



Inhaled corticosteroid prevents the thickening of airway smooth muscle in murine model of chronic asthma

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Abstract

Airway smooth muscle growth contributes to the mechanism of airway hyperresponsiveness (AHR) in asthma. Although current steroid use demonstrates anti-inflammatory activity, there is little reported on the action of corticosteroid on smooth muscle of the asthmatic airway. The present study investigated the effect of inhaled corticosteroid on the thickening of airway smooth muscle in bronchial asthma. We developed a mouse model of airway remodeling including smooth muscle thickening in which ovalbumin (OVA)-sensitized female BALB/c-mice were repeatedly exposed to intranasal OVA administration twice a week for 3 months. Mice were treated intranasally with fluticasone during the OVA challenge. Mice chronically exposed to OVA developed sustained eosinophilic airway inflammation compared with control mice. In addition, the mice chronically exposed to OVA developed features of airway remodeling, including thickening of the peribronchial smooth muscle layer. Intranasal administration of fluticasone inhibited the development of eosinophilic inflammation, and importantly, thickening of the smooth muscle layer. Moreover, intranasal fluticasone treatment reduced the transforming growth factor (TGF)- β 1 level in bronchoalveolar lavage fluid and regulated active TGF- β 1 signaling with a reduction in the expression of phospho-Smad2/3 and the concomitant up-regulation of Smad7 in lung tissue sections. These results suggest that intranasal administration of fluticasone can modulate the remodeling of airway smooth muscle via regulation of TGF- β 1 production and active TGF- β 1 signaling.

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1. Introduction

Asthma represents chronic inflammation of the airway followed by repair. The end result of repeated cycles of inflammation and repair may be imperfect repair resulting in a structurally and functionally abnormal remodeling of the airway. The structural remodeling changes noted in the asthmatic airway include subepithelial fibrosis, increased smooth muscle mass, angiogenesis, and increased mucous glands. These changes increase airway wall thickness and may result in the decline in lung function characteristic of human chronic asthma [1]. Traditionally, the role of the smooth muscle cell in airway inflammation has been regarded to contributing to the pathogenesis of asthma

solely by its contractile properties. Several reports have shown that in addition to its contractile function in asthma, airway smooth muscle can undergo hyperplasia and/or hypertrophy [2] leading to structural changes in the airway wall, consequently contributing to the development of persistent airway obstruction and increased nonspecific airway hyperresponsiveness (AHR) [3]. Woodruff et al. [4] showed that the volume of airway smooth muscle was increased 50–83% in biopsy samples from cases of moderately severe asthma compared with control cases. Baynayoun et al. [5] also reported that airway smooth muscle hypertrophy in proximal airways are selective determinants of severe persistent asthma. Additional reports from cell culture-based studies suggest a further role of airway smooth muscle in airway inflammation by acting as an important source of pro-inflammatory mediators [6].

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Glucocorticoids are the most effective therapy currently available for the treatment of asthma. Glucocorticoids act primarily as anti-inflammatory agents in asthma. Clinically, inhaled corticosteroid has been recommended as the first choice for controller therapy. Current studies focus on effect of corticosteroid on airway remodeling, especially airway fibrosis and goblet cell hyperplasia. Many studies show that inhaled corticosteroid inhibits the subepithelial fibrosis and goblet cell hyperplasia, although effect of steroid on airway fibrosis is controversial [7,8,27]. However, the effect of inhaled corticosteroid on smooth muscle remodeling has not been determined.

Transforming growth factor (TGF)- β 1 is a profibrotic cytokine thought to play an important role in promoting the structural changes of airway remodeling in asthma. Hallmarks of the TGF- β 1 signal transduction pathways are the activation of TGF- β 1 types I and II receptors and the subsequent phosphorylation and translocation of the intracellular effectors Smad2 and Smad3 to the nucleus where they regulate gene transcription. Smad7 is an intracellular inhibitor, which is rapidly induced by TGF- β family members and provides a negative feedback loop. Recent studies on a mouse model of allergic asthma have demonstrated *in situ* activation of these TGF- β signaling pathways [9].

The aim of this study was to determine the effects of corticosteroids on the thickening of the smooth muscle layer. To further explore the mechanism, we investigated the effect of corticosteroid treatment on TGF- β 1 production and active TGF- β 1 signaling in airway lesions. Importantly, we administered fluticasone intranasally, and during the chronic phase after eosinophilic inflammation as is the therapy usually employed on asthmatic patients.

2. Methods

2.1. Sensitization and antigen challenge protocol

Female BALB/c mice ($n = 16$ mice/group; The Dae-Han Experimental Animal Center, Daejeon, Korea), 8–10 wk of age, were immunized by subcutaneous injection on days 0, 7, 14, and 21 with 25 μ g of ovalbumin (OVA, grade V; Sigma-Aldrich, St. Louis, MO) adsorbed to 1 mg of alum (Sigma-Aldrich) in 200 μ l of normal saline. Intranasal OVA challenges (20 ng/50 μ l in phosphate-buffered saline, PBS) were administered on days 27, 29, and 31 under isoflurane (Vedco, St. Joseph, MO) anesthesia. Intranasal OVA challenges were then repeated twice a week for 3 months. Age- and sex-matched control mice were treated in the same way with PBS without OVA. Mice were killed 24 h after the final OVA challenge and bronchoalveolar lavage (BAL) fluid and lungs were analyzed. All animal experimental protocols were approved by The Catholic University of Korea, Animal Subjects Committees.

2.2. Administration of fluticasone

Micronized dry powder of fluticasone (GlaxoSmith-Kline, Brentford, UK) was dissolved in 70% ethanol and diluted in sterile normal saline on the experimental day. Fluticasone was given by intranasal administration (100 μ g/kg in 25 μ l normal saline, daily) starting on day 35, and then continuing during the intranasal OVA challenges for 3 months. The control mice were treated in the same way with normal saline with 3.5% ethanol.

2.3. Bronchoalveolar lavage

Mice were killed by CO₂ asphyxiation after measuring airway responsiveness. The trachea was exposed and cannulated with silicone tubing attached to a 23-gauge needle on an 800 μ l tuberculin syringe. After instillation of 1 ml of sterile PBS through the trachea into the lung, BAL fluid was withdrawn. The total cell counts in BAL fluid were performed using a hemacytometer. The BAL fluid was cytospun (3 min at 1000 rpm) onto microscope slides and stained with Diff-Quick. The percentages of BAL fluid macrophages, eosinophils, lymphocytes and neutrophils were obtained by counting 400 leukocytes on randomly selected portions of the slide by light microscopy. Supernatants were stored at -70°C .

2.4. ELISA

The TGF- β 1 concentrations in the BAL fluid were measured with an ELISA-kit (R&D system, Minneapolis, MN). The protocol was followed according to the manufacturer's instructions.

2.5. Immunohistochemistry

Six-micron-thick sections of lung from each paraffin block were deparaffinized with xylene and hydrated in ethanol. For immunohistochemical detection of α -smooth muscle actin, the lung sections were incubated overnight at 4°C with either a primary monoclonal antibody directed against α -smooth muscle actin (Sigma-Aldrich), or as a negative control mouse serum instead of the primary antibody. Immunoreactivity was detected by sequential incubations of lung sections with a biotinylated secondary antibody, followed by peroxidase reagent and AEC chromogen (3-amino-9-ethylcarbazole). Similar methods were used for incubation of anti-Smad antibodies (anti-phospho-Smad2/3 and Smad7; Santa Cruz Biotechnology, Santa Cruz, CA) for the immunohistochemical detection of Smad protein. Mouse Smad subtypes were detected using DAB (3,3'-diaminobenzidine) chromogen (Vector, Burlingame, CA).

The immunostaining area of α -smooth muscle actin in each paraffin embedded lung was outlined and quantified using a light microscope attached to an image analysis system (BX50, Olympus, Tokyo, Japan). Results are expressed as the area of immunostaining per micron length of basement membrane of

bronchioles 150–200 μm of internal diameter. At least 10 bronchioles were counted in each slide.

2.6. Western blot

Total protein was isolated from lung by homogenization in a buffer containing 50 mM HEPES (pH 7.4), 1% Nonidet P-40, 0.5% deoxycholate, 5 mM EDTA, 1 mM sodium *ortho*-vanadate, 5 mM NaF, and phosphatase and protease inhibitor cocktails (Sigma-Aldrich). The lysates were centrifuged at 14,000*g* for 15 min at 4 °C, the supernatants were collected, their total protein content was determined using a conventional method (Pierce Chemical Co., Rockford, IL), and aliquots were stored at –70 °C until assayed. Equal amounts of sample proteins were resolved using 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane by electroblotting in a buffer containing Tris–HCl (25 mM), glycine (192 mM), and methanol (20%, v/v). After transfer, the blots were incubated in 5% powdered milk in Tween/Tris-buffered saline 10 mM Tris–HCl (pH 7.5), 0.15 M NaCl, and 0.1% Tween 20 at room temperature for 1 h, then incubated with anti-pSmad3 and -Smad7 antibodies overnight at 4 °C, washed with Tween/Tris-buffered saline, and exposed to corresponding horseradish peroxidase-conjugated IgG for 1 h. The labeled band was detected using enhanced chemiluminescence detection kit and developed with Hyperfilm-enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

2.7. Data analysis

Results from each groups were compared by ANOVA with the nonparametric Kruskal–Wallis test, followed by post-testing with Dunn's multiple comparison of means. A statistical software package (Graph Pad Prism, San Diego, CA) was used for the analysis. A *P* value less than 0.05 was considered statistically significant. All results are given as means $\pm$ SEM.

3. Results

3.1. Effect of intranasal fluticasone on airway inflammation

The numbers of total cells, macrophages, lymphocytes, and eosinophils in BAL fluids were significantly increased in the OVA group compared with those in the control group. The administration of intranasal fluticasone significantly reduced the increase in total cells, macrophages, lymphocytes, and eosinophils recovered from the asthmatic airway lumens (*P* < 0.05) (Fig. 1).

3.2. Effect of intranasal fluticasone on thickening of airway smooth muscle layer

The immunostaining area of peribronchial myofibroblast α -smooth muscle actin was quantified by image analysis

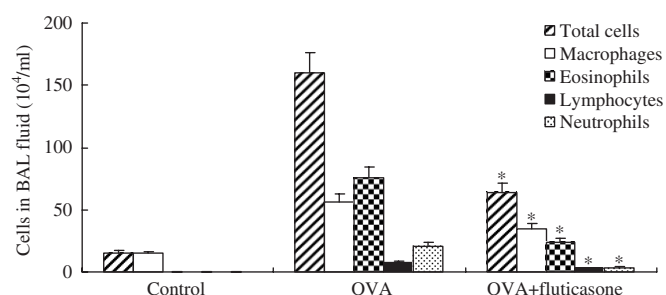


Fig. 1. Effect of intranasal fluticasone on total and differential cell counts in bronchoalveolar lavage (BAL) fluid. Mice were sacrificed 24 h after the final ovalbumin (OVA) challenge, and BAL cells were isolated. Values are expressed as mean $\pm$ SEM, *n* = 10–15/group. **P* < 0.05 in comparison with OVA group.

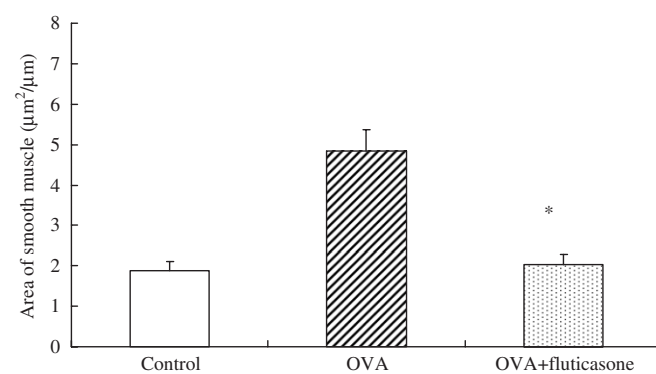


Fig. 2. Effect of intranasal fluticasone on airway smooth muscle area. Smooth muscle area are expressed as the area of immunostaining per micron length of basement membrane of bronchioles using image analyzer. Values are expressed as mean $\pm$ SEM, *n* = 10–15/group. **P* < 0.05 in comparison with OVA group.

and expressed as the stained area in $\mu\text{m}^2/\mu\text{m}$ circumference of a bronchiole. The immunostaining area of peribronchial α -smooth muscle actin in mice repetitively challenged with OVA was significantly greater than that in control non-OVA-challenged mice (4.83 ± 0.53 vs. $1.89 \pm 0.21 \mu\text{m}^2/\mu\text{m}$ circumference of bronchiole) (*P* < 0.05). Intranasal administration of fluticasone in repetitive OVA challenges mice significantly reduced the immunostaining area of α -smooth muscle actin compared with the area of untreated mice challenged repetitively with OVA (2.03 ± 0.24 vs. $4.83 \pm 0.53 \mu\text{m}^2/\mu\text{m}$ circumference of bronchiole) (*P* < 0.05) (Fig. 2).

3.3. Effect of intranasal fluticasone on TGF- β 1

As TGF- β 1 is able to induce smooth muscle hyperplasia, we measured levels of these cytokines in the BAL fluid. Levels of TGF- β 1 were significantly increased in the OVA group compared with those in the control group (789 ± 106 vs. $236 \pm 8 \text{ pg/ml TGF-}\beta$ 1) (*P* < 0.03). Intranasal fluticasone treatment significantly reduced levels of BAL TGF- β 1 compared with that of the OVA group (543 ± 51 vs. $789 \pm 106 \text{ pg/ml TGF-}\beta$ 1) (*P* < 0.05) (Fig. 3). The OVA

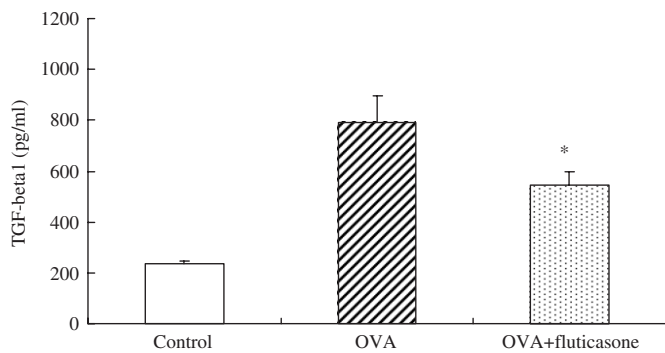


Fig. 3. Effect of intranasal fluticasone on TGF- β 1. TGF- β 1 concentration in bronchoalveolar lavage fluid. Mice were sacrificed 24 h after the final ovalbumin challenge. TGF- β 1 level in BAL fluid were measured with ELISA. Values are expressed as mean $\pm$ SEM, $n = 10$ –15/group. * $P < 0.05$ in comparison with OVA group.

group showed an increase in peribronchial TGF- β 1 positive cells (Fig. 5b) compared with the control group (Fig. 5a) in lung tissue. Intranasal administration of fluticasone reduced the peribronchial TGF- β 1 positive cells (Fig. 5c).

3.4. Effects of intranasal fluticasone on Smad expression

We demonstrated that fluticasone treatment inhibited the thickening of the smooth muscle layer in the airways and reduced TGF- β 1 expression. Recent reports have demonstrated an improved method for investigating the expression of active TGF- β 1 signaling in situ [10,11], which involves examination of the expression of the intracellular effectors, Smads. Therefore, we investigated the expression patterns of phosphor-Smad2/3 (pSmad2/3) and Smad7 in the lung following fluticasone administration in order to investigate any effect on active TGF- β signaling in airway lesions. An increase in expression of pSmad2/3 was observed during prolonged allergen challenge. Administration of fluticasone considerably decreased pSmad2/3 expression. In contrast with pSmad2/3, Smad7 was markedly up-regulated in mice treated with intranasal fluticasone compared with the OVA group (Figs. 4 and 5d–i).

4. Discussion

We have demonstrated that therapeutic intranasal administration of fluticasone is able to ameliorate airway remodeling through inhibition of smooth muscle layer thickening in asthma. The in vitro effects of corticosteroid on smooth muscle cell are well known. Glucocorticoids either directly or indirectly modulate contraction of airway smooth muscle by suppressing agonist-induced increases in intracellular calcium levels or by down-regulating or uncoupling receptors linked to contraction such as muscarinic M2 or M3, histamine H1 receptors [12,13]. In addition to the effects on contraction, glucocorticoids are also effective antiproliferative agents in airway smooth muscle [14,15]. This study demonstrated the therapeutic

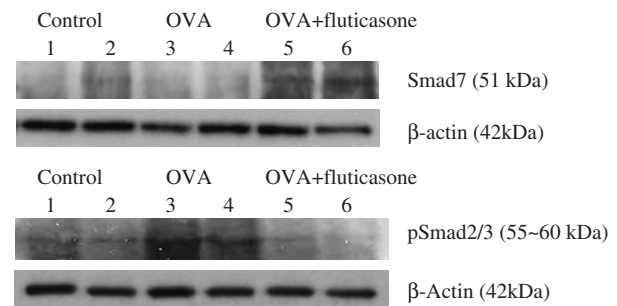


Fig. 4. Effect of intranasal fluticasone on Smad protein. Mice were sacrificed 24 h after the final ovalbumin challenge. Expression of pSmad2/3 and Smad7 in lung tissue were determined by western blotting.

effect of inhaled corticosteroid on airway smooth muscle remodeling in allergic airways disease. Glucocorticoids also suppress induction of cyclooxygenase-2 in human airway smooth muscle cells and the subsequent synthesis and release of arachidonic acid metabolites, particularly prostaglandin E2 [16]. The potential of airway smooth muscle to recruit and activate pro-inflammatory cells such as the eosinophil may also be reduced by glucocorticoids, as they are effective in preventing the release of several cytokines such as RANTES, interleukin-8, and granulocyte macrophage colony-stimulating factor.

TGF- β is a profibrotic cytokine, and the TGF- β isoforms are implicated in the extracellular matrix changes observed in fibrosis. The levels of TGF- β 1 are increased in bronchoalveolar lavage fluids of patients with asthma [17]. Clinical studies using biopsies from asthmatic patients have also demonstrated a correlation between basement membrane thickness and the TGF- β 1 level [18] and the activation of TGF- β 1 signaling through an increased expression of Smad2 in asthmatic airways [19]. Our data showed that expression of TGF- β 1 and the intracellular effector phospho-Smad2/3 is dramatically increased during prolonged allergen challenge in a number of different cell types including bronchial epithelial and endothelial cells, inflammatory leukocytes and in airway smooth muscle cells. This expression occurred concomitant with increased airway smooth muscle thickness. Insulin-like growth factor [20], epidermal growth factor [21], platelet-derived growth factor [22] and TGF- β 1 [23] have been shown to promote an increase in cell number when added to cultured airway smooth muscle cell, although it is not clear which growth factors account for the changes in airway smooth muscle in asthma. Our results suggest that TGF- β 1 play a role on thickening of the smooth muscle layer as well as subepithelial fibrosis in asthma.

Fluticasone treatment significantly decreased the smooth muscle area of bronchioles in α -smooth muscle actin-stained lung tissue sections. In order to investigate the effect of fluticasone treatment on TGF- β 1, we investigated the expression of TGF- β 1 and Smad molecules in the lung. Treatment with inhaled fluticasone reduced the expression of TGF- β 1 and regulated active TGF- β 1 signaling in the airways, as demonstrated by a decrease in pSmad2/3

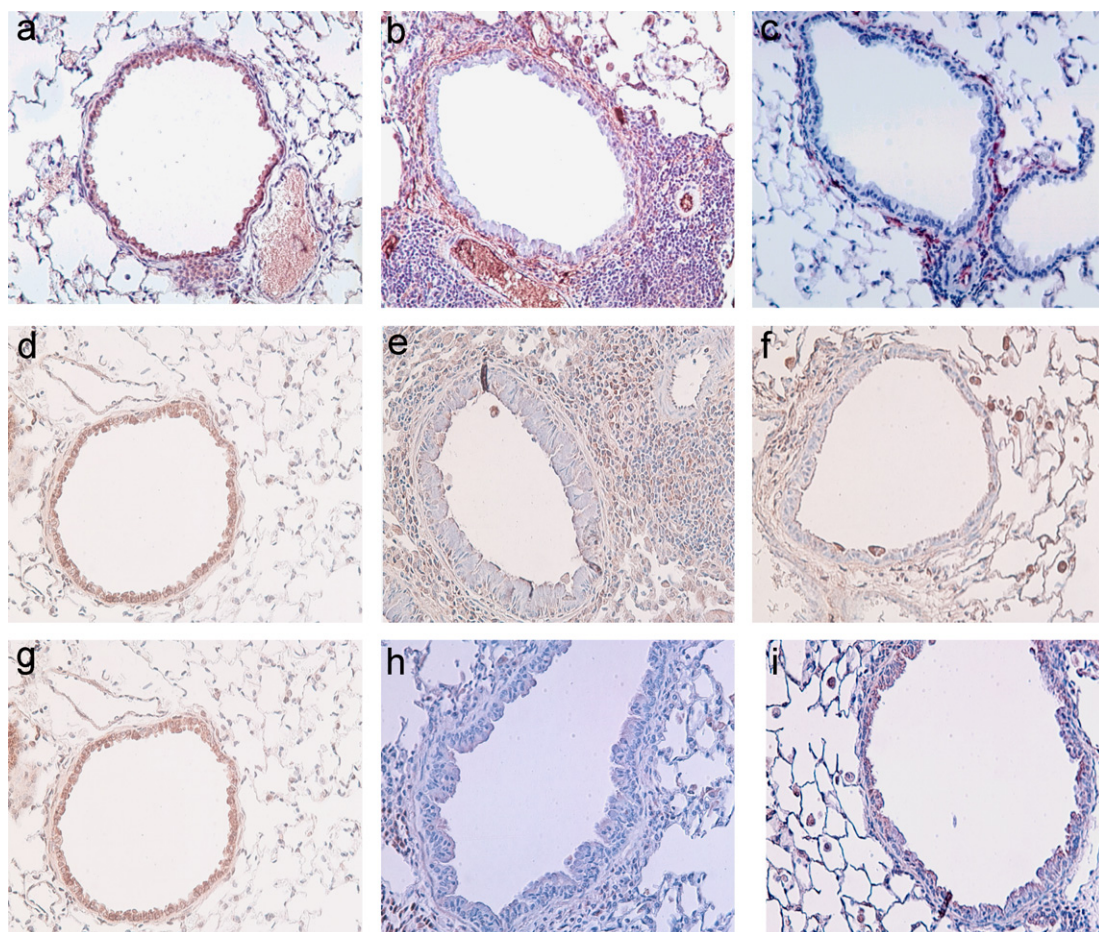


Fig. 5. Modulation of active TGF- β 1 signaling by intranasal fluticasone. Mice were sacrificed 24 h after the final ovalbumin challenge. Paraffin-embedded lung tissue sections were stained with specific antibody to TGF- β 1 (a–c), pSmad2/3 (d–f), Smad7 (g–i) ($\times 200$). Positive staining is depicted in brown. Left panel, control group; middle panel, untreated asthma mice; right panel, asthma mice treated with intranasal fluticasone.

expression concomitant with an increase in the inhibitory molecule, Smad7. Signaling of corticosteroids is mediated by the glucocorticoid receptor. Generally, they act as transcription factors by forming homodimers of two hormone-receptor complexes, which bind to a response element in the promoter region of target genes and modulate gene expression. Additionally, corticosteroids and their receptors are able to suppress gene expression by protein–protein interactions with other transcription factors, e.g., AP-1 or NF- κ B, characterized as a mechanism for their immunosuppressive and anti-inflammatory function [24]. There are several possible mechanisms by which fluticasone inhibited the thickening of smooth muscle in asthmatic airway. Firstly, fluticasone may inhibit directly smooth muscle proliferation. Glucocorticoids have anti-proliferative activity against a broad spectrum of mitogens [25]. Glucocorticoids decrease the transcription and translation of cyclin D1, and consequently arrest the cell cycle progression late in the G1 phase [26]. Secondly, a decrease of TGF- β 1 level is a possible mechanism. We have observed the reduction in BAL fluid TGF- β 1 by ELISA, and the reduction in TGF- β 1 expression in lung tissue by immunohistochemical stain. However, this result is contra-

dictory with a recent study which demonstrated that steroid treatment of asthmatics had no effect on the expression of TGF- β 1 which cause the failure of steroids to decrease collagen deposition [27]. These differences appear to result from the severity of the asthma in the patient population studied, as well as from previous steroids use by the patients. Finally, corticosteroid regulates active TGF- β 1 intracellular signaling. Previously, *in vitro* studies have shown that steroids modulate active TGF- β 1 signaling through a down-regulation of the constitutive TGF- β 1 type II receptor [28] and an up-regulation of Smad7 expression [29]. Here, we observed an induction of Smad7 expression and suppression of pSmad2/3 by fluticasone.

In conclusion, our results suggest that inhaled fluticasone treatment reduces the thickening of airway smooth muscle through mechanisms involving a decrease in the production of TGF- β 1 as well as modulation of active TGF- β 1 signaling in the lung.

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