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MK615 attenuates *Porphyromonas gingivalis* lipopolysaccharide-induced pro-inflammatory cytokine release via MAPK inactivation in murine macrophage-like RAW264.7 cells

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ABSTRACT

The Japanese apricot, known as *Ume* in Japanese, has been a traditional Japanese medicine for centuries, and is a familiar and commonly consumed food. The health benefits of *Ume* are now being widely recognized and have been strengthened by recent studies showing that MK615, an extract of compounds from *Ume*, has strong anticancer and anti-inflammatory effects. However, the potential role of MK615 in the periodontal field remains unknown. Here, we found that MK615 significantly reduced the production of pro-inflammatory mediators (tumor necrosis factor-alpha and interleukin-6) induced by *Porphyromonas gingivalis* lipopolysaccharide (LPS), a major etiological agent in localized chronic periodontitis, in murine macrophage-like RAW264.7 cells. MK615 markedly inhibited the phosphorylation of ERK1/2, p38MAPK, and JNK, which is associated with pro-inflammatory mediator release pathways. Moreover, MK615 completely blocked LPS-triggered NF-κB activation. The present results suggest that MK615 has potential as a therapeutic agent for treating inflammatory diseases such as periodontitis.

Introduction

Periodontitis is a major chronic inflammatory disease that destroys the periodontal tissue and eventually causes loss of teeth. Although periodontitis is a local disease, its chronic status triggers systemic inflammatory diseases including severe type 2 diabetes, heart disease, and atherosclerosis [1–3]. Therefore, the development of periodontitis therapies contributes to effective inhibition of systemic inflammatory diseases.

Periodontitis is initiated by overgrowth of a specific Gram-negative anaerobic bacteria that leads to gingival connective tissue destruction and irreversible alveolar bone resorption. Recently, the lipopolysaccharide (LPS) of *Porphyromonas gingivalis*, a major etiological agent in localized chronic periodontitis [4], was shown to contribute to the processes leading to alveolar bone loss and connective tissue destruction [5,6]. The LPSs of Gram-negative bacteria are potent inducers of pro-inflammatory mediators and can initiate a number of host-mediated destructive processes [7]. Moreover, the continuous high secretion of various cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6, by host cells following stimulation with periodontal pathogens and their products is a critical determinant of periodontal tissue destruction [8,9]. The cytokines produced by *P. gingivalis* LPS-activated macrophages are also potent inducers of inflammatory mediators, and have been identified as major mediators of inflammatory processes [10]. Using gene chip microarrays, a recent study revealed that both TNF-α and IL-6 productions are increased in *P. gingivalis* LPS-stimulated macrophages [11]. Therefore, blockade of TNF-α and IL-6 secreted by *P. gingivalis* LPS-stimulated macrophages may suppress pro-inflammatory responses, and inhibit the development and progression of periodontal disease.

Natural compounds, such as catechins in green tea, naringenin, a major flavanone in grapefruits, and polyphenols in cranberries, may be useful for the prevention and treatment of inflammatory periodontal diseases [12–14]. Consequently, natural compounds endowed with the capacity to modulate host inflammatory responses have received considerable attention, with the suggestion that they

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may be potential new therapeutic agents for the treatment of periodontal diseases [15]. *Prunus mume Sieb. et Zucc.* is a variety of Japanese apricot and is known as *Ume* in Japan [16]. The health benefits of *Ume* are now being widely recognized and have been strengthened by recent studies showing that MK615, an extract of compounds from *Ume*, has strong anticancer and antiproliferative effects in *in vivo* and *in vitro* settings [16–19]. These studies also indicated that MK615 may possess strong anti-inflammatory effects [16–19]. However, the potential role of MK615 in the periodontal field remains unknown.

While all types of blood cells are present in normal gingival connective tissue [20], accumulation of inflammatory cells is observed during the progression and development of chronic periodontitis [21]. Furthermore, the number of leukocytes migrating to the gingival sulcus and periodontal pockets increases during the progression of inflammation [22,23]. Monocytes and macrophages, which are found in higher numbers in active periodontal lesions than in inactive sites [24], play important roles in the host inflammatory responses to periodontopathogens [25].

The aim of the present study was to investigate the effects of MK615 on the productions of inflammatory cytokines (TNF-α and IL-6) by macrophages stimulated with *P. gingivalis* LPS. In addition, the changes in the phosphorylation states of macrophage intracellular kinases induced by *P. gingivalis* LPS and MK615 were characterized by Western blotting. Our data show that MK615 inhibits inflammatory cytokine release via suppression of mitogen-activated protein kinase (MAPK) and NF-κB p65 activation in LPS-stimulated RAW264.7 cells.

Materials and methods

Reagents. LPS was extracted from lyophilized *P. gingivalis* cells with phenol–water at 67 °C for 20 min. The pooled extract in the water phase was dialyzed against distilled water and ultracentrifuged at 140,000*g* for 3 h to separate LPS. The LPS sediment was resuspended in water and ultracentrifuged twice more as described previously [26]. MK615 was a gift from AdaBio (Takasaki, Gunma, Japan). Polyclonal antibodies against phospho (p)-p38MAPK, p-c-Jun N-terminal kinase (JNK), p-extracellular signal-regulated kinase (ERK) 1/2, total-IκBα, p65, and p-p65 were purchased from Cell Signaling Technology (Beverly, MA). An anti-β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). SB203580 and SP600125 were purchased from Calbiochem (San Diego, CA). U0126 was purchased from Promega (Madison, WI).

Cell culture. RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% FBS at 37 °C in a 5% CO_2 atmosphere.

Determination of cytotoxicity. A 3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to test the cytotoxicity of MK615 toward macrophages as described previously [27]. Briefly, RAW264.7 cells were seeded at a density of 2–3 × 10^4 cells/well in 96-well dishes and treated with MK615 (0–10 μl/ml) for 24 h. Subsequently, the cells were incubated with MTT (25 mg/ml) for 3 h. The formazan product was solubilized by treatment with 100 μl dimethyl sulfoxide for 16 h. The dehydrogenase activity was expressed as the ratio between the absorbance at the test wavelength of 570 nm and the absorbance at the reference wavelength of 630 nm.

Stimulation of RAW264.7 cells with LPS. RAW264.7 cells (2 × 10^6 cells/6-cm dish) were starved for 2 h in serum-free Opti-MEM-I medium (Invitrogen, Carlsbad, CA) with or without MK615. Next, 100 ng/ml of LPS was added to the medium. Some RAW264.7 cells were preincubated with MAPK inhibitors (U0126, SB203580, or SP600125) for 1 h before exposure to LPS.

Determination of cytokine production. Enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) were used to quantify the TNF-α and IL-6 concentrations in cell-free supernatants, according to the manufacturer's protocols. The absorbance at 450 nm was measured using a microplate reader with the wavelength correction set at 550 nm. The sensitivities of the commercial ELISA kits were 15.6 pg/ml for TNF-α and 9.3 pg/ml for IL-6.

Western blotting. Aliquots (40 μl) of cell lysates were subjected to 12% SDS–PAGE, and the separated proteins were transferred to a nitrocellulose membrane (GE Healthcare Biosciences KK, Piscataway, NJ) as described previously [27]. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline (TBS; pH 7.4) containing 0.02% Tween 20 (TBST) for 1 h at room temperature (RT) and then incubated with a primary antibody diluted in TBST containing 1% non-fat dry milk overnight at 4 °C. After washing, the membrane was incubated with a horseradish peroxidase-conjugated anti-rabbit IgG polyclonal antibody (Invitrogen) diluted 1:3000 in TBST containing 2.5% non-fat dry milk for 1 h at RT. The membrane was washed again, and the immunoreactive bands were visualized using an ECL detection system (GE Healthcare Biosciences KK).

Immunofluorescence. RAW264.7 cells (1 × 10^5 cells/well) were plated on culture slides, and serum-starved for 2 h with or without MK615. Next, 100 ng/ml of LPS was added to the medium for 1 h. After washing, the cells were fixed with 4% paraformaldehyde, and blocked with 1% BSA in PBS containing 0.1% Triton-X 100. After washing, the cells were incubated with an NF-κB p65 antibody. After further washing, the cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen; 1:200 dilution), washed and stained with 4′,6-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI). The stained cells were visualized under an Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analysis. Differences between means were analyzed for statistical significance using Student's *t*-test and were considered significant at $P < 0.05$.

Results

Effects of MK615 on P. gingivalis LPS-induced pro-inflammatory cytokine productions by RAW264.7 cells

In this study, we focused on determining whether MK615 could inhibit TNF-α and IL-6 release by *P. gingivalis* LPS-induced RAW264.7 cells. MTT assays revealed no obvious cytotoxic effects following treatment of the macrophages with MK615 (0, 0.1, 1, 5, and 10 μl/ml) for 24 h (Fig. 1A), indicating that any decrease in inflammatory mediator production was probably unrelated to cell toxicity. RAW264.7 cells were treated with MK615 (0, 0.1, 1, and 10 μl/ml) for 2 h prior to stimulation with *P. gingivalis* LPS. LPS (100 ng/ml) was then added to the cells and incubated for a further 6 h. The media were analyzed by ELISA. MK615 significantly inhibited TNF-α (Fig. 1B) and IL-6 (Fig. 1C) release by the stimulated RAW264.7 cells in dose-dependent manners, clearly indicating that MK615 contains an inhibitor of TNF-α and IL-6 release.

MK615 inhibits P. gingivalis LPS -induced phosphorylation of MAPKs

LPS possibly induces TNF-α and IL-6 expressions through transient phosphorylation of ERK1/2, JNK, and p38MAPK [28–32]. We examined whether MK615 suppressed the phosphorylation of MAPKs by *P. gingivalis* LPS using western blot analysis. As shown in Fig. 2A, all MAPKs, namely ERK1/2, p38MAPK, and JNK, were phosphorylated by *P. gingivalis* LPS (lane 2). In contrast, MK615 significantly attenuated the phosphorylation of MAPKs, including

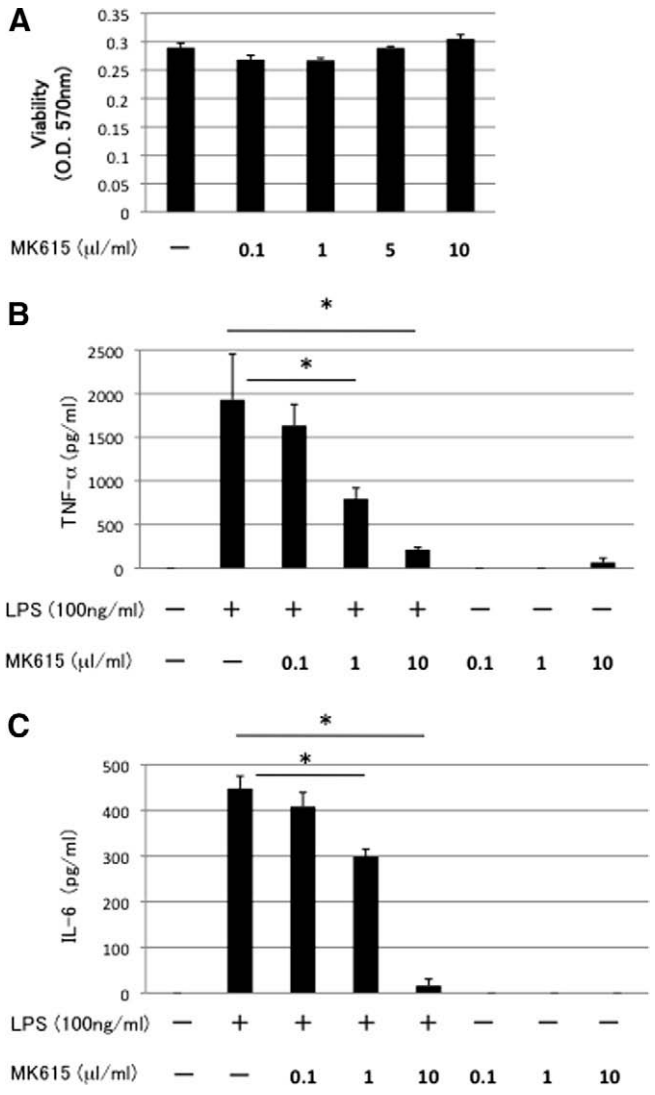

Fig. 1. MK615 suppresses pro-inflammatory cytokine release by *P. gingivalis* LPS-induced RAW264.7 cells. (A) Cell viability as a function of the MK615 concentration. Cells were incubated with various concentrations of MK615 (0.1–10 μl/ml). After 24 h, the cell viability was measured by the MTT assay. (B, C) Cells were pretreated with the indicated MK615 concentrations (0.1, 1, and 10 μl/ml) for 2 h and then stimulated with *P. gingivalis* LPS (100 ng/ml) for 6 h. The concentrations of the pro-inflammatory cytokines released into the supernatants were measured by ELISA. (B) TNF-α. (C) IL-6. Control values were obtained from the corresponding basal medium or after treatment with MK615 alone. Values are expressed as means ± SE of triplicate experiments. $^{*}P < 0.05$.

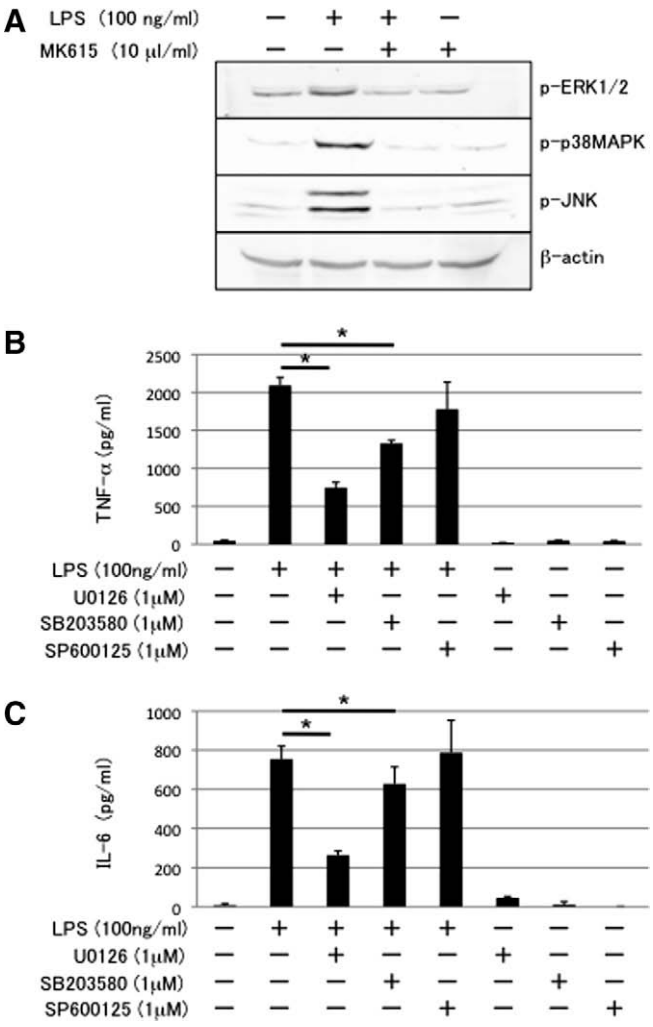

Fig. 2. MK615 inhibits *P. gingivalis* LPS-induced phosphorylation of MAPKs. (A) Cells were preincubated with 10 μl/ml MK615 for 2 h prior to the addition of *P. gingivalis* LPS (100 ng/ml) for 30 min. Lysates of the cells were analyzed by Western blotting using phospho (p)-ERK (top), p-p38MAPK (second panel), and p-JNK (third panel) antibodies. The same membrane was immunoblotted with an anti-β-actin antibody (bottom panel) as a protein loading control. (B, C) Cells were pretreated for 1 h with MAPK inhibitors (U0126, SB203580, and SP600125) at 1 μM and then stimulated with *P. gingivalis* LPS (100 ng/ml) for 6 h. The concentrations of the pro-inflammatory cytokines released into the supernatants were measured by ELISA. (B) TNF-α. (C) IL-6. Values are expressed as means ± SE of triplicate experiments. $^{*}P < 0.05$.

ERK1/2, p38MAPK, and JNK, in the *P. gingivalis* LPS stimulation system (lane 3).

To clarify the pathway of the *P. gingivalis* LPS-mediated stimulation of RAW264.7 cells, we examined the effects of specific MAPK inhibitors (ERK1/2: U0126; p38MAPK: SB203580; JNK: SP600125). Interestingly, TNF-α and IL-6 were reduced following U0126 or SB203580 treatment, whereas SP600125 had no effect (Fig. 2B and C).

MK615 inhibits P. gingivalis LPS-induced NF-κB DNA-binding activity in RAW264.7 cells

We investigated the inhibitory mechanism of MK615 toward cytokine production by LPS-stimulated RAW264.7 cells. The production of inflammatory cytokines requires NF-κB activation [33]. Therefore, to examine the molecular mechanism by which MK615 inhibits NF-κB transcriptional activity, we first investigated the effects of MK615 on LPS-induced IκBα degradation. As shown in Fig. 3A, the degradation of IκBα induced by LPS was inhibited when RAW264.7 cells were pretreated with MK615. Second, to investigate the possibility that MK615 regulates the phosphorylation of the p65 subunit of NF-κB, RAW264.7 cells were treated with LPS (100 ng/ml) alone or in combination with MK615 (10 μl/ml). Phosphorylation of p65 was increased in the total cell lysate of RAW264.7 cells stimulated with LPS for 1 h (Fig. 3B, lane 2). In contrast, MK615 completely suppressed the phosphorylation of p65 induced by LPS (Fig. 3B, lane 3).

MK615 inhibits P. gingivalis LPS-induced nuclear translocation of the p65 subunit of NF-κB

Next, we determined whether MK615 suppressed the nuclear translocation of NF-κB in *P. gingivalis* LPS-stimulated RAW264.7 cells by immunofluorescence. As shown in Fig. 4, LPS stimulation

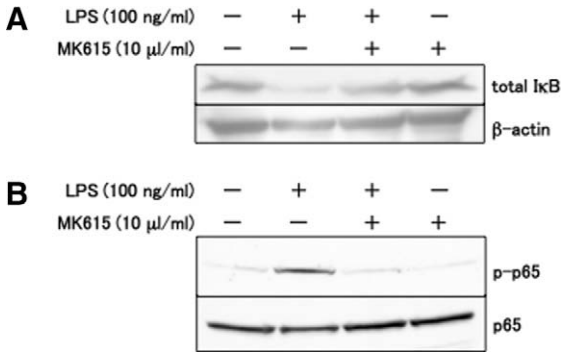

Fig. 3. MK615 suppresses *P. gingivalis* LPS-induced NF-κB activation but not degradation of IκBα in RAW264.7 cells. (A) Cells were pretreated with MK615 for 2 h and then stimulated with LPS for 1 h. Lysates of the cells were analyzed by western blotting with an anti-total-IκBα antibody (upper panel). The same membrane was immunoblotted with an anti-β-actin antibody as a protein loading control (lower panel). (B) Cells were pretreated with MK615 for 2 h and then stimulated with LPS for 1 h. Lysates of the cells were evaluated by western blotting with an anti-p-p65 antibody (upper panel). The same membrane was immunoblotted with an anti-p65 antibody (lower panel).

for 1 h upregulated the activity of p65 and caused its translocation into the nuclei (Fig. 4B). In contrast, a very low level of p65 activity was observed in the control cells (Fig. 4A). Pretreatment with MK615 and subsequent stimulation with LPS attenuated the p65 translocation (Fig. 4C). Incubation with MK615 alone did not affect the p65 translocation (Fig. 4D).

Discussion

The present study demonstrates for the first time that MK615 suppresses cytokine release (TNF-α and IL-6) by RAW264.7 cells in response to *P. gingivalis* LPS stimulation. Furthermore, the inhibitory mechanism of MK615 is mediated by the attenuation of MAPK phosphorylation and the subsequent inactivation of NF-κB to suppress LPS-induced translocation and phosphorylation of the p65 subunit. These findings suggest that MK615 may play an important role during inflammation, and may represent a key molecule with therapeutic potential for periodontitis.

MK615 extracted from *Ume* contains several triterpenoids, including oleanolic acid and ursolic acid, and may thus have anti-inflammatory effects. Recent studies have suggested that triterpenoids have antitumor and anti-inflammatory effects [16–19,34]. Indeed, MK615 induces antiproliferative, pro-apoptotic and pro-autophagic effects in tumor cells [16–19]. Moreover, MK615 inhibits the release of high mobility group box-1 (HMGB1), which is a nuclear protein and a novel cytokine, by LPS-stimulated RAW264.7 cells [34]. The inhibitory mechanism is mediated via the anti-oxidation of heme oxygenase-1 expressed by oleanolic acid, thus strongly suggesting that MK615 may suppress the effects of inflammation. In the present study, MK615 was found to inhibit cytokine release, including TNF-α and IL-6, by *P. gingivalis* LPS-stimulated RAW264.7 cells. MK615 inhibited MAPK signaling, including that of ERK1/2, p38MAPK, and JNK, in the *P. gingivalis* LPS-stimulated RAW264.7 cells. Furthermore, inhibitors of these MAPKs inhibited cytokine release, except for that of JNK. Therefore, the inhibitory mechanism appears to be mediated via ERK1/2 and

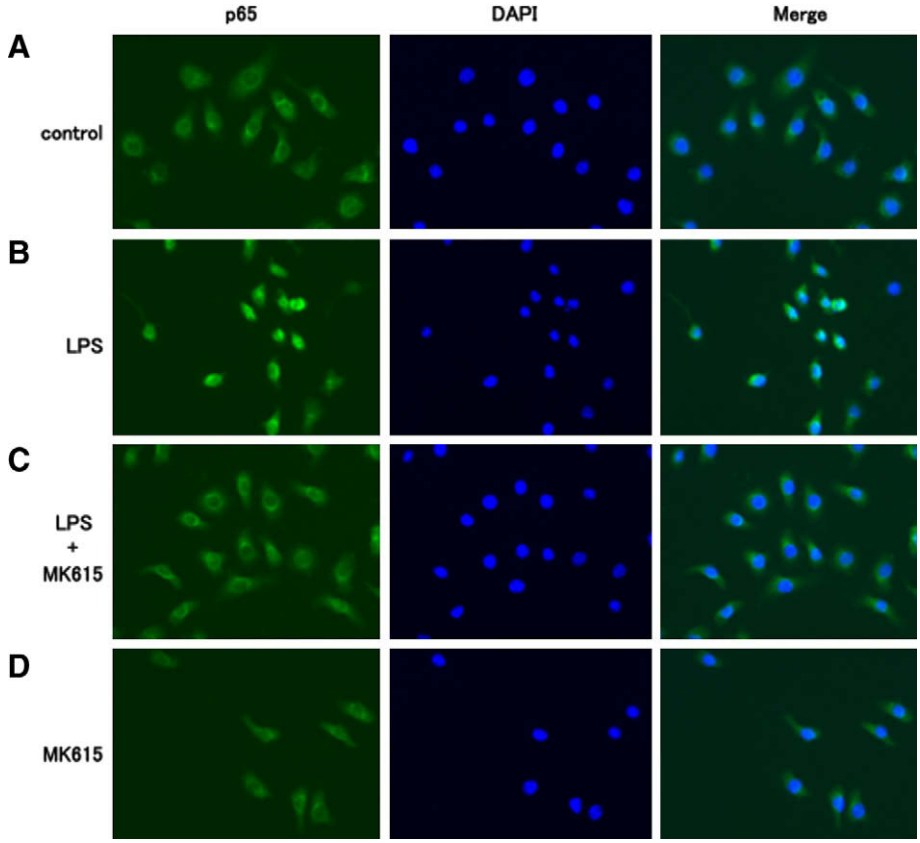

Fig. 4. MK615 suppresses *P. gingivalis* LPS-induced nuclear translocation of NF-κB p65 in RAW264.7 cells. Cells were left untreated or pretreated with MK615 and stimulated with LPS. (A) In untreated cells, NF-κB p65 is limited to the cytoplasm. (B) LPS-stimulated cells show NF-κB p65 (green) translocation into the nucleus. (C) LPS-stimulated cells pretreated with MK615 show a significant reduction in p65 nuclear translocation. (D) Cells treated with MK615 alone show no effect. Cells stained with DAPI were used to verify the nuclear localization (blue). Original magnification: 400×.

p38MAPK signaling in *P. gingivalis* LPS-stimulated RAW264.7 cells, and the downstream of NF-κB finally fails to become activated. These results suggest that MK615 has anti-inflammatory effects.

The host inflammatory responses to periodontal pathogens, notably excessive productions of cytokines, are considered to be major factors contributing to the local tissue destruction observed in periodontitis. Consequently, therapeutic approaches that inhibit cytokine production are receiving increasing attention as options for managing chronic periodontitis. We used cultured macrophages to investigate the capacity of MK615 to inhibit the LPS-induced inflammatory responses. Pro-inflammatory cytokines, such as TNF-α and IL-6, are involved in the initiation and amplification of the inflammatory process [35]. They also contribute to the pathophysiology of many inflammatory diseases, including rheumatoid arthritis, atherosclerosis, and periodontitis [13]. More specifically, TNF-α plays an active role in the progression of periodontitis by inducing the production of adhesion molecules and other mediators that facilitate and amplify the inflammatory responses, stimulate matrix metalloproteinases and enhance bone resorption [36]. IL-6, a multifunctional cytokine, plays important roles in regulating the immune responses and bone resorption during periodontal disease. In the present study, MK615 exhibited a strong potential to reduce the productions of TNF-α and IL-6 by macrophages. Taken together, these lines of evidence suggest that MK615 may contribute to reductions in the impacts of the host-destructive processes mediated by these two cytokines, and may represent a useful therapeutic agent for chronic periodontitis.

In conclusion, the present study has provided evidence that MK615 inhibits cytokine release, including TNF-α and IL-6, by *P. gingivalis*-stimulated RAW264.7 cells via inhibition of MAPK pathways, and may therefore play major roles in regulating chronic periodontitis. Further studies are required to investigate the effects of local application of MK615 as an adjunctive treatment to conventional therapy for periodontitis patients. Such studies may lead to the development of novel periodontal therapies and strategies for public oral health.

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