Effects of Luteolin on the Release of Nitric Oxide and Interleukin-6 by Macrophages Stimulated With Lipopolysaccharide From *Prevotella Intermedia*

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Background: Although a range of biologic and pharmacologic activities of luteolin has been reported, little is known about its potential as an agent to treat periodontal disease. In the present study, we investigated whether luteolin could downregulate the production of proinflammatory mediators in murine macrophage-like RAW264.7 cells stimulated with lipopolysaccharide (LPS) from *Prevotella intermedia (Pi)*, a major cause of inflammatory periodontal disease, and we attempted to elucidate the possible mechanisms of action.

Methods: LPS was prepared from lyophilized *Pi* ATCC 25611 cells by the standard hot phenol-water method. Culture supernatants were collected and assayed for nitric oxide (NO) and interleukin (IL)-6. We used real-time polymerase chain reaction to detect inducible NO synthase (iNOS) and IL-6 mRNA expression. iNOS expression, phosphorylation of JNK and p38, IκB-α degradation, nuclear translocation of nuclear factor-kappaB (NF-κB) subunits, and signal transducer and activator of transcription-1 (STAT1) phosphorylation were characterized via immunoblotting. DNA-binding of NF-κB was also analyzed.

Results: Luteolin strongly suppressed the production of NO and IL-6 at both gene transcription and translation levels in *Pi* LPS-activated RAW264.7 cells. Mitogen-activated protein kinase pathways were not involved in the inhibition of *Pi* LPS-induced NO and IL-6 release by luteolin. Luteolin did not reduce NF-κB transcriptional activity at the level of IκB-α degradation. Luteolin blocked NF-κB signaling through inhibition of nuclear translocation and DNA binding activity of NF-κB p50 subunit and suppressed STAT1 signaling.

Conclusions: Although further research is encouraged to clarify the detailed mechanism of action, flavonoid luteolin may contribute to blockade of the host-destructive processes mediated by these two proinflammatory mediators and could have potential use in the treatment of inflammatory periodontal disease. *J Periodontol 2011;82: 1509-1517.*

KEY WORDS

Interleukin-6; lipopolysaccharide; luteolin; nitric oxide; periodontal disease; *Prevotella intermedia*.

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Periodontal disease is a chronic inflammatory process accompanied by destruction of surrounding connective tissue and alveolar bone, and sometimes loss of teeth.[1] Recent evidence suggests that periodontal disease is a potential risk factor for several systemic diseases including cardiovascular disease, diabetes, stroke, and preterm low birth weight.[2,3] Hence, the treatment of periodontal infection contributes to effective prevention and management of these systemic disorders.

With the current understanding of periodontal disease etiology and pathogenesis, it became apparent that host responses to the specific causative bacteria and their metabolic products are a major determinant of disease pathogenesis. Recent work has demonstrated, in addition to bacterial control, that modulation of the host inflammatory response is a plausible therapeutic strategy for periodontal disease.[4,5]

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Prevotella intermedia (Pi) is a major periodontal pathogen dominant in the periodontal pockets of patients with chronic periodontitis.[6,7] This bacterium has also been frequently recovered from subgingival flora in patients with acute necrotizing ulcerative gingivitis[8] and pregnancy gingivitis.[9] Lipopolysaccharides (LPSs) from periodontal pathogens, including *Pi*, stimulate secretion of host inflammatory mediators, such as nitric oxide (NO) and cytokines in immune cells, and thereby initiate the host inflammatory response associated with periodontal disease.[10-13] Therefore, host modulatory agents directed at inhibiting NO and specific cytokines seem to be beneficial in terms of attenuating periodontal disease progression and potentially enhancing therapeutic responses.

Flavonoids, a group of naturally occurring polyphenolic compounds abundant in plants and vegetables, possess a wide spectrum of biologic activities, including antioxidant, anticarcinogenic, antiangiogenic, anti-inflammatory, antiallergic, and antiviral properties.[14,15] Flavonoids can be divided into five subcategories: 1) flavonols, 2) flavones, 3) flavanols, 4) flavanones, and 5) anthocyanidines. Luteolin (3′,4′,5,7-tetrahydroxyflavone) is a flavone found at high concentrations in celery, green pepper, parsley, perilla leaf and seeds, and chamomile.[16]

Although a range of biologic and pharmacologic activities of luteolin have been reported,[16] little is known about its potential anti-inflammatory efficacy in periodontal disease. Therefore, in the present study we investigated the effects of luteolin on the production of inflammatory mediators by macrophages stimulated with LPS from *Pi*, a major cause of inflammatory periodontal disease, and we sought to determine the underlying mechanisms of action.

MATERIALS AND METHODS

Reagents

The following reagents were purchased: luteolin;§ antibodies against inducible NO synthase (iNOS), c-Jun N-terminal kinase (JNK), phospho-JNK, p38, phospho-p38, IκBα, signal transducer and activator of transcription-1 (STAT1), and phospho-STAT1;‖ and antibodies against nuclear factor-kappaB (NF-κB) p65, NF-κB p50, β-actin, and Poly(ADP-ribose) polymerase (PARP)-1.¶

Bacteria and Culture Conditions

Pi ATCC 25611 was used throughout. It was grown anaerobically on the surface of enriched trypticase–soy agar containing 5% (vol/vol) sheep blood, or in general anaerobic medium (GAM) broth# supplemented with 1 μg/mL menadione and 5 μg/mL hemin. Plate-grown cultures were routinely incubated for 4 days and used as the inoculum for liquid growth. Liquid-grown cells were incubated for ≈24 hours, to late exponential growth phase. They were collected by centrifugation at 12,000 × g for 20 minutes at 4°C; washed three times with phosphate buffered saline (PBS, pH 7.2); and lyophilized. Culture purity was assessed by Gram staining and plating on solid medium.

LPS Isolation

LPS was prepared from lyophilized *Pi* cells by the standard hot phenol-water method, as described previously.[11] LPS extract was treated with DNase (25 μg/mL) and RNase (25 μg/mL) in 0.1 M Tris (pH 8.0) at 37°C overnight to remove nucleic acids. Any contaminating protein was then hydrolyzed with proteinase K (50 μg/mL), followed by heating at 60°C for 1 hour and incubating overnight at 37°C. The protein content of the purified LPS, determined by the method of Markwell et al.,[17] was <0.1%. Coomassie blue staining of overloaded sodium dodecyl sulfate (SDS)–polyacrylamide gels did not reveal any visible protein bands in the purified LPS, confirming the purity of the preparation (data not shown).

Cell Cultures

The murine macrophage cell line RAW264.7** was grown in Nunc flasks in Dulbecco's modified Eagle's medium supplemented with 100 U/mL of penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.2% $NaHCO_3$, 1 mM sodium pyruvate, and 10% (vol/vol) heat-inactivated fetal bovine serum in a humidified chamber with 5% CO_2/95% air at 37°C. At confluence, the medium and non-adherent cells were removed and replaced with fresh culture medium. After an additional 24 hours of culture, the cells were harvested by gentle scraping with a rubber policeman, washed three times, and viable cells counted. The cells were seeded into 24-well culture plates at a density of 5×10^5 cells per well and incubated for ≥12 hours to allow them to adhere to the plates. After washing three times with medium, cells were pretreated with various concentrations of luteolin for 1 hour and incubated with *Pi* LPS for 24 hours, after which culture supernatants were collected and assayed for NO and interleukin (IL)-6.

Cytotoxicity Assay

The cellular toxicity of luteolin was assessed by the MTT assay, which is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by mitochondrial dehydrogenases. Cells were pretreated with various concentrations of luteolin for 1 hour and incubated with *Pi* LPS for 24 hours, after which MTT was added to

§ Sigma-Aldrich, St. Louis, MO.
‖ Cell Signaling Technology, Beverly, MA.
¶ Santa Cruz Biotechnology, Santa Cruz, CA.
# Nissui, Tokyo, Japan.
** American Type Culture Collection, Rockville, MD.

the cultures to a final concentration of 0.5 mg/mL. After incubation at 37°C in 5% CO_2 for 2 hours, the supernatant was removed and the cells were solubilized in dimethyl sulfoxide. The extent of reduction of MTT to formazan within the cells was quantified by measuring absorbance at 570 nm with an enzyme-linked immunosorbent assay (ELISA) reader.†† Cell viability is expressed as a percentage of the control value.

Measurement of NO Production

NO production was assayed by measuring the accumulation of the stable oxidative metabolite, nitrite (NO_2^-), in culture supernatants. Briefly, 5×10^5 cells per well were stimulated in 24-well tissue culture plates for 24 hours, and 100 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% phosphoric acid)‡‡ was added to equal volumes of culture supernatants in a 96-well flat-bottomed microtiter plate and left at room temperature for 10 minutes. Optical densities at 540 nm were read with an ELISA reader,§§ and nitrite concentrations were calculated from a standard curve established with serial dilutions of $NaNO_2$‖‖ in culture medium.

Measurement of IL-6 Production

The amount of IL-6 secreted into the culture medium was determined by ELISA using a commercially available kit¶¶ according to protocols recommended by the manufacturer. The sensitivity of the assay was 3.8 pg/mL, according to the manufacturer.

RNA Extraction and Real-Time Polymerase Chain Reaction for iNOS and IL-6 mRNA

Cells were plated in 100-mm tissue culture dishes at a density of 1×10^7 cells per dish and pretreated with various concentrations of luteolin for 1 hour, followed by incubation with *Pi* LPS for 24 hours. After incubation, they were washed twice with PBS and collected by centrifugation. Total RNA was isolated with a kit## according to the manufacturer's instructions. cDNA was prepared from 1 μg of the total RNA using a kit.*** A real-time polymerase chain reaction (PCR) was performed using a real-time PCR detection system††† with specific primers for mouse iNOS and IL-6. As an endogenous control, β-actin primer was used. PCR was conducted‡‡‡ according to the manufacturer's instruction. Thermal cycler conditions were as follows: after denaturing at 98°C for 30 seconds, PCR was performed for 45 cycles, each of which consisted of denaturing at 95°C for 1 second, annealing and extending at 60°C for 5 seconds. The following PCR primers for iNOS (130 bp), IL-6 (162 bp), and β-actin (149 bp) were used: iNOS sense, 5′-GCACCACCCTCCTCGTTCAG-3′ and antisense, 5′-TCCACAACTCGCTCCAAGATTCC-3′; IL-6 sense, 5′-GCCAGAGTCCTTCAGAGAGATACAG-3′ and antisense, 5′-GAATTGGATGGTCTTGGTCCTTAGC-3′; and β-actin sense, 5′-TGAGAGGGAAATCGTGCGTGAC-3′ and antisense, 5′-GCTCGTTGCCAATAGTGATGACC-3′. Each assay was normalized to β-actin mRNA.

Immunoblotting Analysis

Cells were plated in 60-mm tissue culture dishes at a density of 4×10^6 cells per dish and pretreated with various concentrations of luteolin for 1 hour, followed by incubation with *Pi* LPS for the indicated periods of time. To prepare cell lysates, cells were washed three times with ice-cold PBS and lysed by incubating for 30 minutes on ice with 200 μL of lysis buffer (50 mM Tris·Cl [pH 8.0], 150 mM NaCl, 0.002% sodium azide, 0.1% SDS, and 1% ethylphenyl-polyethylene glycol) containing protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 5 mg/mL aprotinin, 5 mg/mL pepstatin A, and 5 mg/mL leupeptin). The cell lysates were centrifuged at 10,000 × g for 10 minutes to remove insoluble material. The nuclear fraction was prepared from cells using a nuclear extract kit§§§ according to the manufacturer's instructions. Briefly, the cells were washed with ice-cold PBS and phosphatase inhibitors, collected with a cell scraper, and harvested by centrifugation. The cell pellet was then resuspended in hypotonic buffer and kept on ice for 15 minutes. The suspension was then mixed with detergent and centrifuged at 14,000 × g for 30 seconds. The nuclear pellet obtained was resuspended in complete lysis buffer in the presence of the protease inhibitor cocktail, incubated for 30 minutes on ice, and centrifuged at 14,000 × g for 10 minutes. The resulting supernatant, corresponding to nuclear fraction, was collected and stored at –80°C until use. Protein concentrations were determined with the bicinchoninic acid protein assay reagents‖‖‖ according to the manufacturer's instructions. The same amount of protein (30 μg) was then subjected to SDS–polyacrylamide gel electrophoresis on 10% to 12% acrylamide gels with 3% stacking gels. The resolved proteins were transferred to a nitrocellulose membrane by electroblotting, and the blots were blocked for 1 hour in PBS with 0.1% polysorbate 20 containing 3% non-fat dry milk, followed by incubation with specific primary antibodies. They were then washed three times for 10 minutes each with PBS with 0.1% polysorbate 20, incubated with horseradish peroxidase–conjugated secondary antibodies at room temperature for 1 hour, and visualized

†† Spectra Max 250 ELISA Reader, Molecular Devices, Sunnyvale, CA.
‡‡ Sigma-Aldrich.
§§ Spectra Max 250 ELISA Reader, Molecular Devices.
‖‖ Sigma-Aldrich.
¶¶ OptEIA, BD Pharmingen, San Diego, CA.
RNeasy Mini Kit, Qiagen, Valencia, CA.
*** iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA.
††† CFX96 real-time PCR detection system, Bio-Rad.
‡‡‡ SsoFast EvaGreen Supermix, Bio-Rad.
§§§ Active Motif, Carlsbad, CA.
‖‖‖ Pierce, Rockford, IL.

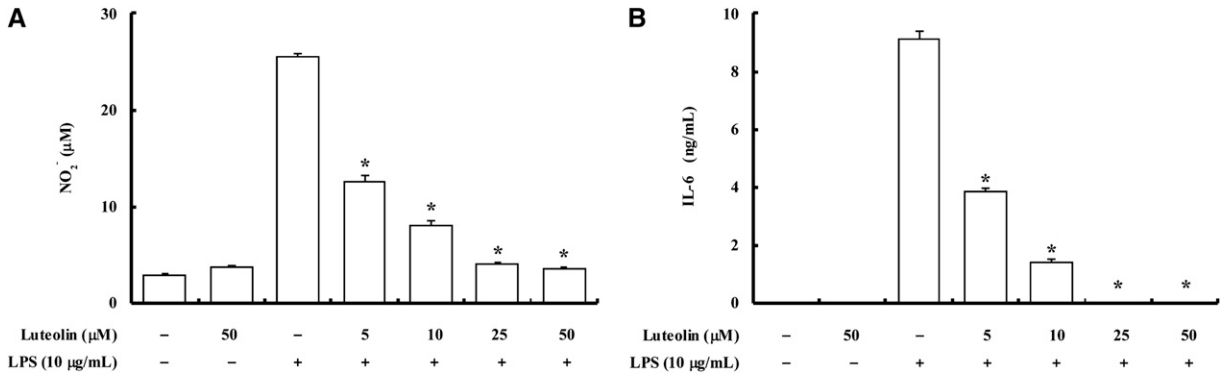

Figure 1.
Effects of luteolin on Pi *LPS-induced production of NO* ***(A)*** *and IL-6* ***(B)*** *in RAW264.7 cells. Cells were pretreated with various concentrations of luteolin (0, 5, 10, 25, and 50 μM) for 1 hour and incubated in the absence or presence of* Pi *LPS (10 μg/mL). Supernatants were removed after 24 hours and assayed for NO and IL-6. The results are means ± SD of three independent experiments.* *P *<0.01 versus* Pi *LPS alone.*

by enhanced chemiluminescence¶¶¶ as recommended. The intensity of each protein-specific band was quantified by densitometer with densitometric software.

DNA-Binding Activity of NF-κB

Cells were plated in 60-mm tissue culture dishes at a density of 4×10^6 cells per dish and pretreated with various concentrations of luteolin for 1 hour, followed by incubation with *Pi* LPS for the indicated periods of time. After extracting the nuclear protein as described previously, the DNA-binding activity of NF-κB in nuclear extract was assayed by using a TransAM NF-κB p65/NF-κB p50 transcription factor assay kits### according to the manufacturer's recommended procedures. Oligonucleotide with the NF-κB consensus binding site (5′-GGGACTTTCC-3′), to which the activated NF-κB contained in nuclear extracts specifically binds, has been immobilized on a 96-strip well plate. The activated NF-κB p65 and p50 specifically bound to this oligonucleotide were detected using specific antibodies to NF-κB p65 and p50, respectively. An ELISA reader**** was used to read the sample absorbance, with results expressed as optical density emitted at 450 nm.

Statistical Analysis

Data are expressed as means ± SD and statistical analysis was performed using Student *t* test with $P < 0.05$ considered statistically significant.

RESULTS

Effects of Luteolin on Pi *LPS-Induced NO and IL-6 Production*

RAW264.7 cells were pretreated with various concentrations of luteolin (0, 5, 10, 25, and 50 μM) for 1 hour and incubated with *Pi* LPS for 24 hours, after which culture supernatants were collected and assayed for NO and IL-6. As shown in Figure 1, *Pi* LPS stimulation led to marked increases of NO and IL-6 levels. Luteolin effectively suppressed the *Pi* LPS-induced production of NO and IL-6, and these effects of luteolin were concentration-dependent. Pretreatment of cells with 50 μM luteolin reduced NO production by 86%. Of note, luteolin completely blocked the IL-6 secretion at a concentration of 25 μM. No notable effects on cell viability were observed when the cells were exposed to up to 50 μM of luteolin as determined by MTT assay (data not shown), indicating that the suppression of NO and IL-6 production could not be attributable to a direct cytotoxic effect by luteolin.

Immunoblot analysis showed that luteolin also reduced *Pi* LPS-induced iNOS protein expression in a concentration-dependent manner, corresponding to 11% inhibition at 5 μM, 31% at 10 μM, 45% at 25 μM, and 100% at 50 μM (Fig. 2). This result indicated that luteolin inhibited the production of NO by reducing iNOS protein expression in LPS-stimulated RAW264.7 cells. Real-time PCR analysis showed that luteolin also reduced *Pi* LPS-induced iNOS and IL-6 mRNA expression in a dose-dependent manner (Fig. 3).

Effects of Luteolin on Pi *LPS-Induced Phosphorylation of JNK and p38*

Our previous study[18] showed that the JNK, p38, NF-κB, and Janus kinases (JAK)2/STAT1 pathways are involved in NO and IL-6 production induced by *Pi* LPS. We then determined whether luteolin inhibited *Pi* LPS-induced NO and IL-6 production by regulation of JNK or p38 pathways activated by LPS. As anticipated, stimulation with *Pi* LPS resulted in the phosphorylation of JNK and p38 (Fig. 4). However, luteolin failed to prevent LPS from activating either JNK or p38 (Fig. 4). These findings suggest that the JNK and p38 pathways

¶¶¶ Cell Signaling Technology.
Active Motif.
**** Spectra Max 250 ELISA Reader, Molecular Devices.

are not involved in the inhibition of *Pi* LPS-induced NO and IL-6 release by luteolin.

Effects of Luteolin on Pi *LPS-Induced NF-κB Activation*

We then investigated whether luteolin inhibited *Pi* LPS-induced production of NO and IL-6 via regulation of NF-κB pathway. To determine whether the inhibitory action of luteolin was caused by its effect on *Pi* LPS-induced degradation of IκB-α, the upstream signaling pathway of NF-κB, the cytoplasmic levels of IκB-α protein were examined by immunoblotting. As shown in Figure 5A, the degradation of IκB-α induced by *Pi* LPS was not inhibited when cells were pretreated with luteolin. We next examined whether luteolin prevents the nuclear translocation of the subunits of NF-κB (i.e., p65 and p50), which immediately occurred downstream IκB-α degradation. Nuclear fractions were isolated and immunoblotted with antibodies against NF-κB p65 and p50. Whereas nuclear translocation of p50 subunit induced with *Pi* LPS was dose-dependently hampered in the presence of luteolin, luteolin did not affect p65 nuclear translocation (Fig. 5B). The treatment with 5, 10, 25, and 50 μM of luteolin reduced 25%, 46%, 59%, and 70%, respectively, of p50 nuclear translocation. Finally, we determined whether luteolin could affect NF-κB-dependent transcription by inhibiting the binding of NF-κB to DNA. The DNA-binding activity of NF-κB in nuclear extract was analyzed by using the ELISA-based NF-κB p65/NF-κB p50 transcription factor assay kits. DNA-binding activities of NF-κB p65 and p50 subunits were markedly increased on exposure to *Pi* LPS (Fig. 5C). Whereas the increased NF-κB p50 binding activity induced by *Pi* LPS was dose-dependently attenuated by pretreatment with luteolin, luteolin did not affect p65 binding activity (Fig. 5C).

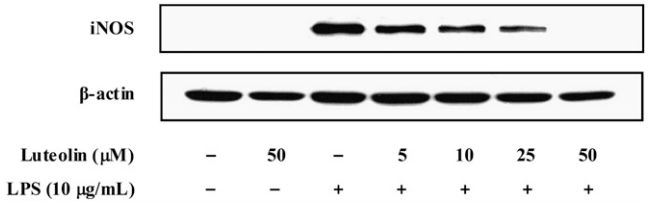

Figure 2.
Effects of luteolin on Pi *LPS-induced expression of iNOS protein in RAW264.7 cells. Cells were pretreated with various concentrations of luteolin (0, 5, 10, 25, and 50 μM) for 1 hour and incubated in the absence or presence of* Pi *LPS (10 μg/mL) for 24 hours. iNOS protein synthesis was measured by immunoblot analysis of cell lysates using iNOS-specific antibody. A representative immunoblot from two separate experiments with similar results is shown.*

Effects of Luteolin on Pi *LPS-Induced Phosphorylation of STAT1*

In addition, we examined whether luteolin regulates *Pi* LPS-induced NO and IL-6 production through inhibiting the STAT1 pathway. Luteolin attenuated *Pi* LPS-induced STAT1 phosphorylation in a dose-dependent manner, corresponding to 21% inhibition at 5 μM, 53% at 10 μM, 99% at 25 μM, and 100% at 50 μM (Fig. 6). This result suggests that luteolin exerts its effects on *Pi* LPS-induced NO and IL-6 production via regulation of the STAT1 pathway.

DISCUSSION

A disequilibrium between oxidative stress and antioxidant activity is associated with the development and progression of periodontitis, and flavonoids may increase the antioxidant activity of saliva and crevicular fluid and help protect against periodontal disease.[19] In the present study, we investigated whether flavonoid luteolin could downregulate the production of inflammatory mediators in macrophages stimulated with

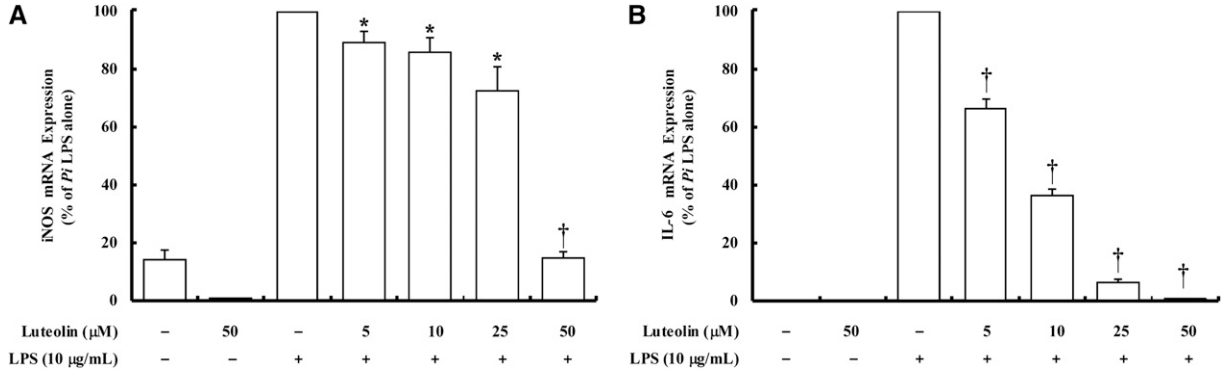

Figure 3.
Effects of luteolin on Pi *LPS-induced iNOS* ***(A)*** *and IL-6* ***(B)*** *mRNA expression in RAW264.7 cells. Cells were pretreated with various concentrations of luteolin (0, 5, 10, 25, and 50 μM) for 1 hour and incubated in the absence or presence of* Pi *LPS (10 μg/mL) for 24 hours. Real-time PCR was performed, β-actin being used as an endogenous control. Data are presented as percentage of* Pi *LPS alone. The results are means ± SD of three independent experiments.* ***** *P <0.05;* † *P <0.01 versus* Pi *LPS alone.*

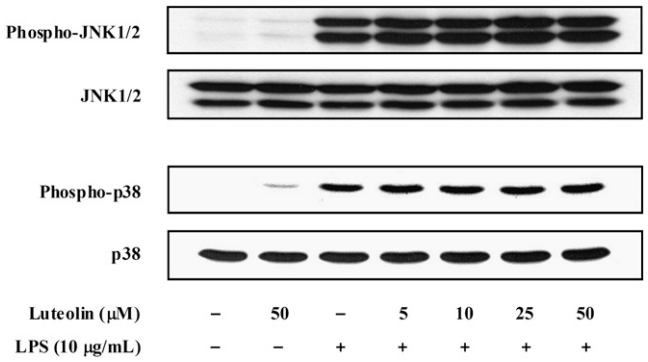

Figure 4.
Effects of luteolin on Pi LPS-induced phosphorylation of JNK and p38 in RAW264.7 cells. Cells were pretreated with various concentrations of luteolin (0, 5, 10, 25, and 50 μM) for 1 hour and incubated in the absence or presence of Pi LPS (10 μg/mL) for 30 minutes (for JNK) or 15 minutes (for p38). Cell lysates were subjected to immunoblot analysis using specific antibodies. A representative immunoblot from two separate experiments with similar results is shown.

LPS from *Pi*, the causative agent of inflammatory periodontal disease, and we attempted to elucidate possible mechanisms of action. LPS is a major component of the outer membrane of Gram-negative bacteria, including *Pi*. It has the ability to trigger a number of host cells, especially mononuclear phagocytes, to produce and release a wide variety of proinflammatory cytokines, including tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8.[20] In addition, LPS can induce significant production of NO in a variety of cell types including macrophages.[21,22] NO has recently received considerable attention as a novel type of mediator;[23] inhibition of NOS activity and NO production frequently limits the progression and severity of experimental inflammatory diseases, such as osteoarthritis, glomerulonephritis, and colitis.[24,25]

LPS preparations extracted from oral black-pigmented bacteria including *Pi* have been reported to possess unique chemical and immunobiologic

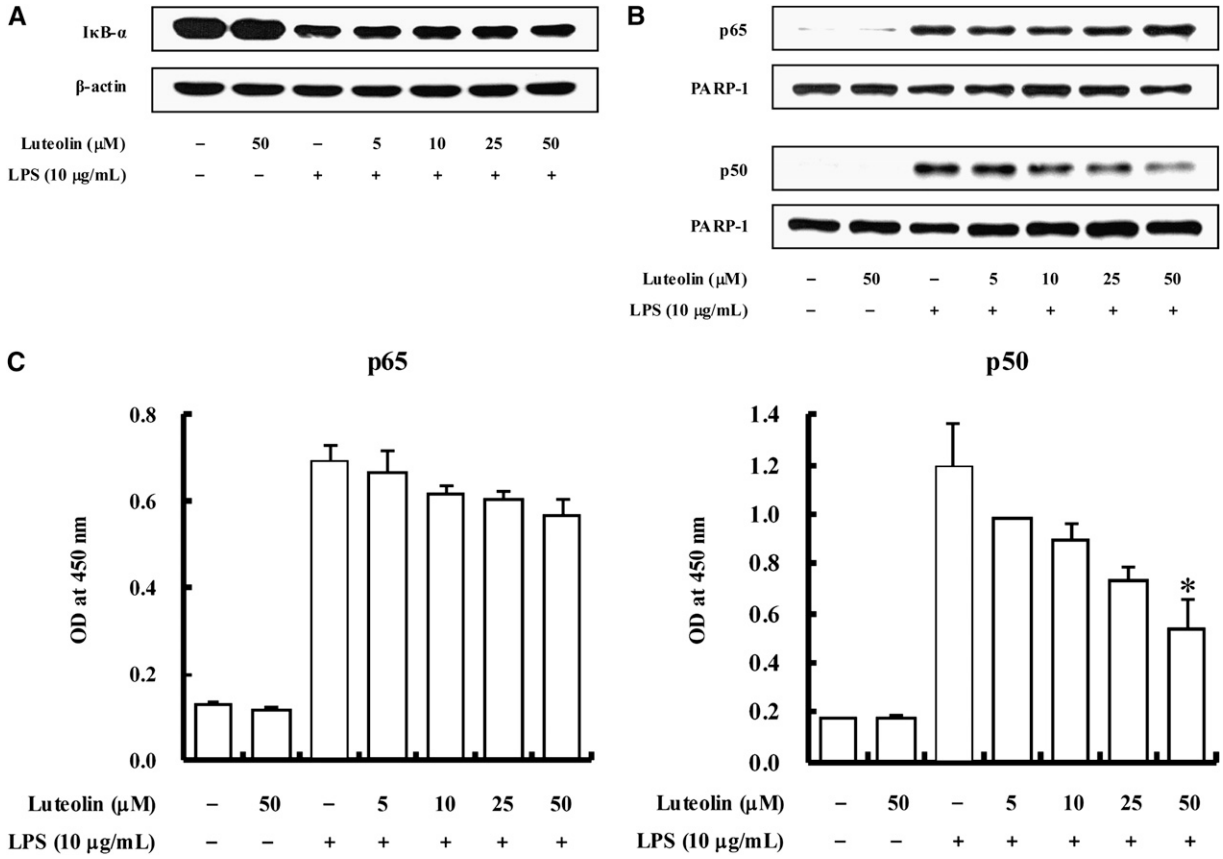

Figure 5.
Effects of luteolin on Pi LPS-induced NF-κB activation in RAW264.7 cells. ***A through C)*** *Cells were pretreated with various concentrations of luteolin (0, 5, 10, 25, and 50 μM) for 1 hour and incubated in the absence or presence of Pi LPS (10 μg/mL). A) After 30 minutes of incubation, IκB-α degradation was determined by immunoblot analysis of cell lysates using antibody against IκB-α. A representative immunoblot from two separate experiments with similar results is shown. B and C) After 30 minutes (for NF-κB p65) or 8 hours (for NF-κB p50) of incubation, the nuclear fraction was isolated from cells. B) Nuclear translocation of NF-κB subunits was assessed by immunoblot analysis using antibodies against NF-κB p65 and p50. A representative immunoblot from two separate experiments with similar results is shown. C) DNA-binding activity of NF-κB in nuclear extracts was assessed by using the ELISA-based NF-κB p65/NF-κB p50 transcription factor assay kits. The results are means ± SD of two independent experiments.* ***** *P <0.05 versus Pi LPS alone. OD = optical density; PARP-1 = poly (ADP-ribose) polymerase.*

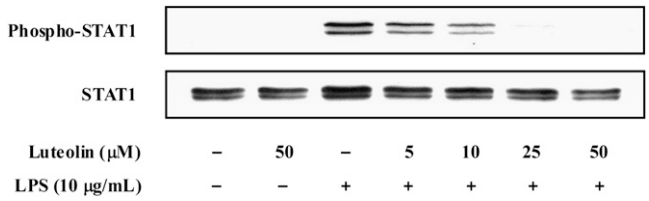

Figure 6.
Effects of luteolin on Pi *LPS-induced phosphorylation of STAT1 in RAW264.7 cells. Cells were pretreated with various concentrations of luteolin (0, 5, 10, 25, and 50 μM) for 1 hour and incubated in the absence or presence of* Pi *LPS (10 μg/mL) for 4 hours. Expression of phospho-STAT1 was measured by immunoblot analysis of cell lysates. Total STAT1 was used as an internal control. A representative immunoblot from two separate experiments with similar results is shown.*

properties quite different from those of the classic LPSs from the family *Enterobacteriaceae*, such as *Escherichia coli* and *Salmonella* species.[26] Kirikae et al.[27] also indicated that the active molecules and mode of action of *Pi* LPS are quite different from those of LPS from *Salmonella*. Hashimoto et al.[28] demonstrated the structure of lipid A from *Pi* ATCC 25611 LPS to be composed of a diglucosamine backbone with a phosphate at the four-position of the nonreducing side sugar and five fatty acids containing branched long chains. They also found that the lipid A activates murine cells through a Toll-like receptor 4–mediated signaling pathway.

It is well known that LPSs from periodontal pathogens induce excess production of inflammatory mediators, such as NO, IL-6, and TNF-α, in immune cells. These proinflammatory mediators are potential targets for the development of new therapeutic approaches to the treatment of periodontitis because these molecules play a significant role in the development of periodontal destruction. LPS signals in macrophages can be blocked by flavonoids, and hence flavonoid luteolin may have potential use in the treatment of periodontal disease.

The results of the present study indicate that luteolin could suppress the production of NO and IL-6 at both gene transcription and translation levels in *Pi* LPS-activated RAW264.7 cells. NO is thought to have an important role in the pathogenesis of inflammatory periodontal disease as it does in other inflammatory diseases. Enhanced production of NO has been demonstrated in periodontal disease,[29] and gingival tissues from patients with chronic periodontitis have higher levels of iNOS protein and mRNA than healthy tissue.[30-33] Macrophages, polymorphonuclear cells, and fibroblasts are the sources of iNOS in periodontal tissues, with endothelial cells also contributing.[30-33] Moreover, LPS from *Aggregatibacter actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*), a major pathogen of aggressive periodontitis, induced significant production of NO in macrophages,[10] and LPSs from *Pi* and *Prevotella nigrescens*, the causative agents of inflammatory periodontal disease, fully induced iNOS expression and NO production in the murine macrophage cell line (RAW264.7) in the absence of other stimuli.[11,12] However, the exact mechanism by which NO regulates the pathogenesis of both periodontitis and subsequent bone loss remains unclear. Excessive amounts of NO, produced by iNOS-expressing cells in periodontal tissues, may lead to activation of matrix metalloproteinases, potentiating matrix degradation.[34] NO is also thought to downregulate the production of tissue inhibitors of matrix metalloproteinases.[34] Additionally, IL-6 is also important in the pathogenesis of periodontal disease. Clinically, IL-6 levels in sites with periodontal disease are higher than those in healthy sites and closely related to the severity of periodontal disease.[35,36] Moreover, it has been well demonstrated that IL-6 is a potent bone resorptive agent, induces osteoclastogenesis, and hence plays an important role in alveolar bone resorption in periodontal disease.[37,38] Blockade of NO and IL-6, therefore, could be a highly efficient tool for blocking the development and progression of inflammatory periodontal disease.

It is generally accepted that multiple signal transduction pathways participate in LPS-induced activation of macrophages and resultant production of proinflammatory mediators, and mitogen-activated protein kinase (MAPK) and NF-κB pathways play critical roles. However, the results of this study suggest that MAPK pathways are not involved in the inhibition of *Pi* LPS-induced NO and IL-6 release by luteolin. NF-κB is a transcription factor that plays a critical role in the expression of proinflammatory cytokines and other mediators.[39-41] NF-κB is comprised of homodimers or heterodimers of five different Rel proteins: 1) p65 (RelA); 2) p50 (NF-κB1); 3) p52 (NF-κB2); 4) c-Rel; and 5) RelB. In unstimulated cells, NF-κB is present in the cytoplasm in an inactive form bound to the inhibitory κB (IκB) proteins. IκB becomes phosphorylated, ubiquitinated, and then degraded on stimulation with a broad range of stimuli, including LPS. Then, the activated NF-κB dimers are translocated into the nucleus, bind to κB-binding sites in the promoter regions of target genes, and induce the transcription of various proinflammatory mediators including iNOS and IL-6.[42,43] Luteolin did not inhibit NF-κB transcriptional activity at the level of IκB-α degradation. Luteolin blocked NF-κB signaling through inhibition of nuclear translocation and DNA binding activity of NF-κB p50 subunit induced with *Pi* LPS. Both NF-κB p65 and p50 accumulate in the nucleus in a variety of cell types in response to LPS. Although NF-κB p65, RelB, and c-Rel have transactivation domains and directly promote gene

transcription, p50 lacks such a domain and does not directly stimulate gene transcription.[44] Therefore, p50 usually forms a heterodimer with other NF-κB subunits and participates in target gene transcription.[43,45] NF-κB has been considered as a potential target molecule for the treatment of inflammatory diseases, and its inhibition by luteolin would be useful in the treatment of periodontal disease.

The STAT signaling pathway plays an essential role in the regulation of inflammatory responses.[46] Seven STAT family members are identified in mammals, and each one binds to a different DNA sequence.[47] Different STATs form homodimers or heterodimers. The STAT family of transcription factors is activated by JAKs.[48,49] Activated STAT dimers translocate into the nucleus and induce the transcription of their target genes. STAT1, downstream of JAK2, seems to be an important transcription factor for LPS-induced gene expressions in macrophages.[50] In this study, luteolin exerted its effects on the inhibition of *Pi* LPS-induced NO and IL-6 production via regulation of the STAT1 pathway. Thus, the STAT1 signaling pathway could be an attractive molecular target for treating inflammatory periodontal disease.

CONCLUSIONS

The present study shows for the first time that luteolin could strongly suppress NO and IL-6 production induced by LPS from *Pi*, a major cause of inflammatory periodontal disease, in macrophages. The underlying mechanisms of luteolin involve the inhibition of NF-κB and STAT1 pathways in LPS-stimulated macrophages. Although further research is required to clarify the detailed mechanism of action, we conclude that luteolin may contribute to blockade of the host-destructive processes mediated by these two proinflammatory mediators, and could be a highly efficient modulator of host response in the treatment of inflammatory periodontal disease. Further in vivo studies are required to evaluate better the potential of luteolin as a therapeutic agent to treat periodontal disease.

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