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Curcumin attenuates ovalbumin-induced airway inflammation by regulating nitric oxide

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ABSTRACT

Curcumin has been strongly implicated as an anti-inflammatory agent, but the precise mechanisms of its action are largely unknown. In this study, we show that curcumin contributes to anti-inflammatory activity in the murine asthma model and lung epithelial cell A549 through suppression of nitric oxide (NO). To address this problem, curcumin was injected into the peritoneum of ovalbumin (OVA)-sensitized mice before the last allergen challenge. OVA challenge resulted in activation of the production of inducible nitric oxide (iNOS) in lung tissue, inflammatory cytokines, recruitment of eosinophils to lung airways, and airway hyper-responsiveness to inhaled methacholine. These effects of ovalbumin challenge were all inhibited by pretreatment of mice with curcumin. Furthermore, supplementation with curcumin in the A549 human airway epithelial cells decreased iNOS and NO production induced by IFN- γ . These findings show that curcumin may be useful as an adjuvant therapy for airway inflammation through suppression of iNOS and NO.

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Asthma is a complex chronic inflammatory lung disease characterized by airway hyper-responsiveness (AHR) to allergens, airway edema, and increased mucus secretion [1,2]. The development and persistence of airway inflammation is accompanied by the infiltration of eosinophils, neutrophils, macrophages, and lymphocytes into the bronchial lumen and lung tissues [3,4]. Airway epithelial cells, smooth muscle cells, endothelial cells, and fibroblasts are capable of liberating inflammatory mediators in the airway [5,6]. To date, more than 50 inflammatory mediators have been identified in airway inflammation [7]. One of these, endogenous nitric oxide (NO) produced by inducible NO synthase (iNOS), is well known for its possible role in inducing airway diseases including asthma [8,9]. NO strongly promotes the chemotaxis of inflammatory cells in the lung and favors the development of a Th2 response [10,11]. There is good evidence that NO inhibitors suppress airway inflammation by suppressing the recruitment of inflammatory cells, and mucus secretion in the lungs [12]. Therefore, NO inhibition may be a good target for the clinical study of asthma.

Previous studies have shown that curcumin strongly inhibited the initiation and promotion of chemical carcinogen-induced tumor formation in mice [13,14], and the proliferation of various cul-

tured tumor cells [15,16]. These potent chemopreventive activities of curcumin may be attributed to the inhibition of certain signal transduction pathways critical for tumor cell growth, such as AP-1 and NF- κ B [17,18]. Several researchers have shown that curcumin inhibits iNOS [19,20] and inflammatory cytokines induced by macrophages and dendritic cells [21,22]. Although many researches have studied the anti-inflammatory effects of curcumin, airway inflammation in the animal model has not been investigated.

In this study, we attempt to characterize the effects of a non-cytotoxic concentration of curcumin in a murine model of asthma. We suggest that administration of curcumin inhibits airway inflammation by blocking the expression of iNOS and NO.

Materials and methods

Reagents. Curcumin, ovalbumin (OVA), and methacholine were purchased from Sigma (St. Louis, MO) and IFN- γ was purchased from Calbiochem (San Diego, CA). Antibodies against iNOS and β -actin were purchased from Upstate Biotechnology (Lake Placid, NY) and Sigma, respectively.

Allergen sensitization and challenge. Female BALB/c mice, 8–10 weeks of age and free of murine-specific pathogens, were obtained from the Charles River Laboratories (Yokohama, Japan), housed

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throughout the experiments in a laminar flow cabinet, and maintained on a standard laboratory diet *ad libitum*. All experimental animals used in this study were treated according to a protocol approved by the Institutional Animal Care and Use Committee of the Pusan National University College of Medicine. Mice were sensitized on days 1 and 14 by intraperitoneal injection of 20 µg OVA emulsified in 1 mg of aluminum hydroxide (Pierce; Rockford, IL) in a total volume of 200 µl. On days 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (w/v) OVA (OVA/OVA) in saline (OVA/PBS) using an ultrasonic nebulizer (NE-U12; Omron, Japan).

Administration of curcumin. Curcumin solution (10 or 20 mg/kg body weight/day) was freshly prepared and administered intraperitoneally 3 times at 24 h intervals on days 21–23, beginning 1 h before the first challenge.

Determination of airway responsiveness to methacholine. Airway responsiveness was measured in mice 48 h after the last challenge in an unrestrained conscious state. Mice were placed in a barometric plethysmographic chamber (All Medicus Co., Seoul, South Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine at increasing concentration (from 2.5 to 50 mg/ml) was nebulized through an inlet of the main chamber for 3 min. Readings were taken and averaged for 3 min after each nebulization. Enhanced pause (Penh), calculated as (expiratory time/relaxation time – 1) × (peak expiratory flow/peak inspiratory flow), according to the manufacturer's protocol, is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. Penh was used as a measure of airway responsiveness to methacholine. Penh values averaged for 3 min after each nebulization were evaluated.

Histology. Before removal of the lungs, the lungs and trachea were filled intratracheally with a fixative (4% paraformaldehyde) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) paraformaldehyde. For histological examination, fixed embedded lungs were sectioned into 4 µm sections and stained with haematoxylin and eosin (H&E). Immunohistochemistry was also performed with anti-iNOS by using the streptavidin–biotin–peroxidase method.

Total cell count. Total cell numbers were counted with a haemocytometer. Smears of bronchoalveolar lavage (BAL) cells prepared with Cytospin II (Shandon, Runcorn, UK) were stained with Diff-Quik solution (Dade Diagnostics, Aguada, PR) for differential cell counting. Two independent, blinded investigators counted the cells using a microscope. Approximately 200 cells were counted in each of four different random locations.

Measurement of cytokines. Levels of IL-4, IL-5, IL-12, and IFN-γ were quantified in the supernatants of BAL fluids (BALF) and IgE in sera by enzyme immunoassays according to the manufacturer's protocol (R&D Systems; Minneapolis, MN).

Cell culture. Human airway epithelial cell line A549 was cultured in a humidified atmosphere of 5% CO₂ at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml of penicillin G, and 100 U/ml of streptomycin.

Western blot. Lung tissues or A549 cells were washed twice with cold PBS and then lysed in PRO-PREP protein extraction solution (iNtRON Biotechnology, Sungnam, South Korea). Total cell extracts were separated on 10% polyacrylamide gels, and then transferred to nitrocellulose membranes using standard procedures. The membranes were developed using an ECL reagent (Amersham, Arlington Heights, IL).

Nitrite assay. NO levels in the culture supernatants were measured by a Griess reaction. After A549 cells were stimulated with IFN-γ for 24 or 48 h, 100 µl of each cultured medium was mixed with the same volume of the Griess reagent (1%

sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H₃PO₄). NO concentration was determined by measuring the absorbance at 540 nm with a microplate spectrophotometer. Nitrite concentration was calculated according to a standard curve of sodium nitrite generated by known concentrations.

Statistical analysis. The images were visualized with Chemi-Smart 2000 (Vilber Lourmat; Cedex, France). Images were captured using Chemi-Capt (Vilber Lourmat) and downloaded to Photoshop. Statistical analyses were conducted using Tukey's Multiple Comparison test, and values were presented as means ± SEM. A value of **p* < 0.05 was accepted as an indication of statistical significance.

Results

Curcumin suppresses AHR and the infiltration of inflammatory cell in lung

To evaluate the effect of curcumin on airway inflammation, AHR first was assessed as the percent of Penh in response to increasing doses of methacholine at 48 h after the last OVA challenges in mice. As shown in Fig. 1A, the percentage of Penh produced by the administration of methacholine was significantly increased in OVA/OVA mice compared with OVA/PBS mice. Peritoneal administration of curcumin (20 mg/kg body weight/day) significantly reduced the percentage of Penh in OVA/OVA mice. These results indicate that administration of curcumin reduces bronchoconstriction in an OVA-induced asthma model. To determine the infiltration of inflammatory leukocytes, we next investigated the histological samples of lung tissues and the number of cells in BALF. As shown in Fig. 1B, many inflammatory cells in the airway and around the blood vessels were observed in the OVA/OVA mice, but not in the OVA/PBS mice. However, the administration of curcumin resulted in a significant improvement of luminal narrowing in the airway. As shown in Fig. 1C, curcumin also significantly decreased the total cell number and eosinophils in OVA/OVA mice. We observed that the iNOS inhibitor, L-NMMA, also significantly decreased the percentage of Penh, infiltration of inflammatory cells. These data indicate that curcumin can suppress airway inflammation in an OVA-induced murine asthma model.

Curcumin decreases iNOS expression in lung

To investigate further whether the levels of NO are related to curcumin-induced anti-asthmatic effects, we measured iNOS expression in the lung. As shown in Fig. 2A, immunohistochemical staining readily provided a quantitative measure of iNOS expression in the lung. We found predominantly iNOS-stained cells in OVA/OVA mice lung tissue compared with those of OVA/PBS mice, however the administration of curcumin decreased the stained cells in OVA/OVA mice lung tissue. To quantify further the inhibitory effect of iNOS, observed by immunohistochemical staining, we investigated iNOS expression using Western blot analysis. As shown in Fig. 2B, the increased iNOS expression observed in OVA/OVA mice was significantly inhibited by curcumin. The administration of L-NMMA as a positive control inhibited iNOS expression in OVA/OVA mice. These results may indicate that curcumin-induced anti-asthmatic effects are related to the down-regulation of iNOS.

Curcumin attenuates the expression of IL-4 and IL-5 in BALF and IgE in serum

Because Th2 cytokines promote airway inflammation in asthma through increased IgE levels, we investigated the expression of inflammatory cytokines in BALF and IgE in serum. All cytokines were determined using ELISA at 48 h after the last OVA challenge.

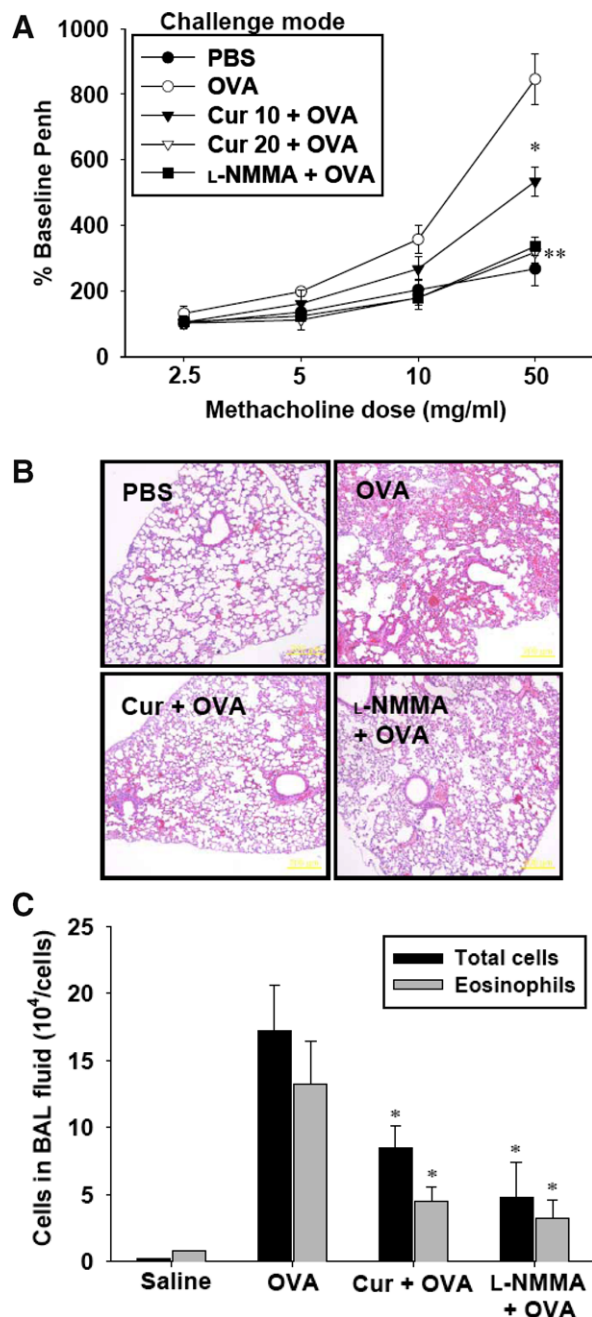


Fig. 1. Curcumin suppresses airway hyper-responsiveness and infiltration of inflammatory cells in the lung. Mice were sensitized and challenged by OVA and/or curcumin (10 or 20 mg/kg body weight/day) as described in the Materials and Methods. Lungs were removed 48 h after the last OVA challenge. (A) Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. Mice were placed in the main chamber and were nebulized first with PBS, then with increasing doses (2.5–50 mg/ml) of methacholine for 3 min for each nebulization. (B) Lung tissue was fixed, embedded, cut into slices, and stained with H&E solution (200 $\times$). (C) BAL cells were collected using a cytospin, and then stained with Diff-Quik. Differential cell counting was performed using standard morphological criteria. (B and C) Data represents at 20 mg/kg body weight/day of curcumin. This experiment used six mice per group ($n = 6$). * $p < 0.05$ vs. OVA/OVA mice.

As shown in Fig. 3A, the levels of IL-4 and IL-5 in BALF were significantly increased in OVA/OVA mice compared with OVA/PBS mice, and a small reverse trend was found in the levels of IFN- γ and IL-12. However, administration of curcumin significantly attenuated IL-4 and IL-5 levels, and slightly restored the levels of IFN- γ . Because IgE favors to Th2 in airway inflammation, we measured

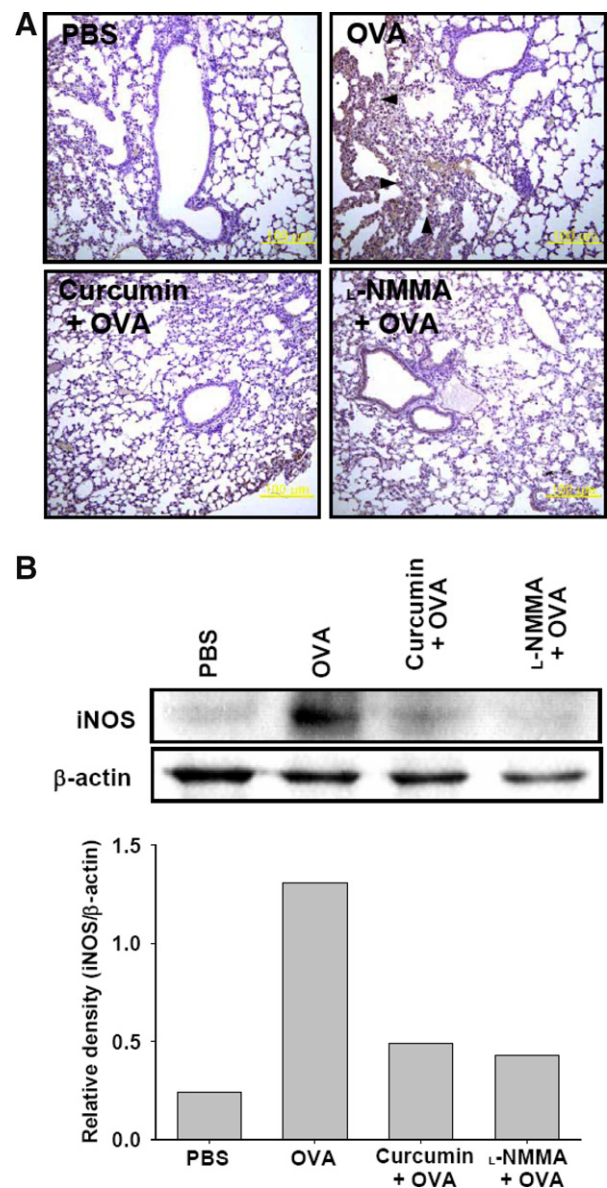


Fig. 2. Curcumin (20 mg/kg body weight/day) decreases iNOS expression in the lung. (A) Lung tissues were fixed, embedded, cut into slices, and stained with iNOS. (B) Total cell lysates were prepared from the lung, and proteins were subjected to SDS-PAGE followed by Western blot analysis with specific antibodies against iNOS. β -Actin was used as an internal control.

how far curcumin modulated the levels of serum IgE in OVA/OVA mice. As shown in Fig. 3B, the levels of serum IgE were found to be significantly increased in OVA/OVA mice compared with those of OVA/PBS mice. However, administration of curcumin significantly decreased the levels of serum IgE. These data indicate that curcumin modulates Th1/Th2 levels in an OVA-induced asthma model.

Curcumin inhibits iNOS and NO expression in IFN- γ -stimulated lung epithelial A549 cells

Because IFN- γ clearly induced iNOS and NO in human lung alveolar epithelial cell line A549 [23], we specifically examined whether curcumin reduces the expression of IFN- γ -induced iNOS and NO using Western blot analysis and Griess reaction. As shown in Fig. 4A, treatment with curcumin significantly inhibited IFN- γ -induced iNOS protein expression at 24 h. This inhibition generally occurred in a dose-dependent manner. β -Actin protein level was

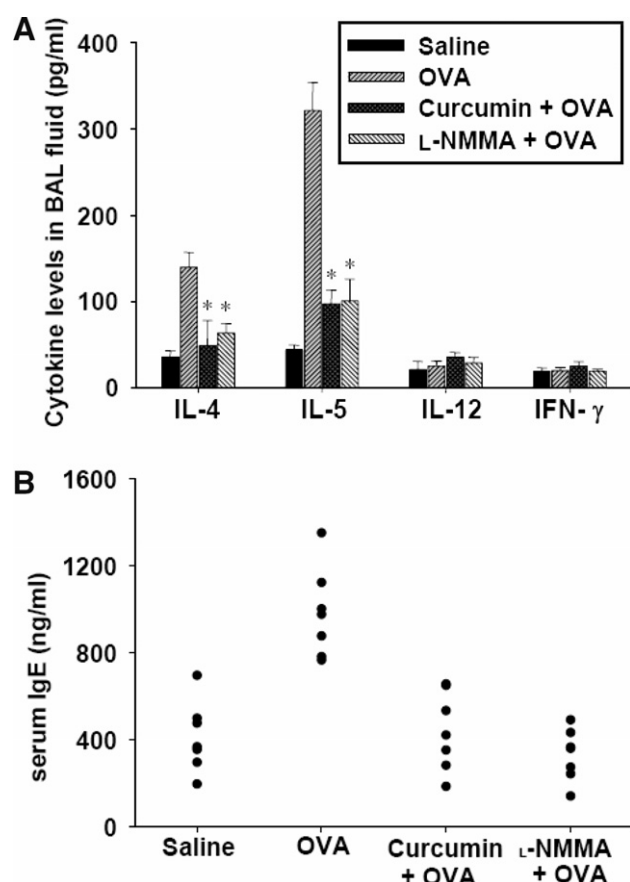


Fig. 3. Curcumin (20 mg/kg body weight/day) attenuates the expression of IL-4 and IL-5 in BALF and the levels of serum IgE. (A) BALF was collected 48 h after the last OVA challenge. (B) Blood was collected by cardiac puncture to measure serum IgE. All cytokines were analyzed using ELISA ($n = 3$). Values shown are means $\pm$ SEM. * $P < 0.05$, significantly different from cells treated with OVA/OVA mice.

not affected by IFN- γ or curcumin treatment. After 24 and 48 h of IFN- γ stimulation, the levels of NO in the culture media were determined using a series of Griess assays. As shown in Fig. 4B, in the absence of curcumin, IFN- γ stimulation resulted in a marked induction of NO production ($27 \pm 7 \mu\text{M}$ at 24 h and $38 \pm 5 \mu\text{M}$ at 48 h, respectively) compared with untreated control cells (approximately $10 \mu\text{M}$). However, pretreatment of curcumin significantly reduced the levels of IFN- γ -induced NO production to $14 \pm 5 \mu\text{M}$ at 24 h and $19 \pm 7 \mu\text{M}$ at 48 h, respectively. These results suggest that curcumin suppresses iNOS and NO levels in IFN- γ -stimulated A549 cells.

Discussion

Curcumin is a yellow pigment isolated from the rhizome of *Curcuma longa* (turmeric), which has been shown to act as a powerful anti-inflammatory [19–22]. Nevertheless, the effect of curcumin on airway inflammation remains largely unknown. In this study, we determined that the administration of curcumin significantly reduced the characteristics of airway inflammation such as AHR, infiltration of inflammatory cells, and expression of inflammatory cytokines. We first demonstrated that curcumin decreases the expression of iNOS and NO in OVA-induced airway inflammation model.

NO is a short-lived, easily diffusible molecule of high biological activity. Endogenous NO is considered to be a major marker of physiological deterioration of airway functions and is closely implicated in airway disease such as asthma, adult respiratory dis-

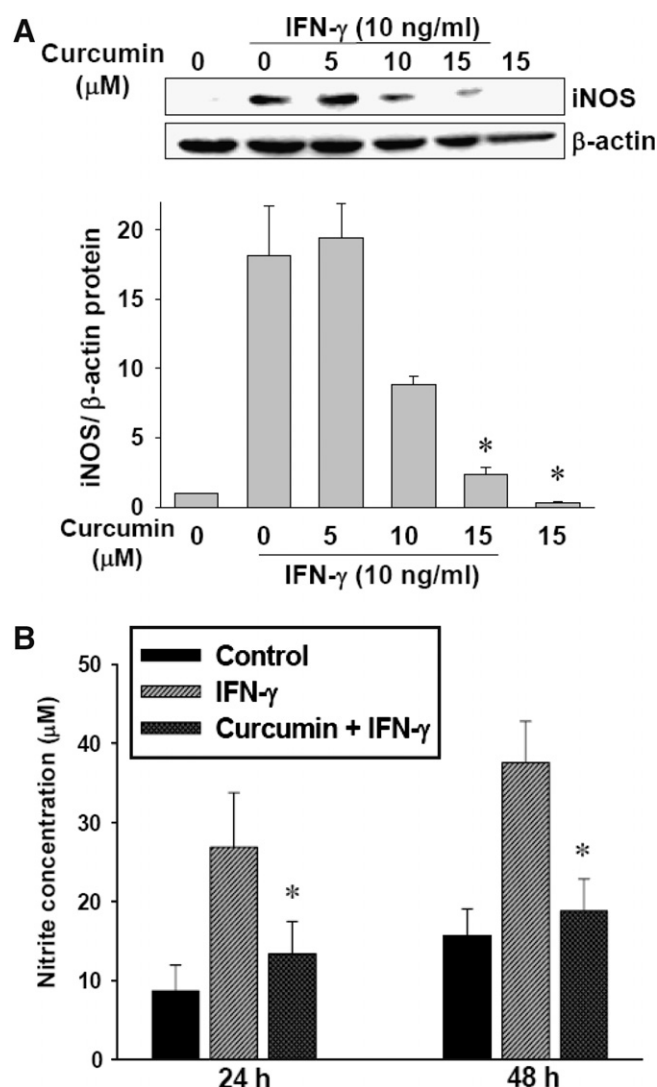


Fig. 4. Curcumin (20 mg/kg body weight/day) inhibits iNOS and NO expression in IFN- γ -stimulated lung epithelial A549 cells. (A) A549 cells were stimulated with IFN- γ (10 ng/ml) 1 h after curcumin pretreatment and incubated for 24 h. Total cell lysates were prepared from the lung and proteins were subjected to SDS-PAGE followed by Western blot analysis with specific antibodies against iNOS. β -Actin was used as an internal control. (B) In parallel experiments, the amounts of NO were determined using Griess reagent and a standard curve created using NaNO_2 . Results are representative of those obtained from three independent experiments. Values are means $\pm$ SEM from three independent experiments, * $P < 0.05$, significantly different from cells treated with IFN- γ in the absence of curcumin.

stress syndrome, and lung fibrosis [24,25]. NO is also known as a potent vasodilator in the bronchial circulation and may play a major role in airway circulation [10,11]. In this process, excessive NO may recruit eosinophils in the airway and shift the balance toward Th2 cells, thus exacerbating in inflammation of the airway. There is much evidence that NO-donors increase airway inflammation through the survival of eosinophils [26]. However NO inhibitors block eosinophil recruitment in the lungs through Fas receptor-mediated apoptosis in these cells [27,28]. Deficient iNOS also suppressed ozone-induced airway tissue injury and LPS-induced acute airway inflammation in mice [29,30]. Therefore, potent iNOS inhibitors have long been considered to be an effective treatment of airway inflammation. In this study, the administration of curcumin significantly attenuated OVA-induced asthmatic inflammation accompanied by the suppression of iNOS and NO expression. Administration of curcumin significantly inhibited leukocyte recruitment into the lung and regulated the Th1/Th2 balance to

suppress OVA-induced airway inflammation. These results suggest that inhibiting NO by curcumin attenuates airway inflammation through the infiltration of inflammatory cells into the lung. Nevertheless, curcumin may inhibit AHR of OVA-induced asthma, regardless of suppression of NO, because AHR is not linked with NO in asthma [12]. Therefore, we consider that the suppression of NO by curcumin is one of many inflammatory effects in asthma.

This study is the first to provide experimental evidence demonstrating that curcumin inhibits OVA-induced airway inflammation in a murine model of asthma through suppression of NO. These findings suggest that curcumin may be useful as an adjuvant therapy for asthmatic patients in the future.

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