amplitude of $[Ca^{2+}]i$ intensity in osteoclasts induced by acid treatment was quantified as the maximal rise of $[Ca^{2+}]i$ above basal levels. $^{\#\#}P < 0.01$ compared with control (pH 7.5); $^{**}P < 0.01$ compared with pH 6.0.

vation of ASIC1a in osteoclasts could result in osteoclastogenesis using the intracellular Ca^{2+} chelator BAPTA. Similar to the effects of the PcTX1 and ASIC1a-specific RNAi, enhancement of osteoclast differentiation at pH 6.0 was abolished by chelation of cytosolic Ca^{2+} with BAPTA (50 μM), but BAPTA did not affect differentiation of osteoclasts cultured at pH 7.5 (Fig. 3D). In summary, these results indicate that the acid-induced increase of $[Ca^{2+}]i$ mediated by ASIC1a is responsible for osteoclastogenesis. To evaluate the role of the calcineurin/NFATc1 pathway in mediating the effects of acidosis on differentiation, we investigated the effect of cyclosporine A (1 mg/ml), a specific calcineurin inhibitor, on acid-induced osteoclast differentiation. As shown in Fig. 3E, cyclosporine A inhibited acid-induced osteoclast differentiation. Similar to BAPTA, cyclosporine A inhibited acid-induced osteoclast differentiation (Fig. 3E). Consistent with the osteoclast differentiation results, administration of PcTX1 and ASIC1a-specific RNAi, but not NC-RNAi, reduced acid-induced osteoclastic bone resorption (Fig. 3F and G).

3.4. ASIC1a is involved in acid-stimulated NFATc1 signaling in osteoclastogenesis

NFATc1 in the nucleus is activated by Ca^{2+} signaling, which explains why activation of the Ca^{2+}–NFATc1 pathway is crucial for osteoclast differentiation [15,19]. Moreover, the Ca^{2+}–calmodulin pathway also induces c-fos expression after c-fos binding to c-jun increases NFATc1 transcription [15].

To gain insight into the mechanism by which the ASIC1a–Ca^{2+}–NFATc1 axis positively regulates osteoclast differentiation via the activation of NFATc1, we treated osteoclasts in the presence of extracellular solution at pH 6.0 or 7.5 for 18 h, and extracted RNA from cultured cells for real-time RT-PCR analysis. As shown in Fig. 4A, we confirmed that mRNA levels of NFATc1 in rat osteoclasts were substantially up-regulated after treatment with extracellular solution at pH 6.0 compared with extracellular solution at pH 7.5 (Fig. 4A). In particular, the up-regulation of NFATc1 induced by pH 6.0 was significantly attenuated in osteoclasts treated with PcTX1 and ASIC1a-specific RNAi, but not NC-RNAi (Fig. 4A). Although it is well known that the Ca^{2+}–calmodulin pathway also induces c-fos expression after c-fos binding to c-jun increases NFATc1 transcription, we did not detect any changes of c-fos or c-jun mRNA expression in osteoclasts treated with PcTX1 and ASIC1a-specific RNAi (Fig. S2).

Next we carried out western blot analyses to investigate whether inhibition of ASIC1a also affects production of NFATc1 protein in acid-mediated NFATc1 signaling in osteoclast differentiation. Whole cell fractions were prepared from osteoclasts treated with PcTX1, ASIC1a-specific RNAi, and NC-RNAi cultured with or without extracellular solution at pH 6.0. As shown in Fig. 4B, NFATc1 protein was significantly up-regulated in response to pH 6.0 treatment compared with controls cultured in extracellular solution at pH 7.5. Moreover, we found that extracellular acidosis induction of NFATc1 protein was reduced in lysates prepared from

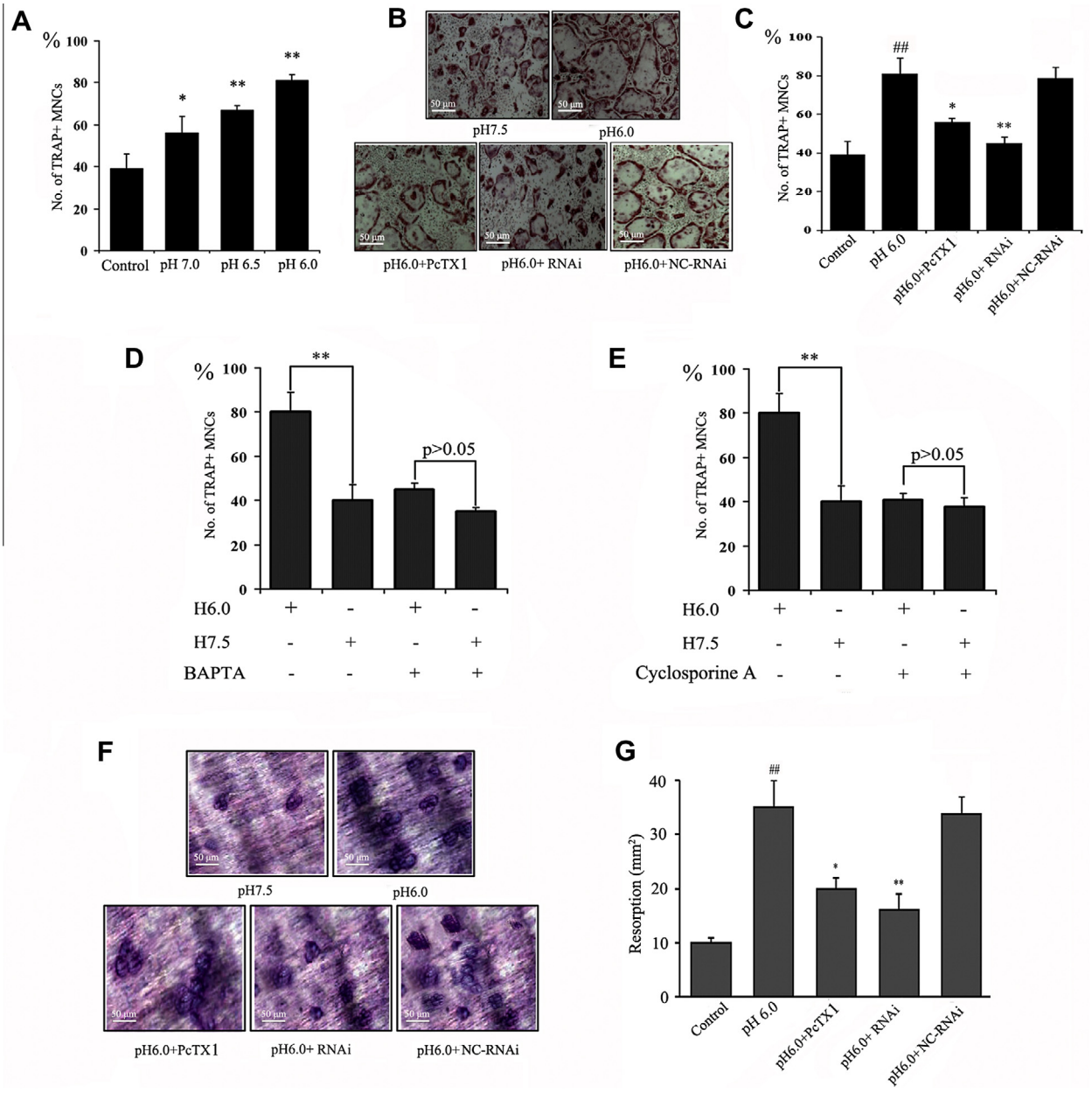

Fig. 3. Acid-induced $[Ca^{2+}]i$ increase via ASIC1a is involved in acid-induced osteoclastogenesis. (A) Acidosis-induced osteoclast differentiation. BMMs were differentiated into osteoclasts in 96-well culture plates, and then osteoclasts were incubated at different pH values for 18 h. After TRAP staining, $TRAP^+$ multinuclear cells ($TRAP^+$ MNCs) with more than three nuclei were scored as osteoclasts. $^*P < 0.05$ compared with control (pH 7.5); $^{**}P < 0.01$ compared with control (pH 7.5). (B) Osteoclast differentiation was determined by counting of TRAP-positive cells with more than three nuclei, which were counted as viable osteoclasts. (C) Acid-induced cell differentiation in an extracellular solution treated with PcTX1 and ASIC1a-specific RNAi. $^{\#\#}P < 0.01$ compared with control (pH 7.5); $^*P < 0.05$ compared with pH 6.0; $^{**}P < 0.01$ compared with pH 6.0. (D) Chelation of cytosolic Ca^{2+} with BAPTA inhibited the effect of extracellular solution at pH 6.0 on osteoclast differentiation, but did not affect osteoclast differentiation at pH 7.5. $^{**}P < 0.01$ compared with pH 6.0. (E) Inhibition of calcineurin with cyclosporine A decreased osteoclast differentiation at pH 6.0, but did not affect differentiation at pH 7.5. $^{**}P < 0.01$ compared with pH 6.0. (F) Bone resorption lacuna stained with toluidine blue. Bar is 50 μm. (G) Quantification of resorption, total area of resorption. $^{\#\#}P < 0.01$ compared with control (pH 7.5); $^*P < 0.05$ compared with pH 6.0; $^{**}P < 0.01$ compared with pH 6.0.

cells treated with PcTX1 and ASIC1a-specific RNAi, but not NC-RNAi (Fig. 4B).

To determine whether ASIC1a is involved in acid-stimulated NFATc1 activity, we investigated the localization of NFATc1 after acid stimulation following pretreatment with PcTX1, ASIC1a-specific RNAi, and NC-RNAi, which is used extensively in evaluating NFATc1 activity in osteoclasts. As shown in Fig. 4C, the majority of NFATc1 was located in the cytoplasm, maintained at pH 7.5. Upon stimulation by extracellular acidification (pH 6.0), nuclear translocation in cells significantly was increased. Interestingly, acid-induced NFATc1 nuclear translocation was blocked by PcTX1 and ASIC1a-specific RNAi. However, the majority of NFATc1 was located in the cytoplasm in NC-RNAi group (date not shown).

Previous reports have shown that NFATc1 regulates osteoclastic genes such as TRAP, cathepsin K, and osteoclast-associated receptor (OSCAR), which are known to be involved in osteoclast differentiation [20]. Thus, we next used real-time RT-PCR to investigate the regulation of these key genes downstream of NFATc1 in osteoclasts treated with PcTX1, ASIC1a-specific RNAi, and NC-RNAi. We found that all three genes are highly up-regulated in response to pH 6.0 (Fig. 4D). However, consistent with the decrease in NFATc1 expression, induction of TRAP, cathepsin K, and OSCAR by pH 6.0 was

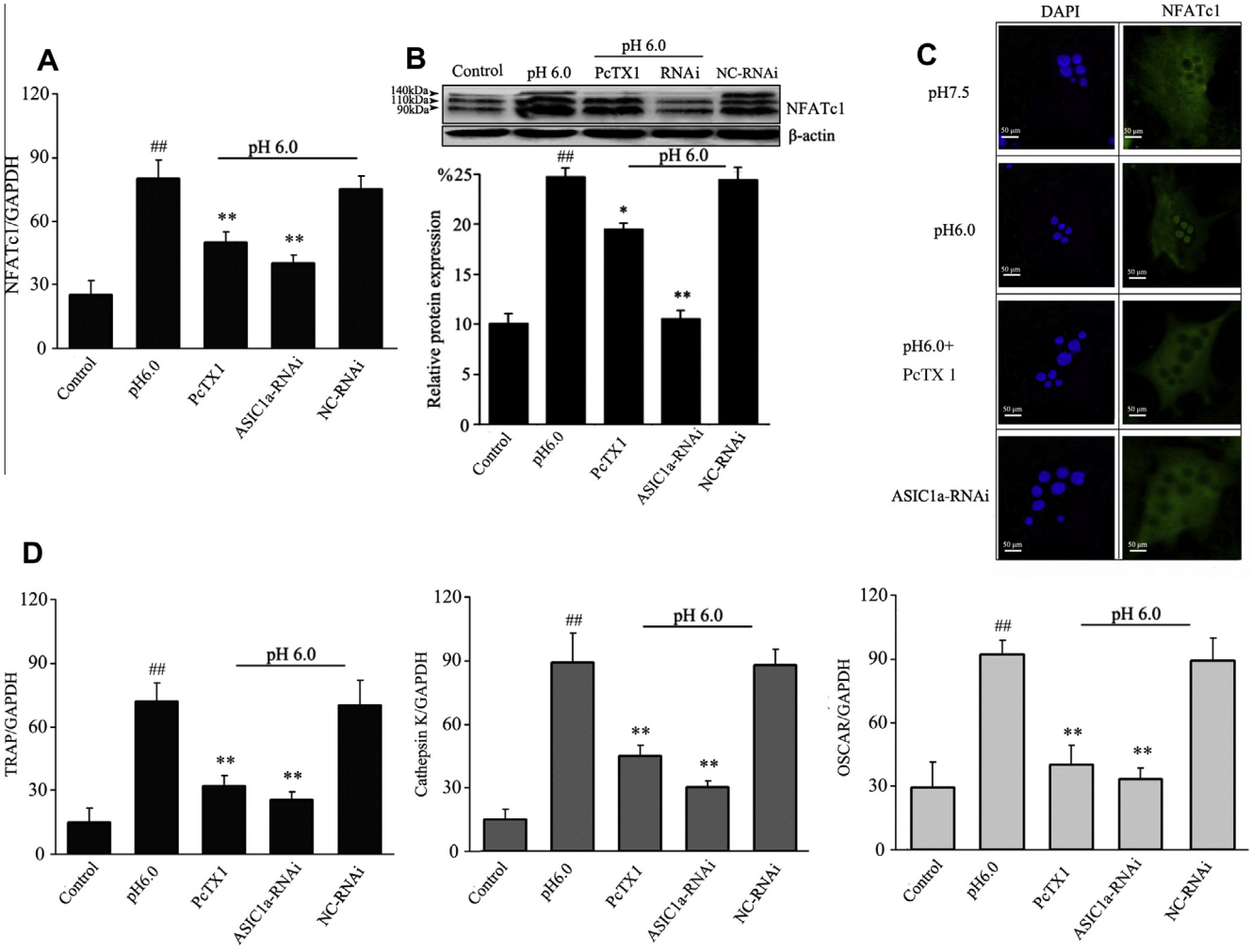

Fig. 4. ASIC1a is involved in acid-stimulated NFATc1 signaling in osteoclastogenesis. (A) Osteoclasts were cultured in an extracellular solution at pH 6.0 for 18 h, and real-time RT-PCR analysis of NFATc1 message level was performed. (B) NFATc1 protein levels were determined by Western blot (upper panel). Molecular mass markers (kDa) are as indicated. Arrows indicate three NFATc1 isoforms. Densitometry of the NFATc1 protein bands (lower panel). $^{\#\#}P < 0.01$ compared with control (pH 7.5); $^{**}P < 0.01$ compared with pH 6.0. (C) NFATc1 localization was assessed by immunofluorescence and nuclei were stained with DAPI. In extracellular solutions at pH 7.5, NFATc1 showed predominantly cytoplasmic localization. Extracellular acidification to pH 6.0 induced nuclear accumulation of NFATc1. Images of osteoclasts plated on PcTX1 and ASIC1a-specific RNAi at pH 6.0 showing NFATc1 localized predominantly in the cytoplasm. Bar is 25 μm. (D) Real-time RT-PCR analysis of expression of the osteoclastic genes TRAP, cathepsin K, and OSCAR, known to be direct transcriptional s of NFATc1 in osteoclast differentiation. $^{\#\#}P < 0.01$ compared with control (pH 7.5); $^{**}P < 0.01$ compared with pH 6.0.

obviously diminished in cells treated with PcTX1, ASIC1a-specific RNAi, but not NC-RNAi (Fig. 4D). These findings suggest that ASIC1a-mediated Ca^{2+} entry contributes significantly to the induction of osteoclast-specific genes by regulating transcription of NFATc1, a master regulator of osteoclastogenesis.

4. Discussion

In the present study, we have shown that ASIC1a is essential for the extracellular acidification-induced increase in $[Ca^{2+}]i$ levels in osteoclasts. More interestingly, ASIC1a provides a pathway for Ca^{2+} to enter osteoclasts in extracellular acidosis, which contributes to the acidosis-induced osteoclastogenesis. ASIC1a activation in osteoclasts enhances their differentiation by inducing NFATc1 translocation.

It has previously been reported that mRNA encoding ASICs was detected in human osteoclasts [16]. Our results show that ASIC1a in particular is highly upregulated in response to acidosis, whereas the level of other ASIC subtypes is unchanged. In acid conditions, the transcript level of ASIC1a isoform is highly increased. Higher expression of ASIC1a in osteoclasts may explain why acidosis does stimulate osteoclast differentiation. This could signify a particular sensing role for ASIC1a during acidosis in osteoclasts. The negative impact of systemic acidosis on the skeleton has long been known in that low pH exerts a direct stimulatory effect on multinucleated bone-resorbing osteoclasts [21]. In the present study, we provide direct evidence that ASIC1a is involved in pH-sensing in osteoclasts.

Over the past several years, considerable progress has been made towards understanding the mechanisms by which osteoclasts might detect and respond to changes in extracellular pH in such an acidosis-sensitive manner [6,21]. It has been reported that the rise of $[Ca^{2+}]i$ induced by extracellular acidosis contributes to regulation of osteoclastogenesis [6]. The molecular identity of the pathways of acid-induced Ca^{2+} entry are not yet known, but might be associated with ASIC1a, as described in the present study. We demonstrated that osteoclasts derived from rat BMMs respond to a decrease in pH by increasing $[Ca^{2+}]i$ levels. This acid-induced rise of $[Ca^{2+}]i$ is inhibited by the ASIC1a antagonist PcTX1, and is suppressed in osteoclasts in which ASIC1a transcripts have been

depleted using RNA interference. These findings support an essential role for ASIC1a in acid-induced Ca^{2+} signaling in osteoclasts.

It has been proposed that lowering extracellular pH induces an increase in osteoclastogenesis (14–16, 18, 19). Moreover, some studies have found that acidosis has a direct effect on osteoclasts, enhancing their differentiation, adhesion, and migration [5]. Nevertheless it remained unclear whether acid-induced $[Ca^{2+}]i$ increased via ASIC1a was involved in acid-induced osteoclastogenesis. Therefore, we measured osteoclast differentiation and bone resorption. In this study, we found that ASIC1a regulates Ca^{2+} influx and contributes to osteoclast formation and bone resorption in osteoclasts.

Recent studies have unveiled the precise molecular mechanism underlying osteoclast differentiation. Notably, the discovery of NFATc1, the major regulator of osteoclast differentiation, has proven to be a breakthrough in this field [5]. NFATc1in the nucleus is activated by Ca^{2+} signaling, which explains why activation of the Ca^{2+}–NFATc1 pathway is crucial for osteoclast differentiation [15]. NFATc1 induces expression of TRAP, cathepsin K, and OSCAR, which are responsible for bone osteoclast differentiation and survival [20]. Komarova et al. [6] previously demonstrated that RANKL and acidification induce osteoclast function through the Ca^{2+}/calcineurin/NFATc1 signaling axis in osteoclasts. Consistent with the previous study [22], our present results confirmed that, although NFATc1 initially accumulate in the nucleus immediately after acid-induced calcium signal, the actual transcriptional response is integrated over substantially longer periods of time that require maintained presence of NFATc1. Our results clearly illustrate that ASIC1a plays a key role in induction of the expression of osteoclastic genes known to be a direct transcriptional target of NFATc1 in osteoclastogenesis. Based on these data, we propose that ASIC1a activation in rat osteoclasts enhances their differentiation, mainly by inducing NFATc1 translocation into the nucleus and activation of NFATc1 transcriptional activity, which is stimulated by $[Ca^{2+}]i$ elevation.

In conclusion, we found that the proton-sensitive ASIC1a was present in rat osteoclasts. We have shown that ASIC1a-mediated elevation of $[Ca^{2+}]i$ explains the effect of acidification on osteoclastogenesis by regulating activation of the NFATc1. Thus, we speculate that ASIC1a may play a role in pH sensing in osteoclasts, which has likely been described previously as a physiological function of proton-activated channels in neurons. Our findings point to ASIC1a as a potential therapeutic target for the treatment of human diseases caused by excessive activity of osteoclasts, such as osteoporosis.

Conflict of interest

All authors state that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.08.017.

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