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ORIGINAL ARTICLE

Treatment with hydrogen molecules prevents RANKL-induced osteoclast differentiation associated with inhibition of ROS formation and inactivation of MAPK, AKT and NF-kappa B pathways in murine RAW264.7 cells

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Abstract The bone protective effects of the hydrogen molecule (H_2) have been demonstrated in several osteoporosis models while the underlying molecular mechanism has remained unclear. Osteoclast differentiation is an important factor related to the pathogenesis of bone-loss related diseases. In this work, we evaluated the effects of incubation with H_2 on receptor activator of NFκB ligand (RANKL)-induced osteoclast differentiation. We found that treatment with H_2 prevented RANKL-induced osteoclast differentiation in RAW264.7 cells and BMMs. Treatment with H_2 inhibits the ability to form resorption pits of BMMs stimulated by RANKL. Treatment with H_2 reduced mRNA levels of osteoclast-specific markers including tartrate resistant acid phosphatase, calcitonin receptor, cathepsin K, metalloproteinase-9, carbonic anhydrase typeII, and vacuolar-type H^+-ATPase. Treatment with H_2 decreased intracellular reactive oxygen species (ROS) formation, suppressed NADPH oxidase activity, down-regulated Rac1 activity and Nox1 expression, reduced mitochondrial ROS formation, and enhanced nuclear factor E2-related factor 2 nuclear translocation and heme oxygenase-1 activity. In addition, treatment with H_2 suppressed RANKL-induced expression of nuclear factor of activated T cells c1 and c-Fos. Furthermore, treatment with H_2 suppressed NF-κB activation and reduced phosphorylation of p38, extracellular signal-regulated kinase, c-Jun-N-terminal kinase, and protein kinases B (AKT) stimulated with RANKL. In conclusion, hydrogen molecules prevented RANKL-induced osteoclast differentiation associated with inhibition of reactive oxygen species formation and inactivation of NF-κB, mitogen-activated protein kinase and AKT pathways.

Keywords Hydrogen molecule · Osteoclast differentiation · Receptor activator of NFκB ligand · Reactive oxygen species · Cell signaling

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Introduction

Recently, it has been proven that hydrogen gas (H_2), a highly flammable gas, had amazing antioxidant property [1] and exerted beneficial effect on bone tissue. In rodents, administration with H_2 attenuated bone loss induced by ovariectomy [2] and modeled microgravity-'hind-limb suspension' [3] and attenuated experimental periodontitis [4]. In patients, consumption of water containing H_2 improved symptoms of rheumatoid arthritis [5]. However, the underlying molecular mechanism of beneficial effect of H_2 on bone tissue remained elusive.

Normal bone remodeling requires a homeostatic balance between the activities of bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoclasts participate in bone

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remodeling by removing the bone's mineralized matrix resulting in bone resorption. It is well known that excessive osteoclast formation is the cause of pathological bone diseases such as rheumatoid arthritis, periodontal disease, and osteoporosis [6]. Receptor activator of NF-κB (nuclear factor-κB) ligand is a key factor stimulating the differentiation and activation of osteoclasts and, therefore, is essential for bone remodeling [7]. Binding of RANKL to its receptor, receptor activator of nuclear factor κB (RANK), begins the induction of TNF receptor-associated factors and activates multiple downstream signaling pathways such as NF-κB, AKT, c-JUN n-terminal kinases (JNK), p38 MAP kinases (p38) and extracellular signal-regulated kinase (ERK) [8–10], which are required for osteoclast differentiation. Reactive oxygen species, generated by stimulation with RANKL, served to regulate RANKL signaling pathways including AKT, MAPK and NF-κB [11, 12], and played an important role in the RANKL-induced osteoclast differentiation.

In this study, we evaluated the effect of H_2 on the RANKL-induced osteoclast differentiation in RAW264.7 cells and BMMs and further investigated the underlying mechanism in RAW264.7 cells stimulated with RANKL.

Materials and methods

Materials and cell culture

Unless otherwise specified, regents were purchased from Sigma-Aldrich (St Louis, MO, USA).

The murine monocyte/macrophage cell line RAW264.7 was used as osteoclast precursor cells. RAW cells differentiate into osteoclast-like cells in the presence of receptor activator of NFκB ligand (RANKL). The cells were grown in DMEM, supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2.

Bone marrow cells were prepared as previously described by Hu et al. [13]. For the experiments of osteoclastogenesis in bone marrow macrophages (BMMs), freshly prepared bone marrow cells were incubated with α-MEM containing 10 % FBS in the presence of 10 ng/ml macrophage colony stimulating factor (M-CSF) for 1 day. The non-adherent cells were collected and counted, and replated with 50 ng/ml M-CSF. After 2 days, non-adherent cells were washed out with fresh media, and the adherent cells were used as BMMs.

Purified osteoclasts

The osteoblasts were isolated from mouse calvariae immediately after dissection. Bones were washed in PBS containing 4 mM EDTA for 10 min at 37 °C and then incubated in a HEPES buffer solution (25 mM HEPES, pH 7.4, 70 mM NaCl, 30 mM KCl, 10 mM $NaHCO_3$, 1.5 mM K_2HPO_4, 1 mM $CaCl_2$, 60 mM sorbitol, 27.8 mM D-(+)-glucose, and 1 mg/ml BSA) containing 2 mg/ml collagenase and 90 μM N-α-tosyl-L-lysyl chloromethyl ketone for three sequential 20-min digestion periods at 37 °C in a shaking water bath. At the end of each digestion, released cells were collected and resuspended in the HEPES buffer also containing 1 mM $MgSO_4$, and all three digests were pooled for plating on 60-mm Primaria culture dishes (Falcon, Becton–Dickinson). Medium was changed every 2–3 days.

Preparation of hydrogen-rich medium (HRM)

H_2 was dissolved into medium (H_2, 0.97 ± 0.15 mg/l) according to method described by Ohsawa et al. [1]. We dissolved O_2 into a second medium by bubbling O_2 gas at the saturated level, and CO_2 into a third medium by bubbling CO_2 gas. All three media were maintained at atmospheric pressure. Then, we combined the three media (H_2 medium:O_2 medium:CO_2 medium) in the proportion 75:20:5 % (vol/vol/vol). For culture, we put the combined medium into multiwell plates seeded with cells in the closed glass culture flask. Then we filled the culture flask with mixed gas consisting of 75 % H_2, 20 % O_2 and 5 % CO_2 (vol/vol/vol), which was re-gassed every 12 h. The medium was changed daily. HRM was freshly prepared every week, which maintained a continuous concentration. As control, the control medium underwent a similar operation to those in the HRS, except that the H_2 was changed to N_2. The pH of the culture media without hydrogen gas was 7.38 ± 0.09, and that of the culture media with hydrogen gas was 7.44 ± 0.07.

Study design

RAW cells (0.5×10^4 cells/well) were cultured on 48-well plates in hydrogen-rich medium or not, in the presence of RANKL (50 ng/ml). After 6 days of culture, the number of osteoclasts was counted. BMMs (2×10^4 cells/well) were cultured on 48-well plates in hydrogen-rich medium or not, in the presence of M-CSF (30 ng/ml) and RANKL (50 ng/ml). After 6 days of culture, the number of osteoclasts was counted.

BMMs (2×10^4 cells/well) were cultured on 48-well tissue culture plates containing one 5-mm dentin disk in each well in hydrogen-rich medium or not, in the presence of RANKL (50 ng/ml). After 14 days of culture, pit formation assay was performed.

Purified osteoclasts (2×10^4 cells/well) were cultured on 48-well tissue culture plates containing one 5-mm

dentin disk in each well in hydrogen-rich medium or not, in the presence of RANKL (50 ng/ml) for 12, 24, or 36 h. TRAP positive multinuclear cells containing more than three nuclei were counted as surviving osteoclasts.

RAW cells (5×10^4 cells/well) were cultured on 6-well plates in hydrogen-rich medium or not, in the presence of RANKL (50 ng/ml). After 6 days of culture, intracellular ROS formation and NO formation were detected; total RNA was extracted for measurement of mRNA levels of tartrate resistant acid phosphatase (TRAP), calcitonin receptor (CalcR), cathepsin K (Cath K), metalloproteinase-9 (MMP9), carbonic anhydrase type II (Car2), and vacuolar-type H+ATPase (H+-ATPase) by RT-PCR; total protein was collected for measurement of proteins expression of Nox1, Rac1, NFATc1, c-Fos, and iNOS; the nucleus proteins were separated for measurement of proteins expression of Nrf2 and Lamin B1. NADPH oxidase activity, HO-1, and iNOS activity were determined. Mitochondria were isolated by differential centrifugation of cellular homogenates for measurement of ROS formation.

RAW cells (5×10^4 cells/well) were cultured on 6-well plates in hydrogen-rich medium or not, in the presence of RANKL (50 ng/ml). After 30 min of culture, total protein was collected for measurement of proteins expression of P-p38, P-ERK, P-JNK, p38, ERK, JNK by Western blotting analysis.

RAW cells were seeded on 24-well culture plates at 2×10^4 cells/well. After transfected with NF-κB-dependent luciferase reporter, cells were cultured in hydrogen-rich medium or not, in the presence of RANKL (50 ng/ml). After 24 h of culture, NF-κB activity was measured.

The in vitro experiments were performed at least three times and each experiment was performed with replicates.

Cytotoxicity assay

The cytotoxic effect of H_2 was evaluated using methyl thiazolyl tetrazolium (MTT). RAW264.7 cells and BMMs were seeded into 96-well plates and treated with H_2 in the presence or absence of RANKL (50 ng/ml) for 72 h. For the MTT assay, 20 ml of MTT solution (5 mg/ml) was added and the cells were continuously incubated for an additional 4 h. Subsequently, the supernatants were removed, and the formazone crystals were dissolved using 150 μl of DMSO. The optical absorbance at 570 nm was read.

TRAP staining

Cells cultured for a given period were washed with PBS and fixed in 10 % neutral formalin. They were then washed with distilled water and stained with Fast Red Violet LB Salt. After washing, TRAP positive cells with more than three nuclei were considered to be osteoclast-like cells and osteoclasts with 8 nuclei or more were considered to be large osteoclasts.

Osteoclast pit formation

Bone marrow cells (BMMs) were cultured, as described previously, in 48-well tissue culture plates containing one 5-mm dentin disk in each well. The cells were stimulated with RANKL (50 ng/ml) and treated with H_2 for 14 days. After the culturing period, cells were removed from the dentine slices by sonication in 0.2-M ammonium hydroxide, stained in hematoxylin for 40 s and washed in distilled water. The surface of each dentine slice was examined by light microscopy. The total pit areas were measured by an image analysis system (LEICA QUIPS, LEICA Imaging Systems, England). The ratio of resorption area to total area was quantified. The final results were normalized to the result of the RANKL group.

Survival assay of purified osteoclasts

Primary osteoblasts and bone marrow cells were co-cultured in 100-mm tissue culture dishes precoated with type I-collagen gel in the presence of $1\alpha,25(OH)_2D_3$ ($10-^8$ M). After the cells were cultured for 7 days, all cells were recovered from the dishes by treatment with 0.2 % collagenase. To purify osteoclasts the crude osteoclast preparation was plated in 100-mm tissue culture dishes. After the cells were cultured for 6 h, osteoblasts were removed by treatment of cells with PBS containing 0.001 % Pronase E and 0.02 % EDTA for 5 min. The purity of osteoclasts in this preparation was about 95 %. For the osteoclast survival assay, purified osteoclasts were further incubated for the indicated periods in the presence or absence of test chemicals and stained for TRAP as described. TRAP-positive multinuclear cells containing more than three nuclei were counted as viable osteoclasts.

Measurement of reactive oxygen species (ROS) production

RAW cells (5×10^3 cells/well) were cultured on 96-well plates. Before the treatment, culture medium was replaced with phenol-red-free DMEM containing 2′, 7′-Dichlorodihydrofluorescein diacetate (10 μmol/l) for 30 min. The ROS production was measured with a fluorescence reader after treatment.

Quantitative real-time PCR analysis (qRT-PCR)

The cells were transferred into a tube containing Trizol (Life Technologies Inc., Gaithersburg, USA) and total RNA was isolated, according to the manufacturer's

Table 1 Oligonucleotide primer pairs used for real-time RT-PCR

Target gene	Sequence (5′-3′)	GenBank ID
TRAP		
Sense	CGATCACAATCTGCAGTACC	NM_007388.2
Antisense	ACCCAGTGAGTCTTCAGTCC	
CalcR		
Sense	TGGTGCGGCGGGATCCTATAAGT	NM_001042725
Antisense	AGCGTAGGCGTTGCTCGTCG	
MMP9		
Sense	GCTGACTACGATAAGGACGGCA	NM_013599.2
Antisense	GCGGCCCTCAAAGATGAACGG	
CathK		
Sense	GCGTTGTTCTTATTCCGAGC	NM_007802.3
Antisense	CAGCAGAGGTGTGTACTATG	
Car2		
Sense	CTCTGCTGGAATGTGTGACCTG	NM_009801.4
Antisense	CTGAGCTGGACGCCAGTTGTC	
H^+-ATPase		
Sense	ACGGTGATGTCACAGCAGACGT	NM_175406.3
Antisense	CCTCTGGATAGAGCCTGCCGCA	
18S		
Sense	CTGCCGTCTGAGTGTATCGC	X00686
Antisense	GCTGGGGCTGAGGAAAGTG	

TRAP tartrate resistant acid phosphatase, *CalcR* calcitonin receptor, *Cath K* cathepsin K, *MMP9* metalloproteinase-9, *Car2* carbonic anhydrase type II, *H^+-ATPase* vacuolar-type H^+ ATPase

protocol. RT-PCR analysis was performed with a QuantiTectTM SYBR® Green PCR (Tiangen, Shanghai, China) according to the manufacturer's instructions. The sequences of primers are listed in Table 1. The highly specific measurement of mRNA was carried out for tartrate resistant acid phosphatase (TRAP), calcitonin receptor (CalcR), cathepsin K (Cath K), metalloproteinase-9 (MMP9), carbonic anhydrase type II (Car2), vacuolar-type H^+ ATPase (H+-ATPase) and 18S using the LightCycler system (Bio-Rad, Carlsbad, USA). Each sample was run and analyzed in duplicate. Target mRNA levels were adjusted as the values relative to 18S, which was used as the endogenous control to ensure equal starting amounts of cDNA. When comparison between two groups was performed, the control group was used as the calibrator with a given value of 1, and the other groups were compared with this calibrator.

Measurement of mitochondrial ROS production

Mitochondria were isolated by differential centrifugation of cellular homogenates. Mitochondrial protein concentration was determined using a DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Mitochondrial ROS production was evaluated by lucigenin chemiluminescence. The final results were corrected for protein content.

Measurement of NF-κB activity

NF-κB activity was determined using the NF-κB luciferase assay. RAW264.7 cells (2×10^4 cells/well) were seeded on 24-well culture plates. Cells were incubated for 1 h with a total of 170 ng plasmids (Promega, Madison, WI, USA) (85 ng NF-κB-dependent luciferase reporter plasmid-pGL4.32[luc2P/NF-kB-RE/Hygro] and 85 ng pcDNA3-β-gal), 1 μl Tfx-50 reagent (Promega), and 200 μl serum-free RPMI. In all, 800 μl RPMI containing FBS was then added, and incubation continued. The pGL4.32 plasmid was a NF-kB reporter vector. It contained NF-kB response elements and firefly luciferase gene. After incubation of 24 h, cells were stimulated with RANKL and treated with H_2 or not for 24 h. NF-κB luciferase activity was measured using a luciferase assay system and normalized against β-galactosidase activity.

Measurement of intracellular NO

Intracellular NO content was measured using a membrane-permeable indicator dye, 4-amino-5-methylamino-2′,7′-difluororescein diacetate (DAF-FM), which reacts with NO to form a green fluorescent product [14]. After treatment, cells were incubated for 30 min at 37 °C in 10 μM DAF-FM in the dark, washed with PBS and incubated for an additional 30 min in medium without the dye. Fluorescence intensity was recorded at the excitation wavelength of 488 nm.

Measurement of iNOS activity

After treatment, the iNOS activity was determined by using a Nitric Oxide Synthase Assay Kit (Biyotime Institute of Biotechnology, Jiangsu, China).

Measurement of NADPH oxidase activity

NADPH-oxidase activity was measured by the lucigenin-enhanced chemiluminescence method as described [15]. After treatment, cells were washed and pelleted in ice-cold PBS and then prepared in 300 μl lysis buffer (20 mM KH_2PO_4, pH 7.0), 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin) by using a Dounce homogenizer (on ice). Homogenates were centrifuged at 1000 rpm at 4 °C for 10 min to remove the unbroken cells and debris, and aliquots were used immediately. To start the assay, 100 μl homogenates were added to 900 μl 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin,

and 100 μM NADPH. Photon emission was measured in a luminometer every 30 s for 10 min. There was no measurable activity in the absence of NADPH. The final results were corrected for protein content.

Measurement of HO-1 enzyme activity

The HO-1 enzyme activity was measured by a previously described method [16]. Briefly, microsomes from the harvested cells were added to a reaction mixture containing NADPH and mouse liver cytosol as a source of biliverdin reductase, and the substrate hemin. The reaction was conducted at 37 °C in the dark for 1 h; it was terminated by the addition of 1 ml of chloroform and the extracted bilirubin was calculated by the difference in absorbance between the wavelengths 464 and 530 nm.

Rac activity assay

Cell lysates (150 μg) obtained from RAW264.7 after treatment were incubated with 15 μg recombinant GST-PBD (glutathione *S*-transferase-p21-binding domain; human Pak1 aa 67–150) for 1 h at 4 °C, and then proteins complexed to the beads were recovered by centrifugation, and the active GTP-Rac1 and Rac1 were detected with an anti-Rac1 antibody by Western blotting analysis. The ratio of GTP-Rac1 to Rac1 expression was calculated to assess Rac1 activity.

Western blotting analysis

Protein concentration determination was performed with a protein assay kit (Bio-Rad Hungary, Budapest, Hungary). Proteins were loaded and resolved on an 8 % SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PerkinElmer, Norwalk, CT, USA). Target proteins were detected using specific antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Then they were blotted with HRP-conjugated secondary antibody (1:5000). Immunoreactive bands were detected by a chemiluminescent reaction (ECL kit, Amersham Pharmacia).

The total cellular proteins were used for analysis of Nox1, Rac1, NFATc1, c-Fos, P-p38, P-ERK, P-JNK, P-AKT, p38, ERK, JNK, AKT, β-actin, and iNOS expressions; the separated nucleus proteins were used for analysis of Nrf2 and Lamin B1.

Statistical analysis

All data are presented as mean ± S.D. A one-way ANOVA with LSD post hoc test was used to detect significant differences between groups. A value of $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed using SPSS 11.0.0 software (SPSS Inc., Chicago, IL, USA).

Results

Effect of treatment with H_2 on osteoclastogenesis

To exclude the possibility that inhibition of H_2 on TRAP activity was due to cytotoxicity, the viability of RAW264.7 cells and BMMs in the presence or absence of RANKL was tested using MTT assay. The results indicated that H_2-induced cytotoxicity was negligible (Fig. 1a, b).

Osteoclastogenesis was induced by RANKL addition in RAW264.7 (Fig. 1c) and by RANKL and M-CSF addition in BMMs (Fig. 1d) and was measured by counting the number of osteoclasts. Treatment with H_2 reduced formation of TRAP positive cells in both groups, which indicated that treatment with H_2 suppressed RANKL-induced osteoclast differentiation.

BMMs stimulated with RANKL had marked ability to resorb dentin discs. When they were co-incubated with H_2, a decrease in the resorbed area on dentine discs was observed (Fig. 1e, f), which indicated that treatment with H_2 inhibiting the ability to form resorption pits of BMMs stimulated by RANKL.

Stimulation with RANKL prolonged the survival of purified osteoclasts, which was partly prevented by treatment with H_2 (Fig. 1g).

Effect of treatment with H_2 on the mRNA expression of osteoclast-specific markers in RAW264.7 cells stimulated with RANKL

Then we investigated the effect of treatment with H_2 on the mRNA expression of genes associated with osteoclast-specific markers. Stimulation with RANKL in RAW264.7 cells markedly induced expressions of TRAP, CalcR, Cath K, MMP9, Car2, and H^+-ATPase. Their expression in the control group was very low or could not be detected, so they were ignored. Treatment with H_2 reduced mRNA levels of TRAP, CalcR, Cath K, MMP9, Car2, and H+-ATPase (Fig. 2), which was consistent with the results from TRAP staining.

Effect of treatment with H_2 on ROS formation induced by RANKL in RAW264.7 cells

Stimulation with RANKL in RAW264.7 cells induced ROS formation (Fig. 3a). NADPH oxidase and mitochondria are main sources of ROS. Stimulation with RANKL in RAW264.7 cells enhanced NADPH oxidase activity

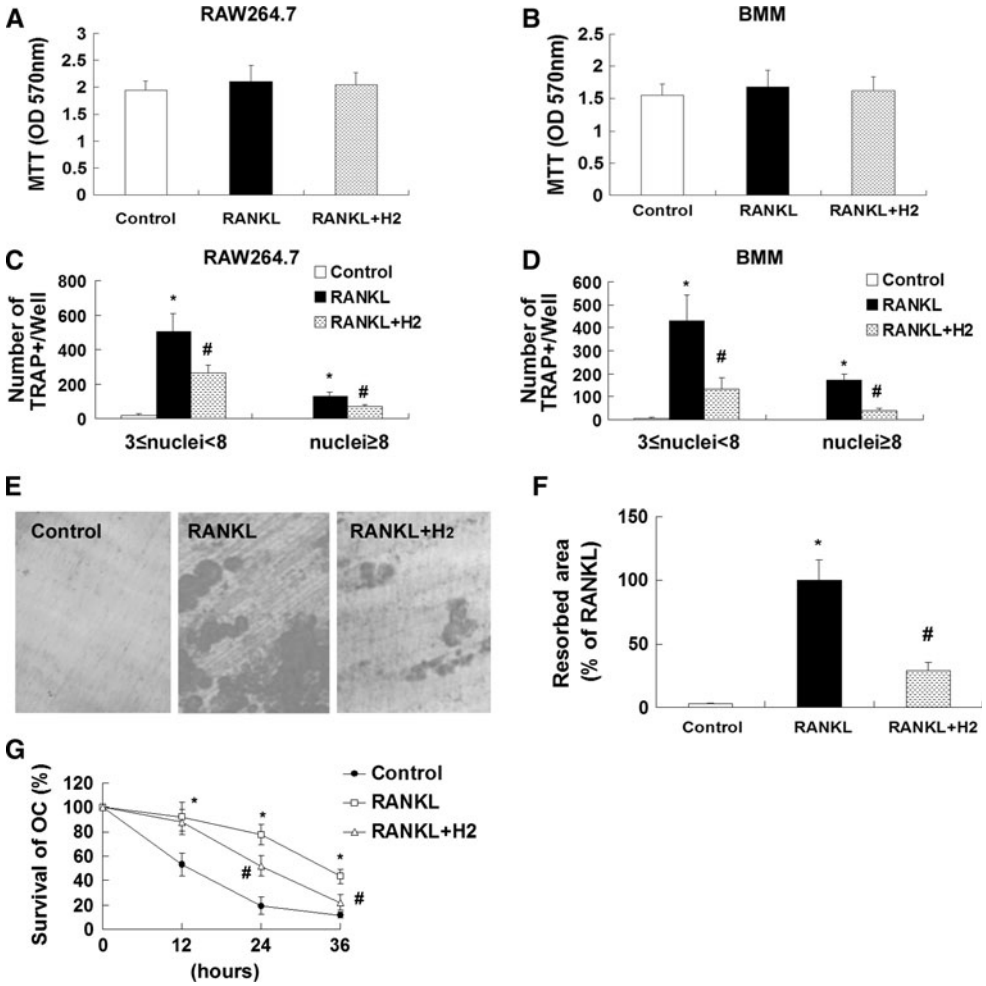

Fig. 1 Effect of treatment with H_2 on osteoclastogenesis induced by RANKL. RAW264.7 cells (**a**) and BMMs (**b**) were treated with H_2 in the presence or absence of RANKL (50 ng/mL) for 72 h. Cytotoxicity was determined by MTT assay. RAW264.7 cells (**c**) were stimulated with RANKL (50 ng/ml) and treated with H_2 or not. BMMs (**d**) were stimulated with RANKL (50 ng/ml) and M-CSF (30 ng/ml) and treated with H_2 or not. After 6 days of culture, the number of TRAP positive osteoclasts was counted. BMMs were stimulated with RANKL and treated with H_2 or not on dentine discs for 14 days, resorption pit assay was determined. Representative images (**e**) of groups were shown. The ratio of resorption area to total area was quantified. The final results (**g**) were normalized to the result of RANKL-treated group. Purified osteoclasts were stimulated with RANKL and treated with H_2 or not, TRAP positive multinuclear cells containing more than three nuclei were counted as surviving osteoclasts (**g**). *RANKL* receptor activator of NFκB ligand, *BMMs* bone marrow macrophages, *M-CSF* macrophage colony stimulating factor, *TRAP* tartrate resistant acid phosphatase, $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the RANKL-treated group

(Fig. 3b) and increased mitochondrial ROS formation (Fig. 3c). The small guanosine triphosphatase (GTPase) Rac1 is a cytosolic component of NADPH oxidase complex and is responsible for the activation of NADPH oxidases. Stimulation with RANKL in RAW264.7 cells upregulated Nox1 expression and enhanced Rac1 activity (Fig. 3e). Nrf2 may contribute to the maintenance of redox homeostasis by serving as endogenous antioxidant systems through the action of proteins such as heme oxygenase-1 (HO-1). Stimulation with RANKL in RAW264.7 cells had no effect on nuclear translocation of Nrf2 (Fig. 3e) but suppressed HO-1 activity (Fig. 3d).

Treatment with H_2 decreased intracellular ROS formation, suppressed NADPH oxidase activity through reducing Rac1 activity and Nox1 expression, reduced mitochondrial ROS formation, and enhanced Nrf2 nuclear translocation and HO-1 activity.

Effect of treatment with H_2 on NO formation induced by RANKL in RAW264.7 cells

Stimulation with RANKL in RAW264.7 cells enhanced NO formation (Fig. 4a) through inducing iNOS expression (Fig. 4c) and increased its activity (Fig. 4b). Treatment

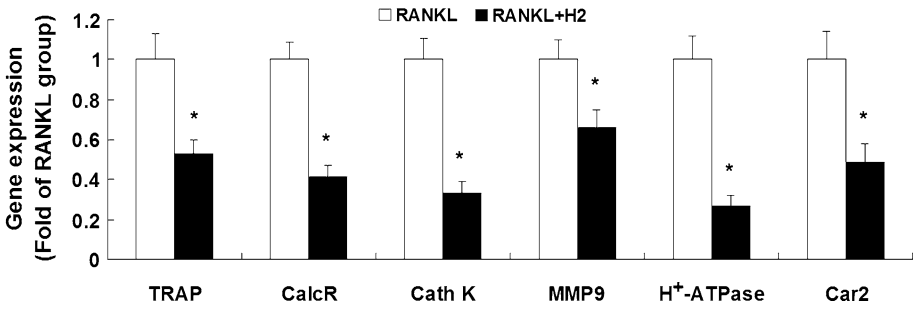

Fig. 2 Effect of treatment with H_2 on the mRNA expression of osteoclast-specific markers in RAW264.7 cells. RAW264.7 cells were stimulated with RANKL (50 ng/ml) and treated with H_2 or not. After 6 days of culture, the mRNA levels of TRAP, CalcR, Cath K, MMP9, Car2, and H^+-ATPase were measured by RT-PCR. *RANKL* receptor activator of NFκB ligand, *TRAP* tartrate resistant acid phosphatase, *CalcR* calcitonin receptor, *Cath K* cathepsin K, *MMP9* metalloproteinase-9, *Car2* carbonic anhydrase type II, *H^+-ATPase* vacuolar-type H^+ ATPase, $^*P < 0.05$ versus the control group, $^{\#}P < 0.05$ versus the RANKL-treated group

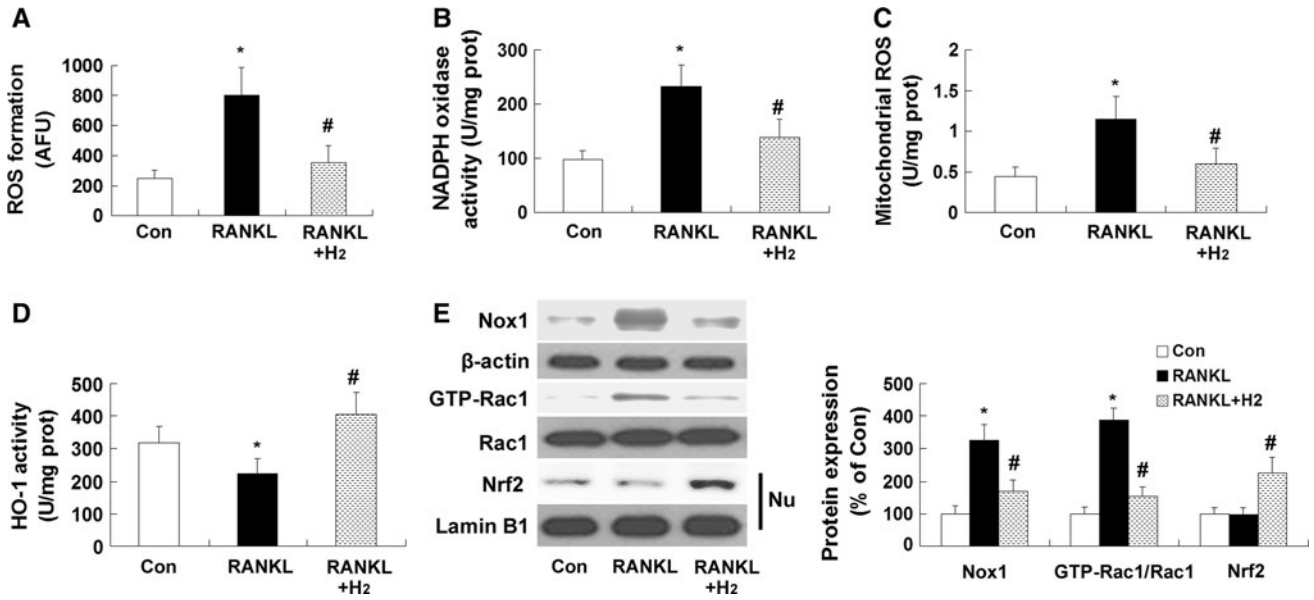

Fig. 3 Effect of treatment with H_2 on ROS formation induced by RANKL in RAW264.7 cells. RAW264.7 cells were stimulated with RANKL (50 ng/ml) and treated with H_2 or not. After 6 days of culture, cellular ROS (**a**), NADPH oxidase activity (**b**), mitochondrial ROS formation (**c**), HO-1 activity (**d**), and Nox1, Rac1, and Nrf2 protein expressions (**e**) were determined. In the Western blotting analysis, the protein levels in the cell and nucleus were adjusted as relative values to β-actin and Lamin B1, respectively. The ratio of GTP-Rac1 to Rac1 expression was calculated to assess Rac1 activity. *RANKL* receptor activator of NFκB ligand, *ROS* reactive oxygen species, *HO-1* heme oxygenase, *Nrf2* nuclear factor E2-related factor 2, $^*P < 0.05$ versus the control group, $^{\#}P < 0.05$ versus the RANKL-treated group

with H_2 prevented RANKL-induced activation of iNOS and suppressed NO formation.

Effect of treatment with H_2 on NFATc1 and c-Fos expression, NF-κB activity and MAPK signaling pathway induced by RANKL in RAW264.7 cells

Stimulation with RANKL in RAW264.7 cells induced protein expression of NFATc1 and c-Fos (Fig. 5a). Treatment with H_2 suppressed NFATc1 and c-Fos expression induced by RANKL.

Stimulation with RANKL in RAW264.7 cells enhanced NFκB activity (Fig. 5b) and increased phosphorylation of p38 (Fig. 5c), ERK (Fig. 5d), JNK (Fig. 5e), and AKT (Fig. 5f), revealing that stimulation with RANKL in RAW264.7 cells activated NFκB, AKT, and MAPK pathways. Treatment with H_2 suppressed NFκB activity and reduced phosphorylation of p38, ERK, JNK, and AKT, indicating that treatment with H_2 prevented activation of NFκB, MAPK, and AKT pathways induced by RANKL.

Discussion

This study showed for the first time that incubation with medium containing hydrogen molecules could inhibit

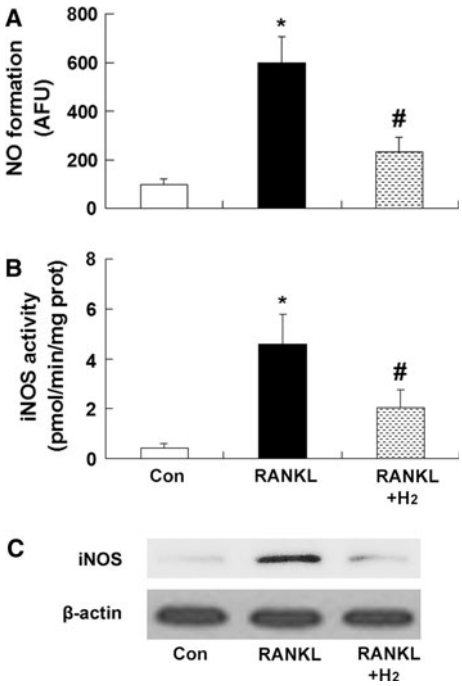

Fig. 4 Effect of treatment with H_2 on NO formation induced by RANKL in RAW264.7 cells. RAW264.7 cells were stimulated with RANKL (50 ng/ml) and treated with H_2 or not. After 6 days of culture, cellular NO formation (**a**), iNOS activity (**b**), and iNOS expression (**c**) were determined. *RANKL* receptor activator of NFκB ligand, *NO* nitric oxide, *iNOS* inducible nitric oxide synthase, $^{*}P < 0.05$ versus the control group, $^{\#}P < 0.05$ versus the RANKL-treated group

RANKL-induced osteoclast differentiation. During osteoclastogenesis, NFATc1 and c-Fos can be identified as the osteoclast-specific transcription factors and the master regulators of osteoclast differentiation [17, 18]. In this study, treatment with H_2 significantly reduced protein expression of NFATc1 and c-Fos induced by RANKL, which might be related to the effects of H_2 on ROS or signal pathways including NF-κB, MAPK and AKT pathways.

ROS are essential for the formation and function of osteoclasts and regulate RANKL-stimulated osteoclast differentiation [11, 19, 20]. In this study, treatment with H_2 significantly reduced intracellular ROS formation induced by RANKL. NADPH oxidase was the major source of intracellular ROS involved in the regulation of osteoclast function and differentiation [21, 22]. Nox1 expression and Rac1 activity were found enhanced when stimulated with RANKL in the current study. MitoQ was more effective than general antioxidants in suppressing the RANKL-induced differentiation of RAW264.7 cells [23], indicating that mitochondria was the another major source of intracellular ROS for osteoclast differentiation. In this study, RANKL stimulated mitochondrial ROS formation, which was attenuated by treatment with H_2. Our result showed an important property of H_2: H_2 was electrically neutral and much smaller than the other antioxidants, and could easily penetrate membranes and enter cells and organelles such as the nucleus and mitochondria, which are relatively impermeable to most commonly-used antioxidants.

In addition, treatment with H2 influenced the antioxidant systems through activating nuclear factor E2-related factor (Nrf2) and enhanced HO-1 activity when stimulated with RANKL. Nrf2 is a master transcription factor that regulates induction of antioxidant gene expression and phase II antioxidant enzymes [24]. Number of osteoclasts in Nrf2(−/−) mice was higher than that in Nrf2(+/+) mice [25]. Induction of HO-1 with hemin or curcumin in bone marrow-derived macrophages or RAW-D murine osteoclast precursor cells inhibited osteoclastogenesis [26]. Therefore, up-regulation of Nrf2 and its downstream protein HO-1 contributed to the inhibitory effect of H_2 on RANKL-induced osteoclast differentiation.

In this study, stimulation with RANKL induced iNOS expression and NO formation, which was consistent with a previous report [27]. The impact of NO on the osteoclast function is bi-directional. High content of NO was toxic to osteoclasts; normal content of NO enhanced the osteoclast generation [28, 29]. However, the reports about the role of iNOS-derived NO in osteoclast differentiation were contradictory. Zheng et al. [27] demonstrated that preventing RANKL-induced NO increased osteoclast formation and bone pit resorption. But cells from iNOS KO mice showed decreased osteoclast growth and fewer resorption pit counts [30]; NOS inhibition prevented alveolar bone resorption in experimental periodontitis in rats [31]. Treatment with H_2 suppressed iNOS expression and reduced NO formation. However, whether reduction of NO production contributed to the inhibitory effect of H_2 on osteoclastogenesis required further investigation.

In this study, stimulation with RANKL in RAW264.7 cells activated NF-κB activity and enhanced phosphorylation of AKT, ERK, p38, and JNK. Osteoclast differentiation entails binding of RANK ligand to its cognate receptor on myeloid progenitor cells and subsequent activation of multiple intracellular pathways including AKT/PI3K, MAPK, and NF-κB [8–10]. The critical role of NF-κB in osteoclast differentiation and bone homeostasis has been widely investigated [32, 33]. NF-κB p50 and p52 expression was essential for RANK-expressing osteoclast precursors to differentiate into $TRAP^{+}$ osteoclasts in response to RANKL and other osteoclastogenic cytokines [34]. It was reported that JNK1-activated c-Jun signaling in cooperation with nuclear factor of activated T cells (NFAT) was key to RANKL-regulated osteoclast differentiation [18]. In addition, stimulation of p38 led to the downstream activation of themi/Mitf (microphthalmia/microphthalmia transcription factor), which controlled the

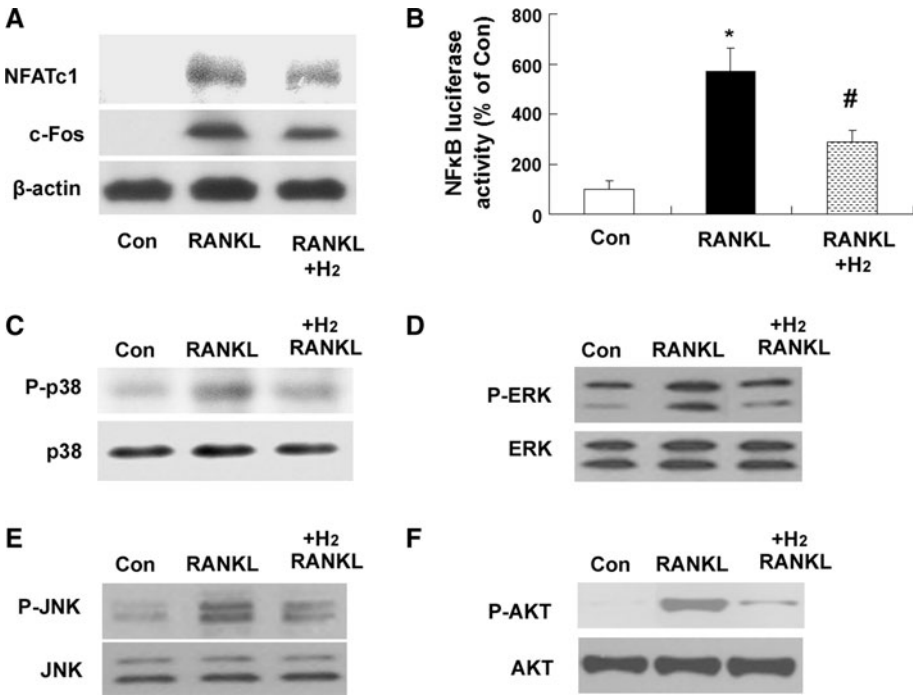

Fig. 5 Effect of treatment with H_2 on NFATc1 and c-Fos expression, NF-κB activity and MAPK and AKT signaling pathway induced by RANKL in RAW264.7 cells. RAW264.7 cells were stimulated with RANKL (50 ng/ml) and treated with H_2 or not. After 6 days of culture, NFATc1 and c-Fos expression (**a**) was determined; after 24 h of culture, NF-κB activity (**b**) was determined; after 30 min of culture, MAPK and AKT signaling including p38 (**c**), ERK (**d**), JNK (**e**), and AKT (**f**) were determined. The ratio of P-p38 to p38 expression, the ratio of P-ERK to ERK expression, the ratio of P-JNK to JNK expression, and the ratio of P-AKT to AKT expression, was calculated to assess p38, ERK, JNK, and AKT signaling, respectively. *RANKL* receptor activator of NFκB ligand, *ERK* extracellular signal-regulated kinase, *NF-κB* nuclear factor κB, *JNK* c-Jun-N-terminal kinase, *NFATc1* nuclear factor of activated T cells c1, *MAPK* mitogen-activated protein kinase, *AKT* protein kinases B, $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the RANKL-treated group

expression of the genes encoding TRAP and cath K, indicating the importance of p38 signaling cascades [6]. In differentiated osteoclasts, ERK activity correlated with cell survival and maintenance of cell polarity, but not with resorption function [35]. AKT could induce osteoclast differentiation through regulating the GSK3β/NFATc1 signaling cascade [36]. Treatment with H_2 attenuated NF-κB activity and phosphorylation of AKT, ERK, p38, and JNK, which was similar with previous studies about H_2 [37, 38].

The direct effects of H_2 on osteoclast differentiation might be attributed to two major molecular mechanisms in this study: scavenging activity of ROS and signal-modulating activities. It was reported that the effect of hydrogen on signaling pathways might be independent from its antioxidant effect [39]. Therefore, the intrinsic effect of H_2 on RANKL-induced osteoclast differentiation required further investigation.

One limitation should be noted in this work. From the pharmacological point of view, we should test at least two concentration of the hydrogen molecule in the medium. But the proportion of H_2 in the mixed gas was same as the proportion of H_2 in medium. When we lowered the proportion of H_2, the mixed gas became highly explosive and very dangerous for our experimenters. So we could not provide the data in this work.

In conclusion, treatment with H_2 prevented RANKL-induced osteoclastogenesis associated with down-regulating RANKL-induced ROS generation and suppressing the activation of NF-κB, MAPK and AKT pathways.

Conflict of interest The authors have stated that they have no conflict of interest.

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