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2-Methoxyestradiol (2-ME) reduces the airway inflammation and remodeling in an experimental mouse model

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KEYWORDS

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Abstract Patients with asthma experience airway structural changes, termed airway remodeling, in response to persistent inflammation. 2-Methoxyestradiol (2-ME) is an anti-angiogenic agent and downregulates hypoxia-inducible factor 1 (HIF-1) and inhibits HIF-1 α -induced transcriptional activation of vascular endothelial growth factor (VEGF) expression. We hypothesized that 2-ME may interfere with the development of the clinical manifestations of asthma. We used a chronic murine model of allergic airway inflammation with subepithelial fibrosis in BALB/c mice. Mice were sensitized with ovalbumin (OVA) that was administered intraperitoneally at days 0–5 and challenged intratracheally (IT) with OVA on days 12–22. The mice received 2-ME IT at days 24, 26 and 28 and sacrificed at day 32. The sensitized/challenged mice developed an extensive cell inflammatory response of the airways. 2-ME administration significantly reduced the cellular infiltrate in the perivascular and peribronchial lung tissues, reduced goblet mucous production, reduced airway fibrosis and thickness of smooth muscle and blood vessels, and reduced eosinophil infiltration. Mice treated with 2-ME had a significant decrease of HIF-1 and VEGF expression in the perivascular, peribronchial, and interstitium of lung tissues. Collagen IV expression was also significantly reduced in 2-ME treated mice compared to untreated mice. The 2-ME treatment was associated with a significant decrease of OVA-specific IgE antibodies. These findings provide the first indication that IT administration of 2-ME is effective in preventing and

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reversing antigen-induced airway remodeling in the OVA allergen inflammatory murine model. The potential role of 2-ME in patients is discussed.

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Introduction

The pathophysiology of asthma involves a number of cell types and mediators. Airway structural changes that occur in patients with asthma in response to persistent inflammation are termed airway remodeling and include airway wall thickening, subepithelial fibrosis, and hyperplasia of mucous glands, myofibroblasts, and smooth muscle vasculature [1]. Chronic airway inflammation plays a key role in asthma. The relationship between inflammation and structural changes is speculative. It is believed that remodeling is a direct result of a switch from acute to chronic inflammation followed by healing [2]. For this reason, anti-inflammatory agents will not necessarily prevent or attenuate the process of remodeling [3]. All current guidelines focus on the treatment of inflammation in asthma although there are differences between the processes of airway wall remodeling for which there is no defined treatment validated [2].

Long-acting β agonists produce bronchial dilation and improve asthma symptoms, effects that are maintained with regular use over time. However, immunotherapy with β agonists has been consistently shown to be inferior to the use of inhaled corticosteroids which can reduce the underlying inflammation associated with asthma. For these reasons, inhaled corticosteroids are recommended as first line maintenance treatment for asthma [4].

2-ME is a naturally occurring derivative of 17β -estradiol and was shown to be a well tolerated small molecule that possesses anti-tumor and anti-angiogenic activities [5]. Studies by Mabeesh et al. [6] reported that 2-ME destabilizes microtubules and blocks HIF1- α nuclear accumulation and HIF1- α activity by an oxygen- and proteasome-independent pathways. Evidence was provided for a link between the microtubule cytoskeleton and HIF regulation. Destruction of the normal function of the microtubule cytoskeleton is required for HIF1- α -induced inhibition by microtubule-targeted drugs such as Taxol and Vincristine.

Several reports have been published suggesting the use of different strategies for the suppression of antigen-induced asthma in mice. For instance, the oral administration of CpG-ODNs was effective in preventing and reversing antigen-induced eosinophilia airway inflammation [7]. Also, low doses of leukotriene receptor antagonist were an effective therapy in the airway remodeling in the ovalbumin mouse model [8].

Angiogenesis supports the development of many diseases such as cancer, rheumatoid arthritis, psoriasis, macular degeneration, and diabetic retinopathy [9–11]. VEGF is a major mediator of angiogenesis, whose expression is induced under hypoxic conditions [9,12]. The induction of VEGF under hypoxic conditions is a multistage process in which the alpha subunit of HIF (HIF-1 α) plays a key role [13]. HIF1- α is rapidly degraded by the proteasome under normoxic conditions. Following hypoxic stabilization, HIF1 α is translocated into the nucleus where it heterodimerizes with HIF1 β and activates the transcription of more than forty genes

important for adaptation and survival under hypoxia [14]. Hence, the anti-angiogenic activity of 2-ME may regulate the inflammatory process in asthma and reduce cell infiltration. Therefore, we hypothesized that the local intratracheal (IT) administration of 2-ME may inhibit airway inflammation and remodeling. The objective of this study was to test this hypothesis and determine the effect of IT 2-ME administration in the development of airway remodeling using a chronic murine model of allergic airway inflammation with subsequent fibrosis. The findings support our hypothesis and demonstrate that 2-ME significantly inhibited antigen-induced inflammation and airway remodeling in the OVA allergen inflammatory murine model.

Materials and methods

Animals

Balb/c mice (males) were obtained and maintained in a pathogen-free environment in the facilities of the Instituto Nacional de Ciencias Medicas y de la Nutricion (INCMN) (Mexico City). The mice were housed in a temperature-controlled room with 12-h dark/light cycles, and allowed food and water ad libitum. All the experiments described below were performed in accordance with the INCMN regulations.

Experimental design

The protocol for sensitization and intratracheal challenge has previously been described by us in details [15] and illustrated in Fig. 1. Briefly, 6 to 8 week-old Balb/c male mice were sensitized on days 0 and 5 by intraperitoneal injection of 100 μ g chicken egg ovalbumin (OVA, grade V, Sigma, USA) emulsified in 1 mg of aluminum hydroxide (alum) (Pierce Chemical, USA) in a total volume of 100 μ L. Seven days after the last sensitization, mice were exposed twice to intratracheal (IT) administration of 0.75% OVA. 2-ME was purchased commercially from Sigma Aldrich (St Louis, MO). The dose of 2-ME chosen for the *in vivo* experiment was derived from a report using 100 mg/kg in a cancer murine model via the intraperitoneal route [3,16]. We have chosen a lesser concentration of 30 mg/kg because 2-ME was administered IT. 6 to 8 week-old Balb/c male mice ($n=18$)

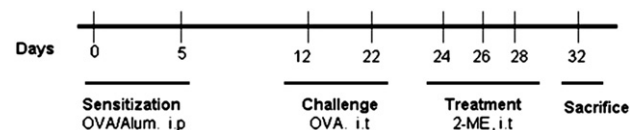


Figure 1 Murine models of airway inflammation and treatment with 2-ME. The effects of IT administered 2-ME and/or OVA were examined in the context of immunotherapy of established atopic lung inflammation.

were divided into three groups. Group one received ovalbumin (OVA) ($n=6$), group two received OVA and then was treated with 2-ME (OVA/2-ME) ($n=6$) and group three ($n=6$) was control and received saline solution (SS). Groups one and two were immunized by intraperitoneal (i.p.) injection of 100 μg (100 μL) of OVA complexed with 1 μg alum on days 0 and 5 and then the mice received an intratracheal (IT) dose of 100 μL of 0.75% of OVA in SS on days 12 and 22. The control group three ($n=6$) received saline solution (SS) with alum i.p. on days 0 and 5 and SS without alum IT on days 12 and 22. A concentrated stock solution of 2-ME was prepared in DMSO and then diluted in SS to prepare a solution of 2-ME (30 mg/kg) that was administered IT to group two beginning on day 24 of the protocol and continuing until day 28 every other day. The mice received 3 administrations every other day, (D 24, D 26 and D 28) of 100 μL (30 mg/kg) of 2-ME IT. Positive control consisted of mice sensitized and OVA-exposed. The non-immunized and OVA-exposed mice were similar to group 1 mice as they did not exhibit detectable airway inflammation. The positive control OVA mice received SS containing the same concentration of DMSO as the one used for treatment with 2-ME. All animals were sacrificed 96 h after the last IT treatment.

Lung histology and morphometric analysis

At 96 h after the last challenge, lungs were removed from the mice after sacrifice. Before the lungs were removed, they were filled intratracheally with a fixative (4% formalin) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 4- μm sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin and eosin (H&E) to assess the inflammatory cell infiltrates. The area (μm^2) was calculated from stained slides, selecting venules with diameter of 100–200 μm and bronchi with 150–300 μm of diameter. Four blood vessels or bronchi from four to six lung sections were analyzed for each experimental group, and results were expressed as average and standard deviations of infiltrated area (μm^2). Mucus production was assessed from lung sections stained with periodic acid Schiff (PAS). The PAS-positive material was measured in μm in bronchi with 150–300 μm . Five bronchi from four to six lung sections were analyzed for each experimental group and the results were expressed as average and standard deviations of PAS-positive area (μm). Mason's trichrome (MT) was used to determine collagen deposition in the lung. The histological analyses were performed by observers who were not aware of the groups of mice from which the samples originated.

Bronchoalveolar lavages (BAL)

After the mice were sacrificed by exsanguination, the tracheas were exposed and intubated with a polyethylene catheter. The BAL fluid was collected by washing with two separated aliquots of 1 ml of SS with 2% of FBS through the trachea. The cells from the first wash were harvested by centrifugation (500 $\times g$ for 10 min at 4 $^{\circ}\text{C}$), counted in a

hemacytometer and adjusted to 1×10^5 cells/ml. Differential counts from four to six H/E stained cytospin preparations for each experimental group were determined by microscopy. Cells were counted by standard procedures at 40 $\times$ magnification. Results are presented as cells/ml averages from all the slides per group (200 cells/slide).

Immunocytochemistry

For immunohistochemistry of VEGF or HIF-1 α and type IV collagen, the deparaffinized 4- μm sections were incubated sequentially in accordance with the instructions of the LSAB kit from DAKO corporation (Carpinteria, CA, USA). In brief, antigen retrieval was performed by immersing the slides in 0.01% sodium citrate pH 6.0 incubated for 5 min in boiling water. The endogenous peroxidase activity was inhibited by immersing the slides in 3% H_2O_2 -methanol for 25 min, and the background non-specific binding was reduced by incubating with 1% BSA in PBS for 60 min. The slides were incubated overnight at room temperature with antibodies against HIF-1 α (1:250) (Santa Cruz Biotechnology, Santa Cruz, CA), VEGF (1:750) (Santa Cruz) or type IV collagen (1:500) (Santa Cruz). Finally, the slides were washed five times in PBS 0.1 M pH 7.4 for 8 min. In order to reduce the variability, all samples from each group were processed at the same time in a single experiment using a single batch of antibody diluted in PBS-normal swine serum. After washing, the slides were incubated with a biotinylated secondary antibody container in the LSAB kit from DAKO corporation for 30 min at r.t. followed by an incubation with a streptavidin-HRP conjugate container in the LSAB kit for 30 min at r.t. and then with 3,3'-diaminobenzidine tetrahydrochloride (liquid DAB, DAKO corporation) for 1 to 5 min in the dark. The reaction was arrested with distilled water and the slides were counterstained with hematoxylin. Thereafter, the tissues were washed in tap water for 5 min, dehydrated through ethanol baths (70, 90 and 100%) in xylene and mounted with E-2 Mount medium (Shandon lab, Pittsburgh, PA, USA). Finally, the slides were analyzed under light microscopy (Olympus BX-40). The analyses were performed by blinded observers.

Determination of serum OVA-specific IgE

Serum levels of specific anti-OVA IgE were determined by ELISA. The ELISA kit was purchased from MD Bioscience, St. Paul, MN (Catalog #OVA IgE 96). The analysis was performed according to the manufacturer's instructions. The specificity of the ELISA was corroborated by demonstrating that serum from naïve non-immunized mice did not reveal any detectable binding to OVA. Color was developed by adding OPD in substrate buffer (Sanofi Diagnostic Pasteur). The reaction was arrested with 50 μL H_2SO_4 and the OD were determined at 495 nm.

Statistical analysis

Data were expressed as mean $\pm$ S.E.M. Statistical comparisons were performed using one-way analysis of variance followed by the Fisher's test. Significant differences between groups were determined using the unpaired Student's test. Statistical significance was set at $p < 0.05$.

Results

Intratracheal administration of 2-ME reduces OVA-induced atopic response

Three groups of mice ($n=6$ /group) were examined and were treated with OVA alone (group one), OVA+ 2-ME (group two) and with saline (SS) (group three) as described in Materials and methods. The mice were rendered allergic to OVA with two i.p. injections of OVA-alum suspension at days 0 and 5 and 2 IT challenges at days 12 and 22. Two days after sensitization group two mice were treated three times with 2-ME administered by the intratracheal route every other day. The intensity of allergic inflammation reaction was evaluated on day 32 at sacrifice. The schematic diagram of the above treatment schedules is also illustrated in Fig. 1.

The tissue cellular infiltration was assessed based on H&E staining of the lung sections. Fig. 2 shows a representative lung section derived from each experimental group. The OVA challenge in the sensitized mice induced dramatic perivas-

cular (Fig. 2A, d) and peribronchial (Fig. 2A, c) infiltration of various types of cells as compared with the SS control mice (Fig. 2A, a and b). The 2-ME treatment markedly reduced the infiltration of inflammatory cells in both the perivascular (Fig. 2A, f) and peribronchial areas (Fig. 2A, e). The quantitative pulmonary inflammation was estimated by morphological analysis in the perivascular and peribronchial areas and was expressed in μm^2 of infiltrate. Allergic mice treated with 2-ME (OVA/2-ME) showed significant reduction of perivascular cell infiltrate (Fig. 2B, upper panel) in comparison to untreated allergic mice (OVA), $x=18 \mu\text{m}^2$ versus $38 \mu\text{m}^2$ ($p<0.05$). Similar results were obtained in the peribronchial areas (Fig. 2B, bottom panel). Mice sensitized and OVA-exposed (positive control) behaved similarly to group 1 mice (data not shown). Mice non-immunized and OVA-exposed did not show any airway inflammation (data not shown).

Mucus production that occurs as a result of allergen exposure is an important characteristic of the pathogenesis of asthma, and this production is believed to be highly associated with Th2-type cytokines namely IL-5 and IL-13 [17]. We have reported in our airway inflammation model the

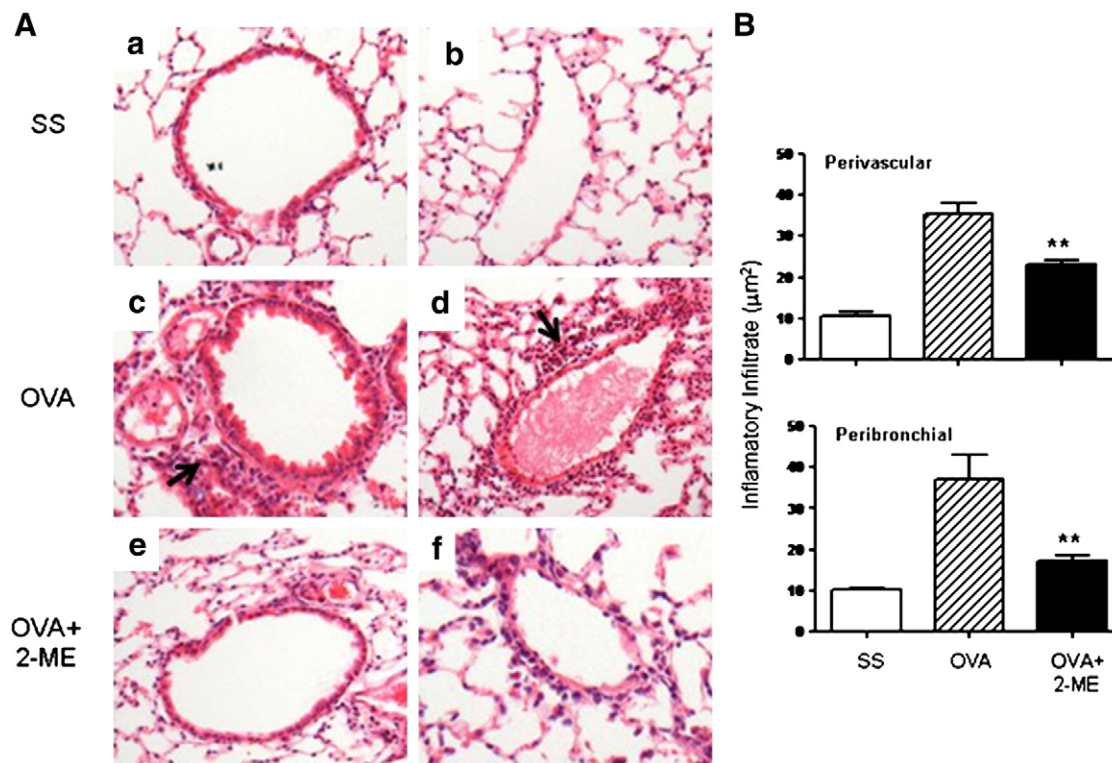


Figure 2 2-ME administered intratracheally reduces airway inflammation in OVA-treated mice. (A) Histology and morphometric analyses of lungs. Representative whole mounts of lung tissues were obtained from OVA-sensitized/challenged mice, in the absence (c and d) or presence (e and f) of 2-ME administered IT at a dose of 30 mg/kg, or saline-treated mice (a, b). The tissues (b, d, f) blood vessels and a, c, e bronchia) were stained with hematoxylin and examined by light microscopy. OVA-treated mice (b and c) exhibit a marked dense cellular infiltrate of eosinophils and other inflammatory cells in the interstitium (arrow) of the lungs. In contrast with OVA treatment alone, OVA-sensitized/challenged mice and administered 2-ME (e and f) have a marked reduction in the cellular infiltration around blood vessels and bronchi. The control mice have blood vessels and bronchi of normal appearance and inflammatory cell infiltration is absent in the lung interstitium (a and b). (B) Lung inflammatory infiltrate in allergic mice to OVA. Lung sections from five to eight mice from each experimental group were stained with H/E and analyzed under light microscopy (40 $\times$), and the inflammatory infiltrate was measured in venules and middle size veins with 100–200 μm of diameter, and bronchi with 150–300 μm diameter using an image analyzer (five venules or bronchi from five to eight lung sections were analyzed for each experimental group). Results are expressed as averages and standard deviations of infiltrate area. (Upper) Perivascular infiltrate; (lower) peribronchial infiltrate. ** $p<0.05$ presence of 2-ME vs. absence of 2-ME (ANOVA).

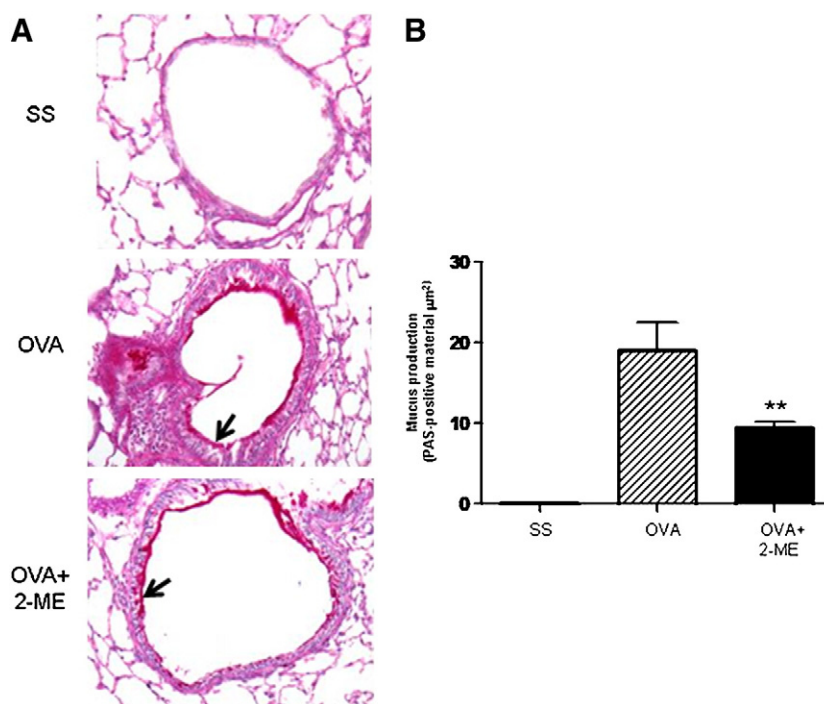


Figure 3 2-ME reduced mucus production in lungs from allergic mice to OVA. (A) Representative microphotography of periodic acid Schiff (PAS) staining observed at magnification of 40 $\times$. Airway goblet cell hyperplasia (arrows) was observed in the OVA-sensitized/challenged mice (middle panel). There were no goblet cell hyperplasia and mucus occlusion in the SS control mice (top panel). 2-ME treatment decreased goblet cell hyperplasia and mucus occlusion in the lung interstitium (arrow) (bottom panel). (B) Mucus production by histological analysis of lung sections from five to eight mice from each experimental group that were stained with PAS and analyzed under light microscopy (40 $\times$). The PAS-positive material was measured in 200 μm squares (40,000 μm^2) using an image analyzer (five squares from five to eight lung sections were analyzed for each experimental group). Results are expressed as averages and standard deviations of PAS-positive area (μm^2). ** $p < 0.05$ presence of 2-ME vs. absence of 2-ME (ANOVA).

involvement of the Th2 cytokines (IL-4, IL-5, IL-13), both at the level of transcription and expression [15]. Lungs from the different experimental groups were stained with PAS to detect mucus-secreting material. Fig. 3A shows representative lung sections stained with PAS from the different experimental groups. Compared to control, the OVA group developed an intense mucus production, affecting the peribronchial regions with marked goblet cell hyperplasia and hypertrophy (Fig. 3A, middle panel) compared to control mice (Fig. 3A, top panel). The allergic mice treated with 2-ME (Fig. 3A, bottom panel) presented significant decrease of mucus production. Moreover, morphometric analysis in the epithelial portion of the airway wall also revealed that chronic allergen exposure increased the amount of mucin present in comparison with the negative control group (SS), which was significantly ($p < 0.001$) decreased following treatment with 2-ME (Fig. 3B).

The remodeling airway tissue and fibrosis are important processes that result from allergen exposure and are important characteristics of the pathogenesis of asthma. We, therefore, determined the effect of 2-ME treatment on airway smooth muscle thickness in our experimental murine model of allergic airway inflammation. Fig. 4A shows representative lung sections from the different experimental groups stained with Mason's trichrome. In OVA-sensitized and challenged mice, dense collagen deposition/fibrosis was seen throughout the lung interstitium surrounding the airways and blood vessels (Fig. 4A, middle panel) as compared with the control SS group (Fig. 4A, top panel). Treatment with 2-ME

markedly reduced the dense collagen deposit/fibrosis (Fig. 4, bottom panel). To measure more quantitatively the smooth muscle layer thickness of the surrounding airways we used the Imagine-pro Plus System. Fig. 4B (upper panel) shows that the thickness of the smooth muscle layer surrounding the airways was markedly increased in the OVA group (11.2 ± 0.4 μm in vessels and 5.5 ± 1.1 μm in bronchials) as compared with the negative control group (SS) ($0.15 \mu\text{m} \pm 0.012$ in vessels and 0.13 ± 0.009 in bronchials). Treatment with 2-ME induced significant reduction in the thickness of smooth muscle content in the airway ($4.8 \mu\text{m} \pm 1.3$ in vessels and $2.65 \mu\text{m} \pm 0.5$ in bronchials) ($p = 0.0001$). To analyze collagen deposition in a more quantitative manner, subepithelial collagen within the bronchial was measured using the Image-pro Plus System and is presented in μm . As shown in Fig. 4B (bottom panel) the mean of the area of airway fibrosis in OVA mice was significantly increased (with 9.9 ± 0.9 μm in vessels and 7.6 ± 0.78 μm in bronchials) as compared to the SS group (0.2 ± 0.01 μm in vessels and 0.15 ± 0.002 μm in bronchials). Administration of 2-ME resulted in a significant reduction in the collagen deposition in the lung including the perivascular and peribronchial areas. The means of the areas of airways collagen in mice treated with 2-ME were $5.0 \mu\text{m} \pm 1.3$ in vessels and 2.7 ± 0.2 μm in bronchi which were significantly different from the OVA group ($p < 0.0001$).

Altogether, these results demonstrate that treatment with 2-ME reduce significantly the inflammation, mucus production and collagen deposits/fibrosis in the allergic mice.

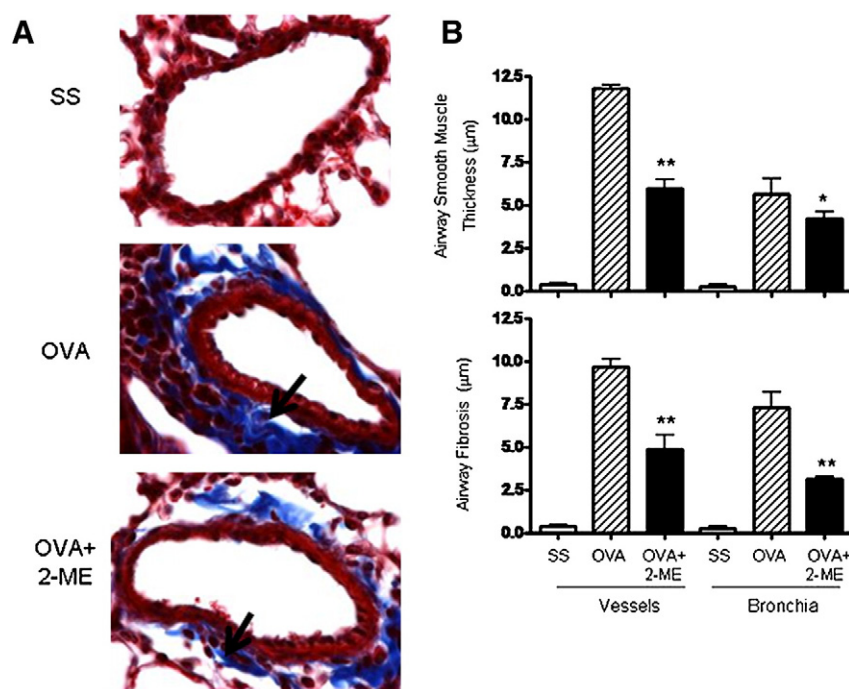


Figure 4 2-ME reduced collagen deposit in lungs from allergic mice to OVA. (A) Representative microphotography of Masson's Trichrome staining (40×). Extensive collagen deposit of airways (arrows) was observed in the OVA-sensitized/challenged mice (middle panel). There was no deposit of collagen in the SS control mice (top panel). 2-ME treatment decreased collagen deposit in the lung interstitium around the airways (arrow) (bottom panel). (B) Collagen deposit histological analysis of lung sections derived from five to eight mice from each experimental group. They were Masson's Trichrome stained and analyzed under light microscopy (40×). The collagen was measured in 200 μm squares (40,000 μm²) using an image analyzer (five squares from five to eight lung sections were analyzed for each experimental group). Results are expressed as averages and standard deviations of blue Masson's Trichrome-positive area (μm²). **p*<0.05 presence of 2-ME vs. absence of 2-ME (ANOVA).

2-ME reduces BAL eosinophils

Cell infiltration was measured in BAL from allergic mice and differential cell counts were determined by microscopy in H/E stained cytospin preparations. The differential cell counts revealed a significantly increase in the number of cells predominantly lymphocytes, neutrophils and eosinophils in the OVA-exposed group ($15.5 \times 10^3 \pm 7.8 \times 10^3$ eosinophils/ml; $9.8 \times 10^3 \pm 1.8 \times 10^3$ lymphocytes/ml and $3.1 \times 10^3 \pm 0.7 \times 10^3$ neutrophils/ml), compared with the SS group ($0.1 \times 10^3 \pm 0.03 \times 10^3$ eosinophils; $0.15 \times 10^3 \pm 0.02 \times 10^3$ lymphocytes and $0.8 \times 10^3 \pm 0.05 \times 10^3$ neutrophils) (Fig. 5). The OVA-exposed group treated with 2-ME showed significant reduction of BAL lymphocytes and eosinophils numbers with $11.2 \pm 2.4 \times 10^3$ eosinophils and $5.1 \pm 1.6 \times 10^3$ lymphocytes/ml in the OVA/2-ME group versus ($15.5 \times 10^3 \pm 7.8 \times 10^3$ eosinophils/ml and $9.8 \times 10^3 \pm 1.8 \times 10^3$ lymphocytes/ml in the OVA group (*p*<0.05).

2-ME reduces the expression of HIF-1α, VEGF and collagen IV in allergic mice

It has been shown that 2-ME blocks the activity of HIF-1α via disruption of microtubules [18]. In order to delineate if the administration of 2-ME also affects HIF-1α expression in the allergic mice, we performed immunohistochemistry to measure the expression protein in lung tissues. Very low immunoreactive HIF-1α localized in epithelial layers around the vessels and bronchioles in the SS group (Fig. 6A, upper panel). In contrast,

the OVA-exposed mice showed a very significant increase of HIF-1α expression in both the perivascular and the peribronchial areas, and that increase was markedly observed in the interstitium areas (Fig. 6A, middle panel). Interestingly, IT

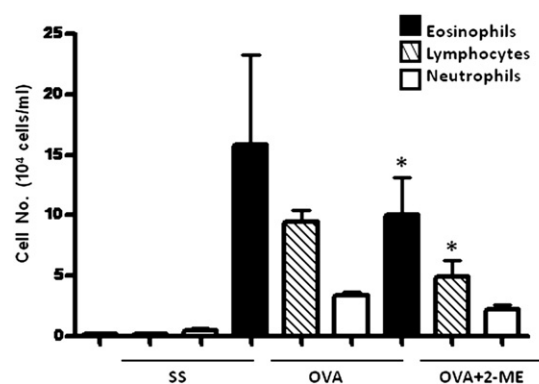


Figure 5 2-ME treatment reduces BAL eosinophils in mice allergic to OVA. Total leukocytes were counted and differential cell analysis per milliliter was determined in BAL from mice allergic to OVA, both treated and untreated with 2-ME. Cytospin BAL preparations were stained with H/E and the differential count of 200 cells per five to eight mice were analyzed for each experimental group (100×). Results are presented as the average and standard deviations (cells/ml). **p*<0.05 presence of 2-ME vs. absence of 2-ME (ANOVA; Kruskal–Wallis).

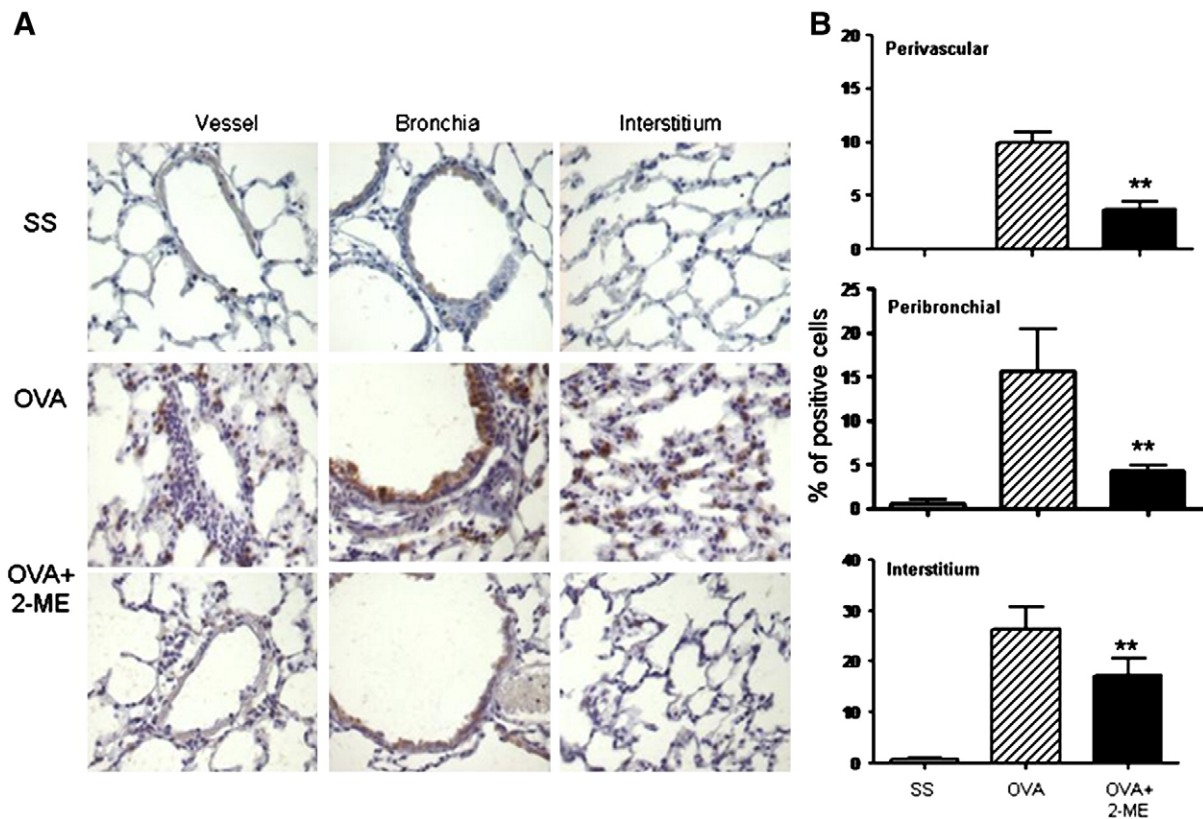


Figure 6 2-ME treatment reduces the HIF-1 α expression in lung tissue. (A) Immunohistochemical comparison of HIF-1 α expression in lung tissue obtained from OVA-sensitized/challenged mice, in the absence or presence of 2ME2 administered IT at a dose of 30 mg/kg, and saline-treated mice. Compared with OVA treatment alone (middle panel); OVA-sensitized/challenged mice and administered 2-ME have a marked reduction in the cellular infiltration HIF-1 α positive numbers cells (perivascular, peribronchial and interstitium) (bottom panel); the control saline-treated mice have very few positive cells (top panel). (B) Percentage of HIF-1 α immunostained cells in several lung compartments, in the absence or presence of 2-ME treatment. Lung sections from five to eight mice from each experimental group were immunostained with anti-HIF-1 α antibodies and analyzed under light microscopy (40 $\times$). The percentage positive cells were measured in surrounded cellular infiltrate in venules, bronchi and interstitium using an image analyzer (five venules or five bronchi from five to eight lung sections were analyzed for each experimental group). Results are expressed as averages and standard deviations of positive cells. (Upper) Perivascular infiltrate, (middle) peribronchial infiltrate, (lower) interstitium. ** $p < 0.05$ presence of 2-ME vs. absence of 2-ME (ANOVA).

administration of 2-ME dramatically decreased the HIF-1 α expression in allergen-induced asthmatic lungs (Fig. 6A, bottom panel). To analyze the HIF-1 expression in a more quantitative manner, we counted the percent of HIF-1 positive cells in several lung areas. As shown in Fig. 6B, the mean percentage of HIF-1 α positive cells in the OVA group mice significantly increased with $10.1 \pm 1.1\%$ (perivascular area), $15.2 \pm 5.2\%$ (peribronchial) and $26.5 \pm 4.1\%$ (interstitium), compared with the SS group that shows almost no expression in the different lung areas. In contrast, treatment with 2-ME resulted in a significant reduction in the HIF-1 expression as compared with the OVA group in the perivascular area ($3.9 \pm 0.6\%$ versus $10.1 \pm 1.1\%$), peribronchial area ($5.0 \pm 0.1\%$), and in the interstitium ($7.2 \pm 2.2\%$) (Fig. 6B).

Recent reports demonstrated that HIF-1 is implicated in the transcriptional regulation of VEGF and it has been shown that VEGF participates in the airway remodeling process that results following allergen exposure [6]. To further understand the role of HIF-1 in this process after allergen exposure, we next determined the effect of 2-ME treatment on VEGF expression

in our experimental murine model of allergic airway inflammation by immunohistochemistry. Low immunoreactive VEGF localized in epithelial layers around the vessels and bronchioles in the SS group (Fig. 7A, upper panels). In contrast, the OVA-exposed mice showed a very significant increase in VEGF expression in both the perivascular and also in the peribronchial areas, and these increases were very significant in the interstitium areas (Fig. 7A, middle panels). The 2-ME treatment dramatically decreased VEGF expression in the allergen-induced asthmatic lungs (Fig. 7A, bottom panels). To quantify the VEGF expression, we counted the percent of VEGF positive cells in several lung areas. Fig. 7B shows that the mean percentage of VEGF positive cells in the OVA group mice significantly increased with $10.5 \pm 1.3\%$ (perivascular area), $7.5 \pm 1.2\%$ (peribronchial) and $38.7 \pm 4.5\%$ (interstitium), compared with the SS group with $3.3 \pm 0.8\%$ (perivascular area), $1.5 \pm 0.2\%$ (peribronchial) and $14.7 \pm 2.5\%$ (interstitium). In contrast, treatment with 2-ME resulted in a significant reduction in VEGF expression as compared with the OVA group in the perivascular area ($3.2 \pm 0.5\%$ versus $10.5 \pm 1.3\%$), peribronchial

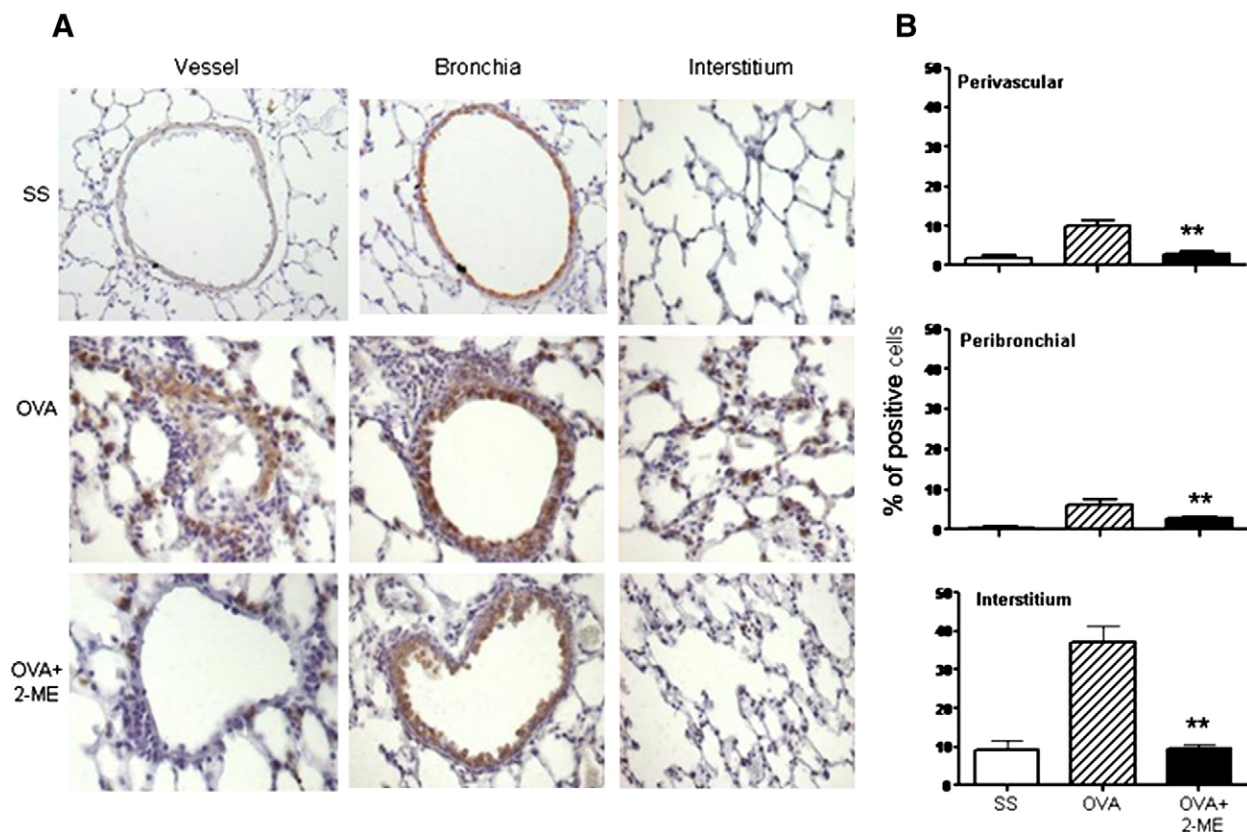


Figure 7 2-ME treatment reduces VEGF expression in lung tissue. (A) Immunohistochemical comparison of VEGF expression in lung tissue obtained from OVA-sensitized/challenged mice, in the absence or presence of 2-ME administered IT at a dose of 30 mg/kg, and saline-treated mice. Compared with OVA treatment alone (middle panel), OVA-sensitized/challenged mice administered 2-ME have a marked reduction in the cellular infiltration of VEGF positive numbers cells (perivascular, peribronchial and interstitium) (bottom panel); the control saline-treated mice have very few positive cells. (B) Percentage of VEGF immunostained cells in several lung compartments, in the absence or presence of 2-ME treatment. Lung sections from five mice from each experimental group were immunostained with anti-VEGF and analyzed under light microscopy (40 $\times$). The percentage positive cells were measured in surrounded cellular infiltrate in venules, bronchi and interstitium using an image analyzer (five venules or five bronchi from five to eight lung sections were analyzed for each experimental group). Results are expressed as averages and standard deviations of positive cells. (Upper) Perivascular infiltrate, (middle) peribronchial infiltrate, (lower) interstitium. ** $p < 0.05$ presence of 2-ME vs. absence of 2-ME (ANOVA).

($2.2 \pm 0.1\%$ versus $7.5 \pm 1.2\%$), and the interstitium ($9.3 \pm 2.2\%$ versus $38.7 \pm 4.5\%$).

In order to determine the effect of 2-ME treatment on collagen IV expression (as a marker of fibrosis) in our experimental murine model of allergic airway inflammation, we performed immunohistochemistry to measure the expression of this protein. The results were very similar to those observed for VEGF expression. Fig. 8 shows very low immunoreactive collagen IV localized in epithelial layers around the vessels and bronchioles in the SS group (Fig. 8A, upper panels). In contrast, the OVA-exposed mice showed a very significant increase in the expression of this protein in both the perivascular and also in the peribronchial areas, and these increases were very significant in the interstitium areas (Fig. 8A, middle panels). The 2-ME treatment dramatically decreased collagen IV expression in the allergen-induced asthmatic lungs (Fig. 8A, bottom panels). To quantify the expression of collagen IV, we counted the percent of collagen IV positive cells in several lung areas. Fig. 8B shows that the mean percentage of collagen IV positive cells in the OVA group

mice significantly increased with $17.2 \pm 3.2\%$ (perivascular area), $10.1 \pm 1.4\%$ (peribronchial) and $36.7 \pm 6.5\%$ (interstitium), compared with the SS group with $2.8 \pm 0.8\%$ (perivascular area), $2.5 \pm 0.2\%$ (peribronchial) and $4.1 \pm 1.5\%$ (interstitium). In contrast, treatment with 2-ME resulted in a significant reduction in collagen IV expression as compared with the OVA group in the perivascular area ($4.2 \pm 0.3\%$ versus $17.2 \pm 3.2\%$), peribronchial ($3.5 \pm 0.1\%$ versus $10.1 \pm 1.4\%$), and the interstitium ($6.7 \pm 1.2\%$ versus $36.7 \pm 6.5\%$). The above results, altogether, demonstrate that 2-ME inhibits significantly the expression of HIF-1 α , VEGF and collagen VI in the OVA asthma murine model.

2-ME inhibits circulating IgE anti-OVA antibody in the sera of OVA allergic mice

We examined whether the marked reduction in eosinophil recruitment into the airways of OVA-challenged mice conferred by 2-ME was associated with a defect in sensitization to OVA and resulting in a defect in IgE production.

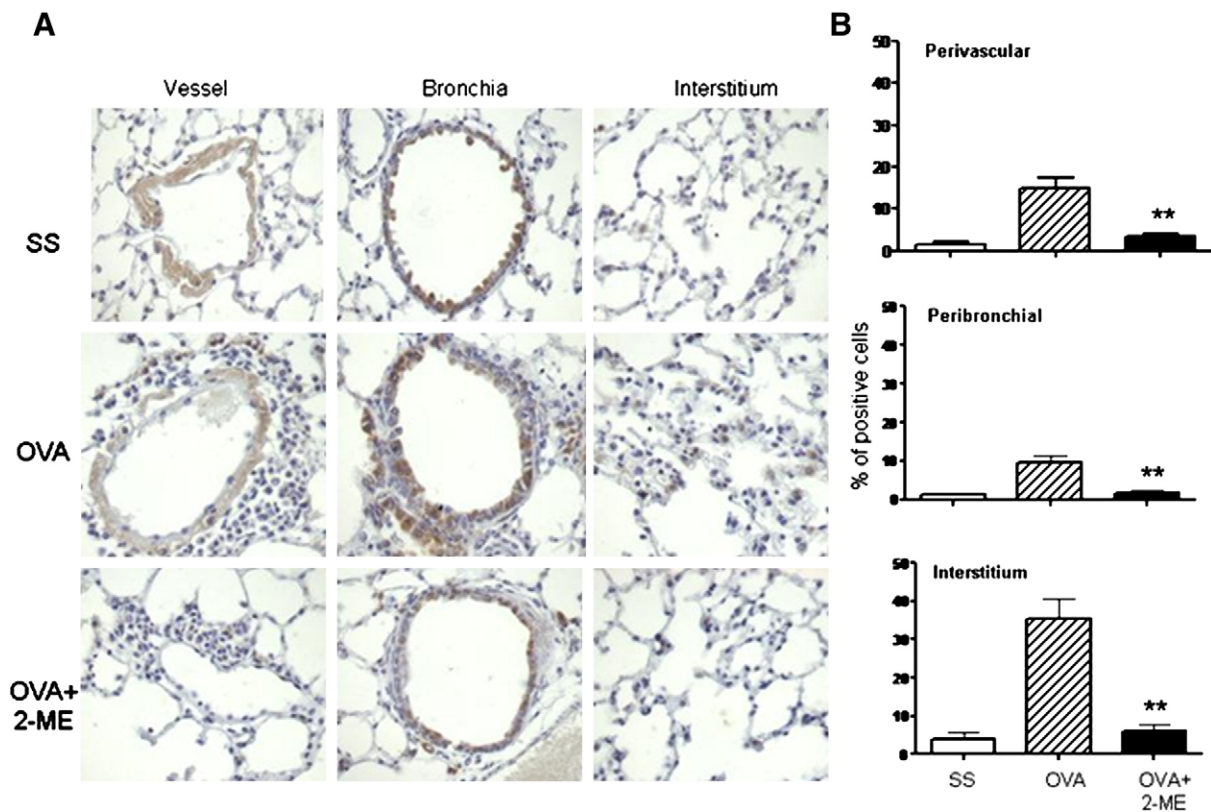


Figure 8 2-ME treatment reduces the collagen IV expression in lung tissue. (A) Immunohistochemical comparison of collagen IV expression in lung tissue obtained from OVA-sensitized/challenged mice, in the absence or presence of 2-ME administered IT at a dose of 30 mg/kg, and saline-treated mice. Compared with OVA treatment alone (middle panel), OVA-sensitized/challenged mice administered 2-ME have a marked reduction in the cellular infiltration collagen IV positive numbers cells (perivascular, peribronchial and interstitium) (bottom panel); the control mice have very few positive cells (top panel). (B) Percentage of collagen IV immunostained cells in several lung compartments, in the absence or presence of 2-ME treatment. Lung sections from five mice from each experimental group were immunostained with anti-collagen IV antibodies and analyzed under light microscopy (40 $\times$). The percentage of positive cells was measured in surrounded cellular infiltrates in venules, bronchi and interstitium using an image analyzer (five venules or bronchi from five lung sections were analyzed for each experimental group). Results are expressed as averages and standard deviations of positive cells. (Upper) Perivascular infiltrate, (middle) peribronchial infiltrate, (lower) interstitium. ** $p < 0.05$ presence of 2-ME vs. absence of 2-ME (ANOVA).

Circulating levels of OVA-specific IgE were measured by ELISA in the sera of allergic mice before and after treatment with 2-ME. Fig. 9 shows that 2-ME caused a slight but significant decrease of specific IgE antibody.

Discussion

The present findings provide for the first time evidence that intratracheal (IT) administration of 2-ME was effective in preventing the pathogenesis of asthma in a murine model of atopic asthma. Administration of 2-ME markedly diminished the inflammatory pulmonary response, eosinophil infiltration in the lung tissue, goblet cell hyperplasia with airway occlusion with mucus and reduction in collagen IV expression. The above findings also correlated with inhibition of circulating level of anti-ovalbumin IgE antibody. In addition, 2-ME inhibited HIF-1 expression concomitantly with inhibition of VEGF expression in the same tissues. It has been well documented that VEGF and HIF-1 α are intimately involved in the pathogenesis of asthma and 2-ME has been reported to inhibit both HIF-1 α and VEGF [16,19]. The present findings

corroborate these results in our murine airway inflammation model. Currently, 2-ME is being clinically tested in several diseases, including cancer, diabetes, rheumatoid arthritis, etc [3,16,20]. In addition, the present findings suggest the potential therapeutic application of 2-ME in the treatment of asthmatic patients with airway inflammation.

Epithelial goblet cells and mucus-secreting sub-mucosal glands are the major sources of luminal mucus. Goblet cell hyperplasia is a feature of the airways inflammation in asthma [3]. 2-ME significantly reduced the allergen-induced goblet cell hyperplasia and mucus occlusion observed in OVA-sensitized/challenged mice. Muz et al. [8] have reported that cysteinyl leukotrienes receptor blockade significantly reduced the allergen-induced goblet cell hyperplasia and mucus occlusion in the airway in OVA-sensitized/challenged mice. Similar findings were previously reported by Henderson et al. [21]. The present findings demonstrate that the mucus in epithelial goblet cell hyperplasia is decreased after 2-ME treatment.

Smooth muscle invasion is usually increased in large and/or small airways in both fatal and non-fatal cases of asthma

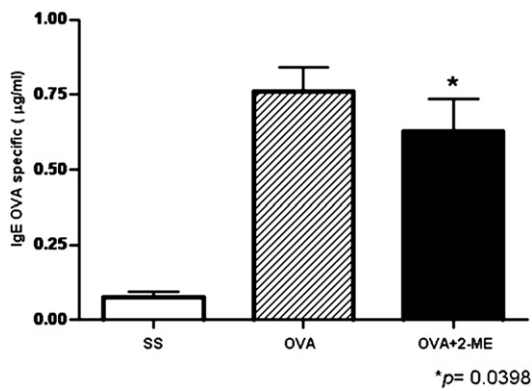


Figure 9 Intratracheal administered of 2-ME reduces IgE. ELISA for serum IgE levels was performed as described in Materials and methods. We analyzed the IgE serum levels (µg/ml) in the experimental and control groups. Each column represents the mean and S.M.E. of five to eight mice in each group. * $p < 0.05$ in comparison with the control group (*U* Mann Whitney).

[22]. In asthma, remodeling is usually present in biopsies as shown by collagen deposits on the reticular basement membrane [22]. Airway thickening beneath the basement membrane occurs with deposits of collagen and other extracellular matrix proteins including: fibronectin, tenascin and laminin [1]. The additional reticulin is largely produced after activation of myofibroblasts leading to fibrosis of the airways [23]. Collagen deposition occurs in the connective tissue layer surrounding the blood vessels and alveolar interstitial. The present findings demonstrate the airway collagen deposition/fibrin is specifically reduced in the 2-ME treated mice.

Exaggerated levels of VEGF are present in persons with asthma. However, the role of VEGF in normal and asthmatic lungs has been documented. Lee et al. [24] generated lung targeted VEGF transgenic mice and evaluated the role of VEGF in TH-2 mediated inflammation. In these mice, induction of VEGF resulted in an asthma-like phenotype with inflammation, parenchymal and vascular remodeling, edema, mucus, metaplasia, hyperplasia and airway hyper-responsiveness. VEGF also enhances respiratory antigen sensitization [24]. In this setting, VEGF was produced by the epithelial cells and by the TH-2 cells suggesting that VEGF regulation may be therapeutic in asthma. Clinical studies suggest, a role for angiogenesis in the development and persistence of chronic asthma. Suzaki et al. [25], studied the use of endostatin which is a potent anti-angiogenic factor in the OVA model, OVA-challenged mice induced immunolocalization of numerous VEGF positive cells around airways and also increased serum level of VEGF. Treatment with endostatin inhibited the airway hyper-responsiveness, pulmonary allergic inflammation, production of IgE specific anti-OVA antibodies and inhibited induction of inflammation. Our findings herein with 2-ME-induced inhibition of VEGF corroborate the role of VEGF in asthma. VEGF regulates angiogenesis and also induces inflammation and remodeling.

The blood vessels formed in asthmatic airways are involved in inflammation and airway remodeling processes in current asthma. VEGF and angiopoietin-1 (Ang-1) are angiogenic factors involved in the formation of blood vessels. VEGF has been reported to contribute to non-specific airway

hyper-responsiveness, has chemotactic effect on eosinophils and enhances airway smooth muscle cell proliferation. Further, TH-2 cells have receptors for VEGF and TH-2-associated cytokines increase VEGF expression. A study examined the relationship between VEGF level and clinical characteristics of VEGF expression and its association with HIF-1 expression. Bronchoscopy was performed in 30 patients and VEGF levels were assessed in alveolar lavage and HIF-1 α and HIF-2 α were examined by immunohistochemistry. The results demonstrated that the levels of VEGF were elevated compared to control. The same was found for HIF-1 α and HIF-2 α . The findings demonstrated that VEGF expression correlated significantly with HIF-1 α and HIF-2 α [23]. Our findings here are in agreement with this report.

2-ME is a naturally occurring metabolite of 17 beta-estradiol which has anti-proliferative, anti-angiogenic and anti-tumor activity. The anti-angiogenic effect of 2-ME has been suggested to be due in part to the inhibition of HIF [6]. HIF is a transcription factor which mediates the adaptation of cells and tissues to low oxygen concentration by upregulating genes involved in angiogenesis and glycolysis [26]. The oxygen-dependent regulation of HIF-1 α occurs at the level of protein stability. In normoxia, HIF-1 α is targeted for rapid degradation [27]. Mabeesh et al. [6], reported that 2-ME inhibits HIF-1 α protein cell disease and proposed that this effect is at the expense of microtubule disruption by 2-ME. Hagen et al. [11], however, reported that 2-ME prevents HIF-1 α stabilization and rapid degradation. 2-ME exhibited anti-proliferative and cytotoxic effects in head and neck squamous cell lines [16]. 2-ME inhibited HIF-1 α DNA binding and affected the expression of downstream genes such as BH3 interactin domain death agonist (BID) and VEGF.

Several mechanisms have been proposed for 2-ME activity including those mediated by its ability to bind to the colchicine-binding site of tubulin and the inhibition of superoxide dismutase enzymatic activity [18,28]. 2-ME destabilizes microtubules at doses that are efficacious *in vivo*. 2-ME blocks HIF-1 α nuclear accumulation and HIF-1 activity by oxygen and proteasome-independent pathways [6,11]. This mechanism is shared by other microtubule-targeting drugs, such as taxol. 2-ME depolymerizes microtubules and downregulates HIF-1 α protein levels and HIF transcriptional activity in an oxygen and proteasome-independent manners. The mechanism by which 2-ME inactivates HIF-1 α is not fully understood. It has been reported that 2-ME induces superoxide radicals [29] and HIF-1 α is shown to be downregulated by oxygen reactive species [30]. 2-ME anti-angiogenic activity is not solely attributed to its inhibition of VEGF but also to other effects. Pharmacological inhibitors of HIF-1 α regulated genes are more advantageous than HIF-1 gene inactivation for therapeutic application [31].

Long-acting β -agonists produce broncho-dilation and improve asthma symptoms, effects that are maintained with regular use over time. However, monotherapy with β agonists has consistently been shown to be inferior to inhaled corticosteroids, which can reduce the underlying inflammation associated with the disease. For these reasons, inhaled corticosteroids are recommended as first line maintenance treatment for asthma. For patients whose condition is inadequately controlled with inhaled corticosteroids alone, the addition of a longer acting β agonist is recommended as

symptoms are improved. However, much controversy exists in the use of β agonists in patients with asthma ever since its application over 50 years ago. Regular β agonists usage is associated with tolerance to the drug and a worsening of disease control. Several deaths have been reported with the long-acting β agonists Salmeterol and there was a four fold increased risk for asthma related death. The FDA in July of 2005 issued a strong warning of increased risk that should be placed on the labels of all long-acting β agonists with a recommendation that they be used only after other drugs have failed. A meta analysis pooled from 19 treatments with 33,826 patients from long-acting β agonists increased exacerbation and life threatening conditions compared with placebo [32]. Similar results were found with Salmeterol and Formoterol and in both children and adults. Concomitant inhaled corticosteroids do not adequately protect against these effects.

Our present findings here demonstrate that 2-ME is an effective agent in the treatment of airway inflammation associated with asthma. 2-ME is currently in clinical trials for other diseases and thus toxicity studies are being performed [33, 34]. A phase 2 trial of 2-ME was conducted in 60 patients with multiple myeloma (MM). 2-ME was administered orally at 1000 mg/daily for the first 39 patients and increased to 800 mg twice daily for the remaining patients. No partial responses were observed, although minor responses and prolonged, stable disease were noted. Plasma level of 2-ME was not adequate [33]. Another study investigated the maximum tolerated dose (MTD) and toxicity of 2-ME administered orally in 20 patients with solid tumors. The MTD for 2-ME was not reached and the trial was closed due to low plasma concentration of 2-ME relative to the doses administered. The investigators are currently examining new formulation of 2-ME with improved bio-availability [34]. A clinical study compared the therapeutic effect of 2-ME alone or in combination with docetaxel in patients with metastatic breast cancer. 2-ME alone or in combination with docetaxel was well tolerated [35]. Although these studies were not promising for 2-ME administered orally, we propose that the preferred route of 2-ME administration in asthma is via inhalation using different carriers. Further, this study suggests that HIF-1 α and VEGF are biomarkers in the development and response to treatment in asthma.

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References

- [1] J.A. Elias, Z. Zhu, G. Chupp, R.J. Homer, Airway remodeling in asthma, *J. Clin. Invest.* 104 (1999) 1001–1006.
- [2] J. Bousquet, P.K. Jeffery, W.W. Busse, M. Johnson, A.M. Vignola, Asthma from bronchoconstriction to airways inflammation and remodeling, *Am. J. Respir. Crit. Care Med.* 161 (2000) 1720–1745.
- [3] P.K. Jeffery, Remodeling in asthma and chronic obstructive lung disease, *Am. J. Respir. Crit. Care Med.* 164 (2001) S28–S38.
- [4] M.J. Abramson, A.J. Crockett, P.A. Frith, C.F. McDonald, COPDX: an update of guidelines for the management of chronic obstructive pulmonary disease with a review of recent evidence, *Med. J. Aust.* 184 (2006) 342–345.
- [5] V.S. Pribluda, E.R. Gubish Jr., T.M. Lavalley, A. Treston, G.M. Swartz, S.J. Green, 2-Methoxyestradiol: an endogenous anti-angiogenic and antiproliferative drug candidate, *Cancer Metastasis Rev.* 19 (2000) 173–179.
- [6] N.J. Mabjeesh, D. Escuin, T.M. Lavalley, V.S. Pribluda, G.M. Swartz, M.S. Johnson, M.T. Willard, H. Zhong, J.W. Simons, P. Giannakakou, 2-ME inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF, *Cancer Cell* 3 (2003) 363–375.
- [7] K. Kitagaki, T.R. Businga, J.N. Kline, Oral administration of CpG-ODNs suppresses antigen-induced asthma in mice, *Clin. Exp. Immunol.* 143 (2006) 249.
- [8] M.H. Muz, F. Deveci, Y. Bulut, N. Ilhan, H. Yekeler, T. Turgut, The effects of low dose leukotriene receptor antagonist therapy on airway remodeling and cysteinyl leukotriene expression in a mouse asthma model, *Exp. Mol. Med.* 38 (2006) 109–118.
- [9] J. Folkman, Angiogenesis-dependent diseases, *Semin. Oncol.* 28 (2001) 536–542.
- [10] C.G. Lee, H. Link, P. Baluk, R.J. Homer, S. Chapoval, V. Bhandari, M.J. Kang, L. Cohn, Y.K. Kim, D.M. McDonald, J.A. Elias, Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung, *Nat. Med.* 10 (2004) 1095–1103.
- [11] T. Hagen, G. D'Amico, M. Quintero, M. Palacios-Callender, V. Hollis, F. Lam, S. Moncada, Inhibition of mitochondrial respiration by the anticancer agent 2-methoxyestradiol, *Biochem. Biophys. Res. Commun.* 322 (2004) 923–929.
- [12] M.A. Goldberg, T.J. Schneider, Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin, *J. Biol. Chem.* 269 (1994) 4355–4359.
- [13] J.A. Forsythe, B.H. Jiang, N.V. Iyer, F. Agani, S.W. Leung, R.D. Koos, G.L. Semenza, Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1, *Mol. Cell Biol.* 16 (1996) 4604–4613.
- [14] M. Hedtjarn, C. Mallard, S. Eklind, K. Gustafson-Brywe, H. Hagberg, Global gene expression in the immature brain after hypoxia–ischemia, *J. Cereb. Blood Flow Metab.* 24 (2004) 1317–1332.
- [15] S.H. Yezep, R.H. Pando, L.S. Argumedo, M.V. Paredes, A.H. Cueto, A. Isibasi, C.R. Bonilla, Therapeutic efficacy of an *E. coli* strain carrying an ovalbumin allergenic peptide as a fused protein to OMPC in a murine model of allergic airway inflammation, *Vaccine* 21 (2003) 566–578.
- [16] J.L. Ricker, Z. Chen, X.P. Yang, V.S. Pribluda, G.M. Swartz, W.C. Van, 2-methoxyestradiol inhibits hypoxia-inducible factor 1 α , tumor growth, and angiogenesis and augments paclitaxel efficacy in head and neck squamous cell carcinoma, *Clin. Cancer Res.* 10 (2004) 8665–8673.
- [17] A. Tomkinson, G. Cieslewicz, C. Duez, K.A. Larson, J.J. Lee, E.W. Gelfand, Temporal association between airway hyperresponsiveness and airway eosinophilia in ovalbumin-sensitized mice, *Am. J. Respir. Crit. Care Med.* 163 (2001) 721–730.
- [18] R.J. D'Amato, C.M. Lin, E. Flynn, J. Folkman, E. Hamel, 2-Methoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site, *Proc. Natl. Acad. Sci. U. S. A.* 91 (1994) 3964–3968.
- [19] C. Chen, Q. Hu, J. Yan, J. Lei, L. Qin, X. Shi, L. Luan, L. Yang, K. Wang, J. Han, A. Nanda, C. Zhou, Multiple effects of 2-ME and D609 on the cortical expression of HIF-1 α and apoptotic genes in a middle cerebral artery occlusion-induced focal ischemia rat model, *J. Neurochem.* 102 (2007) 1831–1841.
- [20] A.C. Issekutz, K. Sapru, Modulation of adjuvant arthritis in the rat by 2-methoxyestradiol: an effect independent of anti-

- angiogenic action, *Int. Immunopharmacology* 8 (2008) 708–716.
- [21] W.R. Henderson Jr., L.O. Tang, S.J. Chu, S.M. Tsao, G.K. Chiang, F. Jones, M. Jonas, C. Pae, H. Wang, E.Y. Chi, A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model, *Am. J. Respir. Crit. Care Med.* 165 (2002) 108–116.
- [22] N. Carroll, J. Elliot, A. Morton, A. James, The structure of large and small airways in nonfatal and fatal asthma, *Am. Rev. Respir. Dis.* 147 (1993) 405–410.
- [23] C.E. Brewster, P.H. Howarth, R. Djukanovic, J. Wilson, S.T. Holgate, W.R. Roche, Myofibroblasts and subepithelial fibrosis in bronchial asthma, *Am. J. Respir. Cell Mol. Biol.* 3 (1990) 507–511.
- [24] S.Y. Lee, S. Kwon, K.H. Kim, H.S. Moon, J.S. Song, S.H. Park, Y.K. Kim, Expression of vascular endothelial growth factor and hypoxia-inducible factor in the airway of asthmatic patients, *Ann. Allergy Asthma Immunol.* 97 (2006) 794–799.
- [25] Y. Suzaki, K. Hamada, M. Sho, T. Ito, K. Miyamoto, S. Akashi, H. Kashizuka, N. Ikeda, Y. Nakajima, M. Iwase, I. Homma, L. Kobzik, H. Kimura, A potent antiangiogenic factor, endostatin prevents the development of asthma in a murine model, *J. Allergy Clin. Immunol.* 116 (2005) 1220–1227.
- [26] G.L. Semenza, HIF-1: mediator of physiological and pathophysiological responses to hypoxia, *J. Appl. Physiol.* 88 (2000) 1474–1480.
- [27] A.C. Epstein, J.M. Gleadle, L.A. McNeill, K.S. Hewitson, J. O'Rourke, D.R. Mole, M. Mukherji, E. Metzen, M.I. Wilson, A. Dhanda, Y.M. Tian, N. Masson, D.L. Hamilton, P. Jaakkola, R. Barstead, J. Hodgkin, P.H. Maxwell, C.W. Pugh, C.J. Schofield, P.J. Ratcliffe, *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation, *Cell* 107 (2001) 43–54.
- [28] M. Cushman, H.M. He, J.A. Katzenellenbogen, C.M. Lin, E. Hamel, Synthesis, antitubulin and antimitotic activity, and cytotoxicity of analogs of 2-methoxyestradiol, an endogenous mammalian metabolite of estradiol that inhibits tubulin polymerization by binding to the colchicine binding site, *J. Med. Chem.* 38 (1995) 2041–2049.
- [29] D. Chauhan, G. Li, M. Sattler, K. Podar, C. Mitsiades, N. Mitsiades, N. Munshi, T. Hideshima, K.C. Anderson, Superoxide-dependent and -independent mitochondrial signaling during apoptosis in multiple myeloma cells, *Oncogene* 22 (2003) 6296–6300.
- [30] Z.Z. Yang, A.Y. Zhang, F.X. Yi, P.L. Li, A.P. Zou, Redox regulation of HIF-1 α levels and HO-1 expression in renal medullary interstitial cells, *Am. J. Physiol. Renal. Physiol.* 284 (2003) F1207–F1215.
- [31] D.G. Nagle, Y.D. Zhou, Natural product-based inhibitors of hypoxia-inducible factor-1 (HIF-1), *Curr. Drug Targets* 7 (2006) 355–369.
- [32] S.R. Salpeter, N.S. Buckley, T.M. Ormiston, E.E. Salpeter, Meta-analysis: effect of long-acting β -agonists on severe asthma exacerbations and asthma-related deaths, *Ann. Intern. Med.* 144 (2006) 904–912.
- [33] S.V. Rajkumar, P.G. Richardson, M.Q. Lacy, A. Dispenzieri, P.R. Greipp, T.E. Witzig, R. Scholssman, C.F. Sidor, K.C. Anderson, M.A. Gertz, Novel therapy with 2-methoxyestradiol for the treatment of relapsed and plateau phase multiple myeloma, *Clin. Cancer Res.* 13 (2007) 6162–6167.
- [34] W.L. Dahut, N.J. Lakhani, J.L. Gulley, P.M. Arlen, E.C. Kohn, H. Kotz, D. McNally, A. Parr, D. Nguyen, S.X. Yang, S.M. Steinberg, J. Venitz, A. Sparreboom, W.D. Figg, Phase I clinical trial of oral 2-methoxyestradiol, an antiangiogenic and apoptotic agent, in patients with solid tumors, *Cancer Biol. Ther.* 5 (2006) 22–27.
- [35] J. James, D.J. Murry, A.M. Treston, A.M. Stornio, G.W. Sledge, C. Sidor, K.D. Miller, Phase I safety, pharmacokinetic and pharmacodynamic studies of 2-methoxyestradiol alone or in combination with docetaxel in patients with locally recurrent or metastatic breast cancer, *Invest. New Drugs* 25 (2006) 41–48.