

Inhibitory Effect of Myricetin on Matrix Metalloproteinase Expression and Activity in Periodontal Inflammation

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(received August 29, 2016; revised November 28, 2016; accepted November 29, 2016)

Flavonoid myricetin, usually found in tea and medicinal plants, has antioxidant and anti-inflammatory effects. Our objectives in this study were to verify the effects of myricetin on periodontal ligament fibroblasts (PDLFs) under inflammatory conditions and to observe its effects on osteoclast generation and on cytokine expression in RAW264.7 cells. To determine the effects of myricetin on PDLFs, we examined the expression and activity of proteolytic enzymes, including MMP-1, MMP-2, and MMP-8, which all play an important role in chronic periodontitis. We observed the effects of myricetin on intracellular signal transduction to verify the molecular mechanism involved. By measuring the formation of TRAP-positive multinucleated cells and the expression and activity of MMP-8, we were able to assess the effects of myricetin on osteoclast generation. In addition, by measuring the secretion of IL-6 and NO, we could evaluate the effects of myricetin on inflammatory mediators. We found that Myricetin had no effect on the viability of the PDLFs in the presence of inflammation, but it did decrease both the expression of MMP-1 and MMP-8 and the enzyme activity of

MMP-2 and MMP-8 in these fibroblasts. Myricetin also decreased the lipopolysaccharide-stimulated phosphorylation of JNK, p38 signaling, IKKB, AKT, and p65RelA in the PDLFs. In the RAW264.7 cells, myricetin inhibited both the expression and the activity of MMP-8. Furthermore, Myricetin not only suppressed the generation of LPS-stimulated osteoclasts, but it also slightly inhibited LPS-stimulated degradation of IκB and decreased the release of LPS-induced IL-6 and NO. These findings suggest that myricetin alleviates the tissue-destructive processes that occur during periodontal inflammation.

Key words: myricetin, matrix metalloproteinase, PDLF, osteoclastogenesis

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Introduction

Periodontitis is a chronic inflammatory disease that induces periodontal tissue destruction. Proteolytic enzymes, such as the matrix metalloproteinases (MMPs), play important roles in inflammatory diseases, including chronic periodontitis. Periodontal tissue is a complex and highly specialized structure consisting of both hard and soft tissues.

Gram-negative bacterial infection is the most frequent cause of periodontal diseases [1]. Lipopolysaccharide (LPS) is present on the outer membrane of gram-negative anaerobic microorganisms such as *Porphyromonas gingivalis*, which causes periodontitis [2]. LPS stimulates the secretion of inflammatory mediators, such as interleukin (IL)-1, prostaglandin E₂, and nitric

oxide (NO) [3,4]. These mediators play important roles in osteoclastogenesis and connective tissue destruction [5,6], metalloproteinases (MMPs), which degrade extracellular matrix components, including collagens, fibronectin, and proteoglycan core proteins [8]. Excess formation of MMPs stimulates the decomposition of the extracellular matrix in the periodontium, which results in the loss of connective tissue adhesion and of supporting bone, which in turn causes tooth loss [8,9].

These proteolytic enzymes are classified into subgroups based on their substrate specificities. Collagenase (MMP-1, MMP-8, and MMP-13), gelatinase (MMP-2 and MMP-9), and stromelysin (MMP-3, MMP-10, and MMP-11) are the primary MMPs that participate in the degradation of matrix components [10,11]. In a study of patients with periodontitis, increased MMP-1 and MMP-2 levels were detected in gingival crevicular fluid, periodontal tissues, and periodontal ligament cells (PDLs) [12].

Based on available reports, we know that LPS stimulates macrophages, activates immune-related mediators such as NO and triggers activity of the signal system for mitogen-activated protein kinase (MAPK). It also plays an important role in the activation of nuclear factor kappa-B (NF κ B), a protein belonging to the Rel protein group that regulates the transcription of target genes by the formation of dimers. Among in the NF κ B transcription factors, RelA/p50 dimers are the most common form. When the signal is transmitted to the cell, inhibitory kappa B (I κ B) is phosphorylated by I κ B kinase (IKK) and subsequently degrades, and the NF κ B that is separated from the I κ B regulates transcription. RelA is also capable of phosphorylation, and it is known that RelA phosphorylation displays a regulatory effect on transcription activity and protein stability [13,14].

The periodontal ligament is a cellular connective tissue that links the cementum to the surrounding bone. Periodontal ligament fibroblasts (PDLFs), the primary cell type in the periodontal ligament, act as immune-responsive and supportive cells for the periodontal tissues. The location of these cells in periodontal pathological conditions suggests that they play key roles in regulating the inflammatory response and in amplifying inflammatory signals [15].

Osteoclasts are bone-resorbing cells derived from the monocyte/macrophage lineage. Two major cytokines—macrophage colony-stimulating factor (M-CSF) [16] and receptor activator of nuclear factor kappa-B (RANK) ligand (RANKL)—are essential for osteoclast formation [17].

RANKL binds to RANK and affects adaptor molecules, such as tumor necrosis factor receptor-associated factor (TRAF) 6. This binding induces several downstream signaling pathways, including Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38, NF κ B, and phosphatidylinositol 3-kinase/Akt [18,19]. Osteoclasts have important physiological functions in bone resorption and pathological functions in diseases that lead to bone-destroying inflammatory diseases [20].

Natural compounds, such as flavonoids, are found in many plants, including edible plants [21,22]. Myricetin, a hexahydroxyflavone, is a flavonoid usually found in grape seeds, tea, fruits, and medicinal plants [23] that has antiproliferative, antioxidant, and anti-inflammatory effects [24]. Although myricetin is known to exert its anti-inflammatory effect by decreasing IL-1 transcription and NO secretion in LPS-stimulated monocytes [25,26], the effect of modulating periodontal inflammation via suppression of MMP production and activity, as well as the basic mechanisms underlying these activities, remains largely unknown.

In this study, we attempted to verify the effects of myricetin on the expression and activity of MMPs and their signaling mechanisms by inducing an inflammatory response in human PDLFs and in mouse mononuclear cells (RAW264.7 cells).

Materials and Methods

Reagents

LPS from *Escherichia coli* was purchased from Sigma-Aldrich (St. Louis, MO, USA). Myricetin (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) (1,000 \times). Antibodies against MMP-1, MMP-2, JNK, p38, ERK, I κ B α , IKK, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against MMP-8 were purchased from Abcam (Cambridge, UK). The antibodies against phospho-JNK, phospho-p38, phospho-ERK, phospho-IKK, p65RelA, phospho-p65RelA, AKT, and phospho-AKT were purchased from Cell Signaling (Danvers, MA, USA). Customized primers for RT-PCR were obtained from Bioneer (Daejeon, Korea).

Cell cultures

Human periodontal ligament tissue was harvested from

healthy teeth extracted from three individuals during orthodontic treatments. The Ethics Committee of the School of Dentistry at Dankook University in Korea approved all protocols, and informed consent was obtained prior to the extractions (IRB No. H-1009/006/002). Periodontal ligament (PDL) tissue was carefully removed from the middle third of the root surface with a surgical blade and was placed in a culture dish. The tissues samples were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics (10,000 U/mL of penicillin G and 10,000 µg/mL of streptomycin) (HyClone, South Logan, Utah, USA). After the explanted cells from the tissue reached confluence, the cells were detached by means of 0.25% trypsin and 0.2% EDTA and were subcultured at a ratio of 1:5. Cell cultures between the 5th and 9th passages were used.

Mouse monocyte/macrophage RAW264.7 cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA) and were cultured and maintained at 37°C in a 5% CO₂ atmosphere in DMEM with 10% FBS. The medium was changed every 3 days.

Cell viability assays

The cells were seeded in 96-well plates at a density of 10⁴ cells/well in DMEM with 10% FBS. After 24 hours, the cells were treated with 1 µg/mL of LPS and serially diluted myricetin for 2 days for the PDLFs or 4 days for the RAW264.7 cells. Cell viability was tested with an MTT assay according to the manufacturer's protocol (Sigma M5655) (Sigma-Aldrich). After the supernatant media were removed, the formazan granules were solubilized with isopropyl alcohol, and the absorbance was then measured using a microplate absorbance reader.

MMP activity assay

To test the effect of myricetin on MMP activity, the PDLFs were seeded in 96-well plates at a density of 10⁴ cells/well in DMEM with 10% FBS. After 24 hours, the cells were treated with 1 µg/mL of LPS and serially diluted myricetin for 24 hours. The levels of MMP-1, MMP-2, and MMP-8 activity in the cell culture supernatants were tested using the fluorometric MMP Assay Kit (AnaSpec, Fremont, CA, USA) according to the manufacturer's protocol. Fluorescence intensity was measured at excitation/emission rates of 340/490 nm using a multi-detection microplate reader (SpectraMax M2e) (Molecular Devices, Sunnyvale, CA, USA).

Induction of multinucleated osteoclasts and tartrate-resistant acid phosphatase-positive [TRAP(+)] staining

To differentiate the RAW264.7 monocyte/macrophage cells into osteoclasts, the cells were pre-incubated with 100 ng/mL of RANKL (PeproTech, Rocky Hill, NJ, USA) for 24 hours and stimulated with 1 µg/mL of LPS for 4 or 7 days. After culturing, the cells were fixed with citrate-acetone-formaldehyde for 5 minutes, and a TRAP staining kit was used to TRAP expression. The TRAP(+) multinucleated cells were observed to be osteoclasts by means of an optical microscope.

TRAP activity assay

RAW264.7 cells were seeded in 96-well plates at a density of 10⁴ cells/well in α -MEM with 10% FBS. The cells were pre-treated with 100 ng/mL of RANKL and cultured with 1 µg/mL of LPS and serially diluted myricetin for 4 days. The cells were then harvested with a 0.1% Triton X-100 solution, and TRAP activity was examined using 50 mM of citrate solution with 100 mM of *p*-nitrophenyl phosphate as a substrate and 10 mM of sodium tartrate using spectrophotometry. The total protein content in the cell lysates was measured using Lowry protein assay reagents (Pierce Biotechnology, Rockford, IL, USA) with bicinchoninic acid (BCA) as the standard.

Western blot analysis

PDLFs or RAW264.7 cells were seeded in 6-well plates at a density of 5×10⁵ cells/well. After 24 hours, the cells were serum-starved for 6 hours, pre-treated with serially diluted myricetin in media including 10% FBS for 2 hours, and then stimulated with 5 µg/mL of LPS for 15 minutes or 2 hours. Next, the cells were collected, and protein homogenates were prepared using a lysis solution. Protein lysates (30 µg/lane) were electrophoresed using SDS-PAGE and were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline and Tween20 (TBST) and probed with antibodies against MMP-1, MMP-2, MMP-8, phospho-ERK1/2 (Thr202/Tyr204), phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38, phospho-IKK, phospho-p65RelA, phospho-AKT, and I κ B α . The blots were then re-probed with the corresponding non-phospho-antibodies for MAPK, IKK, p65RelA, AKT, and I κ B α . The membranes were developed using X-ray film (KOREA FUJI Photo Film, Seoul, Korea) exposure and a chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK).

Interleukin-6 (IL-6) ELISA assay

To test the effect of myricetin on IL-6 release, RAW264.7 cells were seeded in 96-well plates at 10⁴ cells/well in α -MEM with 10% FBS. After 1 day, the cells were incubated with 1 μ g/mL of LPS and serially diluted myricetin for 2 days. The amount of IL-6 released into the culture supernatants was determined using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocols. The absorbance was detected at 450 nm using a spectrophotometer.

Nitrite assay

NO secretion was evaluated by measuring the levels of nitrite, a stable oxidized form of NO, in the culture supernatants. To test the effect of myricetin on inducible nitric oxide synthase (iNOS) activity, RAW264.7 cells were seeded in 96-well plates at 10⁴ cells/well in α -MEM with 10% FBS for 24 hours. Next, the cells were cultured with 1 μ g/mL of LPS and serially diluted myricetin in phenol red-free media for 72 hours. After incubation, 100 μ L of culture supernatant was reacted with 100 μ L of Griess reagent (Sigma-Aldrich) in a 96-well reading plate, and the absorbance was detected at 550 nm using a spectrophotometer. Nitrite levels measured were compared with a sodium nitrite standard.

Statistical analysis

Significance was determined using Student’s t-test. All tests

were performed in triplicate. Each value represents the mean \pm standard error (SE). Differences were considered statistically significant at $p < 0.05$ and $p < 0.01$.

Results

To examine the protective effects of myricetin on periodontal tissue using LPS-stimulated human PDLFs, we determined the expression and enzyme activity of MMPs and the phosphorylation of MAPK and activation of NF κ B-related molecules. In addition, we investigated myricetin’s activity in LPS-stimulated murine RAW264.7 cells under inflammatory conditions, including its effects on the expression and enzyme activity of MMPs, osteoclast generation, and the phosphorylation of MAPK and IL-6 and NO release.

Studies in PDLFs

Myricetin inhibits LPS-stimulated expression of MMPs in PDLFs

Myricetin did not cause any significant changes on viability of the PDLFs (Fig. 1A). We used western blot analysis to examine MMP-1, MMP-2, and MMP-8 protein production. PDLFs were pre-treated with myricetin for 2 hours and

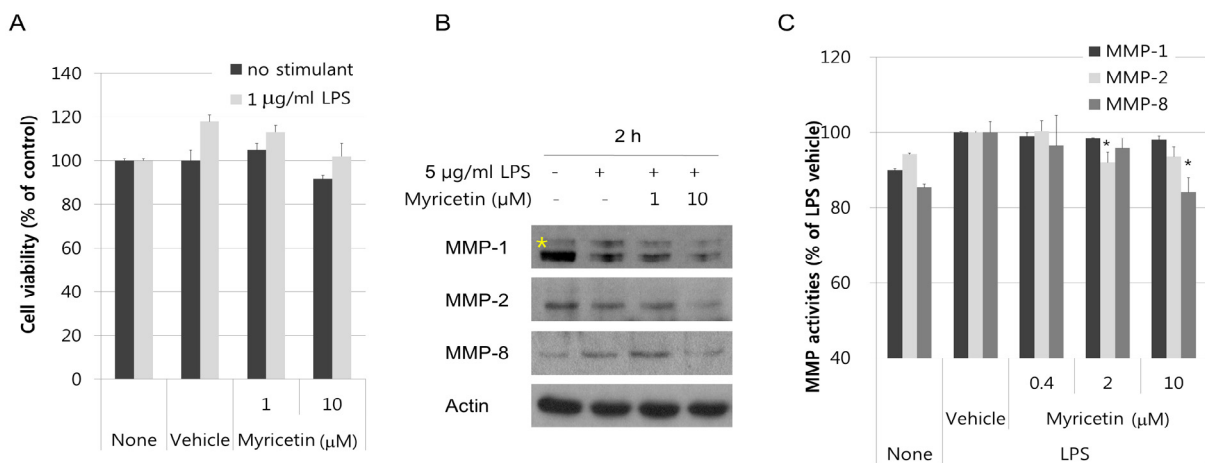


Figure 1. (A) Effect of myricetin on the viability of periodontal ligament fibroblasts (PDLFs). PDLFs were treated with myricetin and 1 μ g/mL of LPS for 48 hours. After removing the media, the formazan granules were solubilized, and the absorbance was measured using a microplate reader. Cell viability is expressed as the absorbance ratio (i.e., absorbance of treated cells/absorbance of control cells, $\times 100$). Values are expressed as means \pm SE (n=3). (B) Effect of myricetin on LPS-induced protein expression. The PDLFs were stimulated with 5 μ g/mL of LPS for 2 hours. Levels of protein expression were determined with the use of western blot analysis. (C) Effect of myricetin on MMP activity. PDLFs were treated with myricetin and 1 μ g/ml of LPS for 24 hours. After culture, the supernatants were evaluated using a fluorescence assay kit. Fluorescence was recorded at excitation/emission rates of 340/490 nm. Values are expressed as means \pm SE (n=3). * $p < 0.05$.

activated with LPS for 2 hours. Myricetin inhibited protein production by MMP-1, MMP-2, and MMP-8 (Fig. 1B).

Myricetin inhibits LPS-activated enzyme activity of MMPs in PDLF

To test the inhibitory effect of myricetin on extracellular matrix destruction, the activities of MMP-1, MMP-2, and MMP-8 were assessed in the culture supernatants from the LPS-stimulated PDLFs using a fluorescent assay. The enzyme activities of MMP-1, MMP-2, and MMP-8 were activated by LPS in the PDLFs. Myricetin decreased the MMP-2 and MMP-8 activities at concentrations of 2 or 10 μM , as compared with the LPS-induced control (Fig. 1C).

Myricetin inhibits phosphorylation of MAPK in LPS-stimulated PDLFs

To determine the kind of effects myricetin has on the signal transduction pathway in PDLFs, we first verified the effects on activation of MAPKs by means of western blot analysis. In this way, PDLFs were pre-treated with myricetin for 2 hours and then activated with LPS for 15 minutes. We measured the degree of protein expression for MAPK. Although

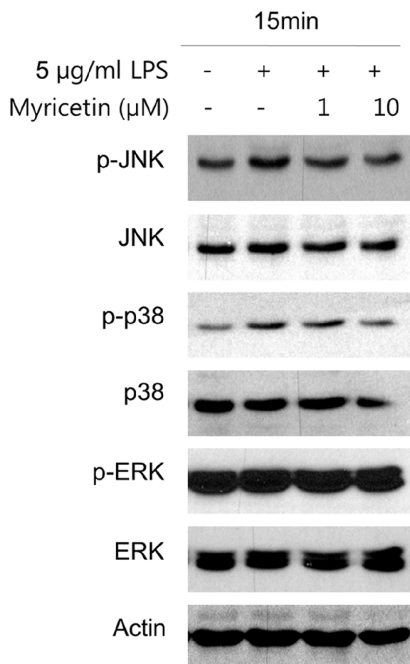


Figure 2. Effect of myricetin on LPS-induced activation of MAPKs. The PDLFs were serum-starved for 30 minutes, pre-treated with myricetin for 2 hours, and stimulated with 1 $\mu\text{g/ml}$ of LPS for 15 minutes. Protein levels of the genes indicated were determined with the use of western blot analysis.

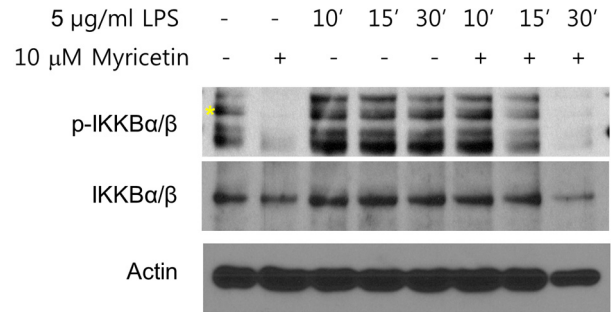


Figure 3. Effect of myricetin on LPS-induced activation of IKK. The PDLFs were serum-starved for 30 minutes, pre-treated with myricetin for 2 hours, and stimulated with 1 $\mu\text{g/ml}$ of LPS for 10, 15, and 30 minutes. Protein levels of the genes indicated were determined with the use of western blot analysis.

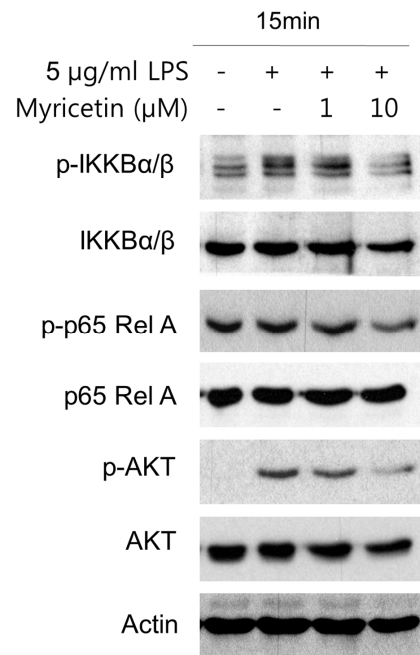


Figure 4. Effect of myricetin on LPS-induced activation of IKK, p65RelA, and AKT. The PDLFs were serum-starved for 30 minutes, pre-treated with myricetin for 2 hours, and stimulated with 1 $\mu\text{g/ml}$ of LPS for 15 minutes. Protein levels of the genes indicated were determined with the use of western blot analysis.

phosphorylation of JNK and p38 was observed following LPS treatment, no marked activation of ERK was observed. Myricetin suppressed JNK and p38 phosphorylation (Fig. 2).

Myricetin inhibits activation of NF κ B-related molecules in LPS-stimulated PDLFs

Since it has been reported that NF κ B can act as an important transcription factor in the expression of inflammatory cytokines by LPS, we investigated whether or not myricetin also has an effect on the activation of NF κ B in PDLFs. We pre-treated

PDLFs with myricetin for 2 hours and then stimulated them with LPS. Subsequently, we prepared protein samples and observed changes in the phosphorylation of IKK. When PDLFs were treated with LPS, an increase in the phosphorylation of IKK was observed after 10 minutes and quantitative changes were observed up to 30 minutes after treatment. In all cases after treatment with LPS and myricetin, the phosphorylation of IKK was suppressed within 15 minutes (Fig. 3). The phosphorylation reaction was observed in IKK, I κ B α , p65RelA, and AKT over a 15-minute period following treatment with LPS. As a result, there was an increase in the phosphorylation of IKK, p65RelA, and AKT in the LPS-treated cells; in the case of myricetin treatment, the increased phosphorylation was suppressed (Fig. 4).

Studies in RAW264.7 cells

Myricetin inhibits osteoclast generation in RAW264.7 cells

Pre-treatment with RANKL and LPS significantly induced osteoclast generation in RAW264.7 cell culture on day 7. Myricetin at concentrations of 0.2, 1, and 5 μ M suppressed the formation of RANKL-pre-treated, LPS-induced multinucleated osteoclasts (Fig. 5A and 5B). TRAP activity was measured in the cell lysates from LPS-stimulated RAW264.7 cells using spectrophotometry. Myricetin inhibited TRAP activity at concentrations of 1 and 5 μ M, as compared with the LPS-induced control (Fig. 5C).

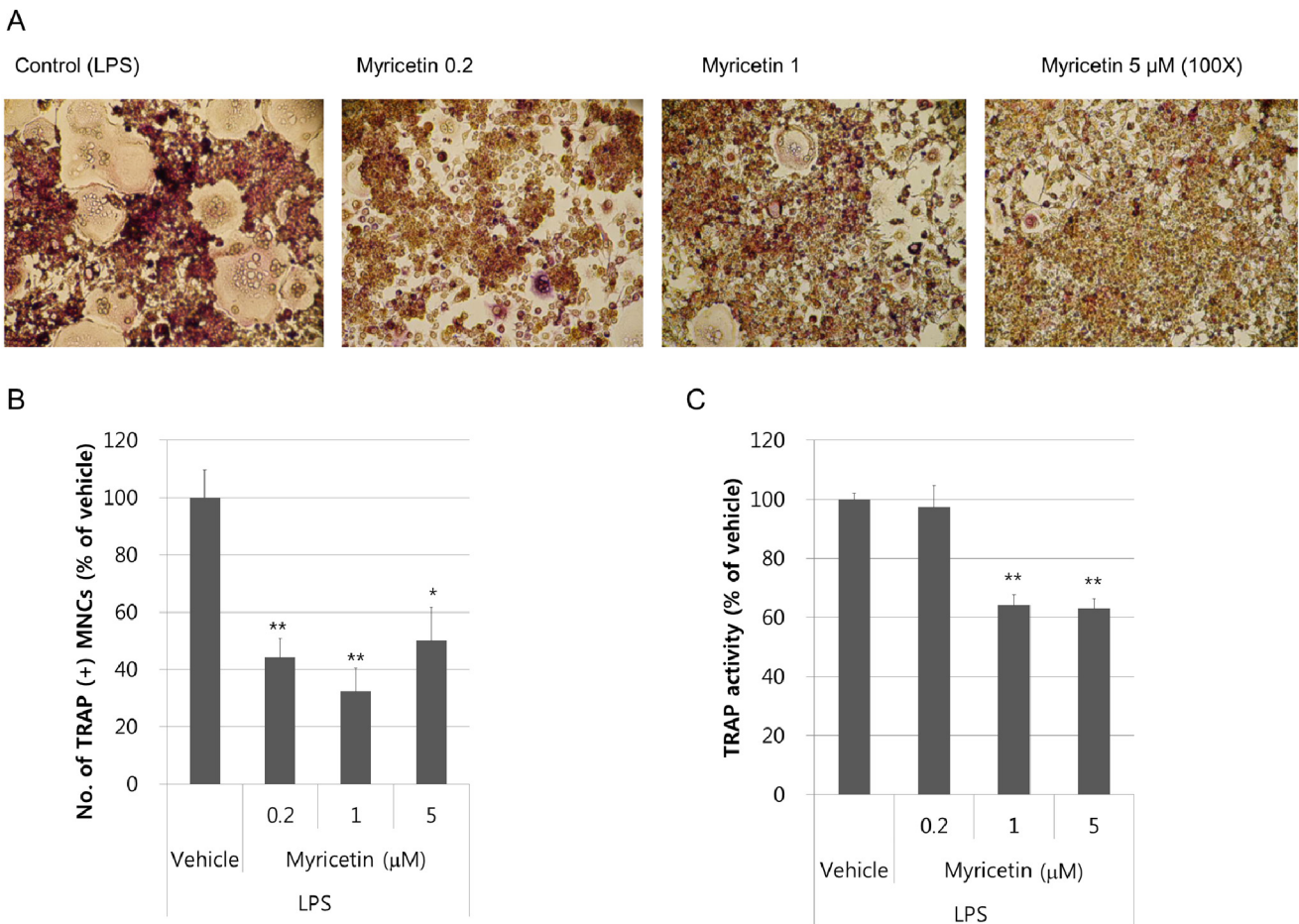


Figure 5. Effect of myricetin on osteoclastogenesis in RAW264.7 cells. RAW264.7 cells were seeded in 96-well plates and cultured for 1 day with 100 ng/mL of RANKL and for 4 to 7 days in the presence of 1 μ g/mL of LPS and various concentrations of myricetin. (A) After culture, the cells were fixed and stained for TRAP expression and photographed at $\times 100$. Arrows indicate multinucleated cells. (B) TRAP-positive multinucleated cells with three or more nuclei were counted. Values are expressed as means \pm SE (n=3). *p<0.05; **p<0.01. (C) Assessment of TRAP activity. After culture, enzyme activity was measured by means of spectrophotometry using *p*-nitrophenyl phosphate as a substrate in tartrate buffer. Total protein content was determined using the BCA protein assay reagent. Values are expressed as means \pm SE (n=3). **p<0.01.

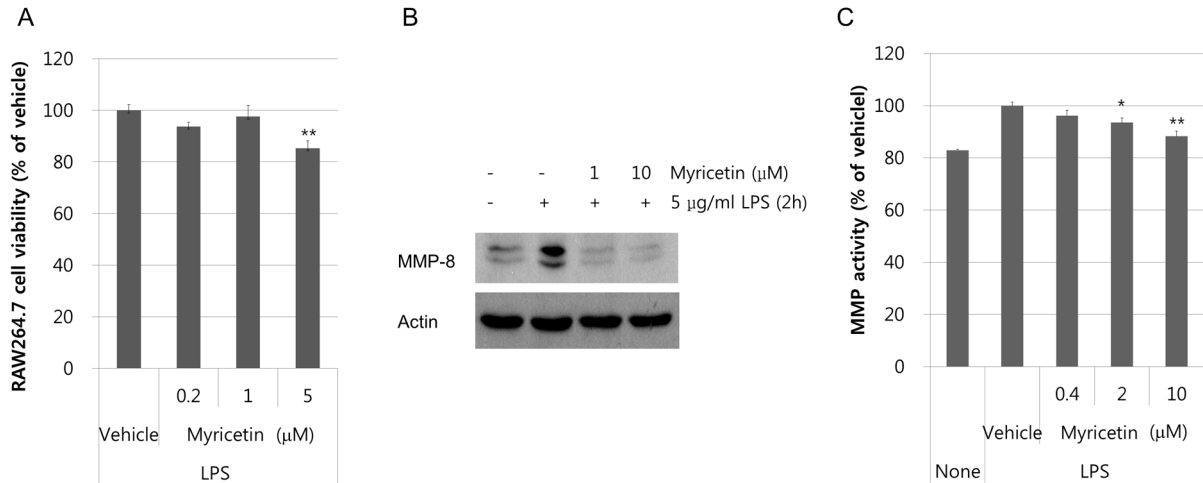


Figure 6. (A) Effect of myricetin on RAW264.7 cell viability. Cell viability was assessed using an MTT assay and is expressed as the absorbance ratio (i.e., absorbance of the treated cells/absorbance of control cells, $\times 100$). Values are expressed as means \pm SE ($n=3$). ** $p<0.01$. (B) Effect of myricetin on LPS-induced protein expression. The RAW264.7 cells were stimulated with 5 $\mu\text{g}/\text{mL}$ of LPS for 2 hours. Levels of protein expression were determined with the use of western blot analysis. (C) Effect of myricetin on LPS-induced MMP-8 activity. RAW264.7 cells were treated with myricetin and 1 $\mu\text{g}/\text{mL}$ of LPS for 24 hours. After culture, the supernatants were assessed using a fluorescence assay kit. Fluorescence was recorded at excitation/emission rates of 340/490 nm. Values are expressed as means \pm SE ($n=3$). * $p<0.05$; ** $p<0.01$.

Myricetin inhibits LPS-activated expression and enzyme activity of MMPs in RAW264.7 cells

Myricetin decreased cell viability at a concentration of 5 μM in RAW264.7 cells (Fig. 6A). The protein expression of the MMP-8 gene was assessed with western blot analysis. The protein expression of MMP-8 was stimulated with LPS. Myricetin inhibited the induction of MMP-8 expression in RAW264.7 cells (Fig. 6B). MMP-8 enzyme activity was measured in the culture supernatants from the LPS-stimulated RAW264.7 cells using a fluorescent assay. Myricetin inhibited the MMP-8 activity at 2 and 10 μM , as compared with the LPS-induced control (Fig. 6C).

Myricetin does not suppress phosphorylation of MAPK in LPS-stimulated RAW264.7 cells

The basic mechanisms of action of myricetin during osteoclast generation were evaluated by assessing myricetin's effect on the phosphorylation of MAPK. RAW264.7 cells were pre-treated with myricetin for 2 hours and then induced with LPS for 15 minutes. The activation of MAPK components JNK, ERK1/2, and p38 was assessed by western blot analysis. LPS induced the phosphorylation of JNK, ERK1/2, and p38 within 15 minutes. LPS-induced phosphorylation of JNK, ERK1/2, and p38 was not changed by myricetin. In addition, LPS induced the degradation of I κ B α , whereas myricetin induced a slight recovery from the LPS-activated degradation of I κ B α (Fig. 7).

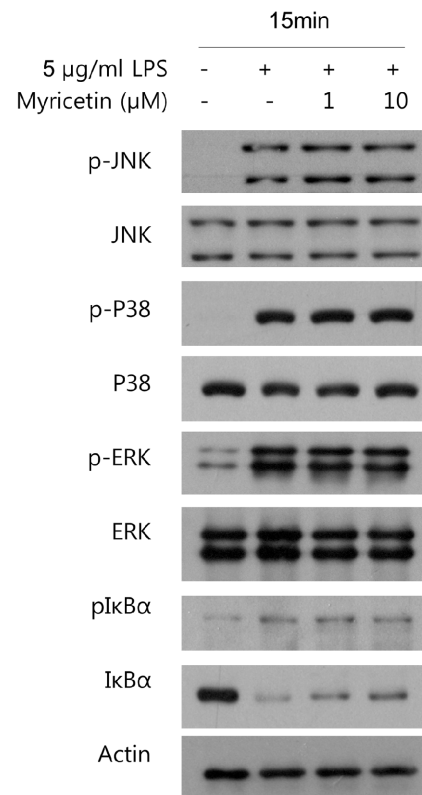


Figure 7. Effect of myricetin on LPS-induced activation of MAPKs and LPS-induced degradation of I κ B α . The RAW264.7 cells were serum-starved for 30 minutes, pre-treated with myricetin for 2 hours, and stimulated with 1 $\mu\text{g}/\text{mL}$ of LPS for 15 minutes. Protein levels of the genes indicated were determined with the use of western blot analysis.

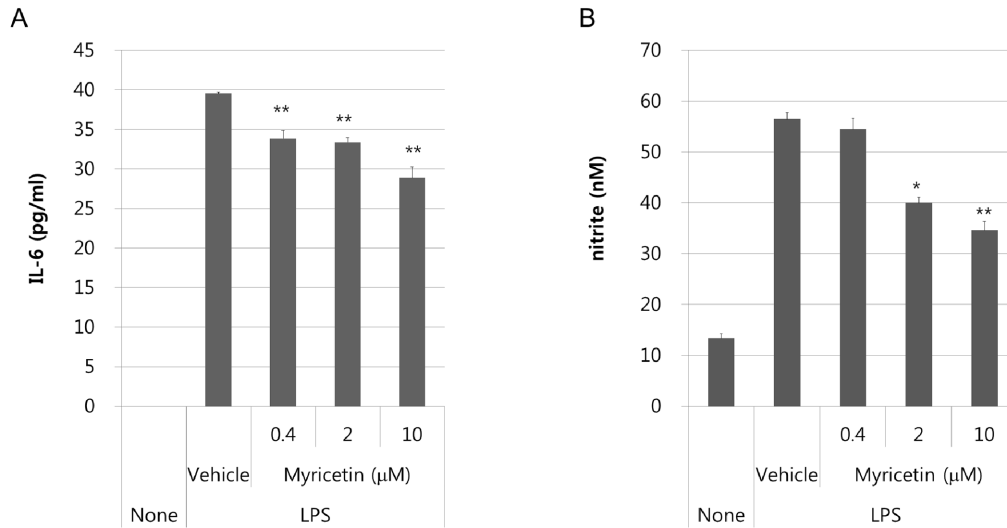


Figure 8. Effects of myricetin on LPS-induced interleuin-6 (IL-6) (A) and nitric oxide (NO) secretion (B). RAW264.7 cells were cultured with 1 $\mu\text{g}/\text{mL}$ of LPS for 48 hours. After culture, the supernatants were measured using ELISA kits and Griess reagent. Values are expressed as means \pm SE (n=3). *p<0.05; **p<0.01.

Myricetin suppresses IL-6 and NO release in LPS-stimulated RAW264.7 cells

To determine whether myricetin was capable of reducing the expression of inflammatory cytokines that was increased when RAW264.7 cells were stimulated with LPS, we treated RAW264.7 cells with LPS and compared the expression levels of IL-6 using the ELISA method and measured NO with the Griess reagent assay. The amounts of secreted IL-6 and NO were assessed by ELISA and Griess reagent assay, respectively. Levels of IL-6 and NO secretion were evaluated to determine whether myricetin inhibited the LPS-stimulated release of these inflammatory cytokines. LPS increased IL-6 and NO production in RAW264.7 cells, whereas myricetin significantly decreased LPS-stimulated IL-6 and NO production (Fig. 8A and 8B).

Discussion

In this study, we assessed the effects of the flavonoid myricetin on the regulation of LPS-activated MMP expression and activity in human PDLFs. We also examined the basic mechanism of action of myricetin in LPS-activated osteoclast generation in murine RAW264.7 cells.

Myricetin is present in many plants and medicinal herbal extracts [23]. Although many studies have shown the efficacy of myricetin, such as its anti-inflammatory effects, in certain

conditions, its targets in periodontal tissues have not been determined.

MMP-1 and MMP-2 transcription levels have been correlated with destructive gingival tissue in patients with chronic periodontitis [27,28], and the amounts of MMP-1 and MMP-2 proteins in gingival crevicular fluid are higher in patients with periodontitis than in periodontally healthy subjects [29]. Accordingly, the levels of MMP-1 and MMP-2 in PDLFs may also be related to the grade of periodontal tissue destruction. Our results showed that myricetin significantly decreased MMP-1 expression and MMP-2 activity in PDLFs when compared with control cells. Therefore, we hypothesized that myricetin may alleviate periodontal disease in gingival tissue.

Previously, MMP-8 was believed to be produced only by polymorphonuclear leukocytes [30]. However, it was more recently reported that MMP-8 is also produced in cells of non-polymorphonuclear lineage, such as gingival crevicular epithelial cells, gingival fibroblasts, and periodontal ligament (PDL) cells [31~33]. Guan et al. [34] reported that infection with *Prevotella intermedia* stimulates MMP-8 expression and activates this protein in PDL cells. In patients with chronic periodontitis, the amount of MMP-8 in the gingival crevicular fluid, gingival tissue, and PDL cells was significantly increased. The amount of MMP-8 was significantly inhibited after periodontal surgical treatment, which suggests that MMP-8 is involved in periodontal tissue degradation [35]. In

our study, myricetin inhibited MMP-8 expression and activity in PDLFs, which suggests that myricetin may suppress connective tissue degradation by repressing MMP-8 activity as well as mRNA and protein expression. In addition, myricetin decreased MMP-8 expression and activity in the RAW264.7 cells. Therefore, inhibition of MMP-8 activity may help manage the progression of periodontal disease.

These results are consistent with an earlier report showing that myricetin decreases LPS-induced MMP-2 and MMP-8 in human gingival fibroblasts (HGFs) [36]. However, a present study showed that myricetin is less effective in decreasing MMP activities in PDLFs, as compared with HGFs. In our report we used western blot analysis to confirm the actions of myricetin on the MMP changes in PDLFs.

Since we were able to verify that myricetin reduced the expression and activity of MMP-1, MMP-2, and MMP-8 in PDLFs, we also investigated its effects on intracellular signal transduction in order to determine what kind of molecular mechanism was responsible for these effects. In observing the changes in the activity of MAPKs in response to LPS, we found that LPS increased the activity of JNK, and these effects were suppressed by myricetin.

In addition, myricetin's effects were observed with respect to the NF κ B-related signal transduction system. The regulation of phosphorylation of RelA is also known to play an important role in activating the NF κ B pathway [37]. It is known that kinases such as casein kinase II, AKT, and IKK are capable of inducing the phosphorylation of RelA [37]. In our study as well, RelA phosphorylation was increased by treatment with LPS, and increases in the phosphorylation of IKK and AKT were also observed. In cases where the PDLFs were treated with myricetin, this increase in phosphorylation was suppressed. From these results, it is believed that the suppression of RelA phosphorylation and the resulting decrease in DNA-binding capacity also act as important mechanisms in the decrease in NF κ B transcription activity in response to myricetin.

We also tested the effects of myricetin on osteoclast generation in murine RAW264.7 cells. Myricetin suppressed the generation of osteoclast-like cells, and there was no evidence of cytotoxic changes in the RAW264.7 cells after treatment with myricetin at concentrations between 0.2 and 1 μ M.

During inflammation, MAPK components participate in the signaling pathways that regulate the synthesis and secretion of pro-inflammatory factors from activated macrophages [38]. In

this study, myricetin did not affect the LPS-activated phosphorylation of MAPK proteins such as JNK, ERK and p38. RANKL induces the activation of NF κ B via TRAF6 signaling [39]. Activation of NF κ B requires the breakdown of I κ B α , an inhibitory protein that complexes with NF κ B dimers [40]. In this study, myricetin led to recovery of the slightly degraded I κ B α .

PDLFs are involved not only in the differentiation of osteoblasts and the formation of bone, but they are also known to be involved in the inflammatory response when periodontal tissue is exposed to bacteria, during which cytokine secretion is promoted [41]. Accordingly, after treatment with myricetin, we observed a reduction in the secretion of cytokines from PDLFs. IL-6 is an activator of bone destruction, and excess production of this cytokine is involved in periodontal tissue destruction [42]. In this study, myricetin suppressed LPS-activated IL-6 release in monocytes.

In addition, inflammatory factors, such as NO, are involved in bone loss [43]. NO is produced by the salivary glands in the mouth and is secreted into the saliva. The microorganisms that induce inflammatory infections are known to further promote the production of NO by oral tissues by stimulating immune cells such as macrophages. Recently, it has been demonstrated that NO is a target for the development of therapies in patients with periodontal disease because NO plays an important role in the tissue degradation associated with such diseases [44]. In the current study, myricetin decreased LPS-activated NO secretion in RAW264.7 cells.

In conclusion, our results showed that myricetin exerts suppressive effects on periodontal tissue degradation and osteoclastogenesis and, in this way, plays a role in the inhibition of NF κ B-related protein. Therefore, inhibition of periodontal tissue destruction may be a plausible therapeutic strategy for the management of patients with inflammatory periodontal disease. However, we believe it will be necessary to study animal models of periodontitis in order to verify whether myricetin can actually be of benefit in the clinical setting.

Acknowledgements

The present research was conducted by the research fund of Dankook University in 2014.

Conflict of interest

The authors declare no conflict of interest.

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