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Isorhamnetin inhibits *Prevotella intermedia* lipopolysaccharide-induced production of interleukin-6 in murine macrophages via anti-inflammatory heme oxygenase-1 induction and inhibition of nuclear factor-κB and signal transducer and activator of transcription 1 activation

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Background and Objective: Interleukin-6 (IL-6) is a key proinflammatory cytokine that has been considered to be important in the pathogenesis of periodontal disease. Therefore, host-modulatory agents directed at inhibiting IL-6 appear to be beneficial in terms of attenuating periodontal disease progression and potentially improving disease susceptibility. In the current study, we investigated the effect of the flavonoid isorhamnetin on the production of IL-6 in murine macrophages stimulated with lipopolysaccharide (LPS) from *Prevotella intermedia*, a pathogen implicated in inflammatory periodontal disease, and its mechanisms of action.

Material and Methods: Lipopolysaccharide from *P. intermedia* ATCC 25611 was isolated using the standard hot phenol–water method. Culture supernatants were collected and assayed for IL-6. We used real-time PCR to quantify IL-6 and heme oxygenase-1 (HO-1) mRNA expression. The expression of HO-1 protein and the levels of signaling proteins were monitored using immunoblot

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analyses. The DNA-binding activity of nuclear factor-κB (NF-κB) was analyzed using ELISA-based assay kits.

Results: Isorhamnetin significantly down-regulated *P. intermedia* LPS-induced production of IL-6 as well as its mRNA expression in RAW264.7 cells. Isorhamnetin up-regulated the expression of HO-1 at both gene transcription and translation levels in cells stimulated with *P. intermedia* LPS. In addition, inhibition of HO-1 activity by tin protoporphyrin IX blocked the inhibitory effect of isorhamnetin on IL-6 production. Isorhamnetin failed to prevent LPS from activating either c-Jun N-terminal kinase or p38 pathways. Isorhamnetin did not inhibit NF-κB transcriptional activity at the level of inhibitory κB-α degradation. Isorhamnetin suppressed NF-κB signaling through inhibition of nuclear translocation and DNA binding activity of NF-κB p50 subunit and attenuated signal transducer and activator of transcription 1 signaling.

Conclusion: Although further research is required to clarify the detailed mechanism of action, we propose that isorhamnetin may contribute to blockade of the host-destructive processes mediated by IL-6 and could be a highly efficient modulator of the host response in the treatment of inflammatory periodontal disease. Further research in animal models of periodontitis is required to better evaluate, the potential of isorhamnetin as a novel agent for treating periodontal disease.

It is well established that host responses to periodontopathic bacteria are a major contributor to periodontal tissue destruction. Therefore, modulation of certain host-response pathways might be a treatment strategy, in addition to suppressing the causative microbial challenge, for periodontal disease.

Prevotella intermedia is a periodontal pathogen that is frequently recovered from subgingival flora in patients with chronic periodontitis (1,2). This bacterium has also been reported to be associated with necrotizing ulcerative gingivitis (3). The results of a recent study indicate that *P. intermedia* can adhere to and invade epithelial cells (4). Lipopolysaccharide (LPS) is an outer membrane component of gram-negative bacteria, including *P. intermedia*. It is a potent inducer of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-6 in a variety of cell types, including macrophages (5).

IL-6 is a key proinflammatory cytokine that has been considered to be important in the pathogenesis of periodontal disease, as in other chronic inflammatory diseases. IL-6 has been found at higher levels in gingival crevicular fluid from periodontally diseased sites in comparison with that from healthy sites (6,7). Moreover, studies have shown that IL-6 has a strong potential to induce osteoclastogenesis and alveolar bone resorption (8,9). Therefore, host-modulatory agents directed at inhibiting IL-6 appear to be beneficial in terms of attenuating periodontal disease progression and potentially improving disease susceptibility.

Plant compounds endowed with the capacity to block specific proinflammatory cytokines could have potential therapeutic values in the prevention and treatment of periodontal diseases. Flavonoids have received considerable attention because of their biological properties (10,11). Isorhamnetin (3'-methoxy-3,4',5,7-tetrahydroxyflavone) is a flavonoid found in apples, blackberries, pears and sea buckthorn (12). It is also an immediate metabolite of quercetin in mammals (13,14). Isorhamnetin has been reported to have many biological properties, including antioxidant, anticarcinogenic and anti-inflammatory activities (15–18), and could be a potential therapeutic agent against periodontal disease. However, to our knowledge, the effects of isorhamnetin on periodontal disease and the underlying mechanisms have not been published to date. In the current study, we investigated the effect of isorhamnetin on the production of IL-6 in murine macrophages stimulated with LPS from *P. intermedia*, a pathogen implicated in inflammatory periodontal disease, and its mechanisms of action.

Material and methods

Reagents

Isorhamnetin, DNase, RNase and proteinase K were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against 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 heme oxygenase-1 (HO-1), nuclear factor-κB (NF-κB) p65, NF-κB p50, β-actin and poly (ADP-ribose) polymerase-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Tin protoporphyrin IX was obtained from Frontier Scientific Inc. (Logan, UT, USA).

Bacteria, culture conditions and LPS isolation

P. intermedia ATCC 25611 was grown anaerobically on the surface of enriched Trypticase soy agar containing 5% (volume by volume) sheep blood, or in GAM broth (Nissui, Tokyo, Japan) supplemented with 1 μg/mL of menadione and 5 μg/mL of hemin, as described previously (19). Then, LPS was isolated from *P. intermedia* ATCC 25611 using the standard hot phenol–water method (19). Nucleic acids were eliminated by treating the LPS suspension with DNase and RNase. Protein contaminants were hydrolyzed with proteinase K.

Cell culture and cytotoxicity assay

The murine macrophage cell line RAW264.7 (American Type Culture Collection, Rockville, MD, USA) was grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in a humidified incubator under 5% CO_2, as described previously (19). The cellular toxicity of isorhamnetin was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, according to the manufacturer's instructions.

Determination of IL-6 production

Cells were plated in 24-well culture plates at a density of 5×10^5 cells/well in 500 μL of culture medium and were incubated for at least 12 h to allow the cells to adhere to the plates. The cells were then treated with various concentrations of *P. intermedia* LPS and isorhamnetin for 24 h, after which the culture supernatants were collected and the level of IL-6 was measured using a commercially available ELISA kit (OptEIA; BD Pharmingen, San Diego, CA, USA).

Purification and quantification of IL-6 and HO-1 mRNA

Cells were plated in 100-mm tissue culture dishes at a density of 1×10^7 cells/dish in 10 mL of culture medium and were treated with various concentrations of *P. intermedia* LPS and isorhamnetin for 24 h. Then, the cells were washed twice with phosphate-buffered saline and harvested by centrifugation. Total RNA was isolated with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Complementary DNA was prepared from 1 μg of total RNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Real-time PCR was carried out on the CFX96 real-time PCR detection system (Bio-Rad) with specific primers for mouse IL-6 and HO-1. As an endogenous control, β-actin primer was used. PCR was conducted according to the manufacturer's instructions. Thermal cycler conditions were as follows: an initial denaturation at 98°C for 30 s, followed by PCR for a total of 45 cycles, each of which consisted of denaturation at 95°C for 1 s and annealing/extension at 60°C for 5 s. The following real-time PCR primers for IL-6 (162 bp), HO-1 (149 bp) and β-actin (149 bp) were used: IL-6 sense, 5′-GCCAGAGTCCTTCAGAGAGATACAG-3′ and antisense, 5′-GAATTGGATGGTCTTGGTCCTTAGC-3′; HO-1 sense, 5′-CAATGTGGCCTTCTCTCTGT-3′ and antisense, 5′-TTTTGGTGAGGGAACTGTGT-3′; and β-actin sense, 5′-TGAGAGGGAAATCGTGCGTGAC-3′ and antisense, 5′-GCTCGTTGCCAATAGTGATGACC-3′. Each assay was normalized to β-actin mRNA.

Preparation of cell extracts and immunoblotting analysis

Cells were plated in 60-mm tissueculture dishes at 4×10^6 cells/dish in 4 mL of culture medium and treated with various concentrations of *P. intermedia* LPS and isorhamnetin for the indicated periods of time. Whole-cell lysates and nuclear fractions were prepared and analyzed as described previously (19). Briefly, cell lysates were prepared using lysis buffer containing 50 mM Tris–Cl, 150 mM NaCl, 0.002% sodium azide, 0.1% sodium dodecyl sulfate, 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). Proteins (30 μg) were electrophoresed in a sodium dodecyl sulfate–polyacrylamide gel. The resolved proteins in the gel were electrotransferred to a nitrocellulose membrane, probed with specific primary antibodies and secondary antibodies conjugated to horseradish peroxidase, and visualized on X-ray film using enhanced chemiluminescence detection reagent (Cell Signaling Technology).

Assay of NF-κB DNA-binding activity

RAW264.7 cells were plated in 60-mm tissue culture dishes, at a density of 4×10^6 cells per dish, and incubated with various concentrations of *P. intermedia* LPS and isorhamnetin for the indicated periods of time. After extracting the nuclear protein as described above, the DNA-binding activity of NF-κB p65 or p50 was assayed using ELISA-based TransAM NF-κB assay kits (Active Motif), as described previously (19).

Statistical analysis

Data are expressed as mean ± standard deviation. Statistical analysis was performed using the Student's *t*-test, with $p < 0.05$ considered statistically significant.

Results

Effect of isorhamnetin on *P. intermedia* LPS-induced IL-6 production

RAW264.7 cells were treated with different concentrations of isorhamnetin (0, 12.5, 25 and 50 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL) for 24 h, and then the culture supernatants were collected for measurement of the IL-6 level. Treat-

ment of the cells with LPS induced a marked increase in the level of IL-6 compared with the control (Fig. 1A). Approximately 18.5 ng/mL of IL-6 was released at a concentration of 10 μg/mL. *Salmonella typhimurium* LPS also stimulated IL-6 production, although IL-6 induction by P. intermedia LPS was significantly greater than with *S. typhimurium* LPS (data not shown). As shown in Fig. 1A, isorhamnetin suppressed, in a dose-dependent manner, the *P. intermedia* LPS-induced secretion of IL-6. When the concentration of isorhamnetin was increased to 50 μM, IL-6 production was reduced by 84%. Isorhamnetin did not affect cell viability, as determined by the MTT assay (data not shown), indicating that its inhibitory effect was not attributable to a direct cytotoxic effect. To determine whether the decrease in the level of *P. intermedia* LPS-induced IL-6 in the presence of isorhamnetin was associated with transcriptional regulation, real-time PCR analysis was performed. *P. intermedia* LPS induced a marked increase in IL-6 mRNA, and treatment with isorhamnetin reduced LPS-induced IL-6 mRNA expression in a concentration-dependent manner (Fig. 1B).

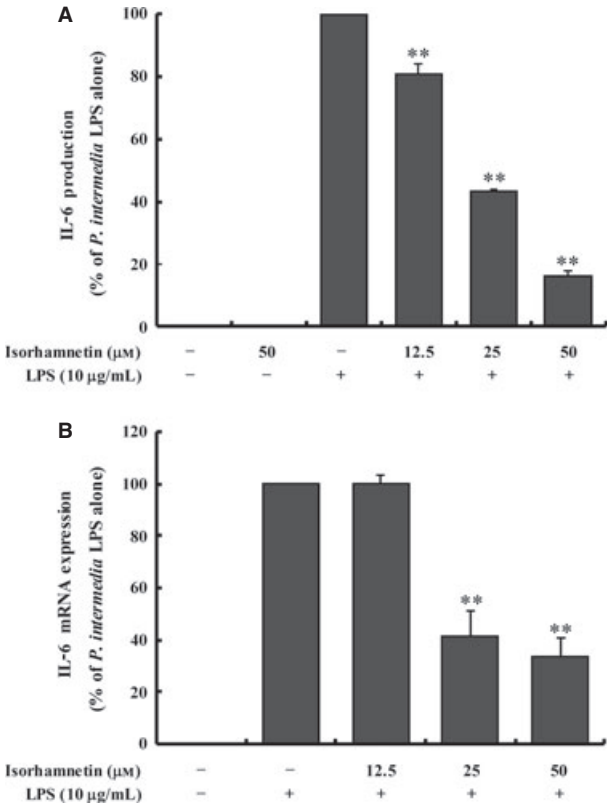

Fig. 1. Effects of isorhamnetin on *Prevotella intermedia* lipopolysaccharide (LPS)-induced interleukin-6 (IL-6) production (A) and IL-6 mRNA expression (B) in RAW264.7 cells. Cells were treated with different doses of isorhamnetin (0, 12.5, 25 and 50 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL) for 24 h. (A) Supernatants were removed after 24 h and assayed for IL-6. Data represent the percentage of *P. intermedia* LPS alone and are expressed as the mean ± standard deviation of three independent experiments. $^{**}p < 0.01$ vs. *P. intermedia* LPS alone. (B) Real-time PCR was performed with EvaGreen Supermix, and β-actin was used as the endogenous control. Data represent the percentage of *P. intermedia* LPS alone and are expressed as the mean ± standard deviation of three independent experiments. $^{**}p < 0.01$ vs. *P. intermedia* LPS alone.

Effect of isorhamnetin on HO-1 induction in cells treated with *P. intermedia* LPS

Cells were incubated with various concentrations of isorhamnetin (0, 12.5, 25 and 50 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL) for 6 h, and the induction of HO-1 protein was determined by immunoblot analysis of cell lysates using a specific antibody against HO-1. Addition of the indicated concentrations of isorhamnetin up-regulated, in a dose-dependent manner, the levels of HO-1 protein in cells treated with *P. intermedia* LPS (Fig. 2A). To determine whether the induction of HO-1 protein by isorhamnetin was associated with transcriptional regulation, real-time PCR was performed. In cells treated with *P. intermedia* LPS, isorhamnetin increased the expression of HO-1 mRNA in a dose-dependent manner (Fig. 2B).

Effect of isorhamnetin-mediated induction of HO-1 on the inhibition of *P. intermedia* LPS-induced IL-6 production

We next examined whether isorhamnetin-mediated HO-1 induction plays a role in modulating the *P. intermedia* LPS-mediated production of IL-6. Cells were treated with isorhamnetin (50 μM) and *P. intermedia* LPS (10 μg/mL) for 24 h in the presence of the indicated concentrations of tin protoporphyrin IX, a competitive HO-1 inhibitor, after which culture supernatants were assayed for IL-6. Tin protoporphyrin IX blocked, in a dose-dependent manner, the inhibitory effect of isorhamnetin on IL-6 production (Fig. 2C).

Effects of isorhamnetin on *P. intermedia* LPS-induced phosphorylation of JNK and p38

Our previous study has shown that the JNK, p38, NF-κB and JAK2/STAT1 pathways are involved in IL-6 production induced by *P. intermedia* LPS (19). We first determined the action of isorhamnetin on *P. intermedia* LPS-induced activation of JNK and p38. LPS induced the phosphorylation of JNK and p38 (Fig. 3). However, as shown in Fig. 3, isorhamnetin failed to prevent

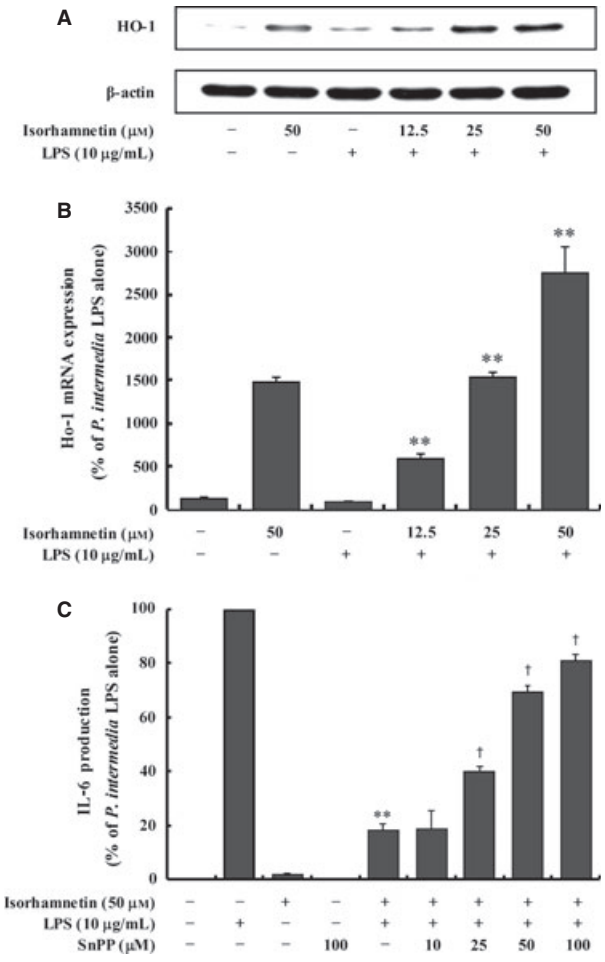

Fig. 2. Involvement of heme oxygenase-1 (HO-1) in the inhibitory effects of isorhamnetin on *Prevotella intermedia* lipopolysaccharide (LPS)-induced production of interleukin-6 (IL-6) in RAW264.7 cells. (A, B) Cells were treated with different doses of isorhamnetin (0, 12.5, 25 and 50 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL) for either 6 h (for HO-1 protein) or 24 h (for HO-1 mRNA). (A) HO-1 protein synthesis was measured by immunoblot analysis of cell lysates using HO-1-specific antibody. A representative immunoblot from two separate experiments with similar results is shown. (B) Real-time PCR was performed using EvaGreen Supermix, with β-actin as the endogenous control. Data represent the percentage of *P. intermedia* LPS alone and are expressed as the mean ± SD of three independent experiments. $^{**}p < 0.01$ vs. *P. intermedia* LPS alone. (C) Cells were treated with isorhamnetin (50 μM) and *P. intermedia* LPS (10 μg/mL) for 24 h in the presence of different doses of tin protoporphyrin IX (SnPP) (0, 10, 25, 50 and 100 μM). Supernatants were removed after 24 h and assayed for IL-6. Data represent the percentage of *P. intermedia* LPS alone and are expressed as the mean ± standard deviation of three independent experiments. $^{**}p < 0.01$ vs. *P. intermedia* LPS alone; $^{\dagger}p < 0.01$ vs. *P. intermedia* LPS plus isorhamnetin.

LPS from activating either JNK or p38 pathways.

Effects of isorhamnetin on *P. intermedia* LPS-induced NF-κB activation

We next investigated the effects of isorhamnetin on the NF-κB signaling pathway, which mediates *P. intermedia* LPS-induced production of IL-6. To determine the effect of isorhamnetin on *P. intermedia* LPS-induced degradation of IκB-α, we performed immunoblot analysis of the cytoplasmic levels of IκB-α protein. As shown in Fig. 4A, isorhamnetin did not interfere with LPS-induced degradation of IκB-α. We then examined the effects of isorhamnetin on the translocation of NF-κB subunits into the nucleus. Immunoblot analysis of nuclear extract was carried out using antibodies against NF-κB p65 and p50. *P. intermedia* LPS strongly promoted the nuclear translocation of NF-κB p65 and p50 subunits, while only p50 nuclear translocation was markedly attenuated by treatment with isorhamnetin (Fig. 4B). Finally, we investigated whether isorhamnetin could affect the binding of NF-κB to DNA. The DNA-binding activity of NF-κB in the nuclear fraction was analyzed using the ELISA-based NF-κB transcription factor assay kits. As anticipated, DNA-binding activities of NF-κB p65 and p50 subunits were induced by *P. intermedia* LPS (Fig. 4C). Whereas isorhamnetin dose-dependently attenuated NF-κB p50 binding activity induced by LPS, it had no effect on p65 binding (Fig. 4C).

Effects of isorhamnetin on *P. intermedia* LPS-induced phosphorylation of STAT1

In addition, we investigated whether isorhamnetin inhibited *P. intermedia* LPS-induced production of IL-6 via regulation of the STAT1 signaling pathway. Immunoblot analysis of whole-cell lysates was carried out with specific antibodies against STAT1 and phospho-STAT1. We found that STAT1 phosphorylation induced by *P. intermedia* LPS was significantly attenuated by isorhamnetin (Fig. 5).

Discussion

Because IL-6 is a key cytokine which has been considered to be important in the pathogenesis of periodontal disease (6–9), the present work investigated whether the flavonoid isorhamnetin could down-regulate the production of this proinflammatory cytokine in macrophages stimulated with *P. intermedia* LPS and also attempted to determine the underlying mechanisms of action. LPS signals can be blocked by flavonoids, and hence isorhamnetin appears to be

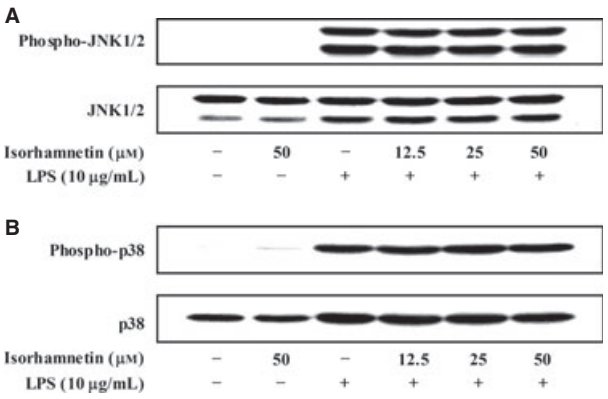

Fig. 3. Effects of isorhamnetin on *Prevotella intermedia* lipopolysaccharide (LPS)-induced phosphorylation of JNK and p38 in RAW264.7 cells. Cells were incubated with different doses of isorhamnetin (0, 12.5, 25 and 50 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL) for either 30 min (for JNK) or 15 min (for p38). Cell lysates were subjected to immunoblot analysis using specific antibodies. A representative immunoblot from two separate experiments with similar results is shown.

helpful in terms of blocking the development and progression of inflammatory periodontal disease.

The periodontium is consistently in contact with LPS produced by gram-negative periodontopathogenic bacteria, and dense infiltration of inflammatory cells, including macrophages, occurs in the gingival connective tissues of patients with periodontal disease (20). The LPS of *P. intermedia* is expected to interact with infiltrating macrophages to induce the production and release of proinflammatory cytokines.

Macrophage cell lines have been used as *in vitro* models to study compounds for potential anti-inflammatory effects. In the present study, we examined the effects of isorhamnetin on *P. intermedia* LPS-induced IL-6 production in the well-characterized RAW264.7 murine macrophage cell line, which can be stimulated with LPS to mimic the condition of inflammation. However, this *in vitro* model system does not take into consideration the complex interactions between different cell types that occur *in vivo* and thus is not a realistic environment for investigating the effects of compounds on the LPS-induced production of cytokines.

In addition, the pharmacokinetics, bioavailability and metabolism of isorhamnetin and its glycosides in humans and animals have not yet been elucidated. Only the subsequent pharmacokinetic and metabolic profiles of isorhamnetin after administration with quercetin or its glycosides are reported (21,22).

The structure and function of *P. intermedia* LPS are distinct from the classical LPSs derived from *Enterobacteriaceae*, such as *Escherichia coli* and *Salmonella* species (23). The active molecule(s) and mode of action of LPS from *P. intermedia* have been shown to differ from those of *Salmonella* LPS (24). It was indicated that the LPS preparation from *P. intermedia* activates the lymphocytes and macrophages of classical LPS-nonresponsive C3H/HeJ mice (24). While polymyxin B destroyed the biological activity of LPS and lipid A isolated from *Enterobacteriaceae*, *P. intermedia* LPS was comparatively resistant to treatment with polymyxin B (24). It was reported that lipid A from *P. intermedia* ATCC 25611 LPS is 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 (25). In addition, *P. intermedia* lipid A activates murine cells through a toll-like receptor 4-mediated signaling pathway (25).

P. intermedia LPS induces significant production of inflammatory mediators, such as nitric oxide, TNF-α and IL-6, in macrophages (19,26,27). In addition, *P. intermedia* LPS dose-dependently inhibited bone formation by reducing alkaline phosphatase activity and calcium incorporation, and induced the release of nitric oxide and IL-6 by fetal mouse osteoblasts in organoid culture (28). The specific cytokines induced by *P. intermedia* LPS play a significant role in the local tissue destruction observed in periodontal disease, and hence these molecules are potential targets for the development of new therapeutic approaches for the treatment of periodontitis. The findings of the present study clearly indicate that isorhamnetin significantly down-regulates *P. intermedia* LPS-induced production of IL-6 as well as expression of IL-6 mRNA in RAW264.7 cells, suggesting that it inhibits this specific proinflammatory cytokine at both protein and gene transcription levels.

The responsiveness to LPS might be species-specific. However, *P. intermedia* LPS has also been found to induce significant production of IL-6 in human cells, including differentiated THP-1 cells and primary gingival fibroblast cultures (S. J. Kim, unpublished observations). Work is in progress to test the effects of isorhamnetin on *P. intermedia* LPS-induced production of IL-6 in human cells.

We also examined whether isorhamnetin plays a role in modulating other proinflammatory mediators induced by *P. intermedia* LPS. However, isorhamnetin failed to inhibit the production of either nitric oxide or TNF-α (S. J. Kim, unpublished observations). Boesch-Saadatmandi *et al.* (18) have recently studied the anti-inflammatory effect of isorhamnetin in murine RAW264.7 macrophages stimulated with *Salmonella enteritidis* LPS. Isorhamnetin significantly decreased both TNF-α mRNA levels and TNF-α secretion. In addition, isorhamnetin significantly decreased the mRNA and protein levels of inducible nitric oxide synthase, as well as IL-1β and IL-6 mRNA.

HO-1 is a rate-limiting enzyme in heme catabolism, leading to the generation of equimolar amounts of

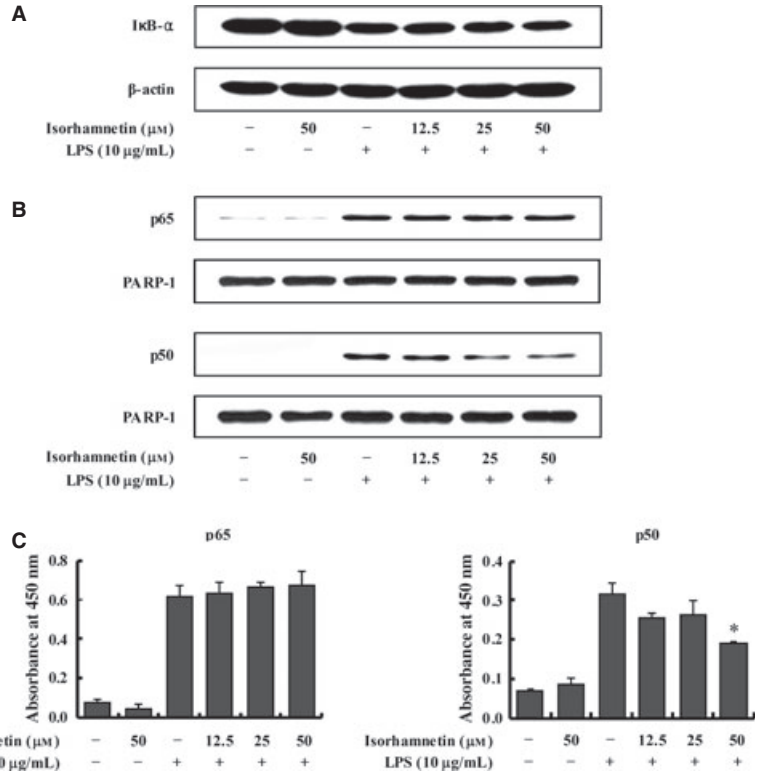

Fig. 4. Effects of isorhamnetin on *Prevotella intermedia* lipopolysaccharide (LPS)-induced nuclear factor-κB (NF-κB) activation in RAW264.7 cells. (A, B, C) Cells were incubated with different doses of isorhamnetin (0, 12.5, 25 and 50 μM) in the absence or presence of *P. intermedia* LPS (10 μg/mL). (A) After 30 min of incubation, inhibitory κB-α (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, C) After either 30 min (for NF-κB p65) or 8 h (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 subunits. A representative immunoblot from two separate experiments with similar results is shown. PARP-1, poly (ADP-ribose) polymerase-1. (C) DNA-binding activity of NF-κB in nuclear extracts was assessed using the ELISA-based NF-κB p65/NF-κB p50 transcription factor assay kits. The results are given as mean ± standard deviation of two independent experiments. $*p < 0.05$ vs. *P. intermedia* LPS alone.

carbon monoxide, free iron and biliverdin, which is rapidly converted to bilirubin by bilirubin reductase (29,30). Studies have shown that HO-1 is inducible in many cell types in response to a variety of stimuli, including proinflammatory mediators, oxidative stress and LPS (31–33). HO-1 is known to have potent anti-inflammatory and antioxidant properties (29,30,34). In animal models, deficiencies of HO-1 expression exhibited increased inflammatory state, but HO-1 overexpression exerted anti-inflammatory effects (30,35). In this study, we investigated the possibility that the inhibitory effect of isorhamnetin on *P. intermedia* LPS-induced IL-6 production is mediated through the induction of anti-inflammatory HO-1. The results indicate that isorhamnetin is a potent inducer of HO-1, and isorhamnetin-mediated induction of HO-1 plays an important role in modulating *P. intermedia* LPS-mediated production of IL-6.

Although the detailed mechanisms involved in the anti-inflammatory effects of HO-1 are not yet clear, several lines of evidence demonstrate that one or more of the enzymatic by-products of heme catabolism contribute to anti-inflammatory responses. For example, carbon monoxide, a key product of HO-1, has been reported to decrease the production of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 in LPS-stimulated macrophages (36,37). In addition, bilirubin, a potent antioxidant, is capable of blocking inflammation (38). As isorhamnetin, as confirmed in this study, can induce the expression of HO-1 in macrophages activated by *P. intermedia* LPS, the suppressive properties of isorhamnetin on *P. intermedia* LPS-induced IL-6 production appear to be mediated by heme metabolites, such as carbon monoxide and bilirubin.

MAPK signal-transduction pathways are known to participate in LPS-induced activation of macrophages and the resultant production of proinflammatory mediators. However, isorhamnetin did not affect either JNK or p38, suggesting that these pathways are not involved in the inhibition, by isorhamnetin, of *P. intermedia* LPS-induced IL-6 production. Our previous studies (39,40) have also shown that JNK and p38 pathways did not lead to the effects of several flavonoids like luteolin and daidzein on regulating *P. intermedia* LPS-induced IL-6 release.

NF-κB is a multi-unit transcription factor that is known to play a critical role in the regulation of various target genes related to the inflammation in LPS-activated macrophages (41–43). In resting cells, NF-κB is present in the cytoplasm in a latent form linked to the IκB proteins. IκB undergoes phosphorylation, followed by ubiquitination and subsequent proteasome-mediated degradation, in response to a variety of stimuli, including LPS. Then, the free NF-κB is translocated to the nucleus, where it induces activation of a wide variety of genes associated with inflammation (44,45). In this study, isorhamnetin did not inhibit NF-κB transcriptional activity at the level of IκB-α degradation. Instead, isorhamnetin suppressed NF-κB signaling through inhibition of nuclear translocation and DNA-binding activity of the NF-κB p50 subunit induced with *P. intermedia* LPS. Isorhamnetin did not affect the nuclear translocation and DNA binding of p65. Both NF-κB p65 and

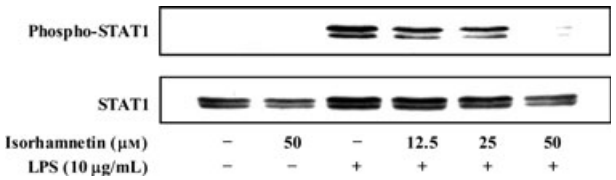

Fig. 5. Effects of isorhamnetin on *Prevotella intermedia* lipopolysaccharide (LPS)-induced phosphorylation of signal transducer and activator of transcription 1 (STAT1) in RAW264.7 cells. Cells were incubated with different doses of isorhamnetin (0, 12.5, 25 and 50 μM) in the absence or presence of *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.

p50 have been shown to accumulate in the nucleus upon stimulation with LPS, where they bind to specific regulated sequences in the DNA to induce transcription. The NF-κB p65 subunit contains a characteristic transcriptional activation domain and directly promotes gene transcription (46). In contrast, the NF-κB p50 subunit does not have a transactivation domain and therefore usually forms a heterodimer with other NF-κB subunits to be transcriptionally active (45–47). NF-κB has been considered as an attractive molecular target for the therapy of inflammatory diseases, and its inhibition by isorhamnetin would be useful in the treatment of inflammatory periodontal disease.

Another transcription factor that plays a crucial role in the regulation of inflammatory responses is STAT (48). There are seven mammalian STAT family members, and each one binds to a different DNA sequence (49). STATs are activated through JAKs, which results in dimerization and translocation into the nucleus where they induce the transcription of target genes (50). STAT1, downstream of JAK2, has been shown to be an essential activator of gene transcription in macrophages exposed to LPS (51). In the present study, isorhamnetin exerted its effects on *P. intermedia* LPS-induced IL-6 production via regulating the STAT1 pathway. Thus, the STAT1 signaling pathway could also be a molecular target for treating periodontal disease.

In conclusion, the present study showed that isorhamnetin strongly down-regulates IL-6 production induced by LPS from *P. intermedia* in murine macrophages. The underlying mechanisms of the inhibitory effect of isorhamnetin involve anti-inflammatory HO-1 induction and inhibition of NF-κB and STAT1 activation. Although further research is required to clarify the detailed mechanism of action, we propose that isorhamnetin may contribute to blockade of the host-destructive processes mediated by IL-6, and could be a highly efficient modulator of host response in the treatment of inflammatory periodontal disease. Further research in animal models of periodontitis is required to better evaluate the potential of isorhamnetin as a novel agent for treating periodontal disease.

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