Arch Pharm Res Vol 28, No 12, 1350-1357, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

DA-9201 Shows Anti-Asthmatic Effects by Suppressing NF-κB Expression in an Ovalbumin-Induced Mouse Model of Asthma

Seung-Ho Lee, Mi Jung Seo, Seul Min Choi, Yong Sung Sohn, Kyung Koo Kang, Byoung Ok Ahn, Jong Won Kwon, and Moohi Yoo

Research Institutes of Dong-A Pharmaceutical Company, Yongin 449-905, Korea

(Received October 15, 2005)

Nuclear factor kappa B (NF-κB) regulates the expression of multiple cytokines, chemokines, and cell adhesion molecules that are involved in the pathogenesis of asthma. We investigated the anti-asthmatic effects and the mechanism of action of DA-9201, an extract of the black rice, in a mouse model of asthma. Mice immunized with ovalbumin (OVA) were administered with DA-9201 (30, 100 or 300 mg/kg) or dexamethasone (DEXA, 3 mg/kg) for 2 weeks and challenged with aerosolized OVA during the last 3 days. Anti-asthmatic effects were assessed by means of enhanced pauses, level of total IgE and Th2 cytokines in plasma or bronchoalveolar lavage fluid (BALF), the percentage of eosinophils in BALF, and histopathological examination. The expression of NF-κB in nuclear and cytoplasmic fraction and its DNA-binding activity in lung tissues were analyzed by means of Western blotting and electrophoretic gel mobility shift assay (EMSA), respectively. DA-9201 significantly reduced airway hyperresponsiveness (AHR), total IgE level in plasma and BALF, IL-4, IL-5, and IL-13 levels in BALF, and the percentage of eosinophils in BALF. Tissue inflammation was significantly improved by DA-9201 treatment. In addition, DA-9201 dramatically suppressed the expression of NF-κB and its DNA-binding activity. These results suggest that DA-9201 may be useful for the treatment of asthma and its efficacy is related to suppression of NF-κB pathway.

Key words: Asthma, DA-9201, NF-κB, Anti-inflammatory, Mice model

INTRODUCTION

The worldwide prevalence and severity of allergic asthma have increased dramatically over the last dacades (Umetsu *et al.*, 2002). Although the reasons for this increased incidence are unknown, allergen specific Th2 cells exert their critical functions by producing a unique repertoire of cytokines, the most important of which are IL-4, IL-5, and IL-13. The pathophysiological characteristics of allergic asthma such as chronic pulmonary eosinophilia, airway hyperresponsiveness (AHR) to a variety of non-specific spasmogenic stimuli, excessive airway mucus production and elevated serum IgE levels have all been linked to aberrant Th2 cell responses (Busse and Lemanske, 2001; Larche *et al.*, 2003; Ray and Cohn, 1999; Wills-Karp, 1999).

NF-κB is a pleiotropic transcription factor that plays an important role in regulating the expression of multiple genes involved in inflammation and immune responses (Ghosh *et al.*, 1998; Pahl, 1999). Especially, NF-κB regulates the expression of multiple cytokines, chemokines and cell adhesion molecules that are involved in asthma pathogenesis (Baldwin, 1996; Sha, 1998). It has been demonstrated that asthmatic inflammation is associated with increased NF-κB activity in lung cells (Bureau *et al.*, 2000; Hart *et al.*, 1998; Yang *et al.*, 1998). Furthermore, NF-κB has a critical role in the expression of GATA3 that regulates the Th2 cytokines, eosinophilic airway inflammation, mucus secretion and IgE production in a murine model of asthma (Das *et al.*, 2001).

The *Oryza sativa L.* var. (Family : Glamineae), which is commonly known as black rice in S. Korea, has been used in folk medicine successfully for the management of various allergic disorders (Kim *et al.*, 1999a, 1999b). DA-9201 is an ethanolic extract of the black rice.

This study was performed to investigate the anti-asthmatic effect of DA-9201 and the mechanism of action in a mouse model of asthma.

Correspondence to: Kyung Koo Kang, Research Institutes, Dong-A Pharmaceutical Company, Yongin 449-905, Korea
Tel: 82-31-280-1385, Fax: 82-31-282-8564
E-mail: kangkk@empal.com

MATERIALS AND METHODS

Mice

Female BALB/c mice (5 weeks old) were purchased from Charles River Japan (Japan) and kept under the standard animal facility at the institutes of Dong-A Pharmaceutical company (temperature 23±2°C, humidity range 40~70%, 12 h light/dark cycle ([lighting:7:00-19:00]). Food and UV-sterilized tap water were provided *ad libitum*. This study was performed in compliance with the institutional "Standard operating procedure for animal care and experiments" of the Dong-A Pharmaceutical Co Ltd. and with the "Principles of laboratory animal care" established by the National Institute of Health.

Reagents

Chicken egg albumin (OVA, grade V), aluminum hydroxide gel (alum) and DEXA-water soluble were purchased from Sigma-Aldrich (St. Louis, MO). Acetyl-β-methylcholine chloride (methacholine) and protease inhibitor cocktail were also purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals or reagents were commercially obtained and of the highest quality.

Preparation of DA-9201

The black rice (*Oryza sativa* L. var japonica) was purchased from the National Agricultural Cooperative Federation (Geochang, S. Korea). The black rice (8.00 kg) was processed by using a household rice-polishing machine (Bio SangSang, Korea) to obtain the rice bran (1.348 kg). Then, the rice bran was extracted with 70% ethanol (6.74 L) at room temperature for 24 h with agitating. The extract was filtered through filter paper (Advantec, Japan), and the filtrate (5.13 L) was concentrated at 60°C under vacuum using an evaporative system and lyophilized at -40°C to dryness; 77.80 g (yield: 5.77%) of extract powder was obtained. The dried extract, DA-9201, was suspended in a 1% hydroxyprophylmethylcellulose (HPMC) solution for oral administration.

The HPLC analysis of DA-9201

The dried ethanol extract, 10 mg of DA-9201, was dissolved in 1 mL of mobile phase followed by filtration using a 0.45 μm syringe filter (PVDF, Waters, Milford, U.S.A.). Standards of several anthocyanins including cyanidine-3-O-β-glucopyranoside, peonidine-3-O-β-glucopyranoside, delpinidine-3-O-β-glucopyranoside, and pelalgonidine-3-O-β-glucopyranoside were obtained from Polyphenols Laborotories (Hanabryggene Technology Centre, Norway). Standards of phenolic acids such as protocatechuic acid and ferulic acid were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). The chromatographic systems consisted of Dionex equipped with a P580 pump, ASI-100 automated sample injector and UV detector, and were monitored and controlled by using the Chromeleon computer program (Dionex). Wavelengths used for the identification of protocatechuic acid and anthocyanins were 254 nm and 520 nm, respectively. To quantify the ferulic acid, the HPLC-MS analyses were carried out by using a Waters Alliance 2975 LC system (Waters Co., U.S.A.) coupled to a mass spectrometer. The amounts of the standard materials in DA-9201 are shown in Table I.

Sensitization

As shown in Fig. 1, mice (n=8/group) were immunized with 50 μg OVA mixed with 1mg alum as an adjuvant in a total volume of 0.1 mL per mouse. Sensitized mice were immunized again 2 weeks after the primary immunization. Normal control mice were injected peritoneally with phosphate buffer saline (PBS, pH 7.0).

Airway OVA challenging

Two weeks after the last sensitization, immunized mice were orally administered with DA-9201 (30, 100, 300 mg/kg, each n=8) or DEXA (3 mg/kg, n=8) for 15 consecutive days. Sham-treated mice were administered HPMC orally and served as control. During the last 3 days, mice were

Table I. The amounts of standard materials in DA-9201

Standard materials	Quantity (%)
cyanidine-3-O-β-glucopyranoside	1.49
peonidine-3-O-β-glucopyranoside	0.30
delpinidine-3-O-β-glucopyranoside	0.00
pelalgonidine-3-O-β-glucopyranoside	0.30
protocatechuic acid	0.47
ferulic acid	0.05

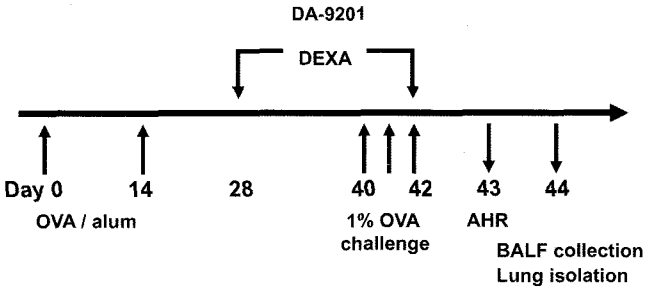

Fig. 1. Schematic diagram of the experimental protocol. Mice were sensitized on day 0 and 14 by intraperitoneal injection of OVA 50 μg emulsified in alum 1 mg. On day 40, 41, and 42 after the primary sensitization, the mice were challenged for 30 min with an aerosol of 1% OVA in saline (or with saline in the case of normal control) using an ultrasonic nebulizer. DA-9201 or DEXA was administered orally at 24 h intervals on day 28~42. All mice were sacrificed 48 hr after the last exposure.

challenged with aerosolized 1% OVA for 30 min/day. OVA challenging was performed by placing mice in a Plexiglas box (29×22×18 cm) and aerosolizing OVA using an ultrasonic nebulizer (NE-U12; Omron, Japan) driven by compressed air for 30 min. Normal control mice were challenged with aerosolized PBS.

AHR measurement

Twenty four hours after the last OVA challenge, AHR was assessed by measuring methacholine-induced airway resistance using whole body plethysmography (OCP-3000, Allmedicus Co., Ltd., Korea). Unrestrained, conscious mice (n=8/group) were placed in separate plethysmographic chambers and stimulated with aerosolized saline followed by incremental doses of aerosolized methacholine (5, 10, 15 mg/mL in saline) for 3 min. Airway resistance, representing airway responsiveness to methacholine stimulation, was monitored and expressed as enhanced pause (Penh).

Bronchoalveolar lavage fluid (BALF) collection

After mice were bled and sacrificed following anesthesia with ether, BALF was collected for differential cell counting and measurement of cytokines. It was carried out by cannulating the upper part of the trachea and lavaging by three times with 0.5 mL of PBS containing 0.05 mM EDTA. The BALF was centrifuged at 400 g at 4°C for 3 min, and the cells were separated from the fluid. The supernatant was stored at -70°C until use. The cells were re-suspended in PBS containing 0.05mM EDTA, and the total number was determined with the use of a hemocytometer. The differential BAL cells were counted by microscopy after cytospin preparations and Giemsa staining (Giemsa stain modified, Sigma).

Cytokines and immunoglobulin measurement

Plasma and BALF samples from each animal were analyzed for total IgE. Complementary capture and detection antibody pairs for mouse IgE antibody were purchased from PharMingen (San Diego, CA), and the IgE enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions. IL-4, IL-5, and IL-13 levels in the BALF were also determined by ELISA according to the manufacturer's instructions (R&D Systems).

Preparation of nuclear and cytoplasmic fraction for Western blot or EMSA

Whole lungs were removed in additional experimental animals (n=8/group) and single cell suspensions were prepared as previously described (Kennedy *et al.*, 1995). Briefly, lung tissue from each mouse was perfused with PBS, minced with scissors, and finally passed through 70 μm nylon mesh (BD Falcon) to obtain the single cell suspension. The cells were washed with ice-cold PBS and then transferred to microtubes. They were allowed to swell after the addition of 100 μL hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride. The lysates were incubated for 10 min in ice and centrifuged at 7200 *g* at 4°C for 5 min. Supernatants containing cytoplasm fraction were stored at -70°C until use. Pellets containing crude nuclear fraction were resuspended in 50 μL of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride and then incubated for 30 min in ice. The samples were centrifuged at 15800 *g* for 10 min to obtain supernatants containing nuclear fraction. They were stored at -70°C until use.

Electrophoretic mobility shift assay (EMSA)

A double-stranded DNA probe for the consensus oligonucleotide of NF-κB (5'-AGT TGA GGG GAG TTT CCC AGG G-3', Santa Cruz Biotechnology, Inc.) was used for gel shift analysis after end-labeling of the probe with [g-^{32}P] ATP and T_4 polynucleotide kinase. The reaction mixtures contained 4 μL of 5X binding buffer containing 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/mL poly dI–dC, 50 mM Tris–HCl (pH 7.5), and 10 μg of nuclear extracts and sterile water in a total volume of 20 μL. Reactions were initiated by addition of 1 μL probe (10^6 cpm) following 10 min preincubation and continued for 20 min at room temperature. The samples were then assayed for NF-κB DNA binding activity by EMSA. For supershift assay, 10 μg of nuclear fraction was incubated with 2 μg each of the highly specific anti-p65, anti-p52 antibody (Santa Cruz Biotechnology, Inc.) at room temperature for 1 h followed by EMSA. In some experiments, the specificity of NF-κB binding to the DNA consensus oligonucleotide was confirmed by the inhibition of NF-κB DNA binding with the cold NF-κB or the cold SP-1. Samples were loaded onto 4% polyacrylamide gels at 100 V. The gels were removed, fixed and dried followed by autoradiography.

Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses were performed. Expression of NF-κB was immunochemically monitored in the nuclear and cytoplasmic fractions of the cells from lungs using anti-rabbit NF-κB antibody (Santa Cruz Biotechnology, Inc.). The secondary antibodies were alkaline phosphatase-conjugated anti-rabbit antibodies. The bands of NF-κB were developed using 5-*bromo*-4-*chloro*-3-indolylphosphate/4-nitroblue tetrazolium chloride.

Lung histology

Another set of experiment with the same scheme except BALF collection was repeated to obtain intact lung tissues for lung histology. The trachea and lungs (n=8/group) were collected 24 h after the last OVA challenging, fixed in 10% buffered formalin, embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin (H&E) to assess the inflammatory cell infiltration and Periodic Acid – Schiff (PAS) to quantify airway goblet cells. Inflammation was examined and scored by two independent blinded investigators. The degree of peribronchial and perivascular inflammation was graded on a subjective scale of 0–3, as described elsewhere (Tournoy *et al.*, 2000). A value of 0 was assigned when no inflammation was detectable, a value of 1 was assigned for occasional cuffing with inflammatory cells, a value of 2 was assigned for most bronchi or vessels surrounded by a thin layer (one to five cells thick) of inflammatory cells, and a value of 3 was given when most bronchi or vessels were surrounded by a thick layer (more than five cells thick) of inflammatory cells. Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation (expressed as inflammation score in arbitrary units; U). Quantification of goblet cells in airway tissue was achieved by counting the numbers of these cells in five fields per slide in PAS stained section.

Statistical analysis

All statistical analyses were performed using SigmaStat® for Windows 2.0 software (Jandel corporation, U.S.A.). An ANOVA test was used to examine the comparisons between the experimental groups and within each test group. All the data is expressed as a mean ± S.E.M. The comparison between the group means was conducted using a Dunnett multiple range test with a significance level of $p = 0.05$.

RESULTS

Effect of DA-9201 on AHR

Animals challenged with OVA showed a significant increased Penh in response to increasing doses of methacholine when compared with saline-challenged animals. Animals treated with DA-9201 300 mg/kg demonstrated statistically significant reductions in Penh at methacholine concentrations of 10 and 15 mg/mL (Fig. 2). At dose levels of 30 or 100 mg/kg, DA-9201 also exhibited a marked decrease in airway responsiveness, but these were not statistically significant. DEXA also significantly reduced AHR to aerosolized methacholine (Fig. 2).

Effect of DA-9201 on eosinophilia in BALF

Differential cell counting revealed a significantly increased

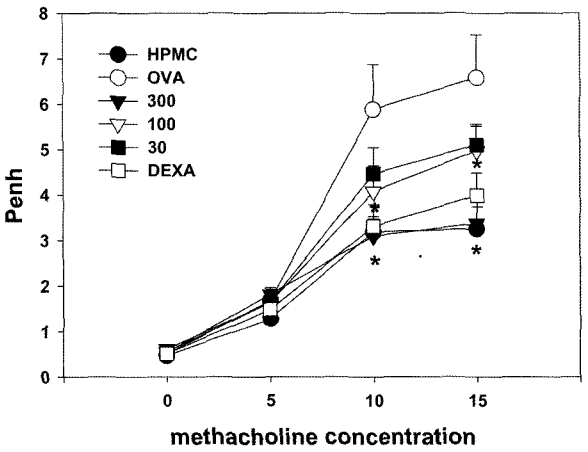

Fig. 2. The effect of DA-921 on airway reactivity assessed by change in Penh in response to increasing concentrations of aerosolized methacholine. HPMC represents saline-inhaled mice administered HPMC; OVA, OVA-inhaled mice administered HPMC; 30, OVA-inhaled mice administered DA-9201 30 mg/kg; 100, OVA-inhaled mice administered DA-9201 100 mg/kg; 300, OVA-inhaled mice administered DA-9201 300 mg/kg; DEXA, OVA-inhaled mice administered DEXA 3 mg/kg. Each bar represents the mean±SEM (n=8/group). * Significantly differences compared with OVA control ($p < 0.05$).

percentage of eosinophil in the OVA-exposed animals (Fig. 3). In contrast to OVA control, a significant and dose-dependent decrease in the number of eosinophils was observed in mice treated with DA-9201 (Fig. 3). In a group treated with DA-9201 300 mg/kg, the percentage of eosinophils was significantly lower than that in the OVA control group.

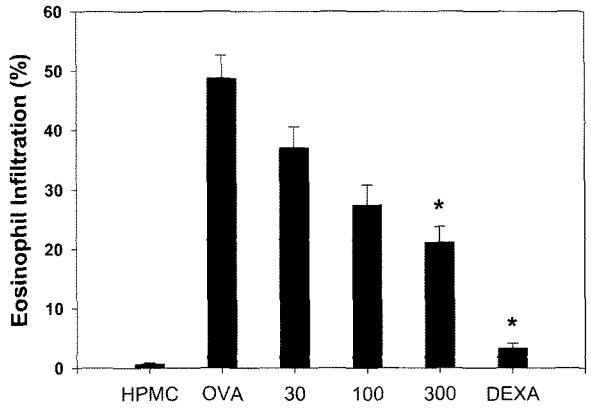

Fig. 3. The effect of DA-9201 on eosinophil infiltration in the airways. One day after the last OVA inhalation, another set of mice were euthanized and their lungs were lavaged. Cells in the BALF were collected, and cytospin preparations were made. Different cell types in the BALF were analyzed. Each bar represents the mean±SEM (n=8/group). * Significantly different from the OVA control group ($p < 0.05$).

Effect of DA-9201 on the level of IgE and Th2 cytokines in plasma or BALF

While levels of IgE in plasma and BALF in the OVA

Table II. The effect of DA-9201 on the level of IgE and Th2 cytokines in plasma or BALF

Groups	plasma	BALF			
	IgE	IgE	IL-4	IL-5	IL-13
HPMC	35.1 ± 5.7	1.9 ± 1.3	8.4 ± 1.4	19.5 ± 2.8	25.6 ± 3.4
OVA	147.0 ± 11.3*	29.0 ± 7.7*	186.2 ± 66.1*	39.6 ± 7.38*	47.7 ± 7.0*
30	131.4 ± 10.7	9.5 ± 2.0**	58.0 ± 37.9	35.0 ± 4.8	51.1 ± 4.1
100	110.1 ± 7.2**	7.0 ± 3.0**	59.9 ± 35.3	30.0 ± 2.6	38.3 ± 6.7
300	106.8 ± 7.2**	7.0 ± 2.4**	34.4 ± 5.4**	24.7 ± 2.5**	32.1 ± 5.1**
DEXA	114.3 ± 9.1**	7.2 ± 2.4**	16.9 ± 2.2**	18.8 ± 1.4**	30.1 ± 1.8**

The level of IgE in plasma and BALF was quantified by sandwich ELISA and expressed as nanogram per mililitter. The level of IL-4, IL-5, and IL-13 in BALF was quantified by sandwich ELISA and expressed as picogram per mililitter. *p<0.05 vs. negative control, **p<0.05 vs. OVA control.

control mice were significantly higher than the level in normal control, DA-9201 significantly and dose-dependently reduced the level of IgE in both plasma and BALF (Table II). Levels of Th2 cytokines were also elevated in sensitized mice challenged with OVA as compared to that in normal control mice. All these cytokines were significantly reduced by the treatment with DA-9201 (300 mg/kg), although the reduction did not show a statistical significance at dose levels of 100 and 30 mg/kg (Table II).

Effect of DA-9201 on the binding activity and expression of NF-κB in lung tissues

EMSA revealed that NF-κB-DNA binding in nuclear extracts from lung tissues was increased in the OVA-sensitized and challenged mice. This elevation in the NF-κB-DNA binding activity was dramatically decreased by

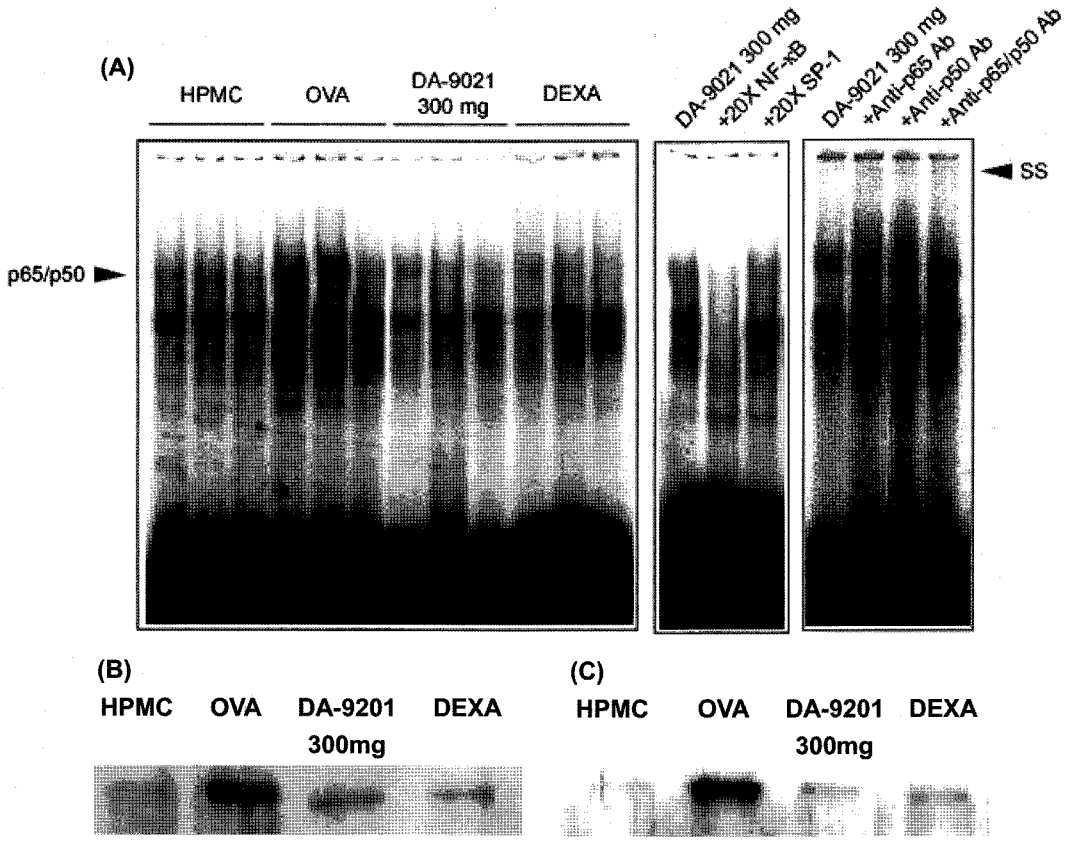

Fig. 4. The effect of DA-9201 on the binding activity and the expression of NF-κB. The DNA-binding activity of NF-κB in the nuclear fraction of lung was determined by EMSA assay (A). To characterize specific NF-κB complex, supershift analysis was conducted with anti-p65, anti-p52 antibody. NF-κB and supershifts (solid arrowheads) are indicated. The specificity of NF-κB binding to the DNA consensus oligonucleotide was also checked by the inhibition of NF-κB DNA binding with the cold NF-κB or the cold SP-1. The expression level of NF-κB in the nuclear (B) and cytoplasmic fraction (C) were quantified by Western blotting.

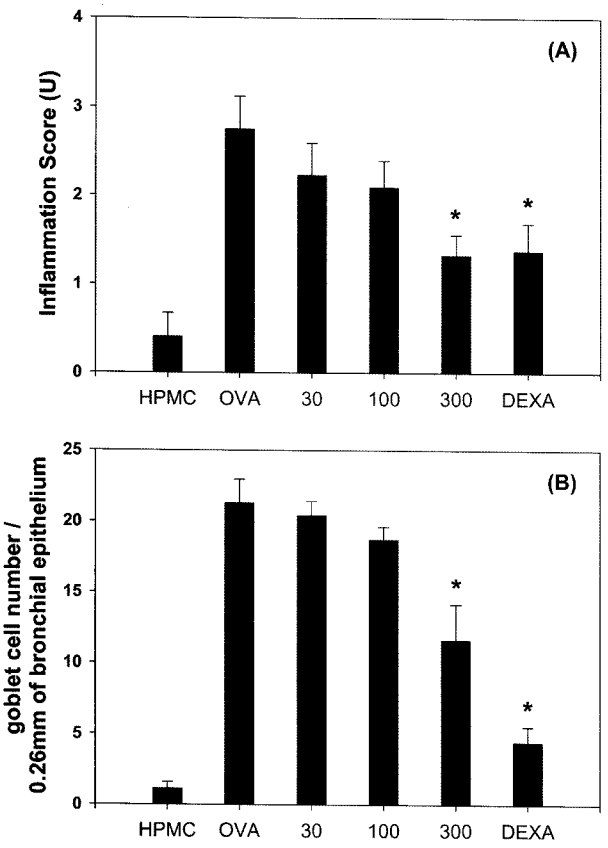

Fig. 5. Effect of DA-9201 on peribronchial and perivascular lung inflammation and the number of goblet cells. Values represent mean ± SEM (n=8/group). Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores (A). The number of goblet cells in the airway wall was visualized by periodic acid-Schiff (PAS) staining (B). * Significantly different from the OVA control ($p < 0.05$).

treatment with DA-9201 (Fig. 4A). Supershift assay confirmed NF-κB-DNA binding activity by the presence of p65 and p50 subunits of NF-κB in nuclear protein extracts. Furthermore, the specificity of NF-κB binding to the DNA consensus oligonucleotide was also confirmed by the inhibition of NF-κB DNA binding with the cold NF-κB or the cold SP-1. Western blotting revealed that the level of NF-κB p65 protein in nuclear and cytoplasmic protein extracts from lung tissues was increased in the OVA-sensitized and -challenged mice. These elevations in NF-κB p65 level in nuclear and cytoplasmic protein extracts were markedly decreased by treatment with DA-9201, respectively (Fig. 4B and C).

Histological examination

The scores of peribronchial, perivascular and total lung inflammation were increased significantly after OVA inhalation compared with scores after saline inhalation. The increased peribronchial, perivascular and total lung inflammation after OVA inhalation was significantly reduced by administration of DA-9201 (Fig. 5A). The number of PAS-stained goblet cells was also increased in the mice OVA-sensitized and -challenged mice, and DA-9201 treatment significantly decreased the number of goblet cells (Fig. 5B).

DISCUSSION

Airway inflammation in asthma and allergic disease is a complex phenomenon driven predominantly by Th2 cells. The inflammation is characterized by the recruitment of leukocytes, predominantly eosinophils from the vasculature into the tissue where they cause severe damage to the bronchiolar epithelium (de Monchy *et al.*, 1985; Walker *et al.*, 1991). Epithelial, and endothelial cells also play major roles in this cascade by secreting cytokines and chemokines and by expressing cell surface adhesion molecules.

The molecular mechanism that regulates eosinophilic inflammation in asthma is an area of intense investigation in many laboratories. Yang *et al.* (1998) investigated the role of NF-κB in the elicitation of eosinophilic inflammation in a mouse model of allergic airway inflammation and showed that NFkB played a critical regulatory role in the development of eosinophilic inflammation in response to inhaled allergens. Therefore, particular attention is now being focused on the mechanisms that mediate anti-inflammatory events at the gene level of NF-κB, which is a key target on account of its role in regulating the transcription of proinflammatory genes known to be involved in the allergic tissue response (Barnes, 1997; Manning and Anderson, 1994).

NF-κB is also considered a master regulator of inflammation and immune processes (Baldwin, 1996; Baeuerle and Baltimore, 1996). Especially, NF-κB plays a critical role in Th2 cell differentiation and is required for the induction of allergic airway inflammation (Das *et al.*, 2001). In fact, the induction of many of the genes for the cytokines, chemokines and adhesion molecules is regulated by NF-κB in asthmatics (Hart *et al.*, 1998; Stutz and Woisetschlager, 1999; Washizu *et al.*, 1998). Furthermore, activation of NF-κB may be the critical step for perpetuation of chronic airway inflammation in asthma (Hart *et al.*, 1998). Accumulating reports have supported that NF-κB is a therapeutic target in asthma and have demonstrated that specific NF-κB inhibition in the lung was associated with strong attenuation of allergic lung inflammation, AHR and mucus production. Therefore, it is believed that specific NF-κB inhibitor has a therapeutic potential in asthma.

In this study, to test the hypothesis that the anti-inflammatory effects of DA-9201 are mediated through NF-κB regulated pathways, we examined the NF-κB expression and indices of airway inflammation after antigen provocation. The results clearly demonstrated that DA-9201 signi-

ficantly reduced airway hyperresponsiveness, Th2 cytokines production and airway eosinophilia and inflammation as well as IgE production. In addition, DA-9201 treatment markedly reduced the binding activity of NF-κB and down-regulated the expression of NF-κB protein in both nuclear and cytoplasmic fraction in lung tissues. These results suggest that anti-asthmatic effects of DA-9201 are closely linked to its suppressive effect on NF-κB pathway.

Black rice (*Oryza sativa L.* var.) is a dark purple colored seed and a major rice crop in the south Asia and China. It is broadly known as enriched rice with various medical effects on a number of allergic disorders, enhancing ability for the spleen, liver, stomach, and intestine function, a hematopioetic effects in pharmacy, anti-mutagenic and anti-cancer activity, and anti-atherosclerotic effects (Ling *et al.*, 2002; Miyazawa *et al.*, 2003). It is well known that rice bran contains a considerable amount of anthocyanins and various types of phenolic acids. Recently two major anthocyanins, cyanidine-3-*O*-β-D-glycoside and peonidine-3-*O*-β-D-glycoside, and various phenolic compounds were identified in the black rice extract cultivated in S. Korea (Chung and Woo, 2001; Han *et al.*, 2004). Anthocyanins have been considered to be one of the biologically active substances. Their anti-inflammatory activity, anti-oxidant activity and other medical benefits have been extensively investigated (Hu *et al.*, 2003; Kowalczyk *et al.*, 2003; Tsuda *et al.*, 2002).

With regards to therapeutic strategies for asthma treatment, corticosteroids are the most potent anti-inflammatory agents and they are known to produce substantial improvement in lung functions of asthmatic subjects. However, due to many undesirable side effects of systemic corticosteroids, inhaled corticosteroids are used as the first line of treatment for asthma and are an effective means of reducing inflammation and bronchial constriction in asthma patients. Although the most frequently reported side effects of inhaled corticosteroids are local effects, systemic side effects such as adrenal suppression, decreased bone metabolism and decreased growth rate also have been reported (Bleecker, 1998). Therefore there is a need for a safe and potent alternative anti-inflammatory agent that does not cause adverse effects on the normal physiological functions.

In this study, using a mouse model of asthma, we found that DA-9201, a black rice extract, has a potential of anti-asthma. At the doses tested, especially at 100 mg or 300 mg, DA-9201 suppressed the pulmonary inflammation, accumulation of eosinophils in airway and AHR. Additionally, Th2 cytokines such as IL-4, IL-5, and IL-13, and IgE production were decreased by administration of DA-9201. Furthermore, DA-9201 is expected to be highly safe, since black rice has been used as food in many Asian countries. These results suggest that DA-9201 has advantages over other anti-inflammatory agents in that it is particularly safe and has the potential benefits in the prevention and treatment of asthma.

In conclusion, DA-9201 significantly inhibited AHR, Th2 cytokines and IgE production, airway inflammation, and eosinophil accumulation. In addition, DA-9201 treatment down-regulated the expression of NF-κB and markedly reduced the binding activity of NF-κB. These results suggest that DA-9201 may be useful for the treatment of asthma and its efficacy is related to suppression of NF-κB pathway.

ACKNOWLEDGEMENT

We would like to thank Professor Sang Geon Kim, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, for participating in EMSA assay.

REFERENCES

Baeuerle, P. A. and Baltimore, D., NF-κB: ten years after. *Cell*, 87, 13-20 (1996).

Baldwin, A. S. Jr., The NF-κB and IkB proteins: new discoveries and insights. *Annu. Rev. Immunol.*, 14, 649-681 (1996).

Barnes, P. J., Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory disease. *N. Engl. J. Med.*, 336, 1066-1071 (1997).

Bleecker, E., Inhaled corticosteroids: current products and their role in patient care. *J. Allergy Clin. Immunol.*, 101, S400-402 (1998).

Bureau, F., Bonizzi, G., Kirschvink, N., Delhalle, S., Desmecht, D., Merville, M. P., Bours, V., and Lekeux, P., Correlation between nuclear factor-κB activity in bronchial brushing samples and lung dysfunction in an animal model of asthma. *Am. J. Respir. Crit. Care. Med.*, 161, 1314-1321 (2000).

Busse, W. W. and Lemanske, R. F., Asthma. *N. Engl. J. Med.*, 344, 350-362 (2001).

Chung, H. S. and Woo, W. S., A quinolone alkaloid with anti-oxidant activity from the aleurone layer of anthocyanin-pigmented rice. *J. Nat. Prod.*, 64, 1579-1580 (2001).

Das, J., Chen, C. H., Yang, L., Cohn, L., Ray, P., and Ray, A., A critical role for NF-κB in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nat. Immunol.*, 2, 45-50 (2001).

de Monchy, J. G. R., Kaufmann, H. F., Venge, P., Koeter, G. H., Jansen, H. M., Sluiter, H. J., and de Vries, K., Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *Am. Rev. Respir. Dis.*, 131, 373-376 (1985).

Ghosh, S., May, M. J., and Kopp, E. B., NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.*, 16, 225-260 (1998).

Han, S. J., Ryu, S. N., and Kang, S. S., A new 2-arylbenzofuran with antioxidant activity from the black colored rice (Oryza sativa L.) bran. *Chem. Pharm. Bull.*, 52, 1365-1366 (2004).

Hart, L. A., Krishnan, V. L., Adcock, I. M., Barnes, P. J., and Chung, K. F., Activation and localization of transcription factor, nuclear factor-κB, in asthma. *Am. J. Respir. Crit. Care. Med.*, 158, 1585-1592 (1998).

Hu, C., Zawistowski, J., Ling, W., and Kitts, D. D., Black rice (Oryza sativa L. indica) pigmented fraction suppresses both reactive oxygen species and nitric oxide in chemical and biological model systems. *J. Agric. Food Chem.*, 27, 5271-5277 (2003).

Kennedy, J. D., Hatfield, C. A., Fidler, S. F., Winterrowd, G. E., Haas, J. V., Chin, J. E., and Richards, I. M., Phenotypic characterization of T lymphocytes emigrating into lung tissue and the airway lumen after antigen inhalation in sensitized mice. *Am.J. Respir. Cell Mol. Biol.*, 12, 613-623 (1995).

Kim, H. M., Kang, C. S., Lee, E. H., and Shin, T. Y., The evaluation of the antianaphylactic effect of Oryza sativa L. subsp. hsien Ting in rats. *Pharmacol. Res.*, 40, 31-36 (1999a).

Kim, H. M., Yi, D. K., and Shin, H. Y., The evaluation of antianaphylactic effect of Oryza sativa L. in rats. *Am. J. Chinese. Med.*, 27, 63-71 (1999b).

Kowalczyk, E., Krzesinski, P., Kura, M., Szmigiel, B., and Blaszczyk, J., Anthocyanins in medicine. *Pol. J. Pharmacol.*, 55, 699-702 (2003).

Larche, M., Robinson, D. S., and Kay, A. B., The role of T lymphocytes in the pathogenesis of asthma. *J. Allergy Clin. Immunol.*, 111, 450-463 (2003).

Ling, W. H., Wang, L. L., and Ma, J., Supplementation of the black rice outer layer fraction to rabbits decreases atherosclerotic plaque formation and increases antioxidant status. *J. Nutr.*, 132, 20-26 (2002).

Manning, A. M. and Anderson, D. C., Transcription factor-κB an emerging regulator of inflammation. *Annu. Rep. Med. Chem.*, 29, 235-244 (1994).

Miyazawa, M., Oshima, T., Koshio, K., Itsuzaki, Y., and Anzai, J., Tyrosinase inhibitor from black rice bran. *J. Agric. Food Chem.*, 51, 6953-6956 (2003).

Pahl, H. L., Activators and target genes of Rel/NF-κB transcription factors. Oncogene, 18, 6853-6866 (1999).

Ray, A. and Cohn, L., Th2 cells and GATA-3 in asthma: new insights into the regulation of airway inflammation. *J. Clin. Invest.*, 104, 985-993 (1999).

Sha, W. C., Regulation of immune responses by NF-κB/Rel transcription factors. *J. Exp. Med.*, 187, 143-146 (1998).

Stutz, A. M. and Woisetschlager, M., Functional synergism of STAT6 with either NF-κB or PU.1 to mediate IL-4-induced activation of IgE germline gene transcription. *J. Immunol.*, 163, 4383-4391 (1999).

Tournoy, K. G., Kips, J. C., Schou, C., and Pauwels, R. A., Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin. Exp. Allergy*, 30, 79-85 (2000).

Tsuda, T., Horio, F., and Osawa, T., Cyanidin 3-*O*-beta-D-glucoside suppresses nitric oxide production during a zymosan treatment in rats. *J. Nutr. Sci. Vitaminol.*, 48, 305-310 (2002).

Umetsu, D. T., McIntire, J. J., Akbari, O., Macaubas, C., and DeKruyff, R. H., Asthma : an epidemic of dysregulated immunity. *Nat. Immunol.*, 3, 715-720 (2002).

Walker, C., Kaegi, M. K., Braun, P., and Blaser, K., Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J. Allerg Clin. Immunol.*, 88, 935-942 (1991).

Washizu, J., Nishimura, H., Nakamura, N., Nimura, Y., and Yoshikai, Y., The NF-κB binding site is essential for transcriptional activation of the IL-15 gene. *Immunogenetics*, 48, 1-7 (1998).

Wills-Karp, M., Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.*, 17, 255-281 (1999).

Yang, L., Cohn, L., Zhang, D. H., Homer, R., Ray, A., and Ray, P., Essential role of nuclear factor-κB in the induction of eosinophilia in allergic airway inflammation. *J. Exp. Med.*, 188, 1739-1750 (1998).