Porphyromonas *gingivalis* LPS-induced IL-1β and IL-8 Expression by Reducing MAPK Activation in THP-1 Cells

I Yiemwattana[1], R Kaomongkolgit[2]

ABSTRACT

Objective: To investigate the inhibitory effects of alpha-mangostin on *Porphyromonas* gingivalis lipopolysaccharide (LPS)-induced *production* of *pro-inflammatory* cytokines and activation of mitogen-activated protein kinase (MAPK) signaling in THP-1 cells.

Methods: The cell viability after P. gingivalis LPS or alpha-mangostin treatment was measured with MTS assay. The ability of alpha-mangostin to modulate the THP-1 cell response to P. gingivalis LPS stimulation was assessed by real-time polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) method for interleukin (IL)-1β and IL-8 expression and by western blotting for the activation of MAPK signaling.

Results: Alpha-mangostin reduced the expression of IL-1β and IL-8 in P. gingivalis LPS-stimulated THP-1 cells. Alpha-mangostin also suppressed P. gingivalis LPS-induced phosphorylation of c-Jun N-terminal kinase (JNK), extracellular-signal-regulated kinase 1/2 (ERK1/2), and p38 mitogen-activated protein kinase (p38 MAPK).

Conclusion: Alpha-mangostin suppresses the production of inflammatory cytokine in P. gingivalis LPS-stimulated THP-1 cells, possibly by reduction the activation of MAPK. These results suggest that alpha-mangostin may be used as an anti-inflammatory agent in adjunctive treatment of periodontal disease.

Keywords: Alpha-mangostin, IL-1β/8, P. gingivalis LPS, THP-1 cells

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From: [1]Departments of Preventive Dentistry, Faculty of Dentistry, Naresuan University, Phitsanulok 65000, Thailand. [2]Department of Oral Diagnosis, Faculty of Dentistry, Naresuan University, Phitsanulok 65000, Thailand

Correspondence: Dr I Yiemwattana, Department of Preventive Dentistry, Faculty of Dentistry, Naresuan University, Phitsanulok 65000, Thailand. Fax: +6655966053, e-mail: ichayak@yahoo.com

INTRODUCTION

Porphyromonas gingivalis is involved in the pathogenesis of periodontitis, a chronic inflammatory disease that destroys the tissues supporting the tooth (1). The LPS of *P. gingivalis* is a virulence factor that plays a crucial role in mediating inflammation and stimulating production of pro-inflammatory cytokines by immune cells (2). Monocytes are part of the innate immunity which constitutes the first line of defense against periodontal pathogens. These cells are involved in the development of periodontal inflammation and destruction (3, 4). Previous studies have shown that *P. gingivalis* LPS stimulates monocytes to exhibit increased production of proinflammatory cytokines such as IL-1β and IL-8 (5, 6).

IL-1β and IL-8 plays a significant role in the pathogenesis of periodontal disease. Previous studies have confirmed that the IL-1β levels in both gingival crevicular fluid (GCF) and periodontal tissue of periodontitis patients are increased in relation to the severity of inflammation (7, 8) and IL-1β level in GCF is reduced following periodontal treatment (9). The chemotactic effects of IL-8 in inflamed gingiva may contribute to the resorption of alveolar bone (10, 11). The level of IL-8 is greatly increased in inflamed periodontal tissue and periodontal therapy reduced the expression of this cytokine (12), thus level of IL-8 is associated with periodontal status.

Alpha-mangostin, the dominant xanthone found in the pericarps of mangosteen, possesses anti-inflammatory activities by decreasing expression of pro-inflammatory mediators and activation of signaling pathways. These effects include the finding that alpha-mangostin decreased LPS-stimulated IL-1β, tumor necrosis factor (TNF)-α, IL-4, and IL-8 expression in human adipocytes (13) and U937 cells (14). Treatment of *E coli* LPS-stimulated U937 cells with alpha-mangostin has shown to decrease MAPK activation (14, 15). In human monocytic cell line THP-1, the inhibitory effect of alpha-mangostin has been report only in *Escherichia coli* LPS-stimulated TNF-α and IL-8 production (16). However, the efficacy of

alpha-mangostin on IL-1β and IL-8 expression and activation of MAPK signaling in *P. gingivalis* LPS stimulated human monocytes has not been elucidated. Therefore, the purpose of this study was to investigate the inhibitory effects of alpha-mangostin on the *P. gingivalis* LPS-induced IL-1β and IL-8 production and the MAPK activation in THP-1 cells.

SUBJECTS AND METHODS

Preparation of alpha-mangostin

Alpha-mangostin was isolated and purified as previously described (17). The yield of alpha-mangostin from the dried pericarp was approximately 0.4% (w/w). The stock solution of alpha-mangostin was dissolved in dimethysulfoxide (DMSO) and sterilized by filtration through 0.2 μm disc filters.

Cell culture

This study was approved by the Naresuan University Institutional Review Board (COE No. 057/2015). THP-1 cells were obtained from the European Collection of Cell Cultures. Cells were maintained in RPMI 1640 (Hyclone, Cramlington, UK) containing 10% heat-inactivate fetal bovine serum (Hyclone), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) in 5% CO_2 at 37 °C.

Cytotoxicity assays

The effect of alpha-mangostin and *P. gingivalis* LPS on the viability of THP-1 cells were examined by using the CellTiter 96 Aqueous One Solution Cell Proliferation Kit (MTS assay; Promega, WI, USA). THP-1 cells were seeded at a density of $1X10^5$ cells per well in 24-well

plates. After 24h, cells were treated with 0, 0.25, 0.5, and 1.0 μg/ml of alpha-mangostin or 0, 0.25, 0.5, and 1.0 μg/ml of *P. gingivalis* LPS (InvivoGen, CA, USA) in serum free medium for 24 h. Then, 20 μl MTS solution was added to each well. After incubation for 4 h, soluble formazan absorbance was recorded using a microplate reader (Bio-Rad Laboratories, CA, USA) at 490 nm. These experiments were repeated three independent times in triplicate wells.

P. gingivalis LPS challenge

THP-1 cells were seeded in a 6-well plate at a density of $3x10^5$ cells per well and incubated in absence (controls) or presence of 0.5, 0.75, and 1.0 μg/ml of *P. gingivalis* LPS. RT-PCR and ELISA were performed.

RT-PCR analysis of IL-1β and IL-8 mRNA levels

THP-1 cells were seeded in a 6-well plate at a density of $3x10^5$ cells per well. Following 3 h of preincubation in the presence or absence of 0.5 μg/ml *P. gingivalis* LPS, cells were treated with 0.25, 0.5, and 1.0 μg/ml of alpha-mangostin for 16 h. Total RNA was then isolated by Nucleospin RNAII (Macherey-Nagel, PA, USA) according to the manufacturer's instruction. Concentration and purity of RNA was determined using a Nano Drop ND-2000c spectrophotometer (Thermo Fisher Scientific, MA, USA). cDNA was synthesized from 1 μg of total mRNA and mixed with LightCycler 480 DNA SYBR Green I Master (Roche). Two-step quantitative real-time PCR was performed using the LightCycler 480 II Real-Time PCR System (Roche). GAPDH served as a control. The sequences of the PCR primers were use as follow: IL-1β sense 5'-GGATATGGAGCAACAAGTGG-3', IL-1β antisense 5'-ATGTAC CAGTTGGGGAACTG-3', IL-8 sense 5'-ACACTGCGCCAACACAGAAATTA-3', IL-8 antisense 5'-TTTCTTGAAGTTTCACTCATC-3', GAPDH sense 5'-TGAAGGGTC GGAGTCAACGGAT-3', and GAPDH antisense 5' -TCACACCATGACGAACATGG-3'.

Data were analyzed with the LightCycler® 480 software. Quantification was calculated using the starting quantity of the cDNA of interest relative to that of GAPDH cDNA in the same sample.

ELISAs for IL-1β and IL-8 determination

The culture supernatants obtained from real-time PCR experiments were collected to quantify cytokine production. Value of IL-1β and IL-8 were measured by sandwich ELISA kit (Biolegend, CA, USA) according to the manufacturer's instructions. All measurements were performed in duplicate and were repeated at three different occasions.

Western blotting analysis

THP1 cells were pretreated with 0.5 μg/ml *P. gingivalis* LPS for 30 min and treated with 0.25, 0.5, and 1.0 μg/ml alpha-mangostin for 3 h. Then, cells were washed with PBS and the whole cells were extracted with protease and phosphatase inhibitors. Mixed proteins were determined the concentration using a bicinchoninic acid kit (Pierce Biotechnology, IL, USA). The volume of extracted protein was determined to 80 μg in each well of 10% SDS polycrylamide gel and transferred to polyvinylidene difluoride membranes. The activations of JNK, ERK1/2 and p38 MAPK were assessed using anti-phospho-ERK1/2 antibody (Upstate Biotechnology, NY, USA), anti-phospho-JNK, and anti-phospho-p38 MAPK antibodies (Cell Signaling Technology, MA, USA). Immunoreactive proteins were observed under under CCD camera (GE Healthcare Life Sciences, PA, USA).

Statistical analysis

Statistical analyses were performed using the SPSS 17.0 software. All data were expressed as mean ± standard deviation (SD). Differences between experimental groups were analyzed ANOVA followed by Tukey HSD comparison test. The level of statistical significance was set at $p < 0.05$.

RESULTS

Cytotoxic effect of *P. gingivalis* LPS and alpha-mangostin on THP-1 cells

The *in vitro* cytotoxicity was analyzed by MTS assay and was expressed as a percentage of control values. *P. gingivalis* LPS (Fig. 1A) and alpha-mangostin (Fig. 1B) showed no cytotoxic effect at the concentrations up to 1 μg/ml ($p < 0.05$).

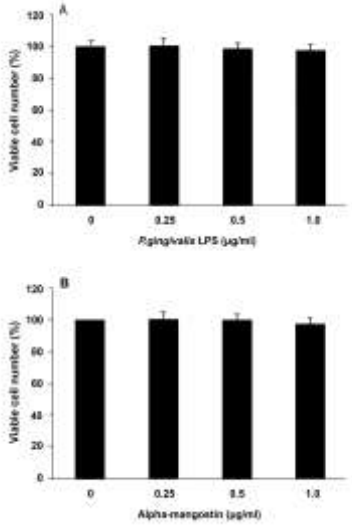

Fig. 1: *P. gingivalis* LPS and alpha-mangostin exhibited no cytotoxicity in THP-1 cells. Cells were treated with various concentrations of *P. gingivalis* LPS (A) and alpha-mangostin (B) for 24 h. The MTS assay was used to measure cell viability. The data (mean ± SD) are the average of three independent experiments performed in triplicate.

Effects of *P. gingivalis* LPS on the mRNA and protein expression level of IL-1β and IL-8 in THP-1 cells

The mRNA expression levels of IL-1β and IL-8 were increased significantly when THP-1 cells were treated with *P. gingivalis* LPS 0.5, 0.75, and 1.0 μg/ml (Fig. 2A and 2B). These results were consistent with the IL-1β and IL-8 protein expression (Fig. 2C and 2D). These dose-dependent experiments suggest that the expression of IL-1β and IL-8 in THP-1 cells

were modulated by *P. gingivalis* LPS. In the following experiment, *P. gingivalis* LPS at concentration of 0.5 μg/ml was applied in all experiments.

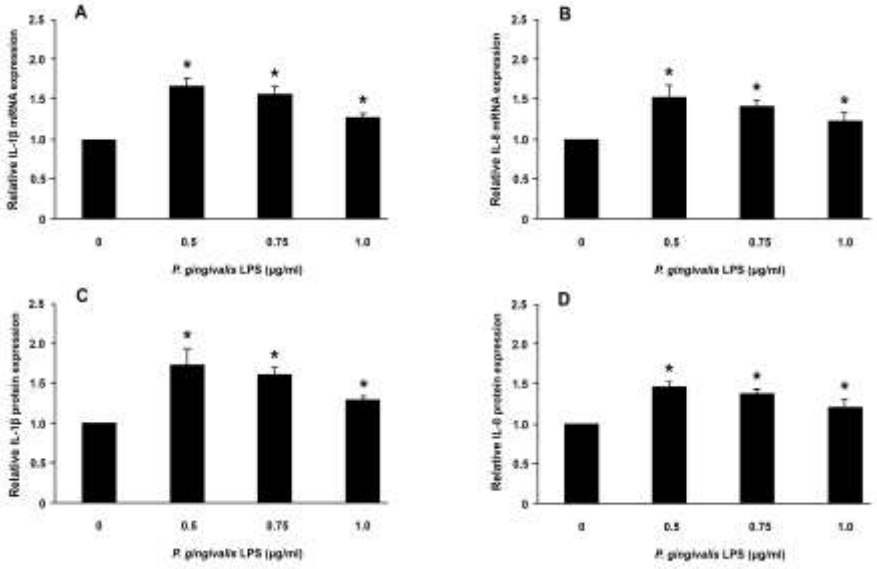

Fig. 2: Stimulation with *P. gingivalis* LPS increased IL-1β (A), IL-8 (B) mRNA, IL-1β (C), and IL-8 (D) protein in THP-1 cells. Cells were incubated with 0.5, 0.75, and 1.0 μg/ml of *P. gingivalis* and extracted for RT-PCR. Conditioned media were subjected to ELISA analysis. Histograms represent mean ± SD of relative quantification from three independent experiments. *indicates $p < 0.05$, when compared with *P. gingivalis* LPS-stimulated untreated cells.

Effects of alpha-mangostin on IL-1β and IL-8 mRNA expression in *P. gingivalis* LPS-stimulated THP-1 cells

To investigate the suppression effect of alpha-mangostin, THP-1 cells were pre-treated with *P. gingivalis* LPS, and subsequently treated with 0.25, 0.5, and 1.0 μg/ml alpha-mangostin. Alpha-mangostin 0.5 and 1.0 μg/ml significantly reduced the mRNA expression of *P. gingivalis* LPS-upregulated IL-1β (Fig. 3A) and IL-8 (Fig. 3B) ($p < 0.05$).

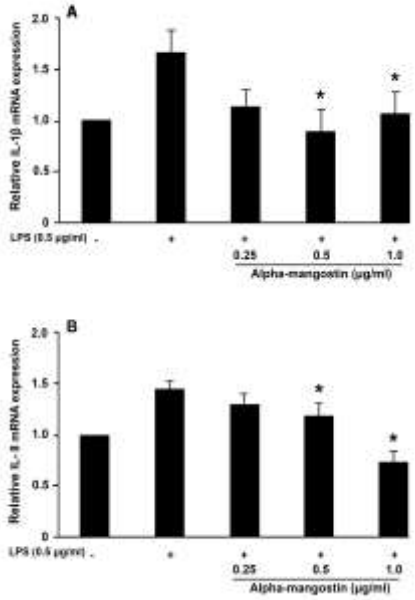

Fig. 3: Alpha-mangostin suppressed IL-1β (A) and IL-8 (B) mRNA expression in *P. gingivalis* LPS stimulated THP-1 cells. Cells were incubated with 0.5 μg/ml of *P. gingivalis* LPS for 3 h, followed by alpha-mangostin treatment for 16 h. Cells were extracted for RT-PCR and levels of IL-1β and IL-8 were normalized to GAPDH. Histograms represent mean ± SD of relative quantification from three independent experiments. *indicates $p < 0.05$, when compared with *P. gingivalis* LPS-stimulated untreated cells.

Effects of alpha-mangostin on IL-1β and IL-8 protein expression in *P. gingivalis* LPS-stimulated THP-1 cells

In order to confirm the suppression effect of alpha-mangostin, the ELISA was used to detect IL-1β and IL-8 protein expression. Similar to previous experiment, treatment with 0.5 and 1 μg/ml alpha-mangostin significantly inhibited *P .gingivalis* LPS-upregulated IL-1β and IL-8 protein expression ($p < 0.05$) (Fig. 4). Again, alpha-mangostin suppressed *P. gingivalis* LPS-induced increases in the expression of IL-8 protein in a dose-dependent manner.

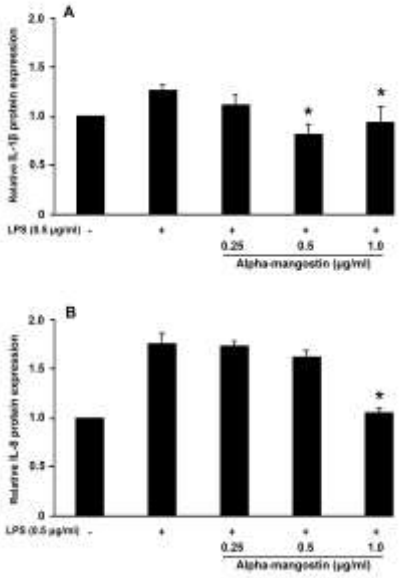

Fig. 4: Alpha-mangostin suppressed IL-1β (A) and IL-8 (B) protein expression in *P. gingivalis* LPS stimulated THP-1 cells. Conditioned media from RT-PCR experiments was subjected to ELISA analysis. Histograms represent mean ± SD of relative quantification from three independent experiments performed in triplicate. *indicates $p < 0.05$, when compared with *P. gingivalis* LPS-stimulated untreated cells.

Effect of alpha-mangostin on *P. gingivalis* LPS-mediated MAPK activation

MAPK is considered to be the central pathway for the regulation of inflammatory cytokine production, expression of three MAPK signaling molecules were examined using immunoblotting. *P. gingivalis* LPS treatment induced the phosphorylation of JNK, ERK1/2 and p38 MAPK, and 0.5 and 1.0 μg/ml alpha-mangostin treatment decreased these responses in a dose-dependent manner (Fig. 5). The lowest protein levels of JNK, ERK1/2 and p38 MAPK phosphorylation were reduced to 63.06%, 61.83% and 46.39%, respectively, compared with that in the *P. gingivalis* LPS-treated cells. These data demonstrate that alpha-mangostin could inhibit the activation of MAPK signaling in *P. gingivalis* LPS stimulated-THP-1 cells.

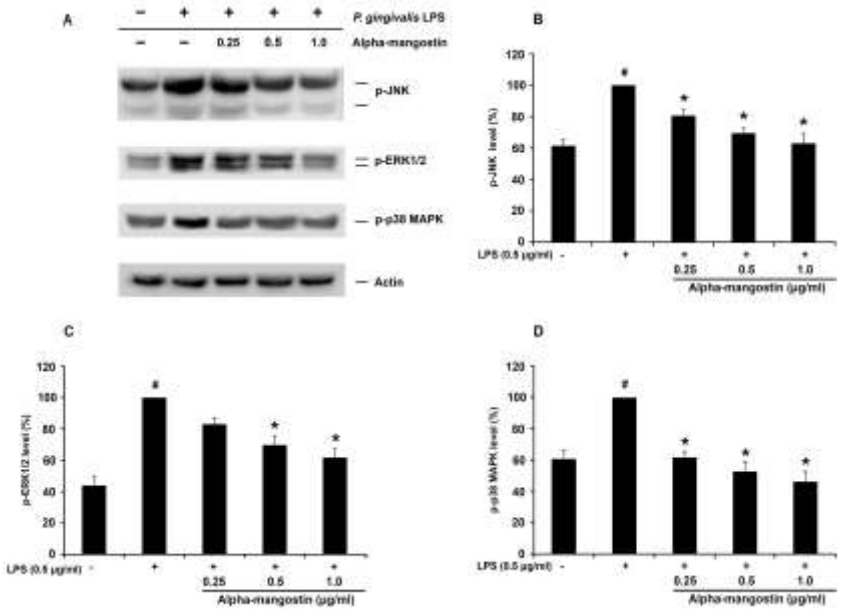

Fig. 5: Alpha-mangostin decreases the activation of MAPK signaling by *P. gingivalis* LPS in THP-1 cells. Cells were incubated with 0.5 μg/ml *P. gingivalis* LPS for 30 min, followed by alpha-mangostin treatment for 3 h. Levels of protein were measured by western blotting (A). The protein expression levels were normalized by the corresponding actin expression levels. Histograms represent mean ± SD of fold JNK (B), ERK1/2 (C), and p38 MAPK (D) phosphorylation normalized to in those treated with *P. gingivalis* LPS only. Data are representative of at three independent experiments. #indicates $p < 0.05$, when compared with untreated cells and *indicates $p < 0.05$, when compared with *P. gingivalis* LPS-stimulated untreated cells.

DISCUSSION

Alpha-mangostin has been discovered to possess anti-inflammatory activities, including inhibition of inflammatory cytokines production in *E. Coli* LPS-stimulated cells (13-16). In the present study, the inhibitory effects of alpha-mangostin on IL-1β and IL-8 expression and MAPK activation were investigated for the first time in *P. gingivalis* LPS activated THP-1 cells. The cytotoxicity of *P. gingivalis* LPS and alpha-mangostin were initially assessed in order to further modulate cytokine expression without the induction of cell death. The results showed that treatment of activated THP-1 cells with noncytotoxic concentrations of alpha-

mangostin inhibited expression of IL-1β and IL-8 and reduced phosphorylation of JNK, ERK1/2 and p38 MAPK.

IL-1β and IL-8 have been shown to play an important role in inflammatory processes of periodontal tissue (10). The results of our study demonstrated that the low (0.5 μg/mL) dose of *P. gingivalis* LPS significantly increased the expression of IL-1β and IL-8, indicating that *P. gingivalis* LPS may promote the periodontal inflammation by the highly expressed proinflammatory cytokine in THP-1 cells. Decreased levels of IL-1β and IL-8 both in mRNA and protein expression were found after alpha-mangostin treatment. IL-1β secretion in this study revealed reduced expression by 65–75 % and the level of IL-8 production was also reduced by 60 %. Consistent with our data, *E. coli* LPS-induced IL-8 secretion was decreased by 30–40 % in THP-cells in response to alpha-mangostin treatment (16). We recently reported the inhibitory effects of alpha-mangostin on the IL-6 and IL-8 expression in *P. gingivalis* LPS-stimulated human gingival fibroblasts (17). These data suggested that treatment of *P. gingivalis* LPS activated THP-1 cells with alpha-mangostin resulted in reduction of IL-1β and IL-8 expression.

LPS stimulation exhibits inflammatory activity by activating several intracellular signaling pathways that include three MAPK pathways, JNK, ERK1/2 and p-38 MAPK. Prior studies have demonstrated that the anti-inflammatory effects of alpha-mangostin against *E. Coli* LPS-stimulated human adipocytes and U937 cells can be partly attributed to preventing the activation of MAPK signaling pathways (14, 15). *P. gingivalis* LPS directly induced activation of MAPK signaling pathways that is especially relevant for expression of inflammatory genes during periodontal disease progression (19). In human monocytes, *P. gingivalis* LPS was reported to induce JNK (5) and p38 MAPK (20) phosphorylation. Our results also indicated that *P. gingivalis* LPS induced phosphorylation of JNK, ERK1/2 and p38 MAPK and alpha-mangostin can reduce the activation of these signaling proteins. Thus,

the inhibition of *P. gingivalis* LPS-induced production of IL-1β and IL-8 by alpha-mangostin involves MAPK signaling in THP-1 cells. However, our experimental design is limited by the expression of two cytokines and three MAPK molecules to assess the anti-inflammatory activity of alpha-mangostin in THP-1 cells.

CONCLUSIONS

These results demonstrate that alpha-mangostin inhibited IL-1β and IL-8 production upon stimulation with *P. gingivalis* LPS in THP-1 cells. These effects were mediated by reducing MAPK activation. This suggests that alpha-mangostin, by reducing inflammatory response in THP-1 cells, may be beneficial in the treatment of periodontal disease.

ACKNOWLEDGEMENTS

The authors would like to thank Associate Professor Suchada Chutimaworapan, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand, for her support. The authors thank Dr. Paiboon Jitprasertwong for kindly providing THP-1 cells. We thank Miss Jirawan Yeesibsan and Miss Niratcha chaisomboon for their technical assistance. We also thank Mr. Arthur Ian Simpson, a former instructor at Naresuan University, for kindly editing the English. This work was supported by the Faculty of Dentistry, Naresuan University (Grant number: R2558C131).

Conflict of interest: The authors declare that they have no conflict of interest.

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