Marein prevented LPS-induced osteoclastogenesis by regulating the NF- κ B pathway *in vitro*

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Abstract

Gram-negative bacterial infection causes many bone diseases such as osteolysis, osteomyelitis and septic arthritis. Lipopolysaccharide (LPS), a bacteria product, played an important role in this process. Drugs that inhibited LPS-induced osteoclastogenesis were urgently needed for the prevention of bone destruction in infective bone diseases. Marein, a major bioactive compound of *Coreopsis tinctoria*, which possesses anti-oxidative, anti-inflammatory, anti-hypertensive, anti-hyperlipidemic and anti-diabetic effects. In this study, the effect of marein on RAW264.7 cells was measured by CCK-8 assay; TRAP staining was used to determine osteoclastogenesis; the levels of osteoclast-related genes and NF- κ B-related proteins were analyzed by WB; the levels of pro-inflammatory cytokines were quantified by ELISA. Our results showed that marein inhibited LPS-induced osteoclast formation from osteoclast precursor RAW264.7 cells. The effect of marein was related to its inhibitory function on expressions of pro-inflammatory cytokines and osteoclast-related genes including RANK, TRAF6, MMP-9, CK and CA α . Besides, marein treatment could inhibit LPS-induced activation of NF- κ B signaling pathway in RAW264.7 cells. Meanwhile, inhibition of NF- κ B signaling pathway decreased the formation of osteoclasts and expression of pro-inflammatory cytokines which were LPS-induced. Collectively, marein could prevent LPS-induced osteoclast formation *in vitro* by regulating NF- κ B signaling pathway. These findings provided evidence that marein might be beneficial as a valuable choice for the prevention and treatment of bacteria-induced bone destruction disease, and gave new insights for understanding its possible mechanism.

Introduction

The osteoclast was a large, multinucleated cell from the monocyte-macrophage lineage [1]. It could degrade bone tissue by secreting H⁺, Cl⁻, cathepsin K (CtsK) and matrix metalloproteinase (MMPs) in the resorption area [2] and played a key physiological role in bone remodeling and a pathological role in bone destruction diseases including osteopetrosis, osteoporosis, rheumatoid arthritis, inflammatory osteolysis, [3] Paget's disease, osteopetrosis, and pycnodysostosis [4]. Inheritance of osteoclast differentiation from hematopoietic progenitors required macrophage colony stimulating factor (M-CSF) and receptor activated nuclear factor kappa-B (NF- κ B) ligand (RANKL) to monitor differentiation [5-7]. These factors were the key determinants in stimulating the differentiation and activation of osteoclast, and were very crucial in bone remodeling [8].

The lipopolysaccharide (LPS) was a central component of the outer membrane in gram-negative bacteria that played a key role in host-pathogen interactions with the innate immune system [9]. LPS was a potent endotoxin and highly resistant to degradation by mammalian enzymes thus providing a persistent inflammatory stimulus [10]. It could cause inflammatory bone loss, leading to severe morbidity and large medical expenses and it was also considered to be a key pathogen of inflammatory osteolytic diseases such as osteomyelitis, septic arthritis, periodontitis, and infection of orthopedic implants [11]. LPS-induced pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , prostaglandin E2 (PGE2) and IL-6, could directly stimulate osteoclast differentiation and ultimately lead

to the destructive bone loss through increasing the expression of receptor activator for nuclear factor- κ B ligand (RANKL) [12-14]. Therefore, exploring the molecular mechanism on how to intervene and regulate the impact of LPS on bone metabolism disorders was of great significance for understanding and treating infectious bone diseases.

Marein was a major bioactive compound of *Coreopsis tinctoria* [15, 16]. The chemical structure formula of marein is shown in Fig.1A. Previous studies have shown that marein possesses several beneficial biological activities including anti-oxidative, anti-inflammatory, anti-hypertensive, anti-hyperlipidemic, and anti-diabetic effects [17]. For instance, marein protects human nucleus pulposus cells against high glucose-induced injury and extracellular matrix degradation through inhibiting ROS/NF- κ B pathway [18]. Correspondingly, NF- κ B signaling pathway was particularly closely related to the regulation of bone metabolism under inflammatory conditions, and it played an important role in regulating osteoclast differentiation, activation and osteoblast activity [19]. Nevertheless, to the best of our knowledge, there were no studies regarding the function of marein on osteoclastogenesis. Herein, the aim of our study was to determine the effects of marein on osteoclastogenesis in RAW264.7 cells and further explore the underlying mechanism.

Materials And Methods

Cell culture and treatment

Osteoclast precursor RAW264.7 cells were obtained from Procell (Wuhan, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/mL penicillin, and 100 μ g/mL streptomycin maintained at 37°C with a 5% CO₂ atmosphere. Cultured RAW264.7 cells were pretreated for 1 h with marein (Wuhan ChemFaces Biochemical Co., Ltd, Wuhan, China) at various concentrations followed by exposure to LPS (100 ng/mL) for 24h. RAW264.7 cells were used to assess if marein had toxic effect on them. For inhibitor studies, sulfasalazine (NF- κ B pathway inhibitor, Med Chem Express) was added to cell culture 30 min before treatment with marein and maintained throughout the experiment.

Assessment of cell viability by CCK-8 assay

The assay cell counting kit-8 (CCK-8) was used to measure whether the marein had toxic effects on the RAW264.7 cells. RAW264.7 cells were cultured to the logarithmic growth phase, and after digestion, 1×10^5 /mL cell suspension was prepared with the pre-warmed medium and inoculated into a 96-well cell culture plate with a volume of 100 μ l per well. After the cells have adhered, the original medium was aspirated, and RAW264.7 cells were added at concentrations of 0 μ M, 5 μ M, 10 μ M, 20 μ M and 40 μ M marein, PBS as a control. The next day, the CCK-8 solution was added 10 μ l per well, and after another 4 hours' culture, the absorbance of each well was measured at 450 nm. This experiment was in quadruplicate.

Osteoclastogenesis assay *in vitro*

To assess whether marein inhibited LPS-induced osteoclast formation, a total of 1×10^3 RAW264.7 cells/well were seeded in a 96-well plate and treated with or without LPS at a concentration of 100 ng/mL alone or in combination with different marein at concentrations of 5, 10, 20 and 40 μ M. That is to say, the assay included six groups: PBS, LPS, LPS+5 μ M marein, LPS+10 μ M marein, LPS+20 μ M marein and LPS+40 μ M marein. Besides, to assess the effect of inhibiting NF- κ B pathways on osteoclast formation *in vitro*, the assay was changed to four groups containing PBS, LPS, LPS+40 μ M marein and LPS+sulfasalazine (NF- κ B inhibitor). Cells were washed twice with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde (pH 7.4) at room temperature (r.t.) for 15 min and stained for TRAP using leukocyte Acid Phosphatase Kit 387-A according to the manufacturer's protocol. TRAP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast under an IX70 microscope (Olympus, Japan).

Western blot analysis

RAW264.7 cells were cultured overnight. After treatment with or without marein (5, 10, 20 and 40 μ M), the cells were stimulated with LPS for 24h to detect receptor activator of nuclear factor kappa B (RANK), TNF receptor associated factor 6 (TRAF6), matrix metalloproteinase 9 (MMP-9), cathepsin K (CK), carbonic anhydrase II (CA II) inhibitor of nuclear factor kappa B α (I κ B α) or phosphorylation-I κ B α (p-I κ B α). The cellular lysates were prepared. The above proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 4% bovine serum albumin (BSA) for 1 h at r.t. and then probed with rabbit anti-mouse antibodies against RANK, TRAF6, MMP-9, CK, CA II , I κ B α , p-I κ B α and β -actin at 4°C overnight. After washing three times, the blots were subsequently incubated with a goat horseradish peroxidase-conjugated secondary antibody for 2 h at r.t. After four times washing for 10 min in Tris-buffered saline with Tween-20 (TBST), the membranes were detected using a chemiluminescence detection system. The intensity of the bands was quantified by ImageJ software. β -actin as a control.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was to detect the pro-inflammatory cytokines tumor necrosis factor- α (TNF- α), interleukin 1 β (IL-1 β), prostaglandin E2 (PGE2) and cyclooxygenase-2 (COX-2) quantitatively. Similarly, PBS, LPS, LPS+5 μ M marein, LPS+10 μ M marein, LPS+20 μ M marein and LPS+40 μ M marein were performed. ELISA plates were coated with purified TNF- α , IL-1 β , PGE2 and COX-2 antibodies in carbonate-bicarbonate buffer at 4°C overnight. The plates were blocked with 200 μ l blocking buffer BSA at 37°C for 1 h. Then, the wells were added samples to be tested and incubated at 37°C for 2 h and washed four times carefully. Horseradish peroxidase (HRP)-conjugated TNF- α , IL-1 β , PGE2 and COX-2 antibodies were added to detect the specific proteins. After reaction at 37 °C for 1 h, the plates were washed four times. Next, the wells were incubated with the substrate for approximately 10-15 min, and the enzyme reaction was terminated with stop solution. The OD value was measured at 450 nm. The assay tested each sample in triplicate. The results are expressed as pg/ml.

Statistical analysis

All the experiments were performed at least three times. Results are expressed as the mean $\pm$ standard deviation (S.D.). Statistical comparisons between groups were carried out by one-way ANOVA with post hoc tests to compare differences between experimental and control group. Statistical analyses were performed using GraphPad Prism 7 software. Statistical significance was defined at $p < 0.05$.

Results

Marein inhibited LPS-induced osteoclast formation

To eliminate the inhibitory effects of marein on osteoclast formation could have been due to its cytotoxicity on RAW264.7 cells, CCK-8 assay was performed. The data showed that the cell viability of RAW264.7 cells was not distinctly reduced by marein with concentrations of 5, 10, 20 and 40 μ M, indicating that marein under 40 μ M was not nontoxic to RAW264.7 cells (Fig. 1B).

Next, for observing the effect of marein on LPS-induced osteoclastogenesis, RAW264.7 cells were treated with different concentrations of marein as mentioned above for 72 h in the presence or absence of LPS and then stained with TRAP, an osteoclast-specific protein. Data showed that LPS could exactly stimulate RAW264.7 cells differentiation into osteoclast. Marein treatment caused osteoclast less rounded and few nuclei than that of LPS-treated group (Fig.1C), and indeed inhibited LPS-induced osteoclast formation in a dose-dependently manner (Fig.1D). Notably, 40 μ M of marein nearly inhibited LPS-induced osteoclast formation, the osteoclast number was counted with three different microscope fields and the average number of osteoclasts was only 12.5 % of the LPS-treated group. Besides, marein at 5 μ M inhibited osteoclastogenesis less severely than the other marein-treated groups.

Marein restrained LPS-induced osteoclast-related gene expression

The expressions of osteoclast differentiation-related genes including RANK, TRAF6, MMP-9, CK, and CA $\square$ were analyzed quantitatively using WB. The WB bands containing all the former proteins were shown in Fig.2A. Notably, the data indicated that LPS at 100 ng/mL significantly up-regulated proteins expression of RANK (Fig.2B), TRAF6 (Fig.2C), MMP-9 (Fig.2D), CK (Fig.2E), and CA $\square$ (Fig.2F) during osteoclast formation. Marein treatment groups obviously inhibited expression of these relevant genes, and the inhibitory effects were also in a dose-dependent manner. Particularly, 40 μ M of marein exhibited the inhibition on these genes stronger than those of the other three concentrations.

Marein suppressed the release of pro-inflammatory cytokines

It is reported that pro-inflammatory cytokines were considered as mediators of bone-loss [20]. These cytokines enhanced osteoclast formation and migration in bone destructive diseases such as rheumatoid arthritis and periodontitis. Consistent with this, RAW264.7 cells incubated with LPS for 24 h resulted in obvious increases in TNF- α , IL-1 β , PGE2, and COX-2 (Fig.3), and their levels were increased to 1.4-, 1.2-, 1.5- and 1.4-fold compared with control group, respectively. Clearly, marein treatment for 24 h suppressed

LPS-induced the secretion of these cytokines with a dose-dependent manner. Importantly, marein at 40 μ M caused a more obvious reduction of TNF- α , IL- β , PGE2, and COX-2 compared with the remaining concentrations (Fig.3).

Marein inhibited LPS-triggered activation of NF- κ B Pathway

The above results demonstrated that marein could suppress LPS-induced osteoclast formation as well as the expression of osteoclast-related genes. Therefore, to illuminate the possible molecular mechanisms underlying the inhibitory effect of marein on LPS-induced osteoclast formation, we explored whether marein affect the NF- κ B pathway which LPS-induced. Given this, the proteins expression of I κ B α and p-I κ B α were analysed by WB. The WB bands were presented in Fig.4A. Stimulation of RAW264.7 cells with LPS could up-regulate the ratio of p-I κ B α /I κ B α , which presented the activation of NF- κ B pathway (Fig.4B). When treated with different concentrations of marein, it could efficiently inhibit LPS-increased NF- κ B-related protein levels as assessed by western blot assay. Notably, the level of p-I κ B α /I κ B α was decreased as the concentration increased. Taken together, these findings illustrated that marein inhibited LPS-induced osteoclastogenesis through suppressing I κ B α phosphorylation of NF- κ B pathway.

Inhibition NF- κ B pathway restrained LPS-induced osteoclast formation and inflammation

Next, the effects of inhibition NF- κ B pathway on LPS-induced osteoclast formation and inflammation need to be elucidated. In TRAP-staining assay, RAW264.7 cells were treated with 40 μ M marein or sulfasalazine (NF- κ B inhibitor) in the presence of LPS. Marein and sulfasalazine treatment caused osteoclast less rounded and few nuclei than that of LPS-treated group and indeed inhibited LPS-induced osteoclast formation (Fig. 5A). Notably, 40 μ M marein could inhibit LPS-induced osteoclast formation, and the number of osteoclast was 64% of the LPS-treated group. Besides, osteoclastogenesis was inhibited in sulfasalazine group compared with LPS-treated group (Fig. 5B). Next, the contents of TNF- α , IL-1 β , PGE2, and COX-2 in the culture supernatants were measured by ELISA. RAW264.7 cells incubated with 40 μ M marein or sulfasalazine in the presence of LPS for 24 h. LPS-treated group caused the release of these pro-inflammatory cytokines. When treated with 40 μ M marein and sulfasalazine, the expressions of LPS-induced TNF- α (Fig.5C), IL-1 β (Fig.5D), PGE2 (Fig. 5E), and COX-2 (Fig.5F) represented a downward trend. These results indicated that suppression of the NF- κ B pathway indeed decreased the LPS-induced osteoclast formation and inflammation.

Discussion

Infectious bone diseases caused by osteomyelitis, trauma, or post-operative infection of implants may cause persistent inflammatory responses in the body, while the affected sites may be secondary to local bone metabolic disorders, severe bone destruction, and bone death [21, 22]. Considering that LPS can induce some inflammatory cytokines and marein can modulate immune system. Therefore, it is worthy of studying whether marein could inhibit LPS-induced inflammation in osteoclasts. According to current results, we found that marein could inhibit LPS-induced osteoclastogenesis through suppressing NF- κ B-mediated inflammatory response in RAW264.7 cells.

Related research confirmed that LPS could stimulate secretion of prostaglandins (PG), histamine and other inflammatory mediators, and activate the release of immune factors such as interleukin (IL), TNF- α , and these inflammatory mediators can be produced by LPS stimulation through different mechanisms to promote the formation and bone resorption of osteoclast activity. Studies have demonstrated that LPS can promote osteoclast differentiation by enhancing COX-2 expression and activating RANK, JNK and ERK1/2 pathways [23]. Besides, LPS can also accelerate osteoclast differentiation by activating NF- κ B-NFATc1 signal pathway [24]. In this study, we found that LPS can increase the expressions of TRAP-positive cells, osteoclasts-related genes and pro-inflammatory cytokines in RAW264.7 cells. However, these events were inhibited by marein, which indicated that marein might be a potential candidate for treating and/or preventing osteoclast-associated diseases, including osteoporosis.

Several studies reported that some plant-derived natural organic compounds, such as isoflavones (coumestrol, daidzein and genistein) showed a direct inhibitory effect on cytokine-induced osteoclast differentiation [25, 26]. Another study illustrated that treatment with puerarin (10-50 μ mol/L) effectively inhibited the production of the inflammatory mediators (TNF- α , IL-1 β , PGE2) induced by LPS [27]. Besides, it is reported that artesunate reduced TNF- α production and prevented LPS-induced bone loss *in vivo* [1]. Desoxyrhapontigenin inhibited the differentiation of bone marrow macrophages into mature osteoclasts by suppressing the RANKL-induced activator protein-1 and NFATc1 signaling pathways [28]. Punicalagin played an important role in the attenuation of LPS-induced inflammatory responses in RAW264.7 macrophages and that the mechanisms involved downregulation of the FoxO3a/autophagy signaling pathway [29]. In accordance with their results, we found that treatment with marein effectively inhibited the production of TNF- α , IL-1 β , PGE2 and COX-2 induced by LPS, which were consistent with the inhibitory effect of marein on osteoclast formation. Therefore, marein could inhibit the production of these pro-inflammatory cytokines which LPS-induced, and prevent later stages of osteoclast differentiation in the infective bone destruction.

NF- κ B was the key transcription factor associated with the regulation of COX-2 and mPGES1 after LPS stimulation [30]. Abnormal activation of NF- κ B signaling induced osteoclast formation by increasing expression of NFATc1 [31]. Islam et al. reported that LPS, as a potent bone resorbing factor, could induce osteoclastic cell differentiation in RAW264.7 cells [32]. However, the molecular mechanisms underlying the LPS-induced RAW264.7-osteoclasts formation still remained unclear. One speculation was that LPS might mimic RANKL-induced osteoclast formation via activation of NF- κ B and SAPK/JNK [33]. Previous studies [34, 35] have indicated that NF- κ B pathway served as a central role in the regulation of osteoclast differentiation and survival. Therefore, NF- κ B pathway could serve as a therapeutic target in inhibition of osteoclastogenesis.

Importantly, we found that marein treatment could markedly decrease the osteoclast numbers and down-regulate the expression of pro-inflammatory cytokines through inhibiting LPS-induced activation of NF- κ B, which were critical for osteoclast differentiation. A primary level of control for NF- κ B was through interactions with an inhibitor protein called I κ B. I κ B retained NF- κ B in the cytoplasm through masking of the nuclear localization sequences. Removal of I κ B activated the NF- κ B NLSs so that NF- κ B rapidly

translocated into the nucleus, bound to select gene promoters in a sequence-specific manner, and activated gene transcription [36]. Activation of NF- κ B to move into the nucleus was controlled by the targeted phosphorylation and subsequent degradation of I κ B α . Therefore, the up-regulated ratio of p-I κ B α /I κ B α could present the activation of NF- κ B pathway [37]. In this study, we found that marein could inhibit NF- κ B pathway by decreasing the ratio of p-I κ B α /I κ B α and down-regulate the levels of pro-inflammatory cytokines including TNF- α , IL-1 β , PGE2, and COX-2. Encouragingly, the inhibitory effect of marein was similar to that of sulfasalazine. These results indicated that marein could inhibit NF- κ B activation in LPS-treated RAW264.7 cells through suppressing the phosphorylation inactivation of I κ B α to increase the transcriptional activity of NF- κ B. Considering the key role of NF- κ B signaling pathway in LPS-induced osteoclast formation [34, 35], we concluded that suppression of NF- κ B could decrease the formation of osteoclasts and marein may serve as an effective therapeutic agent to treat bone loss diseases.

Conclusions

These results demonstrated that marein suppressed LPS-induced osteoclastogenesis *in vitro*. Moreover, marein exhibited inhibitory effects on LPS-induced osteoclastogenesis by inhibiting NF- κ B signaling pathway. Our findings introduced a novel therapeutic approach to control bacteria-induced bone destruction disease, and demonstrated NF- κ B signaling pathway may play an important role in this regulating process. The effect of marein on LPS-induced bone loss *in vivo* still needed to be proved in the near future. Nevertheless, our research may provide a reference on correctly evaluation about the pharmacological effect of these natural plant drugs on bone tissue in inflammatory state and direction for the application of natural plant drugs in the regulation of bone metabolism in infectious bone disease.

Declarations

Acknowledgments

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Conflicts of interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

Marein inhibited LPS-induced osteoclastogenesis. (A) The chemical structure of marein. (B) RAW264.7 cells viability was measured by CCK-8 assay. (C) RAW264.7 cells were stained for TRAP staining and cells containing more than three nuclei were counted as osteoclast under IX70 microscope. Bars=10μm. (D) The osteoclast number was counted per field of microscope and the number of control group was zero. Bars represent the mean $\pm$ S.D. from three independent experiments. ** $p < 0.01$ and **** $p < 0.0001$ compared with LPS-treated group.

Figure 2

Marein restrained LPS-induced osteoclast-related protein expression. RAW264.7 cells were treated with 5, 10, 20 and 40 μ M marein for 24h with or without LPS. (A) Osteoclast-related genes were analyzed by western blotting using antibodies against RANK, TRAF6, MMP-9, CK and CA II. MMP-9 (B), CK (C), the relative density of RANK (D), TRAF6 (E) and CA II (F) were quantified by western blotting analysis and normalized to control. Bars represent the mean $\pm$ S.D. from three independent experiments. #### $p < 0.0001$ compared with control group, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ compared with LPS-treated group.

Figure 3

Marein suppressed the release of pro-inflammatory cytokines. The expressions of pro-inflammatory cytokines including TNF- α (A), PGE2 (B), IL- β (C) and COX-2 (D) were measured by ELISA. All values were given as means $\pm$ S.D. from three independent experiments. ### p <0.001 compared with control group, * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001 compared with LPS-treated group.

Figure 4

Marein inhibited LPS-triggered activation of NF- κ B pathway in RAW264.7 cells. (A) The expression levels of I κ B α and p-I κ B α . (B) The relative level of p-I κ B α /I κ B α . All values are given as means $\pm$ S.D. from three independent experiments. ##### p <0.0001 compared with control group. * p <0.05, *** p <0.001, and **** p <0.0001 compared with LPS-treated group.

Figure 5

Inhibition NF- κ B pathway restrained LPS-induced osteoclast formation and inflammation. (A) Cells were stained for TRAP staining and cells containing more than three nuclei were counted as osteoclast under IX70 microscope. Bars=10 μ m. (B) The numbers of osteoclasts were counted per field of microscope and the number of control group was zero. The expressions of pro-inflammatory cytokines TNF- α (C), IL- β (D), PGE2 (E), and COX-2 (F) were measured by ELISA. All values are given as means $\pm$ S.D. from three

independent experiments. $^{##}p<0.01$, $^{###}p<0.001$, and $^{####}p<0.0001$ compared with control group. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, and $^{****}p<0.0001$ compared with LPS-treated group. Sulfasalazine served as a positive control.