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Silibinin attenuates allergic airway inflammation in mice

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ABSTRACT

Allergic asthma is a chronic inflammatory disease regulated by coordination of T-helper2 (Th2) type cytokines and inflammatory signal molecules. Silibinin is one of the main flavonoids produced by milk thistle, which is reported to inhibit the inflammatory response by suppressing the nuclear factor-kappa B (NF-κB) pathway. Because NF-κB activation plays a pivotal role in the pathogenesis of allergic inflammation, we have investigated the effect of silibinin on a mouse ovalbumin (OVA)-induced asthma model. Airway hyperresponsiveness, cytokines levels, and eosinophilic infiltration were analyzed in bronchoalveolar lavage fluid and lung tissue. Pretreatment of silibinin significantly inhibited airway inflammatory cell recruitment and peribronchiolar inflammation and reduced the production of various cytokines in bronchoalveolar fluid. In addition, silibinin prevented the development of airway hyperresponsiveness and attenuated the OVA challenge-induced NF-κB activation. These findings indicate that silibinin protects against OVA-induced airway inflammation, at least in part *via* downregulation of NF-κB activity. Our data support the utility of silibinin as a potential medicine for the treatment of asthma.

1. Introduction

Allergic asthma is one of the most common respiratory diseases, and is characterized by chronic eosinophilic airway inflammation, reversible airway obstruction, increased mucus production, and non-specific airway hyperresponsiveness (AHR) [1]. These effects are attributed to T-helper2 (Th2) cells, together with other inflammatory factors, including B cells, mast cells, eosinophils, cytokines, and chemokines. In particular, interleukin (IL)-4, IL-5, and IL-13, which are produced by Th2 cells, are all related to AHR and inflammatory changes in the airway through the activation of eosinophils [2]. Because the influx and differentiation of Th2 cells are important factors in the induction and aggravation of asthma, increasing attention has been paid to investigations that target the activation of Th2 cells or the modulation of the Th1/Th2 balance to prevent and treat asthma.

Nuclear factor-kappa B (NF-κB), which is the ubiquitous eukaryotic transcription factor that regulates gene expression of proinflammatory cytokines and enzymes, plays an important role in immune and inflammatory responses, including asthma [3]. Its increased activation has been demonstrated in the lungs after allergen challenge and in airway epithelial cells and macrophages of asthmatic patients [4,5]. Choi et al. also reported that pretreatment of NF-κB p65 antisense results in a significant inhibition of established asthmatic reaction in a murine model [6]. Taken together, the development of a new strategy to inhibit lung specific NF-κB activity might constitute an interesting topic in the management of asthma.

Silibinin is the major active constituent in silymarin that is a mixture of polyphenolic flavonoids isolated from milk thistle (*Silybum marianum*), which has been used to treat liver disease [7]. Cumulating evidence has indicated that silibinin inhibits various inflammatory responses by suppressing NF-κB pathway [8]. In addition, several studies have focused on its anti-allergic properties, and silibinin has been shown to abrogate ovalbumin (OVA)-induced anaphylactic shock in mice as well as to suppress histamine release from human basophils [9,10]. Interestingly, it has been reported that silymarin has a protective effect against the bronchospasm induced by antigen challenge in sensitized guinea pigs, and reduces atopic dermatitis-like skin lesions in NC/Nga mice [11,12]. We have also demonstrated in a previous study that silibinin attenuates IgE-mediated allergic response in

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rodent mast cells [3]. Recently, silibinin has been revealed to decrease antigen-specific IgE production through the modulation of Th1/Th2 balance in OVA-sensitized mice [13]. As such, these findings favor the view that silibinin inhibits allergic airway inflammation in a bronchial asthma model. Nevertheless, to our knowledge, the anti-asthmatic effect of silibinin and its mechanism of action have not yet been investigated. In this study, we examined whether silibinin would exert a suppressive effect on pulmonary inflammation and AHR using OVA-induced asthmatic mice.

2. Materials and methods

2.1. Animals and experimental protocols

Specific 7-week-old pathogen-free (SPF) inbred female BALB/c mice were purchased from House section of Yanbian University Health Science Center (YanJi, China). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments, and provided water and standard chow *ad libitum*. The experiments were performed in compliance with the guidelines approved by Institutional Animal Care and Use Committee of Yanbian University School of Medical Sciences. Mice were immunized intraperitoneally with 10 μg of ovalbumin (OVA; chicken egg albumin from Sigma, St. Louis, MO, USA) plus 1.0 mg of aluminum hydroxide adjuvant (Imject® Alum; Pierce, Rockford, IL, USA). A booster injection of 10 μg of OVA plus 1.0 mg aluminum hydroxide adjuvant was given 10 days later. From day 17 to day 19, the immunized mice were challenged by exposure to an aerosol of 1% OVA in phosphate-buffered saline (PBS) for 20 min. The bronchoprovocation was carried out in the vented plastic chamber ($18 \times 14 \times 8$ cm) adapted for mice. Aerosol particles of approximately 3–5 μm in diameter were created from an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan), directed into the plastic chamber, and vented to a fume hood. Each group consisted of seven animals. The saline-treated mice used as controls were exposed to aerosolized saline. Silibinin [200 mg/kg body weight (BW), Sigma], dissolved in vehicle [0.5% (w/v) carboxy methyl cellulose and 0.025% Tween 20 in distilled water], or dexamethasone (DXM; 0.5 mg/kg BW) was administered by oral gavage to each animal at 24 h intervals on days 17–19, beginning 1 h before the first provocation (challenge).

2.2. Assessment of airway hyperresponsiveness (AHR)

Airway responsiveness was measured 2 days after the last OVA challenge according to the method of Choi et al. [6]. Conscious unrestrained mice were placed in a barometric plethysmographic chamber (All Medicus, Seoul, Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine (Mch) in increasing concentrations (from 2.5 to 50 mg/ml) was then nebulized through an inlet of the main chamber for 3 min, and readings were taken and averaged for 3 min after each nebulization. The bronchopulmonary resistances are expressed as enhanced pauses (Penh), which were calculated as: [expiratory time (Te)/relaxation time (RT) – 1] × [peak expiratory flow (PEF)/peak inspiratory flow (PIF)], according to the manufacturer's protocol. The results are expressed as the percentage increase in Penh over the baseline, following challenge performed with each concentration of Mch, where the baseline Penh (after PBS challenge) is expressed as 100%.

2.3. Collection of bronchoalveolar lavage (BAL) fluid and differential cell count

Immediately following assessment of airway responsiveness, mice were anesthetized and the tracheas were cannulated while gently massaging the thorax. The lungs were lavaged with 0.7 ml of PBS. The BAL fluid samples were collected and the number of total cells in a 0.05 ml aliquot was counted using a hemocytometer (Baxter Diagnostics, Deerfield, IL, USA). The remaining samples were centrifuged, and the supernatants were stored at −70 °C until need for the assay of TNF-α, IL-1β, IFN-γ, IL-4, IL-5, and IL-13 levels. The cell pellets were resuspended in PBS and cytospin preparations of the BAL cells were stained with Diff-Quik solution (International Reagents, Kobe, Japan). The cell differentials were then enumerated based on the cell morphology and staining profile. The counting was done by an observer blind to the experimental treatments.

2.4. Cytokine measurement

TNF-α, IL-1β, IL-4, IL-5, IL-13, and IFN-γ levels in BAL fluids were determined using the mouse enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), as the manufacturer's instructions. The sensitivity for each cytokine is 2.0 pg/ml for TNF-α, IL-1β, IL-4, IL-5, IL-13, and IFN-γ.

2.5. Histological examination of murine lung tissue

Lungs were fixed with 10% formalin, and the tissues were embedded in paraffin. Fixed tissues were cut at 4 μm, placed on glass sides, and deparaffinized. Sections were stained with hematoxylin-eosin and periodic acid-Schiff for light microscopic examinations.

2.6. Western blot analysis

Freshly isolated lung tissues were homogenized in the presence of protease inhibitors and protein concentrations were determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA). A 30 μg sample of protein from the lung homogenates was loaded per lane on a 12% SDS–PAGE gel. Electrophoresis was then performed. The proteins were then transferred to nitrocellulose membranes. Western blot analysis was performed using the polyclonal antibodies against TNF-α, IL-1β, PARP, β-actin (Santa Cruz Biochemicals, Santa Cruz, CA, USA), IL-4, IL-5, IL-13 (R&D Systems), or IFN-γ (Calbiochem, San Diego, CA, USA). The binding of all the antibodies was detected using an ECL detection system (iNtRON Biotechnology, Seoul, Korea), according to the manufacturer's instructions.

2.7. Cytosolic and nuclear protein extractions for analysis of nuclear factor-kappa B (NF-κB)

Cytosolic or nuclear extractions from harvested lung tissues were performed as described previously [14]. For Western blot analysis, samples were processed by the procedure mentioned above. The NF-κB activation was assayed using antibody against NF-κB p65 (Cell Signaling Technology, Danvers, MA, USA), inhibitory kappa B-alpha (IκB-α), or phosphorylated (p)-IκB-α (Santa Cruz).

2.8. Densitometric analysis and statistical analysis

All immunoreactive and phosphorylation signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad, Hercules, CA, USA). Data are expressed as mean ± SEM. Statistical evaluation of the data was performed using ANOVA, followed by Dunnett's *post-hoc* test. Results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Silibinin inhibits OVA-induced chemotaxis and inflammatory reactions in the mouse lung

To examine the effect of silibinin on chemotaxis, that is, recruitment of inflammatory cells into airway, inflammatory cells were counted in BAL fluids. We analyzed the cellular composition of the BAL fluids of mice 48 h after the last OVA challenge. In the saline-treated mice, OVA challenge resulted in a marked increase of eosinophils and slight increases of neutrophils and lymphocytes when compared with control mice (Fig. 1A). However, pretreatment of silibinin or DXM (reference drug) significantly attenuated OVA-induced recruitment of eosinophils ($p < 0.05$). The observed reduction in chemotaxis into the airway correlated with the histological changes of lung parenchyma. Lungs from OVA-challenged mice showed widespread perivascular and peribronchiolar inflammatory cell infiltrates (Fig. 1B). However, administration of silibinin or DXM results in a significant reduction of inflammatory cell infiltration. These results indicate that treatment with silibinin efficiently inhibits the infiltration of inflammatory cells and attenuates allergic airway inflammation.

3.2. Silibinin reduces the levels of proinflammatory and Th2 cytokines in BAL fluids of allergic mice

Allergic asthmatic inflammation is known to be caused by the secretion of a series of proinflammatory (TNF-α, IL-1β) and Th2 cytokines (IL-4, IL-5, and IL-13) [1]. To assess the effect of silibinin on pulmonary inflammation in allergic mice, levels of these cytokines in lung tissues as well as BAL fluids were measured. ELISA showed that the levels of TNF-α, IL-1β, IL-4, IL-5, and IL-13 in BAL fluids were significantly increased in OVA-challenged mice compared with the levels in control mice (Fig. 2A and D). The increased levels of these cytokines were significantly decreased by the administration of silibinin or DXM. Consistent with these results, Western blot analysis revealed that protein expressions of TNF-α, IL-1β, IL-4, IL-5, and IL-13 in lung tissues were significantly upregulated in allergic mice compared with those in control mice (Fig. 2B, C, E, and F). The elevated levels

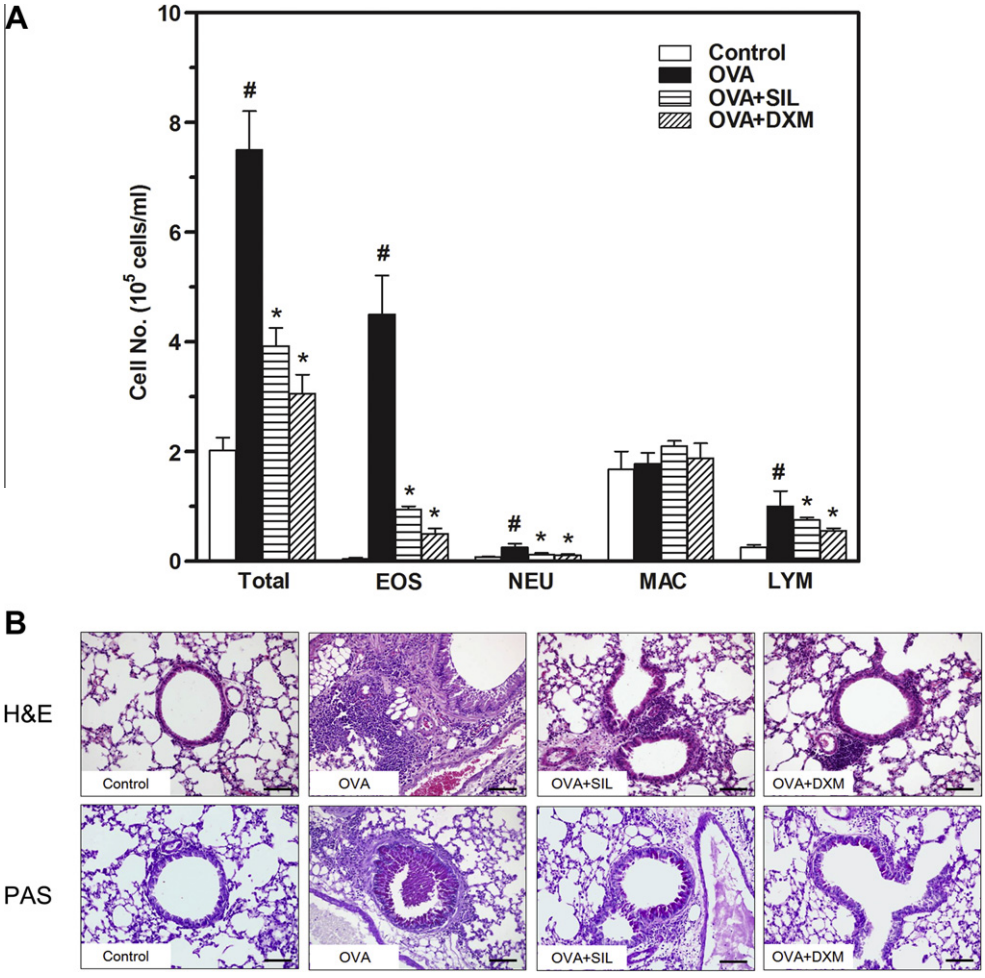

Fig. 1. Differential cell counts in BAL fluids and histological evaluation of lung inflammation following OVA challenge and treatment with silibinin. (A) Mice were orally given with silibinin (SIL) or dexamethasone (DXM: reference drug) daily for 3 consecutive days after the first OVA challenge. The effect of SIL or DXM on OVA challenge-induced differential cell counts in BAL fluid was analyzed 48 h after the last OVA challenge. Results from five independent experiments with 7 mice/group are given as mean ± SEM. $^{\#}p < 0.05$ vs. control; $^{*}p < 0.05$ vs. OVA-sensitized and challenged group. EOS, eosinophil; NEU, neutrophil; MAC, macrophage; LYM, lymphocyte. (B) Paraffin-embedded lung sections were prepared 48 h after the last OVA challenge and stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS). Magnification 200×. Bars indicate 100 μm. Data are representative of five independent experiments. Control, saline-sensitized and challenged mice; OVA, OVA-sensitized and challenged mice; OVA + SIL, OVA-sensitized and challenged mice treated with silibinin; OVA + DXM, OVA-sensitized and challenged mice treated with dexamethasone.

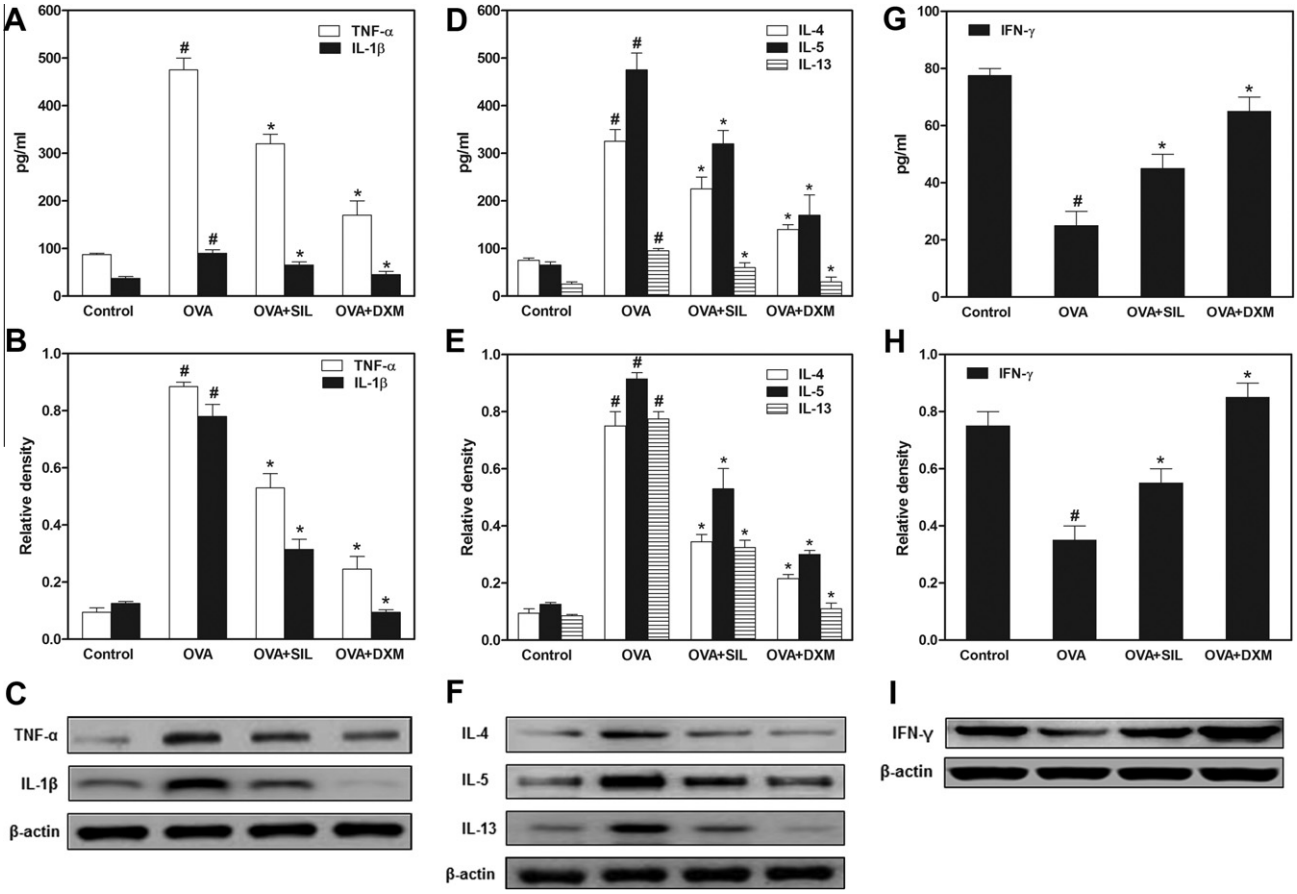

Fig. 2. Assessment of proinflammatory and Th2 cytokines in BAL fluids and lung tissues of OVA-sensitized mice treated with silibinin. BAL fluids were collected 48 h after the last challenge. The levels of TNF-α, IL-1β, IL-4, IL-5, IL-13, and IFN-γ were quantified by specific ELISAs. Protein expressions of these cytokines in lung tissues after 48 h were also measured by Western blot. Density ratio vs. β-actin was measured using a densitometer. A representative immunoblot of five independent experiments is shown. Results from five independent experiments with 7 mice/group are given as mean ± SEM. $^{\#}p < 0.05$ vs. control; $^{*}p < 0.05$ vs. OVA-sensitized and challenged group. Control, saline-sensitized and challenged mice; OVA, OVA-sensitized and challenged mice; OVA + SIL, OVA-sensitized and challenged mice treated with silibinin; OVA + DXM, OVA-sensitized and challenged mice treated with dexamethasone.

of these cytokines after OVA challenge were significantly reduced by silibinin or DXM.

3.3. Silibinin attenuates OVA-induced reduction of IFN-γ levels in BAL fluids of allergic mice

According to the previous reports that Th1 reaction (e.g., through IFN-γ) counterbalances a detrimental Th2 response, the enhancement of Th1 response is likely to be implicated in the prevention of asthma development [15]. To determine the effect of silibinin on Th1 response, we measured IFN-γ levels in BAL fluids using ELISA. The IFN-γ level in OVA-challenged mice was less than that in control mice (Fig. 2G). However, administration of silibinin or DXM increased the reduced IFN-γ level in OVA-challenged mice. In accordance with this data, Western blot assay showed that the IFN-γ protein level in the lung tissue was significantly decreased in allergic mice compared with that in control mice (Fig. 2H and I). The decreased IFN-γ level was increased by the pretreatment of silibinin or DXM.

3.4. Silibinin suppresses the Nuclear Translocation of NF-κB and Phosphorylation of IκB-α in lung tissues of allergic mice

Based on the knowledge that NF-κB plays a key role in allergic inflammation of the lung by inducing the transcription of various proinflammatory mediators [16], we hypothesized that silibinin would attenuate airway inflammatory reactions by suppressing NF-κB activation. To address this issue, we first studied the nuclear translocation of NF-κB in lung tissues after OVA challenge. As shown in Fig. 3, there was an increase in the level of p65 subunit of NF-κB in the nuclei from lung tissues of OVA-challenged mice as compared with control mice. However, nuclear extracts from silibinin-treated mice showed the suppression of nuclear translocation. Next, the effects of silibinin on OVA-induced phosphorylation and degradation of IκB-α were evaluated to clarify the molecular mechanisms by which silibinin inhibits NF-κB transcriptional activity. Silibinin significantly reduced the OVA-induced phosphorylation and degradation of IκB-α in the cytosol from lung tissues. Taken together, these findings indicate that silibinin prevents the translocation of NF-κB by blocking phosphorylation and degradation of IκB-α in OVA-challenged mice.

3.5. Silibinin decreases OVA-induced AHR in response to methacholine

We next investigated the effect of silibinin on the development of AHR in mice. Airway responsiveness was determined by Penh, and was substantially increased in OVA-challenged mice in response to methacholine inhalation as compared with control mice. Silibinin as well as DXM dramatically prevented AHR to inhaled methacholine, as shown in Fig. 4, suggesting that immune-mediated pathology *in vivo* was modified.

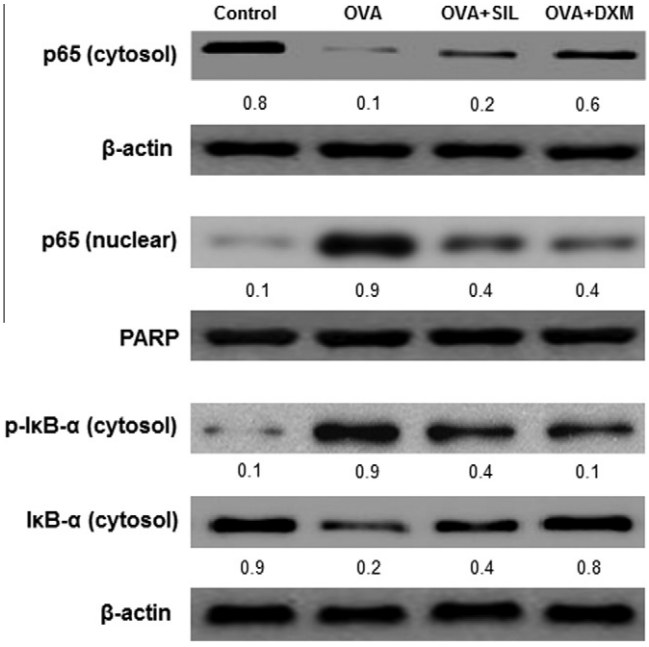

Fig. 3. Effect of silibinin on OVA challenge-induced NF-κB activation. Mice were orally given with silibinin (SIL) and dexamethasone (DXM) daily for 3 consecutive days after the first OVA challenge. Lung homogenates were prepared 48 h after the last OVA challenge. The translocation of p65 to the nucleus, and IκB-α phosphorylation and degradation in cytoplasm were assessed by Western blot. β-actin and PARP were used as internal controls. A representative immunoblot of five independent experiments is shown. Density ratio vs. β-actin was measured using a densitometer. Control, saline-sensitized and challenged mice; OVA, OVA-sensitized and challenged mice; OVA + SIL, OVA-sensitized and challenged mice treated with silibinin; OVA + DXM, OVA-sensitized and challenged mice treated with dexamethasone.

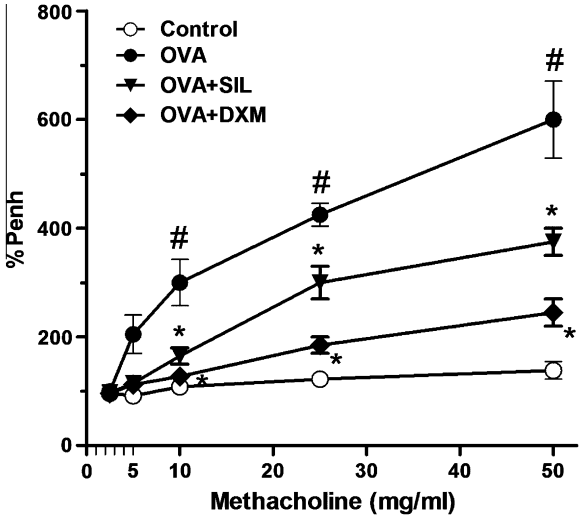

Fig. 4. AHR responses in OVA-sensitized mice treated with silibinin. All animals were nebulized with various concentrations of methacholine (2.5, 5, 10, 25, and 50 mg/ml) as a bronchoconstrictor. Data are shown as the percentage increase in Penh over the baseline, where the baseline Penh of the saline-treated control group is expressed as 100%. Results from five independent experiments with 7 mice/group are given as mean ± SEM. $^{\#}p < 0.05$ vs. control; $^{*}p < 0.05$ vs. OVA-sensitized and challenged group. Control, saline-sensitized and challenged mice; OVA, OVA-sensitized and challenged mice; OVA + SIL, OVA-sensitized and challenged mice treated with silibinin; OVA + DXM, OVA-sensitized and challenged mice treated with dexamethasone.

4. Discussion

Chronic airway inflammation, characterized by increased infiltration of leukocytes, such as eosinophils, and considerable secretion of mucus into the airways, is a major factor in the pathogenesis of asthma. Especially, eosinophil has long been recognized as the principal effector cell and plays pathogenic roles in asthma by its release of cytotoxic granule proteins [17]. Our present findings show that silibinin prevents eosinophilic infiltration into the airways, as evidenced by a significant drop in total cell counts and eosinophil counts in BAL fluid. Likewise, tissue eosinophilia is also inhibited, as revealed by a marked reduction of inflammatory cell infiltration in histological examination. Eosinophilic transmigration into the airways is a multistep process that is orchestrated by not only Th2 cytokines such as IL-4, IL-5, and IL-13 but also proinflammatory cytokines including TNF-α and IL-1β, and coordinated by specific chemokines such as eotaxin and RANTES in combination with adhesion molecules, including VCAM-1 [18,19]. IL-4 is required for B cell maturation and IgE synthesis, and participates in the initiation of Th2 inflammatory responses. IL-5 is pivotal for growth, differentiation, recruitment, and survival of eosinophils. IL-13 can potently induce mucus hypersecretion, eotaxin expression, airway inflammation, and airway hyperresponsiveness [20,21]. TNF-α and IL-1β exert similar responses, which include upregulation of eosinophil chemoattractants and adhesion molecules, recruitment of eosinophil, increase of cytokine release, and enhancement of AHR [22]. The current study shows that silibinin attenuates the release of Th2 and proinflammatory cytokines into the airway. In contrast, IFN-γ levels from untreated asthmatic mice are decreased as compared with control groups. IFN-γ is a Th1 cytokine that downregulates eosinophilic differentiation by suppressing the development of Th2 cells, and has inhibitory effects on the allergic response [23]. As expected from blocking of Th2 cytokine production by silibinin, silibinin impedes OVA-induced reduction of IFN-γ levels, which is in line with the previous report that silibinin treatment shifts immune response toward the Th1-dominant direction by enhancing IFN-γ production in splenocytes of OVA-sensitized mice [13]. Taken together, we speculate that silibinin can prevent the migration of inflammatory cells by diminishing secretion of proinflammatory and Th2 cytokines into lungs as well as by enhancing Th1 immune response (e.g., through IFN-γ).

AHR is a hallmark clinical symptom of asthma, which is defined as the abnormal increase in airflow limitation in response to a provoking stimulus. Although there are less data surrounding the precise mechanisms whereby airway inflammation enhances AHR, it is convincing that inflammatory mediators released during allergic inflammation play a critical role in AHR development [24]. Our data show that administration of silibinin effectively reduces OVA-induced AHR to inhaled methacholine, which is in agreement with the earlier report that silymarin pretreatment reduces bronchospasm in OVA-sensitized and challenged guinea pigs [11]. It has been established that IL-5 plays a crucial role in AHR by mobilizing and activating eosinophils, leading to the release of proinflammatory products such as major basic protein and cysteinyl-leukotrienes, which are closely associated with AHR [25]. Similarly, IL-4 and IL-13 have been shown to induce AHR in murine asthma models in which cysteinyl-leukotrienes might be causative agents of AHR [26]. Moreover, AHR could be brought about by a direct effect of TNF-α on airway smooth muscle [27]. Furthermore, IgE-mediated mast cell activation may contribute to AHR by producing a wide array of inflammatory mediators and cytokines [28], which extends the potential importance of our previous findings [3] in the suggested mechanisms of AHR. As such, the observed reduction of AHR by silibinin may be related to

decrease in Th2 cytokine production, tissue eosinophilia, TNF-α levels, and mast cell degranulation by silibinin.

The transcription factor, NF-κB, has been considered to play a cardinal role in allergic airway disease. Functionally active NF-κB exists in heterodimers, but is usually in an inactive sequestered complex bound to its endogenous inhibitor IκB-α, in the cytoplasm. However, bound IκB-α is rapidly phosphorylated by IκB-α kinase in response to external stimuli, such as inflammatory cytokines, and is subsequently degraded by proteosomes. The free dimeric NF-κB activated by the dissociation of IκB-α translocates into the nucleus where it induces transcription of a wide variety of target genes that encode many inflammatory proteins [16], including cytokines such as TNF-α, IL-1β, IL-4, IL-5, and IL-13, all of which are closely implicated in the pathogenesis of asthma [29]. Our results indicate that silibinin inhibits the activity of NF-κB by suppressing the phosphorylation and degradation of IκB-α, and the subsequent translocation of the p65 subunit of NF-κB from the cytosol to the nucleus in lung tissues of OVA-challenged mice. In addition, decreased NF-κB activity has been shown to be accompanied not only by a reduction in the levels of inflammatory mediators, such as TNF-α, IL-1β, and Th2 cytokines in BAL fluids, but also by improvements in eosinophilic airway inflammation and AHR. From these, it is proposed that the mechanism underlying the anti-asthmatic effects of silibinin may be directly relevant to inhibition of NF-κB transcriptional activity and to subsequent reduction of proinflammatory chemical mediators.

In conclusion, our data indicate that silibinin could ameliorate asthmatic inflammation and AHR by downregulating proinflammatory and Th2 cytokines *via* inhibiting the activity of NF-κB. These findings suggest that silibinin is a potential anti-inflammatory agent in asthma treatment.

Acknowledgments

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