Original Paper

Protective Effects of Pretreatment with Quercetin Against Lipopolysaccharide-Induced Apoptosis and the Inhibition of Osteoblast Differentiation via the MAPK and Wnt/ β -Catenin Pathways in MC3T3-E1 Cells

Chun Guo^{a, b} Rui-Juan Yang^a Ke Jang^c Xiao-Ling Zhou^a Yanyan Liu^a

^aHenan Key Laboratory of Neural Regeneration, the First Affiliated Hospital of Xinxiang Medical University, ^bDepartment of Orthopedics, the First Affiliated Hospital of Henan University, ^cDepartment of Orthopedics, Affiliated Hospital of North Sichuan Medical College, Nanchong, Sichuan, China

Key Words

Quercetin • Lipopolysaccharide • Apoptosis • Osteoblast • Differentiation • MAPK • Wnt/ β -catenin

Abstract

Background/Aims: 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, we found that quercetin treatment reversed lipopolysaccharide (LPS)-induced inhibition of osteoblast differentiation through the mitogen-activated protein kinase (MAPK) pathway in MC3T3-E1 cells. In the present study, we investigated the underlying mechanisms of pretreatment with quercetin on apoptosis and the inhibition of osteoblast differentiation in MC3T3-E1 cells induced by LPS. **Methods:** MC3T3-E1 osteoblasts were treated with quercetin for 2 h; cells were then incubated with LPS in the presence of quercetin for the indicated times. Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay, and cell apoptosis was evaluated using Hoechst 33258 staining. The mRNA expression levels of osteoblast-specific genes, Bcl-2 and caspase-3 were determined by real-time quantitative polymerase chain reaction (RT-PCR). Protein levels of osteoblast-specific genes, caspase-3, Bax, cytochrome c, Bcl-2, Bcl-2 phosphorylated MAPKs and Wnt/ β -catenin were measured using Western blot assays. The MAPK and Wnt/ β -catenin signalling pathways were blocked prior to pretreatment with quercetin. **Results:** Pretreatment with quercetin significantly restored LPS-suppressed bone mineralization and the mRNA and protein expression levels of osteoblast-specific genes such as Osterix (OSX), runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and osteocalcin (OCN) in a dose-dependent manner. Pretreatment with quercetin also inhibited LPS-induced apoptosis. **Conclusion:** Pretreatment with quercetin significantly restored LPS-suppressed bone mineralization and the mRNA and protein expression levels of osteoblast-specific genes such as Osterix (OSX), runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and osteocalcin (OCN) in a dose-dependent manner. Pretreatment with quercetin also inhibited LPS-induced apoptosis. C. Guo, R. Yang and K. Jang contributed equally to this work.

Chun Guo

Henan Key Laboratory of Neural Regeneration,
The First Affiliated Hospital of Xinxiang Medical University, Weihui,
88 Jiankang Road, Henan, (China); E-Mail guospring@126.com

osteoblast apoptosis, significantly restored the down-regulated expression of Bcl-2 and Bcl-XL and decreased the upregulated expression of caspase-3, Bax, and cytochrome c in MC3T3-E1 cells induced by LPS. Furthermore, pretreatment with quercetin not only decreased the abundance of phosphorylated p38 MAPK and increased the abundance of phosphorylated extracellular signal regulated kinase (ERK), but also triggered the Wnt/ β -catenin pathway through enhancing expression of Wnt3 and β -catenin. Pretreatment with MAPK inhibitors or the Wnt/ β -catenin inhibitor XAV939 blocked the protective effects of quercetin against LPS-induced apoptosis and the inhibition of osteoblast differentiation. **Conclusions:** Our findings suggest that pretreatment with quercetin may be a potential drug for preventing abnormal human bone loss induced by LPS in bacteria-induced bone diseases.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

Bone is a dynamic tissue that constantly undergoes remodeling, a life-long coupled process of bone formation by osteoblasts and resorption by osteoclasts. An imbalance between bone formation and bone resorption results in excessive bone loss, a feature of chronic inflammatory diseases such as osteomyelitis, and bacterial arthritis and of infected orthopaedic implants [1].

Lipopolysaccharide (LPS), a component of the outer membrane of all gram-negative bacteria, was shown to be capable of inducing bone resorption *in vivo* and *in vitro* [2-5]. Furthermore, LPS is able to inhibit osteoblast differentiation and to induce apoptosis *in vitro* [6-10]. Currently, effective therapy against bacteria-induced bone destruction is limited to treatment with antibiotic regimens and surgical strategies for infective bone diseases. Therefore, the investigation of potential drugs that restore down-regulated osteoblast function remains a major goal in the prevention of bone destruction in infective bone diseases.

Quercetin, a dietary flavonoid, was shown to inhibit bone loss by affecting osteoclastogenesis and by regulating a variety of systemic and local factors such as hormones and inflammatory cytokines [11-15]. However, the effect of quercetin on osteoblastogenesis remains a matter of controversy [16-21]. In our previous study, we found quercetin reversed LPS-induced inhibition of osteoblast differentiation [22]. However, the effect of pretreatment with quercetin on LPS-induced apoptosis and the inhibition of osteoblast differentiation is still unclear.

Mitogen-activated protein kinase (MAPK) signalling pathways play a vital role in the progression of inflammatory responses and cell apoptosis in osteoblasts [9, 23, 24]. Quercetin has been reported to demonstrate an inhibitory effect on MAPK activation and cyclooxygenase-2 (COX-2) expression induced by LPS [15, 25-27].

The Wnt/ β -catenin signalling pathway also plays a vital role in regulating osteoblast differentiation and bone formation [28-30]. However, the mechanism of the Wnt/ β -catenin signalling pathway in regulating the progression of the inflammatory responses in osteoblasts remains poorly understood.

Therefore, the aim of the present study was to evaluate the effect of pretreatment with quercetin on the apoptosis and osteoblast differentiation of MC3T3-E1 osteoblasts induced by LPS. Furthermore, we also examined the mechanism of pretreatment with quercetin on MAPK phosphorylation and Wnt/ β -catenin signalling in MC3T3-E1 osteoblasts stimulated with LPS.

Materials and Methods

Reagents and antibodies

Escherichia coli LPS (serotype 055: B5), quercetin (Q4951, purity $\geq 95\%$ by HPLC), β -glycerophosphate (β -GP), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ascorbic acid and alizarin red S were

purchased from Sigma Chemical Co (St Louis, MO, USA). The selective MAPK inhibitors SB203580, PD98059 and SP600125 were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). The protease inhibitors cocktail, dexamethasone (Dex), and the selective Wnt/ β -catenin inhibitor XAV939 were purchased from Selleck Chemicals (Houston, TX, USA). Antibodies to Bcl-2 (#2870), Bcl-xL (#2764), p38 MAPK (#9212), phosphorylated (p-)p38 (#9211), extracellular signal-regulated kinase (ERK)1/2 (#4695) and p-ERK1/2 (#4370) were purchased from Cell Signalling Technology, Inc. (Beverly, MA, USA). Antibodies to caspase-3 (sc-7148), Bax (sc-493), cytochrome c (sc-13156), JNK1/2 (sc-571), p-JNK1/2 (sc-12882), osterix (OSX; sc-393325), osteocalcin (OCN; sc-390877), ALP (sc-137213), β -actin (sc-47778), Wnt3 (sc-47778) β -catenin (sc-7963) and GSK3 β (sc-9168) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antibody to runt-related transcription factor 2 (Runx2; ab76956) was purchased from Abcam (Cambridge, UK). The osteoblast-like cell line MC3T3-E1 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All 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% FBS, 1% penicillin-streptomycin solution, and 10 mM HEPES solution and were incubated at 37°C in 5% CO₂ humidified air. To examine the effect of quercetin on osteoblast differentiation stimulated by LPS, MC3T3-E1 cells at 5×10^4 cells/cm² were cultured in osteogenic differentiation medium (DMEM with 10% FBS, 10 mM HEPES, 50 μ g/ml L-ascorbic acid and 10 mM β -GP) for 2 d. On differentiation day 3, cells were treated with quercetin at concentrations of 5, 10, 15, 25, or 50 μ M or were treated without quercetin in osteogenic differentiation medium for 2 h, and then cells were incubated with LPS (100 ng/ml) in the presence of quercetin (5, 10, 15, 25, 50 μ M) for the indicated time.

Cell viability assay

Cell viability was measured with the Cell Counting Kit-8 (CCK-8) assay (Beyotime Institute of Biotechnology, Shanghai, China). MC3T3-E1 cells (4×10^4 cells/cm²) were plated in 96-well culture plates and cultured in 0.1 ml of osteogenic differentiation medium. To evaluate the effect of quercetin on osteoblast viability, cells were treated with 10, 20, 50, or 100 μ M quercetin or without quercetin for 1, 3, 5, or 7 d. To evaluate the effect of quercetin on LPS-induced osteoblast viability, cells were pretreated with 5, 10, 20, 40, 50, 60, 80, 100 or 150 μ M quercetin or without quercetin, and then cells were treated 100 ng/ml LPS or without LPS in the presence of quercetin for 1 or 3 d. At the end of treatment, 10 μ l of CCK-8 was added to each well. Cells were cultured for an additional 1 h. After shaking for 1 min, absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific Inc., Pittsburgh, USA). The experiment was performed in triplicate.

Study of osteoblast apoptosis

To measure apoptosis, we performed Hoechst 33258 staining (Beyotime Institute of Biotechnology) to visualize nuclear morphology and nuclear DNA fragmentation in osteoblasts. MC3T3-E1 cells (4×10^4 cells/cm²) were seeded into 12-well plates and incubated overnight. Cells were pretreated with 5, 10, 15, 25 and 50 μ M quercetin or without quercetin for 2 h, and then cells were treated with 100 ng/ml LPS or without LPS in the presence of quercetin for 1 d. At the end of the treatment, cells were washed with phosphate-buffered saline (PBS) to remove non-adherent cells, and the adherent cells were fixed in 4% paraformaldehyde solution for 10 min. After being washed with PBS, the cells were incubated with 0.2 μ M Hoechst 33258 for 5 min in the dark. Cells with nuclei containing clearly condensed chromatin or cells with fragmented nuclei were scored as apoptotic; the results were expressed as the number of apoptotic cells. The images of apoptotic osteoblasts were obtained under a fluorescence microscope (Olympus IX71, Olympus Optical, Tokyo, Japan).

Determination of mineralized matrix

Mineralization in the MC3T3-E1 cells was determined by staining with alizarin red S, which selectively binds to calcium and yields a dark red stain. MC3T3-E1 cells (4×10^5 cells/cm²) were cultured in 6-well plates and incubated in osteogenic differentiation medium. After pretreatment with quercetin at different concentrations of 10, 25, or 50 μ M or without quercetin for 2 h, MC3T3-E1 cells were incubated with LPS (100

Table 1. List of primers used for real-time PCR

Gene	Primer Sequences(Forward/Reverse) 5'-3'	Accession number	Annealing temperature(°C)	Product size (bp)
ALP	GAGCGTCATCCCAGTGGAG TAGCGGTTACTGTAGACACCC	NM_007433	62	158
Runx2	TTCAACGATCTGAGATTTGTGGG GGATGAGGAATGCGCCCTA	NM_001146038	62	221
OSX	ATGGCGTCCTCTCTGCTTG TGAAAGGTCAGCGTATGGCTT	NM_130458	62	156
OCN	CTGACCTCACAGATCCCAAGC TGGTCTGATAGCTCGTCACAAG	NM_031368	62	177
Bax	AGACAGGGGCCTTTTGCTAC AATTCGCCGGAGACACTCG	NM_007527	60	135
Caspase-3	TGGTGATGAAGGGGTCATTTATG TTCGGCTTTCCAGTCAGACTC	BC038825	60	205
β-actin	GGCTGTATTCCCCTCCATCG CCAGTTGGTAACAATGCCATGT	NM_007393	60	154

ng/ml) for 3 d in the presence of quercetin. Then, cells were cultured only with osteogenic differentiation medium for another 17 d. Staining with 2% alizarin red S (Sigma) was performed, and the images of calcified matrices were photographed under a fluorescence microscope (Olympus IX71) and quantified by the number of calcified nodules formed per unit area.

Quantitative real-time PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen, Grand Island, NY, USA) and quantified by spectrophotometry. After isolation, 3 µl of total RNA from each sample was reverse transcribed (RT) into cDNA utilizing the HiFi-MMLV cDNA Reverse Transcription Kit (Invitrogen) according to the manufacturer's protocol. The primer sequences of osteoblast-specific genes, caspase-3, Bax and β-actin (Generay Biotech Co. Ltd, Shanghai, China) and the annealing temperature used in this study are listed in Table 1. Real-time PCR was performed with a RealSYBR Green I Mix (Beijing CoWin Biotech) according to the manufacturer's protocol. All real-time qPCR reactions were performed in the ABI PRISM 7700 sequence detection system (Applied Biosystems, Grand Island, NY, USA). In each reaction, 1 µl of cDNA, 10 µl of 2×RealSYBR Mixture, and 0.25 µM forward and reverse primers in a total volume of 20 µl were used. The reaction conditions were as follows: 1 cycle at 95°C for 1 min, followed by 40 cycles at 95°C for 15 sec, annealing temperature for 30 sec and extension at 72°C for 30 sec. Real-time qPCR for each sample was run in triplicate. β-Actin was used as the internal control and all results were analysed using the standard $2^{-\Delta\Delta CT}$ method.

Western blot analysis

At the end of treatment, cell culture medium was discarded and cells were detached in PBS by scrapping. Detached cells were centrifuged at 15000 g at 4°C for 10 min. Cell pellets were then lysed in 300 µl of lysis buffer (CytoSuster protein extraction reagent, Novagen, Darmstadt, Germany) with 25 mM NaF, 1 mM Na₂VO₄ and 1×protease inhibitor cocktail. Protein concentrations were quantified by spectrophotometry. For Western blot, an equal amount of protein from each sample was loaded onto SDS-PAGE gels and electrotransferred 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). The secondary antibodies used for detection were horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) or HRP-conjugated goat anti-mouse IgG (both from Beyotime, Shanghai, China). Enhanced chemiluminescence (ECL; Beijing CoWin Biotech Co., Ltd.) was performed in order to detect immunoreactive protein signals. Protein signals were visualized and images were captured with a chemiluminescence detection system (MiniChemil™ III; Beijing Sage Creation Science Co., Ltd., Beijing, China) and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). For re-probing, the PVDF membranes were stripped

Fig. 1. A Effect of quercetin on cell viability in MC3T3-E1 cells at day 1, 3, 5, and 7. B Effect of pretreatment with quercetin on cell viability in MC3T3-E1 cells induced by LPS at day 1 and 3. * $P < 0.05$, ** $P < 0.01$. Data represent the mean $\pm$ SD from three independent experiments.

with 0.2 M NaOH for 15 min prior to blocking with another primary antibody. The expression of the molecules of interest was determined relative to β -actin.

Statistical analysis

The data were expressed as the means $\pm$ standard deviation (s.d.) for three or more independent experiments. Significant differences were determined using a factorial analysis of variance (ANOVA). Statistical analysis was performed using SPSS 13.0 software. A P -value < 0.05 was considered to indicate a statistically significant difference and shown with an asterisk in the bar diagrams.

Results

Effect of quercetin on cell viability MC3T3-E1 cells

MC3T3-E1 cells were treated with 25 or 50 μ M quercetin or without quercetin for 1, 3, 5, or 7 days. Cell viability was not inhibited by quercetin at day 1 and 3 (Fig. 1 A). Quercetin above 60 μ M significantly inhibited cell viability in MC3T3-E1 cells stimulated with LPS on day 1 (Fig. 1 B).

Effect of pretreatment with quercetin on LPS-induced MC3T3-E1 cell apoptosis

MC3T3-E1 cells were pretreated with 5, 10, 15, 25, or 50 μ M quercetin or without quercetin for 1 d, and then the cells were treated with 100 ng/ml LPS or without LPS in the presence of quercetin for 1 d. The number of fluorescence-positive osteoblasts was decreased with pretreatment of quercetin compared to the non-quercetin-treated group or the LPS-treated groups in MC3T3-E1 cells after 24 h (Fig. 2).

Effect of pretreatment with quercetin on the mineralized matrix in LPS-induced MC3T3-E1 cells

The effect of pretreatment with quercetin on the calcified nodules was visualized by alizarin red staining. After pretreatment with

Fig. 2. Effect of pretreatment with quercetin on nuclear condensation in MC3T3-E1 cells induced by LPS at day 1. Hoescht 33258-stained cells were analysed under a fluorescence microscope (magnification, $\times 40$). A Quercetin induced nuclear condensation in MC3T3-E1 cells induced by LPS. B The number of fluorescence-positive osteoblasts was decreased with pretreatment of quercetin compared to the non-quercetin-treated group or the LPS-treated groups in MC3T3-E1 cells after 24 h. * $P < 0.05$; ** $P < 0.01$. Data represent the mean $\pm$ S.D. from three independent experiments.

Fig. 3. Effect of pretreatment with quercetin on the calcified nodules was visualized by alizarin red staining. After pretreatment with quercetin at 10, 25, or 50 μ M or without quercetin for 2 h, MC3T3-E1 cells were incubated with LPS (100 ng/ml) for 3 d in the presence of quercetin. Then, the cells were cultured only in osteogenic differentiation medium. A At day 21 post-induction, the calcified nodules were visualized by alizarin red staining. Magnification $\times 100$. B The number of calcified nodules increased with pretreatment of quercetin compared to LPS-treated osteoblasts. * $P < 0.05$; ** $P < 0.01$. Data represent the mean $\pm$ S.D. from three independent experiments.

quercetin at 10, 25, or 50 μ M or without quercetin for 2 h, MC3T3-E1 cells were incubated with LPS (100 ng/ml) for 3 d in the presence of quercetin. Then, cells were cultured only in osteogenic differentiation medium. At day 21 post-induction, the calcified nodules were visualized by alizarin red staining. The number of calcified nodules was increased with pretreatment of quercetin compared to the LPS-treated osteoblasts (Fig. 3).

Effect of pretreatment with quercetin on the mRNA and protein expression of osteoblast-specific genes in LPS-induced MC3T3-E1 cells

MC3T3-E1 cells were pretreated with 5, 10, 15, 25 or 50 μ M quercetin or without quercetin for 2 h, and then the cells were treated with 100 ng/ml LPS or without LPS in the presence of quercetin for 1 d. The results showed that pretreatment with quercetin significantly regulated the mRNA and protein expression of osteoblast-specific genes such as ALP, Run-2, OSX and OCN in a dose-dependent manner after 1 d compared to the non-quercetin-treated group or the LPS-treated groups of MC3T3-E1 cells (Fig. 4 A, B and C). Following pretreatment with 50 μ M quercetin or without quercetin for 2 h, MC3T3-E1 cells were treated with 100 ng/ml LPS or without LPS in the presence of quercetin for 0, 4, 12, 24, or 48 h. The results also showed that pretreatment with quercetin significantly regulated the protein expression levels of osteoblast-specific genes (Fig. 4 D).

Effect of pretreatment with quercetin on the mRNA and protein expression of caspase-3, Bax, cytochrome c, Bcl-2 and Bcl-XL of LPS-induced MC3T3-E1 cells

MC3T3-E1 cells were pretreated with 5, 10, 15, 25 or 50 μ M quercetin or without quercetin for 2 h, and then the cells were treated with 100 ng/ml LPS or without LPS in the presence of quercetin for 1 d. Pretreatment with quercetin significantly restored the down-regulated expression of Bcl-2 and Bcl-XL while it decreased the upregulated expression of caspase-3, Bax, and cytochrome c compared to the non-quercetin-treated group or the LPS-treated groups of MC3T3-E1 cells (Fig. 5).

Fig. 4. Effect of pretreatment with quercetin on the mRNA and protein expression of osteoblast-specific genes in LPS-induced MC3T3-E1 cells. **A** Effect of pretreatment with quercetin on the mRNA expression levels of OSX, Runx2, ALP and OCN in LPS-induced MC3T3-E1 cells at 1 d using qPCR. **B** and **C** Effect of pretreatment with quercetin on the protein expression levels of OSX, Runx2, ALP and OCN in LPS-induced MC3T3-E1 cells at 1 d using Western-blotting. **D** Effect of pretreatment with quercetin at 50 μM or without quercetin on the protein expression of OSX, Runx2, ALP, OCN and OPN in LPS-induced MC3T3-E1 cells at 0, 4, 8, 12, 24, or 48 h using Western-blotting. * $P < 0.05$, ** $P < 0.01$. Data represent the mean $\pm$ S.D. from three independent experiments.

Effect of pretreatment with quercetin on protein expression of MAPK and Wnt/ β -catenin in LPS-induced MC3T3-E1 cells

MC3T3-E1 cells were pretreated with 5, 10, 15, 25 and 50 μM quercetin or without quercetin for 2 h, and then the cells were treated with 100 ng/ml LPS or without LPS in the presence of quercetin for 30 min. Quercetin significantly enhanced the protein levels of p-ERK1/2, Wnt/3 and β -catenin but decreased the protein levels of p-p38 and GSK-3 β (Fig. 6 and 7).

Effect of MAPK and Wnt/ β -catenin inhibitors on the protein expression of MAPK, Wnt/ β -catenin, osteoblast-specific genes, caspase-3, Bax, cytochrome c, Bcl-2 and Bcl-XL of LPS-induced MC3T3-E1 cells with quercetin pretreatment

After treatment with dexamethasone (Dex) for 2 h, the MAPK inhibitors SP600125, PD98059, and SB203580 and the Wnt/ β -catenin inhibitor XAV939 were applied for 2 h to pretreatment with 50 μM quercetin or without quercetin for 2 h; then cells were incubated with 100 ng/ml LPS or without LPS in the presence of quercetin for 30 min or 1 d. MAPK inhibitors significantly attenuated the quercetin-enhanced protein level of phosphorylated-ERK1/2 (by PD98059) and JNK (by SP600125) and restored the quercetin-induced down-regulation in the protein expression of phosphorylated-p38 (by SB203580) (Fig. 8). Moreover, MAPK inhibitors markedly attenuated the quercetin-enhanced protein expression of osteoblast-specific genes, Bcl-2 and Bcl-XL and restored the quercetin-induced down-regulation in the protein expression of caspase-3, Bax, and cytochrome c (Fig. 9 and 10). The Wnt/ β -catenin inhibitor XAV939 also markedly attenuated the quercetin-enhanced protein expression of osteoblast-specific genes (Fig. 11). Administration of dexamethasone

Fig. 5. Effect of pretreatment with quercetin on the mRNA and protein expression of caspase-3, cytochrome c, Bax, Bcl-2 and Bcl-XL in LPS-induced MC3T3-E1 cells at 1 d. A Effect of pretreatment with quercetin on the mRNA expression levels of caspase-3, and Bax in LPS-induced MC3T3-E1 cells at 1 d using qPCR. B Effect of pretreatment with quercetin on the protein expression levels of caspase-3, cytochrome c, Bax, Bcl-2 and Bcl-XL in LPS-induced MC3T3-E1 cells at 1 d using Western-blotting. C and D Pretreatment with quercetin significantly restored the down-regulated expression of Bcl-2 and Bcl-xL while it decreased the upregulated expression of caspase-3, Bax, and cytochrome c compared to the non-quercetin-treated group or the LPS-treated groups of MC3T3-E1 cells. *P<0.05; **P<0.01. Data represent the mean ± S.D. from three independent experiments.

also markedly attenuated the LPS-enhanced protein expression of MAPKs and the LPS-induced down-regulation of osteoblast-specific genes but reversed the quercetin-induced regulation of the phosphorylation of MAPKs and the protein expression of osteoblast-specific genes in MC3T3-E1 cells stimulated by LPS (Fig. 8-9).

Discussion

Excessive bone resorption in chronic inflammatory diseases such as septic arthritis, osteomyelitis, and infected orthopedic implant failure is at least partially caused by bacteria-induced activation of inflammatory responses [1]. LPS, a pro-inflammatory glycolipid component of the gram-negative bacterial cell wall, is a well-documented mediator of gram-negative bacterial bone destruction [2-5]. Quercetin, a dietary flavonoid, has been reported anti-inflammatory properties [15, 25-27]; however, whether the effect of quercetin on osteoblastogenesis is suppressive [16-18] or stimulatory [19-21] remains a matter of controversy. In this study, we found that cell viability was only inhibited by quercetin at high dosages after 5 and 7 d. In our previous study, we found quercetin treatment reversed LPS-induced inhibition of osteoblast differentiation through the MAPK pathway in MC3T3-E1 cells [22]. In this study, we found that pretreatment with quercetin could have protective

Fig. 6. A and B Effect of pretreatment with quercetin on the protein expression levels of phosphorylation of MAPKs in LPS-induced MC3T3-E1 cells at 30 min using Western-blotting. * $P < 0.05$; ** $P < 0.01$. Data represent the mean $\pm$ S.D. from three independent experiments.

Fig. 7. A and B Effect of pretreatment with quercetin on the protein expression levels of Wnt/ β -catenin pathway at 30 min in LPS-induced MC3T3-E1 cells using Western-blotting. Pretreatment with quercetin at 5, 10, 15, 25, 50 μ M or without quercetin for 2 h, then MC3T3-E1 cells were incubated with LPS (100 ng/ml) for 30 min in the presence of quercetin. * $P < 0.05$; ** $P < 0.01$. Data represent the mean $\pm$ S.D. from three independent experiments.

effects on LPS-induced apoptosis and the inhibition of osteoblast differentiation.

The protective effect of pretreatment with quercetin on the LPS-induced inhibition of osteoblast differentiation is confirmed by evaluating quercetin's effect on the expression of osteoblast-specific genes. Runx2 (Cbfa1) and ALP have been suggested to be involved in the early-stage molecular events of osteoblast differentiation [31, 32], whereas OSX and OCN are involved in the late-stage molecular events [23, 33, 34]. In this study, pretreatment with quercetin significantly restored

Fig. 8. Effect of pretreatment with MAPK inhibitors SP600125, PD98059, and SB203580 or Dex on the protein expression of MAPKs with quercetin pretreatment in LPS-induced MC3T3-E1 cells. **A** Effect of quercetin treatment on the protein expression of phosphorylated-ERK1/2 at 0, 0.25, 0.5, 1, 2, or 4 h in MC3T3-E1 cells. **B** Effect of Dex on the protein expression of MAPKs with quercetin pretreatment in LPS-induced MC3T3-E1 cells. Dex markedly attenuated the LPS- or quercetin-induced regulation of protein expression of MAPKs. **C-E** MAPK inhibitors markedly attenuated quercetin-enhanced protein expression of phosphorylated-ERK1/2 (by PD98059) and JNK (by SP600125) and restored the quercetin-induced down-regulation in the protein expression of phosphorylated p38 (by SB203580).

Fig. 9. Effect of MAPK inhibitors on the protein expression of osteoblast-specific genes at 1 d with quercetin pretreatment in LPS-induced MC3T3-E1 cells. **A** Pretreatment with Dex for 2 h. MAPK inhibitors SP600125, PD98059, SB203580 were applied respectively for 2 h prior to pretreatment with 50 μM quercetin or without quercetin for 2 h; then cells were incubated with 100 ng/ml LPS without LPS in the presence of quercetin for 1 d. MAPK inhibitors markedly attenuated the quercetin-enhanced protein expression of osteoblast-specific genes; while Dex markedly attenuated the LPS-induced down-regulation of osteoblast-specific genes and reversed the quercetin-induced regulation of the protein expression of osteoblast-specific genes in MC3T3-E1 cells stimulated by LPS.

the LPS-suppressed mRNA and protein expression of ALP, Runx2, OSX and OCN in MC3T3-E1 cells in a dose-dependent manner. Administration of dexamethasone also significantly attenuated the LPS-induced down-regulation of the protein expression of osteoblast-specific genes and reversed the quercetin-induced upregulation in protein expression of osteoblast-specific genes stimulated by LPS. Our data indicated that the protective effect of pretreatment with quercetin against LPS-induced inhibition of osteoblast differentiation could be due to

Fig. 10. Effect of MAPK inhibitors on the protein expression of caspase-3, Bax, cytochrome c, Bcl-2 and Bcl-XL at 1 d with quercetin pretreatment in LPS-induced MC3T3-E1 cells. The MAPK inhibitors SP600125, PD98059, and SB203580 were applied respectively for 2 h prior to pretreatment with 50 μ M quercetin or without quercetin for 2 h; then cells were incubated with 100 ng/ml LPS or without LPS in the presence of quercetin for 1 d. MAPK inhibitors markedly attenuated the quercetin-enhanced protein expression of Bcl-2 and Bcl-XL and restored the quercetin-induced down-regulation in the protein expression of caspase-3, Bax, and cytochrome c.

Fig. 11. Effect of the Wnt/ β -catenin inhibitor on the protein expression of osteoblast-specific genes at 24 h with quercetin pretreatment in LPS-induced MC3T3-E1 cells. The Wnt/ β -catenin inhibitor XV-939 were applied for 2 h prior to pretreatment with 50 μ M quercetin or without quercetin for 2 h, and then cells were incubated with 100 ng/ml LPS or without LPS in the presence of quercetin for 24 h. The Wnt/ β -catenin inhibitor markedly attenuated the quercetin-enhanced protein expression of osteoblast-specific genes.

the restoration of osteoblast-specific genes.

The induction of osteoblast apoptosis results in further bacteria-induced bone destruction. In one of our previous studies, we found that LPS had been shown to induce the apoptosis and inhibit osteoblast differentiation through the JNK pathway [9]. We evaluated the effect of pretreatment with quercetin on LPS-induced apoptosis in MC3T3-E1 cells. Our data showed that pretreatment with quercetin inhibited LPS-induced osteoblast apoptosis, using Hoechst 33258 staining, and significantly restored the down-regulated expression of Bcl-2 and Bcl-XL while decreasing the upregulated expression of caspase-3, Bax, and cytochrome c compared to non-quercetin-treated cultures of MC3T3-E1 cells induced by LPS. These results indicated that pretreatment with quercetin could have a protective effect against LPS-induced osteoblast apoptosis. Moreover, pretreatment with quercetin enhanced the protein expression of p-ERK while down-regulating p-p38 MAPK expression in the MC3T3-E1 cells. Based on our results, we suggested that the protective effect of pretreatment with quercetin against LPS-induced osteoblast apoptosis is through the inhibition of the mitochondrial apoptosis pathway.

MAP kinases are activated by various stresses, including LPS, and influence apoptosis either positively or negatively [35]. In many cell types, JNK and p38 MAPK contribute to the induction of apoptosis, whereas ERK inhibits apoptotic processes [36-38]. In this study, pretreatment with quercetin enhanced the protein levels of phosphorylated ERK1/2, but

reduced the protein levels of phosphorylated p38. Furthermore, after pretreatment with MAPK inhibitors for 2 h, the quercetin-enhanced p-MAPKs were attenuated; then, after pretreatment with MAPK inhibitors for 1 d, the quercetin-enhanced expression of osteoblast-specific genes was attenuated by p38-MAPK and ERK inhibitor respectively. MAPK inhibitors also attenuated the quercetin-enhanced expression of Bcl-2 and Bcl-XL, while enhancing the quercetin-down-regulated expression of caspase-3, Bax, and cytochrome c. Administration of dexamethasone also significantly attenuated the LPS-enhanced protein expression of MAPKs, while reversing the quercetin-induced regulation of the phosphorylation of MAPKs in LPS-induced MC3T3-E1 cells. Our study indicated that pretreatment with quercetin could reduce LPS-induced osteoblast apoptosis and restore inhibited osteoblast differentiation through MAPK signalling.

The Wnt/ β -catenin signalling pathway also plays a vital role in regulating osteoblast differentiation and bone formation [28-30]. Wnts are categorized as canonical Wnt signalling, which requires signalling through β -catenin, or non-canonical Wnt signalling, which does not require β -catenin [39, 40]. Evidence suggests that the regulation of Wnt activation of β -catenin in osteoblasts is important for bone formation [41]. In the absence of Wnt ligands, β -catenin forms a multiprotein complex with casein kinase 1 (CK1), glycogen synthase kinase 3 β (GSK3 β), adenomatous polyposis coli (APC), and Axin, which promotes β -catenin ubiquitination and degradation [42, 43]. In the presence of Wnt ligands, Wnt binds to the transmembrane receptor frizzled (Fzd) and co-receptor LRP5/6, GSK3-dependent phosphorylation of β -catenin will be prevented thus leading to the intracellular accumulation of β -catenin, which can then enter the nucleus and interact with the transcription factor T-cell factor/lymphoid enhancer factor (TCF/LEF) to activate the transcription of target genes [44, 45]. In this study, pretreatment with quercetin enhanced the protein levels of Wnt3 and β -catenin but reduced the protein levels of GSK3 β . Furthermore, after pretreatment with a Wnt/ β -catenin inhibitor (XAV939) for 1 d, the quercetin-enhanced expression of osteoblast-specific genes was attenuated. Our study also indicated that pretreatment with quercetin could restore the inhibition of osteoblast differentiation through the Wnt/ β -catenin signalling pathway.

Conclusion

Our data suggests that pretreatment with quercetin could have protective effects against LPS-induced osteoblast apoptosis and against the inhibition of osteoblast differentiation via MAPK and Wnt/ β -catenin signalling pathway. These results indicate that pretreatment with quercetin may be a potential drug for the prevention of human abnormal bone loss induced by LPS in chronic inflammatory diseases.

Acknowledgements

This work was supported by funds from the National Natural Science Foundation of China (81371981, 81672190), the School Fund of Luohe Medical College (2014-DF-002), and the Fund of Henan Provincial Health Bureau (201203081).

Disclosure Statement

We wish to confirm that there are no known conflicts of interest associated with this publication and that there has been no significant financial support for this work that could have influenced its outcome.

References

- Nair SP, Meghji S, Wilson M, Reddi K, White P, Henderson B: Bacterially induced bone destruction: mechanisms and misconceptions. *Infect Immun* 1996;64:2371-2380.
- Mörmann M, Thederan M, Nackchbandi I, Giese T, Wagner C, Hänsch GM: Lipopolysaccharides (LPS) induce the differentiation of human monocytes to osteoclasts in a tumour necrosis factor (TNF) alpha-dependent manner: a link between infection and pathological bone resorption. *Mol Immunol* 2008;45:3330-3337.
- Itoh K, Udagawa N, Kobayashi K, Suda K, Li X, Takami M, Okahashi N, Nishihara T, Takahashi N: Lipopolysaccharide promotes the survival of osteoclasts via Toll-like receptor 4, but cytokine production of osteoclasts in response to lipopolysaccharide is different from that of macrophages. *J Immunol* 2003;170:3688-3695.
- Islam S, Hassan F, Tumurkhuu G, Dagvadorj J, Koide N, Naiki Y, Mori I, Yoshida T, Yokochi T: Bacterial lipopolysaccharide induces osteoclast formation in RAW 264.7 macrophage cells. *Biochem Biophys Res Commun* 2007;360:346-351.
- Hou GQ, Guo C, Song GH, Fang N, Fan WJ, Chen XD, Yuan L, Wang ZQ: Lipopolysaccharide (LPS) promotes osteoclast differentiation and activation by enhancing the MAPK pathway in RAW264.7 cells. *Int J Mol Med* 2013;32:503-510.
- Xing Q, Ye Q, Fan M, Zhou Y, Xu Q, Sandham A: Porphyromonas gingivalis lipopolysaccharide inhibits the osteoblastic differentiation of preosteoblasts by activating Notch1 signaling. *Cell Physiol* 2010;225:106-114.
- Ochi H, Hara Y, Tagawa M, Shinomiya K, Asou Y: The roles of TNFR1 in lipopolysaccharide-induced bone loss: dual effects of TNFR1 on bone metabolism via osteoclastogenesis and osteoblast survival. *J Orthop Res* 2010;28:657-663.
- Kadono H, Kido J, Kataoka M, Yamauchi N, Nagata T: Inhibition of osteoblastic cell differentiation by lipopolysaccharide extracts from Porphyromonas gingivalis. *Infect Immun* 1999;67:2841-2846.
- Guo C, Yuan L, Wang JG, Wang F, Yang XK, Zhang FH, Fang H, Ma XY, Cheng Q, Song GH: Lipopolysaccharide (LPS) induces the apoptosis and inhibits osteoblast differentiation through JNK pathway in MC3T3-E1 cells. *Inflammation* 2014;37:621-626.
- Bandow K, Maeda A, Kakimoto K, Kikuchi J, Shamoto M, Ohnishi T, Matsuguchi T: Molecular mechanisms of the inhibitory effect of lipopolysaccharide (LPS) on osteoblast differentiation. *Biochem Biophys Res Commun* 2010;402:755-761.
- Woo JT, Nakagawa H, Notoya M, Yonekura T, Udagawa N, Lee IS, Ohnishi M, Hagiwara H, Nagai K: Quercetin suppresses bone resorption by inhibiting the differentiation and activation of osteoclasts. *Biol Pharm Bull* 2004;27:504-509.
- Wattel A, Kamei M, Meunier R, Lorget F, Prouillet C, Petit JP, Fardelonne P, Brazier M: Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on *in vitro* osteoclastic bone resorption. *Biochem Pharmacol* 2005;65:35-42.
- Siddiqui A, Swarnkar G, Sharan K, Chakravarti B, Gautam AK, Rawat P, Kumar M, Gupta V, Manickavasagam S, Singh S, Maurya R, Chattopadhyay N: A naturally occurring rare analog of quercetin promotes peak bone mass achievement and exerts anabolic effect on osteoporotic bone. *Osteoporos Int* 2011;22:3013-3022.
- Hittl M, Beyreis M, Tumurkhuu M, Fürst J, Helm K, Pitschmann A, Gaisberger M, Glasl S, Ritter M, Jakob M: Quercetin Stimulates Insulin Secretion and Reduces the Viability of Rat INS-1 Beta-Cells. *Cell Physiol Biochem* 2016;39(1):278-293.
- Guo C, Hou GQ, Li XD, Xia X, Liu DX, Huang DY, Du SX: Quercetin triggers apoptosis of lipopolysaccharide (LPS)-induced osteoclasts and inhibits bone resorption in RAW264.7 cells. *Cell Physiol Biochem* 2012;30:123-136.
- Notoya M, Tsukamoto Y, Nishimura H, Woo JT, Nagai K, Lee IS, Hagiwara H: Quercetin, a flavonoid, inhibits the proliferation, differentiation, and mineralization of osteoblasts *in vitro*. *Eur J Pharmacol* 2004;485:89-96.
- Son YO, Kook SH, Choi KC, Jang YS, Jeon YM, Kim JG, Lee KY, Kim J, Chung MS, Chung GH, Lee JC: Quercetin, a bioflavonoid, accelerates TNF-alpha-induced growth inhibition and apoptosis in MC3T3-E1 osteoblastic cells. *Eur J Pharmacol* 2006;529:24-32.

- 18 Nam TW, Yoo CI, Kim HT, Kwon CH, Park JY, Kim YK: The flavonoid quercetin induces apoptosis and inhibits migration through a MAPK-dependent mechanism in osteoblasts. *J Bone Miner Metab* 2008;26:551-560.
- 19 Prouillet C, Mazière JC, Mazière C, Wattel A, Brazier M, Kamel S: Stimulatory effect of naturally occurring flavonols quercetin and kaempferol on alkaline phosphatase activity in MG-63 human osteoblasts through ERK and estrogen receptor pathway. *Biochem Pharmacol* 2004;67:1307-1313.
- 20 Trivedi R, Kumar A, Gupta V, Kumar S, Nagar GK, Romero JR, Dwivedi AK, Chattopadhyay N: Effects of Egb 761 on bone mineral density, bone microstructure, and osteoblast function: Possible roles of quercetin and kaempferol. *Mol Cell Endocrinol* 2009;302:86-91.
- 21 Fatokun AA, Tome M, Smith RA, Darlington LG, Stone TW: Protection by the flavonoids quercetin and luteolin against peroxide- or menadione-induced oxidative stress in MC3T3-E1 osteoblast cells. *Nat Cell Res* 2015;29:1127-1132.
- 22 Wang XC, Zhao NJ, Guo C, Chen JT, Song JL, Gao L: Quercetin reversed lipopolysaccharide-induced inhibition of osteoblast differentiation through the mitogen-activated protein kinase pathway in MC3T3-E1 cells. *Cell Mol Med Rep* 2014;10: 3320-3326.
- 23 Matsuguchi T, Chiba N, Bandow K, Kakimoto K, Masuda A, Ohnishi T: JNK activity is essential for early expression and late-stage osteoblast differentiation. *J Bone Miner Res* 2003;18:403-410.
- 24 Guo C, Wang SL, Xu ST, Wang JG, Song GH: SP600125 reduces lipopolysaccharide-induced apoptosis and restores the early-stage differentiation of osteoblasts inhibited by LPS through the MAPK pathway in MC3T3-E1 cells. *Int J Mol Med* 2015;35:1427-1434.
- 25 Gutiérrez-Venegas G, Jiménez-Estrada M, Maldonado S: The effect of flavonoid on transduction mechanisms in lipopolysaccharide-treated human gingival fibroblasts. *Int Immunopharmacol* 2007;7:1199-1210.
- 26 Gao M, Wang H, Zhu L: Quercetin Assists Fluconazole to Inhibit Biofilm Formations of Fluconazole-Resistant Candida Albicans in *In vitro* and *In vivo* Antifungal Management of Vulvovaginal Candidiasis. *Cell Physiol Biochem* 2016;40:727-742.
- 27 Gim H, Nam JH, Lee S, Shim JH, Kim HJ, Ha KT, Kim B: Quercetin Inhibits Pacemaker Potentials via Nitric Oxide/cGMP-Dependent Activation and TRPV1 Channels in Cultured Interstitial Cells of Cajal from Mouse Small Intestine. *Cell Physiol Biochem* 2016;38:222-2436.
- 28 Hoepfner LH, Secreto FJ, Westendorp UGJ: Integrin signalling as a therapeutic target for bone diseases. *Expert Opin Ther Targets* 2009;13:485-496.
- 29 Long F: Building strong bone: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol* 2012;13: 27-38.
- 30 Baron R, Kneissel M: WNT signalling in bone homeostasis and disease: from human mutations to treatments. *Nat Med* 2013;19:169-179.
- 31 Kern B, Shen J, Saito T, Marsey G: Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes. *Biochem J* 2001;276:7101-7107.
- 32 Kim HK, Cho SG, Kim Y, Jo TK, Hu QS, Ulhaq R, Song EK, Yoon TR: Mevinolin enhances osteogenic genes (ALP, type I collagen and osteocalcin), CD44, CD47 and CD51 expression during osteogenic differentiation. *J Biosci* 2010;290:290-295.
- 33 Yoshida K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrughe B: The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 2002;109:17-29.
- 34 Fong H, Doll B, McNelis T, Hollinger JO: Osteoblast differentiation *in vitro* and *in vivo* promoted by Osterix. *J Endocrinol* 2007;153:770-778.
- 35 Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH: Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001;22:153-183.
- 36 Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME: Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995;270:1326-1331.
- 37 Fister S, Günthert AR, Aicher B, Paulini KW, Emons G, Gründker C: GnRH-II antagonists induce apoptosis in human endometrial, ovarian, and breast cancer cells via activation of stress-induced MAPKs p38 and JNK and proapoptotic protein Bax. *Cancer Res* 2009;69:6473-6481.

- 38 Park GB, Kim YS, Lee HK, Song H, Cho DH, Lee WJ, Hur DY: Endoplasmic reticulum stress-mediated apoptosis of EBV-transformed B cells by cross-linking of CD70 is dependent upon generation of reactive oxygen species and activation of p38 MAPK and JNK pathway. *J Immunol* 2010;185:7274-7284.
- 39 Lai SL, Chien AJ, Moon RT: Wnt/Fz signalling and the cytoskeleton: potential roles in tumorigenesis. *Cell Res* 2009;19:532-545.
- 40 Fuerer C, Nusse R, Ten Berge D: Wnt signalling in development and disease. *EMBO Rep* 2008;9:134-138.
- 41 Almeida M, Han L, Bellido T, Manolagas SC, Kousteni S: Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta-catenin-dependent and -independent signalling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. *J Biol Chem* 2005;280:41342-41351.
- 42 Ikeda S, Kishida S, Yamamoto H, Murai H, Koyama S, Kikuchi A: Axin, a negative regulator of the Wnt signalling pathway, forms a complex with GSK-3 β and beta-catenin and promotes GSK-3 β -mediated phosphorylation of beta-catenin. *EMBO J* 1998;17:1371-1384.
- 43 Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X, He X: Control of beta-catenin by a phosphorylation/degradation by a dualkinase mechanism. *Cell* 2002;108:837-847.
- 44 Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier C: Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* 1996;382:621-625.
- 45 Novak A, Dedhar S: Signalling through beta-catenin and Lef/Tcf. *Cell Mol Life Sci* 1999;56:523-537.