Quercetin reversed lipopolysaccharide-induced inhibition of osteoblast differentiation through the mitogen-activated protein kinase pathway in MC3T3-E1 cells

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Received November 14, 2013; Accepted June 17, 2014

DOI: 10.3892/mmr.2014.2633

Abstract. Quercetin, a flavonoid found in onions and other vegetables, has potential inhibitory effects on bone resorption in vivo and in vitro. In our previous study it was identified that quercetin triggered the apoptosis of lipopolysaccharide (LPS)-induced osteoclasts and inhibited bone resorption. Currently, little information is available detailing the effect of quercetin on osteoblast differentiation and bone formation in bacteria-induced inflammatory diseases. The present study aimed to investigate the effect of quercetin on osteoblast differentiation in MC3T3-E1 osteoblasts stimulated with LPS. LPS significantly downregulated the mRNA expression of osteoblast-related genes in the MC3T3-E1 cells. By contrast, quercetin significantly restored the LPS-suppressed mRNA expression of osteoblast-related genes in a dose-dependent manner. Quercetin also restored the protein expression of Osterix in MC3T3-E1 cells suppressed by LPS. Furthermore, quercetin selectively triggered the activation of the mitogen-activated protein kinase (MAPK) pathway by enhancing the expression of extracellular signal-regulated kinase and reducing the expression of c-Jun N-terminal kinase. These data suggest that quercetin reversed the inhibition of osteoblast differentiation induced by LPS through MAPK signaling. These findings suggest that quercetin may be of potential use as a therapeutic agent to restore osteoblast function in bacteria-induced bone diseases.

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Key words: quercetin, osteoblast, lipopolysaccharide, osterix, mitogen-activated protein kinase

Introduction

Bone is a dynamic tissue that constantly undergoes remodeling. Bone remodeling is a coupled process of bone formation mediated by osteoblasts and resorption regulated by osteoclasts, which continues throughout life (1). An imbalance between bone formation and resorption may result in excessive bone loss, which is a feature of chronic inflammatory diseases, including rheumatoid arthritis, osteomyelitis, bacterial arthritis and infection of orthopedic implants (2).

Lipopolysaccharide (LPS), a component of the outer membranes of all gram-negative bacteria, has been demonstrated to be capable of inducing bone resorption in vivo and in vitro (3-8). Furthermore, LPS is able to inhibit osteoblast differentiation and function in cell culture (9-12). However, effective therapies against bacteria-induced bone destruction are limited to antibiotic regimens and surgical strategies in chronic inflammatory diseases. Therefore, the investigation and development of potential drugs that restore osteoblast function remains a major goal in the prevention of bone destruction in infective bone diseases.

Quercetin, a dietary flavonoid, has been highlighted as a bioactive substance, due to its biological, pharmacological and medicinal activities. Evidence suggests that quercetin inhibits bone loss by affecting osteoclastogenesis and regulating a variety of systemic and local factors, including hormones and inflammatory cytokines (13-15). By contrast, the effect of quercetin on osteoblastogenesis remains a matter of controversy (16-17).

Mitogen-activated protein kinases (MAPK) pathways, including c-Jun N-terminal kinases (JNK), are involved in the progression of inflammatory responses and cell apoptosis in osteoblasts (18). Quercetin has been reported to demonstrate an inhibitory effect on MAPK activation and cyclooxygenase-2 (COX-2) expression induced by LPS (19). In our previous study it was identified that quercetin triggered apoptosis and inhibited bone absorption of LPS induced-osteoclasts through activation of MAPK p38 and JNK pathways (20). Therefore, the aim of the present study was to examine the effect of quercetin on osteoblast differentiation in MC3T3-E1 osteoblasts stimulated with LPS. Furthermore, the mechanism underlying the effect of

quercetin on MAPK phosphorylation in MC3T3-E1 osteoblasts stimulated with LPS was also examined.

Materials and methods

Reagents. Escherichia coli LPS (serotype 055:B5), quercetin, β-glycerophosphate (β-GP), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The protease inhibitor cocktail, and the selective MAPK inhibitors, PD98059 and SP600125, were purchased from Calbiochem (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco (Gibco, Rockville, MD, USA). Antibodies against JNK, phosphorylated JNK, extracellular signal-regulated kinase (ERK1/2) and phosphorylated ERK 1/2 were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against Osterix (Osx) and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). MC3T3-E1 cells, an osteoblast-like cell line, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Other chemicals and reagents used in the present study were of analytical grade.

Cell culture. MC3T3-E1 cells were grown in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin-streptomycin solution, 10 mM HEPES solution and incubated at 37˚C in 5% CO2 humidified air. To examine the effect of quercetin on osteoblast differentiation stimulated with LPS, MC3T3-E1 cells at 5x104 cells/cm2 were cultured in osteogenic differentiation medium (DMEM with 10% FBS, 10 mM HEPES, 50 μg/ml L-ascorbic acid and 5 mM β-GP) for two days. On differentiation day three, the cells were treated with LPS at 100 ng/ml or without LPS in osteogenic differentiation medium for one day. The cells were then incubated with various concentrations of quercetin (5, 10, 15, 25 or 50 μM) or without quercetin in the presence of LPS (100 ng/ml) for the indicated times.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from MC3T3-E1 cells treated with 10, 50, 100, 200 or 1,000 ng/ml LPS for 24 h, or treated with 5, 10, 15, 25 or 50 μM quercetin in the presence of 100 ng/ml LPS for 24 h. Total RNA was isolated using TRIzol reagent (Invitrogen, Grand Island, NY, USA) and quantified by spectrophotometry (Eppendorf BioSpectrometer; Eppendorf AG, Hamburg, Germany). Following isolation, 3 μg total RNA from each sample was reverse transcribed utilizing the HiFi-MMLV cDNA kit (Beijing CoWin Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. Primers for alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), Osx, bone sialoprotein (BSP), osteocalcin (OCN), type I collagen α1 (Col1α1) and β-actin (Sangon Biotech Co. Ltd, Shanghai, China) and the annealing temperatures used in the present study are listed Table I. In each reaction, 1 μl cDNA, 12.5 μl 2X Taq MasterMix (Beijing CoWin Biotech Co. Ltd., Beijing, China) and 0.4 μM forward and reverse primer in a total volume of 25 μl were used. The initial denaturation was performed at 94˚C for 3 min. Then, the products were 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.

Quantitative (q)PCR. Total RNA was isolated using TRIzol reagent (Invitrogen) and quantified by spectrophotometry. Following isolation, 3 μg total RNA from each sample was reverse transcribed utilizing the HiFi-MMLV cDNA kit (Beijing CoWin Biotech) according to the manufacturer's instructions. The primer sequences of JNK1 and ERK1 (Sangon Biotech Co. Ltd, Shanghai, China) and annealing temperatures used in this study are also listed in Table I. qPCR was performed with a RealSYBR mixture (Beijing CoWin Biotech Co. Ltd.) according to the manufacturer's instructions. All qPCR reactions were performed using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Grand Island, NY, USA). In each reaction, 1 μl cDNA, 10 μl 2X RealSYBR mixture, and 0.25 μM forward and reverse primer in a total volume of 20 μl were used. The reaction condition was as follows: One cycle of 95˚C for 5 min followed by 40 cycles of 95˚C for 15 sec, annealing temperature for 30 sec and extension at 72˚C for 30 sec. qPCR for each sample was run in triplicate. β-actin was used as the internal control and all of the results were analyzed using the standard 2-ΔΔCT method as described previously (21).

Western blot analysis. At the end of treatment, the cell culture medium was aspirated and the cells were detached in phosphate-buffered saline by scrapping. The detached cells were centrifuged at 15,000 x g at 4˚C for 15 min. The cell pellets were then lysed in 300 μl lysis buffers (Cytobuster protein extraction reagent; Novagen, Darmstadt, Germany) with 25 mM NaF, 1 mM Na3VO4 and 1X protease inhibitor cocktail. The protein concentrations were quantified by spectrophotometry. For western blotting, equal quantities of protein from each sample were loaded on SDS-PAGE and electrotransferred onto polyvinylidene fluoride (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 overnight at 4˚C with the following primary antibodies: Rabbit polyclonal anti-JNK, phosphorylated JNK and β-actin, goat polyclonal anti-Osx (all 1:500; Santa Cruz Biotechnology, Inc.) and rabbit polyclonal anti-ERK1/2 and phosphorylated ERK1/2 (1:1,000; Cell Signaling Technology, Inc.) .The secondary antibodies used for detected were horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G (Santa Cruz Biotechnology, Inc.) and HRP-conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology, Inc). Incubation was conducted at room temperature for 2 h (Santa Cruz Biotechnology, Inc.). Enhanced chemiluminescence (Beyotime, Shanghai, China) was used to detect the immunoreactive protein signals. The protein signals were visualized on films, then scanned and quantified using the Image J software (National Institutes of

Table I. Primer sequences and cycle conditions for polymerase chain reaction amplification.

Gene	Primer sequences (5'-3')	Accession number	Annealing temperature (˚C)	Product size (bp)
ALP	F: GAGCGTCATCCCAGTGGAG			
	R: TAGCGGTTACTGTAGACACCC	NM_007433	62	158
Runx2	F: TTCAACGATCTGAGATTTGTGGG			
	R: GGATGAGGAATGCGCCCTA	NM_001146038	62	221
Osx	F: ATGGCGTCCTCTCTGCTTG			
	R: TGAAAGGTCAGCGTATGGCTT	NM_130458	62	156
BSP	F: CAGGGAGGCAGTGACTCTTC			
	R: AGTGTGGAAAGTGTGGCGTT	NM_008318	58	158
OCN	F: GAGGGCAATAAGGTAGTGAA			
	R: CATAGATGCGTTTGTAGGC	NM_001037939	62	160
Col1α1	F: CCCTGCCTGCTTCGTGTA			
	R: TTGAGTTTGGGTTGTTCGTC	BC003198	63	101
JNK1	F: CTCCAGCACCCATACATC			
	R: CATTGACAGACGGCGAAG	BC053027	62	247
ERK1	F: GAGCGGCTGAAGGAGTTG			
	R: GGGATTGGAGTGGGAGAA	NM_011952	62	260
β-actin	F: GGCTGTATTCCCCTCCATCG			
	R: CCAGTTGGTAACAATGCCATGT	NM_007393	60	154

F, Forward; R, Reverse; *ALP*, alkaline phosphatase; *Runx2*, runt-related transcription factor 2; *Osx*, osterix; *Col1α1*, type I collagen; *OSN*, osteocalcin; *BSP*, bone sialoprotein; *ERK*, extracellular signal-regulated kinase; *JNK*, c-Jun N-terminal kinase.

Health Image, Bethesda, MA, USA). For re-probing, PVDF membranes were stripped with 0.2 M NaOH for 10 min prior to blocking with another primary antibody. The expression of the molecules of interest was determined relative to that of β-actin.

Statistical analysis. The data are expressed as the mean ± standard deviation for three or more independent experiments. The significant differences were determined using factorial analysis of variance. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of quercetin on the mRNA expression levels of osteoblast-specific genes in MC3T3-E1 cells stimulated with LPS. Effect of quercetin on the mRNA expression levels of osteoblast-specific genes, including ALP, Osx, Runx2, Col1α1, OCN and BSP in the MC3T3-E1 cells stimulated with LPS was determined by RT-PCR. The mRNA expression levels of osteoblast-specific genes in MC3T3-E1 cells were significantly inhibited by LPS, whereas quercetin significantly restored the LPS-suppressed mRNA expression of osteoblast-related genes in a dose-dependent manner in MC3T3-E1 cells (Fig. 1). Quercetin at 50 μM was demonstrated to completely restore mRNA expression levels of osteoblast-specific genes compared with the non-quercetin-treated cultures in MC3T3-E1 cells stimulated with LPS.

Effect of quercetin on the protein level of Osx in MC3T3-E1 cells stimulated with LPS. The protein level of Osx in MC3T3-E1 cells was significantly inhibited by LPS after cell exposure to LPS >16 h (Fig. 2A). To examine the effect of quercetin on the protein level of Osx in MC3T3-E1 cells stimulated with LPS, the cells were treated with LPS at 100 ng/ml for one day. Then, the cells were incubated with various concentrations of quercetin (5, 10, 15, 25 or 50 μM), or without quercetin, in the presence of LPS (100 ng/ml) for one day. Quercetin significantly upregulated the protein expression of Osx in a dose-dependent manner in MC3T3-E1 cells stimulated with LPS at one day compared with the non-quercetin-treated cultures (Fig. 2B).

Effect of quercetin on the protein levels of MAPKs in MC3T3-E1 cells stimulated with LPS. MC3T3-E1 cells at 5x104 cells/cm2 were cultured in osteogenic differentiation medium for two days. On differentiation day three, the cells were treated with LPS at 100 ng/ml in osteogenic differentiation medium for one day. Then, the cells were incubated with various concentrations of quercetin (5, 10, 15, 25 or 50 μM), or without quercetin, in the presence of LPS (100 ng/ml) for 2 h or 30 min. Quercetin significantly enhanced the protein levels of phosphorylated ERK1/2 and decreased the protein levels of phosphorylated JNK (Fig. 3). Quercetin at 50 μM was demonstrated to inhibit the protein expression of phosphorylated JNK compared with the other quercetin-treated cultures.

MAPK inhibitors, PD98059 and SP600125, were applied for 2 h prior to quercetin treatment and the protein samples were prepared 30 min following quercetin treatment. The results

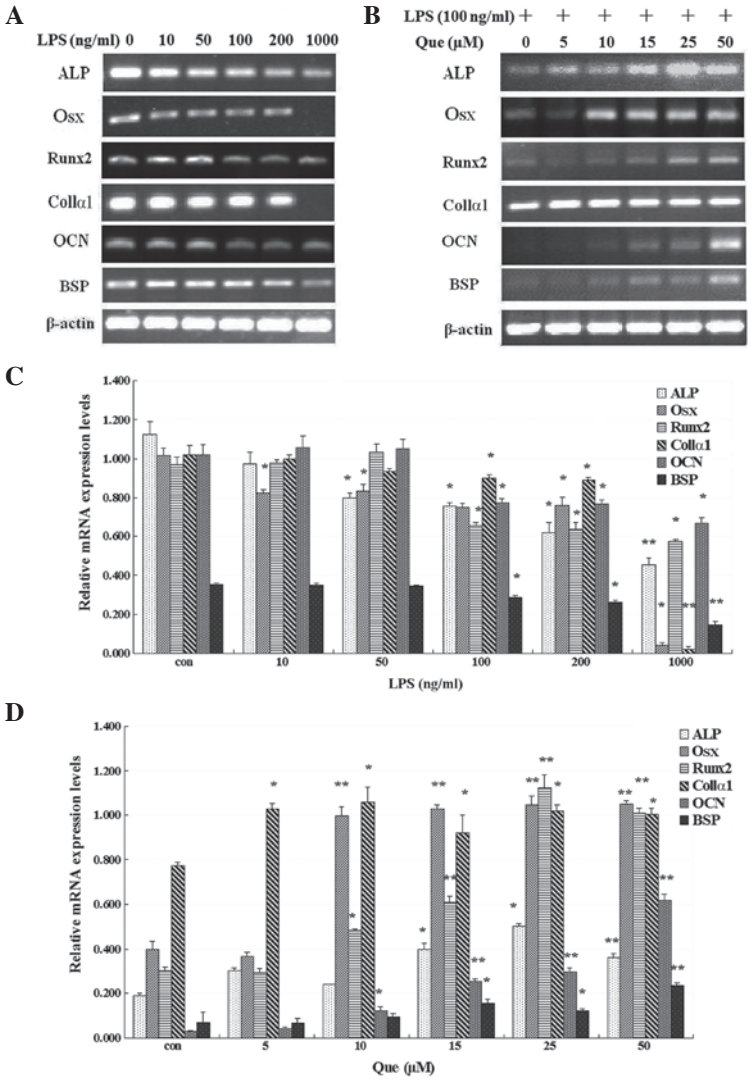

Figure 1. mRNA expression of *ALP*, *OSX*, *Runx2*, *Col1α1*, *OCN* and *BSP* (A) following treatment with LPS at concentrations of 10, 50, 100, 200 and 1,000 ng/ml, or without LPS, for 24 h or (B) with Que treatment (0, 5, 10, 15, 25, or 50 μM) for one day in MC3T3-E1 cells stimulated with LPS (100 ng/ml). (C) MC3T3-E1 cells were treated with LPS (0, 10, 50, 200 and 1,000 ng/ml) for 24 h and (D) with Que at concentrations of 0, 5, 10, 15, 25 and 50 μM. *P<0.05, and **P<0.01, compared with the control and the other groups. Data represent the mean ± standard deviation from three independent experiments. LPS, lipopolysaccharide; Que, quercetin; *ALP*, alkaline phosphatase; *Osx*, osterix; *Runx2*, runt-related transcription factor 2; *Col1α1*, type I collagen α1; *OSN*, osteocalcin; *BSP*, bone sialoprotein.

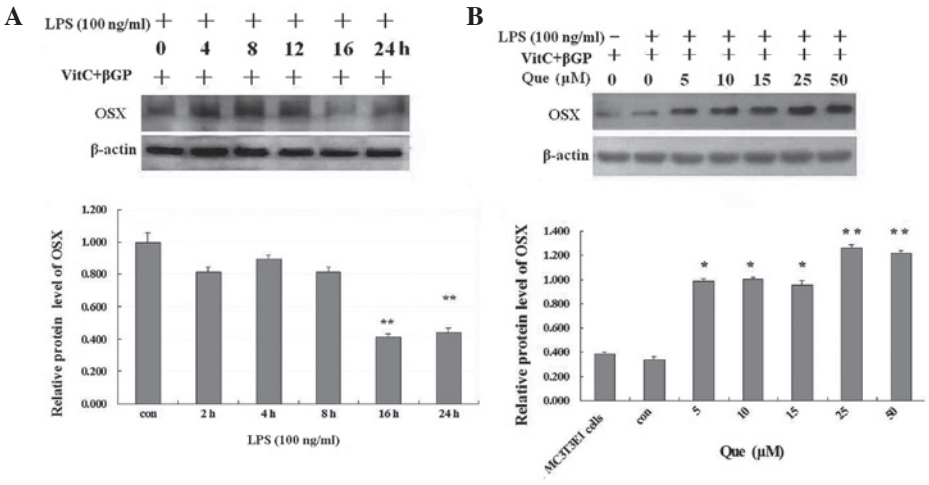

Figure 2. (A) Effect of LPS on the protein expression of Osx in MC3T3-E1 cells. MC3T3-E1 cells were treated with LPS at 100 ng/ml or without LPS for 24 h. (B) Effect of Que on the protein expression of Osx in MC3T3-E1 cells stimulated with LPS. MC3T3-E1 cells were treated with Que at concentrations of 5, 10, 15, 25 and 50 μM, or without Que, for one day in the presence of LPS (100 ng/ml) .*P<0.05 and **P<0.01, compared with the control and the other groups. LPS, lipopolysaccharide; Que, quercetin; Osx, osterix; β-GP, β-glycerophosphate.

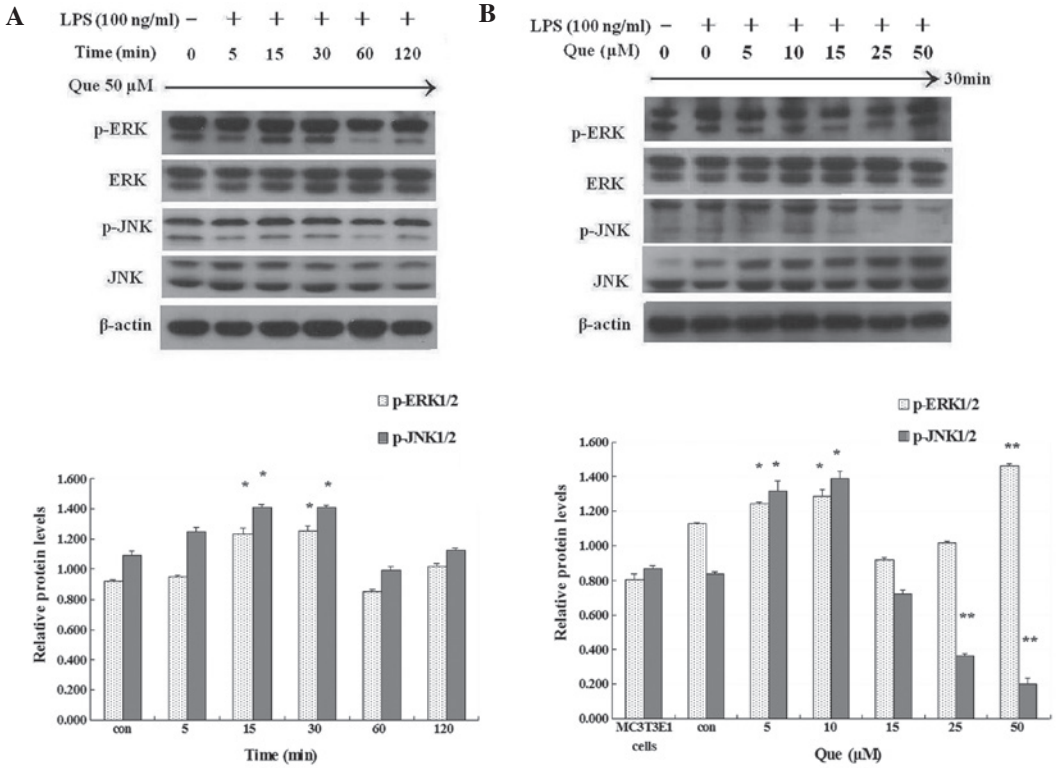

Figure 3. (A) Effect of Que at 50 μM on the protein expression of MAPKs in MC3T3-E1 cells in the presence of LPS (100 ng/ml) for 2 h. (B) Effect of Que at 5, 10, 15, 25 or 50 μM, or without Que, on the protein expression of MAPKs in MC3T3-E1 cells in the presence of LPS (100 ng/ml) for 30 min. *P<0.05 and **P<0.01. Data represent the mean ± standard deviation from three independent experiments. MAPKs, mitogen-activated protein kinases; LPS, lipopolysaccharide; Que, quercetin; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p, phosphorylated.

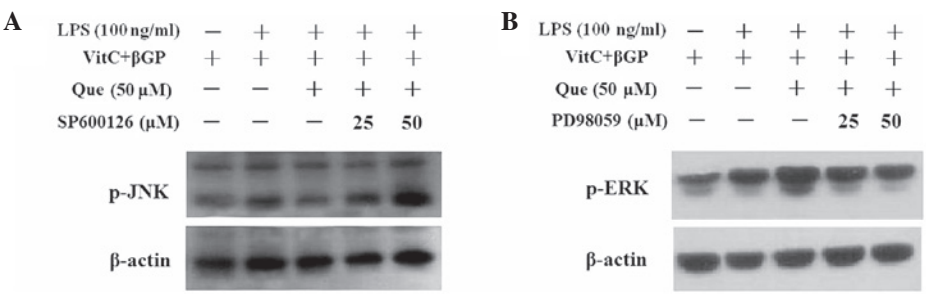

Figure 4. Effect of MAPK inhibitors (A) SP600125 or (B) PD98059 on the protein expression of MAPKs in MC3T3-E1 cells treated with Que in the presence of LPS (100 ng/ml) for 30 min.*P<0.05 and **P<0.01, compared with the control and the other groups. Data represent the mean ± standard deviation from three independent experiments. MAPKs, mitogen-activated protein kinases; LPS, lipopolysaccharide; Que, quercetin; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p, phosphorylated; β-GP, β-glycerophosphate.

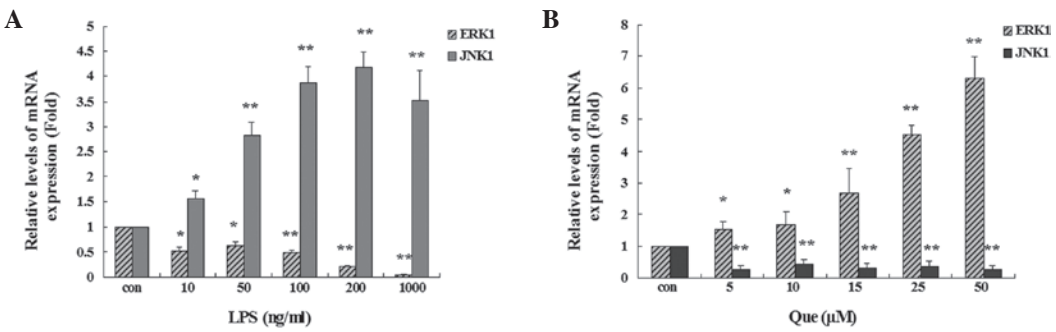

Figure 5. Effect of Que on the mRNA expression of MAPKs in MC3T3-E1 cells stimulated with LPS. (A) The mRNA expression of *ERK1* and *JNK1* following LPS treatment (10, 50, 100, 200 and 1,000 ng/ml), or without LPS, for one day in MC3T3-E1 cells. (B) The mRNA expression of *ERK1* and *JNK1* following Que treatment (5, 10, 15, 25 or 50 μM), or without Que, for one day in the presence of LPS (100 ng/ml) in MC3T3-E1 cells. *P<0.05 and **P<0.01, compared with the control and the other groups. Data represent the mean ± standard deviation from three independent experiments. Que, quercetin; MAPKs, mitogen-activated protein kinases; LPS, lipopolysaccharide; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

demonstrated that MAPK inhibitors selectively decreased the quercetin-enhanced protein level of phosphorylated ERK1/2 (by PD98059), while restored quercetin-induced downregulation of the protein expression of JNK (by SP600125; Fig. 4).

Effect of quercetin on the mRNA expression levels of MAPK in MC3T3-E1 cells stimulated with LPS. MC3T3-E1 cells were cultured in osteogenic differentiation medium with 100 ng/ml LPS for one day. Then, the cells were incubated with various concentrations of quercetin (5, 10, 15, 25 or 50 μM), or without quercetin, in the presence of LPS (100 ng/ml) for one day. The results demonstrated that quercetin enhanced the mRNA expression of ERK1, which was downregulated by LPS, and decreased the mRNA expression of JNK1, which was upregulated by LPS in a dose-dependent manner in MC3T3-E1 cells (Fig. 5).

Discussion

Excessive bone resorption in chronic inflammatory diseases, including septic arthritis, osteomyelitis and infected orthopedic implant failure, is at least partially caused by bacteria-induced activation of inflammatory responses (2). LPS, a pro-inflammatory glycolipid component of the gram-negative bacteria cell wall, is well documented in mediation with gram-negative bacterial bone destruction. LPS stimulates osteoclastic bone resorption in vivo (3,4), and promotes osteoclast differentiation in whole bone marrow cell culture (5) or in preosteoclasts (6,7). Furthermore, LPS also inhibits osteoblast differentiation and function in vitro (9-12). Therefore, the investigation of potential drugs that restore osteoblast function remains a major goal in the prevention of bone destruction in infective bone diseases. In our previous study it was identified that quercetin triggered the apoptosis and inhibited bone absorption of LPS induced-osteoclasts through activation of the MAPK p38 and JNK pathways (20). In the present study, the effect of quercetin on osteoblast differentiation in MC3T3-E1 osteoblasts stimulated with LPS was examined. The results demonstrated that quercetin reversed the inhibition of osteoblast differentiation induced by LPS through MAPK signaling.

The reverse effect of quercetin on LPS-induced inhibition of osteoblast differentiation was confirmed by examining the effect of quercetin treatment on mRNA expression levels of osteoblast-related genes. ALP has been suggested to be involved in the early-stage molecular events of osteoblast differentiation, whereas OCN and BSP are involved in the late-stage molecular events (18,22). Col1, the most abundant and widely distributed type of collagen, is required and sufficient for extracellular matrix mineralization to occur in bone. Col1 is composed of two chains, α1 and α2, encoded by two distinct genes, Col1α1 and Col1α2, which are highly expressed in osteoblasts (22-24). In the present study, the mRNA expression levels of ALP, Osx, Runx2, Col1α1, OCN and BSP in MC3T3-E1 cells were significantly downregulated by LPS. By contrast, quercetin significantly restored the LPS-suppressed mRNA expression of osteoblast-related genes in a dose-dependent manner. The data indicated that the reverse effect of quercetin on LPS-induced inhibition of osteoblast differentiation may be due to the restored expression of osteoblast-related genes.

Bone formation is a highly regulated process involving the differentiation of mesenchymal stem cells to osteoblasts. Runx2 (Cbfa1) is required for mesenchymal cell differentiation into preosteoblasts (25). Osx, downstream of Runx2, is an osteoblast-specific transcription factor essential for osteoblast differentiation and bone formation (26). Forced expression of Osx in vitro induces the expression of several osteoblastic genes, including Col1α1 and osteocalcin (27,28). The present study demonstrated that quercetin restored the expression of Osx and Runx2, which were suppressed by LPS in MC3T3-E1 cells. Based on these results, it is suggested that restoration of osteoblast differentiation induced by quercetin also resulted from enhancing the expression of Osx and Runx2.

MAP kinases are activated by various stresses, including LPS and affect apoptosis either positively or negatively (29). In numerous cell types, JNK and p38 MAPK contribute to the induction of apoptosis, whereas ERK inhibits apoptotic processes (30-32). In the present study, treatment with quercetin enhanced the protein levels of phosphorylated ERK1/2, whereas it reduced the protein levels of phosphorylated JNK. Quercetin also enhanced the mRNA expression of ERK1, while decreased mRNA expression of JNK1 in a dose-dependent manner in MC3T3-E1 cells stimulated with LPS. The quercetin-enhanced phosphorylation of ERK1/2 was attenuated by PD98059; while SP600125 restored quercetin-induced downregulation of phosphorylated JNK. The present study confirms that quercetin was able to restore the inhibition of osteoblast differentiation induced by LPS through MAPK signaling (18).

In conclusion, these data suggest that quercetin may reverse LPS-induced inhibition of osteoblast differentiation through MAPK signaling. These results indicate that quercetin may be an effective drug for the treatment of abnormal human bone loss induced by LPS in chronic inflammatory diseases.

Acknowledgements

The present study was supported by a research grant from the National Natural Science Foundation of China (no. 81371981) and The School Fund of Luohe Medical College (no 2012-DF001).

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