

A phosphodiesterase 4 inhibitor inhibits matrix protein deposition in airways *in vitro*

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Background: Airway smooth muscle (ASM) cells may contribute to airway remodeling through the release of growth factors, cytokines, and extracellular matrix (ECM) proteins. The effect of current asthma therapies on this release is not known.

Objective: We examined the effect of corticosteroids, long-acting β_2 -agonists, and a phosphodiesterase 4 (PDE4) inhibitor on ASM-released connective tissue growth factor (CTGF), collagen I, fibronectin, versican, and IL-6.

Methods: Airway smooth muscle cells from individuals with and without asthma were stimulated with TGF- β with or without the drugs and CTGF and ECM protein expression measured by real-time PCR, cell surface, or matrix-associated ELISA. IL-6 release was measured by ELISA. Bronchial rings from individuals without asthma were incubated with TGF- β with or without the drugs.

Results: Neither corticosteroids nor long-acting β_2 -agonists reduced TGF- β -induced CTGF, collagen I, or fibronectin in either cell type, whereas corticosteroids alone induced the expression of CTGF, collagen I, and fibronectin. These drugs did not prevent the accumulation of TGF- β -induced proteins in bronchial rings, whereas the PDE4 inhibitor roflumilast inhibited TGF- β -induced CTGF, collagen I, and fibronectin.

Conclusion: In our model, current asthma therapies are not able to inhibit matrix protein deposition from ASM cells. The results of this study suggest that the PDE4 inhibitor roflumilast may have a role in regulating the ECM and therefore aspects of airway remodeling in asthma.

Clinical implications: Although current asthma therapies are effective in reducing inflammation and symptoms, reversal or prevention of structural changes contributing to remodeling may require additional therapy, which could include PDE4 inhibitors. (J Allergy Clin Immunol 2006;118:649-57.)

Key words: Asthma, airway remodeling, β -agonists, corticosteroids, phosphodiesterase inhibitors, fibronectin, connective tissue growth factor, human airway smooth muscle cells

Structural changes in the airways are one of the characteristic features of severe persistent asthma. These changes, referred to as *airway remodeling*, include thickening of the laminar reticularis, an increase in the bulk of the airway smooth muscle (ASM), an increase in the number of blood vessels, and alterations in the profile of extracellular matrix (ECM) proteins in the airway wall. The mechanisms leading to airway remodeling are not well understood, but ASM cells may play a role in that they produce and release ECM proteins. James et al¹ reported that the area of smooth muscle correlated with the reticular basement membrane thickness in central airways. We have previously reported that the profile of ECM proteins released *in vitro* from ASM isolated from individuals with asthma is different from that from individuals without asthma. These findings indicate that the ASM could play an important role in the regulation of airway remodeling.

Current asthma therapies are effective at reducing airway hyperresponsiveness, but their effect on airway remodeling is not as evident. Several studies have reported that inhaled corticosteroids (ICSs) were able to reduce the thickness of the basement membrane in asthma,²⁻⁷ whereas several others have shown no effect.⁸⁻¹¹ ICSs have also been reported to have no effect in reversing the reticular basement membrane thickening in lung allografts.¹²

Less is known about the effects of β_2 -agonists on airway remodeling. Early studies by Jeffery et al¹⁸ and Laitinen et al¹¹ showed β_2 -agonists had no effect on the remodeled basement membrane. β_2 -agonists also had no influence on subbasement membrane collagen, fibronectin, or tenascin deposition.^{13,14}

Another class of drugs that is currently being tested as asthma therapeutics are the phosphodiesterase 4 (PDE4) inhibitors.^{15,16} Roflumilast has been shown to be efficacious in the treatment of exercise-induced asthma¹⁷ and allergen-induced asthma¹⁸ and to reduce inflammation,¹⁷ but whether this class of drugs may also play a role in regulation of airway remodeling in the asthmatic airway is not known.

TGF- β is an important profibrotic cytokine, the levels of which are increased in the asthmatic airway.¹⁹⁻²² Many of the profibrotic effects of TGF- β have been shown to be

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Supported by the National Health and Medical Research Council, Australia, and ALTANA Pharma AG. J. K. Burgess is supported by National Health and Medical Research Council Peter Doherty Fellowship #165722.

Disclosure of potential conflict of interest: N. Cox is employed by Centenary Institute. The rest of the authors declared that they had received grant support from Altana Pharma AG.

Received for publication December 12, 2005; revised May 17, 2006; accepted for publication May 19, 2006.

Available online July 25, 2006.

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0091-6749/\$32.00

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doi:10.1016/j.jaci.2006.05.019

Abbreviations used

ASM: Airway smooth muscle
 CTGF: Connective tissue growth factor
 DMSO: Dimethyl sulfoxide
 ECM: Extracellular matrix
 ICS: Inhaled corticosteroid
 LABA: Long-acting β_2 -agonist
 PDE4: Phosphodiesterase 4

mediated via connective tissue growth factor (CTGF), and we have recently reported that TGF- β induces collagen I and fibronectin via CTGF-dependent and CTGF-independent pathways in human ASM cells.²³

Few studies that have examined airway remodeling have looked at the effect of current asthma therapies on the alterations in the vicinity of the ASM. ASM cells express β_2 -adrenoceptors and glucocorticoid receptors and therefore are potential targets for these therapies; however, how these treatments affect ASM production of ECM proteins has not been examined.

In this study, we examined the effect of corticosteroids, long-acting β_2 -agonists (LABAs), and the specific PDE4 inhibitor roflumilast on TGF- β -induced CTGF, collagen I, and fibronectin in human asthmatic and nonasthmatic ASM cells in culture and in nonasthmatic bronchial rings.

METHODS

Cell culture

Approval for all experiments with human lung was provided by the Human Ethics Committees of the University of Sydney and the South West Sydney Area Health Service. Nonasthmatic ASM was obtained from bronchial airways of 23 patients (mean age, 54.8 $\pm$ 19.1 years SD) undergoing resection for either lung transplantation or carcinoma. Asthmatic ASM was obtained from 15 patients (mean age, 24 $\pm$ 4.8 years SD) undergoing either resection for lung transplantation or endobronchial biopsies. The characteristics of the patients are listed in this article's Table E1 in the Online Repository at www.jacionline.org. Pure ASM bundles were dissected free and grown as explants as previously described.²⁴⁻²⁶ ASM cell characteristics were determined by immunofluorescence and light microscopy. Cells were stained with antibodies against α -smooth muscle actin and calponin, whereas omission of the primary antibody was used as a control.²⁷ All experiments were performed with cells between passages 4 and 8.

Airway smooth muscle cell treatment

Airway smooth muscle cells from patients with and without asthma were seeded for 24 hours in 5% FBS Dulbecco modified Eagle medium at a density of 1×10^4 cells per cm^2 . Medium was then changed to 0.1% insulin transferrin and selenium (ITS) Dulbecco modified Eagle medium for 24 hours before addition of TGF- β (1 ng/mL), formoterol (10 and 100 nmol/L), salmeterol (10 and 100 nmol/L), fluticasone (1 and 10 nmol/L), budesonide (1 and 10 nmol/L), roflumilast (1, 10, and 100 nmol/L and 1 $\mu\text{mol/L}$ where appropriate) or the combinations of TGF- β and each of the drugs for the periods described below. All of the drugs were dissolved in aqueous solutions except roflumilast, which was dissolved in dimethyl

sulfoxide (DMSO), for which appropriate controls were included in all experiments.

Real-time PCR

Airway smooth muscle cells from 5 to 7 patients without asthma and 5 to 7 patients with asthma were seeded in 6-well plates and treated as described. Total RNA was extracted from the cells at 8 or 24 hours, and real-time PCR for analysis of CTGF or collagen I was performed as previously described.²⁸

ELISAs

CTGF cell surface ELISA. ASM cells from 6 to 7 patients without asthma and 6 to 7 patients with asthma were seeded in 96-well plates and treated as described for 24 hours. The supernatant was removed and the cells dried overnight at room temperature. The plates were stored at -20°C , in the presence of desiccant, until analysis. After defrosting, the plates were washed twice with 0.05% (vol/vol) Tween-20/PBS and blocked with 2% (wt/vol) BSA/PBS for 1 hour at room temperature. The wells were washed and goat antihuman CTGF (L-20; Santa Cruz Biotechnology, Santa Cruz, Calif) at a final concentration of 1 $\mu\text{g/mL}$ added before overnight incubation at 4°C . The plate was washed 5 times before the rabbit anti-goat horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Carpinteria, Calif) in 1% BSA/PBS/0.05% Tween-20 was added and incubated at room temperature for 1 hour. The plate was washed 7 times and the 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Progen, Darra, Queensland, Australia) added according to the manufacturer's instructions. Color development was stopped by the addition of phosphoric acid and the optical density read at 450 nm.

ECM protein ELISAs. Airway smooth muscle cells from 3 to 7 patients without asthma and 5 to 7 patients with asthma were seeded in 96-well plates and treated as described for 48 hours. ECM free of cells was prepared by treatment with sterile hypotonic ammonium hydroxide. ECM proteins were measured by ELISA as previously described²⁹ by using antibodies to fibronectin (mouse antihuman plasma fibronectin, 2 $\mu\text{g/mL}$, clone 868A11; Chemicon, Temecula, Calif), versican (mouse antihuman large proteoglycan [versican], 2 $\mu\text{g/mL}$, clone 2-B-1; Calbiochem, San Diego, Calif), and a purified mouse IgG₁ isotype control (2 $\mu\text{g/mL}$, clone MOPC-31C; Becton & Dickinson Pharmingen, San Jose, Calif). In a separate series of experiments, cells were treated with TGF- β (1 ng/mL) in the presence or absence of roflumilast (1, 10, and 100 nmol/L and 1 $\mu\text{mol/L}$) for 48 hours and ECM free of cells prepared as described. Fibronectin deposition was measured as described.

Immunohistochemistry

Human lung tissue was obtained from lung specimens resected for carcinoma or transplantation. Bronchial rings (2-5 mm in diameter and 3 mm in length) were dissected free from surrounding parenchymal tissue. The bronchial rings were incubated in treatments as described. After 24 hours, tissues were frozen in optimal cutting temperature embedding medium (Fronine Laboratory Supplies, Riverstone, Australia) and sectioned on a cryostat, and immunohistochemistry was performed by using rabbit antihuman connective tissue growth factor (CTGF) (4 $\mu\text{g/mL}$; Torrey Pines Biolabs, Houston, Tex), mouse antifibronectin (1 $\mu\text{g/mL}$; Chemicon, Temecula, Calif), and mouse anticollagen I (1 $\mu\text{g/mL}$; Chemicon) coupled with a peroxidase-labeled polymer horseradish peroxidase. To help identify the morphology of the tissue, hematoxylin and eosin staining was performed on serial sections. Image analysis was performed as described previously.³⁰ Full details of this method are provided in the Online Repository at www.jacionline.org. In a separate series of experiments, bronchial rings were treated with TGF β (1 ng/mL)

TABLE I. Summary of the major findings*

	Formoterol	Salmeterol	Fluticasone	Budesonide	Roflumilast
CTGF protein					
A ASM	—	—	↑	↑	—
A ASM + TGF	—	—	—	—	—
NA ASM	—	—	↑	↑	—
NA ASM + TGF	—	—	↑	↑	—
Fibronectin protein					
A ASM	—	—	↑	↑	—
A ASM + TGF	—	—	—	—	↓
NA ASM	—	—	↑	↑	—
NA ASM + TGF	—	—	↑	—	↓
Versican protein					
A ASM	—	—	—	—	—
A ASM + TGF	—	—	↓	—	—
NA ASM	↑	—	—	—	—
NA ASM + TGF	—	↑	↓	↓	—
CTGF protein					
NA BR	ND	ND	ND	ND	—
NA BR+ TGF	—	—	—	—	↓↓↓
Fibronectin protein					
NA BR	ND	ND	ND	ND	—
NA BR + TGF	—	—	—	—	↓↓↓
Versican protein					
NA BR	ND	ND	ND	ND	—
NA BR + TGF	—	—	—	—	↓↓↓

*Effect of formoterol, salmeterol, fluticasone, budesonide, and roflumilast on ECM protein deposition, both in the absence and in the presence of TGF- β (+TGF β) in asthmatic (A) and nonasthmatic (NA) ASM and bronchial rings (BR). No change (—), increase (↑), and decrease (↓) compared with the appropriate control (ITS or TGF- β alone). Data are not available for some combinations of treatments (ND).

Mechanisms of asthma and allergic inflammation

in the presence or absence of roflumilast (1, 10, and 100 nmol/L and 1 μ mol/L) for 24 hours and the deposition of fibronectin measured as described.

Analysis of data

Results from triplicate wells from each individual patient for cycle number for real time RT-PCR were averaged, and an overall mean and SEM were calculated for cycle number. Results from duplicate wells from each individual patient for absorbance at 450 nm for ECM ELISAs were averaged, and the absorbance from cells alone at 450 nm was subtracted before an overall mean and SEM were obtained from patients with and without asthma. ANOVA repeated measures with Dunnett multiple comparison or Bonferroni post tests or Student paired *t* tests were performed on the results for real time RT-PCR, ECM ELISAs, and immunohistochemistry analysis where appropriate. In all cases, a *P* value of less than or equal to 0.05 was considered significant.

RESULTS

We have summarized the major findings of our current studies in Table I.

Effect of corticosteroids, LABAs, and a phosphodiesterase inhibitor on CTGF and collagen I mRNA expression

At 8 hours, formoterol and salmeterol (100 nmol/L) caused a significant increase in collagen I mRNA in the

asthmatic cells (see this article's Table E2 in the Online Repository at www.jacionline.org). At 24 hours, budesonide (1 nmol/L) caused a significant increase in CTGF and collagen I mRNA expression in the asthmatic cells (see this article's Table E3 in the Online Repository at www.jacionline.org). Formoterol alone significantly inhibited CTGF mRNA expression in both asthmatic and nonasthmatic ASM cells at 24 hours (see this article's Table E3 in the Online Repository at www.jacionline.org). Roflumilast (1 μ mol/L) alone did not have any effect on the expression of CTGF or collagen I mRNA in either asthmatic or nonasthmatic ASM cells in culture. TGF- β significantly upregulated CTGF and collagen I mRNA expression after 8 and 24 hours in both asthmatic and nonasthmatic ASM cells (*P* < .05). The addition of formoterol (10 and 100 nmol/L), salmeterol (10 and 100 nmol/L), fluticasone (1 and 10 nmol/L), or budesonide (1 and 10 nmol/L) did not reduce the TGF- β -induced expression of CTGF or collagen I mRNA at 8 or 24 hours in either asthmatic or nonasthmatic cells (see this article's Table E2 and Table E3 in the Online Repository at www.jacionline.org) with the exception of formoterol 10 nmol/L, which reduced collagen I in asthmatic cells at 8 hours. Budesonide 10 nmol/L, in the presence of TGF- β , increased collagen I mRNA in asthmatic ASM cells at 24 hours. The addition of roflumilast (1 μ mol/L) also did not decrease the TGF- β -induced CTGF or collagen I mRNA.

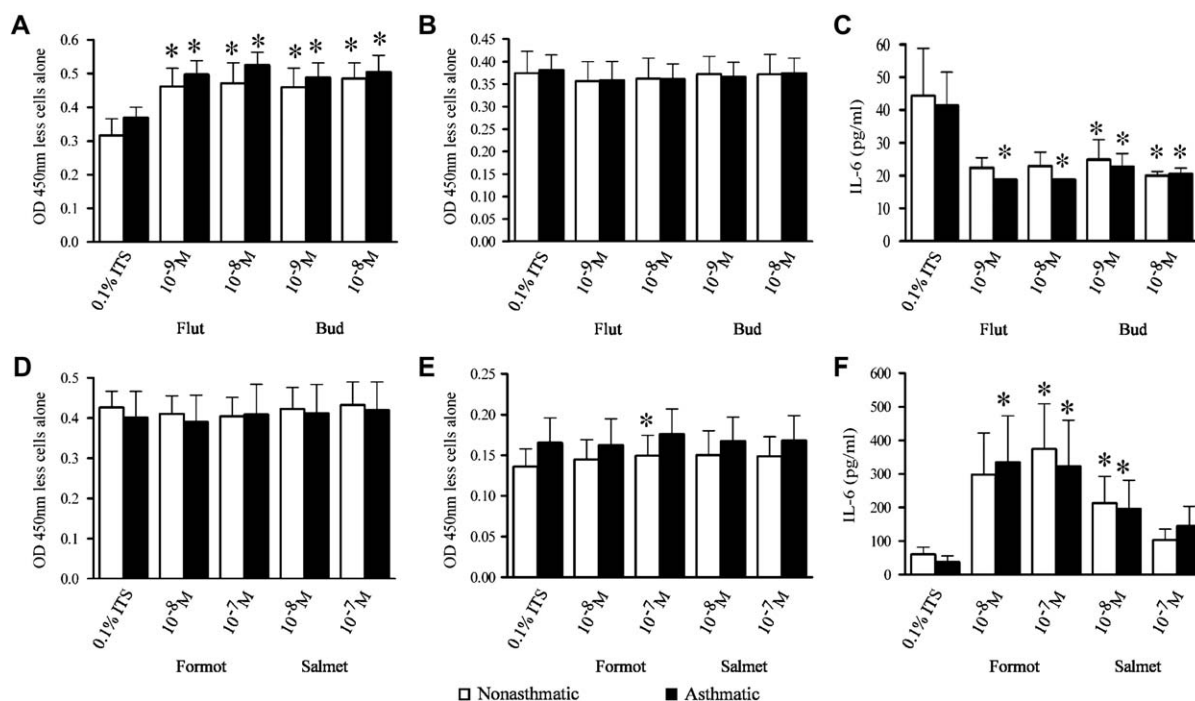


FIG 1. Corticosteroids (A-C) and LABAs (D-F) induction of fibronectin (A and D), versican (B and E), and IL-6 release (C and F). Data are means $\pm$ SEMs from $n = 6-7$ asthmatic and $n = 6-7$ nonasthmatic. *Significantly greater than 0.1% ITS, $P < .02$. Bud, Budesonide; Flut, fluticasone; Formot, formoterol; Salm, salmeterol.

Effect of corticosteroids, LABAs, and a phosphodiesterase inhibitor on cell associated CTGF protein

Transforming growth factor- β significantly increased the cell-associated CTGF protein in both asthmatic and nonasthmatic ASM cells ($P < .05$). This induction was not inhibited by any of the drugs tested (see this article's Table E4 in the Online Repository at www.jacionline.org). In addition, fluticasone (10 nmol/L) and budesonide (1 and 10 nmol/L), in the presence of TGF- β , significantly increased cell-associated CTGF in the nonasthmatic cells (see this article's Table E4 in the Online Repository at www.jacionline.org). Both corticosteroids alone at both concentrations tested (with the exception of budesonide 1 nmol/L in nonasthmatic cells) significantly increased the cell-associated CTGF.

Effect of corticosteroids, LABAs, and a phosphodiesterase inhibitor on ECM protein deposition

The corticosteroids fluticasone and budesonide alone at both concentrations tested caused significant deposition of fibronectin, and formoterol (100 nmol/L) increased deposition of versican in nonasthmatic cells (Fig 1). TGF- β significantly increased the deposition of fibronectin and versican in the ECM produced by both asthmatic and nonasthmatic ASM cells (Fig 2). Neither the corticosteroids nor the LABAs were able to inhibit the TGF- β -induced fibronectin deposition (Fig 2). Fluticasone (1 and 10

nmol/L), budesonide (10 nmol/L), and salmeterol (100 nmol/L) inhibited the deposition of TGF- β -induced versican in nonasthmatic cells, but only fluticasone (10 nmol/L) was effective in asthmatic cells. Roflumilast (1 μ mol/L) significantly inhibited TGF- β -induced fibronectin in asthmatic and nonasthmatic ASM cells (Fig 3) but had no effect on versican deposition. Lower concentrations of roflumilast (1-100 nmol/L) were unable to inhibit the TGF- β -induced fibronectin deposition from asthmatic and nonasthmatic ASM cells (data not shown).

Effect of corticosteroids, LABAs, and a phosphodiesterase inhibitor on IL-6 release

Both corticosteroids alone inhibited IL-6 release in the asthmatic ASM cells, but only budesonide was effective in the nonasthmatic cells (Fig 1). In contrast, the LABAs alone induced significant release of IL-6 from both cell types (both concentrations of formoterol in asthmatic and 100 nmol/L in nonasthmatic cells; salmeterol 10 nmol/L in both cell types; Fig 1), as reported previously in nonasthmatic cells.³¹ TGF- β significantly increased the release of IL-6 from both asthmatic and nonasthmatic ASM cells, as has been reported previously in nonasthmatic ASM cells³² (Fig 2). When the drugs were combined with TGF- β , both corticosteroids at either concentration tested were able to inhibit IL-6 release in both cell types, whereas the LABAs had no effect (Fig 2). Roflumilast (1 μ mol/L) alone had no effect on basal or TGF- β -induced IL-6 release (Fig 3).

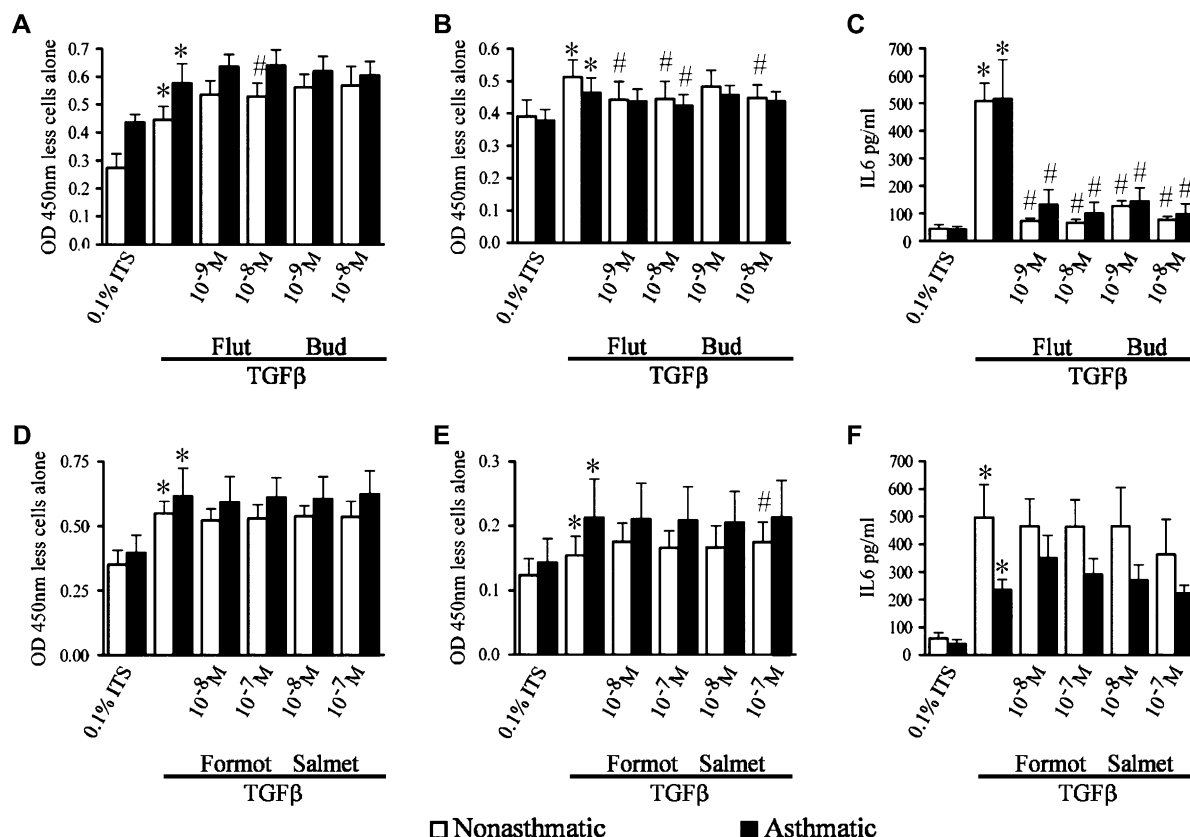


FIG 2. Effect of corticosteroids (A-C) and LABAs (D-F) on TGF- β -induced deposition of fibronectin (A and D), versican (B and E), and IL-6 release (C and F). Data are means $\pm$ SEMs from $n = 6-7$ asthmatic and $n = 6-7$ nonasthmatic. *Significantly greater than 0.1% ITS, $P < .04$; #significantly different from TGF- β , $P < .05$. Bud, Budesonide; Flut, fluticasone; Formot, formoterol; Salmet, salmeterol.

Effect of corticosteroids, LABAs, and a phosphodiesterase inhibitor on CTGF, collagen I, and fibronectin expression in bronchial tissue rings

Incubation of nonasthmatic bronchial rings in the presence of TGF- β resulted in a significant increase in CTGF, collagen I, and fibronectin protein in the tissue sections (Fig 4). Neither the corticosteroids nor the LABAs (at either concentration tested) were able to inhibit this TGF- β -induced protein expression (Fig 4). In contrast, the addition of roflumilast (100 nmol/L and 1 μ mol/L) significantly inhibited the TGF- β -induced expression of CTGF, collagen I, and fibronectin at 24 hours (Fig 4). Lower concentrations of roflumilast (1 and 10 nmol/L) were ineffective (data not shown).

DISCUSSION

In this study, we have examined the effectiveness of current asthma therapies in inhibiting TGF- β -induced ECM proteins produced by ASM cells *in vitro*. Neither the corticosteroids nor the LABAs tested were effective in reducing TGF- β -induced CTGF, collagen I, or fibronectin

mRNA expression or protein deposition from asthmatic or nonasthmatic ASM cells. There was a limited effect on the deposition of versican. In addition, the corticosteroids alone induced the expression of fibronectin protein deposition. Furthermore, these drugs were unable to prevent the accumulation of these proteins in nonasthmatic bronchial rings stimulated with TGF- β . In contrast, the PDE4 inhibitor roflumilast was effective at inhibiting the TGF- β -induced CTGF, collagen I, and fibronectin in nonasthmatic bronchial rings.

Few patients with asthma ever achieve a normal level of airway reactivity to histamine or methacholine, often despite years of ICS treatment.^{9,33} This suggests that if airway hyperresponsiveness and airway remodeling are directly related, then at least a component of airway remodeling is corticosteroid-insensitive. The lack of effectiveness of corticosteroids in our study supports this observation, although whether this is true for ECM deposition in areas other than near the ASM is not known. There is a need to identify other classes of drugs that can be used in concert with the current mainline asthma therapies that are able to control the components of airway remodeling not addressed by corticosteroids. The results of our study suggest that the PDE4 inhibitors may have potential in fulfilling this role.

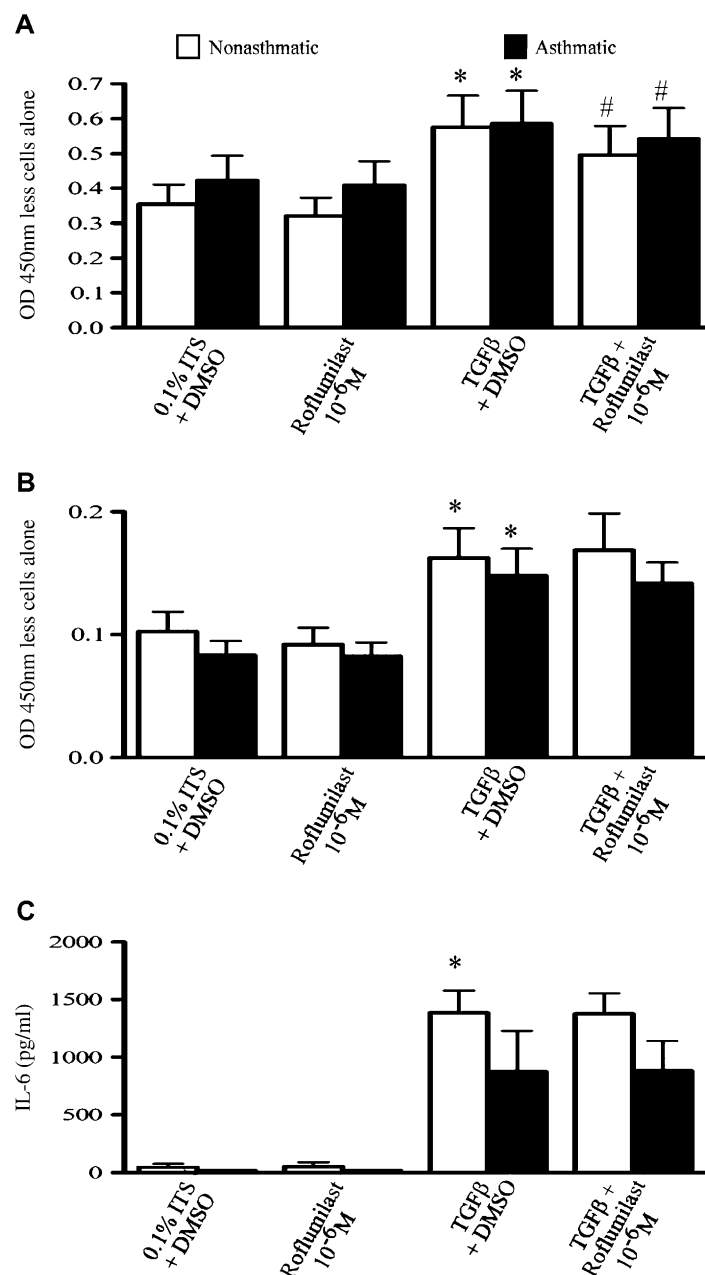


FIG 3. Effect of roflumilast on TGF- β -induced fibronectin and versican deposition and IL-6 release from ASM cells. Roflumilast effect on TGF- β -induced deposition of fibronectin (**A**), versican (**B**), and IL-6 release (**C**). Data are means $\pm$ SEMs from $n = 5$ asthmatic and $n = 3$ nonasthmatic. *Significantly greater than 0.1% ITS, $P < .03$; #significantly different from TGF- β + DMSO, $P < .04$.

We found that the corticosteroids themselves were able to induce the expression of CTGF, collagen I, and fibronectin. This is in agreement with our previous study in which we reported that beclomethasone induced ECM protein production from nonasthmatic ASM cells exposed to atopic and nonatopic serum.²⁹ In addition, other investigators have reported the induction of CTGF³⁴ and collagen^{35,36} by corticosteroids. However, these effects are not universal, because corticosteroids can reduce the production of ECM proteins from bovine tracheal ASM cells,³⁷ fibroblasts,³⁸

and sputum cells.³⁹ The regulation of these ECM proteins appears to be, at least partially, regulated at the level of mRNA transcription, because we observed increased CTGF and collagen I mRNA in the asthmatic ASM cells in the presence of budesonide alone. In addition, we observed for the first time that LABAs also induce the expression of collagen I mRNA in the asthmatic ASM cells, although this was not the case for either CTGF or fibronectin.

There is very little information available about the effectiveness of β_2 -agonists at inhibiting airway remodeling.

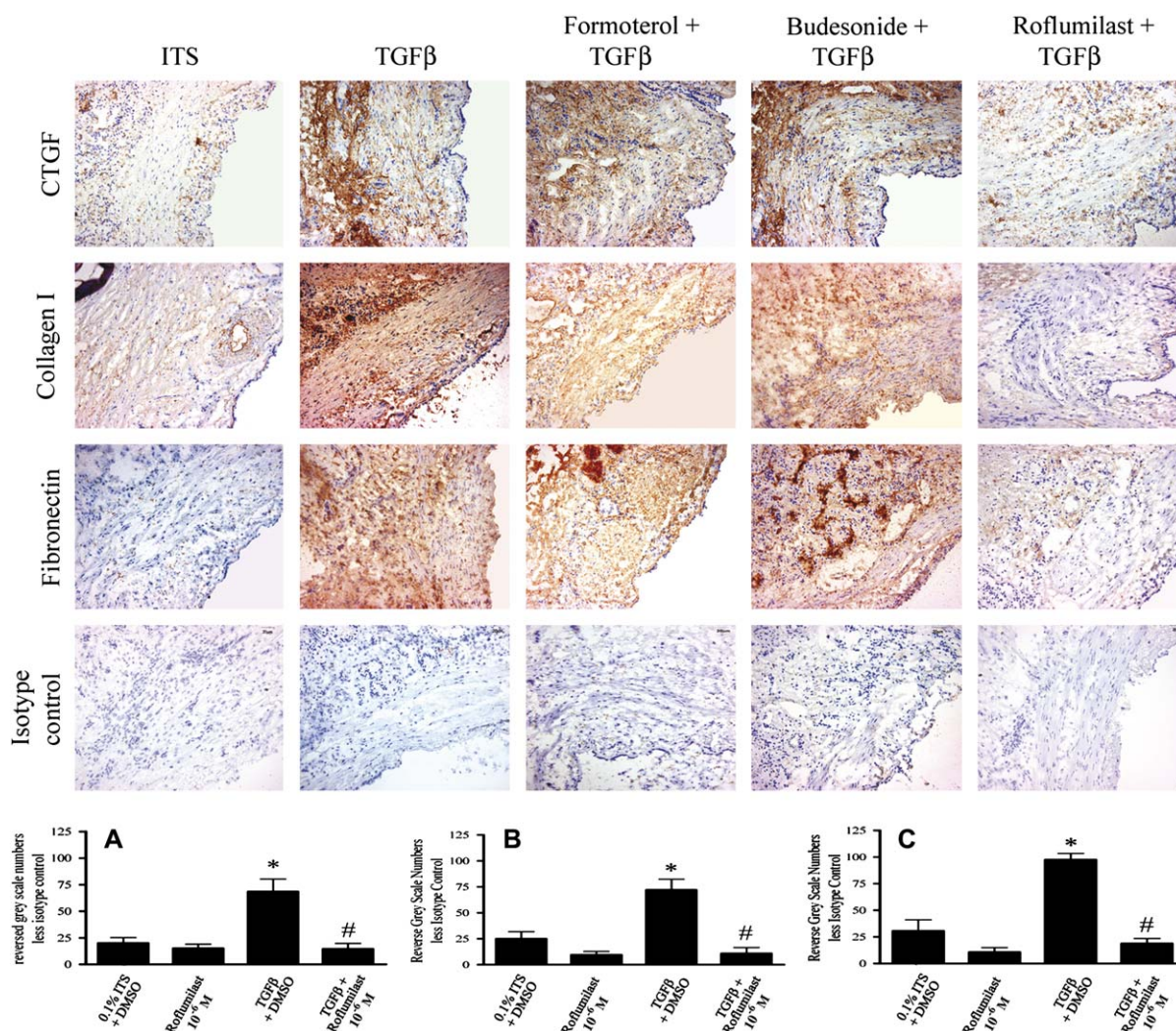


FIG 4. Effect of corticosteroids, LABAs, or roflumilast on TGF- β -induced CTGF, collagen, and fibronectin in nonasthmatic bronchial rings. Immunohistochemical detection of CTGF, collagen I, and fibronectin (brown staining) after stimulation with TGF- β in the presence or absence of drugs in nonasthmatic tissue sections. These changes are shown graphically in **A**, **B**, and **C**. Roflumilast effect on TGF- β -induced CTGF (**A**), collagen I (**B**), and fibronectin (**C**) in nonasthmatic bronchial rings. Data are means $\pm$ SEMs from $n = 5$. *Significantly greater than 0.1% ITS, $P < .01$, #significantly different from TGF- β + DMSO, $P < .003$.

Short-acting β_2 -agonists have been reported to increase airway inflammation *in vivo*,⁴⁰ but the effect on remodeling was not examined. LABAs are known to have anti-inflammatory effects,⁴¹⁻⁴³ but only 1 study has shown an effect on the increased vascularity in the remodeled asthmatic airway.⁴⁴ The findings in our *in vitro* model would be consistent with the lack of an inhibitory effect of LABAs on airway remodeling.

The effectiveness of the corticosteroids at inhibiting and the LABAs at inducing IL-6 release in our study indicates that these drugs were active and the concentrations examined in our study were able to effect responses in the ASM cells. Corticosteroids have previously been reported to inhibit the release of FBS-induced IL-6 from asthmatic and nonasthmatic cells⁴⁵ and to partially inhibit TNF- α -induced IL-6 from nonasthmatic ASM cells.³¹

This, to our knowledge, is the first report that LABAs induce release of IL-6 from asthmatic ASM cells, although this has previously been reported for nonasthmatic cells.³¹

The length of time that the airways have been exposed to ICS also plays a role in the effectiveness of the drugs in inhibiting airway remodeling. Short-term studies (3 months or less) generally have not reported a reduction in the basement membrane thickness or the deposition of ECM proteins in the airways, whereas longer studies with continuous treatment with high-dose corticosteroids for 6 to 12 months have reported significant reductions.⁴⁶ Our selection of time points in the current study may have altered the responses of the ASM cells. We examined only the early time points of 24 and 48 hours and did not continue to expose the cells for longer periods. It is possible the corticosteroids may have been able to inhibit

the TGF- β -induced ECM protein production with longer exposure times. Moreover, we acknowledge that it may not be possible to extrapolate observations *in vitro* to events *in vivo*.

In a mouse model of asthma, corticosteroids inhibited TGF- β -induced Smad2 signaling and collagen deposition.⁴⁷ We have previously shown that TGF- β -induced collagen and fibronectin in human ASM uses other pathways in addition to Smad signaling.²³ This difference in signaling pathways may explain why the corticosteroids were not effective at inhibiting ECM protein deposition in our study.

Roflumilast inhibited fibronectin deposition by both the asthmatic and the nonasthmatic ASM cells. This effect was not evident for the deposition of versican. These proteins are regulated by different signaling pathways and also play different roles in the ECM. Further work is necessary to understand the contribution of these different proteins to functional changes in the asthmatic airway.

In this study, we have shown that the ASM cells have the potential to contribute to the processes regulating airway remodeling by influencing the ECM composition in their direct vicinity, and that current asthma drugs are not effective at regulating the release of growth factors and ECM proteins from the ASM cells. Our results demonstrate that roflumilast has the potential to regulate at least 1 of the ECM proteins viz fibronectin produced by these cells.

The current experiments in which we exposed isolated bronchial rings to TGF- β in the presence or absence of the drugs were conducted only in nonasthmatic tissue, because it was not possible to obtain fresh asthmatic tissue, as opposed to cells from biopsies. Whether these effects would be reproduced in asthmatic tissue remains to be verified. It was noticeable that the inhibitory effect of roflumilast was strong in the tissue sections and less marked in the isolated ASM cells. The reason for this is unclear, but it may reflect intercellular cross-talk between the ASM and other resident cell types. The inhibitory effect of roflumilast in these experiments was dramatic and in sharp contrast to the lack of effect by corticosteroids and LABAs. The effects of roflumilast depend on its potent metabolite roflumilast N-oxide, which has activity at 0.3 to 10 nmol/L *in vivo* (Dr H. Tenor, Personal communication, February 2006). Presumably its mechanism of action is related to an increase in cyclic AMP, which raises the issue of why there was no such response for LABA. The high potency and local concentration of the roflumilast metabolite may explain this discrepancy.

In summary, in this particular model in cell and tissue systems, we have shown that corticosteroids and long-acting β_2 -agonists have little or no effect in inhibiting TGF- β -induced changes that may be relevant to airway remodeling. By contrast, the inhibitory effect of the PDE4 inhibitor roflumilast was marked. Bearing in mind the limitations inherent in *in vitro* studies, we suggest that these results indicate PDE4 inhibitors, such as roflumilast, may have a role in reversing these changes.

We acknowledge the collaborative effort of the cardiopulmonary transplant team and the pathologists at St Vincent's Hospital,

Sydney, and the thoracic physicians and pathologists at Royal Prince Alfred Hospital, Concord Repatriation Hospital and Strathfield Private Hospital and Rhodes Pathology, Sydney. We also acknowledge the contribution of Drs Gregory King and Melissa Baraket at the Woolcock Institute of Medical Research for supplying the asthmatic biopsies and Joanne Thompson for excellent technical assistance.

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