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**Phloretin inhibits interleukin-1 $\beta$ -induced COX-2 and ICAM-1 expression through inhibition of MAPK, Akt, and NF- $\kappa$ B signaling in human lung epithelial cells**

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**ABSTRACT**

Phloretin, a flavonoid isolated from the apple tree, is reported to have anti-inflammatory, anti-oxidant, and anti-adiposity effects. In this study, we evaluated the suppressive effects of phloretin on intercellular adhesion molecule 1 (ICAM-1) and cyclooxygenase (COX)-2 expression in IL-1 $\beta$ -stimulated human lung epithelial A549 cells. The cells were pretreated with various concentrations of phloretin (3–100  $\mu$ M), followed by induced inflammation by IL-1 $\beta$ . Phloretin inhibited levels of prostaglandin E<sub>2</sub>, decreased COX-2 expression, and suppressed IL-8, monocyte chemoattractant protein 1, and IL-6 production. It also decreased ICAM-1 gene and protein expression and suppressed monocyte adhesion to inflammatory A549 cells. Phloretin also significantly inhibited Akt and mitogen-activated protein kinase (MAPK) phosphorylation and decreased nuclear transcription factor kappa-B (NF- $\kappa$ B) subunit p65 protein translocation into the nucleus. In addition, ICAM-1 and COX-2 expression was suppressed by pretreatment with both MAPK inhibitors and phloretin in inflammatory A549 cells. However, phlorizin, a derivative of phloretin, did not suppress the inflammatory response in IL-1 $\beta$ -stimulated A549 cells. These results suggest that phloretin might have an anti-inflammatory effect by inhibiting proinflammatory cytokine, COX-2, and ICAM-1 expression via blocked NF- $\kappa$ B and MAPK signaling

pathways.

## Introduction

Inflammation is an important physiological response, and asthma is a common, complex, chronic inflammatory pulmonary disease.<sup>1,2</sup> Patients in an acute asthma attack that causes airway constriction, wheezing, coughing, and excessive mucus secretion experience difficulty breathing or suffocation and death.<sup>3</sup> IL-1 $\beta$ , secreted by activated macrophages and T cells, plays an important role in respiratory disease and allergic asthma.<sup>4,5</sup> In IL-1 $\beta$  receptor knockout mice, airway hyperresponse and eosinophil infiltration of the lungs are significantly suppressed compared with wild-type asthmatic mice.<sup>6</sup> Hence, in the asthmatic patient, suppression of IL-1 $\beta$  levels in the lung could decrease the lung inflammatory response. Many studies have found that lung epithelial cells in asthmatic patients release proinflammatory cytokines, which induce a severe inflammatory response.<sup>6,7</sup> In addition, lung epithelial cells also release chemokines to induce eosinophil, neutrophil, or macrophage infiltration into the lung, and these inflammatory cells release more inflammatory mediators to increase the inflammatory response.<sup>8</sup>

Many previous studies have found that nuclear transcription factor kappa-B (NF- $\kappa$ B) is part of an important inflammation signaling pathway.<sup>9</sup> In the inactive state, NF- $\kappa$ B is suppressed by I $\kappa$ B; inflammatory signals stimulate I $\kappa$ B phosphorylation,

leading to separation of the two NF- $\kappa$ B subunits p65 and p50 and their transposition into the nucleus, where they interact with the promoters of inflammatory mediator and cytokine genes.<sup>10</sup> Mitogen-activated protein kinase (MAPK) signaling pathways are also activated in inflammatory conditions of human lung epithelial cells.<sup>11</sup> Thus, suppressing NF- $\kappa$ B and MAPK activity is hypothesized to ameliorate the inflammatory response in lung disease.

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in asthma and respiratory inflammation.<sup>12</sup> Studies indicate that during inflammation, lung epithelial cells also express high levels of cyclooxygenase (COX)-2 proteins, leading to increased injury in the lung.<sup>2</sup> The selective COX-2 inhibitor celecoxib, an NSAID, significantly suppresses COX-2 expression and decreases prostaglandin E2 (PGE<sub>2</sub>) production; the resulting decreased activity of the NF- $\kappa$ B or MAPK pathway leads to beneficial effects on respiratory disease.<sup>12</sup> Furthermore, inflammatory lung epithelial cells also express adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), to adsorb more leukocytes and infiltrate into the lungs.<sup>13, 14</sup> Suppressing ICAM-1 expression and inhibiting inflammatory mediators thus could ameliorate asthma or other respiratory inflammatory diseases.

Previous work has shown that phloretin and phlorizin regulate glucose transporters and that phloretin also increases adiponectin expression in 3T3-L1 adipocytes.<sup>15</sup> We have reported that phloretin has an anti-inflammatory effect in lipopolysaccharide (LPS)-stimulated macrophages and increases lipolysis in adipocytes.<sup>16,17</sup> In this study, we evaluated the anti-inflammatory effect of phloretin and the signaling mechanism of the NF- $\kappa$ B and MAPK pathways in IL-1 $\beta$ -induced A549 human lung epithelial cells.

## **Materials and methods**

### **Materials**

The chemical structures of phloretin and phlorizin (from apple wood,  $\geq 99\%$  by HPLC) were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA) (Fig. 1A). The concentration of the stock solution was 100 mM, with DMSO as the solvent. DMSO was  $\leq 0.1\%$  in culture medium, as previously described.<sup>17</sup>

### **Cell line and culture medium**

The A549 human lung epithelial cell line was purchased from the Bioresource Collection and Research Center (BCRC, Taiwan) and cultured in F12 medium (Invitrogen-Gibco<sup>TM</sup>, Paisley, Scotland) supplemented with 10% fetal bovine serum

(Biological Industries, Haemek, Israel), 2 mM glutamine, and 100 U/ml penicillin and streptomycin. The cells were incubated in 5% CO<sub>2</sub> humidified air at 37°C, and cells were subcultured twice each week.

### **Cell viability assay**

Cell viability was measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay, as previously described.<sup>18</sup> In brief, A549 cells (10<sup>4</sup> cells/well) were seeded in 96-well plates and treated with various concentrations of phloretin or phlorizin for 24 h. MTT solution (5 mg/ml) was added and incubated for 4 h, followed by isopropanol dissolution of formazan crystals. OD was spectrophotometrically measured at 570 nm with a microplate reader (Multiskan FC, Thermo, Waltham, MA, USA).

### **ELISAs for proinflammatory cytokines, chemokines, and PGE<sub>2</sub> production**

ELISAs were performed as previously described<sup>18</sup>. Briefly, cells (10<sup>6</sup> cells/ml) were pretreated with or without phloretin and phlorizin (0–100 μM) in 24-well plates for 1 h, stimulated with IL-1β (1 ng/ml), and cultured for 24 h to assay IL-8, IL-6, monocyte chemoattractant protein (MCP)-1, and PGE<sub>2</sub> production using specific ELISA kits (R&D

Systems, Minneapolis, MN, USA). OD was spectrophotometrically measured at 450 nm with a microplate reader (Multiskan FC, Thermo).

### **Preparation of total and nuclear proteins**

A549 cells ( $10^6$  cells/ml) were seeded in 6-well plates and pretreated with or without phloretin for 1 h. Then, cells were stimulated with or without IL-1 $\beta$  (1 ng/ml) for 30 min to detect protein phosphorylation or for 24 h to evaluate total protein assays. Cells also cultured for 1 h to detect NF- $\kappa$ B. The cells were collected, and the proteins extracted through lysis in 300  $\mu$ l of protein lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5% NP40, and 0.1% SDS) containing a protease inhibitor cocktail and phosphatase inhibitors (Sigma). Nuclear proteins were extracted using the NE-PER<sup>®</sup> nuclear and cytoplasmic extraction reagent kits (Pierce, Rockford, IL, USA). All proteins were quantified with the BCA protein assay kit (Pierce).

### **Western immunoblot analysis**

Equal amounts of protein (10–30  $\mu$ g) were separated on 8–10% SDS polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight at 4°C with primary antibodies, including COX-2, Akt, phosphorylated-Akt, I $\kappa$ B- $\alpha$ , phosphorylated-I $\kappa$ B- $\alpha$ , Lamin B1,

and p65 (Santa Cruz, CA, USA); ERK1/2, p38, JNK, phosphorylated-ERK 1/2, phosphorylated-p38, and phosphorylated-JNK (Millipore); and ICAM-1 and  $\beta$ -actin (Sigma). The membrane was washed with TBST buffer (150 mM NaCl, 10 mM Tris pH 8.0, 0.1% Tween 20), and incubated with horseradish peroxidase–conjugated secondary antibodies for 1 h at room temperature. Finally, the membranes were washed and incubated with Luminol/Enhancer Solution (Millipore) for detection and quantification of the specific protein using the BioSpectrum 600 system (UVP, Upland, CA, USA). MAPK inhibitors (JNK inhibitor SP600125, ERK inhibitor PD98059, and p38 inhibitor SB203580) (Enzo Life Sciences, Inc., Farmingdale, NY, USA) were cultured with phloretin to detect ICAM-1 and COX-2 protein expression.

#### **RNA isolation and real-time PCR for gene expression**

RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA); cDNA was synthesized using a cDNA synthesis kit (Life Technologies); and cDNA gene expression was assayed by real-time PCR with a spectrofluorometric thermal cycler (iCycler; Bio-Rad Laboratories, Hercules, CA, USA). Specific primers were designed as follows: ICAM-1, forward 5'-AGA CGC AGA GGA CCT TAA-3' and reverse 5'-CAC ACT TCA CAG TTA CTT GG-3'; COX-2, forward 5'- TCT GGT GCC

TGG TCT GAT G-3' and reverse 5'- GTA TTA GCC TGC TTG TCT GGAA -3';  
MUC5AC, forward 5'-CTG TTA CTA TGC GAT GTG TAG-3' and reverse 5'-GTG  
GCG TGG TAG ATG TAG-3'; and  $\beta$ -actin, forward 5'- AAG ACC TCT ATG CCA  
ACA CAG T -3' and reverse 5'- AGC CAG AGC AGT AAT CTC CTT C -3'.

### **Cell–cell adhesion assay**

A549 cells were treated with phloretin and incubated with 1 ng/ml IL-1 $\beta$  for 24 h. Then, THP-1 cells (a human monocytic cell line) in calcein AM solution (Sigma) were co-cultured with A549 cells for 1 h. Cells were washed and adhesion of THP-1 cells to A549 cells was observed under fluorescence microscopy (Olympus, Tokyo, Japan).

### **Statistical analysis**

All experimental data were analyzed by one-way analysis of variance and post hoc analysis with Dunnett's test. Data are presented as the mean $\pm$ SD of at least three independent experiments, and statistical significance was set at  $p<0.05$ .

## Results

### **Inhibition of proinflammatory cytokine and chemokine production by phloretin and phlorizin pretreatment of IL-1 $\beta$ -stimulated A549 cells**

Phloretin or phlorizin did not significantly exhibit cytotoxic effects at concentrations  $\leq 100$   $\mu\text{M}$  (data not shown). First, we investigated the inhibitory effects of phloretin on IL-1 $\beta$ -induced proinflammatory cytokine and chemokine production. A549 cells were pretreated with various concentrations of phloretin and stimulated with IL-1 $\beta$  for 24 h. Phloretin had a dose-dependent inhibitory effect on levels of IL-6, IL-8, and MCP-1 in IL-1 $\beta$ -stimulated A549 cells (Fig. 1). Phloretin concentrations of  $\geq 10$   $\mu\text{M}$  significantly inhibited IL-6 and MCP-1 levels, and phloretin concentrations  $\geq 30$   $\mu\text{M}$  significantly inhibited IL-8 production. However, for phlorizin, a derivative of phloretin, only 100  $\mu\text{M}$  of phlorizin could suppress IL-6, IL-8, and MCP-1 expression.

### **Effects of phloretin and phlorizin on IL-1 $\beta$ -induced COX-2 protein expression**

A549 cells were pretreated with various concentrations of phloretin, followed by stimulation with IL-1 $\beta$  (1 ng/ml) for 24 h. Pretreatment with phloretin significantly suppressed COX-2 protein expression in a concentration-dependent manner compared with IL-1 $\beta$ -stimulated cells (Fig. 2A); however, phlorizin did not decrease COX-2

production. Phloretin also significantly decreased the level of PGE<sub>2</sub> (3 μM phloretin, 8.89±0.48 ng/ml, *p*=0.67; 10 μM phloretin, 8.38±0.25 ng/ml, *p*=0.33; 30 μM phloretin, 7.45±0.75 ng/ml, *p*<0.05; 100 μM phloretin, 6.27±0.79 ng/ml, *p*<0.01; vs. IL-1β alone, 9.05±0.72 ng/ml) (Fig. 2B); however, phlorizin did not significantly decrease PGE<sub>2</sub> levels.

### **Inhibition by phloretin of NF-κB activation in IL-1β-activated human lung epithelial cells**

Phloretin proved to be a better suppressor than phlorizin of proinflammatory cytokine, chemokine, and COX-2 expression in IL-1β-activated human lung epithelial cells. We also evaluated whether or not phloretin suppressed the NF-κB and MAPK pathways in IL-1β-induced A549 cells. In unstimulated cells, NF-κB p65 was mostly distributed in the cytoplasm, and A549 cells treated with IL-1β showed increased p65 levels in the nucleus (Fig. 3). Interestingly, phloretin suppressed p65 translocation into the nucleus compared with IL-1β-induced A549 cells. Phloretin also significantly suppressed IκB-α phosphorylation and IκB-α degradation to regulate NF-κB activity compared with IL-1β-induced A549 cells.

### **Effect of phloretin on phosphorylation of Akt and MAPK pathways in IL-1 $\beta$ -activated human lung epithelial cells**

Previous studies have shown that IL-1 $\beta$  promotes Akt signaling expression to activate transcription factors of NF- $\kappa$ B for inflammatory cytokine and chemokine production in A549 cells<sup>19</sup>. Here, we found that phloretin significantly suppressed Akt phosphorylation in IL-1 $\beta$ -activated human lung epithelial cells (Fig. 4). In addition, we investigated whether phloretin affects the MAPK pathway in IL-1 $\beta$ -activated A549 cells and found that it decreased phosphorylation of ERK1/2 and p38 and JNK compared with IL-1 $\beta$ -activated A549 cells. Next, MAPK inhibitors were used to evaluate the inhibitory effect of phloretin on COX-2 expression (Fig. 6). We found that phloretin could also enhance ERK, p38, and JNK inhibitors to suppress COX-2 expression.

### **Suppression by phloretin of ICAM-1 and MUC5AC expression in A549 cells**

We used real-time PCR to assess gene expression and found that phloretin significantly decreased levels of MUC5AC and ICAM-1 in A549 cells (Fig. 5A, B). In addition, the ICAM-1 protein assay showed that phloretin significantly decreased ICAM-1

production (Fig. 5D) and suppressed its release into medium (Fig. 5C). Next, we found that phloretin could enhance MAPK inhibitors to suppress ICAM-1 expression (Fig. 6).

### **Inhibition of monocyte adhesion to IL-1- $\beta$ -activated A549 cells by phloretin**

In the present study, phloretin decreased expression of ICAM-1 proteins in inflammatory A549 cells. We also investigated whether phloretin could suppress the ability of monocyte THP-1 cells to adhere to inflammatory A549 cells (Fig. 7).

Using THP-1 cells stained with calcein AM and co-cultured with IL-1 $\beta$ -induced A549 cells, we found that more THP-1 cells adhered to IL-1 $\beta$ -activated A549 cells and that pretreatment with phloretin significantly reduced the adhesion of THP-1 cells to A549 cells.

### **Discussion**

Microorganism infection or inhaled allergens into airways can cause a respiratory inflammatory response.<sup>20</sup> During respiratory inflammation, lung epithelial cells and macrophages are activated and release proinflammatory cytokines to enhance acute lung injury and secrete chemokines to induce leukocyte lung infiltration, leading to a more serious inflammatory response.<sup>14</sup> Adult respiratory distress syndrome (ARDS) involves

pulmonary inflammation and injury with hypoxic damage, and IL-1 $\beta$  is the major cytokine that maintains lung inflammation in ARDS.<sup>21</sup> IL-1 $\beta$  also plays an important role in lung inflammation, fibroproliferation, fibrotic remodeling, and epithelial repair processes in idiopathic pulmonary fibrosis.<sup>5</sup> During idiopathic pulmonary fibrosis, early expression of IL-1 $\beta$ , IL-23, and IL-17A leads to pulmonary inflammation and fibrosis, and using IL-1 $\beta$  antagonists suppresses IL-23 and IL-17A production.<sup>22</sup> Treatment with IL-1 $\beta$  antagonists also can improve airway hyperresponsiveness, eosinophil and neutrophil infiltration, and airway remodeling in a mouse model of asthma.<sup>23,24</sup> IL-1 $\beta$  antagonists also reduce the levels of Th2 cytokines and chemokines in bronchoalveolar lavage fluid in a mouse model of allergic asthma.<sup>24</sup>

Phloretin and phlorizin are found in apple and pear trees.<sup>15</sup> Phloretin 2'-O-glycosyltransferase metabolizes phloretin to produce phlorizin.<sup>25</sup> Recent studies have shown that phloretin has greater anti-inflammatory effects than phlorizin in LPS-induced murin macrophage.<sup>16</sup> Phloretin could decrease inflammatory cytokine and chemokine productions in human peripheral blood mononuclear cells.<sup>26</sup> It is proposed that phloretin decreased the levels of IFN- $\gamma$  on activated CD4 T cells, and suppressed LPS-induced dendritic cells maturation.<sup>27</sup> Some researchers previously thought that phloretin could induce apoptosis of human HepG2 cells through suppressed the

function of glucose transporter 2.<sup>28</sup> Interestingly, phloretin could promote activity of  $\gamma\delta$  T cells to kill human colon cancer cells.<sup>29</sup> We also found that phloretin enhances lipid metabolism and has greater anti-obesity effects than phlorizin.<sup>17</sup> However, the anti-inflammatory effect of phloretin and phlorizin in human lung epithelial cells has not been previously established. In this study, we demonstrated that phloretin can down-regulate COX-2 production and decrease IL-6, IL-8, and MCP-1 production in IL-1 $\beta$ -stimulated lung epithelial cells. In particular, the activation of NF-kB nuclear translocation and phosphorylation of Akt and MAPK also were suppressed. Phloretin decreased ICAM-1 expression in adherent monocytes, as well. Thus, we suggest that phloretin may dampen the inflammatory effect in IL-1 $\beta$ -stimulated lung epithelial cells.

Some researchers previously thought that glycosylation of phlorizin could decrease the stability of free radicals and prevent electron transfer for antioxidant and anti-inflammatory effects,<sup>30</sup> which led us to evaluate the anti-inflammatory molecular mechanisms of phloretin. Meloxicam is a selective COX-2 inhibitor with the effect of suppressing arachidonic acid conversion into PGE<sub>2</sub>.<sup>31</sup> Meloxicam reduces pulmonary inflammation in asthma and chronic obstructive pulmonary disease (COPD).<sup>10</sup> However, long-term oral administration of meloxicam or other COX-2 inhibitors can lead to an allergic response or drug tolerance. Thus, researchers are still looking for additional

alternative treatments for pulmonary inflammatory disease. In the current work, phloretin significantly reduced COX-2 and PGE<sub>2</sub> production in LPS-stimulated macrophages and IL-1 $\beta$ -activated human lung epithelial cells. Phloretin thus may be useful as a COX-2 inhibitor to attenuate inflammatory response.

IL-6 plays an important role in inflammatory lung injury and acute asthma attacks. IL-8, which is released by monocyte and epithelial cells, is the major neutrophil chemotactic factor in the respiratory system.<sup>32</sup> Neutrophils are essential in the acute inflammatory defense against bacterial and fungal infections. In COPD, MCP-1 stimulates collagen and transforming growth factor  $\beta$  production in fibroblast lung tissue.<sup>4</sup> In addition, bronchoalveolar lavage fluid from patients with interstitial lung disease contains more MCP-1, IL-8, IL-6, and IL-1 $\beta$  compared with normal tissues.<sup>33</sup> We found that phloretin significantly decreased IL-6, IL-8, and MAP-1 in IL-1 $\beta$ -stimulated lung epithelial cells, indicating that phloretin might suppress the inflammatory response of lung epithelial cells and improve lung disease symptoms.

As part of the inflammatory response, lung epithelial cells express cell adhesion molecules, including ICAM-1 and VCAM-1, for adherent monocyte and neutrophil migration and infiltration into lung tissue.<sup>34</sup> Activated monocytes and neutrophils release more inflammatory mediators, increasing lung inflammation and lung injury. In

the current work, real-time PCR and Western blot analysis showed that phloretin suppressed ICAM-1 gene expression and protein production. To understand whether the phloretin-based decrease in ICAM-1 expression could also decrease lymphocyte adhesion to inflammatory lung epithelial cells, we used monocyte THP-1 cells co-cultured with IL-1 $\beta$ -stimulated A549 cells. Phloretin decreased THP-1 adherence to A549 cells compared with IL-1 $\beta$ -stimulated A549 cells. Inflammatory lung epithelial cells secrete excessive mucus, which obstructs the airway and leads to difficulty breathing and even suffocation.<sup>35</sup> Phloretin might prevent ICAM-1 expression and decrease inflammatory leukocyte infiltration into lung tissue, reducing mucus production.

In the present study, phloretin inhibited IL-1 $\beta$ -induced NF- $\kappa$ B nuclear translocation as a result of suppressed COX-2 and PGE<sub>2</sub> expression. Previous studies found that PI3K-Akt signaling activates NF- $\kappa$ B nuclear translocation in IL-1 $\beta$ -induced A549 cells.<sup>19</sup> PI3K-Akt signaling also inhibits apoptosis and proliferation in cancer tumor cells.<sup>36</sup> We found that treatment with phloretin significantly decreased phosphorylation of Akt and MAPK. The MAPK and PI3/Akt pathways are important regulatory factors in prolonged NF- $\kappa$ B activation for COX-2 and ICAM-1 expression.<sup>37, 38</sup> To reconfirm that phloretin could inhibit MAPK activity, we treated A549 cells with MAPK

inhibitors and phloretin and found that phloretin addition decreased COX-2 and ICAM-1 expression significantly more. The inhibition of MAPK activation could explain why phloretin inhibits COX-2 synthesis and ICAM-1 expression in IL-1 $\beta$ -stimulated A549 cells.

## **Conclusion**

In conclusion, phloretin significantly inhibited proinflammatory cytokine and COX-2 expression by suppressing activation of the NF- $\kappa$ B and MAPK pathways. Phloretin also inhibited ICAM-1 expression in adherent leukocytes interacting with IL-1 $\beta$ -activated human lung epithelial cells. We believe that phloretin shows promise as a natural anti-inflammatory drug to attenuate lung inflammatory disease.

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## **Conflicts of interest**

The authors declare that there are no conflicts of interest.

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### Figure Legends

**Figure 1** Chemical structure (A) and effects of phloretin and phlorizin on the IL-1 $\beta$ -induced production of IL-8 (B), IL-6 (C), and MCP-1 (D). Cells ( $10^6$  cells/well) were pretreated with the indicated concentrations of phloretin (PT) and phlorizin (PZ) for 1 h and then stimulated with IL-1 $\beta$  (1 ng/ml) for 24 h. The presented data are mean $\pm$ SD; \*  $p<0.05$ , \*\*  $p<0.01$ , compared with the IL-1 $\beta$ -treated group.

**Figure 2** Effects of phloretin and phlorizin on IL-1 $\beta$ -induced production of COX-2 (A) and PGE<sub>2</sub> (B). A549 cells ( $10^6$  cells/ml) were pretreated with the indicated concentrations of phloretin (PT) and phlorizin (PZ) for 1 h and then stimulated with IL-1 $\beta$  (1 ng/ml) for 24 h. COX-2 protein levels were detected using  $\beta$ -actin expression as an internal control. Data are presented as mean $\pm$ SD; \*  $p<0.05$ , \*\*  $p<0.01$ , compared with the IL-1 $\beta$ -treated group.

**Figure 3** Inhibitory effects of phloretin on the nuclear translocation of NF- $\kappa$ B in A549 cells. Effects of phloretin on the IL-1 $\beta$ -induced phosphorylation of I $\kappa$ B- $\alpha$  (A), with total

I $\kappa$ B- $\alpha$  levels used as internal controls. For the nuclear translocation of NF- $\kappa$ B, cells were pretreated with different concentrations of phloretin for 1 h and then incubated with IL-1 $\beta$  (1 ng/ml) for 1 h (B). The internal controls were Lamin B1 in the nucleus and  $\beta$ -actin in the cytosol. The densitometry values of three independent experiments were analyzed and compared with the IL-1 $\beta$ -treated group.

**Figure 4** Effect of phloretin on IL-1 $\beta$ -induced phosphorylation of MAPK and Akt.

A549 cells were pretreated with varying concentrations of phloretin (PT) for 1 h and then incubated with or without IL-1 $\beta$  (1 ng/ml) for 30 min. Protein samples were analyzed using Western blots with phospho-specific antibodies. Total MAPK and Akt levels were used as internal controls. The densitometry values of three independent experiments were analyzed and compared with the IL-1 $\beta$ -treated group.

**Figure 5** Effects of phloretin on the IL-1 $\beta$ -induced production of ICAM-1 and

MUC5AC. A549 cells ( $10^6$  cells/ml) were pretreated with the indicated concentrations of phloretin (PT) for 1 h and then stimulated with IL-1 $\beta$  (1 ng/ml) for 4 h to assay ICAM-1 (A) and MUC5AC gene expression levels, determined using real-time

RT-PCR. The cells were stimulated with IL-1 $\beta$  (1 ng/ml) for 24 h to assay ICAM-1 of the supernatant by ELISA (C) and ICAM-1 protein levels by Western blot (D).

**Figure 6** Inhibitory effects of MAPK inhibitors or phloretin on IL-1 $\beta$ -induced COX-2 and ICAM-1 protein expression in A549 cells. A549 cells were pretreated with MAPK inhibitors (5  $\mu$ M PD98059, 5  $\mu$ M SB203580, and 5  $\mu$ M SP600165) with or without 30  $\mu$ M phloretin (PT) for 1 h, followed by IL-1 $\beta$  stimulation for 24 h. COX-2 and ICAM-1 protein levels were detected, and  $\beta$ -actin expression was used as an internal control. The densitometry values of three independent experiments were analyzed and compared with the IL-1 $\beta$ -treated group.

**Figure 7** Phloretin inhibition of THP-1 cells from adhering to active A549 cells. THP-1 cells were labeled with calcein AM and mixed with A549 cells, followed by observation using fluorescence microscopy. Note the adherence of THP-1 cells to Normal (A) and IL-1 $\beta$ -activated A549 cells (B). THP-1 cells treated with 3  $\mu$ M phloretin (C), 10  $\mu$ M phloretin (D), 30  $\mu$ M phloretin (E), and 100  $\mu$ M phloretin (F), respectively.

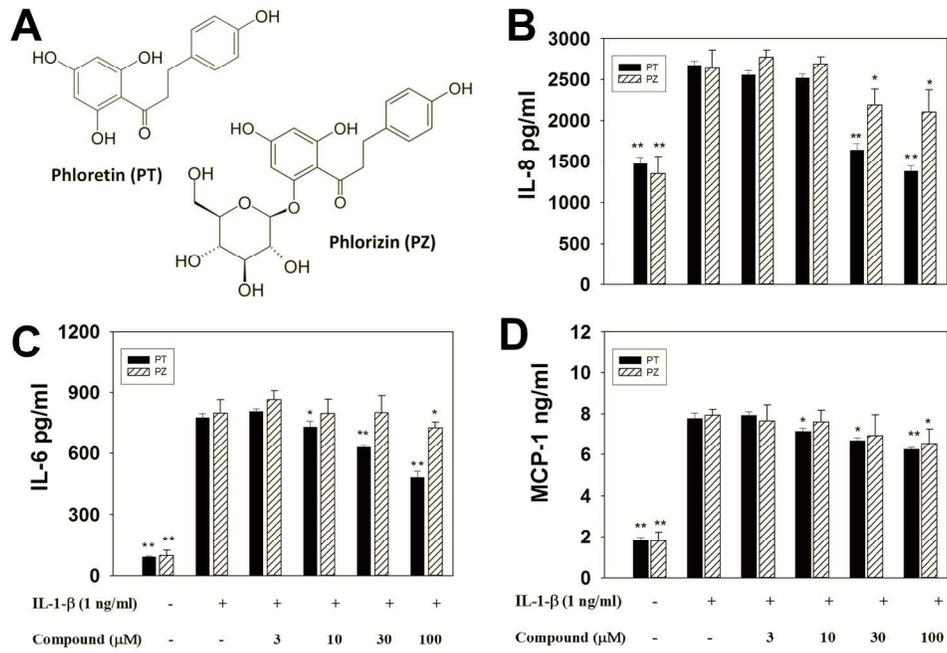


Fig. 1

Figure 1  
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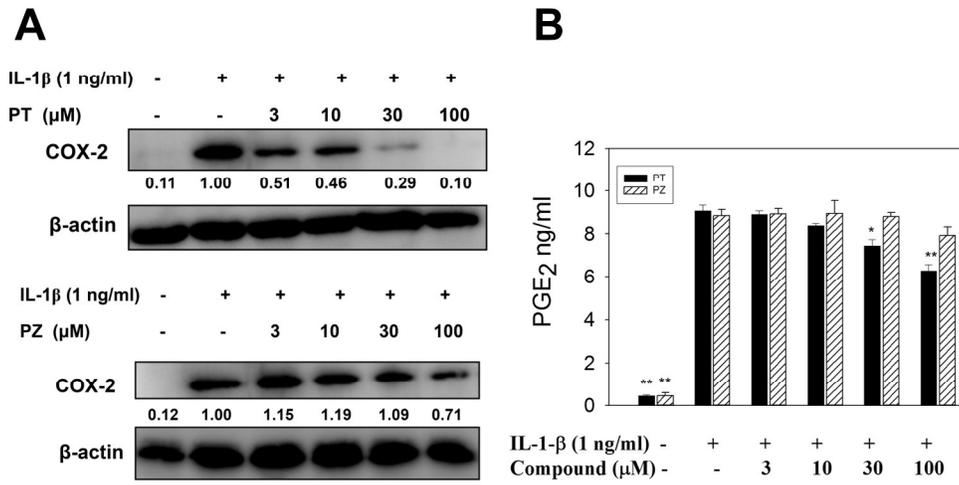


Fig. 2

Figure 2  
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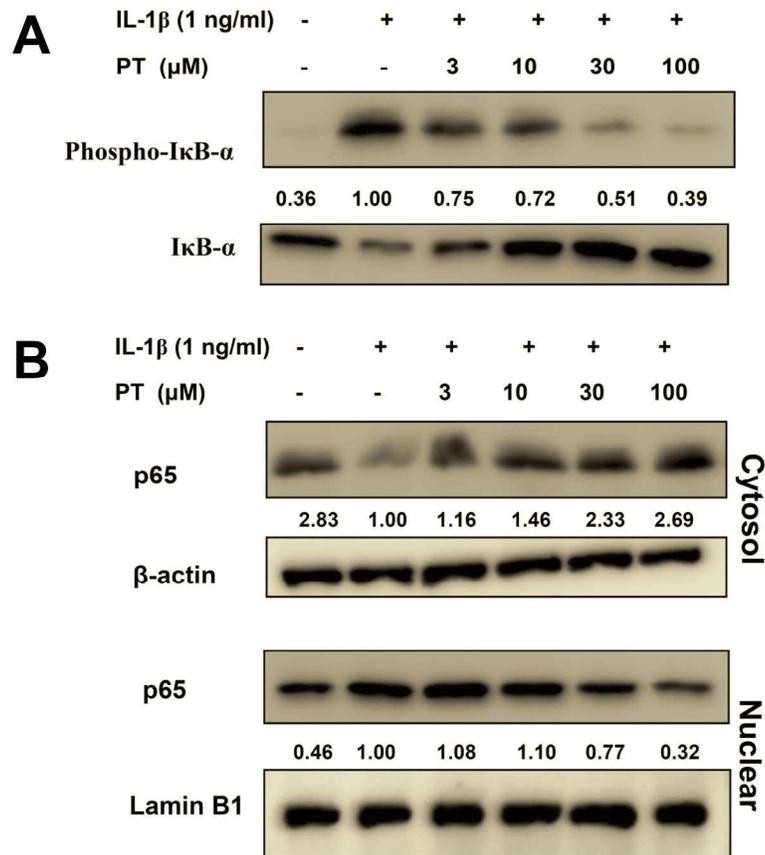


Fig. 3

Figure 3  
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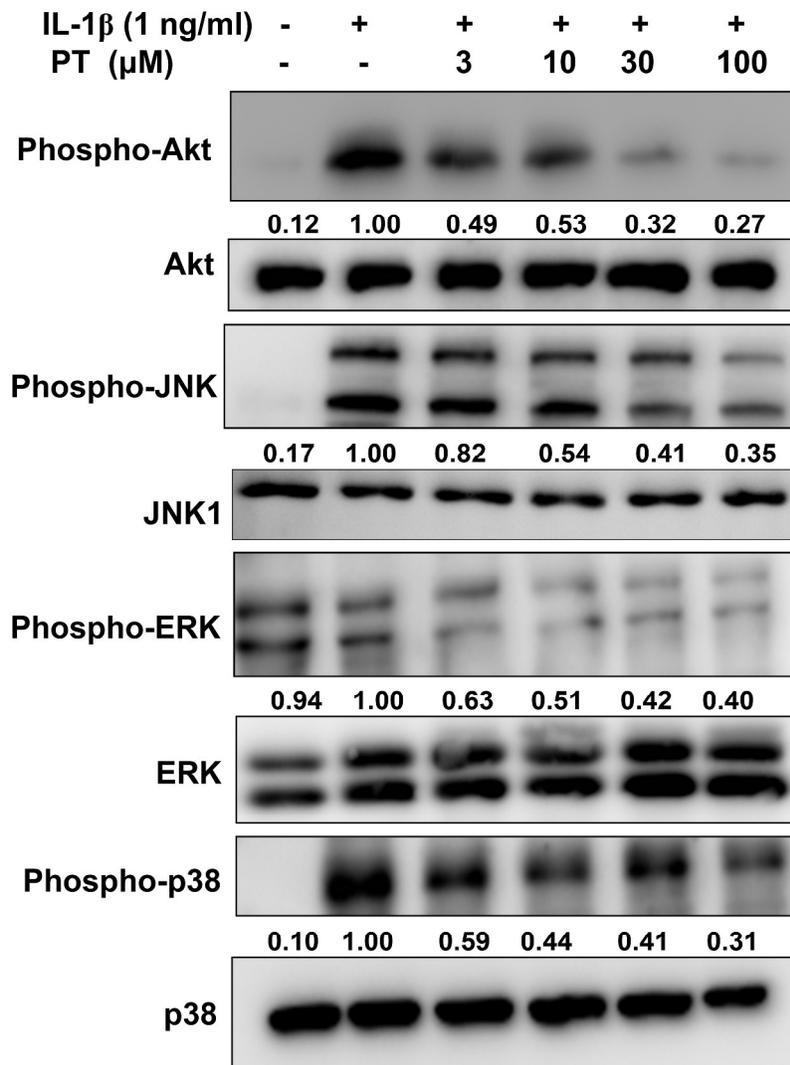


Fig. 4

Figure 4  
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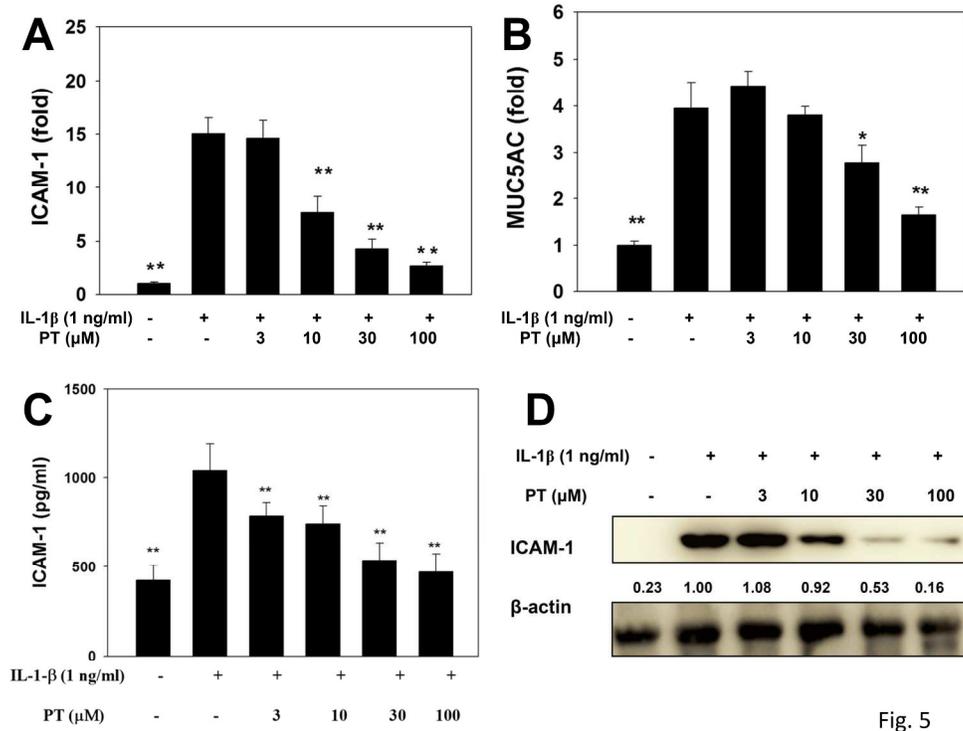


Figure 5  
127x97mm (600 x 600 DPI)

Fig. 5

IL-1 $\beta$ (1 ng/ml)	-	+	+	+	+	+	+	+	+
PT (30 $\mu$ M)	-	-	+	-	+	-	+	-	+
PD98059 (5 $\mu$ M)	-	-	-	+	+	-	-	-	-
SB203580 (5 $\mu$ M)	-	-	-	-	-	+	+	-	-
SP600125 (5 $\mu$ M)	-	-	-	-	-	-	-	+	+

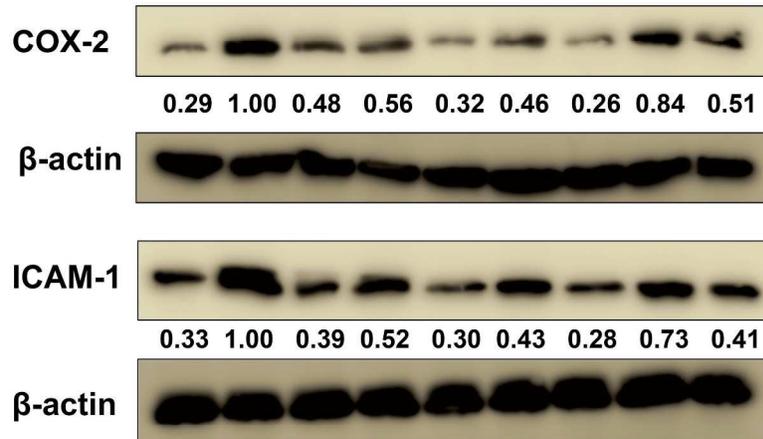


Fig. 6

Figure 6  
125x109mm (600 x 600 DPI)

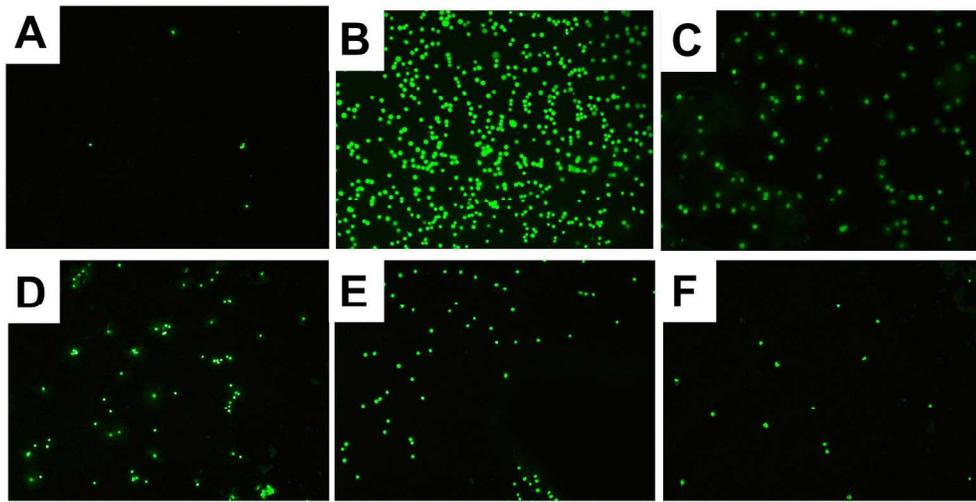


Fig. 7

Figure 7  
105x66mm (600 x 600 DPI)