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Short communication

MYRICETIN BLOCKS LIPOTEICHOIC ACID-INDUCED COX-2 EXPRESSION IN HUMAN GINGIVAL FIBROBLASTS

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Abstract: Periodontitis is an infectious disease caused by microorganisms present in dental bacterial plaque. Lipoteichoic acid (LTA) is a component of the external membrane of Gram-positive bacteria. It causes septic shock. Ingested flavonoids have been reported to directly affect the regulation of cyclooxygenase-2 (COX-2) expression induced by bacterial toxins. In this study, we examined the effects of four flavonoids (luteolin, fisetin, morin and myricetin) on the activation of ERK1/2, p38 and AKT, and on the synthesis of COX-2 in human gingival fibroblasts treated with LTA from *Streptococcus sanguinis.* We found that luteolin and myricetin blocked AKT and p38 activation and that myricetin blocked LTA-induced COX-2 expression. The results of our study are important for elucidating the mechanism of action of flavonoid regulation of inflammatory responses.

Keywords: Periodontitis, Fisetin, Morin, Myricetin, Luteolin, Human gingival fibroblasts, Lipoteichoic acid, Cyclooxygenase

INTRODUCTION

Periodontitis is a progressive disease that affects the dentition-supporting tissues, including the gums, periodontal ligaments and alveolar bone [1–3]. The principal result of advanced periodontitis is tooth loss. Periodontitis is an infectious disease caused by an accumulation of certain microorganisms that live

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Abbreviations used: COX-2 – cyclooxygenase-2; HGF – human gingival fibroblasts; LTA – lipoteichoic acid; MAPK – mitogen-activated protein kinases; PAMPS – pathogen-associated molecular patterns, SDS – sodium dodecyl sulfate; TLR – Toll-like receptors

in dental bacterial plaque. The response of the host to these oral pathogens is a key determinant in the development of periodontitis [4, 5].

Human gingival fibroblasts (HGFs), the predominant cell type in periodontal connective tissue, form a framework that anchors the teeth. They play an active role in the host defense response. Exposing gingival fibroblasts to pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide, lipoteichoic acid or peptidoglycan, promotes the expression of inflammatory cytokines such as the interleukins IL-1β, IL-6 and IL-8, and the enzyme cyclooxygenase [6–11]. HGFs are thought to play an important role in promoting inflammatory responses.

Toll-like receptors (TLRs) play a significant role in PAMP recognition and the induction of molecules that act primarily to reduce the action of pathogens, but also favor the production of pro-inflammatory molecules and the development of chronic periodontitis [12–15]. Lipoteichoic acid (LTA) is a pathogen-associated molecular pattern obtained from Gram-positive bacteria. It associates with TLR-2 and is responsible for septic shock via its promotion of the expression of pro-inflammatory cytokines. The premise that periodontal pathogens or their components are associated with the development of periodontal disease has spurred extensive research into ways to control the disease by regulating activated intracellular signaling pathways [16–19].

There is growing evidence that plant-derived foods and beverages contribute to the healthy development and status of blood vessels and connective tissues. In particular, the large family of plant-derived polyphenols has potential benefits, including putative positive effects on obesity and cardiovascular diseases. Fisetin (3,3',4',7-tetrahydroxyflavone) is a polyphenolic flavonoid found in various fruits and vegetables, including strawberries, mangoes, apples, grapes, cucumbers and onions [20, 21]. Fisetin has been reported to show antioxidant [22], anti-tumor [23], anti-diabetic [24], anti-aging [25] and neuroprotective [25] properties. Morin (3,2',4',5,7-pentahydroxyflavone) and myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromenone) are structurally related flavonols, a common flavonoid type. Morin is abundant in fig and almond shells. Like fisetin, it has been reported to have anti-tumor [26], anti-oxidant [27] and anti-inflammatory [28] properties. Myricetin, the predominant flavonoid in grapes, blackberries, onions and red wine [29], has been shown to inhibit COX-2 synthesis, AP-1 activation and cyclin expression in epidermal cells treated with phorbol esters [31–35].

Among its other functions, the carotenoid luteolin (3',4',5,7-tetrahydroxy-flavone) protects the endothelium from superoxide anions and high glucose levels [36]. It has inhibitory effects on tumor growth [37] and can reduce inflammation [38, 39]. In previous studies, we showed that luteolin blocked AKT-mediated nitric oxide production in gingival fibroblasts treated with lipopolysaccharide. Here, we sought to evaluate the effects of morin, myricetin, fisetin and luteolin on the actions of LTA in HGFs.

MATERIALS AND METHODS

Compounds

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and SuperScript One-Step RT-PCR reagents were purchased from Invitrogen. Luteolin, morin, myricetin, fisetin, phenylmethylsulfonyl fluoride, sodium dodecyl sulfate, ethylenediaminetetraacetic acid (EDTA), tetrazolium salt, and LTA purified from *Streptococcus sanguinis* were obtained from Sigma Aldrich. All four flavonoids were dissolved in ethanol. Antibodies agonist p38, AKT, γ-tubulin, p50, phospho-ERK (Thr 202/Tyr204), phospho-p38 (Tyr 182), phospho-AKT (within the C-terminal Ser 473) and phospho-AKT (Thr 308), and luminol reagent were purchased from Santa Cruz Biotechnology.

Cell culture

HGFs were obtained from healthy tissue samples from patients at the Exodontia Clinic after informed consent was obtained (FBQ-LIFO-001 ISO 9001:2008). The Ethical Committee of the Universidad Nacional Autónoma de México approved the protocol for our study. Gingival tissue was isolated at the cement–enamel junction of the extracted tooth using a surgical blade. The harvested tissue was rinsed several times in DMEM containing antibiotics (100 U/ml penicillin, 125 μg/ml streptomycin and 5 μg/ml amphotericin). The tissue was cut into small pieces and cultured with a medium containing 10% fetal bovine serum. When the cells that grew from the explants had reached confluence, they were detached using 0.025% (w/v) trypsin in PBS for 10 min and subcultured in flasks. The cells that remained attached to the bottom of the flask were discarded in order to avoid contamination with epithelial cells, which are not as easily detached as fibroblasts. The HGFs were cultured in a humidified atmosphere of 5% CO_2 at 37ºC. The cell cultures used in all of the experiments were between passages 5 and 10 [40].

Western blot analysis

At the end of treatment, the cell culture medium was aspirated and the cells were detached in PBS with 1 mM Na_3VO_4 by scraping. Detached cells were transferred to fresh microcentrifuge tubes and centrifuged at 5000 rpm and 4ºC for 10 min. The cell pellets were then lysed in 10 μl lysis buffer consisting of 0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, 1% Nonidet P-40, 0.5 M phenyl-methylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM sodium pyrophosphate (all obtained from Sigma Chemical Co.). The protein concentrations were determined using the standard Bradford assay. For western blotting, an equal amount of protein from each sample was loaded onto SDS-PAGE gel and electrotransferred onto PVDF membranes (Invitrogen Life Technologies). These membranes were then blocked with 5% (w/v) nonfat milk in TBST consisting of 10 mM Tris, 150 mM NaCl and 0.1% (v/v) Tween 20 (pH 7.5) for 1 h at room temperature and incubated with primary antibodies overnight at 4ºC. Secondary

antibody incubation was at room temperature for 2 h (Santa Cruz Biotechnology). Chemiluminescence ECL (Amersham) was used to detect immunoreactive protein signals. Protein signals were then visualized on films and scanned and quantified using the Digi-Doc software. For re-probing, PVDF membranes were stripped with 0.2 M NaOH for 10 min before blocking with another primary antibody. The expression of the protein of interest was determined relative to the basal level.

RT-PCR analysis of inducible COX-2

Total cellular RNA was isolated from human gingival fibroblasts using the method described by Chomczynski and Sacchi [41]. Total cell RNA (1 μg) was reverse transcribed using the One Step RT-PCR kit (Invitrogen). PCR was performed using the oligonucleotides 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' (coding sense) and 5'-AGATCATCTCTGCCTGAGTATCTT-3' (anticoding sense) derived from the COX-2 gene and 5'-AGATCCACAACGGATACATT-3' (anticoding sense) derived from the glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene [42]. PCR amplification conditions included denaturing at 94ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 1.5 min; PCR was carried out for 35 cycles. Fragment identity was characterized based on the apparent fragment size using ethidium bromide-stained agarose gels. Five independent experiments were performed for each treatment. Data were analyzed with LabsWorks 4.0 commercial software. Each densitometric value was expressed as mean ± S.D.

Statistical analysis

Each experiment was repeated at least three times. The quantified results are presented as the means ± SD. Significant differences were determined using factorial analysis of variance (ANOVA). $p < 0.05$ was considered significant difference.

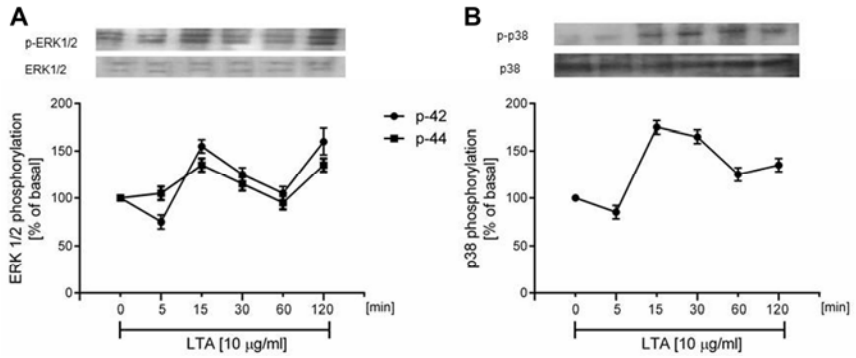

Fig. 1. LTA treatment promotes the phosphorylation of ERK1/2 and p38 in HGFs. The fibroblasts were incubated with LTA (10 μg/ml) for the time intervals indicated. The cell lysates were separated by electrophoresis in SDS-PAGE gels, then transferred to PVD membranes and immunoblotted with (A) anti-phosphorylated ERK1/2 and (B) anti-phosphorylated p38. The membranes were then stripped and incubated with anti-ERK1/2 and anti-p38. The results shown are representative of three separate experiments. SEMs were obtained by densitometry. *$p < 0.05$; significantly different from the values for cells treated with LTA only.

RESULTS

LTA from *S. sanguinis* promotes MAPK activation

When HGF cultures were exposed to LTA (10 μg/ml), a time-dependent increase occurred in the phosphorylation patterns of the MAPKs (mitogen-activated protein kinases), ERK1/2 and p38. The maximum LTA effects were observed 15 min after the LTA treatment (Fig. 1). Densitometric analysis showed that treatment with LTA promoted phosphorylation of ERK1/2 (Fig. 1A) and p38 (Fig. 1B) to levels that were twofold higher than the basal activity levels.

LTA from *S. sanguinis* induces the activation of AKT and degradation of IκB

We studied the effects of LTA on the activation of AKT, which is a serine/threonine kinase that plays a key role in diverse cellular events, such as

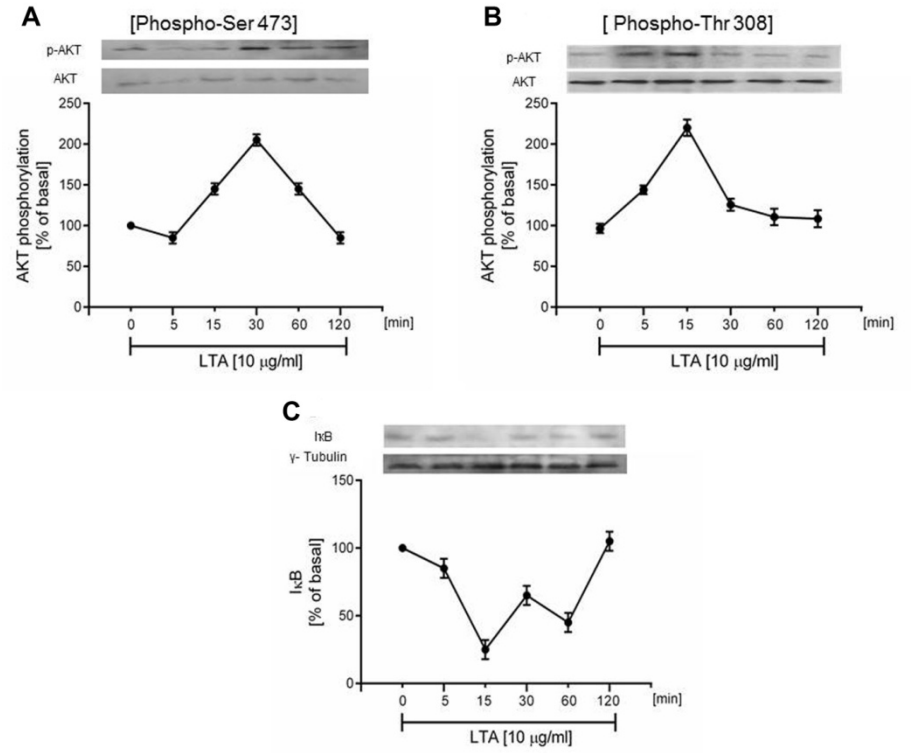

Fig. 2. LTA treatment promotes phosphorylation of AKT and degradation of IκB in HGFs. The fibroblasts were incubated with LTA (10 μg/ml) for the time intervals indicated. The cell lysates were separated by electrophoresis in SDS-PAGE gels, transferred to Hybond-P membranes and immunoblotted with (A) – anti-phosphorylated-AKT (Ser 473), (B) – anti-phosphorylated-AKT (Ser 308) and (C) – anti-IκB antibodies. The membranes were stripped and incubated with AKT or α-tubulin. The results shown are representative of three separate experiments. SEMs were obtained by densitometry. *$p < 0.05$; significantly different from the values for cells treated with LTA only.

apoptosis and cell proliferation. We found an increase in AKT phosphorylation at serine 308 for 15 min after the exposure to LTA. Maximal phosphorylation was detected after 30 min and phosphorylation levels returned to basal levels by 120 min after LTA treatment (Fig. 2A). Similar results were obtained with AKT phosphorylation at serine 473 (Fig. 2B). We then studied the effects of LTA on IκB. LTA promoted the degradation of IκB 15 min after initiation of the treatment. This degradation response was sustained for up to 60 min and returned to basal levels by 120 min (Fig. 2C).

Flavonoids inhibit LTA-induced MAPK phosphorylation

In order to evaluate the effects of the flavonoids on LTA-induced ERK1/2 phosphorylation, we incubated cells with luteolin, fisetin, morin and myricetin (10 μM). All of the tested flavonoids blocked ERK1/2 and p38 activation after

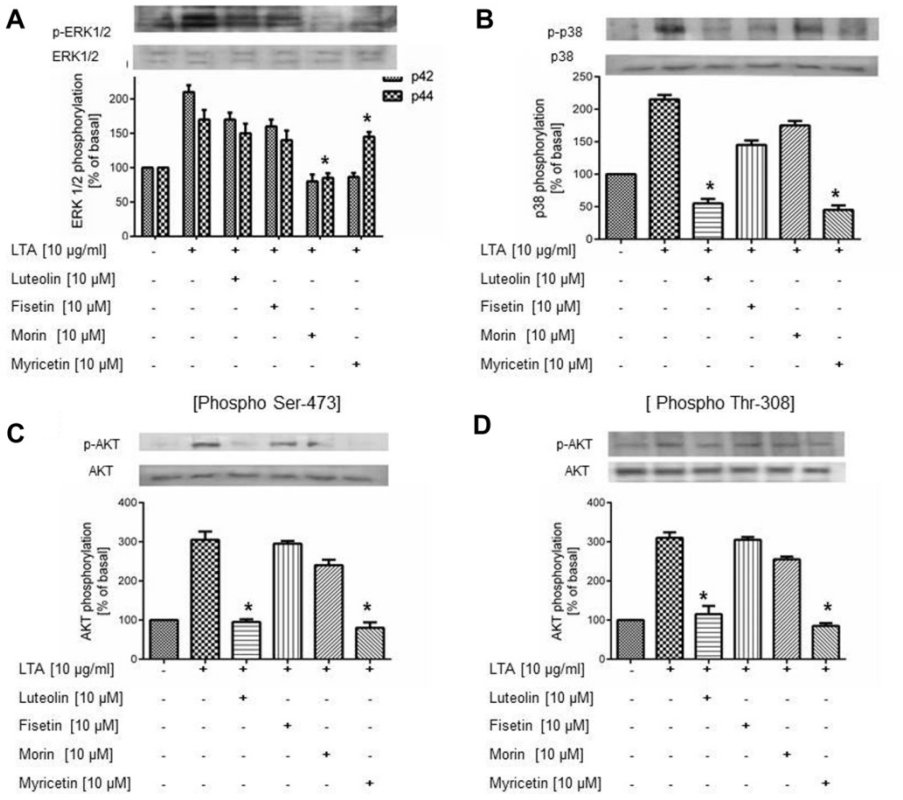

Fig. 3. Flavonoids inhibit LTA-induced phosphorylation of ERK1/2, p38 and AKT in HGFs. The cells were incubated with the flavonoids (10 μM) for 30 min and then treated with LTA (10 μg/ml) for 15 min. Cell lysates were separated in SDS-PAGE gels, transferred to Hybond-P membranes, and incubated with anti-phosphorylated ERK1/2 (A), anti-phosphorylated-p38 (B) and anti-phosphorylated-AKT (C) antibodies. The results are representative of three separate experiments. SEMs were obtained by densitometry. *$p < 0.05$; significantly different from the values for cells treated with LTA only.

15 min of treatment. Morin and myricetin treatment significantly reduced ERK1/2 phosphorylation (Fig. 3A). We found that luteolin, fisetin and myricetin inhibited p38 phosphorylation (Fig. 3B) and that luteolin and myricetin inhibited the AKT phosphorylation (Ser-473 and Ser 308) induced by LTA (Fig. 3C and D).

Flavonoids inhibit LTA-induced increases in COX-2 transcription and translation

Incubating HGFs with LTA significantly increased COX-2 transcription and translation. The increase in COX-2 transcription induced by LTA was markedly attenuated by treatment with the studied flavonoids. Treatment with luteolin caused a reduction in COX-2 transcription to below basal levels (Fig. 4A). Likewise, as shown in Fig. 4B, LTA stimulated COX-2 protein expression to a degree up to three times over the basal levels. Treatment with the flavonoids significantly attenuated LTA-mediated COX-2 expression.

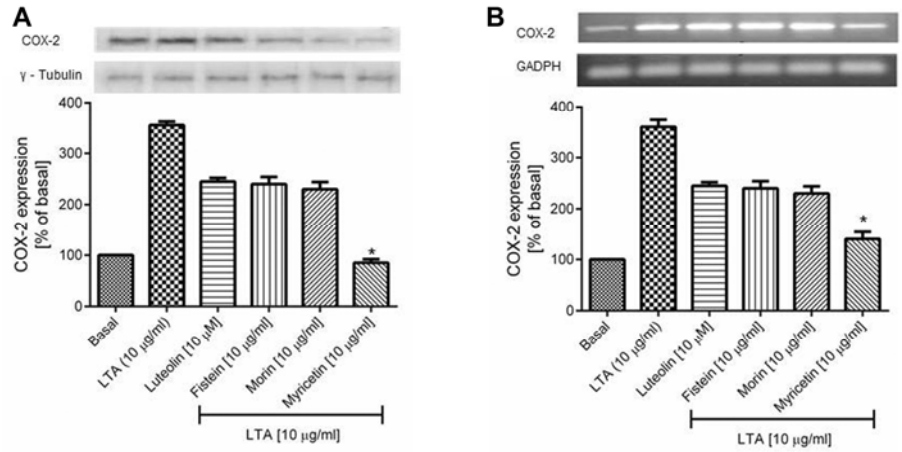

Fig. 4. Flavonoids inhibit LTA effects on the transcription and translation of COX-2 in HGFs. A – Cells were pretreated with flavonoids (10 μM) for 30 min before being treated with LTA (10 μg/ml) for 4 h. Cellular lysates were processed by SDS-PAGE and membranes were blocked and incubated with antibodies that recognized COX-2. To confirm that the same protein concentration was used, the membranes were stripped and incubated with γ-tubulin. The results are representative of three separate experiments. SEMs were obtained by densitometry. *$p < 0.05$; significantly different from the values for cells treated with LTA only. B – Cells were treated with a flavonoid (10 μM) for 30 min and subsequently treated with LTA (10 μg/ml) for 4 h. Total RNA was extracted and COX-2 mRNA induction was determined using RT-PCR. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as a control. Densitometric analyses represent the means and SEMs of five separate experiments. *$p < 0.05$; significantly different from the values for cells treated with LTA only.

DISCUSSION

In this study, we examined the effects of four flavonoids (luteolin, fisetin, myricetin and morin) on the regulation of MAPK activity and LTA-induced COX-2 expression in HGFs Our results showed that that luteolin and myricetin inhibit ERK1/2, p38 and AKT phosphorylation in HGFs. We also found that myricetin blocks COX-2 expression.

LTA plays a key role in Gram-positive bacteria and induces various inflammatory mediators responsible for pathophysiological changes. In the periodontium, LTA promotes inhibition of cell growth and proliferation of epithelial cells in the gingival tissue [43], and induces higher levels of IL-1β than those induced by LPS [43]. LTA-induced iNOS expression, NO release in RAW 264.7 macrophages and LTA-stimulated ERK1/2 activation are mediated through the TLR2 receptor and involve MEK and PI3K activation and NF-κB activation [44]. Here, we found that LTA promotes the activation of ERK1/2 and p38 after 15 min of treatment with LTA (10 μg/ml). Kuo reported similar results [46]. Activation of p38 MAPK has been demonstrated to be involved in LTA-induced COX-2 expression and PGE2 release in human pulmonary epithelial cells [45]. Chiang et al. [46] found that LTA-induced COX-2 synthesis and PGE2 expression were mediated by the activation of PKC and ERK1/2 in macrophages. We previously reported that p38 is involved in COX-2 synthesis and PGE2 release in human gingival fibroblasts [48] and that prostaglandin production is involved in gingival inflammatory processes.

Inhibition of the inflammatory response induced by PAMPs is an attractive therapy target in the treatment of periodontal disease. Natural plant-derived products have been shown to inhibit the expression of the enzyme nitric oxide synthase [49] and to inhibit COX-2 expression in HGFs treated with lipopolysaccharide [3].

On the other hand, flavonoids have anti-inflammatory actions, so it is interesting to investigate the relationship between those actions and the regulation of LTA actions. Huang et al. [50] found that wogonin inhibits LTA-induced nitric oxide gene expression and nitric oxide production in macrophages. We found that luteolin inhibits nitric oxide expression induced by lipopolysaccharide.

Streptococcus sanguinis is a Gram-positive bacterial species that acts as a primary colonizer and promotes plaque aggregation. LTA is a structural component of the external wall and cytoplasmic membrane of Gram-positive bacteria. It is made up of glycerol phosphate polymers and repeating units esterified with D-alanine and α-D-N-acetylglucosamine. The D-alanine content is directly related to the inflammatory potential of LTA. Numerous recent articles have reported that LTA stimulates immune cells to synthesize TNF-α [51], IL-6 [52], IL-8 [53] and nitric oxide [50]. LTA associates with type 2 TLRs and activates kinases such as mitogen-activated protein kinase [54, 55] and AKT [52].

HGFs play an important role in the host defense mechanisms. They recognize and respond to various PAMPs by synthesizing and releasing mediators that

promote inflammatory responses, such as prostaglandins, IL-8, IL-1, IL-6 and TNF-α [50]. Activation of HGF by LTA promotes the secretion of IL-8, IL-1β and COX-2 [43].

Previous studies have shown that flavone derivatives inhibit the IL-1β synthesis induced by *S. sanguinis*-derived LTA in H9c2 mouse cardiomyocyte-derived cells [56]. Likewise, a number of studies have suggested that flavones can affect the production of inflammatory cytokines in activated cells. Treatment with luteolin or quercetagetin has been shown to inhibit the production of IL-1β in H9c2 cells and the activation of mitogen-activated proteins such as ERK1/2, JNK, p38 and AKT.

In conclusion, our experiments showed that flavonoids inhibit the effect of LTA on the activation of various kinases in HGFs. In particular, we found that myricetin inhibits LTA-induced COX-2 expression. These findings provide a good reason to continue studying the effects of flavonoids, especially myricetin, on inflammatory responses in the context of the development of periodontal disease.

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