

Calcitonin gene-related peptide reduces *Porphyromonas gingivalis* LPS-induced TNF- α release and apoptosis in osteoblasts

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Abstract. Periodontal diseases comprise mixed bacterial infections mainly caused by Gram-negative anaerobic bacteria. Lipopolysaccharides (LPS) are important virulence factors and periodontal pathogens, which change local cytokine levels and promote osteoblast apoptosis, thereby leading to an imbalance in bone remodeling mechanisms and accelerating bone loss. Calcitonin gene-related peptide (CGRP) is a vasoactive neuropeptide that is released from sensory nerves and has a positive effect on osteoblast proliferation and differentiation. In addition, this small molecule peptide is an important immune regulator in the inflammatory response. The aim of the present study was to assess the *in vitro* effects of CGRP on *Porphyromonas gingivalis* (Pg)LPS-induced osteoblast apoptosis. Osteoblast cultures were stimulated either with various concentrations of PgLPS (0, 25, 50, 100, 500 and 1,000 ng/ml) for 48 h or with 500 ng/ml PgLPS for various lengths of time (0, 6, 12, 24, 48 and 72 h). The PgLPS-stimulated cells were pretreated with different concentrations of CGRP (0, 1, 10, 100 and 1,000 nM) and cell viability and apoptotic rates were measured by Cell Counting kit-8 assays and flow cytometry, respectively. CGRP, cleaved (c)-Caspase-8 and c-Caspase-3 protein expression levels were analyzed by western blotting. Changes in cytokine expression levels, which included tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, monocyte chemotactic protein (MCP)-1 and MCP-2, were measured by ELISA. PgLPS was demonstrated to inhibit osteoblast viability and promote apoptosis in a time- and concentration-dependent manner. CGRP

expression was revealed to reduce PgLPS-induced cytostatic activity and apoptosis in osteoblasts. CGRP also suppressed the PgLPS-induced release of TNF- α and inhibited the activation of c-Caspase-3 and c-Caspase-8, thus preventing apoptosis in osteoblasts. CGRP may be an important neuropeptide in bone remodeling and may reduce osteoblast apoptosis in inflammatory conditions. These results may provide a solid foundation for CGRP to serve as a new target for the treatment of periodontitis.

Introduction

Bone loss may be caused by the imbalance of bone remodeling. Pathological changes in bone tissue not only depend on the number of osteoclasts and osteoblasts but also are closely associated with the apoptosis-mediated lifecycle of these cells (1). A number of studies have reported that apoptosis participates in the development and progression of periodontitis (2,3). Periodontal pathogens mainly include Gram-negative anaerobic bacteria. Lipopolysaccharides (LPSs) are a major component of the cell wall of Gram-negative bacteria and are considered to be a pivotal factor for inducing the destruction of alveolar bone tissue and for the development and progression of periodontitis (4,5). LPS has previously been demonstrated to directly inhibit the differentiation of periodontal target cells and to increase the absorption of periodontal tissues (6-8). Therefore, LPS is of growing concern for its role in alveolar bone resorption and periodontitis incidence. Previous studies have revealed that LPS and the metabolites of periodontal pathogens may affect matrix formation and cellular apoptosis. (9,10). Regarding this imbalanced process, the biological behavior of osteoblasts is particularly important. LPS may induce inflammatory responses in osteoblasts and abnormal osteoblast apoptosis, which may lead to a disorder in the number of cells coupled between bone resorption and formation, thereby causing bone loss (10). Currently, there are few effective treatments for bone destruction caused by bacteria, and a major objective of bone-loss prevention is to investigate the protection of osteoblast function and activity in the imbalanced state and to search for potential drug targets.

Calcitonin gene-related peptide (CGRP) was first extracted from medullary thyroid carcinoma tissue in 1971 (11). CGRP comprises 37 amino acids and includes two isomers, α -CGRP

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and β -CGRP. CGRP was revealed to be stored as secretory granules in sensory nerve endings (12), and recent studies have demonstrated that CGRP is widely distributed in the nervous, cardiovascular, respiratory and digestive systems (13-16). CGRP exhibits diverse physiological effects in various tissues and in the immune response; exogenous CGRP may increase the number and size of osteoblast colonies (17). In bone tissues, CGRP is not only produced in sensory nerve fibers and endings, but also generated by osteoblasts, and it functions in both autocrine and paracrine signaling (18). It has been recently reported that CGRP may have a potential role in regulating inflammation, as it lies at the intersection of the nervous and immune systems (19,20). CGRP was reported to directly affect CD4⁺ T helper cells and influence the function of antigen-presenting cells to regulate adaptive immune responses (21). Additionally, CGRP was revealed to suppress the release of inflammatory cytokines, such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α and carbon tetrachloride, from monocyte-macrophage cells and dendritic cells upon stimulation by inactivated bacteria or Toll-like receptor agonists (22,23). Numerous studies have provided an understanding that CGRP may be a potential regulator of inflammation that might inhibit the production of pro-inflammatory cytokines, as CGRP serves a major regulatory role in the inflammatory process (24,25). However, the effects of CGRP on *Porphyromonas gingivalis* (Pg) LPS-induced osteoblast apoptosis remained unclear. The present study demonstrated that CGRP blocks PgLPS-induced cytostatic activity and apoptosis, whereas TNF- α serves an important opposing role in this process.

Materials and methods

Cell culture and reagents. Primary osteoblasts were obtained from the calvaria of BALB/C mice according to the method described previously (26) and cultured in Dulbecco's modified Eagle's medium (DMEM; cat. no. 11965; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (cat. no. 10100; Gibco; Thermo Fisher Scientific, Inc.), 1.5 g/l sodium bicarbonate (cat. no. 25080; Gibco; Thermo Fisher Scientific, Inc.), 0.11 g/l sodium pyruvate (cat. no. 11360; Gibco; Thermo Fisher Scientific, Inc.) and 100 μ g/ml penicillin/streptomycin (cat. no. 15140; Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Peptide α -CGRP (cat. no. C0167) and TNF- α (cat. no. T6674) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). PgLPS (Invitrogen; Thermo Fisher Scientific, Inc.) was reconstituted in distilled and deionized water, according to the manufacturer's protocol.

Cell viability assay. Cell viability was evaluated using a Cell Counting kit-8 (CCK-8; cat. no. C0038; Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. Osteoblasts (1.0 \times 10⁴ cells/well) were seeded in 96-well plates and cultured at 4°C overnight. Cells were treated with PgLPS at different concentrations (0, 25, 50, 100, 500 and 1,000 ng/ml) in the aforementioned culture medium for different lengths of time (0, 6, 12, 24, 48 or 72 h). Cells cultured in medium with 0 ng/ml PgLPS or for 0 h incubation were used as the controls. Following treatment, 10 μ l of

CCK-8 was added to each well and incubated for 2 h, and the absorbance of each well was measured with an iMark 680 Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm. All experiments were performed independently and repeated three times. The cell viabilities were normalized to the control group using SPSS software for Windows (version 18.0; SPSS, Inc., Chicago, IL, USA).

ELISA. Osteoblasts (1 \times 10⁴ cells/well) were pretreated with 100 nM CGRP at 37°C for 30 min, followed by treatment with 500 ng/ml PgLPS at 37°C for 48 h. Cells cultured in medium with 0 ng/ml PgLPS or for 0 h incubation were used as the controls. ELISA kits for TNF- α (cat. no. MTA00B), IL-1 β (cat. no. MLB00C), IL-6 (cat. no. M6000B), monocyte chemotactic protein 1 (MCP-1; cat. no. MJE00) and MCP-2 (cat. no. DY790) were all purchased from R&D Systems China Co., Ltd. (Shanghai, China) and used to determine the concentrations in cell culture supernatants according to the manufacturer's protocols.

The cells were centrifuged at 71.04 \times g for 5 min at 25°C, and then 100 μ l supernatant was collected and assessed for concentration of the above proteins. The optical density of each well was measured with a microplate reader at 450 nm and normalized to the control group. The level of absorbance for each tested sample was measured using the Microplate Reader 550 (Bio-Rad Laboratories, Inc.). The data were analyzed by using SPSS software for Windows (version 18.0; SPSS, Inc., Chicago, IL, USA).

Apoptosis assay. Osteoblasts (1 \times 10⁴ cells/well) were pretreated with 100 nM CGRP at 37°C for 30 min, followed by treatment with 500 ng/ml PgLPS at 37°C for 48 h. Cells (1 \times 10⁴ cells/well) were treated with PgLPS at different concentrations (0, 25, 50, 100, 500 and 1,000 ng/ml) in the aforementioned culture medium for different lengths of time (0, 6, 12, 24, 48 or 72 h). Cells cultured in medium with 0 ng/ml PgLPS or for 0 h incubation were used as the controls.

Apoptosis was assessed by flow cytometry using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining kit (cat. no. 556547; BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Briefly, cells were collected and washed twice with cold PBS, resuspended in staining buffer containing Annexin V-FITC (0.025 μ g/ml) and PI (1 μ g/ml) and incubated for 15 min at 25°C in the dark. Cells were washed twice with PBS and apoptotic cells were analyzed by FACScan flow cytometer and CELLQuest software (version 4.0.2; BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. Osteoblast cultures (1 \times 10⁴ cells/well) were stimulated with 500 ng/ml PgLPS at 37°C for various lengths of time (0, 6, 12, 24, and 48 h). CGRP protein expression was assessed. Osteoblasts (1 \times 10⁴ cells/well) were pretreated with 100 nM CGRP at 37°C for 30 min, followed by treating with 500 ng/ml PgLPS at 37°C for 48 h. Cleaved (c)-Caspase-8, (c)-Caspase-3 and TNF- α protein expression was assessed.

Cells were treated and collected by centrifugation at 71.04 \times g for 1 min at 4°C. Following washing with PBS, cells

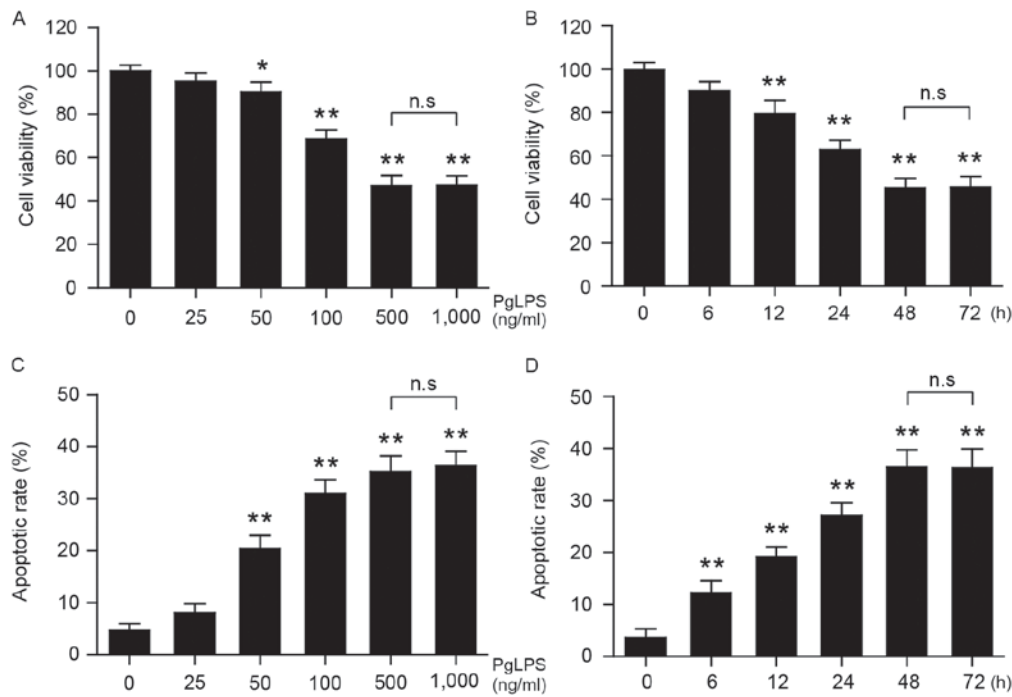


Figure 1. Effects of PgLPS on osteoblast cell viability and apoptosis. Osteoblasts were treated with either (A) 0, 25, 50, 100, 500 or 1,000 ng/ml PgLPS for 48 h, or (B) 500 ng/ml PgLPS for 0, 6, 12, 24, 48 or 72 h. Cell viability was measured by Cell Counting kit-8 assay, and the data were normalized to the control group (0 ng/ml PgLPS or 0 h incubation, respectively). (C and D) Osteoblasts were treated as indicated in A and B, and apoptosis was evaluated by flow cytometry. Data are present the mean \pm standard deviation from three separate experiments; * $P < 0.05$ and ** $P < 0.01$ vs. the respective control (0 h or 0 ng/ml PgLPS). PgLPS, *Porphyromonas gingivalis* lipopolysaccharide.

were lysed in radioimmunoprecipitation assay buffer [50 mM Tris, (pH 7.4) 150 mM NaCl; 1% sodium deoxycholate, 1 mM sodium orthovanadate; 1% Triton X-100; 0.1% SDS; 10 μ g/ml aprotinin; 10 μ g/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride] on ice for 30 min. Supernatants were collected by centrifugation at 18,759 \times g for 5 min at 4°C. Protein concentrations were measured using an Enhanced BCA Protein Assay Reagent (cat. no. P0010; Beyotime Institute of Biotechnology). A total of 30 μ g cellular protein was boiled for 10 min and separated by 12% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (cat. no. 162-0177; Bio-Rad Laboratories, Inc.) at 50 V for 3 h at 4°C. Following blocking with 5% non-fat dried milk for 2 h at room temperature, membranes were incubated with primary antibodies as follows: CRGP (1:1,000; cat. no. 14959; CST Biological Reagents Company Ltd., Shanghai, China), cleaved (c)-Caspase-8 (1:500; cat. no. 9505; CST Biological Reagents Company Ltd.), c-Caspase-3 (1:500; cat. no. 9664; CST Biological Reagents Company Ltd.), TNF- α (1:1,000; cat. no. ab6671; Abcam, Shanghai, China) and actin (1:1,000; cat. no. sc-8432; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C. Membranes were washed three times in 1X TBS +0.1% Tween-20 (TBST), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:2,000; cat. no. 474-1506; Kirkegaard & Perry Laboratories, Inc.; SeraCare Life Sciences, Milford, MA, USA) or HRP-conjugated goat anti-mouse (1:2,000; cat. no. 474-1806; Kirkegaard & Perry Laboratories, Inc.; SeraCare Life Sciences) secondary antibodies for 2 h at room temperature. Following washing by 1X TBST, protein bands were visualized using the Enhanced Chemiluminescence kit

(cat. no. 170-5061; Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. Densitometric analysis of the blots was performed using Quantity One Software (version 4-2; Bio-Rad Laboratories, Inc.) and normalized to actin expression levels.

Statistical analysis. All data are expressed as the mean \pm standard deviation from at least three independent experiments. Statistical analysis was performed using one-way analysis of variance, followed by a Newman-Keuls Student's t-test for multiple comparisons. The analysis was conducted using SPSS software for Windows (version 18.0; SPSS, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PgLPS induces the cell viability inhibition and apoptosis. To assess the effects of PgLPS on osteoblast viability, osteoblasts were first cultured with different concentrations of PgLPS for 48 h and Cell viability was assessed by CCK-8 and apoptosis was assessed by flow cytometry assay as stated above.

Osteoblast viability was significantly reduced by PgLPS treatment at concentrations of 50, 100, 500 and 1,000 ng/ml ($P < 0.05$; Fig. 1A); no significant difference was identified between cells treated with 500 or 1,000 ng/ml PgLPS. Osteoblasts were subsequently stimulated with 500 ng/ml PgLPS for 0-72 h, followed by measurement of viability. The results indicated that as the stimulation time increased, the viability of the osteoblasts significantly decreased within 48 h ($P < 0.01$; Fig. 1B), no significant difference was identified between 48 and 72 h treatment.

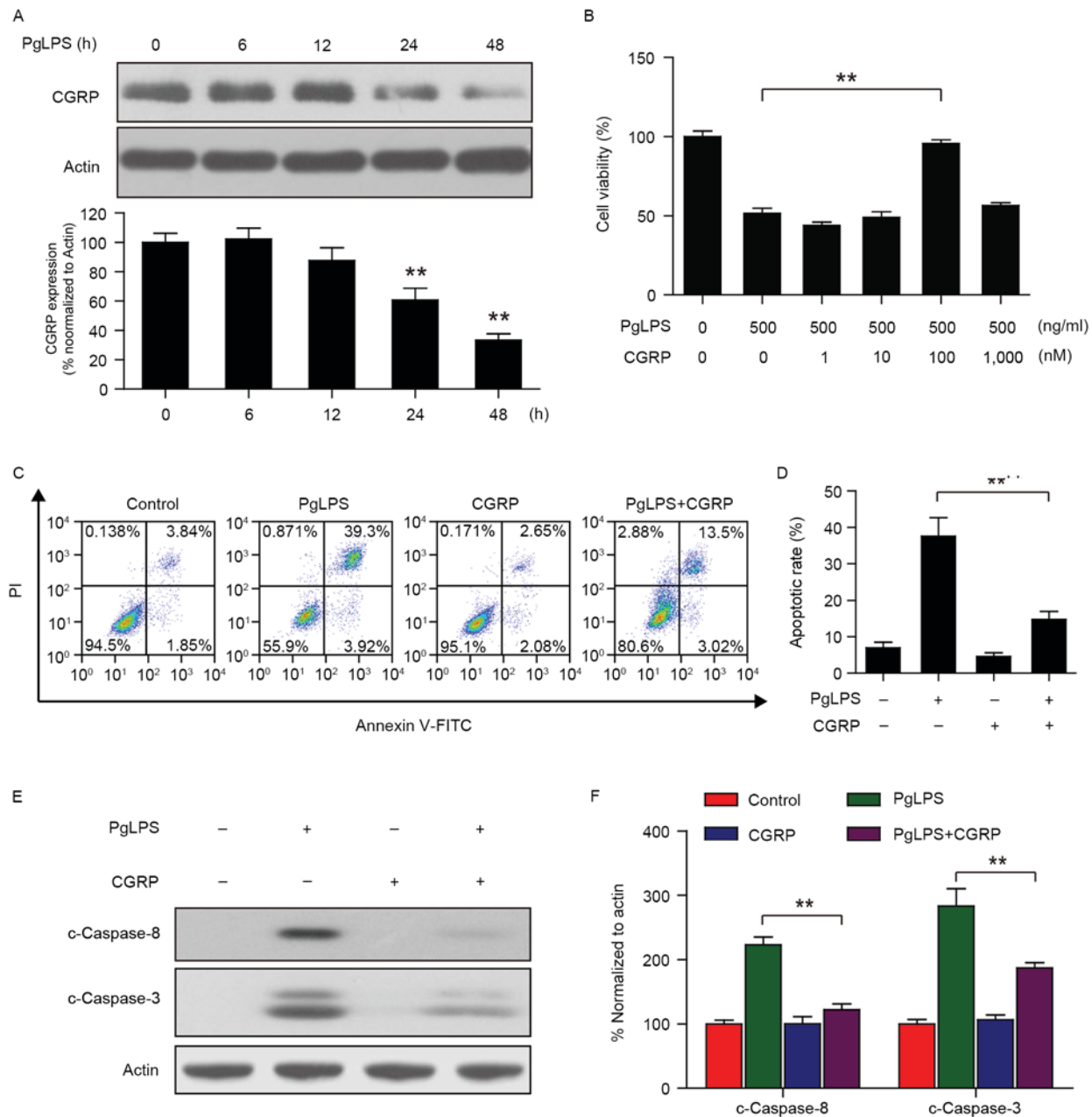


Figure 2. Effects of CGRP pretreatment on PgLPS-induced cell viability inhibition and apoptosis. (A) Osteoblasts were treated with 500 ng/ml PgLPS for 0, 6, 12, 24 and 48 h, and the expression of CGRP protein levels were detected by western blot analysis. Actin was used as a loading control; band intensities normalized to actin are represented as the mean \pm standard deviation from three separate experiments; ** P <0.01 vs. untreated control. (B) Osteoblasts were pretreated with 0, 1, 10, 100 and 1,000 nM CGRP for 1 h, followed by treatment with or without 500 ng/ml PgLPS for 48 h, and cell viability was measured by Cell Counting kit-8 assay. Data are presented as the mean \pm standard deviation (n=3) and normalized to the untreated control group; ** P <0.01. (C and D) Osteoblasts were pretreated with 100 nM CGRP for 30 min, followed by treatment with 500 ng/ml PgLPS for 48 h. Apoptotic rates were measured by flow cytometry and data are present the mean \pm standard deviation from three separate experiments; ** P <0.01 vs. 0 h control. (E and F) Cells were treated as indicated in C; whole cell lysates were prepared and subjected to immunoblotting using antibodies against c-Caspase-8, c-Caspase3 and Actin. Band intensities were quantified by densitometric analysis and normalized to Actin. Data are presented as the mean \pm standard deviation; ** P <0.01. C, cleaved; CGRP, calcitonin gene-related peptide; FITC, fluorescein isothiocyanate; PgLPS, *Porphyromonas gingivalis* lipopolysaccharide; PI, propidium iodide.

Analysis of apoptotic rates revealed that osteoblast apoptosis increased with increasing concentrations of PgLPS and with increasing treatment time (Fig. 1C and D, respectively).

CGRP attenuates cell viability inhibition and apoptosis induced by PgLPS. Osteoblast cultures (1×10^4 cells/well) were stimulated with 500 ng/ml PgLPS at 37°C for various lengths of time (0, 6, 12, 24, and 48 h). Western blot analysis demonstrated that a transient increase occurred in CGRP protein

expression at 6 h following PgLPS stimulation (Fig. 2A); thereafter, CGRP protein levels gradually and significantly decreased over time, between 12 and 48 h.

Osteoblasts (1×10^4 cells/well) were pretreated with 100 nM CGRP at 37°C for 30 min, followed by treating with 500 ng/ml PgLPS at 37°C for 48 h. Results from the CCK-8 assay indicated that PgLPS-stimulated (500 ng/ml) osteoblasts pretreated with CGRP (100 nM) significantly reduced the cytostatic activity of PgLPS on osteoblasts (Fig. 2B). Apoptotic rates were measured

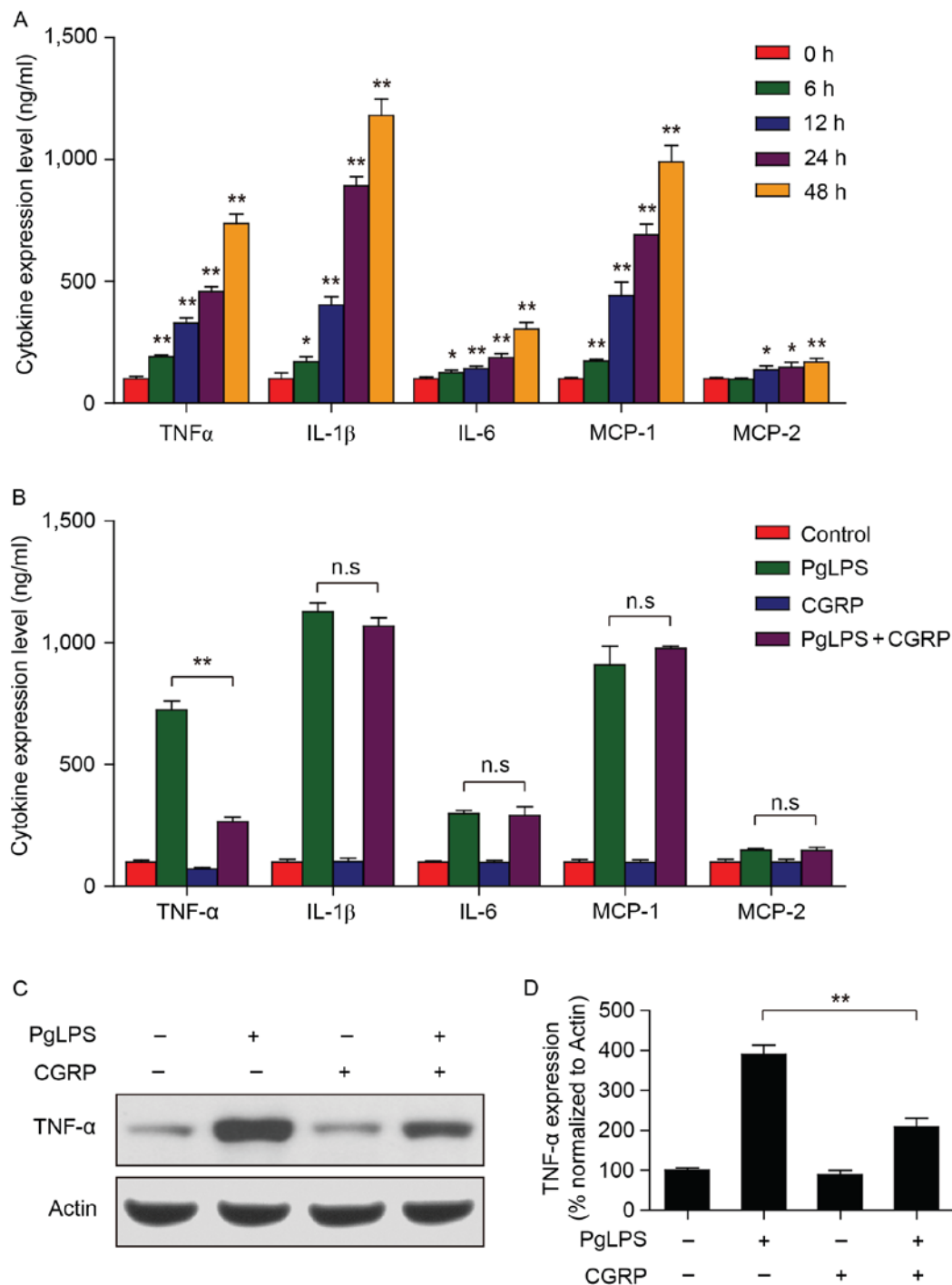


Figure 3. Effects of CGRP pretreatment on PgLPS-induced cytokine expression. (A) Osteoblasts were treated with 500 ng/ml PgLPS for 0, 6, 12, 24 and 48 h. The protein expression levels of TNF- α , IL-1 β , IL-6, MCP-1 and MCP-2 in cell culture supernatants were detected by ELISA. Data are presented as the mean \pm standard deviation; * P <0.05 and ** P <0.01 vs. 0 h control. (B) Cells were pretreated with 100 nM CGRP for 30 min, followed by treatment with 500 ng/ml PgLPS for 48 h. The expression of TNF- α , IL-1 β , IL-6, MCP-1 and MCP-2 in cell culture supernatants were detected by ELISA. Data are presented as the mean \pm standard deviation; * P <0.05 and ** P <0.01. (C and D) Cells were treated as indicated in B, the expression of TNF- α was detected by western blot analysis. Band intensities were quantified by densitometric analysis and normalized to actin. Data are presented as the mean \pm standard deviation; ** P <0.01. CGRP, calcitonin gene-related peptide; IL, interleukin; MCP, monocyte chemotactic protein; n.s., not significant; PgLPS, *Porphyromonas gingivalis* lipopolysaccharide; TNF, tumor necrosis factor.

by flow cytometry and, consistent with the above results, 100 nM CGRP pretreatment markedly suppressed the 500 ng/ml PgLPS-induced apoptosis in osteoblasts (Fig. 2C and D). In addition, CGRP (100 nM) pretreatment was demonstrated to suppress the PgLPS-induced upregulation of c-Caspase-3 and c-Caspase 8 protein expression levels (Fig. 2E and F).

CGRP blocks PgLPS-induced TNF- α expression in osteoblasts. PgLPS is a classic endotoxin and has long been considered a trigger of periodontal diseases (27). PgLPS can also induce cells to release large amounts of inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, and cause a series of inflammatory reactions (28). Cells (1×10^4 cells/well) were

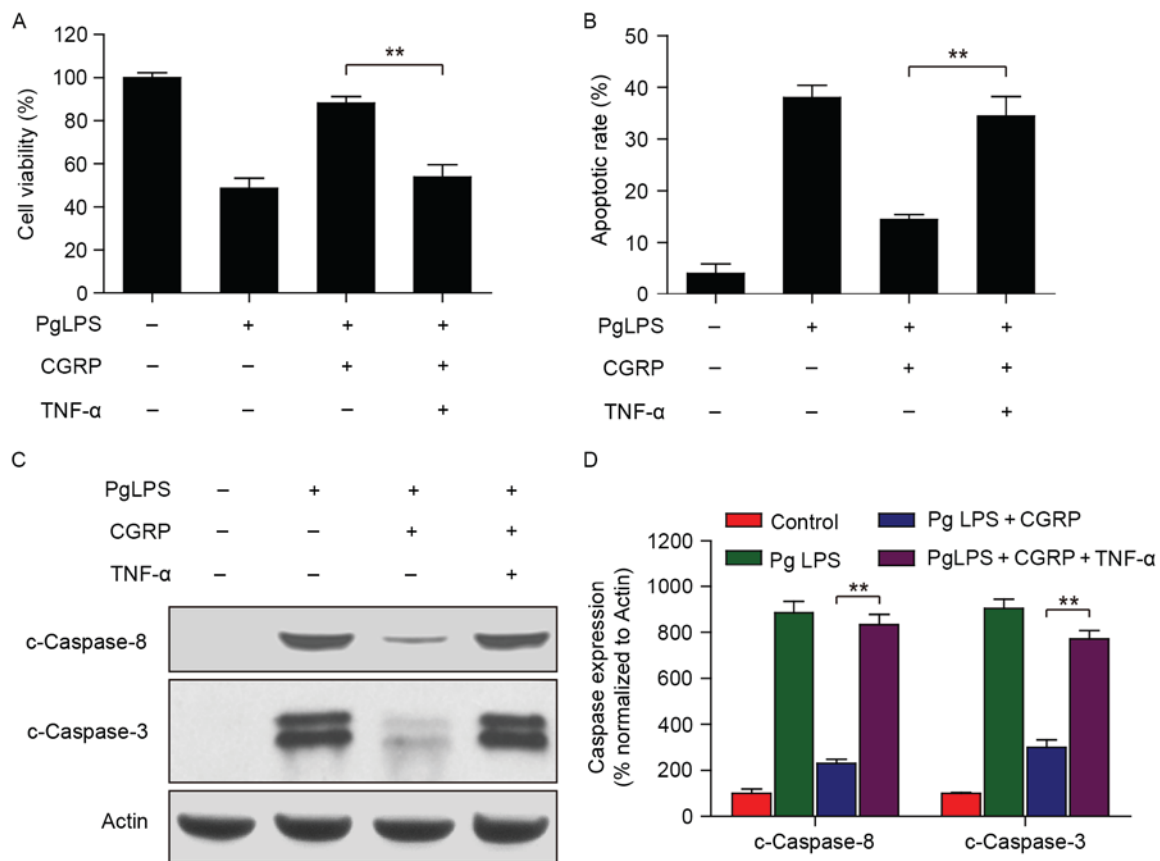


Figure 4. Effects of CGRP pretreatment on PgLPS-induced cell viability inhibition, apoptosis and c-Caspase-8 and c-Caspase-3 expression. (A) Osteoblasts were pretreated with 100 nM CGRP for 30 min, followed by treatment with 500 ng/ml PgLPS for 48 h and 100 ng/ml TNF- α for 24 h. Cell viability was measured by Cell Counting kit-8 assay. Data are presented as the mean \pm standard deviation (n=3) and were normalized to the untreated control group. **P<0.01. (B) Cells were treated as indicated in A; apoptotic rates were measured by flow cytometry. Data are presented as the mean \pm standard deviation from three separate experiments; **P<0.01. (C and D) Expression levels of apoptosis-related proteins c-Caspase-8 and c-Caspase-3 were detected by western blot analysis. Band intensities were quantified by densitometric analysis and normalized to Actin. Data are presented as the mean \pm standard deviation; **P<0.01. C, cleaved; CGRP, calcitonin gene-related peptide; PgLPS, *Porphyromonas gingivalis* lipopolysaccharide; TNF, tumor necrosis factor.

treated with PgLPS (500 ng/ml) in the aforementioned culture medium for different lengths of time (0, 6, 12, 24 or 48 h). Cells cultured in medium with 0 ng/ml PgLPS or for 0 h incubation were used as the controls. Results from the ELISA assays demonstrated that PgLPS (500 ng/ml) treatment promoted the expression of TNF- α , IL-1 β , IL-6 and MCP-1 production in osteoblasts in a time-dependent manner (Fig. 3A). Although no significant changes were identified for MCP-2 expression between 0 and 6 h, expression significantly increased at 12, 24 and 48 h following PgLPS stimulation. In addition, pretreatment with CGRP did not effectively reduce IL-1 β , IL-6, MCP-1 and MCP-2 production in osteoblasts treated with PgLPS, whereas TNF- α production was significantly inhibited (Fig. 3B). Osteoblasts (1×10^4 cells/well) were pretreated with 100 nM CGRP at 37°C for 30 min, followed by treating with 500 ng/ml PgLPS at 37°C for 48 h. Western blot analysis also demonstrated that the PgLPS-induced increase in TNF- α protein expression was suppressed by CGRP pretreatment (Fig. 3C and D).

TNF- α is a key molecule in osteoblasts viability inhibition and apoptosis induced by PgLPS and reversed by CGRP. CCK-8 cell viability assay and flow cytometric results revealed that CGRP pretreatment reversed the PgLPS-induced inhibition

of osteoblast cell viability and increase in apoptosis; however, these effects were not observed in the additional presence of exogenous TNF- α (Fig. 4A and B). Furthermore, western blot protein expression analysis confirmed that CGRP only inhibited the PgLPS-induced upregulation of c-Caspase-8 and c-Caspase-3 levels; whereas, CGRP treatment was not able to inhibit the upregulation of c-Caspase-8 and c-Caspase-3 expression in the presence of both PgLPS and TNF- α . TNF- α is an important pro-inflammatory cytokine and a major bone resorption factor. TNF- α mainly acts on osteoclasts and osteoblasts, and it may cause osteoblast apoptosis (29). The current results demonstrate that CGRP inhibited PgLPS-induced apoptosis; however, this phenomenon was reversed by TNF- α expression. The present study hypothesized that TNF- α was the key factor serving an opposing role in the CGRP-induced inhibition of PgLPS-stimulated osteoblast apoptosis.

Discussion

The present study assessed the effects of CGRP on PgLPS-induced osteoblast apoptosis *in vitro*. The results revealed that PgLPS may inhibit osteoblast viability and promote apoptosis in a time- and concentration-dependent

manner. CGRP expression was demonstrated to reduce PgLPS-induced cytostatic activity and apoptosis in osteoblasts, suggesting that CGRP may be a potential agent for the prevention and treatment of periodontitis.

A number of previous reports have focused on the effects of CGRP on cultured osteoblasts and have revealed that CGRP can regulate bone metabolism and stimulate osteoblasts differentiation (17,30,31). Nevertheless, only a few reports have reported on LPS-induced osteoblast apoptosis. A study published in 1997 (32) demonstrated that CGRP can inhibit LPS-induced TNF- α production in osteoblasts, which is in line with the results of the present study. However, the present study also further assessed the expression of several cytokines induced by LPS and evaluated the effect of CGRP on them. The present study successfully established a LPS-treated osteoblast cell model and elucidated more mechanisms associated with apoptosis.

Inflammation is characterized by an increase in the expression of inflammatory cytokines produced by cells of the activated innate and adaptive immune systems. It has been previously reported that the inflammatory response is closely related to the extent of systemic and local bone loss (33). LPS exposure was reported to stimulate osteoclastic bone resorption *in vivo* and inhibit osteoblast differentiation (34). An increasing amount of data has also demonstrated that PgLPS may directly induce cell death or apoptosis in osteoblasts (10,34). Additional studies have demonstrated that apoptosis serves an essential role in the development and progression of infectious diseases, autoimmune diseases and tumors (35). Osteoblasts cells are capable of secreting matrix and mineralizing into bone tissue; they are the major cells involved in bone remodeling (36). Osteoblast proliferation and apoptosis are of great importance to maintaining the balance of bone formation (37). In the course of periodontitis, bacterial-induced osteoblast apoptosis may be a major contributor to bone loss. A number of studies have revealed that LPS is present in plaque, saliva, gingival crevicular fluid, inflammatory cavities and diseased cementum, with high toxicity to periodontal tissues (38-40). In addition, a previous study suggested that during alveolar bone resorption and periodontitis, bacteria do not directly invade gingival tissue; instead, the destruction of gingival tissue may be mediated by LPS cytotoxicity in the gingival crevice (37). A number of inflammatory cytokines are synthesized and secreted by periodontal tissues and cells in response to LPS exposure, and research has shown that LPS may inhibit osteoblast proliferation and differentiation, and suppress bone formation (34). A previous study reported that PgLPS was able to significantly inhibit alkaline phosphatase (ALP) activity in osteoblasts and decrease the formation of mineralization nodules in a dose- and time-dependent manner (7). Additionally, PgLPS was revealed to increase the protein expression levels of CD14 and LPS receptors, and the mRNA expression level of the bone resorption factor IL-1 β . Similarly, LPS exposure was demonstrated to significantly inhibit ALP activity and collagen synthesis in the osteoblast cell line MC3T3-E1 (34). LPS expression has also been reported to facilitate osteoclast differentiation and activation by activating the mitogen-activated protein kinase signaling pathway (41), as well as induce osteoblast apoptosis by activating the c-Jun N-terminal

kinase pathway (42). The present study demonstrated that PgLPS stimulation inhibited the viability and induced apoptosis in osteoblasts, which was in agreement with previous reports. In addition, CGRP pretreatment was revealed to reduce apoptotic rates that were induced by PgLPS exposure. Following PgLPS stimulation, osteoblasts released a large amount of inflammatory cytokines, including TNF- α , IL-1 β , IL-6, MCP-1 and MCP-2; PgLPS stimulation also increased the expression of apoptotic signals c-Caspase-3 and c-Caspase-8. A number of previous studies reported that the change in cytokine expression levels, including IL-1 β , IL-6, TNF- α , interferon- γ and leukemia inhibitory factor, was an important cause of bone loss (43,44). Among these, the increase of TNF- α expression was identified as one of the characteristics of bone loss (45). It is worth noting that, results from the present study demonstrated that TNF- α was markedly suppressed by CGRP pretreatment, whereas CGRP inhibited the PgLPS-activated caspase signal, which ultimately led to apoptosis. These results indicated that CGRP was able to block PgLPS-induced initiation of inflammation and thus may have a protective effect on osteoblasts.

In the serum of patients suffering from postmenopausal osteoporosis, the level of TNF- α expression was reportedly increased (46). Similarly, TNF- α expression was also revealed to be increased in the serum of patients with type 2 diabetes, which indicated that the change in TNF- α level may be an important cause for bone metabolism diseases (47). TNF- α has many physiological functions in the regulation of cell proliferation, differentiation and survival. In models of pathological bone loss, such as senile osteoporosis and bone resorption caused by chronic inflammation, the concentrations of TNF- α are increased, indicating that TNF- α may also be an important factor in regulating bone metabolism (48). Indeed, TNF- α alone was reported to cause osteoblast apoptosis and, in addition, TNF- α was revealed to inhibit collagen synthesis, ALP activity and osteocalcin synthesis (49). TNF- α may directly promote osteoclast maturation and differentiation by inducing the expression of macrophage colony-stimulating factor and receptor activator of nuclear factor κ B ligand (RANKL) in osteoblasts (50). Additionally, TNF- α may directly promote RANKL-exposed precursor cells to differentiate into osteoclasts, activate mature osteoclasts and inhibit osteoclast apoptosis (51). According to the present results, CGRP pretreatment inhibited PgLPS-induced osteoblast apoptosis; however, this phenomenon was reversed by the addition of TNF- α . Although an accurate explanation of this phenomenon is difficult, it may be speculated that TNF- α serves a pivotal role in CGRP inhibition of PgLPS-induced apoptosis in osteoblasts; however, the exact mechanism involved requires further investigation.

Current treatments for periodontitis mainly consist of antibiotics; despite the therapeutic effects, long-term use of antibiotics may lead to oral dysbiosis, increased bacterial resistance to these antibiotics and gastrointestinal irritation, among other side effects. These factors have affected the clinical application of antibiotics for periodontitis (52). The present *in vitro* study demonstrated that the vasoactive peptide CGRP reduced the expression of inflammatory cytokines and osteoblast apoptosis. In particular, its significant inhibition of TNF- α suggested a new potential target of action for this

peptide in the treatment of periodontitis. In addition, it may be also interesting to integrate the present results with the effects of the neuropeptidergic innervation in dental pulp as, in pulpitis, CGRP-positive fibers in the pulp increased, which also suggested that CGRP may serve a potential role in the regulation of this process. The present study on CGRP may also lead to a new direction for the treatment of pulpitis, which will be the focus of future research.

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