Lipopolysaccharide (LPS) promotes osteoclast differentiation and activation by enhancing the MAPK pathway and COX-2 expression in RAW264.7 cells

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Abstract. Bone degradation is a serious complication of chronic inflammatory diseases such as septic arthritis, osteo-myelitis and infected orthopedic implant failure. At present, effective therapeutic treatments for lipopolysaccharide (LPS)-induced bone destruction are limited to antibiotics and surgical repair in chronic inflammatory diseases. The present study aimed to evaluate the mechanism of LPS on osteoclast differentiation and activation. RAW264.7 cells were non-induced, or induced by the receptor activator of nuclear factor-κB (RANK) ligand (RANKL) and macrophage-colony stimulating factor (M-CSF), and then treated with LPS. Following treatment, the number of osteoclasts and cell viability were measured. The expression of osteoclast-related genes including tartrate-resistant acid phosphatase (*TRAP*), matrix metalloproteinase-9 (*MMP-9*), cathepsin K (*CK*), carbonic anhydrase II (*CAII*) and cyclooxygenase-2 (COX-2) was determined by RT-PCR. Protein levels of RANK, tumor necrosis factor receptor-associated factor 6 (TRAF6), COX-2 and mitogen-activated protein kinases (MAPK) were measured using western blotting assays. LPS promoted osteo-clast differentiation of RAW264.7 cells and differentiated osteoclasts. LPS significantly increased mRNA expression of osteoclast-related genes in RAW264.7 cells. Differentiated osteoclasts were treated with LPS (100 ng/ml) and the results showed a significantly increased mRNA expression of osteo-clast-related genes and protein levels of RANK, TRAF6 and COX-2. Furthermore, LPS at 100 ng/ml significantly promoted the MAPK pathway including increasing the phosphorylation of c-Jun N-terminal kinases (JNK) and the phosphorylation of the extracellular signal-regulated kinase (ERK1/2). In conclu-sion, LPS promoted osteoclast differentiation and activation by enhancing RANK signaling and COX-2 expression. LPS also promoted osteoclast differentiation via activation of the JNK and ERK1/2 cell proliferation pathways.

Introduction

Bone is a dynamic tissue that constantly undergoes remod-eling and involves a continuous process of bone formation and resorption (1). This process allows physiological bone growth and repair of damaged bones. Chronic osteomyelitis, an infectious bone disease, is a dysregulation of this process that results in excessive bone resorption (2). The receptor acti-vator of nuclear factor-κB ligand (RANKL) is a key factor in regulating the process of osteoclast differentiation and main-taining the survival of mature osteoclasts (3). RANK signaling plays a critical role in regulating osteoclast differentiation and bone resorptive activity. RANK is activated by RANKL via the tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which in turn induces the activation of mitogen-activated protein (MAP) kinases (MAPK) (4).

Lipopolysaccharide (LPS), a component of the outer membranes of all Gram-negative bacteria, is the first bacterial component shown to be capable of inducing bone resorption *in vitro* (5). LPS induces the production of cytokines including TNF-α by fibroblasts, macrophages, and other cells, which are able to induce osteoclast formation and activation (6). Moreover, LPS can promote osteoclast differentiation, fusion, survival and activation independent of IL-1, TNF-α, and RANKL (7-10). However, at present, effective therapeutic treatments for LPS-induced bone destruction are limited to antibiotics and surgical repair in chronic inflammatory diseases. Moreover, the role of LPS on osteoclast differentiation and activation through RANK signaling and COX-2 expression remains to be determined. Therefore, the research and development of LPS induced osteolysis remains a key objective for evaluating potential drugs in the prevention of bone destruction in infec-tious bone diseases.

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The aim of the present study was to evaluate the effect of LPS on osteoclast differentiation and activation. We also aimed to clarify the mechanism of LPS on MAPK phosphorylation, as well as on the expression of RANK/TRAF6 and COX-2 in preosteoclasts.

Materials and methods

Reagents. Escherichia coli LPS (serotype 055:B5), Leukocyte Acid Phosphatase kit 387-A, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma Co. (St. Louis, MO, USA). α modified-minimum essential medium (α-MEM), 10% fetal bovine serum (FBS), penicillin/streptomycin and TRIzol® reagent were purchased from Gibco (Rockville, MD, USA). RANKL and macrophage-colony stimulating factor (M-CSF) were purchased from Peprothec (Rocky Hill, NJ, USA). Primary antibodies against total c-Jun N-terminal kinase (JNK), phosphorylated JNK, extracellular signal-regulated kinase (ERK1/2), phosphorylated ERK1/2 and COX-2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies against RANK, TRAF6 and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The mouse macrophage RAW264.7 cell line that can be induced to differentiate into osteoclasts was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Other chemicals and reagents used in this study were of analytical grade.

Cell culture. RAW264.7 cells were grown in α-MEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin solution, 10 mM HEPES solution and incubated at 37°C in 5% CO_2 humidified air. The medium was changed every 3 days. For the cell viability assay, cells were seeded at $1x10^4$ cells/cm^2; for other experiments, cells were seeded at $4x10^5$ cells/cm^2. RAW264.7 were seeded into culture dishes and incubated overnight prior to treatment.

To evaluate the effect of LPS-induced osteoclastogenesis, the cells were treated with 10, 50, 100, 200 or 1000 ng/ml LPS or without LPS for 4 days until differentiation. To evaluate the effect of LPS on differentiated osteoclasts, RAW264.7 cells were treated with 50 ng/ml RANKL and 50 ng/ml M-CSF for 4 days. Then, 10, 50, 100, 200 or 1000 ng/ml LPS or without LPS were administered into cells in the presence of RANKL (50 ng/ml) and M-CSF (50 ng/ml) for 24 h.

Tartrate-resistant acid phosphatase staining (TRAP). Cells were washed with phosphate-buffered saline (PBS) and fixed in 4% (v/v) paraformaldehyde at room temperature for 15 min. The cells were stained with TRAP using the Leukocyte Acid Phosphatase kit 387-A at 37°C for 60 min. Stained cells were then applied for microscopy (Olympus IX71, Olympus Optical, Tokyo, Japan). Osteoclasts were identified as TRAP-positive cells with ≥3 nuclei in each cell.

Cell viability assay. Cell viability was measured using MTT assay. RAW264.7 cells ($1x10^3$ cells/cm^2) were seeded into 96-well flat bottom plates and incubated overnight prior to the experiment. To evaluate the effect of LPS on cell viability, RAW264.7 cells were treated with 10, 50, 100, 200 or 1000 ng/ml LPS or without LPS for 1 and 4 days. At the end of treatment, 20 μl MTT (5 mg/ml) was added to each well. Cells were cultured for an additional 4 h, the supernatant was removed, and 100 μl dimethyl sulfoxide was added to each well. Following agitation for 30 sec, absorbance was measured at 570 nm using a microplate reader (TECAN, Salzburg, Austria). The experiment was performed in triplicate.

Reverse transcription-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen) and quantified by spectrophotometry. After isolation, 2 μg total RNA from each sample was reverse transcribed (RT) utilizing the M-MLV First Strand kit (Invitrogen) according to the manufacturer's instructions. The primer sequences of *TRAP*, matrix metalloproteinase-9 (*MMP-9*), cathepsin K (*CK*) and carbonic anhydrase II (*CAII*) and *β-actin* (Generay Biotech Co., Ltd., Shanghai, China) and annealing temperatures used in this study are listed Table I. In each reaction, 1 μl cDNA, 12.5 μl 2X Taq Master Mix (Beijing CoWin Biotech Co., Ltd., Beijing, China) and 0.4 μM forward and reverse primers in a total volume of 25 μl were used. The initial denaturation was performed at 94°C for 3 min. The products were then subjected to denaturation at 94°C for 30 sec, annealing temperature for 30 sec, extension at 72°C for 30 sec for 32 cycles, and a final elongation at 72°C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis, photographed using Gel-Doc (Bio-Rad, Hercules, CA, USA), and quantified by density determination using Quantity One image analysis software (Bio-Rad). β-actin was used as the internal control. Results were normalized to β-actin, and data were expressed as a ratio of gene to β-actin expression.

Western blot analysis. At the end of treatment, the cell culture medium was aspirated and cells were detached in PBS by scrapping. Detached cells were transferred to fresh microcentrifuge tubes and centrifuged at 12,500 x g at 4°C for 10 min. Cell pellets were then lysed in 300 μl lysis buffer (Cytobuster protein extraction reagent, Novagen, Darmstadt, Germany) with 25 mM NaF, 1 mM Na_3VO_4, 1X protease inhibitor cocktail. Protein concentrations were determined using a standard Bradford assay. For western blotting, equal amounts of protein from each sample were loaded on SDS-PAGE and electro-transferred onto PVDF membranes (Millipore, Bedford, MA, USA). These membranes were then blocked with 5% (w/v) bovine serum albumin in TBST [10 mM Tris, 150 mM NaCl, and 0.1% (v/v) Tween-20, pH 7.5] for 1 h at room temperature, and incubated with primary antibodies overnight at 4°C. Secondary antibody incubation was at room temperature for 2 h (Santa Cruz Biotechnology, Inc.). Chemiluminescence ECL (Amersham, Arlington Heights, IL, USA) was used to detect immunoreactive protein signals. Protein signals were then visualized on films and scanned and quantified using the ImageJ software (National Institutes of Health Image, USA). For re-probing, the PVDF membranes were stripped with 0.2 M NaOH for 10 min before blocking with another primary antibody. The expression of molecules of interest was determined relative to β-actin.

Statistical analysis. Each experiment was repeated at least three times. Quantified results were presented as the mean ± SD. Significant differences were determined using

Table I. Primer sequences used in the RT-PCR analysis.

Gene	Accession no.	Sequences (5'→3')	PCR product size (bp)
TRAP	NM_007388	F: CACTCCCACCCTGAGATTTGT R: CATCGTCTGCACGGTTCTG	118
MMP-9	NM_013599	F: CTGGACAGCCAGACACTAAAG R: CTCGCGGCAAGTCTTCAGAG	145
CK	NM_007802	F: GAAGAAGACTCACCAGAAGCAG R: TCCAGGTTATGGGCAGAGATT	102
CAII	NM_009801	F: TCCCACCACTGGGGATACAG R: CTCTTGGACGCAGCTTTATCATA	170
COX-2	NM_022415	F: GGATGCGCTGAAACGTGGA R: CAGGAATGAGTACACGAAGCC	133
β-actin	NM_007393	F: GGCTGTATTCCCCTCCATCG R: CCAGTTGGTAACAATGCCATGT	154

TRAP, tartrate-resistant acid phosphatase; MMP-9, matrix metalloproteinase-9; CK, cathepsin K; CAII, carbonic anhydrase II; COX-2, cyclo-oxygenase-2; F, forward; R, reverse.

factorial analysis of variance (ANOVA). P<0.05 was considered to indicate a statistically significant difference.

Results

LPS promoted osteoclast differentiation. The population of osteoclasts in LPS-treated cultures was higher than that in non-treated cultures (Fig. 1). LPS at 100 ng/ml induced the most osteoclast formation compared with other concentrations of LPS-treated cultures in RAW264.7 cells (P<0.01) and differentiated osteoclasts (P<0.01).

To ensure the effect of LPS on osteoclast differentiation, we evaluated cell viability in RAW264.7 cells. LPS at 100 ng/ml significantly increased cell viability at 24 h, while 1000 ng/ml LPS decreased cell viability at 24 h and 4 days (Fig. 2).

LPS promoted the mRNA expression of osteoclast-specific genes in RAW264.7 cells and differentiated osteoclasts. On differentiation day 4, LPS at 100 ng/ml significantly increased mRNA expression of *TRAP, MMP-9, CK, CAII* and *COX-2* compared to other concentrations of LPS-treated groups as well as the non-treated group in RAW264.7 cells and differentiated osteoclasts (Fig. 3).

LPS promoted the expression of RANK, TRAF6 and COX-2 protein in differentiated osteoclasts. On differentiation day 4, LPS at 100 ng/ml significantly increased the protein expression of RANK, TRAF6 and COX-2 in differentiated osteoclasts. However, higher concentrations of LPS inhibited the protein expression of these molecules (Fig. 4).

LPS selectively regulated the activation of MAPK in RAW264.7 cells. Since MAP kinases are important regulators of inflammatory mediators and osteoclast differentiation, the effect of LPS on the activation of MAPKs in RAW264.7 cells were examined by western blotting. LPS treatment at 50, 100, 200

or 1000 ng/ml enhanced the protein levels of phosphorylated forms of ERK1/2 and JNK in RAW264.7 cells at 2 h (Fig. 5). When MAPK inhibitors SP600125 and PD98059 were added 2 h prior to LPS treatment, the enhanced phosphorylation of ERK1/2 and JNK in RAW264.7 cells was selectively attenuated (Fig. 6). This suggests LPS-induced osteoclast differentiation through selective activations of ERK1/2 and JNK, which were involved in the cell proliferation pathway.

Discussion

Bacteria-induced inflammatory response is an important contributor to excessive bone resorption in chronic inflammatory diseases such as septic arthritis, osteomyelitis, and infected orthopedic implant failure (2). LPS, a pro-inflammatory glycolipid and main component of the cell wall of Gram-negative bacteria, is well documented in Gram-negative bacteria-induced bone destruction. LPS stimulates osteoclastic bone resorption *in vivo* (11,12) and promotes osteoclast differentiation in whole bone marrow cell culture (13) and in preosteoclasts (7,8). However, LPS on osteoclast differentiation and activation through RANK signaling and COX-2 expression remains to be determined. In the present study, LPS increased the protein expression of RANK, TRAF6 and COX-2 and mRNA expression of osteoclast-related genes in differentiated osteoclasts (100 ng/ml LPS). In our previous study, LPS increased the protein expression of RANK, TRAF6 and COX-2 in RAW264.7 cells (14). Those results confirmed that LPS was able to stimulate osteoclast differentiation directly.

The effect of LPS on osteoclast resorptive activity is confirmed by evaluating mRNA expression levels of osteoclast-related genes. TRAP has been suggested to be involved in bone resorptive activity in osteoclasts (15,16). The proteolytic enzymes such as MMP-9, CAII and CK are highly expressed in osteoclasts and are involved in degradation of the bone matrix during bone resorption (17-22). In the present study,
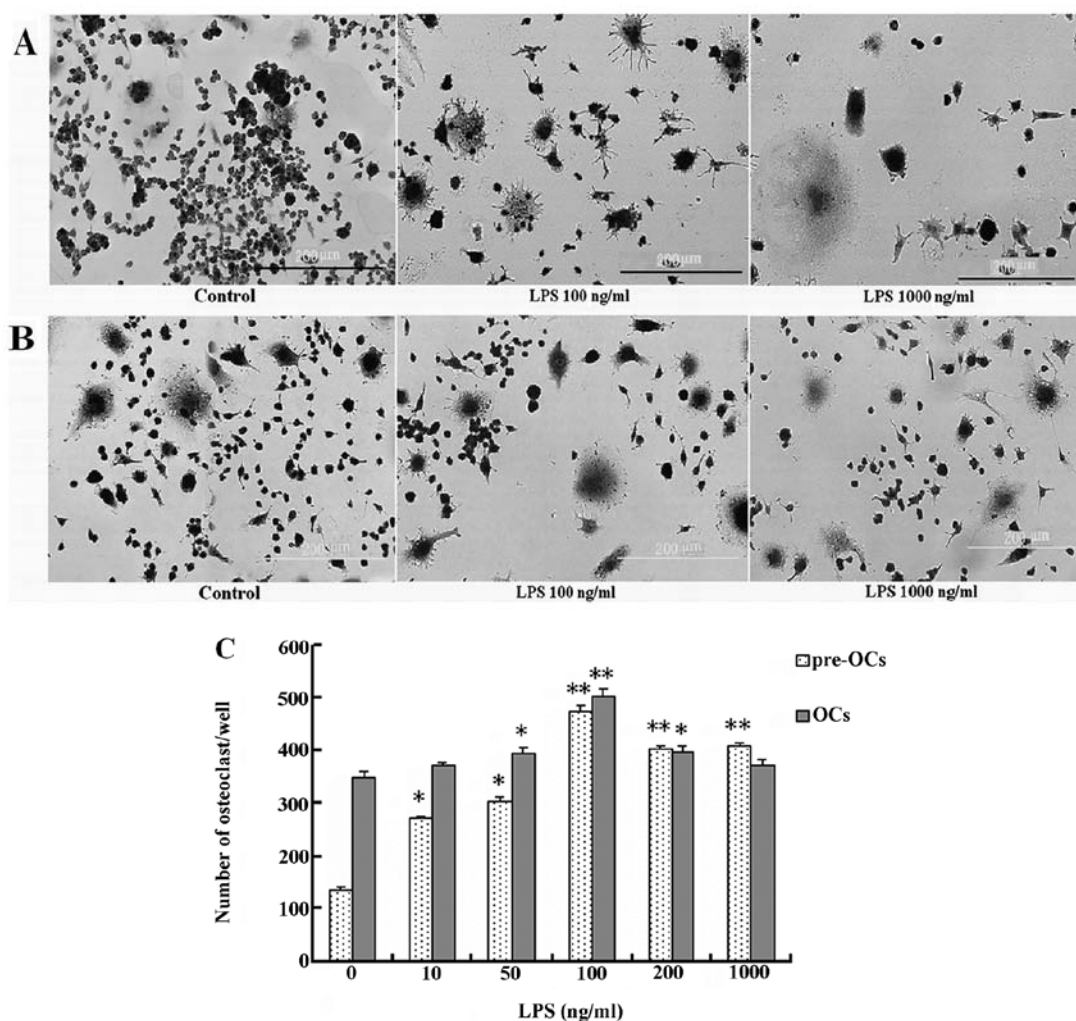
Figure 1. Effect of lipopolysaccharide (LPS) treatment on osteoclast formation. (A) The number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells at 4 days after LPS treatment alone in RAW264.7 cells. (B) The number of TRAP-positive multinucleated cells at 1 day after LPS treatment in differentiated osteoclasts (induced by M-CSF and RANKL) cultures. (C) Effect of LPS treatments on osteoclast survival in RAW264.7 cells and differentiated osteoclasts. *P<0.05; **P<0.01. Data show the mean ± SD from three independent experiments.

the mRNA expression levels of *TRAP*, *MMP-9*, *CAII* and *CK* in RAW264.7 cells and mature osteoclasts were significantly enhanced by LPS treatment. Taken together, our data indicate that LPS promoted osteoclast differentiation and activity due to the upregulated osteoclast-related genes.

COX-2 plays a critical role in osteoclastogenic signaling pathways (23,24). Inhibition of COX-2 blocks osteoclast formation *in vitro* (25,26). In a previous study, we found that LPS upregulated the protein expression of COX-2 of RAW264.7 cells (14). In the current study, LPS upregulated the protein expression of COX-2 of differentiated osteoclasts. Taken together, these findings indicate that LPS promoted osteoclast differentiation by enhancing COX-2 expression.

RANK signal transduction has been reported to be essential for osteoclast differentiation, bone resorptive function, and survival (27,28). Mice deficient in *RANKL* or *RANK* genes exhibit severe osteopetrosis as they do not form osteoclasts (29-32). The selective inhibiton of RANK with RANK:Fc or RANK receptor inhibitor blocks osteoclast maturation and function *in vivo* or *in vitro* (33-35). The selective inhibition of TRAF6 also blocks osteoclast maturation and function (36,27). Taken together with our previous study (14), results
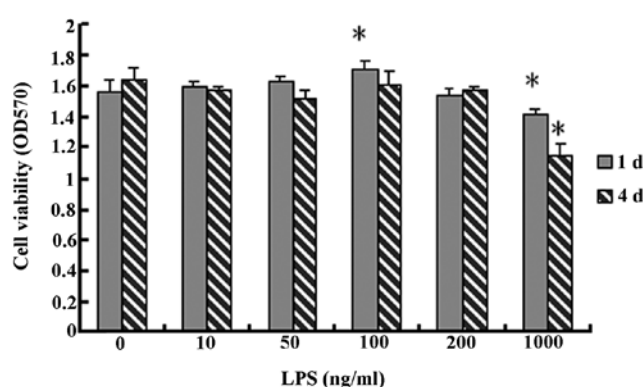

Figure 2. Effect of lipopolysaccharide (LPS) treatment on cell viability in RAW264.7 cells at 24 h and 4 days. *P<0.05; **P<0.01. Data show the mean ± SD from three independent experiments.

of the present study have shown that LPS enhanced the protein expression of RANK and TRAF6 in RAW264.7 cells and differentiated osteoclasts. These results suggest that RANK/TRAF6 signaling plays a prominent role in osteoclastogenesis downstream of LPS.
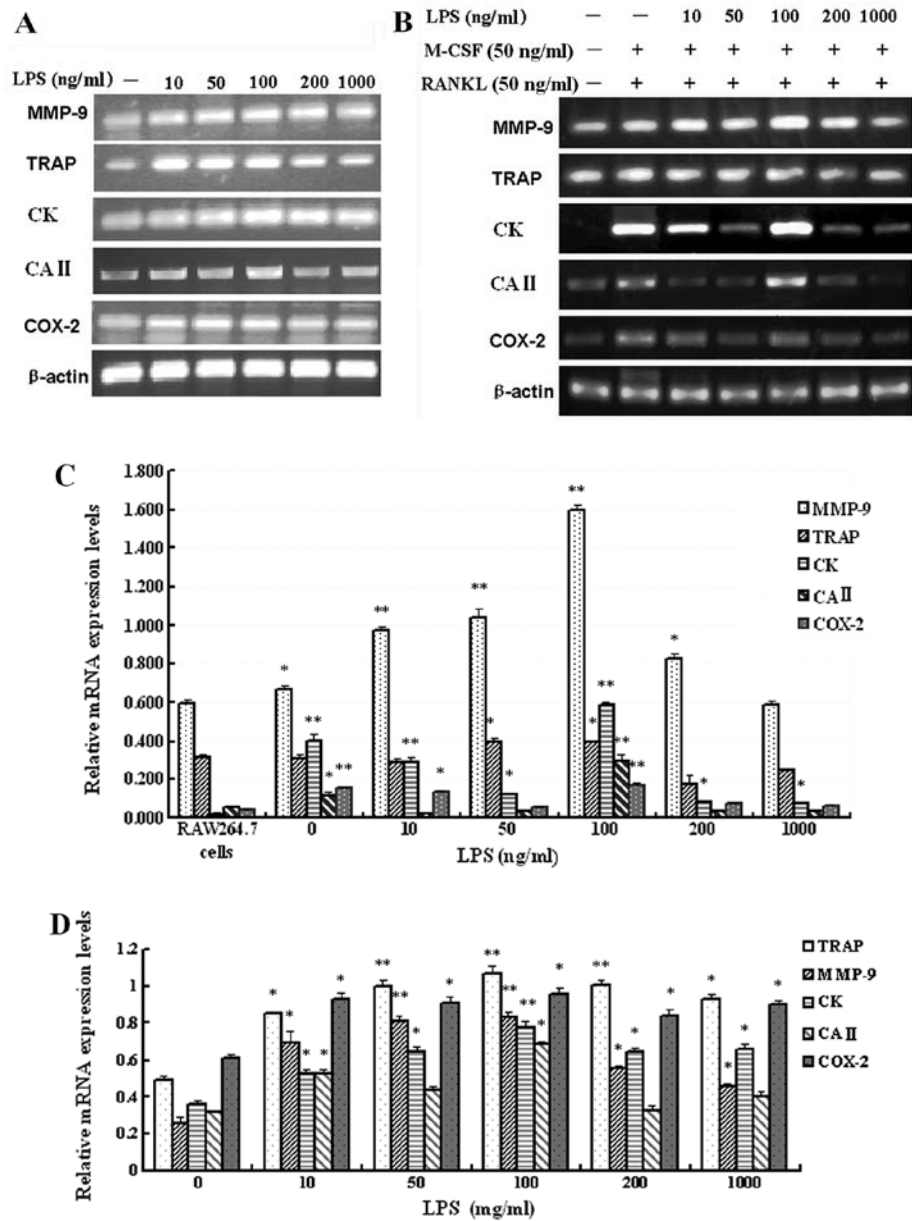
Figure 3. Messenger RNA expression of tartrate-resistant acid phosphatase (*TRAP*), matrix metalloproteinase-9 (*MMP-9*), cathepsin K (*CK*), carbonic anhydrase II (*CAII*) and cyclooxygenase-2 (*COX-2*) following treatment with lipopolysaccharide (LPS) (0, 10, 50, 100, 200 or 1000 ng/ml) (A and C). RAW264.7 cells at day 4, and (B and D) in differentiated osteoclasts at 24 h. *P<0.05; **P<0.01. Data show the mean ± SD from three independent experiments.
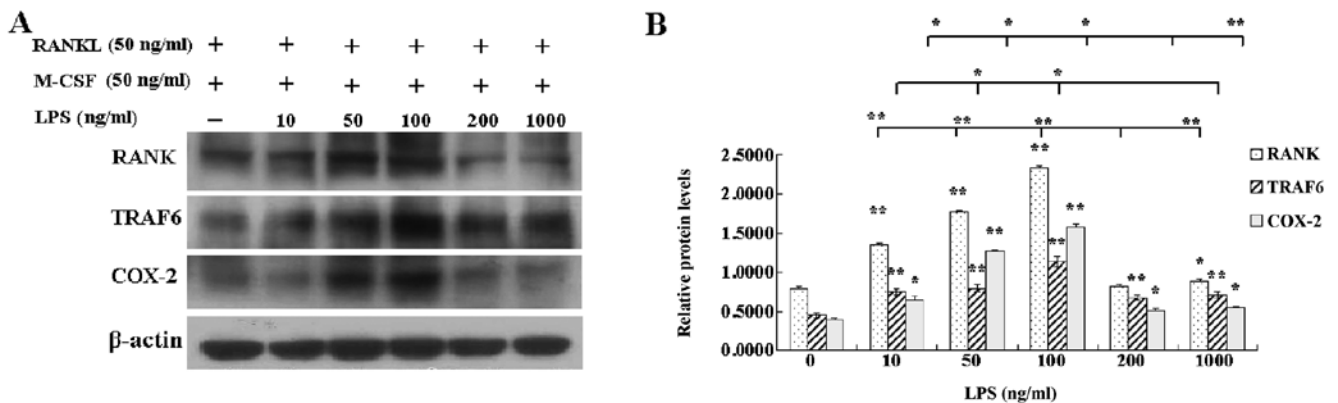

Figure 4. (A) Protein expression of receptor activator of nuclear factor-κB (RANK), tumor necrosis factor receptor-associated factor 6 (TRAF6) and cyclooxygenase-2 (COX-2) at 24 h after lipopolysaccharide (LPS) treatment in differentiated osteoclasts. (B) Effect of LPS on protein expression of RANK and COX-2 at 24 h in differentiated osteoclasts. *P<0.05; **P<0.01. Data show the mean ± SD from three independent experiments.
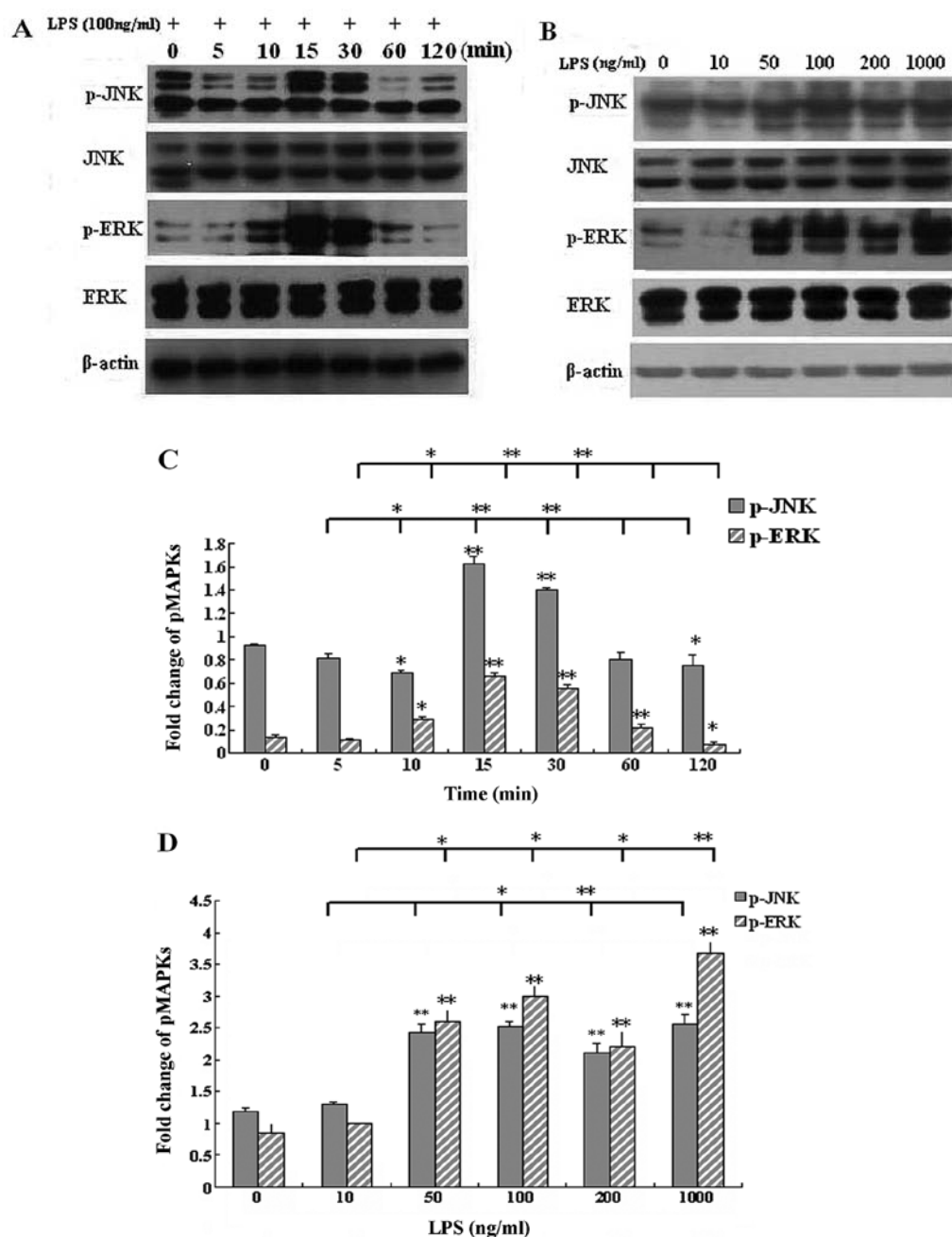
Figure 5. (A and C) Effect of lipopolysaccharide (LPS) at 100 ng/ml on the protein expression of mitogen-activated protein kinases (MAPKs) in RAW264.7 cells at 2 h. (B and D) LPS at 0, 10, 50, 100, 200 or 1000 ng/ml was administered to RAW264.7 cells for 15 min and the protein expression of MAPKs was examined. Data show the mean ± SD from three independent experiments.
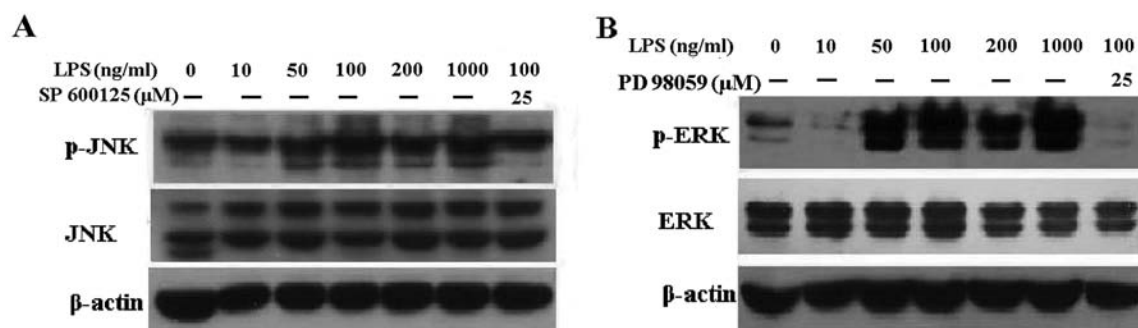

Figure 6. Effect of lipopolysaccharide (LPS) on the protein expression and phosphorylation of mitogen-activated protein kinases (MAPKs) of RAW264.7 cells in the presence of MAPK inhibitors (A) SP600125 and (B) PD98059 at 15 min after LPS treatment. $^{*}P<0.05$; $^{**}P<0.01$. Data show the mean ± SD from three independent experiments.

MAPKs are activated by a variety of stresses including LPS and then affect apoptosis either in a positive or negative manner (37). In many cell types, ERK1/2 is involved in cell proliferation and cell cycle progression, while the JNK pathway has been involved in both apoptosis and survival signaling (38). A novel ERK to JNK cross-activation has been reported, with JNK being the final mediator for the stimulation of cell proliferation by ERK (39). In the current study, LPS promoted osteoclast differentiation through the enhanced phosphorylation of ERK1/2 and JNK which were involved in the cell proliferation pathway.

In conclusion, our data suggest that LPS promoted osteoclast formation and activation by enhancing RANK signaling and COX-2 expression. LPS also promoted osteoclast differentiation through an enhanced cell proliferation pathway of ERK1/2 and JNK. Our findings indicate that blocking RANK signaling and COX-2 expression may be used as an evaluation agent to treat bacteria-induced bone resorption.

Acknowledgements

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