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Inhibition of osteoclast differentiation and bone resorption by sauchinone

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ABSTRACT

Osteoclasts are bone-specific multinucleated cells generated by differentiation of monocyte/macrophage lineage precursors. Regulation of osteoclast differentiation is considered an effective therapeutic approach to the treatment of bone-lytic diseases. In this study, we investigated effects of sauchinone, a lignan from *Saururus chinensis*, on osteoclastogenesis induced by the differentiation factor RANKL (receptor activator of nuclear factor kappa B ligand). Sauchinone strongly inhibited the osteoclastogenesis from primary bone marrow-derived macrophages (BMMs). This effect was accompanied by a significant decrease in the level of carbonic anhydrase II, calcitonin receptor, MMP9, and TRAP, which are normally upregulated during osteoclast differentiation. For the induction of osteoclastogenesis-associated genes, RANKL activates multiple transcription factors through mechanisms involving mitogen-activated protein kinases (MAPK) and reactive oxygen species (ROS). Sauchinone greatly attenuated the activation of ERK and, less prominently, that of p38 MAPKs by RANKL. The RANKL-stimulated induction of c-Fos and NFATc1 transcription factors was also abrogated by sauchinone. In addition, the activation of AP-1, NFAT, and NF-κB transcription factors was alleviated in sauchinone-treated cells. Sauchinone also diminished the RANKL-stimulated increase of ROS production in BMMs. Consistent with the in vitro anti-osteoclastogenic effect, sauchinone inhibited bone destruction and osteoclast formation caused by lipopolysaccharide in an animal model. Taken together, our data demonstrate that sauchinone inhibits RANKL-induced osteoclastogenesis by reducing ROS generation, which attenuates MAPK and NF-κB activation, ultimately leading to the suppression of c-Fos and NFATc1 induction. Also the in vivo effect of sauchinone on bone erosion strengthens the potential usefulness of this compound for diseases involving bone resorption.

1. Introduction

Osteoclasts are cells responsible for the dissolution of inorganic hydroxyapatite and the cleavage of organic collagen fibers in bone matrix. Through the bone resorbing activity, osteoclasts play both a crucial physiological role in bone remodeling and also a pathological role in diseases involving abnormal bone lysis such as osteoporosis, rheumatoid

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Abbreviations: BMM, bone marrow-derived macrophage; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; M-CSF, macrophage colony-stimulating factor; NFAT, nuclear factor of activated T cell; RANKL, receptor activator of nuclear factor kappa B ligand; ROS, reactive oxygen species; TRAF6, tumor necrosis factor receptor-associated factor 6; TRAP, tartrate-resistant acid phosphatase; VtD_3, Vitamin D_3

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arthritis, and periodontal bone erosion. Osteoclasts are multinucleated cells generated from hematopoietic monocyte/macrophage precursor cells under the control of two primarily cytokines, macrophage colony stimulation factor (M-CSF) and receptor activator of NF-κB ligand [RANKL; also called tumor necrosis factor (TNF)-related activation-induced cytokine (TRANCE), osteoclast differentiation factor (ODF) and osteoprotegerin ligand (OPGL)] [1–4]. The binding of RANKL to its receptor RANK leads to recruitment of TNF receptor-associated factor 6 (TRAF6) to the cytoplasmic domain of RANK [5,6]. The downstream targets of TRAF6 include transcription factors such as nuclear factor kappa B (NF-κB), activator protein-1 (AP-1), and nuclear factor of activated T cells (NFAT), as well as the mitogen-activated protein kinases (MAPK) such as p38 MAP kinases, c-Jun N-terminal kinases (JNK), and extracellular-signal regulated kinases (ERK). The phosphatidyl-inositol-3-kinase (PI3K)/Akt pathway is also activated by RANKL [7,8]. Recently, reactive oxygen species (ROS) level was shown to increase and to function upstream in RANKL-stimulated activation of MAPKs [9].

During the differentiation process of monocyte/macrophage cells to mature osteoclasts, transcription factors such as c-Fos, NF-κB, and NFATc1 play a critical and specific role [10]. Stimulation of RANKL results in strong NF-κB activation. NF-κB forms a complex with the inhibitory κB (IκB) in unstimulated conditions. When cells are stimulated by RANKL, the IκB kinase (IKK) complex phosphorylates IκB and phosphorylated IκB is targeted by proteasome and subsequently degraded, leading to the activation of NF-κB signaling [11]. NF-κB subunits are then released from IκB in the cytosol and translocates into the nucleus where they enhance transcription of target genes. In addition to this classical NF-κB activation pathway, NF-κB is synthesized as an inactive precursor (p100), which can be processed to the active form (p52) in a manner dependent on NF-κB-inducing kinase (NIK) in RANKL-stimulated cells [11]. The c-Fos and NFATc1 transcription factors are induced in osteoclast precursors by RANKL stimulation and both of the transcription factors are autoamplified [12]. Mice deficient in c-Fos were shown to develop osteopetrosis due to a failure to commit to osteoclast lineage [13]. c-Fos also controls the induction of NFATc1 by RANKL [12]. For the autoamplification of NFATc1, intracellular calcium oscillation is an important stimulus for calcineurin-mediated NFAT activation [14].

Bone-resorbing osteoclasts are important effector cells in inflammation-induced bone loss. The RANK/RANKL pathway was shown to be essential for osteoclast differentiation in inflammatory bone destruction [15]. In addition, in vitro and in vivo studies have demonstrated that many cytokines elaborated by inflammation, including the proinflammatory cytokines TNF-α and interlukin-1 (IL-1), may contribute to osteoclast differentiation and activation [4,16–18]. Recently, treatment strategies on bone disease focus on the suppression of bone destruction and inflammation-associated bone loss.

To find anti-inflammatory compounds that may inhibit osteoclastogenesis, we screened natural compounds with a bone marrow culture system. During the screening process, we found that osteoclast formation was suppressed by sauchinone, a diastereomeric lignan compound isolated form *Saururus chinensis* (Saururaceae). *S. chinensis* has been traditionally used as folk medicine for the treatment of edema and liver diseases [19]. Sauchinone has been shown to have cytoprotective and anti-oxidant activities in cultured hepatocytes [20,21]. It was also shown to inhibit LPS-induced expression of iNOS, TNF-α and COX-2 in murine macrophages [22].

In this study, we found that sauchinone inhibited osteoclast differentiation from primary precursor cells without cytotoxicity. Sauchinone suppressed transcription activity of NF-κB, NFATc1, and AP-1, transcription factors essential for osteoclast differentiation. In addition, sauchinone inhibited RANKL-induced ROS generation and blocked the RANKL activation of ERK and p38 MAPKs. Furthermore, sauchinone suppressed LPS-induced bone destruction in vivo.

2. Materials and methods

2.1. Reagents and antibodies

RANKL was purchased from PeproTech (Rocky Hill, NJ). Macrophage-colony stimulating factor (M-CSF) was from R&D Systems (Minneapolis, MN). Leukocyte Acid Phosphatase Assay Kit was obtained from Sigma (St. Louis, MO). Antibodies for AKT, ERK, p38, JNK, IκB, phospho-AKT, phospho-ERK, phospho-p38, phospho-JNK, and phospho-IκB were purchased from Cell Signaling Technology (Beverly, MA). Lipofectamine 2000 was from Invitrogen Life Technologies (Carlsbad, CA). The isolation and structural determination of sauchinone was previously described [20].

2.2. Mice

For preparation of primary osteoblasts, 1-day-old ICR mice were used. Bone marrow cells were obtained from tibiae of 6-week-old ICR mice. All animal experiments were approved by the animal care committee of the Institute of Laboratory Animal Resources of Seoul National University.

2.3. Osteoclast culture

Osteoclast cultures were carried out as previously described [23]. For the coculture system of osteoclastogenesis, mouse bone marrow cells and calvarial osteoblasts were cultured together. Primary osteoblasts were obtained by growing calvarial cells from 1-day-old ICR mice in α-minimum essential medium (α-MEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Bone marrow cells were obtained by flushing tibiae from 6-week-old ICR mice with α-MEM and suspended in α-MEM/10% FBS. Then (1–2) $\times 10^7$ bone marrow cells and 1×10^6 osteoblasts were seeded on 48 well plates and incubated for 6–7 days in the presence of 10^{-8} M $VitD_3$ and 10^{-6} M PGE_2. In the single culture system of osteoclastogenesis, osteoclasts were generated by culturing bone marrow-derived macrophages (BMMs) in the presence of M-CSF and RANKL. To generate BMMs, bone marrow cells were flushed from 6-week-old ICR mice and cultured for 24 h in α-MEM/10% FBS. Nonadherent cells were collected and cultured for 3 days in the presence of 20 ng/ml M-CSF. The floating cells were discarded and

adherent cells were considered as BMMs. For osteoclastogenesis, BMMs were seeded at 1×10^6 cells/ml in α-MEM/10% FBS, and cultured in the presence of 20 ng/ml M-CSF and 200 ng/ml RANKL for 4 days. The BMM culture did not generate any osteoclasts in the absence of RANKL. In addition, no alkaline phosphatase (ALP) activity was detected in the BMM cells cultured under an osteogenic condition where calvarial osteoblast cells showed a clear ALP-positivity. Therefore, the BMM preparations used in our study do not contain contaminating osteoblasts, at least to the level enough to influence the osteoclastogenesis.

2.4. Cell proliferation assay

Cell proliferation assays were performed using the CCK-8 kit (Cell Counting Kit-8, Dojindo Molecular Technology, Japan) following the manufacturer's instruction. The principle of this assay is based on the MTT method.

2.5. RT-PCR and real-time PCR analyses

Total RNA was prepared using Trizol (Invitrogen, Carlsbad, CA) and cDNAs were synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen). One microliter of cDNA was amplified with specific primers indicated. The sequences of primers were designed for mouse genes: carbonic anhydrase II, 5′-CTCTCAGGACAATGCAGTGCTGA-3′ and 5′-ATCCAGGTCACACATTCCAGCA-3′; calcitonin receptor, 5′-CCAACTATACTCTGTGCAACG -3′ and 5′-GTAGTAGAGGGCAC-GAGTGAT-3′; MMP9, 5′-CTGTCCAGACCAAGGGTACAGCCT-3′ and 5′-GAGGTATAGTGGGACACATAGTGG-3′; TRAP, 5′-ACTTCCCCAGCCCTTACTACCG-3′ and 5′- TCAGCACATAGCC-CACACCG-3′; GAPDH, 5′-ACTTTGTCAAGCTCATTTCC-3′ and 5′-TGCAGCGAACTTTATTGATG-3′. PCR reaction was consisted of 22–30 cycles of 40 s at 94 °C, 60 s at 58 °C, and 40 s at 72 °C. The number of cycles for each gene was decided to be in the range of linear amplification through an optimization experiment. The PCR product was separated on 1.2% agarose gel and visualized by ethidium bromide staining. Quantitative real-time PCR was performed with the Applied Biosystems 7300 instrument (Applied Biosystems) in triplicates using standard curve method. The thermal cycling condition was one time 5 min incubation at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The PCR primer sequences used are as follows: carbonic anhydrase II, 5′-CT HPRT, 5′-CCTAAGATGAGCG-CAAGTTG-3′ and 5′-CCACAGGGACTAGAACACCT-3′; carbonic anhydrase 2 (CA II), 5′-TGCGGCCTTTGCTAACTTCG-3′ and 5′-GCAGAGGCGGAGTGGTCAGA-3′; calcitonin receptor (CTR), 5′-CAGCAACAACGAAGGCGAGG-3′ and 5′-CAATGCTTGGGGT-GGCTTCA-3′; matrix metallopeptidase 9 (Mmp9), 5′-AGTT-GTGGTCGCTGGGCAAA-3′ and 5′-AGGAGCGTCCCTCGAAGG-TG-3′; tartrate resistant acid phosphatase 5 (TRAP), 5′-AGATTTGTGGCTGTGGGCGA-3′ and 5′-CTGCACGGTTCTGGC-GATCT-3′. Quantitation of the amount of target in unknown samples is accomplished by measuring the fractional cycle number (Ct) using the standard curve. All quantitations were normalized to a housekeeping gene HPRT. The relative quantitation value for each target gene compared to the calibrator for that target is expressed as 2-(Ct-Cc) (Ct and Cc are the mean threshold cycle differences after normalizing to HPRT). The relative expression levels of samples are presented by semilog plot.

2.6. Western blotting

Cells were lysed in a buffer containing 20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors. The lysates (30–40 μg) were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk, the membrane was probed with anti-phospho Akt, ERK, JNK, p38, and Iκ-B. The same membranes were stripped and reprobed with anti-Akt, ERK, JNK, p38, Iκ-B, and actin.

2.7. Tartrate-resistant acid phosphatase staining

Cells were washed with PBS and fixed with 3.7% formaldehyde for 10 min. After washing with PBS, cells were incubated with 0.1% Triton X-100 for 1 min. Cells were washed, and then incubated for 40 min at 37 °C in dark with the mixture of solutions Fast Garnet GBC, sodium nitrite, naphtol AS-BI phosphoric acid, acetate, and tartrate of the Leukocyte Acid Phosphatase Assay kit (Sigma) following the manufacturer's instruction. Cells were washed with water and TRAP-positive multinucleated cells containing three or more nuclei were counted under a light microscope. The data were expressed as mean ± S.D. of triplicate samples.

2.8. Electrophoretic mobility shift assay (EMSA)

Cells were lysed in a hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) containing 0.1% NP-40 and microcentrifuged at $4000 \times g$ for 15 min. The pellet was lysed in 10 μl of a high salt buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) for 20 min on ice. After adding 40 μl of storage buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT), samples were agitated for 10 s by vortexing and then microcentrifuged at $12{,}000 \times g$ for 20 min. Eight micrograms of the nuclear extracts were incubated with approximately 20,000 cpm of ^{32}P-labeled NF-κB binding site oligomer 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (Promega) for 30 min at 20 °C. For supershift experiments, 1 μl of anti-p50 and anti-p65 antibodies were added and incubation was continued for another 30 min. Fifty-fold excess unlabeled probe was added in the negative control experiments. The samples were subjected to electrophoresis on a 6% polyacrylamide gel followed by autoradiography.

2.9. Luciferase reporter assay

To examine NF-κB activation, BMMs were plated at 5×10^5 cells/well in six-well plates. Next day, cells were infected with adenoviruses harboring NF-κB-dependent luciferase gene (Gene Transfer Vector Core, University of Iowa, IA) at 100 MOI. After incubation for 30 min, cells were washed and incubated for 24 h at 37 °C in a CO_2 incubator. Cells were then stimulated with 200 ng/ml RANKL in the absence or presence of sauchinone for 24 h. Cells were lysed in Glo Lysis Buffer

(Promega) and luciferase activity was measured using a luminometer. To examine the effect of sauchinone on RANKL-induced NFATc1 and AP-1 activation, RAW 264.7 cells were transiently transfected with luciferase reporter constructs. Transfected cells were plated in 24-well plates at a density of 1×10^5 cells/well and treated with RANKL in the absence or presence of sauchinone for 24 h. Cells were lysed in Glo Lysis Buffer and luciferase activity was measured.

2.10. Assay of intracellular reactive oxygen species (ROS)

The intracellular production of ROS was assayed as described [9]. In brief, after stimulation with RANKL, dishes of confluent cells were washed with α-MEM lacking phenol red and then incubated in the dark for 10 min in Krebs-Ringer solution containing 5 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA; invitrogen). The DCF fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 515–540 nm under the Zeiss Axiovert 135 inverted microscope (Carl Zeiss) equipped with a 20 × Neofluor objective and Zeiss LSM 410 confocal attachment. After collection of the fluorescence image, the cells were identified by digital interference contrast (DIC), and the mean relative fluorescence intensity for each group of cells was measured by Carl Zeiss vision system (KS400, version 3.0).

2.11. Ca^{2+} oscillation measurement

Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured using the fluorescent dye fura-2/AM (Molecular Probes). BMMs on non-coated glass coverslips were incubated with 30 ng/ml M-CSF and 200 ng/ml RANKL for 48 h in the presence or absence of sauchinone. To load the calcium indicator, the cells were incubated for 40 min at room temperature in culture medium containing 5 μM fura-2/AM and 0.05% pluronic F127 (Molecular Probes). After the incubation, cells were washed three times with Hank's balanced salt solution (GIBCO). The cell ensembles were illuminated at wavelengths of 340 and 380 nm, and the emitted light passed through a 510 nm interference filter was detected with an intensifier charge coupled device camera (International Ltd., Sterling, VA). Images were recorded at every 500 ms and analyzed using image analysis software (Metafluor; Universal Imaging, West Chester, PA).

2.12. Endotoxin-induced bone resorption

To study the effects of sauchinone on in vivo bone destruction, we challenged mice with LPS and administered sauchinone. ICR mice at 6 weeks of age were divided into three groups ($n = 5$ per group). The control group mice received PBS instead of LPS at day 0 and day 4. Other mice received intraperitoneal injections of LPS (5 mg/kg body weight) at day 0 and day 4. Half of the LPS-treated mice also received intraperitoneal injections of sauchinone a day before and every day after LPS injection ("LPS + Sau" group) and the other half of the LPS-treated mice received vehicle (1% DMSO) instead of sauchinone ("LPS" group). All mice were sacrificed 7 day after the first LPS injection. The left femur of each animal was scanned with a high-resolution micro-CT (Skyscan 1072 microCT system; SkyScan, Belgium). The micro-CT system used an X-ray charge-coupled device camera with a cooled 1024 × 1024-pixel and 12-bit sensor. The

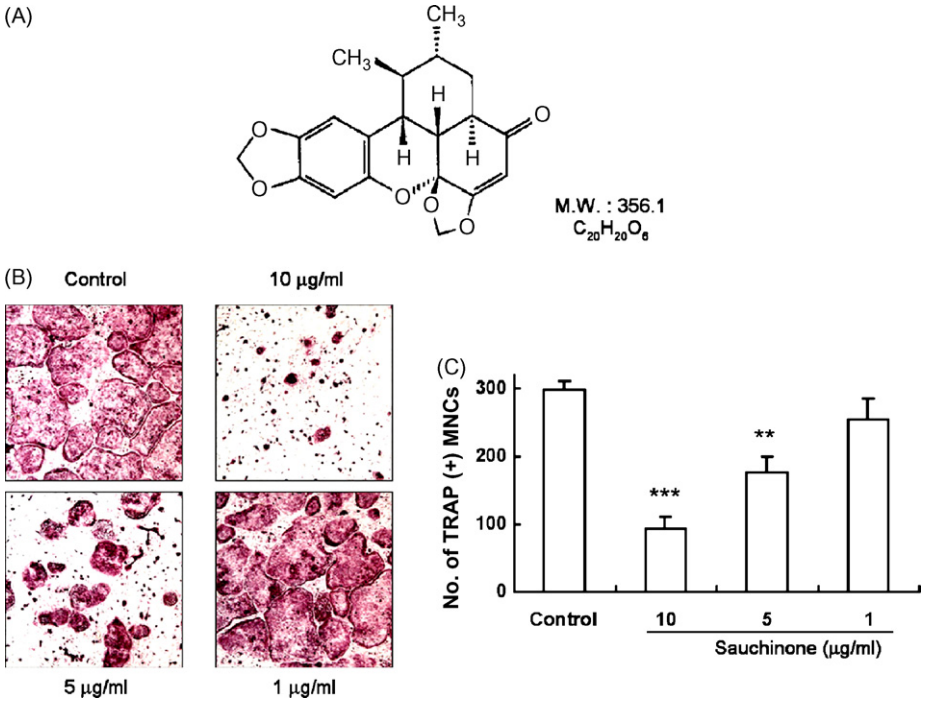

Fig. 1 – Effect of sauchinone on osteoclast differentiation in coculture system. (A) Chemical structure of sauchinone. (B and C) Bone marrow cells and osteoblasts were incubated for 6–7 days in the presence of 10^{-8} M $VitD_3$ and 10^{-6} M PGE_2. The indicated concentration of sauchinone was added to the culture. Cells were fixed and stained for TRAP (B). TRAP-positive multinuclear cells (MNC) containing three or more nuclei were counted as osteoclasts (C). $^{**}p < 0.01$; $^{***}p < 0.001$ vs. control.

right femurs were fixed in 4% paraformaldehyde, decalcified with 12% EDTA, and then embedded in paraffin. Histological sections were prepared and stained for TRAP or hematoxylin-eosin (H&E). Bone histomorphometric analyses were performed with micro-CT data and section images by using Skyscan™ CT-analyser and KAPPA ImageBase (KAPPA opto-electronics Inc., Monrovia, CA), respectively.

2.13. Statistical analysis

Each experiment was performed three to five times. All quantitative data are presented as mean ± S.D. of three to five replicates. Statistical differences were analyzed by Student's *t*-test.

3. Results

3.1. Inhibitory effects on osteoclast differentiation

We screened anti-inflammatory natural compounds to identify ones that inhibit osteoclast differentiation using coculture systems of mouse bone marrow cells and osteoblasts. In this culture system, differentiated osteoclasts with distinct features of multinucleated and TRAP-positive nature were efficiently generated (Fig. 1B). Through this culture-based screening, we found that sauchinone has an activity to suppress osteoclast formation. Sauchinone reduced the number of osteoclasts generated with 40.6 ± 6.25% inhibition at 5 μg/ml concentration (Fig. 1C). In the coculture system, osteoblasts support the differentiation of osteoclast progenitors by surface RANKL expression in response to VtD_3 and PGE_2. Thus, it is possible that sauchinone might have affected osteoblasts' ability to support osteoclastogenesis resulting in reduced osteoclast formation. To determine whether the anti-osteoclastogenic activity of sauchinone was direct to osteoclast precursor cells or indirect through the supporting osteoblasts, we selected another culture system that does not involve osteoblasts. In this single culture system, bone marrow-derived macrophages (BMMs) are induced to differentiate to osteoclasts in the presence of M-CSF and RANKL. Sauchinone reduced the formation of TRAP-positive MNC in this culture system in a dose-dependent manner (Fig. 2A and B). Almost complete inhibition of osteoclastogenesis was observed at 5 μg/ml concentration (Fig. 2B). If sauchinone exerts cytotoxicity and subsequent cell death of the precursor cells during the culture, osteoclast formation could be decreased irrespective to its effect on osteoclast differentiation program. To exclude this possibility, we performed cell proliferation assay after the culture period. Sauchinone did not inhibit cell proliferation of BMMs at the concentrations used in this study (Fig. 2C).

3.2. Effects on RANKL-induced gene regulation

Osteoclast differentiation is associated with up-regulation of specific genes in response to RANKL. We next investigated the

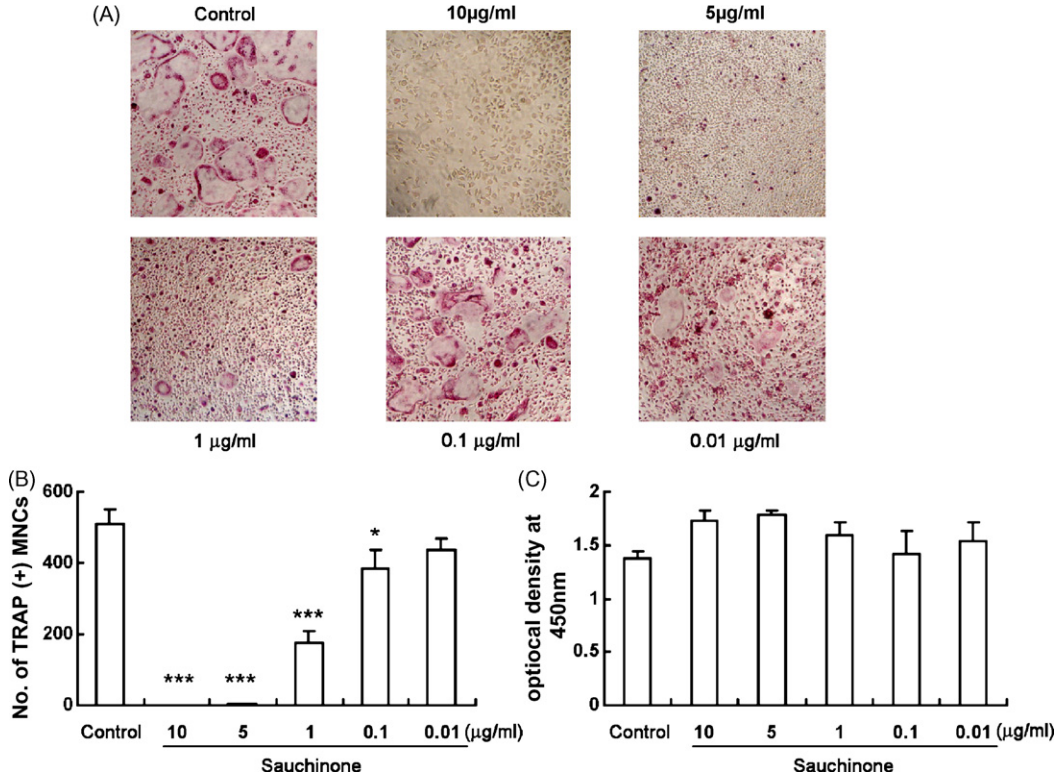

Fig. 2 – Inhibition of osteoclast differentiation by sauchinone in BMM single culture. (A) BMMs were cultured in the presence of 20 ng/ml of M-CSF and 200 ng/ml of RANKL for 4 days. The indicated concentration of sauchinone was added to the culture. Cells were fixed and stained for TRAP. (B) TRAP-positive multinuclear cells containing three or more nuclei were scored. *$p < 0.05$; ***$p < 0.001$ vs. control. (C) BMMs were cultured for 4 days as in (A). The cell proliferation was assessed by CCK-8 assay as described in Section 2.

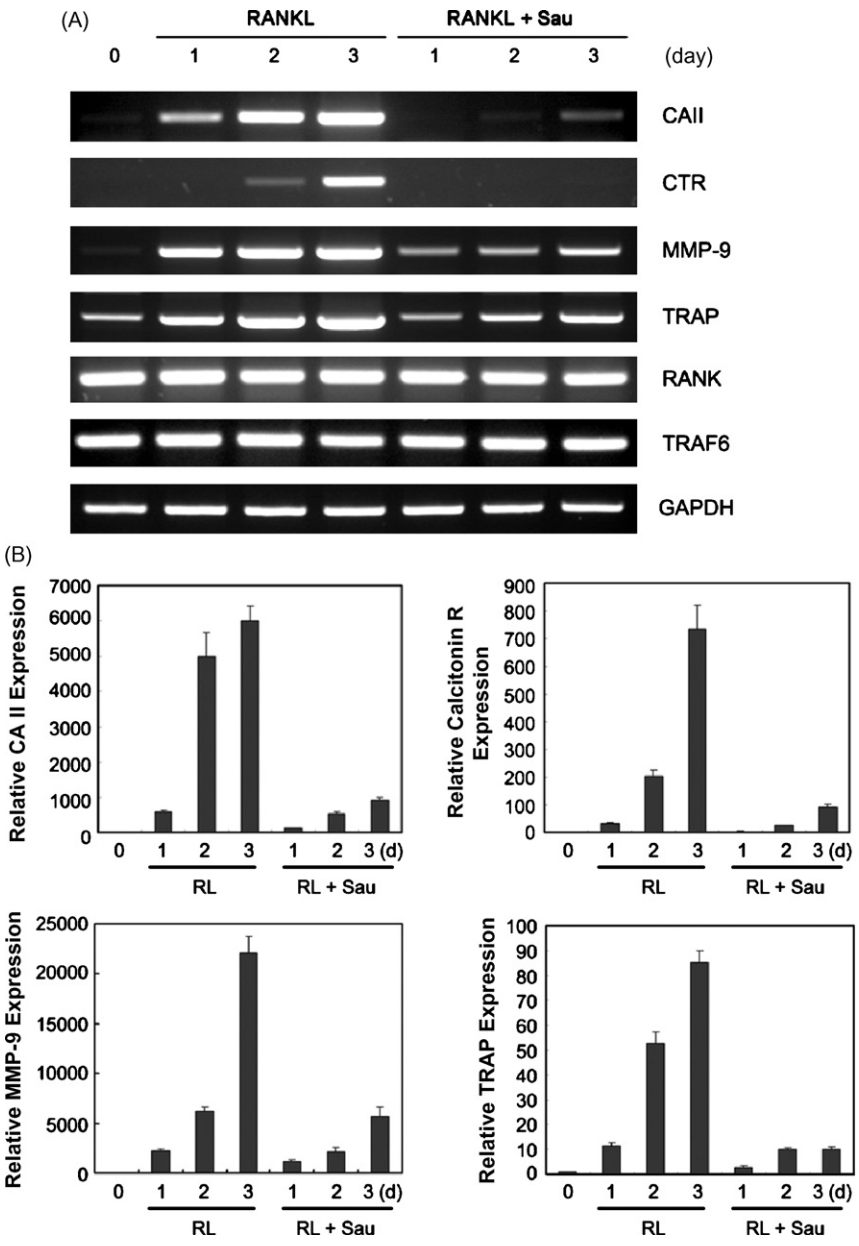

Fig. 3 – Suppression of RANKL-induced gene expression by sauchinone. BMMs were cultured with 20 ng/ml of M-CSF and 200 ng/ml of RANKL in the presence or absence of 10 μg/ml sauchinone for 3 days. The mRNA expression levels of the indicated genes were determined by RT-PCR (A) or quantitative real-time PCR (B).

effect of sauchinone on the expression of those genes. BMMs were cultured in the osteoclastogenic medium containing RANKL and M-CSF in the presence or absence of sauchinone. The expression levels of carbonic anhydrase II (CA II), calcitonin receptor (CTR), matrix metalloproteinase 9 (MMP-9), and tartrate-resistant acid phosphatase (TRAP) mRNA were assessed by semi-quantitive RT-PCR during the culture. As shown in Fig. 3A, addition of sauchinone to the culture almost completely inhibited the induction of CA II and CTR, and also strongly reduced the induction of MMP-9 and TRAP. However, sauchinone had little effects on the expression of RANK and TRAF6 (Fig. 3A). The inhibitory effect of sauchinone on CA II, CTR, MMP-9, and TRAP was also confirmed by quantitative real-time PCR (Fig. 3B). These data show that sauchinone has an effect on the regulation of some genes that are induced during osteoclast differentiation.

3.3. *Suppression of RANK signaling to ERK and p38 MAPKs*

To gain insights into molecular mechanisms by which sauchinone inhibits osteoclast differentiation, we next investigated intracellular signaling pathways engaged by RANK. Osteoclast precursor cells were stimulated with RANKL in the presence or absence of sauchinone and the MAPK signaling pathways were examined with antibodies specific for phosphorylated active MAPKs. The activation of ERK, JNK and p38 MAPKs was observed within 5 min after RANKL stimulation of

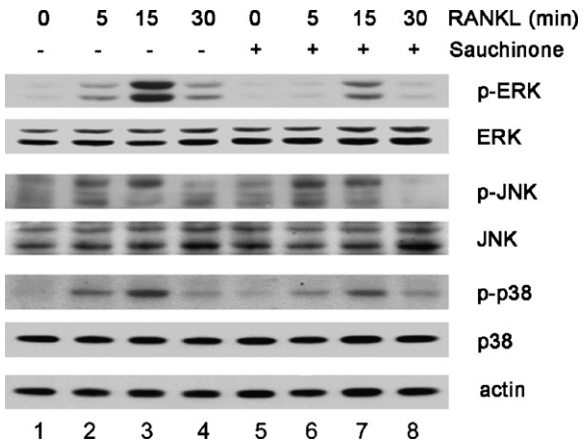

Fig. 4 – Effect of sauchinone on the activation of MAPKs by RANKL. BMMs were serum-deprived in medium containing 0.1% FBS for 5 h, pretreated with 10 μg/ml of sauchinone for 30 min, and then stimulated with 200 ng/ml of RANKL for the indicated times. Cell lysates were prepared and subjected to Western blotting with the indicated phospho-ERK, phospho-JNK, and phosho-p38 specific antibodies. The same membranes were stripped and reprobed with ERK, JNK, and p38 antibodies. The actin blot is shown as a loading control.

BMMs and the activation was maximal at 15 min after RANKL treatment (Fig. 4, lanes 1–4). The presence of sauchinone resulted in a prominent reduction in the activation of ERK by RANKL and a modest attenuation of p38 activation (Fig. 4, lanes 5–8). The activation of JNK by RANKL was not affected by sauchinone (Fig. 4, lanes 5–8).

3.4. *Interference with RANKL-induced expression and activation of NFATc1 and c-Fos*

Activated MAPKs can translocate into the nucleus and regulate various transcription factors. As the ERK activation by RANKL was most prominently affected by sauchinone (Fig. 4) and c-Fos is one the well-characterized target of ERK, we wondered whether sauchinone would influence the c-Fos regulation by RANKL in BMMs. RANKL has been shown to elevate c-Fos level in osteoclast precursor cells [12]. When BMMs were cultured in the differentiation medium containing RANKL and M-CSF for 2 days, c-Fos expression was greatly increased (Fig. 5A, top panel, lane 2). The c-Fos expression was absent in BMMs cultured in the medium containing only M-CSF (Fig. 5A, top panel, lane 1), indicating the c-Fos induction was provoked by RANKL. The presence of sauchinone decreased the extent of c-Fos induction (Fig. 5A, top panel, lanes 3–5). Because c-Fos functions as a component of the activator protein-1 (AP-1) transcription complex, we next determined the AP-1 transcription activity in cells treated with RANKL and sauchinone. For this experiment, RAW 264.7 cells, myeloid line of cells that can undergo osteoclastic differentiation upon RANKL treatment, were transfected with a luciferase reporter construct. As shown in Fig. 5B, RANKL strongly stimulated AP-1 activity while sauchinone attenuated the RANKL-dependent activation of AP-1.

RANKL also induces the expression of NFATc1 transcription factor, which has been postulated as the master gene for osteoclast differentiation [12]. NFATc1 is under the control of AP-1. Therefore, we examined effects of sauchinone on NFATc1 induction and transcription activity in RANKL-treated cells. In BMMs cultured in the presence of RANKL, the expression level of NFATc1 was greatly increased (Fig. 5A, middle panel, lane 2). Sauchinone significantly inhibited the RANKL-dependent induction of NFATc1 (Fig. 5A, middle panel, lanes 3–5). The transcription activity of NFATc1 was also suppressed by sauchinone (Fig. 5C). Recently, intracellular calcium oscillation was shown to be critical for the activation of NFATc1 in osteoclastogenesis [12]. However, the Ca^{2+} oscillation response was not affected by sauchinone (Fig. 5D).

3.5. *Inhibition of transcription activity with no effect on DNA-binding capacity of NF-κB*

Activation of the NF-κB transcription factor is an essential step for osteoclast differentiation. Mice deficient in both the p50 and p52 subunits of NF-κB fail to generate mature osteoclasts and develop severe osteoporosis [24]. Furthermore, a recent study suggested that NF-κB is another upstream transcription factor modulating NFATc1 expression [25]. Therefore, we next determined whether sauchinone affects NF-κB activation by RANKL. BMMs were stimulated with RANKL with or without sauchinone and the phosphorylation and degradation of IκB was assessed by Western blotting. The phosphorylation of IκB was observed at the maximal level at 5 min after RANKL stimulation (Fig. 6A, lanes 1–4). IκB degradation began within 5 min and reached to near completeness at 15 min after RANKL treatment (Fig. 6A, lanes 1–4). Sauchinone increased the basal level of IκB in BMMs (Fig. 6A, lane 1 versus lane 5) and appeared to have little effect on the extent of IκB degradation (Fig. 6A, lanes 5–8). We next evaluated whether the DNA binding activity of NF-κB was modulated by sauchinone. Nuclear extracts from BMMs stimulated with RANKL was incubated DNA oligonucleotides containing consensus NF-κB binding site and the DNA binding ability was assessed by gel mobility shift. The DNA binding of NF-κB was observed in BMMs stimulated with RANKL for 15 min (Fig. 6B, lane 2). Treatment of the cells with sauchinone did not inhibit the RANKL-induced NF-κB DNA binding (Fig. 6B, lane 4). The specificity of NF-κB binding was verified by the competition with excessive unlabeled probe and with p50 plus p65 NF-κB antibodies (Fig. 6B, lanes 5 and 6). Finally, we checked potential effects of sauchinone on transcriptional activity of NF-κB. BMMs were infected with adenoviruses harboring an NF-κB-dependent luciferase reporter gene and stimulated with RANKL in the presence of sauchinone. As shown in Fig. 6C, sauchinone suppressed NF-κB transcription activity in a dose-dependent manner (Fig. 6C). $63.5 \pm 7.9\%$ inhibition was achieved by 5 μg/ml sauchinone. These data indicate that sauchinone inhibited the RANKL-induced NF-κB transcription activity in a manner irrespective of IκB degradation and NF-κB DNA binding.

3.6. *Effects on ROS generation stimulated by RANKL*

Reactive oxygen species (ROS) play a role as a second messenger in the various receptor signaling pathways [26].

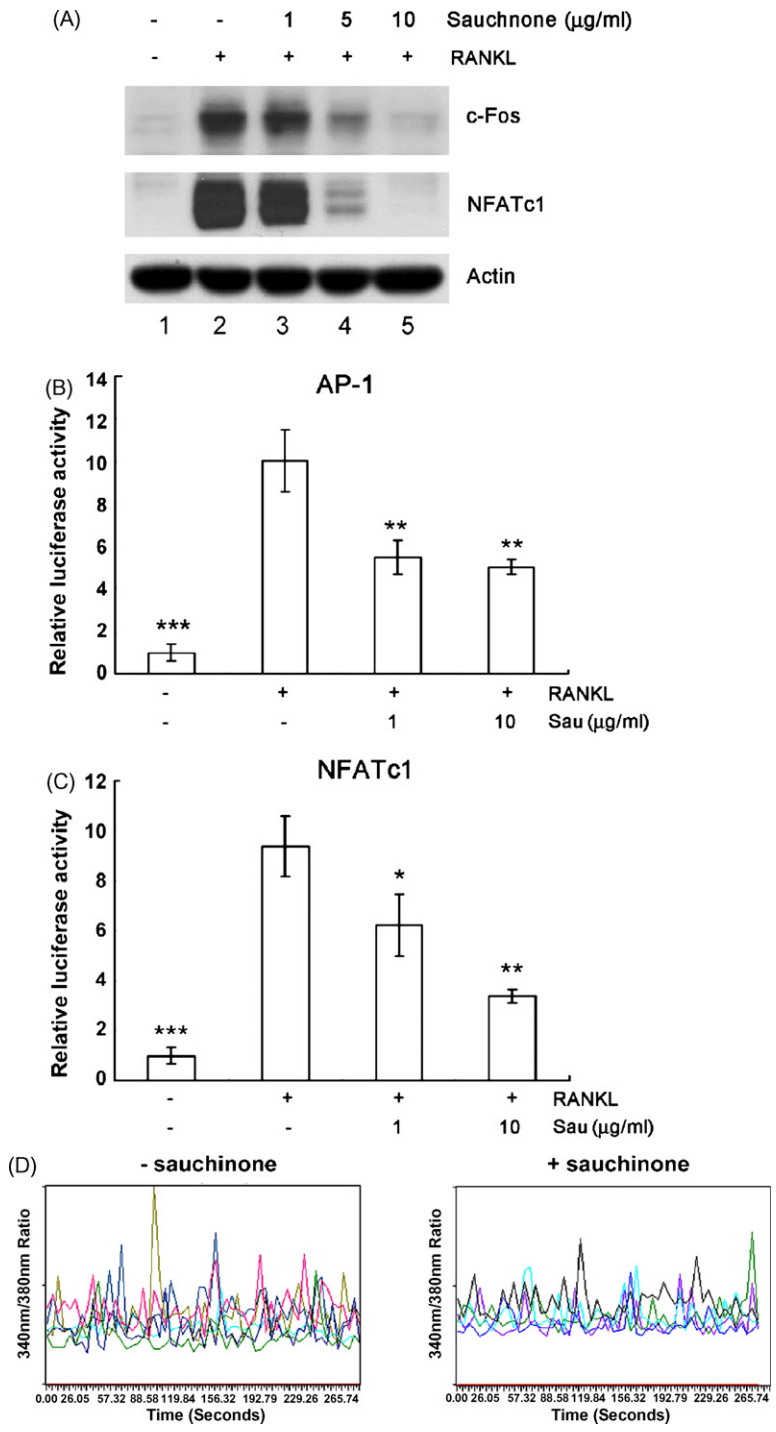

Fig. 5 – Inhibition of NFATc1 and c-Fos expression and activity by sauchinone. (A) BMMs were cultured in the presence of indicated concentration of sauchinone with 20 ng/ml of M-CSF plus 200 ng/ml of RANKL for 2 days. Cell lysates were prepared and subjected to Western blotting with the indicated antibodies. (B and C) RAW 264.7 cells transfected with an AP-1- or NFATc1-dependent luciferase reporter gene construct were treated with RANKL (200 ng/ml) in the absence or presence of sauchinone for 24 h. Cells were lysed and luciferase activity was measured. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. RANKL control. (D) BMMs were cultured in the presence of 10 μg/ml sauchinone with 20 ng/ml of M-CSF plus 200 ng/ml of RANKL for 2 days. Ca^{2+} oscillation was measured as described in Section 2.

In recent reports, ROS was shown to be increased in response to RANKL and act as an intracellular mediator for ERK signaling pathway in osteoclast differentiation and activation [9,27]. In addition, several transcription factors including NF-κB are sensitive to redox state of cells [28]. Therefore, we tested whether sauchinone would affect ROS generation by RANKL in osteoclast precursor cells. Intracellular ROS was measured using the cell-permeant, oxidation-sensitive dye DCFH-DA by

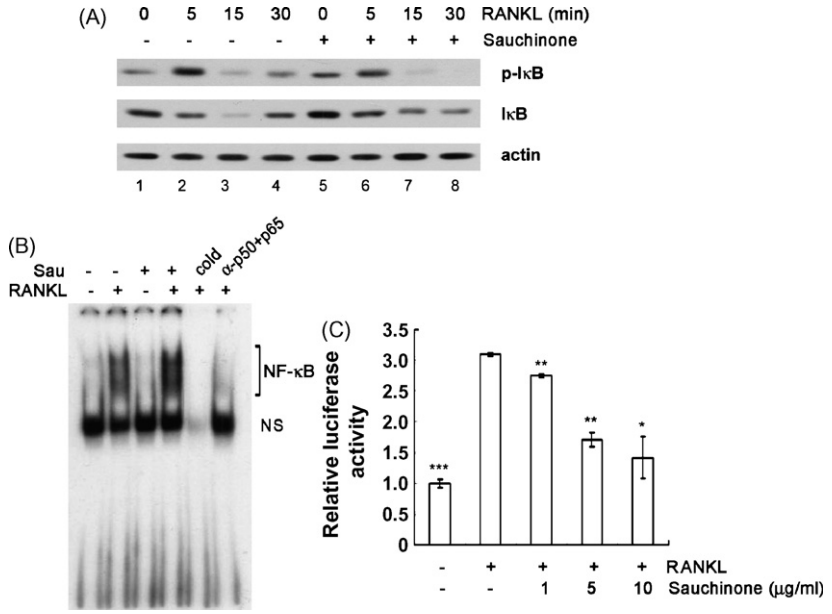

Fig. 6 – Interference with NF-κB transcription activity by sauchinone. (A) BMMs were serum-starved in medium containing 0.1% FBS for 5 h, pretreated with 10 μg/ml of sauchinone for 30 min, and then stimulated with 200 ng/ml of RANKL for the indicated times. Cell lysates were prepared and subjected to Western blotting with phospho-IκB antibody. The membrane was stripped and reprobed with anti-IκB and anti-actin antibodies. (B) BMMs were serum-starved for 5 h, pretreated with 10 μg/ml sauchinone for 30 min, and then stimulated with 200 ng/ml of RANKL for 15 min. Nuclear extracts were prepared and EMSA was performed as described in Section 2. (C) BMMs were infected with adenoviruses harboring NF-κB-dependent luciferase gene and then stimulated with 200 ng/ml RANKL in the absence or presence of sauchinone for 24 h. Cells were lysed and luciferase activity was measured. $^{**}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. RANKL control.

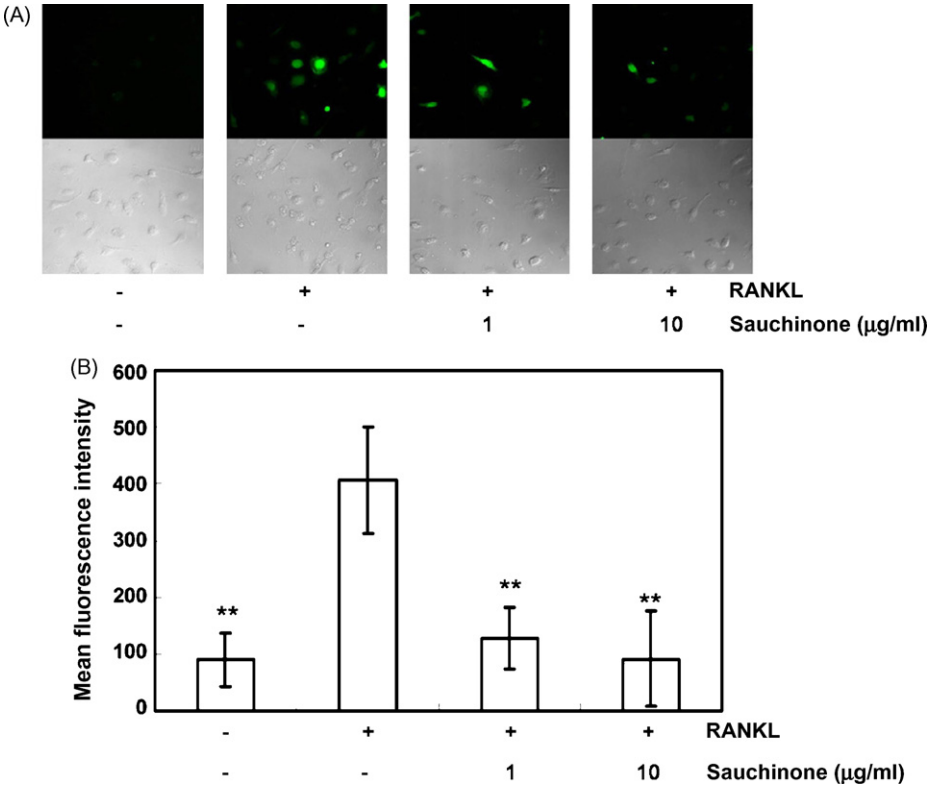

Fig. 7 – Decrease in RANKL-induced ROS production by sauchinone. (A) BMMs were pretreated with different concentrations of sauchinone for 30 min, preloaded with DCFH-DA, and treated with RANKL (100 ng/ml) for 10 min. DCF fluorescence was detected with a confocal microscope. The mean fluorescence intensity was measured by the confocal system. $^{**}p < 0.01$ vs. RANKL control.

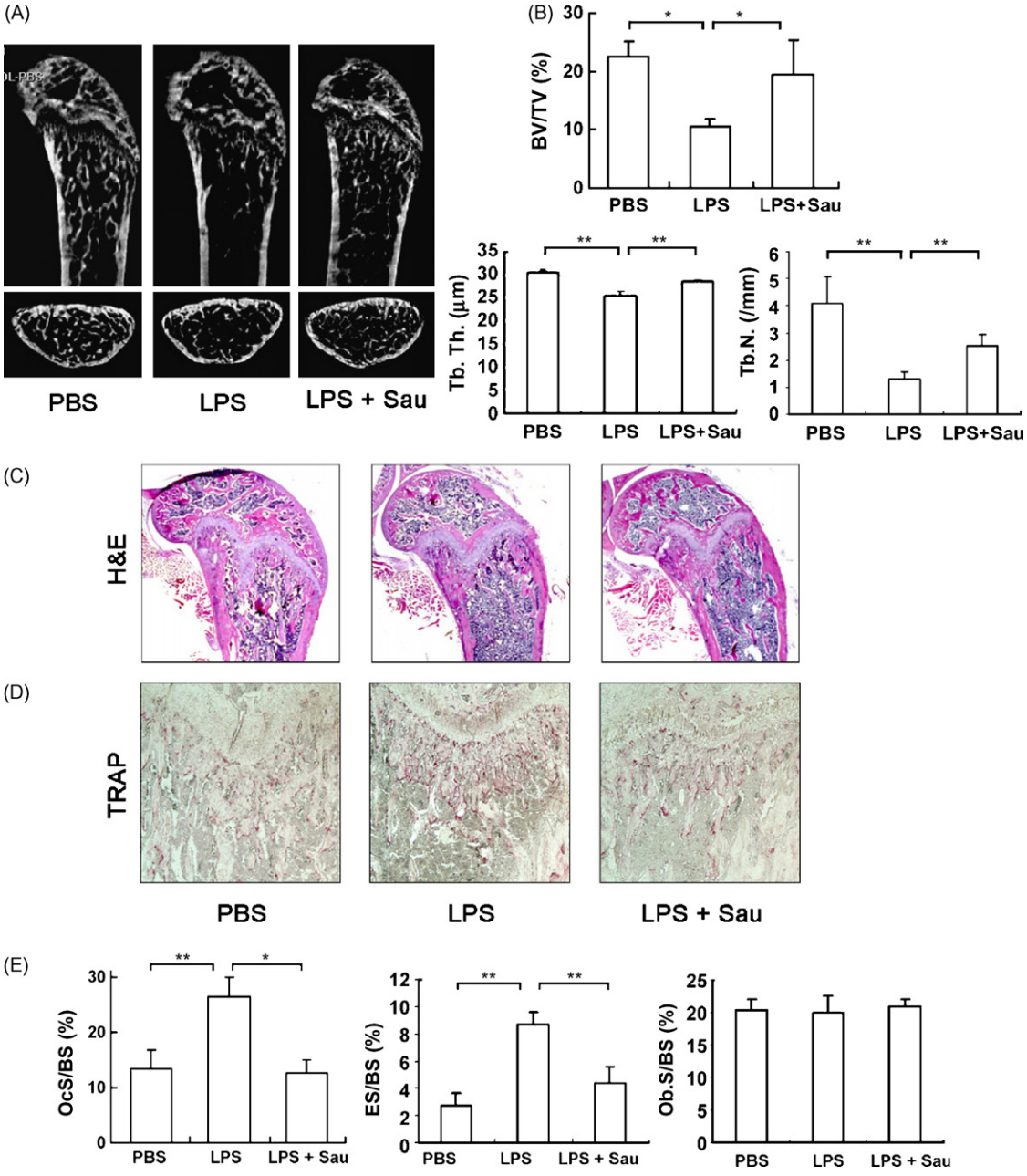

Fig. 8 – In vivo effects of sauchinone on LPS-induced bone destruction. Mice received i.p. injections of LPS or PBS on days 0 and 4. Sauchinone or vehicle was administered for 8 days as described in Section 2. (A and B) The distal metaphysis of femur was scanned with a micro-CT. *$p < 0.05$ between indicated groups. (C) Femurs were decalcified and embedded in paraffin. The paraffin blocks were sectioned and stained with hematoxylin and eosin (H&E). (D) The femur sections were stained for TRAP and counter-stained with methyl green to identify osteoclasts. (E) Percentage of osteoclast surface (OcS) per bone surface (BS), eroded surface (ES) per BS, osteoblast surface (Ob.S) per BS were analyzed in the TRAP- and H&E-stained sections. *$p < 0.05$; **$p < 0.01$ between indicated groups.

confocal microscopy. In BMMs, ROS was increased by stimulation with RANKL and its increase was inhibited by sauchinone (Fig. 7).

3.7. *Suppression of endotoxin-induced bone resorption in vivo*

Next we explored whether the in vitro effect of sauchinone on osteoclast differentiation could be confirmed in in vivo context. For this experiment, an animal model for endotoxin-induced bone destruction was employed. Mice were challenged with LPS and treated with sauchinone. Micro-computer tomography analyses revealed prominent reduction in bone trabecules in the femur of LPS-challenged mice, whereas the reduction was much lower in sauchinone-treated mice (Fig. 8A). The BV/TV decreased by $53.2 \pm 4.3\%$ in LPS-injected mice while no significant reduction was observed in mice injected with LPS plus sauchinone (Fig. 8B). The

LPS-induced decrease in trabecular thinckness (Tb.Th.) and trabecular number (Tb.N.) were also attenuated by sauchinonr (Fig. 8B). The decrease in bone volume indicates that bone resorption by osteoclasts was stimulated. The histological sections of the femur stained for H&E also reveal the protective effect of sauchinone on trabecular bone loss induced by LPS (Fig. 8C). To assess osteoclast differences in bone, the sections were also stained for TRAP. Percentage of osteoclast surface (OcS) and eroded surface (ES) in trabecular bone near growth plate of femur was significantly increased in the LPS-treated mice and this increase was blocked in sauchinone-treated mice (Fig. 8D and E). In contrast, osteoblast surface (Ob.S) was not affected (Fig. 8E). Based on these observations, we conclude that sauchinone has inhibitory effect on osteoclastogenesis and subsequent bone resorption in vivo.

4. Discussion

Osteoclasts are tissue-specific multinucleated cells generated from the differentiation of monocyte/macrophage precursors. The TNF family cytokine RANK ligand (RANKL) is critical for osteoclast precursor differentiation to multinucleated osteoclasts and is increased on osteoblasts in response to several factors. It is also essential for the activation and survival of osteoclasts. M-CSF is required for proliferation, survival, and the expression of RANK in osteoclast precursors. The interaction of RANKL and RANK results in the activation of various signaling cascades during osteoclast development and activation. Molecular genetic studies have identified that several transcription factors including NF-κB, c-Fos and NFATc1 are essential for osteoclast differentiation [7,29].

Several reports have shown that bone destruction was associated with inflammatory diseases such as periodontitis and rheumatoid arthritis. Inflammatory cytokines and prostaglandins up-regulates RANKL in osteoblasts, synovial fibroblasts and activated T cells. The enhanced RANKL expression then stimulates osteoclast formation, resorption activity, and survival [30]. Therefore, we screened for anti-osteoclastogenic components from a focused pool of anti-inflammatory compounds. Sauchinone isolated from *S. chinensis* (Saururaceae) displayed inhibitory effects on osteoclast differentiation (Fig. 1). The BMM single culture system where osteoclast formation is governed by soluble RANKL without supporting osteoblasts, we also observed even more potent inhibitory effect of sauchinone on osteoclast formation (Fig. 2). These observations indicate that the anti-osteoclastogenic activity of sauchinone is direct to the precursor cells rather than being indirect via affecting osteoblasts' function for supporting osteoclast differentiation.

In experiments designed to include sauchinone only for certain duration of the whole culture period, we found that the effect of sauchinone was weak when it was included in the late stage of differentiation (Supplementary Fig. 1). This finding suggests that sauchinone exerts its effect mainly during the early stage of osteoclast differentiation from BMMs. During the differentiation process, osteoclast specific or selective markers, such as carbonic anhydrase II, calcitonin receptor, MMP-9, and TRAP, are profoundly up-regulated. In the BMM culture system, expression of those genes was abolished or greatly attenuated in the presence of sauchinone (Fig. 3). However, the expression of RANK and TRAF6 was not inhibited by sauchinone (Fig. 3), supporting that the effect of sauchinone was specific.

Upon binding of RANKL to RANK, various intracellular signaling pathways involving ERK, JNK, p38, and Akt/protein kinase B (PKB) are activated in osteoclast precursor cells and differentiating osteoclasts [7]. An intricate interplay of these pathways appears to determine specific changes in gene expression and cytoskeletal reorganization for differentiation, function, and survival of osteoclasts. Akt primarily plays a role in promoting osteoclast survival [31]. In our study, the phosphorylation of Akt induced by RANKL was suppressed by sauchinone (Supplementary Fig. 2). Therefore, we tested osteoclast survival by measuring apoptosis. In mature osteoclasts, osteoclast survival was not inhibited by sauchinone (Supplementary Fig. 3). Thus, it is further likely that sauchinone has inhibitory effect on osteoclast precursor cells rather than differentiating or mature osteoclasts. It was reported that BMMs derived from JNK-1 deficient mice, but not from JNK-2 deficient mice, had reduced osteoclastogenesis, suggesting that JNK-1 is more important than JNK-2 for osteoclast differentiation [32]. In the present study, the activation of both JNK-1 and JNK-2 by RANKL was not affected by sauchinone (Fig. 4). The other two MAPK family members (ERK and p38) are also involved in osteoclast differentiation and/or survival. ERK plays a functional role not only in osteoclast differentiation but also in survival [31,33]. In contrast, p38 was shown to be only involved in osteoclastogenesis [33,34]. In our study, sauchinone suppressed the activation of both ERK and p38 in response to RANKL (Fig. 4). Collectively, the ERK and p38 MAPKs seem to be major signaling pathways targeted by sauchinone for its osteoclastogenesis inhibitory activity.

Several transcription factors have been suggested to mediate induction of genes implicated in osteoclastic differentiation in response to RANKL. The NF-κB transcription factor is the first one characterized to be activated downstream of RANK. Interestingly, RANK can activate NF-κB through both the canonical pathway involving IκB phosphorylation and subsequent degradation, and the noncanonical pathway involving NIK-mediated p100 processing [11,35]. Sauchinone decreased the transcriptional activation of NF-κB induced by RANKL without affecting the degradation of IκB nor DNA binding of NF-κB (Fig. 5). The transcription activity of NF-κB can also be modulated by phosphorylation of NF-κB proteins themselves [36]. In deed, we have previously shown that the TAK1-MKK6-p38 pathway regulates p65 phosphorylation at Ser-536 and NF-κB activity in RANKL-stimulated BMMs [37]. As sauchinone reduced RANKL activation of p38, it is possible that the inhibition of NF-κB transcription activity by sauchinone might have involved p65 phosphorylation. NFATc1 is transcriptional factor involved in T cell maturation and has been reported recently as the master switch regulator of osteoclast formation and function [12]. Recently, it was reported that NFATc1 is also a target of p38 MAPK signaling initiated by RANK [38]. Therefore, p38 may be involved in the sauchinone-induced suppression of both NF-κB and NFATc1 in the RANKL-stimulated osteoclast precursor cells. The

RANKL-induced calcium oscillation response, which elicits calcineurin-mediated NFAT activation, was not affected by sauchinone (Fig. 5D). Sauchinone also inhibited the induction of c-Fos the activation of AP-1 by RANKL in osteoclast precursor cells. c-Fos is activated upon phosphorylation by activated ERK and forms the AP-1 complex together with a member of Fos or Jun families. AP-1 in turn regulates c-Fos and other target gene expression. Therefore, the effect of sauchinone on c-Fos and AP-1 may be attributed to its inhibition of ERK activation by RANKL. The suppressive effect of sauchinone on these multiple transcription factors are likely to contribute to its potent inhibition of osteoclast differentiation.

Recent reports have shown that in BMMs stimulated with RANKL, intracellular level of ROS was increased through RANKL-TRAF6-Rac1-NADPH oxidase (Nox) 1 pathway [9]. The generated ROS act as an intracellular signal mediator for the activation of MAPKs by RANKL in osteoclast differentiation [9]. However, the precise mechanisms that RANKL-induced ROS activates MAP kinases have not been elucidated. The RANKL-stimulated ROS production was confirmed in our study and sauchinone was found to decrease the ROS production (Fig. 7). In line with the role of ROS for RANKL signaling, antioxidants, such as *N*-acetyl-L-cystein and glutathione, have been shown to inhibit RANKL-induced activation of Akt, ERK, and NF-κB [27]. In addition, homocystein that increased intracellular ROS level also enhanced RANKL-induced osteoclast formation, which was blocked by SB203580, a p38 MAPK inhibitor [39]. The inhibition of osteoclast differentiation by sauchinone shown in the present study, therefore, may reflect its anti-oxidant activity, which attenuated the activation of ERK and p38 MAPKs and subsequent stimulation and induction of NF-κB, NFATc1, and c-Fos transcription factors.

Previously, sauchinone was reported to inhibit LPS-induced iNOS, TNF-α and COX-2 expression in vitro [22]. LPS, a cell wall component of Gram-negative bacteria, induces fusion of preosteoclasts, supports the survival of mature osteoclast, and stimulates osteoclastic bone resorption by provoking production of inflammatory factors [40]. ROS has been implicated in the stimulation of inflammatory cytokine production by LPS [41,42]. In our in vivo test, sauchinone could reduce osteoclast formation and bone loss induced by LPS (Fig. 8). It is possible that the anti-oxidant property of sauchinone might have reduced both LPS-stimulation of inflammatory cytokine production and elevation in osteoclast generation. The in vivo result enhances the probability of its successful application to diseases with inflammatory bone destruction.

In this study, we showed that sauchinone has inhibitory effect on osteoclastogenesis from primary precursor cells and provided molecular mechanisms for the inhibition that involved ROS, MAPKs, and transcription factors (NF-κB, NFATc1 and c-Fos) downstream of the osteoclast differentiation factor receptor RANK. Furthermore, the in vivo efficacy of sauchinone was confirmed by using an animal model of inflammatory bone loss. Therefore, we suggest the possibility of developing sauchinone or its derivatives for potential use in preventing and treating inflammatory bonelytic diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.06.044.

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