Article

Inhibition of NF-κB Expression and Allergen-induced Airway Inflammation in a Mouse Allergic Asthma Model by Andrographolide

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Andrographolide from traditional Chinese herbal medicines previously showed it possesses a strong anti-inflammatory activity. In present study, we investigated whether Andrographolide could inhibit allergen-induced airway inflammation and airways hyper-responsiveness and explored the mechanism of Andrographolide on allergen-induced airway inflammation and airways hyper-responsiveness. After sensitized and challenged by ovalbumin, the BALB/c mice were administered intraperitoneally with Andrographolide. Hyper-responsiveness was recorded. The lung tissues were assessed by histological examinations. NF-κB in lung was determined by immunofluorescence staining and Western blotting. Treatment of mice with Androqrapholide displayed lower Penh in response to asthma group mice. After treatment with Andrographolide, the extent of inflammation and cellular infiltration in the airway were reduced. Andrographolide interrupted NF-κB to express in cell nucleus. The level of NF-κB expression was inhibited by Andrographolide. The data indicate that Andrographolide from traditional Chinese herbal medicines could inhibit extensive infiltration of inflammatory cells in lung and decrease airway hyperreactivity. Andrographolide could inhibit NF-κB expression in lung and suppress NF-κB expressed in the nucleus of airway epithelial cells. *Cellular & Molecular Immunology*. 2009;6(5):381-385.

Introduction

Asthma is inflammatory lung disorder associated with Th2 cytokines, allergen-specific IgE, and airways hyper-responsiveness. Corticosteroid treatment remains the first preference of treatment, however steroids are not always completed effective for asthma (1). Some data show that high levels of NF-κB and AP-1 attenuate Glucocorticiod receptor (GR) function. A failure to respond may therefore result from reduced GC binding to GR, reduced GR expression, and enhanced activation of inflammatory pathways (2).

Andrographolide from traditional Chinese herbal medicines has been shown to possess a strong anti-inflammatory activity and has the potential to be used in allergic lung inflammation (3). Andrographolide has been commonly shown to inhibit NF-κB activity (4). The aim of the present study was to investigate that effect of Andrographolide on allergen-induced airway inflammation and airways hyperresponsiveness. Experiments in an asthma model showed that Andrographolide inhibits the production of NF-κB expression and allergen-induced airway inflammation. Andrographolide can decrease airway hyperresponsiveness.

Materials and Methods

Reagents and mice

Rabbit anti-mouse NF-κB /P65 antibody was purchased from NeoMarker (US). Andrographolide was gift from Chendu-Kanghong Pharmaco Company. A total of 24 specific pathogen-free, 8-week-old male BALB/c mice, weighed 20-25 g, were obtained from the Experiment Animal Center in Sichuan Academy of Medical Sciences. All the mice were under standard conditions surviving on distilled water and rodent laboratory food.

Animal experimental protocols

Twenty-four mice were divided into 3 groups, normal control group, asthma group and Andrographolide treatment group. Mice were anesthetized by ether. For asthma group and Andrographolide treatment group, the mice were sensitized

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with intraperitoneal injection of 20 μg ovalbumin (OVA) solution adsorbed onto 5 mg aluminum potassium sulfate (alum), on day 1 and day 8. Normal control group mice were only injected with PBS, on day 1 and day 8. From 15 days to 22 days, asthma group and Andrographolide treatment group mice were boosted again with intranasal instillation of 20μg OVA in 100 μl PBS, but this time Androqrapholide treatment group mice were administered intraperitoneally at a dose of 2 μg/g body weight for 7 days at the same time. Twenty four hours after the last challenge by Methacholine (Meh), all the mice airway hyperresponsiveness is recorded by the double-chambered w*hole*-body plethysmograph (Buxco, USA). Three group model were sacrificed under ether anesthesia on day 23. Lung was removed to use for HE stain, immuno-fluorescence and western blotting.

Pulmonary function assessment
Airway hyperresponsiveness (AHR) was measured in unrestrained mice by the double-chambered whole-body plethysmograph (Buxco, USA). AHR was expressed as an enhanced minute pause (Penh), a calculated value that reflects pulmonary resistance. Each mouse was placed in an un-restrained whole body plethymographs without anesthetization and exposed to nebulized physiologic saline for 3 min. Increasing concentrations of nebulized methacholine (0, 1.5, 3, 6, 12, 24, 48) were administered by the use of an ultrasonic nebulizer. Measurements were obtained for 3 min after the completion of each nebulization. Penh values measured during this period were averaged and expressed as absolute Penh values. Data from regular ventilation was collected and recorded for each mouse. These data can be automatic analysis by Fine Point software.

Histologic evaluation
Following sacrifice and BAL collecting, the part of lung tissues was fixed with 10% neutral-buffer formalin in PBS for 10 min before embedding in paraffin. Then 6-μm sections were cut onto 3-amino propyltriethoxy silence-coated APES-coated slides and stained with hematoxylin-eosin (HE) staining. The tissues can be assessed for general morphology and cellular infiltration was performed as described previously (5). The standard of this method discriminates between the presence of mononuclear cells around brochioli (Score 0-3) and around blood vessels (Score 0-3), and the number of patchy cellular infiltration (Score 0-3). Each sample were examining 10 chosen at a magnification of ×200.

Indirect immunofluorescence
Lungs part of each mouse was frozen by liquid nitrogen and placed into Cryostat plat (Thermo shandon, Germam), preparation of 6-μm frozen sections. Slides were fixed for 1 h with 4% paraformaldehyde, washed by PBS 2 min, dried at room temperature. It was then blocked with 10% goat serum in PBS for 10 min. Slices were incubated at room temperature with Ab recognizing NF-κB p65 (NeoMarkers RB-1638-P1, German) for 2 h with 1 : 500 ratio. Following

three washes with PBS (3 × 2 min), slides were incubated with biotinylated secondary Ab 1 : 100 ratio for 30 min (room temperature), then washed three times with PBS (3 × 2 min). At last, slides were incubated for 30 min with a 1 : 100 dilution of SABC-Cy3 labeled Goat Anti-Rabbit IgG at room temperature and washed 5 × 4 min with PBS. DAPI (C1005, Beyotime) a nuclear counter stain can be used to evaluate nuclear localization of NF-κB. Slices were incubated with 100 μl for 3 min at room temperature, washed with PBS 4 × 4 min and covered by glycerin. Sections were scanned by laser scanning confocal fluorescence microscope (Leisa, USA) using a 40× objective and an iris setting of 2 (depth < 2 μm). DAPI staining of nuclei was detected by stimulated fluorescence with the 408 nm laser line, and SABC-Cy3 was detected following stimulation with the 554 nm laser line. Control slides were treated with an unrelated IgG2 mouse antibody of the same IgG isotype.

Detection of NF-κB/p65 in the lung tissue by Western blot
Lung tissue weighing 200 mg from each group of mice and the lung tissue was homogenated with a lysis buffer (1,000 μl RIPA add 10 μl PMSF, Beyotime). Centrifuge sample for 30 min on high speed (10,000 rpm) at 4°C and the supernatant was collected. BCA Concentration measurement kit (P0012, Beyotime) was used for measuring the three group sample protein level and loading of 20 μg protein from each group sample 20 μg protein was separated by 10% SDS-PAGE (100 v 1.5 h) and electrotransferred into a nitrocellulose membrane with Amersham Ecl Semi-Dry Transfer Unit (15 v 30 min). The membrane was blocked with 3% BSA (3 g BSA was dissolve in 100 ml SPBS) for 1 h at 37°C and incubated with polyclone rabbit anti-NF-κB (1 : 300) overnight at 4°C. The membrane was washed with TBST (Tris-HCl pH7.5 5 ml, 20% tween-20 1.18 ml, NaCl 4.4 g 500 ml dd Water) for 2 × 10 min, incubated with HRP-labeled secondary monoclone antibody (1 : 1200) for 1.5 h at 37°C, washed with TBST 2 × 10 min. Chemiluminescent substrate (supersignal, Pierce) was added to the membrane and exposed strip from Kodak Image Station 4000MM (USA)
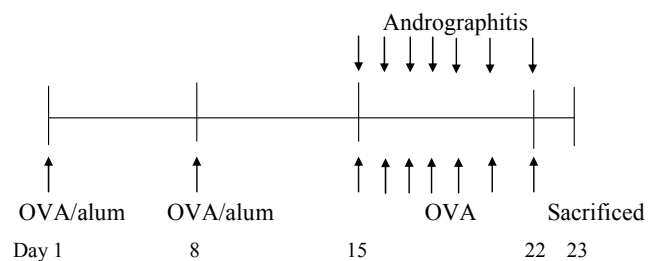

Figure 1. Experiment scheme for treatment of allergic airway inflammation by Andrographolide. On day 1 and 8, mice were sensitized with intraperitoneal injection OVA. From day 15 to 22, mice were challenged with intranasal instillation OVA. Androqrapholide treatment group mice were administered intraperitoneally for 7 days at the same time.
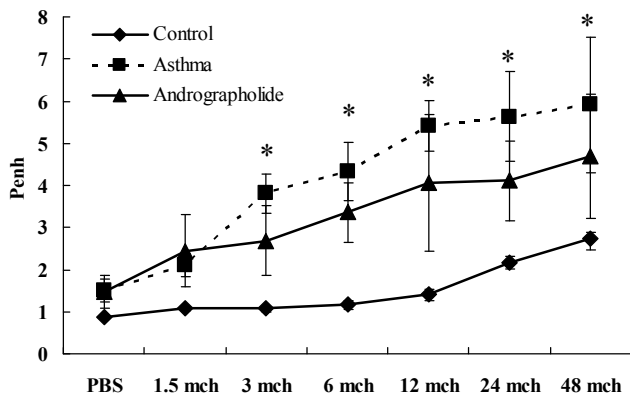
Figure 2. Assessment of airways hyperresponsivenes following allergen sensitization and challenge. Pulmonary hyperresponsivenes related to Penh parameter was analyzed in asthma group (broken line) or treatment group (solid line, triangle) or control group（solid line, diamond） after last stimulation by different dosage of Meh (Buxco, USA). $^*p < 0.05$ compared with asthma group.

was obtained.

Statistical analysis
Data were analyzed with SPSS13.0 and presented as the mean ± standard error (SE) of measurement. Statistical significance was determined by ANOVA and the significant level was defined as $p < 0.05$.

Result

Effects of Andrographolide on airway hyper reactivity
Penh is derived from the expiratory side of the respiratory waveform in a flow whole body plethysmograph. With chambers WBP system (BUXCO, USA), baseline reading was collected and mice were challenged with aerosol control followed by increasing dosages of Meh. According to the table which is analyzed by Fine Point software. A mice in control group, PBS did not show an alter baseline Penh values or airway responses to increasing concentrations of methacholine (Figure 2). Asthma group mice resulted in significant airway hyperresponsivene. However, treatment of mice with Androqrapholide displayed lower Penh in response to asthma group mice ($p < 0.01$). So we conclude that Andrographolide can decrease airway hyperreactivity.

Effects of Andrographolide on allergen-induce airway inflammation
Twenty-four hours after last allergen challenge with intranasal instillation of OVA, histological analysis showed that asthma group mice resulted in the development of significant airway inflammation. The degree of cellular infiltration around the central bronchi, alveoli and blood vessels is put in histological scores (Table 1). Contrast to control group (Figure 3A), the asthma group histological results showed extensive infiltration of inflammatory cells
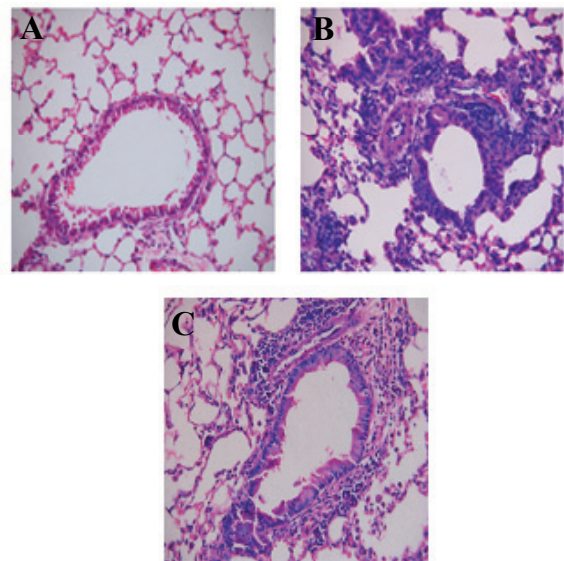

Figure 3. Histological examination of peripheral airway tissue. Lung samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 6 μm thickness were affixed to slides, and stained with hematoxylin and eosin. (A) Control group; (B) Asthma group; (C) Andrographolide treatment group. (original magnification ×200).

around bronchioles, blood vessels and alveoli, such as eosinophils (Figure 3B). After treatment with Andrographolide, the extent of inflammation and cellular infiltration in the airway were reduced (Figure 3C), as compared with asthma group ($p < 0.05$) (Table 1). But the extent of inflammation and cellular infiltration in the airway from treatment were still obvious, as compared with control group ($p < 0.01$) (Table 1). The data show that Andrographolide only partly inhibited allergen-induce airway inflammation.

Andrographolide inhibited airway epithelial NF-κB activation
It was demonstrated that NF-κB rapidly translocates from cytoplasm to the nucleus of airway epithelial cells of sensitized mice exposed to allergen. In our study, the cells around bronchiole were scanned by confocal microscopy, and nuclear of a cell was bright blue fluorescence in all the

Table 1. Degree of inflammatory cellular infiltration in lung tissue (mean ± SD)

Group	bronchioles	blood vessels	alveoli
Control	1.14 ± 0.69	0.71 ± 0.48	0.85 ± 0.38
Asthma	2.53 ± 0.54	2.20 ± 0.38	2.20 ± 0.42
Andrographolide	2.04 ± 0.49*[#]	1.71 ± 0.31*[#]	1.80 ± 0.42*[#]

The degree of cellular infiltration was expressed as a histological score that is described in Materials and Methods. $^*p < 0.05$ compared with asthma group, $^{\#}p < 0.01$ compared with control group.
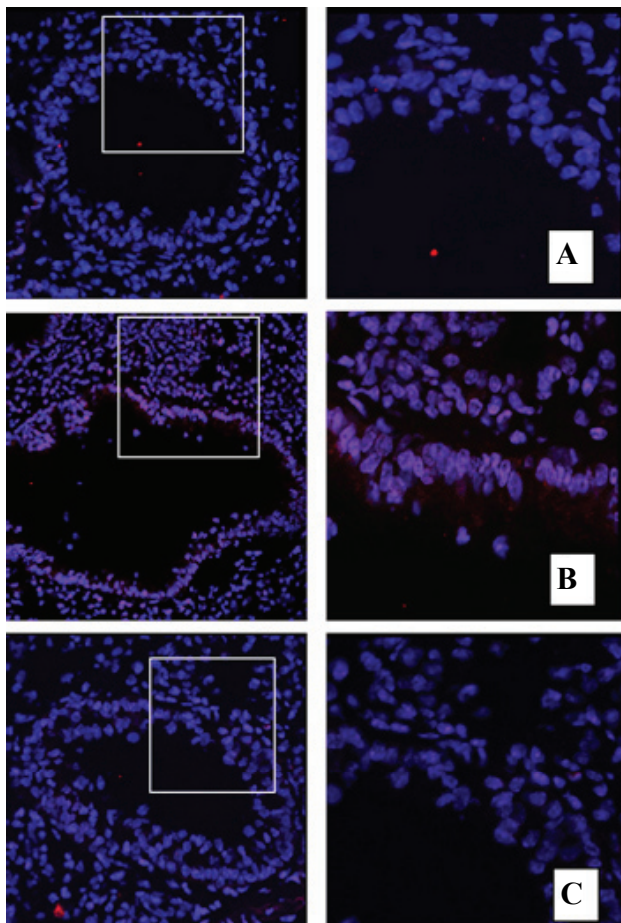
Figure 4. Immunolocalization of NF-κB P65 in airway epithelial cell of allergen sensitized and challenged mice. Frozen lung sections were prepared following intranasal instillation of OVA. These sections were stained with an antibody directed against NF-κB and the biotinylated antigen-antibody complex followed by incubation with an Alexa-554-conjugated antibody (bright red). A nuclear counter stain (blue) was used to evaluate nuclear localization of NF-κB, in which case red and blue merge to create purple. Sections were scanned by confocal microscopy. Original magnification of the parts of three groups of animal model sections and its center images, ×200. (A) Control group; (B) Asthma group; (C) Andrographolide treatment group. Images are representative of results from eight mice.

Figure 5. Detection of NF-κB expression in lung. Lung tissues were collected and analysed by Western blot. Lane 1, Control group; Lane 2, Asthma group; Lane 3, Andrographolide treatment group.

NF-κB to express in cell nucleus. Meantime, NF-κB expression level was analyzed by Western blot (Figure 5). The NF-κB in the asthma group (Lane 2) was increased as compared with that in control group (Lane 1) and treatment group (Lane 3). The level of NF-κB expression of treatment group was inhibited by Andrographolide (Lane 2), so we conclude that Andrographolide treatment of mice exhibits reduced airway epithelial NF-κB activation.

Discussion

Allergic asthma is currently considered a chronic airway inflammatory disorder associated with the presence of activated CD4^{+} Th2-type lymphocytes, eosinophils, and mast cells. Previously, our study has demonstrated that mice developed allergic airway inflammation in response to inhaled allergen, extensive infiltration of inflammatory cells were found around the central bronchi, alveoli and blood vessels (6). Ala'a A reported that Andrographolide was as efficacious as dexamethasone. The effect correlated with reduced expression of mRNA for TNF-α and GM-CSF (7). These results provide evidence that Andrographolide is an effective anti-inflammatory drug with the potential to be used for the treatment of asthma as well as other inflammatory diseases. Recent studies showed that a potential therapeutic value of Andrographolide in the treatment of asthma and it may act by inhibiting the NF-κB pathway at the level of inhibitory κB kinase-β activation. Andrographolide blocked p65 nuclear translocation and DNA-binding activity in the nuclear extracts from lung tissues of OVA-challenged mice (8). But the drug may affect asthmatic inflammation and airway hyperreactivity has not been explored. In present study, histological analysis showed that asthma group mice resulted in the development of significant airway inflammation. Andrographolide from traditional Chinese herbal medicines could inhibit extensive infiltration of inflammatory cells in the airway. Meantime, Andrographolide can decrease airway hyperreactivity. The data that Andrographolide partly inhibited allergen-induce airway inflammation and decrease airway hyperreactivity.

Andrographolide has been used to treat acute upper respiratory tract infection. Andrographolide could control or release the symptoms of acute upper respiratory tract infection. The anti-inflammatory mechanisms of Andro-

pictures (Figure 4). NF-κB displayed red fluorescence in the cell. NF-κB activated exists in the nuclear is purple (red and blue merge). The airway epithelial cells of asthma group has obvious purple color in which case red and blue merge to create (Figure 4C). The results showed that NF-κB translocated from the cytoplasm to the nucleus of airway epithelial cells of sensitized mice exposed to Ag. But in Andrographolide group picture (Figure 4B), the result is similar to that of normal group picture. It only showed less NF-κB activated existing in the nuclear, compared to asthma group. The data suggested that Andrographolide interrupted
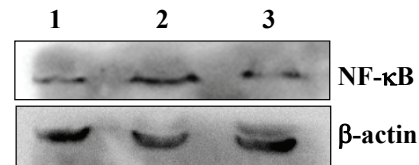
grapholide are associated with preventing NF-κB. It has been confirmed that Andrographolide covalently modifies the reduced cysteine 62 of p50- a major subunit of NF-κB transcription factors, thus blocking the binding of NF-κB transcription factors to the promoters of their target genes, preventing NF-κB activation and inhibiting inflammation *in vitro* and *in vivo* (9). NF-κB plays a central role in asthma. NF-κB activation within airway epithelium is necessary to fully induce the recruitment of inflammatory cells. NF-κB activation in airways drives a majority of the Ag-induced inflammation and is a significant contributor to adaptive immune responses and structural remodeling of the airway wall (10). Previously, our study has confirmed that expression of NF-κB in lung was high. Its increased activation has been demonstrated in lungs after allergen challenge and in airway epithelial cells and macrophages (11). In our study, expression of NF-κB in airway epithelial cells was high. NF-κB translocated from the cytoplasm to the nucleus of airway epithelial cells of sensitized mice exposed to Ag. Andrographolide inhibited NF-κB expression in lung and suppressed NF-κB expressed in nucleus of airway epithelial cells. The result showed that Andrographolide inhibited NF-κB expression in lung and suppressed NF-κB translocation from the cytoplasm to the nucleus of airway epithelial cells.

In summary, this study demonstrates that Andrographolide from traditional Chinese herbal medicines could inhibit extensive infiltration of inflammatory cells in lung and decrease airway hyperreactivity. Andrographolide could inhibit NF-κB expression in lung and suppress NF-κB translocation from the cytoplasm to the nucleus of airway epithelial cells. Our findings implicate the anti-inflammatory mechanisms of Andrographolide are associated with NF-κB expression in lung and suppress NF-κB translocation from the cytoplasm to the nucleus of airway epithelial cells.

Acknowledgement

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