Protective effects of neem (*Azadirachta indica* A. Juss.) leaf extract against cigarette smoke- and lipopolysaccharide-induced pulmonary inflammation

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Abstract. Neem (*Azadirachta indica* A. Juss.) leaf has been reported to exert anti-inflammatory, antibacterial and antioxidant effects. The purpose of this study was to investigate the protective effects of neem leaf extract (NLE) against cigarette smoke (CS)- and lipopolysaccharide (LPS)-induced pulmonary inflammation. Treatment with NLE significantly attenuated the infiltration of inflammatory cells, such as neutrophils and macrophages in bronchoalveolar lavage fluid (BALF). NLE also reduced the production of reactive oxygen species and the activity of neutrophil elastase in BALF. Moreover, NLE attenuated the release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 in BALF. NLE inhibited the recruitment of inflammatory cells and the expression of monocyte chemoattractant protein-1 (MCP-1) in the lungs of mice with CS- and LPS-induced pulmonary inflammation. NLE also decreased the expression of inducible nitric oxide synthase (iNOS) in the lungs of the mice CS- and LPS-induced pulmonary inflammation. Furthermore, treatment with NLE significantly attenuated the activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) in the lungs mice exposed to CS and LPS. NLE also inhibited the phosphorylation of nuclear factor (NF)-κB and inhibitor of NF-κB (IκB) in the lungs of mice expose to CS and LPS. These findings thus suggest that NLE has potential for use in the treatment of chronic obstructive pulmonary disease.

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Abbreviations: COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; LPS, lipopolysaccharide; BALF, broncho-alveolar lavage fluid; ROS, reactive oxygen species; NE, neutrophil elastase; NLE, neem leaf extract; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; iNOS, inducible nitric oxide synthase; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; IκB, inhibitor of NF-κB

Key words: neem, chronic obstructive pulmonary disease, cigarette smoke, lipopolysaccharide, neutrophil, mitogen-activated protein kinases, nuclear factor-κB

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic airway disease that leads to difficulties breathing (1), and is characterized by chronic inflammation of the respiratory tract with increased numbers of inflammatory cells and molecules (2). The worldwide incidence, prevalence and mortality of COPD are increasing (3). Cigarette smoke (CS) is a complex mixture of chemicals generated from the burning of tobacco (4), and is the main cause of COPD (5). CS affects the recruitment of inflammatory cells, including neutrophils into the lungs and is associated with chronic inflammation of the airways and a decline in lung function (6).

Neutrophils are the host defense inflammatory cells that are rapidly recruited to sites of infection (7). However, neutrophilic inflammation is the major cause of pulmonary inflammation in COPD pathophysiology (8). Activated neutrophils produce several cytotoxic mediators, including reactive oxygen species (ROS) and neutrophil elastase (NE), which aggravate pulmonary inflammation and emphysema (9). The increased production of ROS accelerates the development of COPD through the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) (10). NE activity is increased in the lungs affected by COPD, which enhances the destruction of alveolar structure (11). Tumor necrosis factor-α (TNF-α) is a central inflammatory cytokine that is associated with

many immune-mediated diseases, including COPD (12). It is well known that the constitutive overexpression of TNF-α affects the recruitment of inflammatory cells and promotes emphysema in the lungs of animals (13). Interleukin (IL)-6 is a pro-inflammatory cytokine that plays a pivotal role in the pathogenesis of COPD by modulating pulmonary function (14). Monocyte chemoattractant protein-1 (MCP-1) is one of the key chemokines that contributes to the recruitment of inflammatory cells, such as neutrophils (15) and macrophages (16). MCP-1 levels are significantly increased in patients with COPD compared with non-smokers (17). Inducible nitric oxide synthase (iNOS) expression is induced by neutrophils and macrophages in response to pro-inflammatory stimuli (18,19) and is known to have anti-inflammatory activity (20,21). However, the continuous expression of iNOS is associated with pulmonary inflammation and emphysema (22). Recently, it has also been reported that iNOS expression is higher in the lungs of patients with COPD than non-smokers (23). The MAPK signaling pathway promotes the inflammatory response by enhancing inflammatory gene transcription (6,24). NF-κB is a central transcription factor that plays an important role in the expression of inflammatory genes, such as iNOS, TNF-α and IL-6 (25). CS has been shown to affect the activation of MAPKs (26) and NF-κB (27).

Neem (*Azadirachta indica* A. Juss.) belonging to the family, Meliaceae is an evergreen tree, cultivated in various parts of the Indian subcontinent (28). The neem leaf has been reported to exhibit various pharmacological activities, including anti-inflammatory (29), antioxidant (30,31), antimicrobial (32) and antiviral properties (33). Active constituents of the neem leaf include nimbin, nimbidine, isomeldenin, β-sitosterol and quercetin (34). Quercetin (35), β-sitoserol (36) and nimbidine (37) have been shown to exert anti-inflammatory effects. These effects are due to the inhibition of pro-inflammatory molecules, such as TNF-α, iNOS and NF-κB. Recently, neem leaf extract (NLE) has been reported to protect against endotoxemia in mice exposed to lipopolysaccharide (LPS) (38). However, to date, at least to the best of our knowledge, the protective effects of NLE have not been demonstrated in CS- and LPS-induced pulmonary inflammation. Thus, the aim of this study was to investigate the protective effects of NLE against cigarette smoke (CS)- and lipopolysaccharide (LPS)-induced pulmonary inflammation.

Materials and methods

Preparation of NLE. Neem leaf was collected from ward no. 11, Hetauda, Nepal (latitude 27˚27'11.7", longitude 85˚00'11.1" and 531 m above sea level), and identified by Mr. M.R. Poudeyal of the Ethnobotanical Society of Nepal (ESON). Voucher specimens recorded as KRIB 0059759 and 760 have been deposited in the herbarium of the Korea Research Institute of Bioscence and Biotechnolgy (KRIB). After drying and grinding the leaves of neem, the powder (52 g) was added to 100 liters of methanol. The extraction was carried out using the method of repercolation at room temperature. The extract was filtered and concentrated by a rotavapor under reduced pressure, thereby obtaining 2.99 g of neem methanolic extract. In the following experiment, the neem leaves were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/ml, and then diluted to various concentrations prior to use.

Model of CS- and LPS-induced pulmonary inflammation. CS- and LPS-induced pulmonary inflammation was induced using a modification of the procedure described by Lee *et al* (6). Briefly, a total of 30 C57BL/6 mice (6 weeks old; weight, 20 g; n=6/group) were whole-body exposed to room fresh air or CS of 7 cigarettes for 50 min a day for 9 days. CS was generated by 3R4F research cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA). LPS was instilled intranasally on day 8 (5 μg dissolved in 50 μl distilled water). The mice were randomly divided into 5 groups as follows: the normal control (NC), the CS + LPS (CS with intranasal LPS instillation) group, the ROF (CS with intranasal LPS instillation) + roflumilast [10 mg/kg, *per os* (p.o)] group, and the NLE 10 or 20 (CS with intranasal LPS instillation) + NLE (10 or 20 mg/kg, p.o) groups. All the animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology and performed in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and National Animal Welfare Law of Korea.

Measurement of inflammatory cells in bronchoalveolar lavage fluid (BALF). BALF collection was performed using the method of Shin *et al* (5). In brief, the mice were administered an intraperitoneal injection of a pentobarbital (50 mg/kg; Hanlim Pharm, Co., Seoul, Korea) 24 h after the final challenge, and a tracheostomy was performed. To obtain the BALF, ice-cold phosphate-buffered saline (PBS) (0.7 ml) was infused into the lung and withdrawn via tracheal cannulation twice (total volume, 1.4 ml). To determine differential cell counts, 100 μl of BALF were centrifuged at 1,500 rpm for 5 min and the number of neutrophils and macrophages was counted using Diff-Quik® staining reagent according to the manufacturer's instructions (IMEB Inc., Deerfield, IL, USA).

Measurement of ROS and NE in BALF. The effects of NLE on the production of ROS were determined using 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, St. Louis, MO, USA). Briefly, the inflammatory cells were isolated from BALF and incubated with 20 μM DCFH-DA for 10 min at 37˚C. The level of intracellular ROS was then determined using a fluorescence microscope at 488 nm excitation and 525 nm emission (8). The activity of NE was examined using N-succinyl-(Ala)3-p-nitroanilide (Sigma-Aldrich) in 37˚C for 90 min, according to the protocol described by Sakuma *et al* (39).

Measurement of the level of pro-inflammatory cytokines in BALF. The levels of pro-inflammatory cytokines (TNF-α and IL-6) in BALF were determined using ELISA according to the manufacturer's instructions (R&D Systems, Shanghai, China). The absorbance was measured at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA), as previously described (4).

Western blot analysis. Lung tissues were homogenized using a homogenizer with a lysis buffer (Intron Biotechnology, Inc., Seoul, Korea). Protein samples were denatured and resolved on 10% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. The membrane was incubated with blocking solution for 1 h. Specific antibodies against

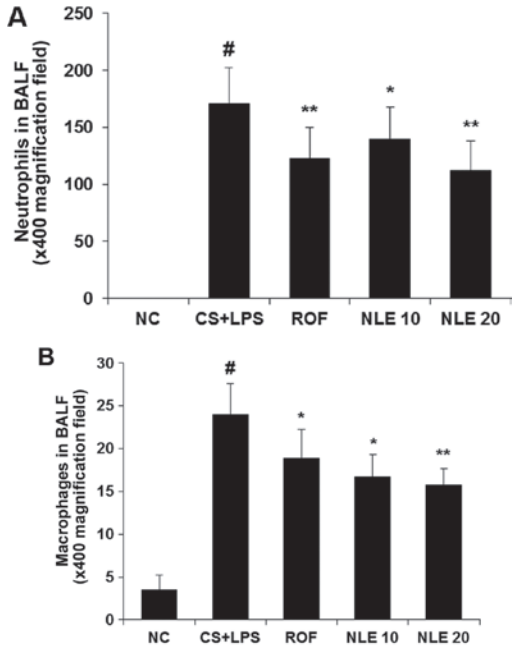

Figure 1. Effect of neem leaf extract (NLE) on the infiltration of neutrophils and macrophages in the bronchoalveolar lavage fluid (BALF) of mice with cigarette smoke (CS)- and lipopolysaccharide (LPS)-induced pulmonary inflammation. (A and B) The BALF differential cell count was determined using the Diff-Quick® staining reagent (x400 magnification). The values are expressed as means ± SD (n=6 mice per group). NC, normal control mice with PBS only; CS + LPS, cigarette smoke (CS) and lipopolysaccharide (LPS); ROF, roflumilast (10 mg/kg) + CS and LPS; NLE 10 or 20, NLE (10 or 20 mg/kg) + CS and LPS. $^{\#}$p<0.01 indicates a statistically significant difference from the normal control group. *p<0.05 and **p<0.01 indicate statistically significant differences compared to the CS and LPS group.

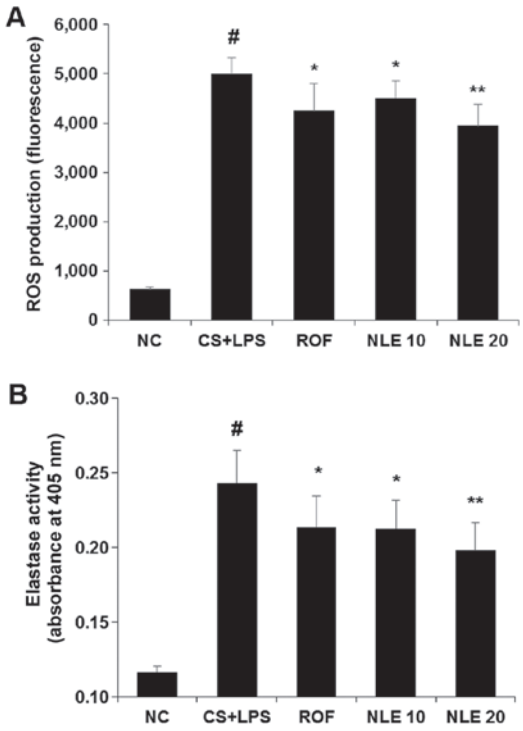

Figure 2. Effect of neem leaf extract (NLE) on the production of reactive oxygen species (ROS) and neutrophil elastase (NE) in bronchoalveolar lavage fluid (BALF). (A) ROS production and (B) NE activity. Data are expressed as the means ± SD. $^{\#}$p<0.01 indicates a statistically significant difference from the normal control group. *p<0.05 and **p<0.01 indicate statistically significant differences compared to the cigarette smoke (CS) and lipopolysaccharide (LPS) group.

MCP-1 (1;1,000; ab25124; Abcam, Cambridge, MA, USA), iNOS (1;1,000; ADI-905-431; Enzo Life Sciences, Farmingdale, NY, USA), p-ERK (1:1,000; #9101; Cell Signaling Technology, Inc., Danvers, MA, USA), ERK (1:1,000; sc-154; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-JNK (1:1,000; KAP-SA011; Enzo Life Sciences), JNK (1:1,000; sc-474; Santa Cruz Biotechnology), p-p38 (1:1,000; ADI-KAP-MA022; Enzo Life Sciences), p-38 (1:1,000; sc-7149; Santa Cruz Biotechnology), p-p65 (1:1,000; #3033; Cell Signaling Technology, Inc.), p65 (1:1,000; sc-372; Santa Cruz Biotechnology), p-inhibitor of NF-κB (IκB; 1:1,000; sc-371; Santa Cruz Biotechnology) and β-actin (1;2,500; #4967; Cell Signaling Technology, Inc.) were incubated overnight at 4˚C with 5% skim milk. The membranes were washed in Tris-buffered saline with Tween 20 (TBST) and incubated with the Peroxidase-AffiniPure goat anti-mouse IgG (H+L) (1:2,000; 115-035-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and the Peroxidase-AffiniPure goat anti-rabbit IgG (H+L) (1:2,000; 111-035-003; Jackson ImmunoResearch Laboratories, Inc.) for 2 h at room temperature. The blots were washed 3 times with TBST, and then developed with an enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ, USA).

Histological analysis. After the BALF samples were collected, lung tissues were fixed in 10% (v/v) neutral-buffered formalin solution. For histological examination, the lung tissues were embedded in paraffin, sectioned at 4 μm thickness, and stained with a hematoxylin and eosin (H&E) solution (Sigma-Aldrich) to estimate the inflammatory response.

Statistical analysis. All values shown in the figures are expressed as the means ± SD obtained from at least 3 independent experiments. Statistical significance was carried out using a two-tailed Student's t-test. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

NLE inhibits the infiltration of inflammatory cells in the BALF of mice with CS- and LPS-induced pulmonary inflammation. Given the fact that the infiltration of inflammatory cells, such as neutrophils and macrophages is increased in the BALF of mice with CS- and LPS-induced pulmonary inflammation (9), we investigated whether NLE inhibits the infiltration of neutrophils and macrophages in BALF. As shown in Fig. 1, we observed that increased numbers of neutrophils and macrophages were detected in the BALF of mice in the CS and LPS group compared with those in the normal control group. However, treatment with NLE significantly attenuated the numbers of neutrophils and macrophages in BALF, compared with the CS and LPS group in a concentration-dependent manner (Fig. 1). The effect of 20 mg/kg NLE was similar to that of treatment with 10 mg/kg ROF.

NLE attenuates the production of ROS and NE in BALF. It is well known that ROS production and NE activity are

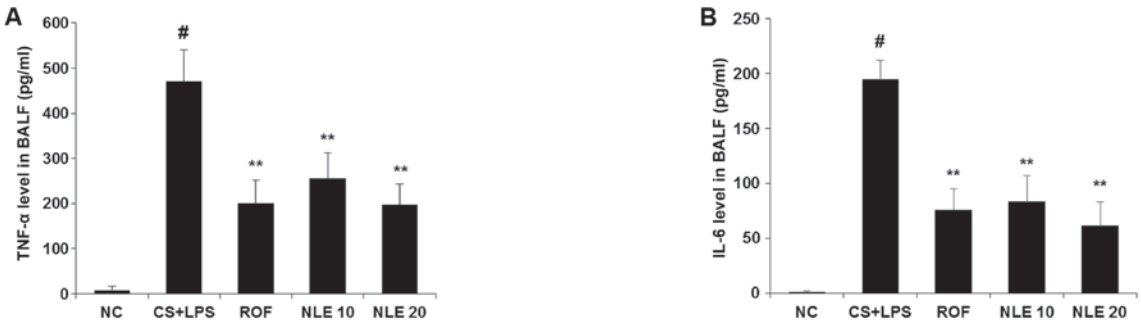

Figure 3. Effect of neem leaf extract (NLE) on the levels of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in bronchoalveolar lavage fluid (BALF). (A) The levels of TNF-α and (B) IL-6 were measured by ELISA. The absorbance was measured at 450 nm using a microplate reader. Data are expressed as the means ± SD. $^{\#}$p<0.01 indicates a statistically significant differencefrom the normal control group. **p<0.01 indicate statistically significant differences compared to the cigarette smoke (CS) and lipopolysaccharide (LPS) group.

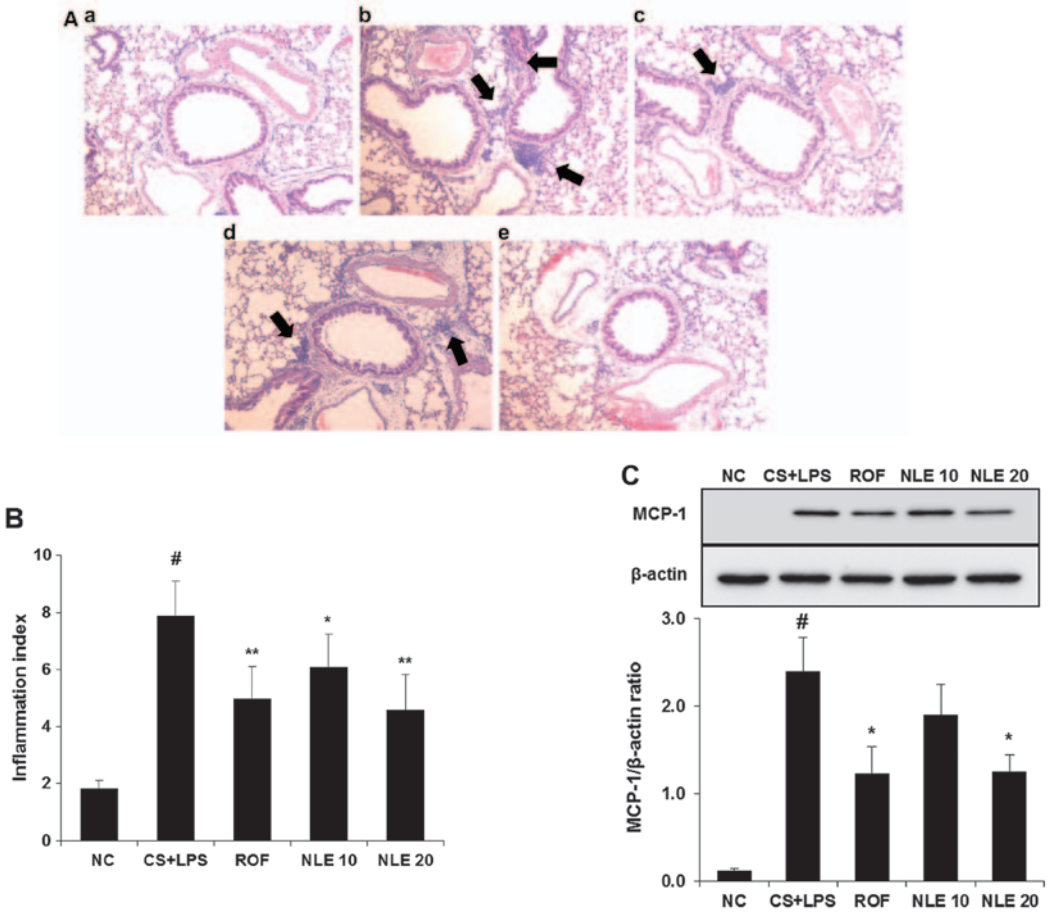

Figure 4. Effect of neem leaf extract (NLE) on the infiltration of inflammatory cells in lungs of mice with cigarette smoke (CS)- and lipopolysaccharide (LPS)-induced pulmonary inflammation. (A) Peribronchial lesion (x400 magnification): (a) negative control, (b) CS + LPS, (c) roflumilast, (d) NLE 10 mg/kg and (e) NLE 20 mg/kg. (B) Quantitative analysis of airway inflammation in lung tissue stained with H&E solution. (C) Monocyte chemoattractant protein-1 (MCP-1) expression was detected by western blot analysis. NC, normal control mice with PBS only; CS + LPS, cigarette smoke (CS) and lipopolysaccharide (LPS); ROF, roflumilast (10 mg/kg) + CS and LPS; NLE 10, NLE (10 mg/kg) + CS and LPS; NLE 20, NLE (20 mg/kg) + CS and LPS. Data are expressed as the means ± SD. $^{\#}$p<0.01 indicates a statistically significant difference from the ormal control group. *p<0.05 and **p<0.01 indicate statistically significant differences compared with the CS and LPS group.

increased in the BALF of mice with CS- and LPS-induced pulmonary inflammation (5,6). Thus, in this study, the levels of ROS and NE were examined in the BALF of mice with CS and LPS-induced pulmonary inflammation. As shown in Fig. 2, the levels of ROS and NE were significantly increased in the CS and LPS group. However, treatment with NLE significantly decreased the levels of ROS and NE (Fig. 2). In particular, treatment with 20 mg/kg NLE more effectively attenuated the levels of those molecules compared with 10 mg/kg ROF.

NLE decreases the levels of TNF-α and IL-6 in BALF. The increased release of TNF-α and IL-6 in BALF is one of the major characteristics of COPD (5). Thus, to determine whether NLE affects the release of pro-inflammatory cytokines in

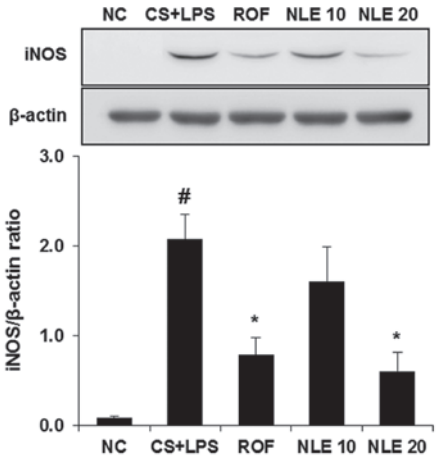

Figure 5. Effect of neem leaf extract (NLE) on the expression of inducible nitric oxide synthase (iNOS) in lungs of mice. The expression of iNOS was detected by western blot analysis. Data are expressed as the means ± SD. #p<0.01 indicates a statistically significant difference from the normal control group. *p<0.05 indicate statistically significant differences compared to the cigarette smoke (CS) and lipopolysaccharide (LPS) group.

BALF, the levels of TNF-α and IL-6 were examined by ELISA. As shown in Fig. 3, treatment with NLE effectively inhibited the release of these cytokines in BALF.

NLE reduces the recruitment of inflammatory cells and the expression of MCP-1 in the lungs of mice with CS- and LPS-induced pulmonary inflammation. To examine whether NLE affects the recruitment of inflammatory cells and the expression of MCP-1 in the lungs of mice with CS- and LPS-induced pulmonary inflammation, the infiltration of inflammatory cells was determined by H&E staining. As shown in Fig. 4A and B, the mice in the CS and LPS group exhibited an increased infiltration of inflammatory cells. However, treatment with NLE significantly reduced the recruitment of inflammatory cells in a concentration-dependent manner. Consistent with the decrease in inflammatory cell recruitment, treatment with NLE also significantly decreased the expression of MCP-1 in the lungs, suggesting that NLE attenuated the recruitment of inflammatory cells (Fig. 4C). Similar to the results shown above, the effect of 20 mg/kg NLE was similar to that of treatment with 10 mg/kg ROF.

NLE inhibits the expression of iNOS in lungs of mice with CS- and LPS-induced pulmonary inflammation. As the increased expression of iNOS induced by neutrophils (40) and macrophages (2) is an important in the pathologenesis of COPD, we investigated whether NLE affects the level of iNOS in the lungs of mice with CS- and LPS-induced pulmonary inflammation. As shown in Fig. 5, iNOS expression was increased in the lungs of mice in the CS and LPS group. However, treatment with NLE effectively inhibited the expression of iNOS, compared with normal control mice.

NLE attenuates the activation of ERK and JNK in the lungs of mice with CS- and LPS-induced pulmonary inflammation. MAPK activation plays an important role in the inflammatory response regulating the release of pro-inflammatory cytokines and mediators. Thus, we investigated whether NLE treatment

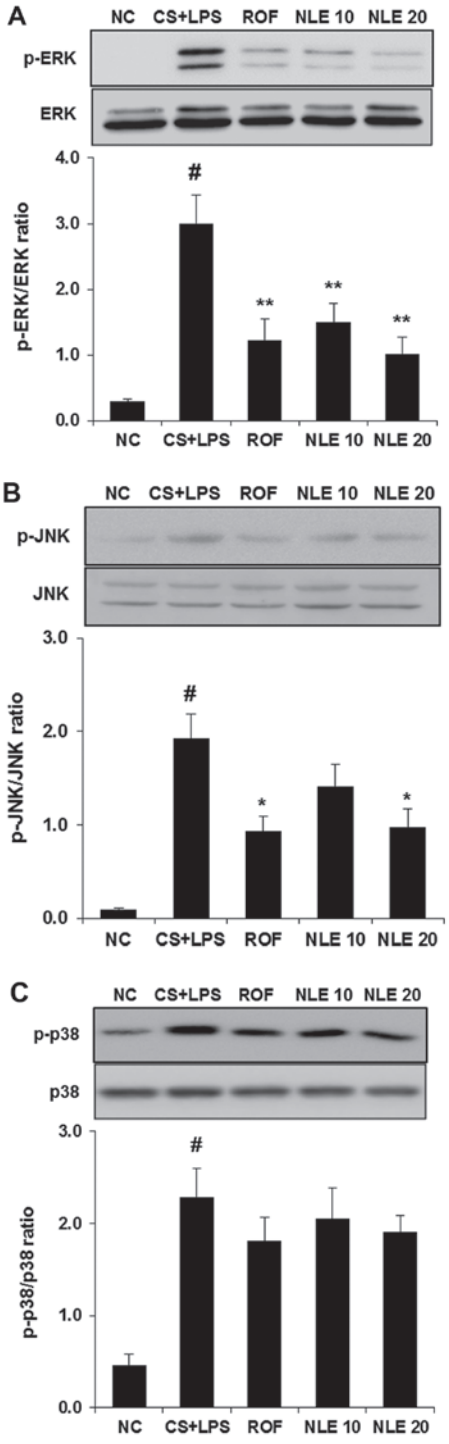

Figure 6. Effect of neem leaf extract (NLE) on the activation of ERK and JNK in lungs of mice. (A) The activation of ERK, (B) JNK and (C) p38 was detected by western blot analysis. Data are expressed as the means ± SD. #p<0.01 indicates a statistically significant difference from the normal control group. *p<0.05 and **p<0.01 indicate statistically significant differences compared to the cigarette smoke (CS) and lipopolysaccharide (LPS) group.

attenuates the activation of MAPKs in the lungs of mice with CS- and LPS-induced pulmonary inflammation. As shown in Fig. 6, the activation of MAPKs (ERK, JNK and p38) was significantly increased in the lungs of mice in the CS and LPS group. However, treatment with NLE significantly decreased the activation of ERK and JNK in a concentration-dependent manner (Fig. 6A and B). The inhibitory effect of 20 mg/kg

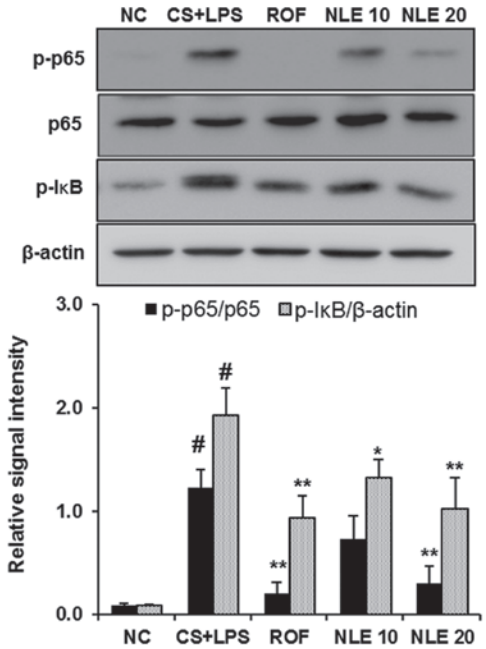

Figure 7. Effect of neem leaf extract (NLE) on the activation of nuclear factor-κB (NF-κB) in lungs of mice. The phosphorylation of NF-κB and inhibitor of NF-κB (IκB) was detected by western blot analysis. Data are expressed as the means ± SD. $^{\#}p<0.01$ indicates a statistically significant difference from the normal control group. $^{*}p<0.05$ and $^{**}p<0.01$ indicate statistically significant differences compared to the cigarette smoke (CS) and lipopolysaccharide (LPS) group.

NLE on ERK and JNK activation was similar to that of treatment with 10 mg/kg ROF. No significant attenuation of p38 activation was observed with NLE (Fig. 6C).

NLE decreases the phosphorylation of NF-κB and IκB in lungs of mice with CS- and LPS-induced pulmonary inflammation. NF-κB is activated by a number of stimuli, including pro-inflammatory mediators and LPS. In response to these molecules, IκB is phosphorylated, ubiquitinated and degraded, resulting in the phosphorylation and nuclear translocation of NF-κB (20,41). In the present study, treatment with NLE inhibited the phosphorylation of NF-κB and IκB in the lungs of mice with CS- and LPS-induced pulmonary inflammation (Fig. 7).

Discussion

In the present study, we examined the protective effects of NLE against CS- and LPS-induced pulmonary inflammation. NLE significantly inhibited the infiltration of inflammatory cells, such as neutrophils and macrophages in BALF. NLE also reduced the production of ROS and NE, and decreased the release of pro-inflammatory cytokines in BALF. NLE attenuated the accumulation of inflammatory cells and the expression of MCP-1 in the lungs of mice with CS- and LPS-induced pulmonary inflammation. Furthermore, NLE inhibited the expression of iNOS in the lungs of mice with CS- and LPS-induced pulmonary inflammation. NLE also attenuated the activation of MAPKs (ERK and JNK) and NF-κB in the lung tissue.

COPD is a global health epidemic the incidence of which is increasing (42), and it is associated with a high risk of morbidity and mortality (43). COPD is characterized by chronic airway inflammation and mucus hypersecretion (44). It is also well known that CS exposure and bacterial infection are associated with the development of COPD (4,45,46). CS is the most important risk factor that increases the recruitment of inflammatory cells in the lungs and the number of goblet cells in the small airway (47). LPS is a major constituent of the Gram-negative bacterial cell wall that stimulates the inflammatory response (48). The recruitment of inflammatory cells, such as neutrophils and macrophages in the airways is a characteristic sign of COPD (6,49,50). ROS production promote the inflammatory response in the lungs via the activation of transcription factors, such as NF-κB and MAPK signal transduction pathways (10). Increased ROS production induced by neutrophils has been reported to promote the oxidation of proteins, DNA and lipids which leads to lung damage (10,51). A number of studies have reported that NE levels are increased in response to CS (52,53) or CS and LPS (5,6), which increases the inflammatory cell recruitment, emphysema and the production of mucus in the lungs (54). CS is the most important source of elevated levels of ROS and NE in COPD (55). In the present study, treatment with NLE significantly inhibited inflammatory cell infiltration in BALF and in the lungs of mice with CS- and LPS-induced pulmonary inflammation (Figs. 1 and 4A and B). NLE also attenuated the production of ROS and the activity of NE (Fig. 2).

Pro-inflammatory cytokines, including TNF-α and IL-6 play an important role in the pathological processes of COPD (56). TNF-α is a central cytokine that regulates inflammation through neutrophil recruitment and endothelial activation (57). IL-6 is involved in the pathogenesis of lung diseases, such as COPD (58). It has also been reported that exposure to CS increases macrophage accumulation that contributes to the development of COPD by increasing the levels of IL-6 (59). Recently, TNF-α and IL-6 were identified to be involved in CS- and LPS-induced COPD (4,5,60). In the present study, NLE decreased the release of TNF-α and IL-6 in BALF (Fig. 3). MCP-1 is a chemokine that plays a key role in the migration of neutrophils and macrophages (15,16). Recently, the increased expression of MCP-1 was detected in the lungs of mice exposed to CS (61). The present data demonstrated beneficial effects of NLE against the CS- and LPS-induced expression of MCP-1 (Fig. 4C). iNOS has been implicated in the pathophysiology of inflammatory diseases, including COPD (23), and the high expression of iNOS has been reported to affect pulmonary inflammation (62). It has also been reported that the inhibition of iNOS exerts protective effects in a wide variety of respiratory diseases (63). The present study demonstrated that NLE significantly suppressed the expression of iNOS in the lungs of mice with CS and LPS-induced pulmonary inflammation in a concentration-dependent manner (Fig. 5).

MAPKs have been reported to regulate pro-inflammatory molecules (64,65) and have been widely studied in pulmonary inflammation (4,5,66). MAPKs (ERK, JNK and p38) mediate pro-inflammatory gene transcription in response to cytokines and LPS (5,67). CS leads to the activation of MAPKs (68-71). It has also been reported that MAPK activation affects ROS production in lungs affected by COPD (10). The present data demonstrated that the activation of MAPKs (ERK, JNK and p38) was induced by CS and LPS in the lungs of mice. However, NLE treatment significantly inhibited the activation or ERK and JNK (Fig. 6A and B). No significant inhibition of p38 activation was observed with NLE treatment (Fig. 6C).

NF-κB is a key transcription factor in the inflammatory response, and is activated by numerous extracellular stimuli, including pro-inflammatory cytokines, such as TNF-α and IL-6 (10). The NF-κB-dependent production of these cytokines affects the recruitment of inflammatory cells, such as neutrophils and macrophages to lung tissue, causing lung injury or emphysema (9,72,73) Therefore, NF-κB signaling is considered to be an important therapeutic target for pulmonary inflammation induced by CS (74). In this study, NLE treatment significantly inhibited the elevated phosphorylation levels of NF-κB and IκB induced by CS and LPS in lung tissue (Fig. 7).

The neem tree (*Azadirachta indica* A. Juss.; Meliaceae) is indigenous to India, and now this tree is cultivated widely in areas of the world (75). *Azadirachta indica* A. Juss has been widely used as neem and has been used in medicine for over 2,000 years (76). Various parts of the neem tree have been used in medicines and food, as well as as insecticides, and many bioactive constituents, including limonoids (tetra-nortriterpenoids) have been isolated and identified (77). NLE has been reported to possess antibacterial activity (78-80). It has also been demonstrated that NLE induces apoptosis in the breast cancer cells (81). Neem leaf fraction has been reported to possess antioxidant properties (82,83). Recently, it has also been shown that NLE protects LPS-induced endotoxemia (38). However, to date, at least to the best of our knowledge, the protective effects of NLE have not been investigated in CS- and LPS-induced pulmonary inflammation.

In conclusion, the present data demonstrated that NLE significantly inhibited the infiltration of inflammatory cells, such as neutrophils and macrophages in the lungs of mice with CS- and LPS-induced pulmonary inflammation. NLE also attenuated the production of inflammatory mediators, including ROS, NE, TNF-α and IL-6 in BALF. Furthermore, NLE decreased the expression of MCP-1 and iNOS in the lungs of mice with CS- and LPS-induced pulmonary inflammation. NLE also inhibited the activation of MAPKs (ERK and JNK) and NF-κB in the lungs of mice. These results thus suggest that NLE may have potential for use as a valuable therapeutic agent in the treatment of COPD.

Acknowledgements

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