

# Myricetin Inhibition of Peptidoglycan-Induced COX-2 Expression in H9c2 Cardiomyocytes

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**ABSTRACT:** Peptidoglycan (PGN) is a cell wall constituent in dental plaque bacteria that triggers inflammatory responses. PGN binds Toll-like receptors, leading to increases in prostaglandin E2 and interleukin-1 $\beta$ , which play crucial roles in the inflammatory response and tissue destruction. Dental surgery can give plaque bacteria access to blood circulation, thereby creating a risk of septic inflammation of the endocardium. Plant-derived flavonoids have been reported to reduce inflammatory cytokine secretion by host cells. In the present study, we investigated the effects of flavonoid myricetin on expression of cyclooxygenase 2 (COX-2) in the H9c2 cells treated with PGN from *Streptococcus sanguinis*, a bacterial constituent of dental plaque associated with infective endocarditis. Myricetin exposure resulted in dose-dependent suppression of PGN-induced COX-2 expression, diminished phosphorylation of p38, extracellular signal regulated kinase 1/2, and c-Jun N-terminal kinase, and reduced I $\kappa$ B- $\alpha$  degradation, consistent with decreased COX-2 activity. In conclusion, the aforementioned results suggest that myricetin is useful for moderating the inflammatory response in infective endocarditis.

**Keywords:** infective endocarditis, peptidoglycan, flavonoids, myricetin, COX-2

## INTRODUCTION

Dental plaque is a complex biofilm made up of an amalgamation of more than 500 kinds of Gram-positive and -negative microbial species (Dewhirst et al., 2010), early colonizing species are associated with dental caries (Hamada and Slade, 1980; Yamashita et al., 1993). Although *Streptococcus sanguinis* has been reported to have preventative effects (Kuramitsu et al., 2007; Nyvad and Kilian, 1987; Xu et al., 2007), it is often a late colonizer of subgingival plaque, particularly when homeostasis in the oral environment has been disrupted (McNicol and Israels, 2010). *S. sanguinis* produce pathogen-associated molecular patterns (PAMPs), such as peptidoglycan (PGN) and lipoteichoic acid (LTA), which stimulate the immune system through binding Toll-like receptors (TLRs).

PGN, which is composed of  $\beta$ -1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid cross-linked by peptides, is a major bacterial cell wall constituent (Paik et al., 2005; Ge et al., 2008). TLRs are a highly conserved family of transmembrane pathogen recognition receptors. To

date, 13 mammalian TLRs have been characterized, each of which responds to different pathogen molecules (Xu et al., 2007; Paik et al., 2005). TLRs are broadly distributed on immune cells and their activation signals production of proinflammatory cytokines, histamine and prostaglandin E2 (Paik et al., 2005; Ge et al., 2008; Lindner et al., 2009; Chi et al., 2011). All TLRs activate common signaling pathways, leading to activation of the transcription factor, such as nuclear factor (NF)- $\kappa$ B (Müller-Anstett et al., 2010). TLR signaling is mediated by two pathways, a myeloid differentiation primary response 88 (MyD88)-dependent one that triggers proinflammatory cytokine expression and an MyD88-independent one [Toll/interleukin (IL)-1 receptor-domain-containing adaptor protein inducing interferon- $\beta$  (TRIF)/TRIF related adaptor molecule (TRAM)-mediated] responsible for interferon type I production. TLR4 is unique since it recognizes both MyD88-dependent and MyD88-independent pathways (Müller-Anstett et al., 2010). Binding of ligands to TLR4 enhances activation of downstream signaling molecules, such as mitogen-activated protein kinase (MAPK), extracellular signal regulated kinase (ERK) 1/2, p38, and c-Jun

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N-terminal kinase (JNK) 1/2 (Into et al., 2004; Darieva et al., 2004).

Signaling through the TLR2 pathway is one of the main molecular mechanisms involved in immune cell responses to the Gram-positive pathogen PGN (Dammermann et al., 2013). A fast response to PGN is beneficial to the host at moderate levels by promoting inflammation and priming the immune system to eliminate the invading pathogen; however, an excessive response to PGN can promote chronic inflammation (Sanchez-Lopez et al., 2014; Lee et al., 2015). Dental surgery can enable the spread of bacteria through the bloodstream, which can lead to formation of vegetations in the lining of heart chambers and valves, thereby promoting septic inflammation of the endocardium (Ito, 2006). *S. sanguinis* appears to play an important role in infective endocarditis by increasing proinflammatory cytokines and cyclooxygenase-2 (COX-2), an enzyme that synthesizes proinflammatory prostaglandins, thereby enhancing chronic inflammatory responses (Valera et al., 2007; Chen et al., 2009; Veltrop et al., 1999; Banks et al., 2002).

Flavonoids are a broadly distributed class of plant pigments, universally present in vascular plants, that are responsible for much of the coloring in plants. A variety of flavonoids have been reported to promote anti-inflammatory activity without cytotoxicity (Grenier et al., 2015; Lee and Lee, 2016; Gutiérrez-Venegas et al., 2013; Gutiérrez-Venegas et al., 2017), and high flavonoid dietary intake has been associated with anti-inflammatory benefits (Veltrop et al., 1999). The flavonoid myricetin, which is abundant in tea, berries, fruits, and vegetables (Peterson and Dwyer, 1998), has diverse biological activities, including antioxidative, antiproliferative, and anti-inflammatory effects, as well as purported antidiabetic, anticarcinogenic, antibacterial, neuroprotective, and hepatoprotective activities (Gutiérrez-Venegas et al., 2013, Ong and Khoo, 2000; Yokomizo and Moriwaki, 2006; Puupponen-Pimiä et al., 2001; Molina-Jiménez et al., 2004; Kielian et al., 2005).

Myricetin has been shown to exhibit anti-inflammatory effects through inhibiting of IL-1 transcription and nitric oxide production in PGN-treated RAW 246.7 cells (Gutiérrez-Venegas and González-Rosas, 2017; Gutierrez and Hoyo-Vadillo, 2017; Hiermann et al., 1998; Peterson and Dwyer, 1998). Myricetin suppresses PGN-induced COX-2 expression by H9c2 cells, which are derived from rat heart tissues, suggesting that myricetin exerts anti-inflammatory activity in periodontal disease (Lee and Lee, 2016; Gutiérrez-Venegas et al., 2013; Gutiérrez-Venegas et al., 2017). Additionally, we showed that myricetin had a protective influence on human gingival fibroblasts (HGFs) following LTA-induced activation of inflammatory cytokines (Gutiérrez-Venegas et al., 2013).

In light of these findings and given that PGN is involved in the activation of multiple signaling pathways downstream of TLR2 including MAPK pathways (Gutiérrez-Venegas et al., 2013), the aim of the present study was to evaluate the effects of myricetin on PGN-induced inflammatory responses in H9c2 cardiomyocytes. The effects of myricetin on PGN-activated signaling pathways were assessed with western blot analysis. Because PGN-induced nuclear translocation of NF- $\kappa$ B in response to phosphorylation and degradation of I $\kappa$ B- $\alpha$  promotes expression of inflammatory molecules (Gutiérrez-Venegas et al., 2013; Gutiérrez-Venegas et al., 2017), we also assessed the effect of myricetin on the phosphorylation of I $\kappa$ B- $\alpha$  in whole cell extracts.

## MATERIALS AND METHODS

Super Script One-Step reverse transcription polymerase chain reaction (RT-PCR) reagents were purchased from Invitrogen (Carlsbad, CA, USA). Myricetin (3,5,7,3',4',5'-hexahydroxyflavon, <96%), phenylmethyl sulfonyl fluoride, sodium dodecyl sulfate, ethylenediamine tetraacetic acid, PD98059, SB203580, SP600125, and tetrazolium salt were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies agonist phospho-ERK (Thr 202/Tyr 204), phospho-p38 (Tyr 182), phospho-JNK (Thr 183/Tyr 186), phospho-I $\kappa$ B- $\alpha$ ,  $\beta$ -actin, anti-COX-2, and luminol reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### H9c2 cell culture

Cells were obtained from American Type Culture Collection (lot # 63781507; Manassas, VA, USA) grown in Dulbecco's modified Eagle medium (Sigma-Aldrich Co.) with 10% fetal bovine serum (Invitrogen) and supplemented with penicillin (120 unit/mL), streptomycin (75  $\mu$ g/mL), gentamycin (160  $\mu$ g/mL), and amphotericin B (3  $\mu$ g/mL) in a 5% CO<sub>2</sub> environment. Cells were used between 5 to 9 passages (Ito, 2006).

### Cell treatment

Myricetin was dissolved in dimethyl sulfoxide in a stock concentration 1 mM and a final concentration of 10  $\mu$ M, and was incubated in fresh culture medium 30 min prior to PGN stimulation. Cells were incubated in fresh media and reagents for different periods, then were divided into control group (basal) with a vehicle, myricetin, and myricetin plus PGN. The concentration was used based on previous results by Gutiérrez-Venegas et al. (2013). Cells were pre-incubated with PD98059 (30  $\mu$ M), SB203580 (20  $\mu$ M), SP600125 (30  $\mu$ M), or H89 (10  $\mu$ M) for 60 min before being stimulated with PGN (10  $\mu$ g/mL) for 6 h.

### RT-PCR

Total RNA was isolated from H9c2 cells using Trizol. Total cell RNA (1  $\mu$ g) was reversed transcribed using One Step RT-PCR kit (Invitrogen). PCR was performed using oligonucleotides 5'-TTC AAA TGA GAT TGT GGG AAA TTG CT-3' (coding strand) and 5'-GTA GAG GCA GGG ATG ATG TT-3' derived from COX-2; 5'-GTA GAG GCA GGG ATG ATG TT-3' (anticoding strand) derived from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. PCR amplification conditions included denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min: PCR was carried out for 35 cycles. Amplification was characterized by fragment size on ethidium bromide-stained agarose gels. Three independent experiments were performed for each treatment. Data were analyzed by using LabWorks 4.0 (Labworks LLC., Lehi, UT, USA).

### Western blotting

Cell homogenates were centrifuged at a speed of 11,290 *g* for 10 min. Proteins were extracted with lysis buffer and pulse sonicated. Lysates were cleared by centrifugation and the resulting supernatant was transferred to a new tube. Protein concentrations of the supernatant were quantified with Bradford protein assay reagent kit (Bio-Rad Laboratories, Hercules, CA, USA). Samples (30  $\mu$ g) were separated using sodium dodecyl sulfate (SDS)-pol-

yacrilamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline Tween-20 (TBST) for 1 h, washed, and probed with the respective polyclonal antibodies. Primary antibodies used were anti-phospho-p38 (1:10,000), anti-ERK 1/2 (1:10,000), anti-JNK (1:10,000), anti-I $\kappa$ B- $\alpha$  (1:10,000), and anti-COX-2 (1:10,000) (Santa Cruz Biotechnology). After incubation with primary antibody, membranes were washed with TBST and incubated with secondary antibodies for 1 h. Proteins were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

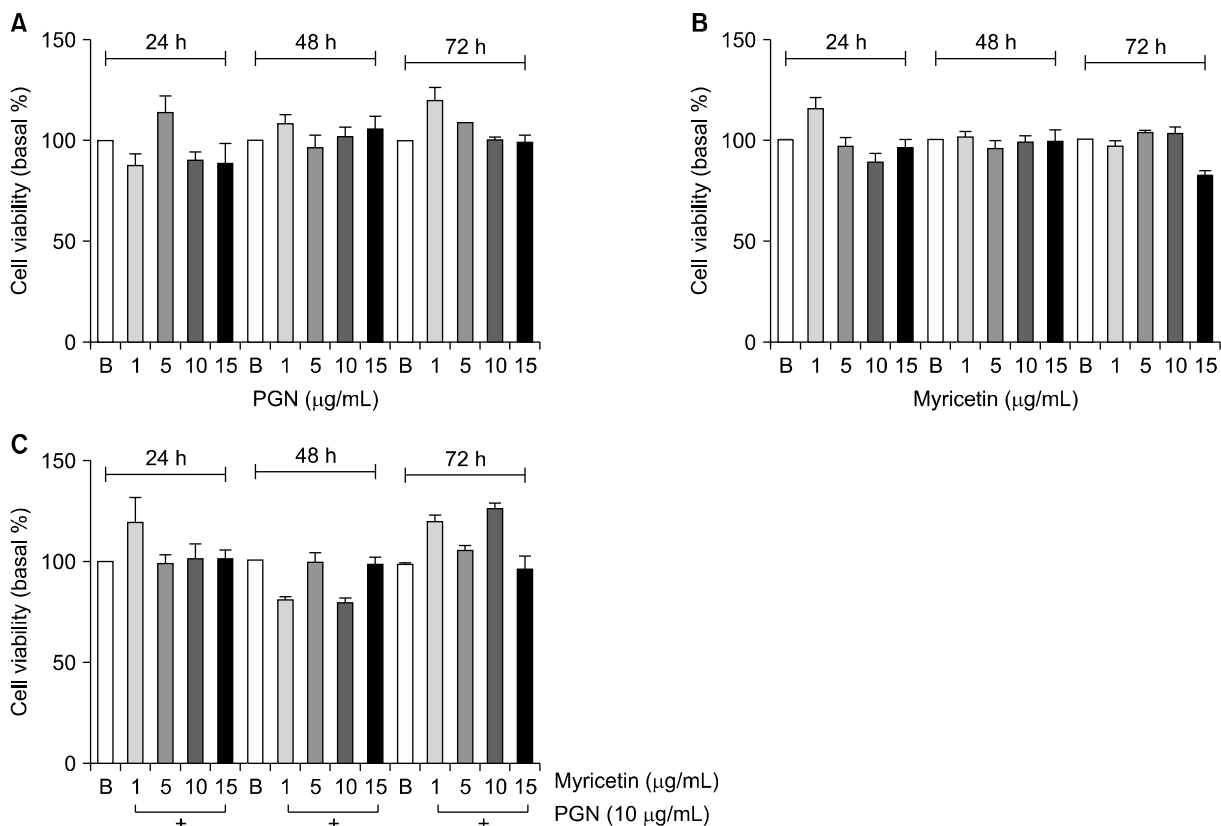
### Statistical analysis

Each experiment was repeated at least three times. Quantified results are presented as mean  $\pm$  standard deviation (SD). Significant differences were determined using factorial analysis of variance.  $P < 0.05$  was considered significant difference.

## RESULTS

### Myricetin and PGN did not disrupt H9c2 cell viability.

The effects of PGN (1 ~ 15  $\mu$ g/mL) and myricetin (1 ~ 15  $\mu$ M) on the viability of H9c2 cells were assessed after 24 h, 48 h, and 72 h. 3-(4,5-Dimethyl-diazol-2-yl)-2,5-di-



**Fig. 1.** Cell Viability in the presence of (A) peptidoglycan (PGN), (B) myricetin, and (C) PGN and/or myricetin. Data are mean  $\pm$  SD (n=3).

phenyltetrazolium bromide (Sigma-Aldrich Co.) assays showed that cell viability was not altered by any of these treatments, confirming that neither compound was cytotoxic at the concentrations employed (Fig. 1).

### PGN promotes MAPK phosphorylation and myricetin inhibited PGN-induced MAPK phosphorylation in H9c2 cells.

PGN (10  $\mu\text{g}/\text{mL}$ ) induced time-dependent MAPK phosphorylation. Maximal pERK1/2 was obtained 15 min into the PGN treatment and ERK1/2 levels were decreasing after 30 min. Maximal p-JNK levels were obtained 30~45 min into the PGN treatment and maximal p-p38 levels were seen after 45 min (Fig. 2). Western blotting confirmed that PGN strongly induced phosphorylation of ERK1/2, p38, and JNK in H9c2 cells, and that these phosphorylation effects were blocked by myricetin in a concentration-dependent manner (Fig. 3).

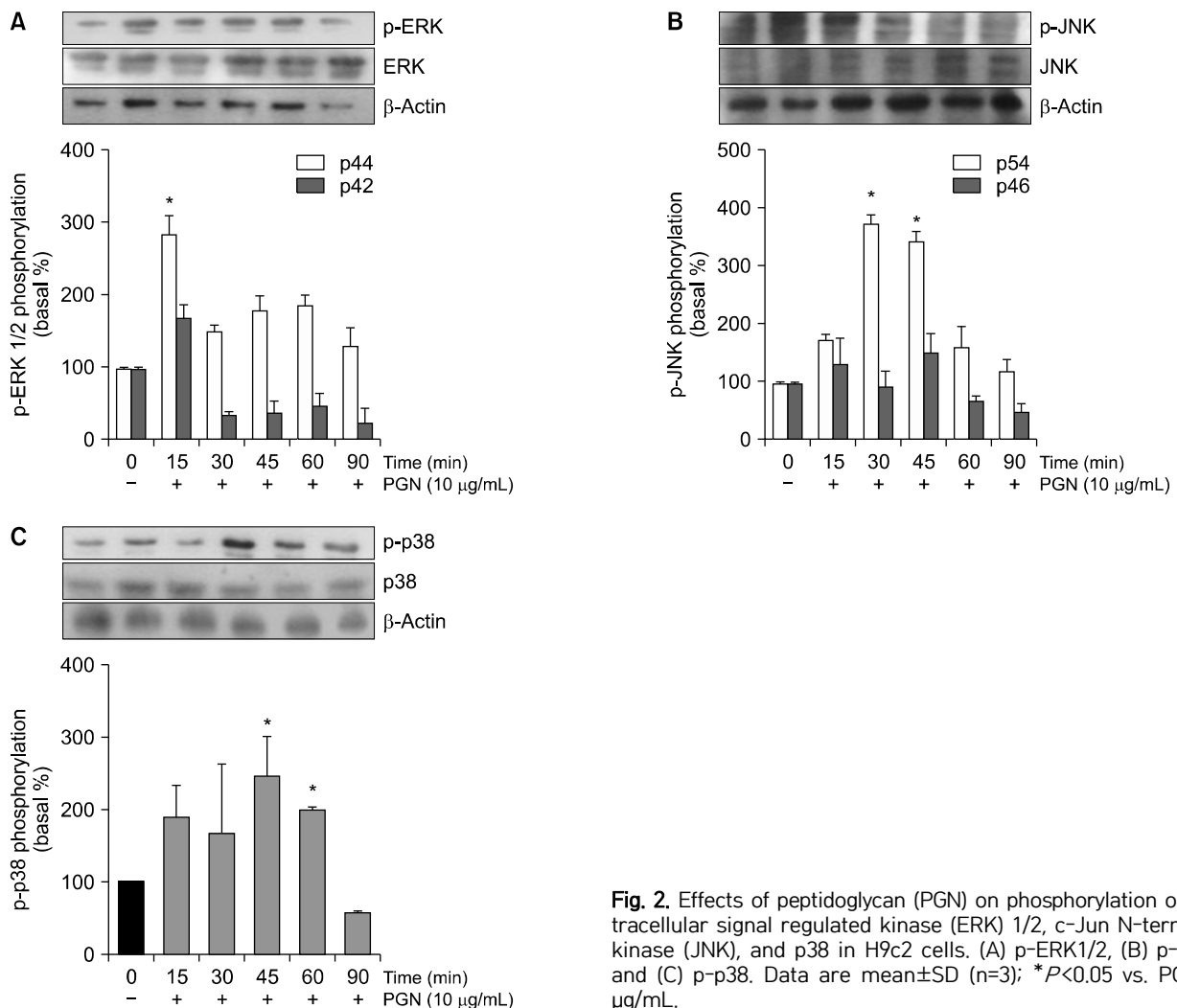
### Myricetin inhibits PGN-induced I $\kappa$ B- $\alpha$ degradation in H9c2 cells.

Extracts of H9c2 cells pretreated with myricetin (1~15

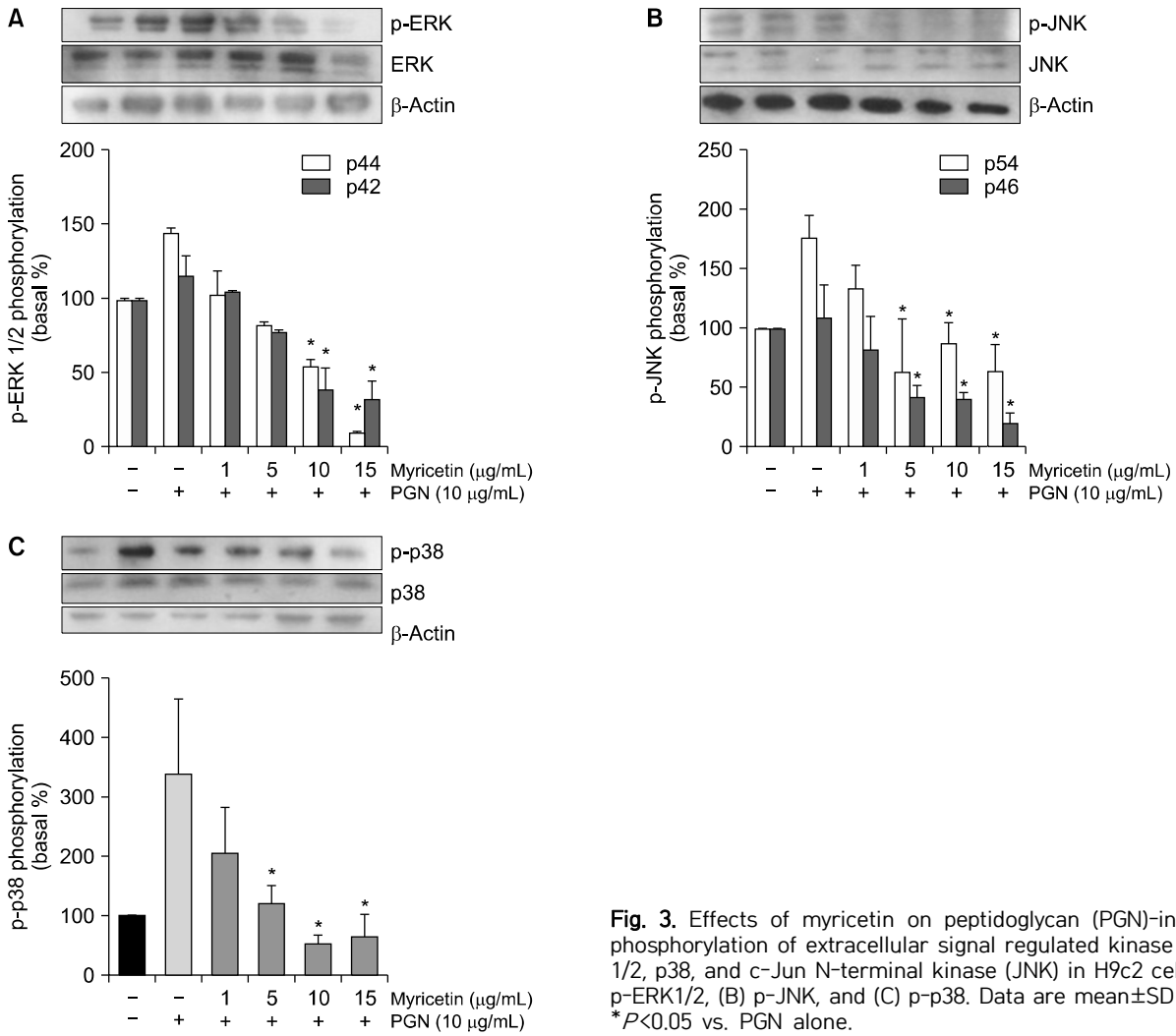
$\mu\text{M}$ ) for 1 h followed by stimulation with PGN (10  $\mu\text{g}/\text{mL}$ ) for 120 min were analyzed for I $\kappa$ B- $\alpha$  levels. As shown in Fig. 4, PGN-stimulated H9c2 cells had increased I $\kappa$ B- $\alpha$  levels at 120 min ( $P<0.05$ ). Myricetin (15  $\mu\text{M}$ ) prevented I $\kappa$ B- $\alpha$  phosphorylation and degradation in a concentration-dependent manner.

### Inhibitory effect of myricetin on PGN-induced COX-2 transcription and translation

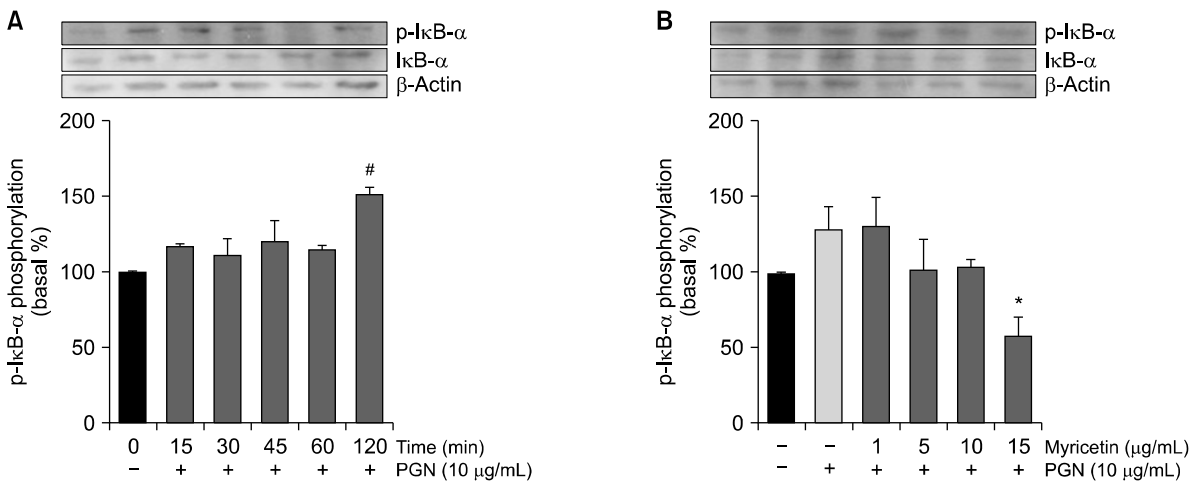
Myricetin (1~15  $\mu\text{M}$  for 30 min) decreased COX-2 expression in H9c2 cells appreciably. Subsequent stimulation of myricetin-pretreated (1~15  $\mu\text{M}$  for 30 min) H9c2 cells with 10  $\mu\text{g}/\text{mL}$  PGN for 6 h promoted a 4-fold increase in expression of COX-2. PGN-stimulated COX-2 secretion decreased by about 90% (Fig. 5). PGN induced a dose-dependent increase in COX-2 protein synthesis, and this expression was modulated by inhibition of the MAPK/ERK kinase (MEK) inhibitor PD98059, indicating that ERK1/2 is involved in COX-2 expression in H9c2 cells (Fig. 5E).



**Fig. 2.** Effects of peptidoglycan (PGN) on phosphorylation of extracellular signal regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK), and p38 in H9c2 cells. (A) p-ERK1/2, (B) p-JNK, and (C) p-p38. Data are mean $\pm$ SD (n=3); \* $P<0.05$  vs. PGN 0  $\mu\text{g}/\text{mL}$ .



**Fig. 3.** Effects of myricetin on peptidoglycan (PGN)-induced phosphorylation of extracellular signal regulated kinase (ERK) 1/2, p38, and c-Jun N-terminal kinase (JNK) in H9c2 cells. (A) p-ERK1/2, (B) p-JNK, and (C) p-p38. Data are mean±SD (n=3); \**P*<0.05 vs. PGN alone.

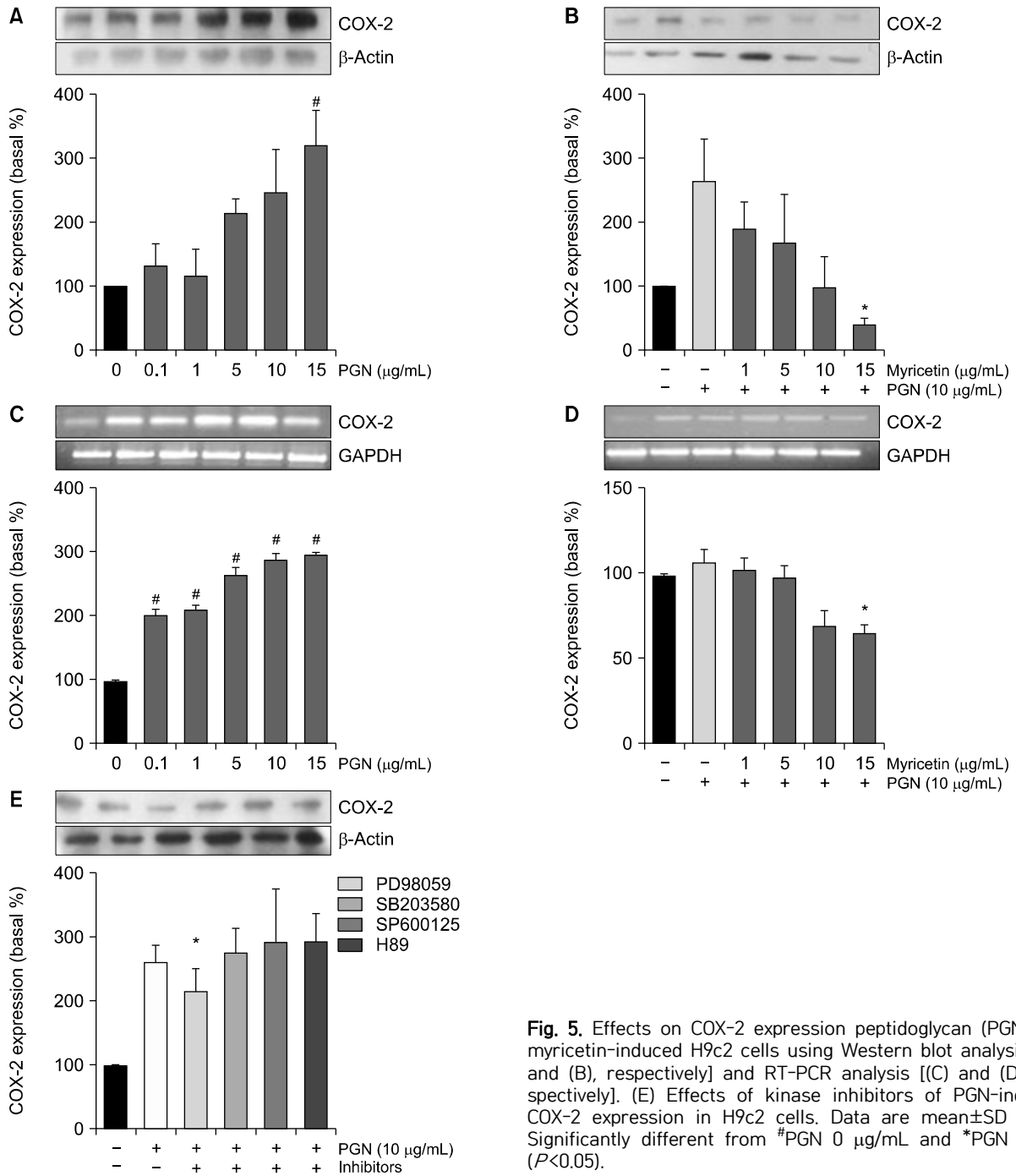


**Fig. 4.** Western blot analysis of IκB-α in H9c2 cells treated with (A) peptidoglycan (PGN) or (B) myricetin. Data are mean±SD (n=3). Significantly different from #0 min and \*PGN alone (*P*<0.05).

**DISCUSSION**

In this study, we evaluated, for the first time, the effect of myricetin on the inflammatory response induced by PGN in H9c2 cells. We showed that myricetin blocks

MAPK phosphorylation stimulated by PGN. In addition, we confirmed that myricetin can attenuate COX-2 expression and found that myricetin blocked activation of NF-κB, probably by arresting IκB-α degradation and translocation of the NF-κB subunit p65 in PGN-stimulated H9c2



**Fig. 5.** Effects on COX-2 expression peptidoglycan (PGN)- or myricetin-induced H9c2 cells using Western blot analysis [(A) and (B), respectively] and RT-PCR analysis [(C) and (D), respectively]. (E) Effects of kinase inhibitors of PGN-induced COX-2 expression in H9c2 cells. Data are mean $\pm$ SD (n=3); Significantly different from #PGN 0  $\mu$ g/mL and \*PGN alone ( $P<0.05$ ).

cells. PGN binding of TLR2 promotes phosphorylation of I $\kappa$ B- $\alpha$ , subsequent I $\kappa$ B- $\alpha$  degradation, and NF- $\kappa$ B translocation to the nucleus, where it regulates expression of proinflammatory mediators (Cho et al., 2007; Ma et al., 2010; Wu et al., 2003).

Our recent study demonstrated that myricetin inhibits induction of COX-2 and prostaglandin E2 in HGFs activated with LTA (Gutiérrez-Venegas et al., 2013). The COX-2 gene promoter contains two major regions that function synergistically in binding regulatory transcription factors, one of which binds NF- $\kappa$ B following PGN activation. A crucial step in NF- $\kappa$ B activation is rapid depletion of cytoplasmic I $\kappa$ B- $\alpha$  protein by proteolytic deg-

radation, which is triggered by phosphorylation of I $\kappa$ B- $\alpha$  at two amino-terminal serine residues. Subsequent ubiquitination marks phosphorylated I $\kappa$ B- $\alpha$  for degradation via the ubiquitin-proteasome pathway. In this study, we demonstrated that myricetin, like other flavonoids (Bali et al., 2014), strongly inhibits I $\kappa$ B- $\alpha$  activity.

MyD88 mediates MAPK activation and NF- $\kappa$ B signaling; MAPK signaling pathways play important roles in PGN transduction pathways. In this study, p-ERK1/2, p-JNK, and p-p38 levels were decreased by myricetin in PGN-stimulated H9c2 cells. These data agree with findings in macrophages, lung fibroblasts, epidermal cells, and umbilical vein endothelial cells (Chen et al., 2009;

Veltrop et al., 1999; Gutiérrez-Venegas and González-Rosas, 2017). Our findings that PGN-induced increases in COX-2 could be blocked the MEK—the enzyme the phosphorylates MAPKs including ERK1/2—suggest that ERK1/2 is involved in COX-2 expression in H9c2 cells and support that notion that MAPK activity is important in the upregulation of proinflammatory cytokines expression via NF- $\kappa$ B in PGN-stimulated H9c2 cells. Additionally, expression of IL-6 and -8 are controlled by the phosphoinositide 3-kinase pathway. Inhibition of either phosphoinositide 3-kinase or MAPK promotes expression of proinflammatory cytokines (Gutiérrez-Venegas et al., 2013; Gutiérrez-Venegas et al., 2017; Gutiérrez-Venegas and González-Rosas, 2017).

The present findings showing that myricetin can modulate PGN triggered cyto-immunological responses in H9c2 cells are consistent with prior reports showing that other flavonoids, including apigenin, epigallocatechin-3-gallate, luteolin, kaempferol, and quercetin exert immune-modulatory effect in H9c2 cells (Hsieh et al., 2013; Feng et al., 2017). Clinical studies are needed to assess the suitability of myricetin for periodontal disease treatment.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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