Glucocorticosteroids and β2-adrenoceptor agonists synergistically prevent the induction of a proliferative, hypocontractile airway smooth muscle phenotype

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Abstract

Airway smooth muscle (ASM) accumulation in the airway wall may contribute to increased airway narrowing in asthma. ASM accumulation is in part explained by hyperplasia, and a number of mitogenic stimuli - including the growth factor PDGF and the extracellular matrix protein collagen I - induce a proliferative ASM phenotype, characterized by increased proliferation and decreased contractile function. Glucocorticosteroids and β2-adrenoceptor agonists have been shown to synergistically increase glucocorticosteroid receptor translocation in ASM cells, but the functional impact of this synergism with regard to phenotype modulation remains to be established. Using bovine tracheal smooth muscle, we investigated the effects of the glucocorticosteroids fluticasone, budesonide, dexamethasone, the β2-agonist fenoterol and the combination of 100-fold lower concentrations of fluticasone and fenoterol on the induction of a proliferative, hypocontractile phenotype by 4 days exposure to PDGF or collagen I. The results demonstrate that the glucocorticosteroids inhibited phenotype switching, the effects induced by collagen I being less susceptible to glucocorticosteroid action than those of PDGF. Treatment with fenoterol also inhibited proliferation induced by both stimuli. Fenoterol decreased the sensitivity and maximal contraction in response to methacholine and KCl, by itself, which was not further affected by PDGF and collagen I. At 100-fold lower concentrations, fluticasone and fenoterol synergistically prevented the induction of a hypocontractile, proliferative phenotype by both mitogens, and reversed the collagen I-induced glucocorticosteroid insensitivity. Collectively, our results indicate that glucocorticosteroids and β2-agonists synergize to inhibit ASM phenotype modulation, which may contribute to the therapeutic effectiveness of combined treatment with these drugs.

Introduction

Allergic asthma is a chronic inflammatory airways disease, associated with allergen-induced early and late bronchial obstructive reactions, airway hyperresponsiveness (AHR) and airway remodelling [1]. Airway remodelling is characterized by changes in the airway tissue architecture, including increased extracellular matrix (ECM) deposition and airway smooth muscle (ASM) accumulation [2-4]. Increased ASM mass is considered to be the most important factor contributing to AHR and to decline in lung function in asthma [5,6], and may comprise ASM hypertrophy and/or hyperplasia [7-9]. In keeping with hyperplasia, proliferation of ASM cells in vitro is increased by various mitogenic stimuli, including growth factors and ECM proteins [10]. Prolonged exposure of ASM to mitogens also induces a switch from a contractile to a hypocontractile phenotype, associated with increased proliferative capability [11,12](Chapter 3). Removal of mitogenic stimuli, in the presence of insulin or TGF-β, results in the
reintroduction of a (hyper)contractile phenotype [13,14](Chapter 5), emphasizing the reversible nature of phenotype plasticity.

Inhaled glucocorticosteroids and β₂-agonists are currently the most effective therapy for asthma control [15,16]. Moreover, combined treatment with glucocorticosteroids and β₂-agonists results in better therapeutic management compared to monotherapy [17,18]. In addition to their potent anti-inflammatory effects, there is evidence that glucocorticosteroids may inhibit ASM remodelling in allergic asthma [19]. In line, in vitro studies have indicated that glucocorticosteroids may inhibit ASM cell proliferation [20-22]. In ASM cells, glucocorticosteroids accelerate the nuclear translocation of the glucocorticosteroid receptor and CCAAT/enhancer binding protein α (C/EBPα) and subsequently increase the expression of the cell cycle inhibitor p21\textsuperscript{waf1/cip1} [21]. Inhibition of ASM cell proliferation is also associated with the downregulation of growth factor-induced increases in cyclin D1 expression and phosphorylation of retinoblastoma protein (pRb) [22]. In ASM cells cultured on the ECM protein collagen type I, however, inhibition of proliferation by glucocorticosteroids is hampered [23-25], suggesting that changes in the ECM environment may impair glucocorticosteroid action. In addition to their anti-mitogenic effects, glucocorticosteroids inhibit TGF-β-induced sm-α-actin mRNA translation [26], indicating that glucocorticosteroids may also affect ASM contractile properties.

β₂-Adrenoceptor agonists have also been shown to attenuate ASM cell proliferation induced by various stimuli [27], via a mechanism involving β₂-adrenoceptor activation and subsequent activation of adenyl cyclase, which triggers the cAMP/protein kinase A (PKA) signalling cascade [28]. Recently, however, it was suggested that not PKA, but another downstream effector of cAMP, exchange protein directly activated by cAMP (Epac) was responsible for the inhibition of ASM proliferation [29]. When combined, β₂-agonists and glucocorticosteroids synergize and synchronize nuclear translocation of the glucocorticosteroid receptor and C/EBPα, resulting in a faster and longer activation of p21\textsuperscript{waf1/cip1} and inhibition of ASM proliferation [21].

To elucidate whether glucocorticosteroids and β₂-agonists synergize to modulate ASM phenotype plasticity, we investigated the effects of the glucocorticosteroids fluticasone, budesonide and dexamethasone, the β₂-agonist fenoterol and the combination of fluticasone and fenoterol on the induction of a hypocontractile bovine tracheal smooth muscle (BTSM) phenotype by PDGF and collagen I. The effects of the glucocorticosteroids and β₂-agonists were also assessed on PDGF- and collagen-induced BTSM cell proliferation.
Materials and methods

Tissue preparation and organ-culture procedure.
Bovine tracheal smooth muscle (BTSM) strips were prepared as described (Chapter 3 and 5). Tissue strips were washed in Medium Zero (sterile DMEM, supplemented with sodium pyruvate (1 mM), non-essential amino-acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1.5 μg/ml), apo-transferrin (5 μg/ml, human) and ascorbic acid (0.1 mM)) and transferred into suspension culture flasks. Strips were maintained in culture in an Innova 4000 incubator shaker (37°C, 55 rpm) for 4 days. When applied, PDGF-AB (10 ng/ml) and collagen type I (50 μg/ml) were present during the entire incubation period. Fluticasone (100 pM – 10 nM), budesonide (30 nM), dexamethasone (100 nM-1 μM) and/or fenoterol (10 nM – 1 μM) were applied 1 hr before and during stimulation with mitogens.

Isometric tension measurements.
Isometric tension measurements were performed as described (Chapter 3 and 5). In short, tissue strips were washed with several volumes of Krebs Henseleit (KH) buffer (composition (mM): NaCl 117.5, KCl 5.60, MgSO$_4$ 1.18, CaCl$_2$ 2.50, NaH$_2$PO$_4$ 1.28, NaHCO$_3$ 25.00 and glucose 5.50, pregassed with 5% CO$_2$ and 95% O$_2$; pH 7.4 at 37°C). Subsequently, strips were mounted for isometric recording in organ baths containing KH buffer. During a 90-min equilibration period, with washouts every 30 min, resting tension was gradually adjusted to 3 g. Subsequently, BTSM strips were precontracted with 20 and 40 mM isotonic KCl solutions. Following washout, maximal relaxation was established by the addition of (-)-isoproterenol (0.1 μM). Collectively, the total washout period before the start of the isometric tension experiments was at least 3 h. After washing, tension was readjusted to 3 g and cumulative concentration response curves were constructed to stepwise increasing concentrations of isotonic KCl (5.6-50 mM) or methacholine (1 nM – 0.1 mM). When maximal tension was reached, the strips were washed and maximal relaxation was established using isoproterenol.

Isolation of bovine tracheal smooth muscle cells.
BTSM cells were isolated as described (Chapter 3 and 5). In short, after removal of the mucosa and connective tissue, tracheal smooth muscle was chopped. Tissue particles were washed and enzymatic digestion was performed in Medium Plus (DMEM supplemented with sodium pyruvate (1 mM), non-essential amino-acid mixture (1:100), gentamicin (45 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1.5 μg/ml) and FBS (0.5%)), supplemented with collagenase P (0.75 mg/ml), papain (1 mg/ml) and soybean trypsin inhibitor (1 mg/ml). After digestion, cells were filtered and washed three times in Medium Plus, supplemented with 10% FBS. For all protocols, cells were used in passage 1-3.
Alamar blue proliferation assay
Collagen I-coated 24-well culture plates were prepared as described (Chapter 3). BTSM cells were plated on uncoated or collagen I-coated plastic culture plates at a density of 30,000 cells/well. Cells were washed twice with PBS and made quiescent by incubation in Medium Zero, supplemented with insulin (1 μM) for 3 days. Cells were then incubated with or without PDGF-AB (10 ng/ml) for 4 days in Medium Zero. Thereafter, cells were washed twice with PBS and incubated with HBSS containing 5% vol/vol Alamar blue solution. Conversion of Alamar blue into its reduced form by mitochondrial cytochromes was quantified by fluorimetric analysis, as indicated by the manufacturer. When used, cells were pretreated for 1 hr with fluticasone, budesonide, dexamethasone and/or fenoterol before stimulation with PDGF-AB.

Western analysis of contractile protein expression
To obtain whole BTSM tissue homogenates, tissue strips were cultured as described above. Western analysis was performed as described (Chapter 5). In short, homogenates were prepared by pulverizing the tissue under liquid nitrogen, followed by sonification in homogenization buffer. Equal amounts of protein were subjected to electrophoresis and transferred onto nitrocellulose membranes. Membranes were subsequently blocked in blocking buffer for 60 minutes at room temperature. Next, membranes were incubated overnight at 4 °C with primary antibodies (anti-sm-α-actin 1:2000, GAPDH 1:400, all dilutions in blocking buffer). After three washes with tris-buffered saline + 0.1% tween (0.1% TBST) of 10 min each, membranes were incubated with horseradish peroxidase-labelled secondary antibodies (dilution 1:2000 in blocking buffer) at room temperature for 60 min, followed by another three washes with 0.1% TBST. Antibodies were then visualized on film using enhanced chemiluminescence reagents and analyzed by densitometry (Totallab™). All bands were normalized to GAPDH expression.

Materials
Platelet-derived growth factor (human, PDGF-AB) was from Bachem (Weil am Rhein, Germany). Monomeric collagen type I (calf skin) was from Fluka (Buchs, Switzerland). Dulbecco’s modification of Eagle’s medium (DMEM), FBS, gentamicin solution, non-essential amino acid mixture, penicillin/streptomycin solution, sodium pyruvate solution and amphotericin B solution (Fungizone) were obtained from Gibco BRL Life Technologies (Paisley, U.K.). Anti-sm-α-actin, apo-transferrin (human), bovine serum albumin, dexamethasone, fluticasone, insulin (bovine pancreas), (-)-isoproterenol hydrochloride and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Anti-GAPDH was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alamar blue was from Biosource (Camarillo, CA, USA). Methacholine was obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Collagenase P and papain were from Boehringer (Mannheim, Germany). Fenoterol was from...
Boehringer Ingelheim (Ingelheim, Germany). Budesonide was a gift of Prof. H.W. Frijlink, University of Groningen. All chemicals used were of analytical grade.

Data analysis
All data are presented as mean ± SEM. Statistical differences between means were calculated using one-way ANOVA for repeated measures, followed by a Newman–Keuls multiple comparisons test. Significance was reached at $P<0.05$.

Results
Effects of glucocorticosteroids on PDGF- and collagen I-induced BTSM hypocontractility and proliferation.
To assess whether glucocorticosteroids inhibit the induction of a hypocontractile ASM phenotype, BTSM strips were incubated with PDGF or collagen I in the absence and presence of fluticasone, budesonide or dexamethasone. In accordance with previous studies [11](Chapter 3), we found that culturing of strips in the presence of PDGF-AB (10 ng/ml) or collagen I (50 μg/ml) significantly ($P<0.05$) decreased maximal contractile force ($E_{\text{max}}$) to methacholine compared to vehicle-treated controls (Figures 1-3).

Figure 1: The induction of a hypocontractile, proliferative phenotype induced by PDGF or collagen I is inhibited by fluticasone. (A,B) Concentration-response curves of methacholine-induced contractions of BTSM strips, pretreated with vehicle (control), (A) PDGF (10 ng/ml), or (B) collagen I (50 μg/ml), in the absence or presence of fluticasone (10 nM) for 4 days. Data represent means ± SEM of 5 independent experiments, each performed in duplicate. (C) Effects of fluticasone (10 nM) on basal and PDGF (10 ng/ml) or collagen I (50 μg/ml)-stimulated increases in cell number of cultured BTSM cells. Data represent means ± SEM of 7 independent experiments each performed in triplicate. **$P<0.01$, ***$P<0.001$ compared to vehicle-treated (control). $P<0.05$, ##$P<0.01$, ###$P<0.001$ compared to mitogen in the absence of fluticasone.
Fluticasone (10 nM, Figure 1) and budesonide (30 nM, Figure 2) both inhibited the decrease in $E_{\text{max}}$ induced by PDGF-AB ($P<0.01$, both glucocorticosteroids) and collagen I ($P<0.05$, both glucocorticosteroids).

Figure 2: The induction of a hypocontractile, proliferative phenotype induced by PDGF or collagen I is inhibited by budesonide. (A,B) Concentration-response curves of methacholine-induced contractions of BTSM strips, pretreated with vehicle (control), (A) PDGF (10 ng/ml), or (B) collagen I (50 μg/ml), in the absence or presence of budesonide (30 nM) for 4 days. Data represent means ± SEM of 5-6 independent experiments each performed in duplicate. (C) Effects of budesonide (30 nM) on basal and PDGF (10 ng/ml), or collagen I (50 μg/ml)-stimulated increases in cell number of cultured BTSM cells. Data represent means ± SEM of 6 independent experiments each performed in triplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to vehicle-treated (control). #*$P<0.05$, ###$P<0.01$, ####$P<0.001$ compared to mitogen in the absence of budesonide.

100 nM Dexamethasone attenuated the decrease in $E_{\text{max}}$ induced by PDGF (Figure 3A, $P<0.001$), whereas the decrease induced by collagen I was not affected (Figure 3B). At 1 μM, however, dexamethasone did inhibit the decrease in $E_{\text{max}}$ induced by collagen I (Figure 3D, $P<0.01$). No apparent effects of the glucocorticosteroids were observed under control conditions. Similar effects were observed for KCl-induced contractions (data not shown). The sensitivity for both contractile stimuli was unaffected by all treatments.
Figure 3: Effects of dexamethasone on the induction of a hypocontractile, proliferative phenotype by PDGF or collagen I. (A,B) Concentration-response curves of methacholine-induced contractions of BTSM strips, pretreated with vehicle (control), (A) PDGF (10 ng/ml), or (B) collagen I (50 μg/ml), in the absence or presence of 100 nM dexamethasone for 4 days. Data represent means ± SEM of 4-6 independent experiments, each performed in duplicate. (C) Effects of 100 nM dexamethasone on basal and PDGF (10 ng/ml) or collagen I (50 μg/ml)-stimulated increases in cell number of cultured BTSM cells. Data represent means ± SEM of 5 independent experiments each performed in triplicate. (D) Concentration-response curves of methacholine-induced contractions of BTSM strips, pretreated with vehicle (control) or collagen I (50 μg/ml) in the absence or presence of 1 μM dexamethasone for 4 days. Data represent means ± SEM of 5 independent experiments, each performed in duplicate. (E) Effects of 1 μM dexamethasone on basal and PDGF (10 ng/ml), or collagen I (50 μg/ml)-stimulated increases in cell number of cultured BTSM cells. Data represent means ± SEM of 6 independent experiments, each performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle-treated (control). *P<0.05, **P<0.01, ***P<0.001 compared to mitogen in the absence of dexamethasone.
As we have previously shown that the decrease in E\textsubscript{max} in response to methacholine and KCl was inversely correlated with the proliferative responses of BTSM cells to growth factors or ECM proteins [11] (Chapter 3), we also investigated whether glucocorticosteroids inhibit the proliferative responses in BTSM cells. PDGF and collagen I matrices increased BTSM cell number (Figures 1-3, P<0.01). Both fluticasone (Figure 1C) and budesonide (Figure 2C) inhibited the increase in cell number induced by PDGF (P<0.001, both glucocorticosteroids) and collagen I (P<0.05 and P<0.001, respectively). In agreement with the effects on contractility, PDGF-induced proliferation was decreased by 100 nM dexamethasone (P<0.05), but collagen I-induced proliferation was not affected by this concentration of the glucocorticosteroid (Figure 3C). At a concentration of 1 \mu M, however, dexamethasone inhibited proliferation induced by both PDGF and collagen I (P<0.01 and P<0.05, respectively, Figure 3E). No effects of the glucocorticosteroids were observed on basal cell number.

Effects of the \beta\textsubscript{2}-adrenoceptor agonist fenoterol on PDGF- and collagen I-induced hypocontractility and proliferation.
To assess whether \beta\textsubscript{2}-adrenoceptor activation affects the induction of a hypocontractile BTSM phenotype, strips were incubated with PDGF or collagen I in the absence and presence of fenoterol. Treatment with fenoterol (1 \mu M) for 4 days, followed by prolonged (approximately 3 h) and repeated washout, significantly (P<0.01) decreased the E\textsubscript{max} to methacholine and reduced the sensitivity to the agonist in control strips (pEC\textsubscript{50}=6.92±0.01 (control) and 6.53±0.08 (fenoterol), P<0.05, Figure 4A and 4B). Both PDGF and collagen I also reduced the E\textsubscript{max} to methacholine, however, without an effect on the sensitivity (Figure 4A and 4B), as also shown in Figure 1-3. Combined treatment of PDGF or collagen I with fenoterol did not further decrease maximal methacholine-induced contractions or sensitivity. Similar effects were observed for KCl-induced contractions (data not shown). To investigate whether changes in contractility were accompanied by changes in contractile protein abundance, expression of sm-\alpha-actin was determined. In accordance with previous findings (Chapter 3), it was found that 4 days of treatment with PDGF decreased sm-\alpha-actin expression by approximately 40\% (Figure 5). Treatment with fenoterol (1 \mu M) tended to decrease sm-\alpha-actin expression by about 25\% compared to controls; however, this did not reach statistical significance. Combined treatment with PDGF did not further decreased sm-\alpha-actin expression.

Treatment of BTSM cells with fenoterol fully inhibited both PDGF- and collagen I-induced increases in cell number (P<0.001, both mitogens, Figure 4C), whereas no effects were observed on cell number in the absence of mitogenic stimuli.
Figure 4: Effects of fenoterol on the induction of a hypocontractile, proliferative phenotype induced by PDGF or collagen I. (A,B) Concentration-response curves of methacholine-induced contractions of BTSM strips, pretreated with vehicle (control), (A) PDGF (10 ng/ml), or (B) collagen I (50 μg/ml), in the absence or presence of fenoterol (1 μM) for 4 days. Data represent means ± SEM of 5 independent experiments, each performed in duplicate. (C) Effects of fenoterol (1 μM) on basal and PDGF (10 ng/ml), or collagen I (50 μg/ml)-stimulated increases in cell number of cultured BTSM cells. Data represent means ± SEM of 7 independent experiments, each performed in triplicate. **P<0.01, ***P<0.001 compared to vehicle-treated (control).

Figure 5: Fenoterol decreases the expression of the contractile protein sm-α-actin. Western analysis of sm-α-actin protein expression in homogenates of BTSM strips pretreated with vehicle (control) or PDGF (10 ng/ml) in the absence or presence of fenoterol (1 μM) for 4 days. (A) Graph shows means ± SEM of 4 independent experiments after densitometric analysis. *P<0.05 compared to control. (B) Representative immunoblots of sm-α-actin (upper panel) and GAPDH (lower panel).
Fenoterol and fluticasone synergize to prevent the induction of a hypocontractile, proliferative phenotype by PDGF and collagen I. To assess whether β2-agonists and glucocorticosteroids synergize to inhibit the induction of a hypocontractile ASM phenotype, BTSM strips were incubated with PDGF or collagen I in the absence and presence of 100-fold lower concentrations of fenoterol (10 nM), fluticasone (100 pM) or the combination of both. At these concentrations, fenoterol and fluticasone, by themselves, only slightly diminished the PDGF- and collagen I-induced decrease of the maximal methacholine-induced contractions, which did not reach statistical significance. However, combined treatment with fenoterol and fluticasone fully inhibited the reduction in E_max induced by PDGF and collagen I (P<0.01 and P<0.05, respectively, Figure 6A and 6B). Of note, under control conditions no effects were observed for fenoterol, fluticasone or the combination (data not shown). For KCl-induced contractions, the reduction in E_max by both agonists appeared to be additive. Moreover, fenoterol significantly reduced the effects of collagen I (P<0.05). The sensitivity for either contractile stimuli was unaffected by all treatments.

Figure 6: Low concentrations of fenoterol and fluticasone synergistically prevented the induction of a hypocontractile, proliferative phenotype by PDGF or collagen I. (A,B) Concentration-response curves of methacholine-induced contractions of BTSM strips, pretreated with vehicle (control), (A) PDGF (10 ng/ml), or (B) collagen I (50 µg/ml), in the absence or presence of fenoterol (10 nM), fluticasone (100 pM) or the combination of both for 4 days. Data represent means ± SEM of 5 independent experiments, each performed in duplicate. (C) Effects of fenoterol (10 nM), fluticasone (100 pM) or the combination of both on basal and PDGF (10 ng/ml), or collagen I (50 µg/ml)-stimulated increases in cell number of isolated BTSM cells. Data represent means ± SEM of 5 independent experiments, each performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 compared to control treatment with mitogen in the absence of fluticasone and fenoterol.
Treatment of BTSM cells with fenoterol (10 nM) did not inhibit PDGF- or collagen-induced proliferation (Figure 6C). Fluticasone (100 pM) significantly (P<0.01) reduced the effects of PDGF on BTSM cell proliferation, while no effects of were observed on collagen I-induced proliferation. Combined treatment with both fenoterol and fluticasone completely prevented the increase in cell number induced by both mitogens (P<0.001, both), in case of collagen I in a synergistic fashion. No effects of fenoterol, fluticasone or the combination were observed under control conditions.

**Discussion**

In the present study, we demonstrate for the first time that glucocorticosteroids and \( \beta_2 \)-agonists synergize in preventing ASM phenotype switching. It was found that the induction of a proliferative, hypocontractile ASM phenotype by prolonged exposure to PDGF and collagen I was inhibited by glucocorticosteroids fluticasone, budesonide and dexamethasone. The effects of collagen I were relatively resistant to glucocorticosteroid action seen both in intact ASM tissue (contractility) and ASM cells (proliferation). Treatment with fenoterol also inhibited PDGF- and collagen I-induced BTSM cell proliferation. When applied in low concentrations, fenoterol and fluticasone synergized to prevent ASM phenotype switching by both mitogens.

ASM accumulation is a characteristic feature of airway wall remodelling in asthma, which is considered to be a major contributor to AHR and to decline in lung function [5,6]. Exposure of ASM to mitogens in vitro induces a switch from a contractile to a hypocontractile phenotype, characterized by increased proliferative rates and decreased contractile responses [11](Chapter 3). Glucocorticoids have been shown to inhibit ASM proliferation [20-22], but the effects on ASM contractility remained to be determined. In our current studies, we found that glucocorticosteroids inhibit PDGF-induced hypocontractility of intact BTSM strips as well as BTSM cell proliferation, indicating that ASM phenotype switching by the growth factor was inhibited. Similarly, collagen I-induced BTSM hypocontractility and proliferation were inhibited, although these effects were more resistant to glucocorticosteroid treatment as indicated by the reduced effects of dexamethasone (100 nM) and fluticasone (100 pM), compared to the effects on PDGF-induced phenotype switching. These findings correspond to previous observations, showing that bFGF-induced proliferation of human ASM cells cultured on collagen I, but not on laminin matrices, was resistant to dexamethasone (100 nM) and fluticasone (1 nM) [23,24]. In a subsequent study, it was demonstrated that the failure of dexamethasone to inhibit ASM proliferation was associated with a failure to decrease cyclin D1 expression and pRb levels [25]. In bovine ASM, however, dexamethasone did inhibit bFGF-induced proliferation on collagen I [30], suggesting that ASM cells of bovine origin may remain more sensitive to steroid treatment.
In addition to their anti-mitogenic effects, glucocorticoids have also been shown to inhibit sm-α-actin accumulation in response to TGF-β [26]. In the current study, glucocorticosteroids prevented BTSM hypocontractility and proliferation without effects in the absence of mitogenic stimuli. Collectively, these findings suggest that glucocorticoids inhibit switching both towards a hypocontractile and towards a hypercontractile ASM phenotype, which may both be present in asthmatics [7,31], to maintain a (normo)contractile ASM phenotype. In support of the maintenance of such a phenotype in vivo, budesonide has been shown to inhibit both ASM hyperplasia and hypercontractility in a guinea pig model of chronic allergic asthma [19].

β2-Adrenoceptor agonists are the primary treatment for the relief of bronchospasm in asthma [32]. In addition, β2-agonists attenuate ASM cell proliferation in response to various stimuli in vitro [27,32]. In the present study, we found that both PDGF- and collagen I-induced BTSM cell proliferation were inhibited by the β2-agonist fenoterol. Interestingly, pretreatment of BTSM strips with fenoterol for 4 days decreased maximal contractions induced by the receptor-dependent agonist methacholine and the receptor-independent stimulus KCl, and reduced the sensitivity to both stimuli. These effects are unlikely to be due to the presence of residual fenoterol as the strips have been washed for at least 3 h, but may be partly explained by increased constitutive β2-adrenoceptor activity, as reported previously by this laboratory [33,34]. In these studies, pretreatment with fenoterol time- and concentration-dependently increased constitutive β2-adrenoceptor activity, resulting in decreased maximal contractile responses as well as reduced sensitivity of the tissue in response to KCl [33]. However, although in these studies methacholine sensitivity after 18 h of fenoterol pretreatment was attenuated, no effects were observed on maximal contractile responses [34], suggesting that additional effects may underlie this decrease. Indeed, treatment of BTSM strips for 4 days with fenoterol tended to decrease sm-α-actin expression, which corresponds with previous findings showing that treatment of human ASM with the long acting β2-agonist salmeterol reduced sm-α-actin expression [26].

Combined treatment with glucocorticosteroids and β2-agonists results in more effective therapeutic management of asthma and COPD than monotherapy [17,18]. In human lung, glucocorticoids have been shown to increase transcription of the β2-adrenoceptor gene [64]. In ASM glucocorticosteroids and β2-agonists synergize to accelerate nuclear translocation of the glucocorticoid receptor and C/EBPα, resulting in the synergistic activation of the cell cycle inhibitor p21\textsuperscript{waf1/cip1} [21]. In addition, β2-agonists synergistically enhance glucocorticosteroid response element (GRE)-dependent transcription, amplifying the transcription of anti-inflammatory genes, including mitogen activated protein kinase phosphatase (MKP-1) and the cyclin-dependent kinase inhibitor p57\textsuperscript{kip2} [35], of which the latter has been shown to be involved in the antiproliferative effects of glucocorticosteroids [36].
In our current study, we showed that combined treatment with low concentrations of fenoterol and fluticasone synergistically inhibited PDGF- and collagen I-induced phenotype switching. No effects of combined treatment were observed in the absence of mitogenic stimuli, suggesting that fenoterol increased the activity of the glucocorticosteroid and not vice versa, as this would have resulted in decreased maximal contractions and reduced sensitivity in response to the contractile stimuli. These findings are in agreement with clinical studies, in which monotherapy with β2-agonists did not suppress inflammation, but enhanced the anti-inflammatory effects of inhaled glucocorticosteroids [17]. Studies on the contribution of the different aspects of airway remodelling in asthmatics have indicated that increased ASM mass is likely to be the most important factor in increased airway resistance and persistent AHR [5,6]. Our current studies may contribute to the increased therapeutic efficacy of combined treatment with β2-agonists and glucocorticosteroids [17], by effectively reducing the increase in ASM mass and development of persistent AHR.

In addition to increased ASM mass, airway remodelling in asthmatics is characterized by increased deposition of ECM proteins, including collagen I, beneath the epithelial basement membrane and surrounding the ASM bundles [37,38]. Collagen I may not only contribute to ASM accumulation by increasing proliferative responses [30,39] (Chapter 3), but also because it renders ASM cells resistant to the anti-mitogenic actions of glucocorticosteroids [23,24]. This could provide an explanation, why a subgroup of severe asthmatics, is poorly controlled by glucocorticosteroids [40]. Our current findings, showing that combined treatment with fluticasone and fenoterol synergistically normalizes not only PDGF-, but also collagen I-induced ASM phenotype switching, suggests that combination therapy may be not only be more beneficial in the therapeutic management of asthma by increasing asthma control and duration of bronchodilation, but also by normalizes the sensitivity of the ASM to lower doses of glucocorticosteroid.

In conclusion, our results indicate that glucocorticoids and β2-agonists synergize to prevent PDGF- and collagen I-induced ASM phenotype switching. As increased ASM mass is considered to contribute importantly to AHR in asthma, these findings may explain the enhanced efficacy of β2-adrenoceptor agonist/glucocorticosteroid combination therapy in controlling asthma.

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References


