Apios americana Medik Extract Alleviates Lung Inflammation in Influenza Virus H1N1- and Endotoxin-Induced Acute Lung Injury

Sung-Hwa Sohn†, Sang-Yeon Lee†, Jun Cui‡, Ho Hee Jang‡, Tae-Hoon Kang‡, Jong-Keun Kim‡, In-Kyoung Kim‡, Deuk-Ki Lee†, Seulgi Choi†, Il-Sub Yoon©, Ji-Woo Chung©, and Jae-Hwan Nam*†

1Department of Biotechnology, The Catholic University of Korea, Bucheon 14662, Republic of Korea
2Department of Molecular Medicine, Lee Gil Ya Cancer and Diabetes Institute, Gachon University, Incheon 21990, Republic of Korea
3Health Care Institute, Phytomecca Co., Ltd, Incheon 21990, Republic of Korea
4Sungkyun Biotech Co., Ltd R&D Center, Sungkyunkwan University, Suwon 15425, Republic of Korea
5Division of Respiratory, Department of Internal Medicine, Seoul St. Mary’s Hospital, The Catholic University of Korea, Seoul 06 591, Republic of Korea

Introduction

Acute lung injury is characterized by the excess production of inflammatory factors in the lung, and is a leading and increasing cause of high morbidity and mortality worldwide [15, 27]. Acute lung injury, which is accompanied by leukocyte influx [24], can be triggered by both infectious and noninfectious stimuli, including influenza virus (e.g., pandemic H1N1 2009 influenza A virus, H1N1) and endotoxin (e.g., lipopolysaccharide, LPS). Neutrophils in particular play an important role in acute lung injury by infiltrating the lung and increasing expression of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 [1]. These cytokines are associated with the development of acute lung inflammation [7, 19, 21]. In this study, we selected LPS-treated and H1N1-infected animal models to better understand the therapeutic effects of Apios americana Medik (hereinafter Apios) on acute lung injury.

Apios americana Medik (hereinafter Apios) has been reported to treat diseases, including cancer, hypertension, obesity, and diabetes. The therapeutic effect of Apios is likely to be associated with its anti-inflammatory activity. This study was conducted to evaluate the protective effects of Apios in animal models of acute lung injury induced by lipopolysaccharide (LPS) or pandemic H1N1 2009 influenza A virus (H1N1). Mice were exposed to LPS or H1N1 for 2–4 days to induce acute lung injury. The treatment groups were administered Apios extracts via oral injection for 8 weeks before LPS treatment or H1N1 infection. To investigate the effects of Apios, we assessed the mice for in vivo effects of Apios on immune cell infiltration and the level of pro-inflammatory cytokines in the bronchoalveolar lavage (BAL) fluid, and histopathological changes in the lung. After induction of acute lung injury, the numbers of neutrophils and total cells were lower in the Apios-treated groups than in the non-Apios-treated LPS and H1N1 groups. The Apios groups tended to have lower levels of tumor necrosis factor-α and interleukin-6 in BAL fluid. In addition, the histopathological changes in the lungs were markedly reduced in the Apios-treated groups. These data suggest that Apios treatment reduces LPS- and H1N1-induced lung inflammation. These protective effects of Apios suggest that it may have therapeutic potential in acute lung injury.

Keywords: Apios, acute lung injury, influenza virus, lipopolysaccharide, inflammation
chronic constipation, hypertension, obesity, and diabetes [10, 14, 29]. The therapeutic effect of Apios is likely to be associated with its anti-inflammatory activity. However, the preventative effects of Apios on acute lung injury and their underlying mechanisms have not yet been evaluated. This study aimed to determine the potential effects of Apios and whether its ability to suppress excessive neutrophil infiltration confers protection against acute lung injury. We provide evidence that Apios has potential preventative effects on acute lung injury.

**Materials and Methods**

**Preparation of Apios Extracts**

Apios was added to three volumes of ethanol, homogenized in a blender, and then filtered using a 25 μm sieve. All filtrates were collected, evaporated, and freeze-dried to obtain an alcohol-soluble solid fraction. This Apios extract was dissolved in dimethyl sulfoxide at a concentration of 100 mg/ml as the stock solution and was stored at -30°C.

**Animals**

Twelve-week-old C57BL/6 male mice (Dae Han Bio Link, Korea) were housed in a controlled environment (inverted 12-h daylight cycle) with free access to food and water. All animal experimental procedures were approved by the Animal Care and Use Committee of the Catholic University of Korea [4].

**Animal Treatment and Induction of Acute Lung Injury**

The mice were randomly divided into seven groups (n = 5/group). (i) Control group: negative control mice were treated with PBS alone; (ii) LPS group: mice were exposed by the intratracheal route to 0.5 mg/kg of LPS (Sigma, USA) on day 56; (iii) LPS+RG group: mice were administered red ginseng (RG) (100 mg/kg body weight (wt)) via oral injection every day for 8 consecutive weeks prior to LPS treatment; (iv) LPS+Apios group: mice were administered Apios extracts (50 mg/kg body wt) via oral injection every day for 8 consecutive weeks prior to LPS treatment; (v) H1N1 group: mice were exposed by the intratracheal route to 2.5 LD50 of Influenza A/California/04/2009 (H1N1) virus, which originated from swine influenza H1N1 viruses, on day 56; (vi) H1N1+RG group: mice were administered RG (100 mg/kg body wt) via oral injection every day for 8 consecutive weeks prior to H1N1 infection; and (vii) H1N1+Apios group: mice were administered Apios extract (50 mg/kg body wt) via oral injection every day for 8 consecutive weeks prior to H1N1 infection. On day 58 or 60, the mice were sacrificed, and various tissues were collected for analyses.

**Analysis of Lung Inflammatory Cells**

PBS was slowly infused into the lungs and then withdrawn via a cannula that had been inserted into the trachea. The cell numbers were counted using a hemocytometer, and differential cell counts were performed on slides prepared by cytocentrifugation at 200 xg for 3 min and subsequent Diff-Quick staining. Approximately 500 cells were counted. The BAL (bronchoalveolar lavage) fluids were then centrifuged, and the supernatants were stored at -80°C until needed.

**Measurements of TNF-α and IL-6 in BAL Fluids**

Mouse TNF-α and IL-6 ELISA kits were purchased from eBioscience (USA), and cytokines in the culture supernatant were measured as described in the manufacturer’s protocol. Enzyme immunoassay 96-well plates (Corning Life Sciences, USA) were coated with TNF-α and IL-6 monoclonal antibodies at 4°C overnight. Nonspecific binding sites were blocked with 1× blocking buffer for 1 h at room temperature (RT), and then 100 μl of a standard was added. BAL fluids were loaded into each well and incubated at 4°C for 24 h. Secondary peroxidase-labeled biotinylated anti-mouse TNF-α and IL-6 monoclonal antibodies were then added and incubated at RT for 1 h. The plates were developed using a chromogenic 3,3',5,5'-tetramethylbenzidine substrate (BD Biosciences, USA), and the reaction was stopped with 2N H2SO4. Optical density was determined at 450 nm using a Multiskan EX spectrophotometer (Thermo Fisher Scientific, USA).

**Histology**

The lung tissues were fixed in 10% neutral formalin. After fixation, these samples were embedded in paraffin and stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS).

**Statistical Analysis**

Statistical analysis of the data was performed using Prism 5 software (GraphPad Software, USA). All data values are expressed as the mean ± standard error of the mean (SEM). The significance of differences between groups was analyzed by a one-way analysis of variance followed by Newman–Keuls post hoc test. Differences of p < 0.05 were considered significant.

**Results**

**Effect of Apios Extracts on Total and Inflammatory Cell Levels in BAL Fluids of LPS-Treated and H1N1-Infected Mice**

To determine whether Apios affects immune cells, mice were subjected to a short-term exposure to LPS (2 days) or H1N1 (4 days, Fig. 1). At 2 or 4 days after LPS treatment or H1N1 infection, the LPS and H1N1 groups showed significantly increased numbers of total cells in the BAL fluid compared with that of the control group. In particular, the influx of neutrophils was markedly higher in the LPS and H1N1 groups than in the control group (Fig. 2). The LPS+Apios and H1N1+Apios groups exhibited markedly decreased numbers of total cells and neutrophils in BAL.
fluid compared with the LPS and H1N1 groups (Fig. 2). The H1N1+RG group showed significantly decreased numbers of neutrophils and total cells compared with the H1N1 group (Fig. 2B). However, the LPS+RG group showed no inhibition of the influx of neutrophils and total cells compared with the LPS group (Fig. 2A).

Effect of Apios Extracts on Pro-Inflammatory Cytokine Production in BAL Fluids of LPS-Treated and H1N1-Infected Mice
To demonstrate the effect of Apios on BAL fluids, we measured the secretion of the pro-inflammatory cytokines TNF-α and IL-6, which contribute to LPS- and H1N1-induced lung inflammation. Secretion of TNF-α and IL-6 was significantly elevated in the LPS and H1N1 groups compared with that in the control group (Fig. 3). Treatment with Apios reduced the levels of TNF-α and IL-6 in the LPS+Apios and H1N1+Apios groups compared with those in the LPS and H1N1 groups, although these differences were not significant (Fig. 3). However, treatment with RG failed to inhibit pro-inflammatory cytokine release (Fig. 3A).

Effect of Apios Extracts on Histological Changes in Lung Tissues of LPS-Treated and H1N1-Infected Mice
To evaluate the effect of treatment with Apios on LPS- and H1N1-induced lung damage, lung sections were stained with H&E. Those from the LPS and H1N1 groups showed LPS- and H1N1-induced inflammatory cell infiltration (Fig. 4). However, Apios treatment led to a marked reduction in the infiltration of inflammatory cells induced by LPS treatment or H1N1 infection (Fig. 4). Interestingly,

Fig. 1. Schematic diagram of the experimental protocol.
Animals were treated with *Apis americana* Medik (Apios) extracts every day for 8 consecutive weeks. On day 56, animals were exposed by the intratracheal route to 0.5 mg/kg of lipopolysaccharide (LPS) or 2.5 LD_{50} of H1N1. (A) The mice were sacrificed at 2 days after LPS treatment. (B) The mice were sacrificed at 4 days after H1N1 infection.

Fig. 2. Effect of *Apis americana* Medik (Apios) extract on immune cell profiles in bronchoalveolar lavage (BAL) fluid. The number of total cells, macrophages, neutrophils, and lymphocytes in BAL fluid were determined. Con: saline treated; LPS: lipopolysaccharide treated; LPS+RG: LPS + red ginseng treated; LPS+Apios: LPS + Apios treated; and H1N1: influenza A/California/04/2009 (H1N1) treated. Data are expressed as the mean number of cells ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001 versus control; and #p < 0.05, ###p < 0.001 versus LPS or H1N1; n = 5).
RG treatment did not significantly protect against LPS-induced lung damage (Fig. 4A). These results of the histological examination of H&E-stained lung tissue paralleled those for the cell numbers in BAL fluid (Figs. 3 and 4).

**Effect of Apios Extracts on Goblet Cell Hyperplasia in Lung Tissues of LPS-Treated and H1N1-Infected Mice**

To demonstrate the effect of Apios on goblet cell hyperplasia, lung tissues were stained with PAS (Fig. 5). The H1N1 group showed no goblet cell hyperplasia (data not shown).
not shown). The LPS group showed more abundant PAS-positive goblet cells around the bronchial airway epithelium than did the control group. By contrast, the LPS+RG and LPS+Apios groups showed significantly fewer PAS-positive goblet cells around the bronchial airway epithelium than did the LPS group (Fig. 5). These findings indicate that treatment with Apios had a preventative effect on the induced acute lung disease.

Discussion

The Spanish influenza pandemic of 1918 resulted in an estimated 675,000 deaths in the United States [11]. H1N1 influenza virus has been documented to cause acute lung injury [2, 12, 26]. Acute lung injury can be triggered by both infectious and noninfectious stimuli [24]. Thus, we selected the LPS-treated and H1N1-infected animal models to better understand the preventative effects of Apios on acute lung injury. In the mouse models of LPS- or H1N1-induced lung injury, the intratracheal administration of LPS or H1N1 induces pulmonary inflammation, resulting from leukocyte recruitment [5, 22, 23].

Apios has traditionally been considered to have health benefits in several diseases [10, 14, 29]. The components of Apios, including the novel isoflavone genistein-7-O-gentiobioside, have been used to explain these various biological effects [3, 14, 25]. In addition, glycoprotein, one of the components of Apios, possesses anti-inflammatory activity, which inhibits the triggering of Toll-like receptor-4 signaling in the LPS-induced inflammatory response [6]. Moreover, another component of Apios, a trypsin inhibitor, possesses anticancer activity [17, 29]. Therefore, Apios seems to be a beneficial food source for alternative healthcare. In the present study, we investigated the effects of oral treatment with Apios extracts for 8 weeks on the pulmonary dysfunction processes that in previous reports [1, 8, 18] have been reported to lead to the resolution of inflammation. We hypothesized that neutrophils play an important role in these processes. We demonstrated an association between inflammation and immune cell infiltration, particularly of neutrophils *in vivo*, using a mouse model of acute lung injury induced by LPS or H1N1 influenza virus. We found that neutrophils rapidly accumulated in the lungs after LPS treatment or H1N1

**Fig. 5.** Effect of *Apis americana* Medik (Apis) extract on goblet cells. Mouse lung sections were stained with periodic acid Schiff (magnification ×200). The arrow indicates the PAS-positive cells. Con: saline treated; LPS: lipopolysaccharide treated; LPS+RG: LPS + red ginseng treated; and LPS+Apios: LPS + Apios treated.
infection, and increased the levels of the pro-inflammatory cytokines, including TNF-α and IL-6, in the BAL fluid. An excessive neutrophil response was associated with higher pathogen burdens and decreased survival when mice were administered H1N1 or LPS [28]. Neutrophils may play a role in modulating the inflammatory pulmonary processes associated with LPS- and H1N1-induced acute lung injury. However, Apios treatment ameliorated neutrophil infiltration and pro-inflammatory cytokine secretion in the mice exposed to LPS or H1N1 (Figs. 2 and 3). These results suggest that Apios may be a useful preventative agent that can suppress the main inflammatory factors associated with acute lung injury. As a consequence of this serious inflammatory response, the lung structures change and alveolar and endothelial permeability increases, ultimately impairing lung function [16, 20]. In our study, exposure to LPS and H1N1 induced several pathological changes that are typical of acute lung injury, including diffuse lung inflammation, goblet cell hyperplasia, and airway remodeling in the bronchi and alveoli. However, Apios treatment ameliorated these pathological lung changes in LPS-treated or H1N1-infected mice (Figs. 4 and 5).

Overall, our results suggest that Apios confers protection against LPS- or H1N1-induced acute lung injury by reducing neutrophil infiltration and inflammation in the lung. The anti-inflammatory effects of Apios indicate that it has therapeutic potential for acute lung inflammatory disease.

Acknowledgments

This study was supported by a grant from the Korean Healthcare Technology R&D project of the Ministry of Health & Welfare (A103001 and HI13C0826), the Ministry of Science & ICT Future Planning (2015M3A9B5030116), and the Technological Innovation R&D Program (S2226237) funded by the Small and Medium Business Administration.

References

18. Orme J Jr, Romney JS, Hopkins RO, Pope D, Chan KJ,


