Physalis peruviana L. inhibits airway inflammation induced by cigarette smoke and lipopolysaccharide through inhibition of extracellular signal-regulated kinase and induction of heme oxygenase-1

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Abstract. *Physalis peruviana* L. (PP) is a medicinal herb that has been confirmed to have several biological activities, including anticancer, antioxidant and anti-inflammatory properties. The aim of the present study was to evaluate the protective effect of PP on cigarette smoke (CS)- and lipopolysaccharide (LPS)-induced pulmonary inflammation. Treatment with PP significantly reduced the influx of inflammatory cells in the bronchoalveolar lavage fluid (BALF) and lung of mice with CS- and LPS-induced pulmonary inflammation. PP also decreased the levels of reactive oxygen species (ROS) and pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in the BALF. PP effectively attenuated the expression of monocyte chemoattractant protein-1 (MCP-1) and the activation of extracellular signal-regulated kinase (ERK) in the lung. In addition, nuclear factor erythroid 2-related factor 2 (Nrf2) activation and heme oxygenase-1 (HO-1) expression were increased by PP treatment. In an *in vitro* experiment, PP reduced the mRNA expression of TNF-α and MCP-1, and the activation of ERK in CS extract-stimulated A549 epithelial cells. Furthermore, PP increased the activation of Nrf2 and the expression of HO-1 in A549 cells. These findings suggest that PP has a therapeutic potential for the treatment of pulmonary inflammatory diseases, such as chronic obstructive pulmonary disease.

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Abbreviations: COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; ERK, extracellular signal-regulated kinase; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; PP, *Physalis peruviana* L.

Key words: Physalis peruviana L., chronic obstructive pulmonary disease, airway inflammation, neutrophils, heme oxygenase-1

Introduction

Chronic obstructive pulmonary disease (COPD) is a global public health concern and a major cause of morbidity and mortality worldwide (1). COPD is characterized by oxidative damage and chronic airway inflammation that results in airflow obstruction and emphysema (2). The exposure to cigarette smoke (CS) is the main cause of COPD, as it alters the inflammatory mechanisms by increasing the influx of inflammatory cells, including neutrophils, and the production of inflammatory molecules (3). Recent studies reported that CS contains harmful particles and trace amounts of microbial cell components, including bacterial lipopolysaccharide (LPS), which play an important role in lung diseases and respiratory infections (4,5).

Airway inflammation is one of the major characteristics of COPD and is associated with an increase in inflammatory cell recruitment (6). Neutrophil influx is a major pathophysiological characteristic of COPD, and persistent activation of neutrophils

leads to lung tissue damage by increasing the production of reactive oxygen species (ROS) and neutrophil elastase (7). Macrophages affect airway inflammation via production of inflammatory cytokines and chemokines in the pathogenesis of COPD (8). High levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were detected in COPD patients and CS exposure animal models (9,10). Monocyte chemoattractant protein-1 (MCP-1), a member of the C-C chemokine subfamily, is produced by macrophages and airway epithelial cells, and affects the influx of inflammatory cells, including neutrophils, in airway inflammatory response (11,12). The administration of antioxidants effectively ameliorated the airway inflammation with MCP-1 inhibition (13). Downregulation of extracellular signal-regulated kinase (ERK) reduces the production of inflammatory cytokines and chemokines in CS extract (CSE)-stimulated pulmonary epithelial cells and in animal models with CS-induced airway inflammation (13,14). The antioxidant defense protein heme oxygenase-1 (HO-1) exerts a protective effect against CS-induced lung inflammation (15). The levels of inflammatory cell influx and inflammatory molecules are decreased by HO-1 induction in animal models of CS-induced airway inflammation (16).

Cape gooseberry [*Physalis peruviana* L. (PP)] is a species within the Solanaceae family, which has been widely used in folk medicine (17). PP displays a wide range of biological properties, including neuroprotective, antioxidant and anti-inflammatory properties (18-20). However, the protective effect of PP against airway inflammation induced by CS and LPS has not been extensively investigated.

Materials and methods

Preparation of PP. PP was collected from the Katu Village, Lore Lindu National Park, Central Sulawesi, Indonesia. Plant samples were collected and identified by the Center for Pharmaceutical and Medical Technology (Tangerang, Indonesia) and verified by Herbarium Bogoriense (Bogor, Indonesia). Voucher specimens were recorded as KRIB 0049496 and PMT 1884 have been deposited at the herbarium of the Korea Research Institute of Bioscence and Biotechnolgy and at the Center for Pharmaceutical and Medical Technology and Herbarium Bogoriense. After drying and grinding the leaves of PP, 150 g of powder was added to 150 ml of methanol and extraction was performed by maceration at room temperature for 18 h. The extract was filtered and concentrated by a rotary evaporator (Laborota 4000; Heidolph, Jakarta, Indonesia) under reduced pressure, thereby obtaining 7.05 g of PP methanolic extract. In the following experiment, the extract was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 20 mg/ml, and then diluted to various concentrations before use.

Mouse models of airway inflammation induced by CS and LPS. Male 6-week-old C57BL/6N mice were purchased from Koatech Co. (Pyeongtaek, Korea) and used after 1 week of quarantine and acclimatization in a specific pathogen-free system. The mice were randomly divided into 4 groups (n=7 per group) as follows: i) The normal control (NC) group; ii) the CS + LPS group; iii) the roflumilast (ROF; 10 mg/kg) group (used as a positive control); and iv) the PP group (administered 10 and 20 mg/kg PP). CS exposure was applied as previously described (7). In brief, the mice were whole-body exposed to room air or CS of 8 cigarettes for 1 h a day for 10 days. CS exposure was generated by 3R4F research cigarette, containing 11.0 mg of total particulate matter, 9.4 mg of tar, and 0.76 mg of nicotine/cigarette (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA). ROF and PP were dissolved with 1% DMSO + 1% Tween-20 in phosphate-buffered saline (PBS), and were administered orally on days 0-9. The mice were administered LPS (5 μg dissolved in 30 μl distilled water) intranasally 1 h after the final ROF and PP treatment. All the experimental procedures were performed in accordance with the procedures approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology, and in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and Korean National Laws for Animal Welfare.

Collection of bronchoalveolar lavage fluid (BALF) and determination of inflammatory cell counts. The collection of the BALF and determination of inflammatory cell counts were performed as previously described (13). In brief, ice-cold PBS (700 μl) was infused into the lungs via tracheal cannulation and extraction was performed twice (total volume, 1,400 μl). In order to count the number of different cells, 100 μl BALF was centrifuged onto glass slides for 5 min at 264 x g, and the slides were dried and stained using Diff-Quik® staining reagent according to the manufacturer's protocol (IMEB Inc., Deerfield, IL, USA).

Measurement of ROS and pro-inflammatory cytokines in the BALF. The intracellular levels of ROS were determined using 2',7'-dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich, St. Louis, MO, USA) as previously described (21). Briefly, BALF cells were washed with PBS and incubated with 20 μM DCF-DA for 10 min at 37˚C. Subsequently, the intracellular ROS levels were detected under fluorescence at 488 nm excitation and 525 nm emission on a fluorescence plate reader (Perkin-Elmer, Waltham, MA, USA). The levels of pro-inflammatory cytokines (TNF-α, IL-6 and MCP-1) were determined using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). The absorbance was measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis. The lung tissues were collected at 6 or 24 h after the last administration of PP. The levels of ERK and nuclear factor erythroid 2-related factor 2 (Nrf2) activation were evaluated using lung tissues that were obtained 6 h after the last administration of PP. The expression of MCP-1, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and HO-1 were evaluated using lung tissues that were collected 24 h after the last administration of PP. The lung tissues were homogenized (1/10, w/v) in tissue lysis/extraction reagent, containing a protease inhibitor cocktail (Sigma-Aldrich). Equal amounts of the total cellular protein were resolved by 8-12% SDS-polyacrylamide gels and transferred to Hybond PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were incubated in blocking solution [5% skimmed milk in Tris-buffered saline + 0.1% Tween-20

(TBST)] for 1 h and incubated overnight at 4˚C with the appropriate primary antibody. A rabbit polyclonal MCP-1 antibody (sc-28879, 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), a rabbit polyclonal p-ERK antibody (cat. no. 9101, 1:1,000; Cell Signaling Technology Inc., Danvers, MA, USA), a rabbit polyclonal iNOS antibody (SC-651, 1:1,000; Santa Cruz Biotechnology, Inc.), a goat polyclonal COX-2 antibody (SC-1747, 1:1,000), a rabbit polyclonal ERK antibody (sc-154, 1:1,000), a rabbit polyclonal Nrf2 antibody (sc-722, 1:1,000), a rabbit polyclonal p-Nrf2 antibody (ab76026, 1:1,000; Abcam, Cambridge, UK), a rabbit polyclonal HO-1 antibody (cat. no. 5061, 1:1,000), and a rabbit polyclonal anti-β-actin antibody (cat. no. 4967, 1:2,500) (both from Cell Signaling Technology Inc.) were diluted in 5% skimmed milk. The membranes were washed in TBST and incubated with the Peroxidase-AffiniPure goat anti-rabbit IgG (H+L) (111-035-003, 1:2,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and the Peroxidase-AffiniPure goat anti-mouse IgG (H+L) (115-035-003, 1:2,000; Jackson ImmunoResearch Laboratories, Inc.) for 2 h at room temperature. The membranes were washed with TBST and developed using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). All bands were visualized using a LAS-4000 luminescent image analyzer (Fujifilm, Tokyo, Japan) and quantified by densitometry (Fuji Multi Gauge software, version 3.0).

CSE-stimulated inflammatory molecules in human airway epithelial cells. A549 human airway epithelial cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) supplemented with 10% FBS in the presence of penicillin (100 U/ml) and streptomycin (100 μg/ml), and were incubated in a 5% CO_2 incubator. CSE was purchased from Kentucky Reference 3R4F research blend cigarettes (University of Kentucky) and administered as previously described (22). The A549 human lung epithelial cells were incubated with the indicated concentration of PP prior to the addition of CSE (10 μg/ml).

Reverse transcription-polymerase chain reaction (RT-PCR). The A549 cells were treated with PP in the absence or presence of CSE (10 μg/ml) for 6 h. Total RNA was isolated using TRIzol™ reagent and reverse-transcribed into cDNA, according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). The PCR conditions for MCP-1 were as follows: 94˚C for 10 min (1 cycle), 94˚C for 30 sec, 58˚C for 30 sec, and 72˚C for 1 min (25 cycles); for TNF-α, 94˚C for 10 min (1 cycle), 94˚C for 30 sec, 57˚C for 30 sec, and 72˚C for 1 min (25 cycles); for β-actin, 94˚C for 10 min (1 cycle), 94˚C for 30 sec, 59˚C for 30 sec, and 72˚C for 1 min (25 cycles); and then a final extension phase at 72˚C for 10 min. The specific forward and reverse primers for TNF-α and MCP-1 were as follows: TNF-α forward, 5'-TCAACCTCCTCTCTGCCATC-3' and reverse, 5'-CCTAAGCCCCCAATTCTCTT-3'; MCP-1 forward, 5'-TCTGTGCCTGCTGCTCATAG-3' and reverse, 5'-CAGATCTCCTTGGCCACAAT-3'; and β-actin forward, 5'-CATGTACGTTGCTATCCAGGC-3' and reverse, 5'-CTCCTTAATGTCACGCACGAT-3'. The housekeeping gene β-actin was used for normalization of RT-PCR. The PCR products were separated on agarose gel.

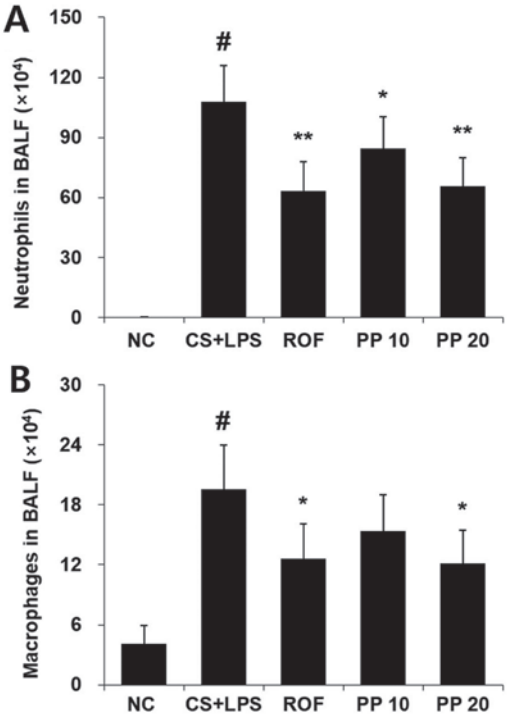


Figure 1. *Physalis peruviana* L. (PP) suppresses the recruitment of inflammatory cells, such as (A) neutrophils and (B) macrophages in the bronchoalveolar lavage fluid (BALF) of mice with cigarette smoke (CS)- and lipopolysaccharide (LPS)-induced inflammation. BALF differential cell count was evaluated with the Diff-Quik® staining reagent. NC, normal control mice; CS + LPS, mice exposed to CS and LPS; ROF, mice administered roflumilast (10 mg/kg) + CS + LPS; PP 10 and 20, mice administered 10 or 20 mg/kg PP + CS + LPS. Data are expressed as the means ± standard deviation. $^{\#}$P<0.01 indicating a statistically significant difference from the NC group; *P<0.05 and **P<0.01 indicating statistically significant differences from the CS + LPS group.

Histological analysis. To evaluate the protective effect of PP, lung tissues were obtained at 24 h after PP administration, washed with PBS and fixed in 10% (v/v) neutral buffered formalin solution. The lung tissues were then embedded in paraffin, sectioned at 4 μm using a rotary microtome, and stained with hematoxylin and eosin (H&E; Sigma-Aldrich) to estimate inflammatory response.

Statistical analysis. The data are expressed as the means ± standard deviation obtained from at least three independent experiments. Statistical significance was determined using two-tailed Student's t-test. A value of P<0.05 was considered to indicate statistically significant differences.

Results

Treatment with PP attenuates the recruitment of neutrophils in the BALF of CS- and LPS-induced airway inflammation animal models. The increased number of neutrophils and macrophages in the BALF is a cornerstone characteristic of CS- and LPS-induced airway inflammation. Thus, the effect of PP on the infiltration of these cells was evaluated using Diff-Quik® staining. As shown in Fig. 1, increased numbers of neutrophils and macrophages were detected in the CS + LPS group, whereas decreased numbers of these cells were detected in the PP group, and this effect was

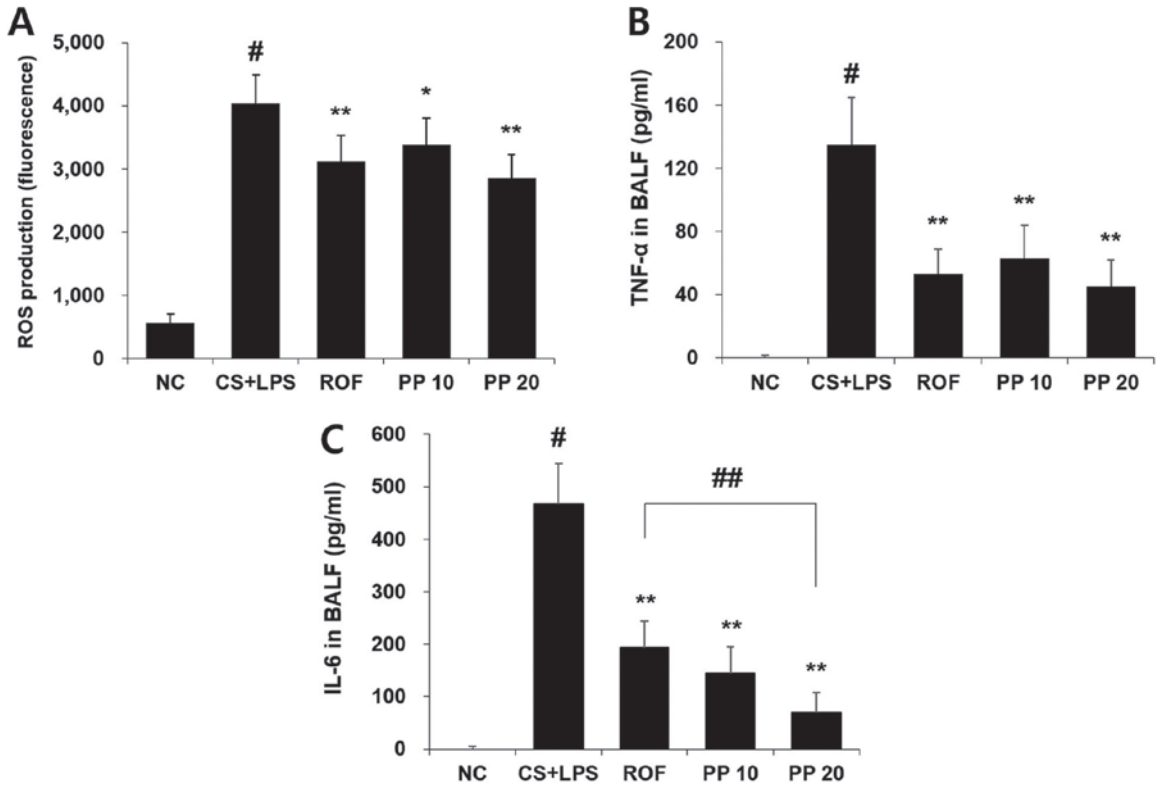


Figure 2. *Physalis peruviana* L. (PP) decreases the levels of reactive oxygen species (ROS) and pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 in the bronchoalveolar lavage fluid (BALF) of mice with cigarette smoke (CS)- and lipopolysaccharide (LPS)-induced inflammation. (A) The production of ROS was determined using 2',7'-dichlorofluorescein diacetate. (B and C) The levels of TNF-α and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). The absorbance was measured at 450 nm using a microplate reader. NC, normal control mice; CS + LPS, mice exposed to CS and administered LPS; ROF, mice exposed CS and administered roflumilast (10 mg/kg) + LPS; PP 10 and 20, mice exposed to CS and administered 10 or 20 mg/kg PP + LPS. Data are expressed as the means ± standard deviation; $^{\#}$P<0.01 indicates a statistically significant difference from the NC group; *P<0.05 and **P<0.01 indicate statistically significant differences from the CS + LPS group; $^{\#\#}$P<0.01 indicates statistically significant differences from the ROF group.

concentration-dependent (Fig. 1). ROF was used as positive control. The effect of 20 mg/kg PP was similar to that of treatment with 10 mg/kg ROF.

Treatment with PP reduces the levels of ROS and pro-inflammatory cytokines in the BALF. Intranasal administration of LPS markedly increased the levels of ROS and inflammatory cytokines, including TNF-α and IL-6. However, treatment with PP significantly reduced these levels in a concentration-dependent manner (Fig. 2). In particular, 20 mg/kg PP attenuated the production of IL-6 more effectively compared with the ROF or CS + LPS groups (Fig. 2C).

Treatment with PP inhibits the infiltration of inflammatory cells and the expression of MCP-1 in the lungs. It was next examined whether PP affects the infiltration of inflammatory cells and the production of MCP-1 in the lungs of animal models with CS- and LPS-induced airway inflammation. As shown in Fig. 3A, increased influx of inflammatory cells was observed around peribronchial lesions in the CS + LPS group, whereas a significant reduction of the influx of these cells was detected in the PP group. Treatment with PP also significantly attenuated the expression of MCP-1 compared with the ROF or CS + LPS groups (Fig. 3B). Similar to the results obtained by PP in the lung, a decreased level of MCP-1 in the BALF was confirmed following PP administration (Fig. 3C).

Treatment with PP decreases the expression of iNOS and COX-2, and reduces the activation of ERK in the lung. LPS administration markedly increased the expression of iNOS and COX-2 compared with the NC group (Fig. 4). However, treatment with PP significantly decreased the levels of iNOS and COX-2 compared with the CS + LPS group in a concentration-dependent manner (Fig. 4A). Similar to the results for iNOS and COX-2, ERK phosphorylation was also effectively decreased with PP administration. In particular, the effect of 20 mg/kg PP was similar to that of 10 mg/kg ROF (Fig. 4B).

Treatment with PP increases the expression of HO-1 in the lungs. HO-1 induction exerts a protective effect in airway inflammatory diseases, including COPD. Thus, western blot analysis was used to investigate whether PP promotes the expression of HO-1. There was an increase in HO-1 expression in the PP group compared with the NC or ROF groups (Fig. 5B). Activation of Nrf2, which is a major transcription factor of HO-1, was also observed following PP administration (Fig. 5A).

Pretreatment with PP reduces the mRNA expression of inflammatory molecules and the activation of ERK in A549 airway epithelial cells. CSE treatment significantly increased the mRNA expression of TNF-α and MCP-1 in A549 cells. However, these effects were effectively attenuated by PP

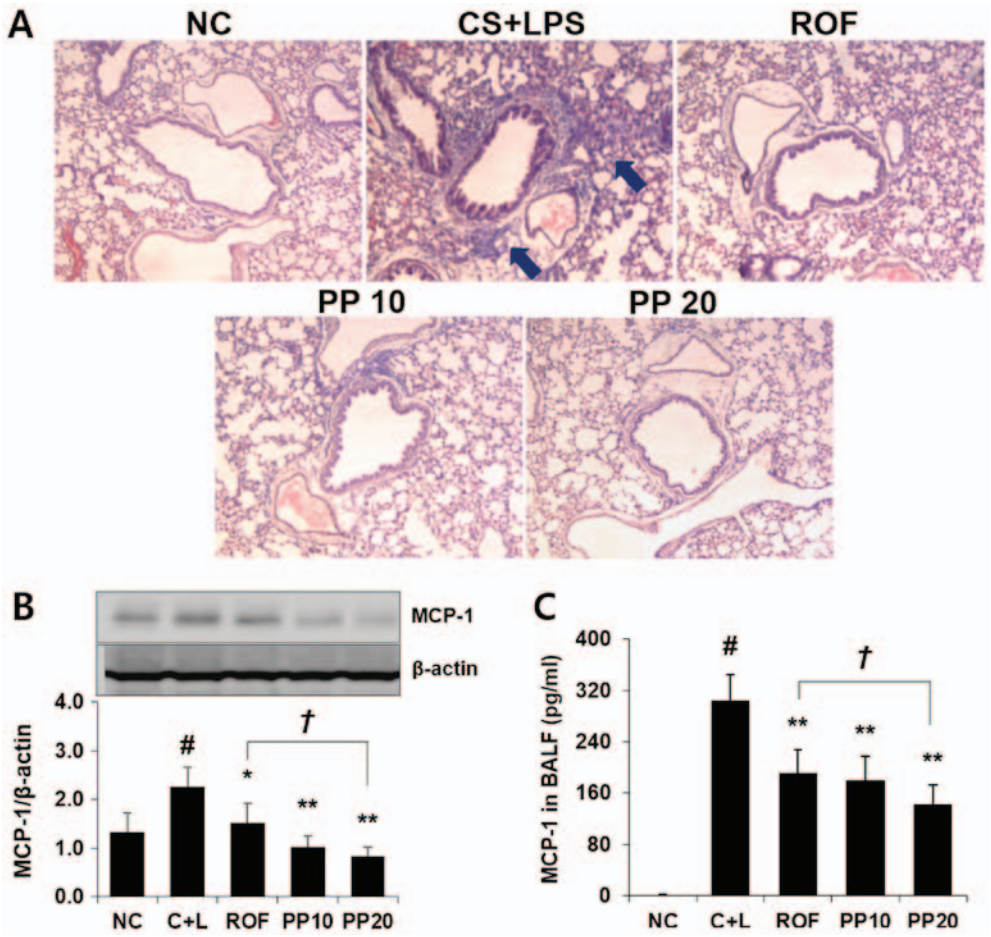


Figure 3. *Physalis peruviana* L. (PP) inhibits the influx of inflammatory cells and the expression of monocyte chemoattractant protein-1 (MCP-1) in the lungs of mice with cigarette smoke (CS)- and lipopolysaccharide (LPS)-induced inflammation. (A) A peribronchial lesion was stained with hematoxylin and eosin to evaluate the airway inflammation (magnification, x200). (B and C) The levels of MCP-1 were detected using western blot analysis and enzyme-linked immunosorbent assay (ELISA). NC, normal control mice; CS + LPS, mice exposed to CS and administered LPS; ROF, mice exposed to CS and administered roflumilast (10 mg/kg) + LPS; PP 10 and 20, mice exposed to CS and administered 10 or 20 mg/kg PP + LPS. Data are expressed as the means ± standard deviation; $^{\#}$P<0.05 indicates a statistically significantly difference from the NC group; *P<0.05 and **P<0.01 indicate statistically significant differences from the CS + LPS group; †P<0.05 indicates statistically significant differences from the ROF group.

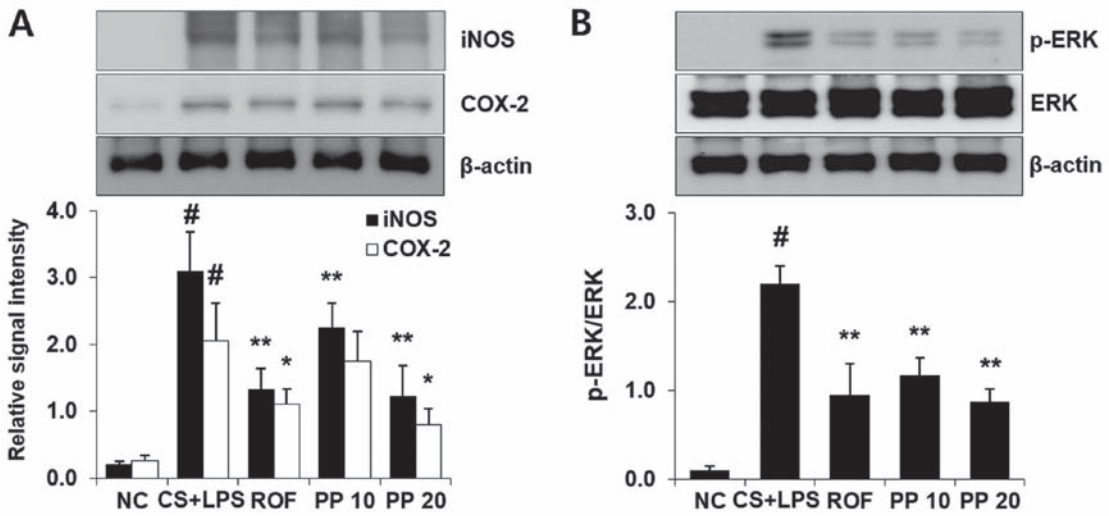


Figure 4. (A and B) *Physalis peruviana* L. (PP) suppresses the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and the activation of extracellular signal-regulated kinase (ERK) in the lungs of mice with cigarette smoke (CS)- and lipopolysaccharide (LPS)-induced inflammation. The levels of iNOS, COX-2 and phosphorylated ERK expression were measured by western blot analysis. Quantitative analysis was performed by a densitometric method. β-actin was used as an internal control. NC, normal control mice; CS + LPS, mice exposed to CS and administered LPS; ROF, mice exposed to CS and administered roflumilast (10 mg/kg) + LPS; PP 10 and 20, mice exposed to CS and administered 10 or 20 mg/kg PP + LPS. Data are expressed as the means ± standard deviation; $^{\#}$P<0.01 indicates a statistically significant difference from the NC group; *P<0.05 and **P<0.01 indicate statistically significant differences from the CS + LPS group.

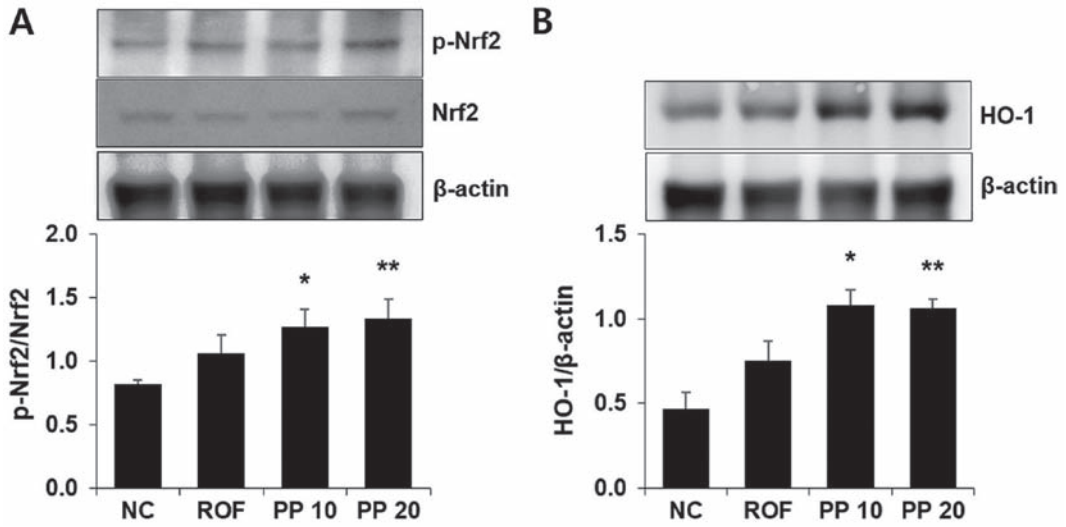


Figure 5. *Physalis peruviana* L. (PP) upregulates the expression of heme oxygenase-1 (HO-1) in the lungs of mice with cigarette smoke (CS) and lipopolysaccharide (LPS)-induced inflammation. (A) Nuclear factor erythroid 2-related factor 2 (Nrf2) activation and (B) HO-1 expression in the lungs were measured by western blot analysis. Quantitative analysis was performed by a densitometric method. β-actin was used as an internal control. NC, normal control mice; CS + LPS, mice exposed to CS and administered LPS; ROF, mice exposed to CS and administered roflumilast (10 mg/kg) + LPS; PP 10 and 20, mice exposed to CS and administered 10 or 20 mg/kg PP + LPS. Data are expressed as the means ± standard deviation; *P<0.05 and **P<0.01 indicated statistically significant differences from the NC group.

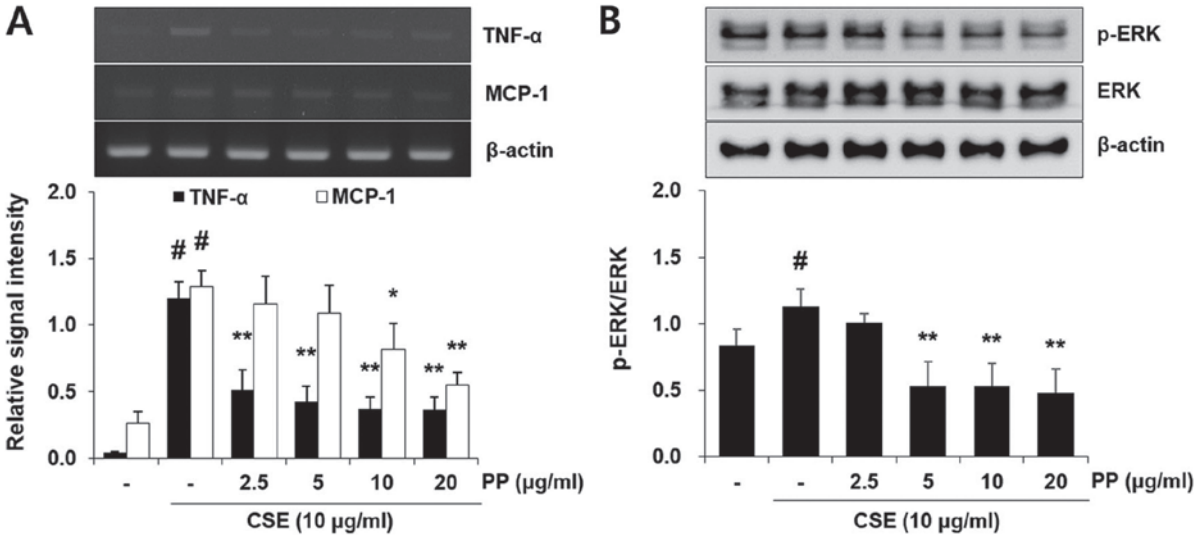
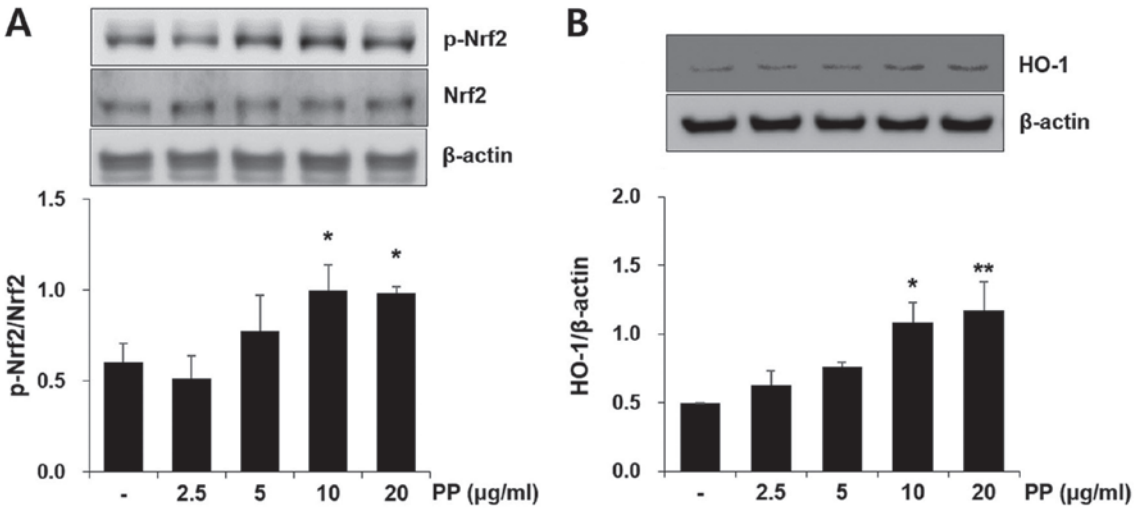


Figure 6. *Physalis peruviana* L. (PP) downregulates the levels of inflammatory molecules and extracellular signal-regulated kinase (ERK) activation in cigarette smoke (CS) extract (CSE)-stimulated A549 human airway epithelial cells. (A) The levels of tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) mRNA were determined using reverse transcription-polymerase chain reaction. (B) The phosphorylation of ERK was determined with western blot analysis. Data are expressed as the means ± standard deviation; $^{\#}$P<0.05 indicates a statistically significantly difference from the normal control group; *P<0.05 and **P<0.01 indicate statistically significant differences from the CS + LPS group. LPS, lipopolysaccharide.

pretreatment (Fig. 6A). It was also confirmed that pretreatment with PP significantly decreased the activation of ERK in CSE-stimulated A549 cells (Fig. 6B). In addition, PP increased Nrf2 activation and HO-1 expression in A549 cells (Fig. 7).

Discussion

COPD is respiratory condition characterized by expiratory airway obstruction, the major cause of which is chronic airway inflammation (23). Given the importance of airway inflammation in COPD, the anti-inflammatory activity of PP was investigated using CS- and LPS-induced airway inflammation animal models. As increased influx of neutrophils is a cornerstone characteristic of airway inflammation in COPD, it was investigated whether treatment with PP attenuates this influx in the airway inflammatory response. As shown in Fig. 1A, there was a distinct reduction in the neutrophil influx in the BALF of the PP group compared with the CS + LPS group. In particular, the effect of 20 mg/kg PP was similar to that of 10 mg/kg ROF. Oxidative stress is implicated in airway inflammation and recognized as a one of the major predicting factors in the pathogenesis of COPD (24). Increased amounts of ROS are generated by neutrophils against CS and



Fiugre 7. *Physalis peruviana* L. (PP) upregulates the expression of heme oxygenase-1 (HO-1) in A549 human airway epithelial cells. (A) Nuclear factor erythroid 2-related factor 2 (Nrf2) activation and (B) HO-1 expression in A549 cells were determined by western blot analysis. Quantitative analysis was performed by a densitometric method. β-actin was used as an internal control. Data are expressed as the means ± standard deviation; *P<0.05 and **P<0.01 indicate statistically significant differences from the normal control group.

contribute to oxidative stress (25). Therefore, it was investigated whether PP inhibits the increase in the level of ROS. As shown in Fig. 2A, ROS production was upregulated in the BALF of the CS + LPS group compared with the NC group (P<0.01), whereas treatment with PP significantly decreased ROS production in a concentration-dependent manner, indicating that PP exerts an antioxidant effect in the airway inflammatory response (Fig. 2A). Similar to the results for ROS, PP effectively attenuated the levels of TNF-α and IL-6 in the BALF (Fig. 2B and C). In particular, 20 mg/kg PP inhibited the production of IL-6 more effectively compared with 10 mg/kg ROF (Fig. 2C). The inhibition rates of IL-6 production were 58.6% (ROF), 69.0% (PP 10 mg/kg) and 84.9% (PP 20 mg/kg). Increased recruitment of inflammatory cells into the lung is the major pathophysiological mechanism underlying COPD (26). Based on this result, H&E staining was used to determine whether PP exerts an inhibitory effect on the recruitment of inflammatory cells. As shown in Fig. 3A, exposure to CS and LPS markedly upregulated the recruitment of inflammatory cells, whereas treatment with PP markedly reduced this recruitment (Fig. 3A). These results indicate that PP plays an antioxidant and anti-inflammatory role in airway inflammation induced by CS and LPS.

Ng *et al* demonstrated that the inhibition of MCP-1 may be valuable in the inhibition of CS-induced airway inflammation (27). Our recent study also confirmed that antioxidant treatment attenuates airway inflammation induced by CS through the inhibition of MCP-1 (13). As airway inflammatory response is accompanied by increased production of MCP-1, it was investigated whether PP acts as a MCP-1 inhibitor. As shown in Fig. 3B, treatment with PP significantly decreased the expression of MCP-1 compared with the CS + LPS group, in a concentration-dependent manner (P<0.01) (Fig. 3B). Similar to those results, the increased level of MCP-1 was effectively reduced with PP administration (Fig. 3C). In particular, 20 mg/kg PP decreased these levels more effectively compared with the ROF or CS + LPS groups (Fig. 3B and C). These results indicate that PP affects the influx of inflammatory cells as well as the expression of MCP-1, suggesting that PP may be a useful inhibitor of MCP-1.

High levels of iNOS are associated with a variety of pathological conditions, including COPD (28). Gupta *et al* reported that CS exposure leads to iNOS expression, which results in production of toxic NO metabolites, leading to severe lung damage (29). The levels of COX-2 are significantly increased in small airway epithelium of patients with COPD, and the increase in the levels of this molecule contribute to airway remodeling and inflammation, which are major characteristics of COPD (30). Therefore, inhibition of iNOS and COX-2 may be important for reducing airway inflammation. In the present study, there was significant increase in iNOS and COX-2 expression in the CS + LPS group compared with the NC group (P<0.01). However, treatment with PP significantly decreased these levels in a concentration-dependent manner (Fig. 4A and B). The inhibitory effect of 20 mg/kg PP on these molecules was similar to that of 10 mg/kg ROF.

The ERK signaling cascade is involved in the airway inflammatory response. Li *et al* reported that CSE causes an inflammatory response through the activation of ERK in human bronchial epithelial cells (31). In our recent study, it was also confirmed that the increased levels of inflammatory cytokines, such as TNF-α and IL-6, were attenuated with downregulation of ERK activation in CSE-stimulated human airway epithelial cells (22). In an *in vivo* study in airway inflammation animal models, treatment with antioxidants effectively inhibited airway inflammation via inhibition of ERK activation (13,32). Therefore, controlling the activity of the ERK pathway may be a valuable therapeutic approach to airway inflammatory diseases, including COPD. Thus, the effect of PP on ERK activation was evaluated. As shown in Fig. 4, the phosphorylation of ERK was markedly increased in the CS + LPS group, whereas decreased phosphorylation was detected in the PP group. This result indicates that PP may be a useful inhibitor of ERK activation in airway inflammation.

Antioxidant enzymes, such as HO-1, NAD(P)H quinone oxidoreductase 1 and superoxide dismutase exert protective

effects against endotoxin-induced inflammation (33,34). Among those, it is well known that HO-1 exerts anti-inflammatory effects in inflammatory conditions by controlling nuclear factor-κB activation and production of inflammatory molecules (35,36). Treatment with antioxidants ameliorates the CS-induced neutrophil influx and airway inflammation through the induction of HO-1 (13). Li *et al* reported that antioxidants downregulate the expression of inflammatory cytokines and upregulate the expression of HO-1 in CSE-stimulated macrophages and bronchial epithelial cells (15). Therefore, HO-1 induction may be useful in the treatment of CS-induced airway inflammation. In the present study, it was confirmed that PP administration upregulated the expression of HO-1 compared with the CS + LPS group. PP more effectively increased HO-1 expression compared with the NC group ($P<0.01$) (Fig. 5). This result suggests that PP may be useful in the induction of HO-1 in CS-induced airway inflammation.

Airway epithelial cells are first-defense cells and an important source of inflammatory cytokines and chemokines against CS (12). Similar to the results obtained by PP *in vivo*, the increased release of inflammatory cytokines and chemokines was markedly decreased with PP administration in CSE-stimulated A549 airway cells (Fig. 6). Nrf2 activation and HO-1 expression were also upregulated with PP treatment in A549 cells (Fig. 7).

Antioxidants have therapeutic as well as preventive potential in the airway inflammatory response of COPD and, therefore, may prove to be a useful therapeutic approach to the treatment of COPD (37). In several studies, antioxidants have been reported to protect against the airway inflammatory response by reducing the influx of inflammatory cells and the levels of inflammatory molecules, and by upregulation of HO-1. Therefore, it was evaluated whether PP, which is a strong antioxidant, exerts a protective effect in airway inflammation. In the present study, PP effectively attenuated airway inflammation by inhibition of neutrophil influx and of inflammatory toxic molecules, such as ROS, which are important pathophysiological characteristics in COPD. These effects of PP were accompanied by Nrf2 activation and HO-1 induction, *in vitro* as well as *in vivo*. Therefore, our results suggest that PP may be a valuable therapeutic adjuvant in airway inflammatory diseases, including COPD.

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