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# Anti-inflammatory effects and the underlying mechanisms of action of daidzein in murine macrophages stimulated with *Prevotella intermedia* lipopolysaccharide

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*Background and Objective:* Host modulatory agents directed at inhibiting specific proinflammatory mediators could be beneficial in terms of attenuating periodontal disease progression and potentially enhancing therapeutic responses. The aim of this study was to investigate whether daidzein could modulate the production inflammatory mediators in macrophages stimulated with lipopolysaccharide (LPS) from *Prevotella intermedia*, a pathogen implicated in periodontal disease, and to delineate underlying mechanisms of action.

*Material and Methods:* LPS was extracted from *P. intermedia* ATCC 25611 cells by the standard hot phenol–water method. The amounts of nitric oxide (NO) and interleukin-6 (IL-6) secreted into the culture medium were assayed. A real-time PCR was performed to quantify inducible nitric oxide synthase (iNOS) and IL-6 mRNA expression. We used immunoblot analysis to characterize iNOS protein expression, phosphrylation of c-Jun N-terminal kinase (JNK) and p38, degradation of inhibitory κB-α (IκB-α), nuclear translocation of nuclear factor-κB (NF-κB) subunits and phosphorylation of signal transducer and activator of transcription 1 (STAT1). The DNA-binding activity of NF-κB was assessed by using ELISA-based kits.

*Results:* Daidzein significantly inhibited the production of NO and IL-6, as well as their mRNA expression, in *P. intermedia* LPS-treated RAW264.7 cells. The JNK and p38 pathways were not involved in the regulation of LPS-induced NO and IL-6 release by daidzein. Daidzein inhibited the degradation of IκB-α induced by *P. intermedia* LPS. In addition, daidzein suppressed NF-κB transcriptional activity via regulation of the nuclear translocation and DNA-binding activity of NF-κB p50 subunit and blocked STAT1 phosphorylation.

*Conclusion:* Although additional studies are required to dissect the molecular mechanism of action, our results suggest that daidzein could be a promising agent for treating inflammatory periodontal disease. Further research in animal models of periodontitis is necessary to better evaluate the potential of daidzein as a novel therapeutic agent to treat periodontal disease.

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With the current advances in the understanding of the initiation and progression of periodontal disease, it is well established that host immune and inflammatory responses to the specific periodontopathogens and their metabolic products mediate local tissue destruction observed in periodontitis. Thus, the use of host modulatory regimens as an adjunctive therapy may be a novel treatment approach for managing periodontal disease (1,2).

Natural compounds, such as flavonoids, may be useful for the prevention and treatment of inflammatory periodontal diseases. Flavonoids are a group of naturally occurring polyphenolic compounds abundant in plants, including vegetables. Flavonoids are known for a variety of biological activities, and some flavonoids have been reported to have anti-inflammatory properties (3,4).

Lipopolysaccharide (LPS) is an outer membrane constituent of gram-negative bacteria, including *P. intermedia*. It stimulates the production of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 in a number of host cells (5). In addition, LPS is a potent inducer of nitric oxide (NO) in a variety of cell types, including macrophages (6,7).

LPSs derived from oral black-pigmented bacteria, including *Porphyromonas gingivalis* and *Prevotella intermedia*, possess chemical and immunobiological properties quite different from those of the classical LPSs from the family *Enterobacteriaceae*, such as *Escherichia coli* and *Salmonella* species (8). Kirikae *et al*. (9) indicated that the active molecule(s) and mode of action of LPS from *P. gingivalis* and *P. intermedia* are quite different from those of LPS from *Salmonella*. It was reported that *P. intermedia* and *P. gingivalis* LPS activate the lymphocytes and macrophages of classical LPS-nonresponsive C3H/HeJ mice (9). Polymyxin B destroys the biological activity of LPS and lipid A isolated from *Enterobacteriaceae*. In contrast, LPS preparations from oral black-pigmented bacteria were comparatively resistant to polymyxin B treatment (9). Hashimoto *et al*. (10) demonstrated the structure of lipid A from *P. intermedia* ATCC 25611 LPS to be composed of a diglucosamine backbone with a phosphate at the 4-position of the nonreducing side sugar, as well as five fatty acids containing branched long chains.

*P. intermedia* is a pathogen that is prevalent in chronic periodontitis (11,12). *P. intermedia sensu lato* has also been implicated in the etiology of necrotizing ulcerative gingivitis (13). Gursoy *et al*. (14) suggested that among the *P. intermedia* group bacteria, *P. intermedia* and *P. nigrescens* type strains can adhere to and invade epithelial cells, the capability of *P. intermedia* ATCC 25611 being highest in this context.

Studies have shown that LPSs derived from periodontal pathogens can activate immune cells to induce production of molecules that act as proinflammatory mediators, such as NO and specific cytokines, and thereby initiate the host inflammatory response associated with periodontal disease (15–18). Therefore, host modulatory agents targeting the components involved in LPS mechanisms of action appear to be helpful in attenuating periodontal disease.

We have previously shown that the flavonoid luteolin strongly suppresses the production of inflammatory mediators in *P. intermedia* LPS-activated macrophages (19). The present study is a continuation of our ongoing work to identify flavonoids that may be beneficial to individuals with periodontal disease. Isoflavones, a class of phytoestrogens, are flavonoid compounds that have been shown to have benefits in the prevention of various chronic diseases, such as atherosclerosis, neurodegenerative diseases and osteoporosis (20–22).

Daidzein is an isoflavone found at high concentrations in soybeans, kudzu root and red clover (23). A large body of evidence supports therapeutic properties of daidzein. As far as we know, however, there is no report presenting the potential of daidzein as a therapeutic agent for treating periodontal disease. These findings prompted us to investigate the effects of daidzein on the production of inflammatory mediators by macrophages stimulated with LPS from *P. intermedia*, a pathogen implicated in inflammatory periodontal disease, and to determine the underlying mechanisms of action.

## Material and methods

### Reagents

Daidzein, DNase, RNase and proteinase K were obtained from Sigma-Aldrich (St.Louis, MO, USA). Antibodies against inducible nitric oxide synthase (iNOS), c-Jun N-terminal kinase (JNK), phospho-JNK, p38, phospho-p38, inhibitory κB-α (IκB-α), signal transducer and activator of transcription 1 (STAT1) and phospho-STAT1 were obtained from Cell Signaling Technology (Beverly, MA, USA), while antibodies against nuclear factor-κB (NF-κB) p65, NF-κB p50, β-actin and poly (ADP-ribose) polymerase-1 (PARP-1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### Bacteria, culture conditions and isolation of LPS

*P. intermedia* ATCC 25611 was used in this study. It was grown in an anaerobic chamber on the surface of enriched Trypticase soy agar containing 5% (v/v) sheep blood, or in general anaerobic medium broth (Nissui, Tokyo, Japan) supplemented with 1 μg/mL menadione and 5 μg/mL hemin as previously described (24). Culture purity was assessed by Gram staining and plating on solid medium. LPS from this periodontopathogen was prepared by the standard hot phenol–water method as described in our previous work (24). Nucleic acids were removed by treating the LPS suspension with DNase and RNase. Any contaminating protein was then hydrolyzed with proteinase K.

### Cell culture and cytotoxicity assay

RAW264.7 murine macrophages obtained from American Type Culture Collection (Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin) at

37°C in a humidified incubator with 5% $CO_2$/95% air. The cellular toxicity of daidzein was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (24).

### Measurement of NO and IL-6 production

RAW264.7 cells were seeded into 24-well culture plates at a density of $5 \times 10^5$ cells per well and incubated for at least 12 h to allow them to adhere to the plates. Cells were then treated with various concentrations of *P. intermedia* LPS and daidzein for 24 h, after which culture supernatants were harvested. The amounts of NO and IL-6 secreted into the culture medium were determined as we have reported (24). Briefly, the amount of NO was assayed by measuring the accumulation of the stable oxidative metabolite, nitrite ($NO_2^-$) (25). IL-6 production was determined by ELISA using a commercially available kit (OptEIA; BD Pharmingen, San Diego, CA, USA), following the manufacturer's instructions.

### Extraction of RNA and real-time PCR for iNOS and IL-6 mRNA

The experiment was conducted as described in our previous work (24). In brief, cells were plated in 100 mm tissue culture dishes at a density of $1 \times 10^7$ cells per dish and treated with various concentrations of *P. intermedia* LPS and daidzein for 24 h. Total RNA was isolated with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Complementary DNA was prepared from 1 μg of the total RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). A real-time PCR was performed using the CFX96 real-time PCR detection system (Bio-Rad) with specific primers for mouse iNOS and IL-6 as described previously (24).

### Preparation of cell extracts and immunoblotting analysis

Cells were plated in 60 mm tissue culture dishes, at a density of $4 \times 10^6$ cells per dish, and treated with various concentrations of *P. intermedia* LPS and daidzein for the indicated periods of time. Whole-cell lysates and nuclear fractions were prepared and analyzed according to our previous paper (24). Briefly, the cell lysates were prepared using lysis buffer containing 50 mM Tris–Cl, 150 mM NaCl, 0.002% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40 and protease inhibitor cocktail. The nuclear fraction was prepared from cells using the nuclear extract kit (Active Motif, Carlsbad, CA, USA). The same amount of protein (30 μg) was then subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to standard protocols. The resolved proteins were electrotransferred to a nitrocellulose membrane, followed by incubation with specific primary antibodies. They were then incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence (Cell Signaling Technology).

### Determination of NF-κB DNA-binding activity

Cells were plated in 60 mm tissue culture dishes, at a density of $4 \times 10^6$ cells per dish, and incubated with daidzein (200 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL) for the indicated periods of time. After preparing the nuclear protein as described above, the DNA-binding activity of

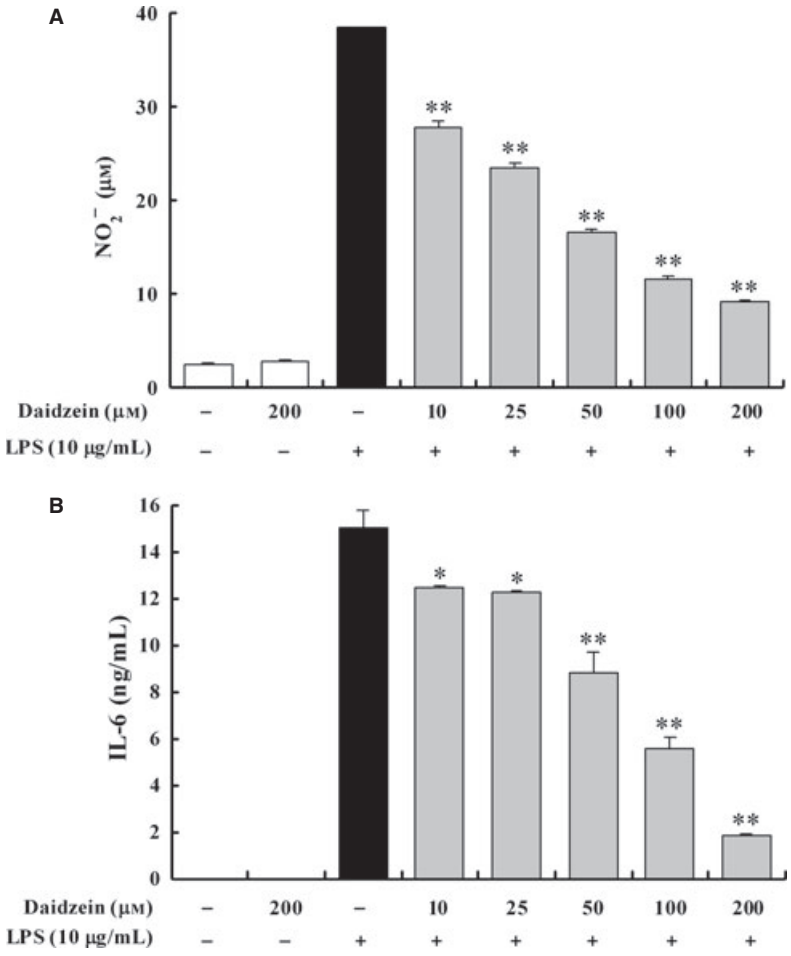


*Fig. 1.* Effects of daidzein on *Prevotella intermedia* lipopolysaccharide (LPS)-induced production of nitric oxide (NO; A) and interleukin-6 (IL-6; B) in RAW264.7 cells. Cells were incubated with different doses of daidzein (0, 10, 25, 50, 100 and 200 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL). Supernatants were removed after 24 h and assayed for NO and IL-6. The results are shown as means ± SD of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ vs. *P. intermedia* LPS alone.

NF-κB was measured using a TransAM NF-κB p65/NF-κB p50 transcription factor assay kits (Active Motif) as previously described (24).

### Statistical analysis

Data are expressed as means ± SD, and statistical analysis was performed using Student's *t*-test, with $p < 0.05$ considered statistically significant.

## Results

### Daidzein downregulates *P. intermedia* LPS-induced production of NO and IL-6

To evaluate the effects of daidzein on *P. intermedia* LPS-induced release of proinflammatory mediators, we treated RAW264.7 cells with different doses of daidzein in the absence or presence of *P. intermedia* LPS (10 μg/mL) for 24 h, and the levels of NO and IL-6 secreted into the culture medium were assayed. The results showed that LPS stimulation of the cells induced significant increases of NO and IL-6 compared with the control cultures (Fig. 1). Incubation of cells with the indicated concentrations of daidzein attenuated NO and IL-6 release induced by *P. intermedia* LPS treatment in a concentration-dependent manner (Fig. 1). At the highest concentration tested (200 μM), daidzein suppressed NO and IL-6 generation by 76 and 87%, respectively. In addition, daidzein did not influence cell viability as evaluated by MTT assay (data not shown), indicating that its inhibitory effects were unrelated to a direct cytotoxicity.

Immunoblot analysis was performed to determine whether the inhibition of NO by daidzein was related to changes in iNOS protein expression. As shown in Fig. 2, daidzein reduced *P. intermedia* LPS-induced iNOS expression in a dose-dependent manner, and complete abolition of expression was observed at 200 μM. Real-time PCR analysis showed that daidzein also inhibited *P. intermedia* LPS-induced iNOS and IL-6 mRNA expression in a concentration-dependent manner (Fig. 3).

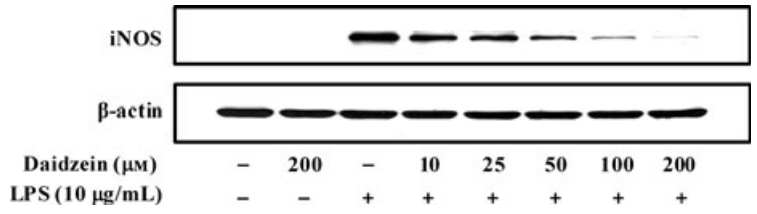


*Fig. 2.* Effects of daidzein on *P. intermedia* LPS-induced expression of inducible nitric oxide synthase (iNOS) protein in RAW264.7 cells. Cells were incubated with different doses of daidzein (0, 10, 25, 50, 100 and 200 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL) for 24 h. The 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.

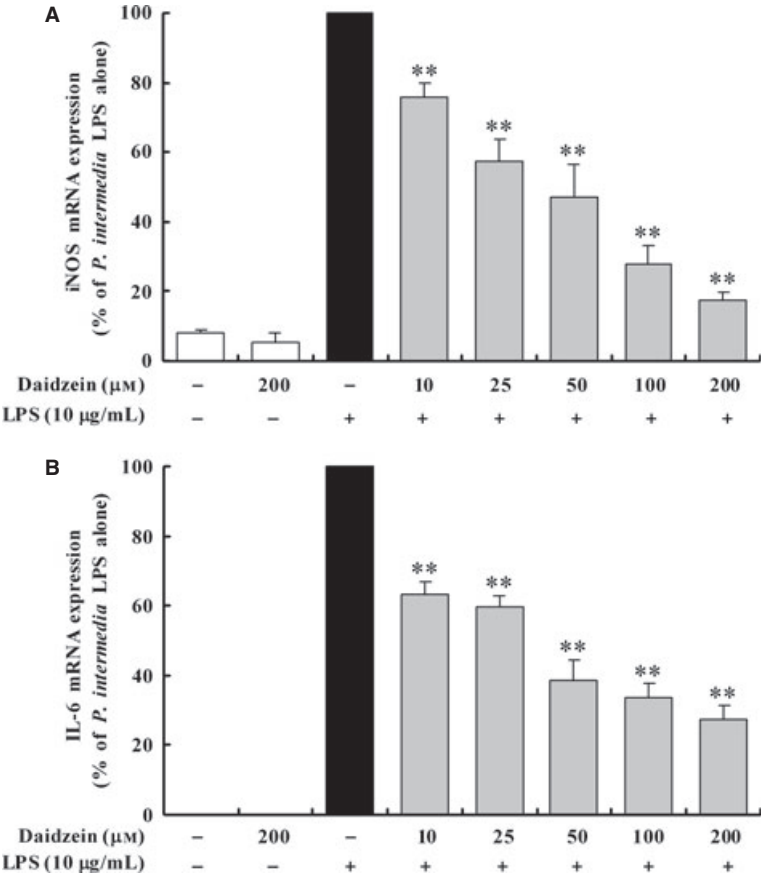


*Fig. 3.* Effects of daidzein on *P. intermedia* LPS-induced iNOS (A) and IL-6 mRNA expression (B) in RAW264.7 cells. Cells were incubated with different doses of daidzein (0, 10, 25, 50, 100 and 200 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL) for 24 h. Real-time PCR was performed with EvaGreen Supermix, with β-actin as an endogenous control. Data are presented as percentages of the value for *P. intermedia* LPS alone. The results are shown as means ± SD of three independent experiments. $^{**}p < 0.01$ vs. *P. intermedia* LPS alone.

### JNK and p38 pathways are not involved in the regulation of *P. intermedia* LPS-induced NO and IL-6 release by daidzein

Our previous study (24) showed that the JNK, p38, NF-κB and JAK2/STAT1 pathways are involved in NO and IL-6 release induced by *P. intermedia* LPS (Fig. S1). We first tested whether the JNK and p38 pathways lead to the effects of daidzein on regulating *P. intermedia* LPS-induced NO

and IL-6 release. Stimulation with *P. intermedia* LPS resulted in the phosphorylation of JNK and p38 (Fig. 4). However, daidzein failed to prevent LPS from activating either JNK or p38 (Fig. 4).

### Daidzein inhibits *P. intermedia* LPS-induced degradation of IκB-α

Next, we examined the effects of daidzein on the NF-κB signaling pathway, which mediates *P. intermedia* LPS-induced NO and IL-6 production. To test whether daidzein affects the *P. intermedia* LPS-induced degradation of IκB-α, the upstream signaling pathway of NF-κB, the cytoplasmic levels of IκB-α protein were determined by immunoblot analysis. The 30 min treatment with LPS was determined to be optimal in a preliminary study that examined IκB-α degradation (data not shown). As expected, treatment with *P. intermedia* LPS resulted in marked degradation of IκB-α (Fig. 5). Daidzein inhibited the degradation of IκB-α induced by *P. intermedia* LPS (Fig. 5).

### Daidzein inhibits nuclear translocation and DNA-binding activity of NF-κB p50 subunit

NF-κB translocates from the cytosol to the nucleus when it is activated. We next investigated the nuclear translocation of the subunits of NF-κB, i.e. p65 and p50, which is immediately occurred downstream of IκB-α degradation. Nuclear fractions were prepared and immunoblotted with antibodies against NF-κB p65 and p50. PARP-1 was used as an internal control. *P. intermedia* LPS significantly induced the nuclear translocation of NF-κB subunits (Fig. 6A). The maximal effects were found 30 min (for NF-κB p65) or 8 h (for NF-κB p50) after LPS addition (data not shown), and those time points were chosen for subsequent studies. While p50 nuclear translocation induced by LPS was attenuated by treatment with daidzein in a dose-dependent manner, daidzein did not affect nuclear translocation of p65 subunit (Fig. 6A). Finally, we examined the DNA-binding activity of

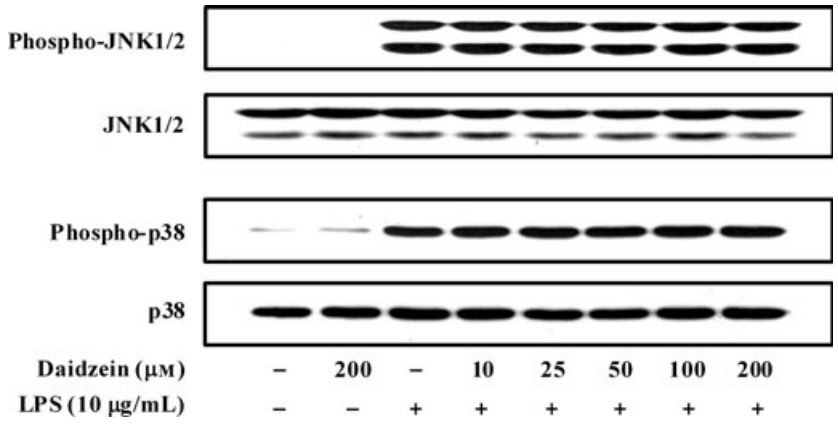


*Fig. 4.* Effects of daidzein on *P. intermedia* LPS-induced phosphrylation of c-Jun N-terminal kinase (JNK) and p38 in RAW264.7 cells. Cells were incubated with different doses of daidzein (0, 10, 25, 50, 100 and 200 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL) for 30 min (for JNK) or 15 min (for p38). Cells lysates were subjected to immunoblot analysis using specific antibodies. A representative immunoblot from two separate experiments with similar results is shown.

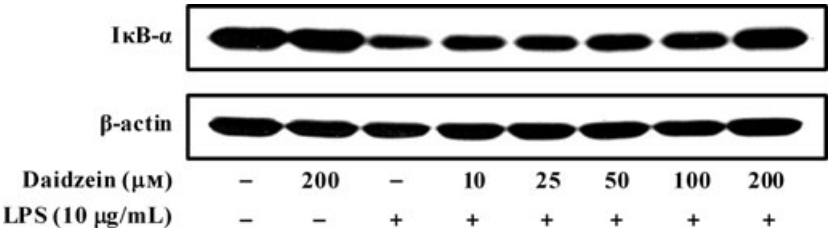


*Fig. 5.* Effects of daidzein on *P. intermedia* LPS-induced degradation of the inhibitory κB-α (IκB-α) in RAW264.7 cells. Cells were incubated with different doses of daidzein (0, 10, 25, 50, 100 and 200 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL). After 30 min of incubation, IκB-α 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.

NF-κB in nuclear extract by using the ELISA-based NF-κB p65/NF-κB p50 transcription factor assay kits (Active Motif). The maximal DNA-binding activities were found 30 min (for NF-κB p65) or 8 h (for NF-κB p50) after LPS addition (data not shown). Stimulation with *P. intermedia* LPS strongly induced DNA binding of NF-κB p65 and p50, whereas only the DNA-binding activity of NF-κB p50 was significantly attenuated by treatment with daidzein (Fig. 6B).

### Daidzein inhibits STAT1 phosphorylation

As the JAK2/STAT1 pathway is involved in *P. intermedia* LPS-mediated NO and IL-6 production, we next examined whether daidzein exerts its effects on *P. intermedia* LPS-induced NO and IL-6 production via regulation of this signaling pathway. Cell lysates were prepared and immunoblotted with antibodies against STAT1 and phospho-STAT1. Daidzein significantly inhibited STAT1 phosphorylation induced by *P. intermedia* LPS (Fig. 7).

## Discussion

Periodontal disease is a chronic inflammatory process that leads to the destruction of gingival connective tissue and alveolar bone, and eventually causes loss of teeth (26). Although periodontal disease is a local disorder, its chronic inflammatory changes could have the potential to affect organs distant from the periodontium. There is evidence indicating that periodontal

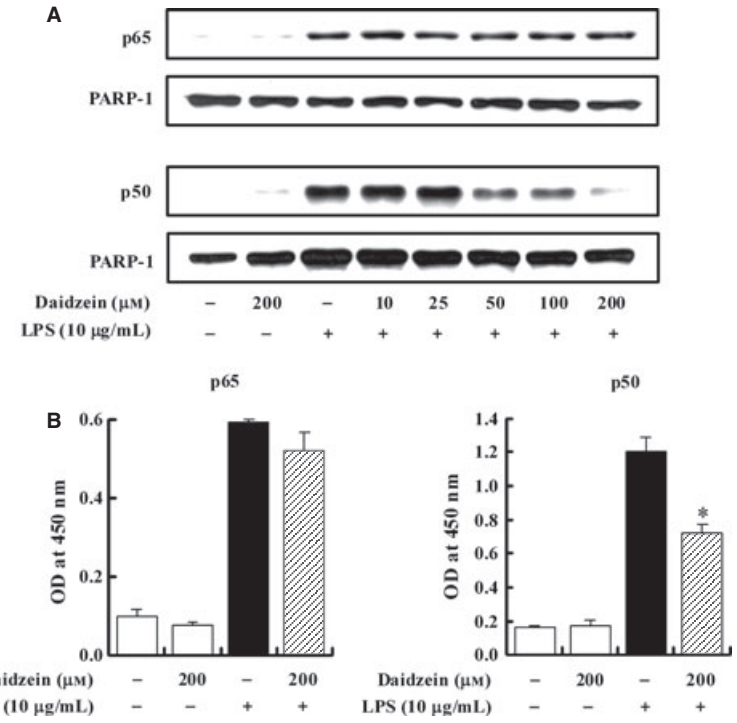


*Fig. 6.* Effects of daidzein on nuclear translocation (A) and DNA-binding activity (B) of nuclear factor-κB (NF-κB) subunits induced with *P. intermedia* LPS in RAW264.7 cells. (A) Cells were incubated with different doses of daidzein (0, 10, 25, 50, 100 and 200 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL). After 30 min (for NF-κB p65) or 8 h (for NF-κB p50) of incubation, the nuclear fraction was isolated from cells. Nuclear translocation of NF-κB subunits was assessed by immunoblot analysis using antibodies against NF-κB p65 and p50. Poly (ADP-ribose) polymerase-1 (PARP-1) was used as an internal control. A representative immunoblot from two separate experiments with similar results is shown. (B) Cells were incubated with different doses of daidzein (0 and 200 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL). After 30 min (for NF-κB p65) or 8 h (for NF-κB p50) of incubation, the 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 shown as means ± SD of two independent experiments. $*p < 0.05$ vs. *P. intermedia* LPS alone.

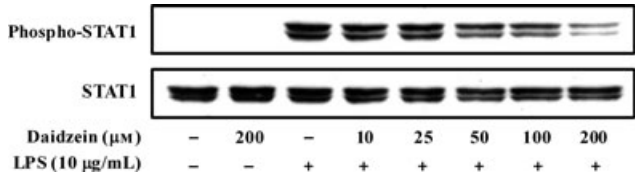


*Fig. 7.* Effects of daidzein on *P. intermedia* LPS-induced phosphorylation of signal transducer and activator of transcription 1 (STAT1) in RAW264.7 cells. Cells were incubated with different doses of daidzein (0, 10, 25, 50, 100 and 200 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL) for 4 h. 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.

disease may represent a significant risk factor for diverse systemic conditions, including cardiovascular disease, diabetes, stroke and preterm low-birth-weight infants (27–29); therefore, the control of periodontal disease is essential in the prevention and management of these systemic diseases.

Our findings clearly indicate that daidzein significantly inhibits the production of NO and IL-6, as well as their mRNA expressions, in LPS-treated RAW264.7 cells, suggesting that it downregulated these proinflammatory mediators at both protein and gene transcription levels. NO and IL-6 are thought to have an important role in the pathogenesis of inflammatory periodontal disease (30–36). In addition, IL-6 is a potent bone resorptive agent and induces osteoclastogenesis; hence, it plays important roles in alveolar bone resorption in periodontal disease (37,38). Blockade of these two proinflammatory mediators, therefore, could be a plausible therapeutic strategy for inflammatory periodontal disease.

It has been reported that daidzein inhibits production of TNF-α, as well as NO and IL-6, in macrophages activated by classical enterobacterial LPS (39–42). In our study, daidzein did not suppress the *P. intermedia* LPS-induced production of TNF-α (data not shown). These results indicate that, unlike enteric LPS, TNF-α induction by *P. intermedia* LPS is mainly via signaling pathways not affected by daidzein. *P. intermedia* LPS has been shown to differ from LPS derived from *Enterobacteriaceae* in structure and function (9,10); therefore, the Toll-like receptors and the inflammatory signaling pathways are different accordingly. The effects of daidzein are also thought to be dose dependent, because in some cases very low doses of daidzein upregulated enteric LPS-induced NO production and iNOS activation (43).

MAPK and NF-κB signal transduction pathways play critical roles in LPS-induced activation of macrophages and the resultant production of proinflammatory mediators. However, daidzein did not affect either JNK or p38, suggesting that MAPK pathways are not involved in the inhibition of *P. intermedia* LPS-induced NO and IL-6 production by daidzein. NF-κB is one of the most important transcription factors that controls a number of inflammation-related genes (44–46). In unstimulated conditions, NF-κB is maintained in the cytoplasm in a latent form bound to IκB proteins. The IκB

becomes phosphorylated and degraded in response to a broad range of stimuli, including LPS. The free NF-κB is then translocated into the nucleus, where it activates the transcription of various proinflammatory enzymes, such as iNOS and cyclooxygenase-2, and cytokines (47,48). In this study, daidzein inhibited NF-κB transcriptional activity at the level of IκB-α degradation. In addition, daidzein suppressed NF-κB transcriptional activity via regulation of the nuclear translocation and DNA-binding activity of NF-κB p50 subunit. In contrast, previous research by Hämäläinen *et al*. (41) has shown that daidzein effectively inhibits nuclear translocation of p65 subunit along with its inhibitory effect on iNOS expression and NO production in murine J774 macrophages activated by enterobacterial LPS. The reason for the discrepancy between our finding and that of Hämäläinen *et al*. (41) is not clear. The difference in the cell line used is perhaps a factor.

Upon stimulation by LPS, both NF-κB p65 and p50 translocate into the nucleus, where they bind to specific regulated sequences in the DNA to induce transcription. NF-κB p65 has a transcriptional activation domain and directly promotes gene transcription, whereas p50 lacks such a domain and does not directly stimulate gene transcription (49); therefore, p50 usually forms a heterodimer with other NF-κB subunits to become transcriptionally active (48,50). NF-κB has been considered as a potential target molecule for the treatment of inflammatory diseases, and its inhibition by daidzein would be beneficial in the treatment of periodontal disease.

Another transcription factor involved in the regulation of inflammatory responses is the STAT (51). The STAT family of transcription factors are activated through the Janus kinases (JAKs) (52,53). STAT1, downstream of JAK2, is an essential activator of LPS-mediated gene transcription in macrophages (54). In this study, daidzein exerted its effects on *P. intermedia* LPS-induced NO and IL-6 production through inhibition of the STAT1 pathway. Thus, the STAT1 signaling pathway is also considered a good potential molecular target for the therapy of inflammatory periodontal disease. Daidzein also inhibited STAT1 activation, along with its inhibitory effect on iNOS expression and NO production in macrophages activated by enterobacterial LPS (41).

In summary, we clearly demonstrated, to our knowledge for the first time, that daidzein could significantly downregulate *P. intermedia* LPS-induced NO and IL-6 production, as well as their mRNA expression. We also provided evidence that the mechanism of the inhibitory effect of daidzein is mediated by NF-κB and STAT1 pathways. Although additional studies to dissect the molecular mechanism of action are required, our results suggest that daidzein could be a promising agent for treating inflammatory periodontal disease. Further research in animal models of periodontitis is necessary to better evaluate the potential of daidzein as a novel therapeutic agent to treat periodontal disease.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Involvement of MAPKs, NF-κB and JAK2/STAT1 pathways in *Prevotella intermedia* LPS-induced production of NO (A) and IL-6 (B) in RAW264.7 cells. Cells were pretreated with various kinase inhibitors for 1 h or 30 min, and then stimulated with *P. intermedia* LPS (10 μg/mL) for 24 h. Supernatants were removed and assayed for NO and IL-6. Data are presented as percentage of *P. intermedia* LPS alone. The results are means ± SD of three independent experiments. $^{**}p < 0.01$ vs. *P. intermedia* LPS alone.

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