Glucocorticosteroids and β2-adrenoceptor agonists synergize to inhibit airway smooth muscle remodelling

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Nonstandard abbreviations: ASM, airway smooth muscle; BTSM, bovine tracheal smooth muscle; C/EBPα, CCAAT/enhancer binding protein α; DMEM, dulbecco's modified eagle's medium; E\textsubscript{max}, maximal contraction; Epac, exchange protein directly activated by cAMP; FBS, fetal bovine serum; PDGF, platelet-derived growth factor; PKA, protein kinase A; TGF-β, transforming growth factor-β.

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Abstract

Airway remodelling, including increased airway smooth muscle (ASM) mass and contractility, contributes to increased airway narrowing in asthma. Increased ASM mass may be caused by exposure to mitogens, including platelet-derived growth factor (PDGF) and collagen type I, which induce a proliferative, hypocontractile ASM phenotype. By contrast, prolonged exposure to insulin induces a hypercontractile phenotype. Glucocorticosteroids and β₂-adrenoceptor agonists synergize to increase glucocorticosteroid receptor translocation in ASM cells; however, the impact of this synergism on phenotype modulation is unknown. Using bovine tracheal smooth muscle, we investigated the effects of the glucocorticosteroids fluticasone (10 nM), budesonide (30 nM), dexamethasone (0.1-1 μM) and the combination of low concentrations of fluticasone (3-100 pM) and fenoterol (10 nM) on ASM phenotype switching in response to PDGF (10 ng/ml), collagen type I (50 μg/ml) and insulin (1 μM). All glucocorticosteroids inhibited PDGF- and collagen I-induced proliferation and hypocontractility, the effects of collagen I being less susceptible to glucocorticosteroid action. At 100-fold lower concentrations, fluticasone (100 pM) synergized with fenoterol to prevent PDGF- and collagen I-induced phenotype switching. This inhibition of ASM phenotype switching was associated with a normalization of the PDGF-induced decrease in the cell cycle inhibitors p21^{WAF1/CIP1} and p57^{KIP2}. At this concentration, fluticasone also prevented the insulin-induced hypercontractile phenotype. At even lower concentrations, fluticasone (3 pM) synergized with fenoterol to inhibit this phenotype switch. Collectively, these findings indicate that glucocorticosteroids and β₂-agonists synergistically inhibit ASM phenotype switching, which may contribute to the increased effectiveness of combined treatment with glucocorticosteroids and β₂-agonists in asthma.
Introduction

Airway remodelling is a feature of chronic asthma, which is characterized by changes in the airway tissue architecture, including increased extracellular matrix deposition and airway smooth muscle (ASM) accumulation (Jeffery, 2001; Lloyd and Robinson, 2007; Postma and Timens, 2006). ASM accumulation is considered to be the most important factor contributing to airway hyperresponsiveness and lung function decline in asthma (Lambert et al., 1993; Oliver et al., 2007), and may comprise ASM hypertrophy as well as hyperplasia (Ebina et al., 1993; Benayoun et al., 2003; Woodruff et al., 2004). In keeping with hyperplasia, various mitogenic stimuli, including growth factors, like platelet-derived growth factor (PDGF), and extracellular matrix proteins, like collagen type I, increase ASM cell proliferation in vitro (Gosens et al., 2008; Dekkers et al., 2007; Dekkers et al., 2010). Prolonged exposure to mitogens also induces a switch from a contractile to a hypocontractile phenotype (Gosens et al., 2002; Hirst et al., 2000b; Dekkers et al., 2007; Dekkers et al., 2012). Removal of mitogenic stimuli, in the presence of insulin or transforming growth factor-β (TGF-β), results in the reintroduction of a (hyper)contractile phenotype (Ma et al., 1998; Schaafsma et al., 2007; Dekkers et al., 2009), emphasizing the dynamic and reversible nature of ASM phenotype plasticity.

Inhaled glucocorticosteroids and β₂-agonists are currently the most effective therapy for asthma control (Barnes, 2006; Penn, 2008). Moreover, combined treatment with glucocorticosteroids and β₂-adrenoceptor agonists results in better therapeutic management (Giembycz et al., 2008; Barnes, 2002). In addition to their anti-inflammatory effects, glucocorticosteroids may also inhibit ASM remodelling as shown in an animal model of chronic allergic asthma (Bos et al., 2007). In line, in vitro studies have indicated that glucocorticosteroids inhibit ASM cell proliferation (Stewart et al., 1995; Roth et al., 2002; Fernandes et al., 1999). 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} (Roth et al., 2002), whereas growth factor-induced increases in cyclin D1 expression and phosphorylation of retinoblastoma protein are reduced (Fernandes et al., 1999). In ASM cells cultured on collagen type I, however, inhibition of proliferation by glucocorticosteroids is hampered (Bonacci et al., 2003b; Bonacci and Stewart, 2006; Bonacci et al., 2006). In addition to their anti-mitogenic effects, glucocorticosteroids inhibit TGF-β-induced translation of \textit{sm}-α-actin (Goldsmith et al., 2007), indicating that glucocorticosteroids may also affect ASM contractility. When combined, glucocorticosteroids synergize with β\textsubscript{2}-agonists in ASM cells, which involves synchronization of translocation of the glucocorticosteroid receptor and C/EBPα, resulting in a faster and stronger activation of glucocorticoid response element (GRE)-dependent transcription of various genes including the cell cycle inhibitors p21\textsuperscript{WAF1/CIP1} and p57\textsuperscript{KIP2}, and the subsequent inhibition of ASM cell proliferation (Roth et al., 2002; Kaur et al., 2008).

To elucidate whether glucocorticosteroids and β\textsubscript{2}-agonists synergize to inhibit phenotype plasticity in intact ASM, we investigated the effects of the glucocorticosteroids fluticasone, budesonide, dexamethasone and the combination of fluticasone and fenoterol on the induction of a proliferative, hypocontractile bovine tracheal smooth muscle (BTSM) phenotype by PDGF and monomeric (denatured) collagen type I, as well as on the induction of a hypercontractile phenotype by insulin. The results demonstrate that glucocorticosteroids and β\textsubscript{2}-agonists synergize to inhibit ASM phenotype switching, both to a hypocontractile and to a hypercontractile phenotype, which may explain the enhanced efficacy of the combined therapy with glucocorticosteroids and β\textsubscript{2}-agonists in asthma.
Methods

Tissue preparation and organ culture procedure.

Bovine tracheal smooth muscle (BTSM) strips of macroscopically identical length (1 cm) and width (2 mm) were prepared as described (Dekkers et al., 2007; Dekkers et al., 2009). Subsequently, strips were washed in Medium Zero (sterile DMEM (Gibco BRL Life Technologies, Paisley, UK), supplemented with sodium pyruvate (1 mM, Gibco), non-essential amino-acid mixture (1:100, Gibco), gentamicin (45 µg/ml, Gibco), penicillin (100 U/ml, Gibco), streptomycin (100 µg/ml, Gibco), amphotericin B (1.5 µg/ml, Gibco), apo-transferrin (5 µg/ml, human, Sigma-Aldrich, Zwijndrecht, The Netherlands) 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 (platelet-derived growth factor-AB (PDGF-AB) and collagen type I) or 8 days (insulin), refreshing the medium on day 4 (Dekkers et al., 2009). Fluticasone (3 pM – 10 nM, Sigma-Aldrich), budesonide (30 nM, gift of Prof. H.W. Frijlink, University of Groningen), dexamethasone (100 nM – 1 µM, Sigma-Aldrich) and/or fenoterol (10 nM, Boehringer Ingelheim, Germany) were applied 1 hr before and during stimulation with PDGF-AB (10 ng/ml, human, Bachem, Weil am Rhein, Germany), monomeric collagen type I (50 µg/ml, calf skin, Fluka, Buchs, Switzerland) or insulin (1 µM, bovine, Sigma-Aldrich). Effective concentrations were based on previous reports (Dekkers et al., 2007; Dekkers et al., 2009; Fernandes et al., 1999; Ten Berge et al., 1995).

Isometric tension measurements.

Isometric tension measurements were performed as described (Dekkers et al., 2007; Dekkers et al., 2009). In short, tissue strips were washed with several volumes of Krebs-Henseleit buffer (composition (mM): 117.5 NaCl, 5.60 KCl, 1.18 MgSO4, 2.50 CaCl2, 
1.28 NaH₂PO₄, 25.00 NaHCO₃, and 5.50 glucose, pregassed with 5% CO₂ and 95% O₂, pH 7.4), pregassed with 5% CO₂ and 95% O₂; pH 7.4 at 37°C. Subsequently, strips were mounted for isometric recording in organ baths containing Krebs-Henseleit 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, Sigma-Aldrich). 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, ICN Biochemicals, Costa Mesa, USA). 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 (Dekkers et al., 2007; Dekkers et al., 2009). 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, Gibco), non-essential amino-acid mixture (1:100, Gibco), gentamicin (45 μg/ml, Gibco), penicillin (100 U/ml, Gibco), streptomycin (100 μg/ml, Gibco), amphotericin B (1.5 μg/ml, Gibco) and FBS (0.5%, Gibco)), supplemented with collagenase P (0.75 mg/ml, Boehringer, Mannheim, Germany), papain (1 mg/ml, Boehringer Mannheim) and soybean trypsin inhibitor (1 mg/ml, Sigma-Aldrich). 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 type I-coated 24-well culture plates were prepared as described (Dekkers et al., 2007; Dekkers et al., 2010). BTSM cells were plated on uncoated or collagen I (50 μg/ml)-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. Cells were pretreated for 1 h with fluticasone, budesonide or dexamethasone, in the absence or presence of fenoterol, before stimulation with PDGF. After the 4 day incubation, cells were washed twice with PBS and incubated with HBSS containing 5% vol/vol Alamar blue solution (Biosource, Camarillo, USA). Conversion of Alamar blue into its reduced form by mitochondrial cytochromes was quantified by fluorimetric analysis, as indicated by the manufacturer. Cell numbers were calculated as percentage of Alamar blue conversion by untreated, unstimulated cells (basal, control).

RNA isolation and real-time quantitative RT-PCR

BTSM cells were plated in 6-well plates and grown until confluence. After serum deprivation for 3 days hours in Medium Zero supplemented with insulin (1 μM), the cells were stimulated with fluticasone (100 pM) alone or in combination with fenoterol (10 nM) for 19 hours, the last 18 hours in the absence or presence of PDGF (10 ng/ml). Total cellular RNA was isolated using the Nucleospin RNA II kit (Machery-Nagel, Düren, Germany). RNA concentration was determined by Nanodrop ND1000 (Thermo Scientific, Wilmington, USA). Total RNA was reverse transcribed using the Promega cDNA synthesis kit. Real-time quantitative PCR for p21WAF1/CIP1 (cyclin-dependent kinase inhibitor 1A) and p57KIP2 (cyclin-dependent kinase inhibitor 1C) was performed using an Eco Personal qPCR System (Illumina, Eindhoven, The Netherlands) using the specific primers listed in Table 1. Cycle parameters were: denaturation at 94°C for 30 seconds,
annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds for 40 cycles. Real-time PCR data were analyzed using the comparative cycle threshold ($C_q$ = amplification cycle number) method. The amount of target gene normalized to an endogenous reference (18S rRNA, designated as $\Delta C_q$) and relative to a calibrator (basal control) is given by the equation $2^{-\Delta\Delta C_q}$

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 Student-Newman–Keuls multiple comparisons test. Significance was reached at $p<0.05$. 
Results

Effects of glucocorticosteroids on PDGF- and collagen I-induced hypocontractility and proliferation.

To assess whether glucocorticosteroids inhibit the induction of a hypocontractile ASM phenotype, BTSM strips were incubated with PDGF-AB (10 ng/ml) or monomeric collagen I (50 μg/ml) in the absence and presence of fluticasone (10 nM), budesonide (30 nM) or dexamethasone (100 nM-1 μM). PDGF and collagen I significantly decreased maximal contractile force (E_max) to methacholine compared to vehicle-treated controls (Figures 1-2, Figure 1 of the online data supplement). Fluticasone (Figure 1) and budesonide (Figure 1 of the online data supplement) both inhibited the decrease in E_max induced by PDGF (p<0.01, both glucocorticosteroids) and collagen I (p<0.05, both glucocorticosteroids). Dexamethasone (100 nM) attenuated the PDGF-induced decrease in E_max (Figure 2A, p<0.001), whereas the collagen I-induced decrease was unaffected (Figure 2B). At 1 μM, however, dexamethasone did inhibit the collagen I-induced decrease in E_max (Figure 2D, p<0.01). No effects of the glucocorticosteroids were observed under control conditions. Similar effects were observed for KCl-induced contractions (Table 1-3 of the online data supplement). The sensitivity for both contractile stimuli was unaffected by all treatments.

As we have previously shown that the decreases in E_max inversely correlate with the proliferative responses of BTSM cells (Dekkers et al., 2007; Gosens et al., 2002), we investigated whether glucocorticosteroids also inhibit BTSM cell proliferation. PDGF and monomeric collagen I increased BTSM cell number (Figures 1-2, p<0.01). Both fluticasone (Figure 1C) and budesonide (Figure 2 of the online data supplement) inhibited the PDGF- induced proliferation. In agreement with the effects on contractility,
PDGF-induced proliferation, but not collagen I-induced proliferation, was decreased by 100 nM dexamethasone ($p<0.05$, Figure 2C). At 1 µM, however, dexamethasone inhibited both PDGF- and collagen I-induced proliferation ($p<0.01$ and $p<0.05$, respectively, Figure 2E). To assure that potentially remaining steroid did not affect Alamar blue conversion by itself, BTSM cells were plated and serum deprived for 3 days, after which Alamar blue conversion was assessed in the absence and presence of fluticasone (10 nM), budesonide (30 nM) or dexamethasone (1 µM). No significant effects of any of the treatments were observed, indicating that these compounds did not affect Alamar blue conversion directly (Figure 2 of the online data supplement) and that the observed effects were due to inhibition of ASM proliferation. No effects of the glucocorticosteroids were observed on basal proliferation either.

**Fenoterol and fluticasone synergize to inhibit 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 a low concentration of fenoterol (10 nM), a 100-fold lower concentration of 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 in methacholine-induced $E_{\text{max}}$. However, combined treatment with fenoterol and fluticasone fully inhibited the PDGF- and collagen I-induced reduction in $E_{\text{max}}$ ($p<0.01$ and $p<0.05$, respectively; Figures 3A and 3B). Of note, no effects were observed for fenoterol, fluticasone or the combination under control conditions (Figure 3 of the online data supplement). For KCl-induced contractions, the reduction in $E_{\text{max}}$ by fenoterol and fluticasone appeared to be additive, whereas fenoterol by itself significantly reduced the effect of collagen I ($p<0.05$, Table 4 of the online data...
The sensitivity to either contractile stimulus was unaffected by all treatments.

Treatment of BTSM cells with fenoterol (10 nM) did not inhibit PDGF- or collagen-induced proliferation (Figure 3C). Fluticasone (100 pM) significantly ($p<0.01$) reduced PDGF-induced proliferation, while no effects were observed on collagen I-induced proliferation. Combined treatment with fenoterol and fluticasone completely prevented the increase in proliferation 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.

**Fenoterol and fluticasone synergize to prevent the PDGF-induced decrease in expression of the cell cycle inhibitors.**

Previous studies have shown that glucocorticosteroids and β2-agonists synergize to increase the expression of the cell cycle inhibitors p57$^\text{KIP2}$ and P21$^\text{WAF1/CIP1}$ (Kaur et al., 2008; Roth et al., 2002). To investigate whether these inhibitors also contributed to the inhibition of PDGF-induced phenotype switching, BTSM cells were pretreated with low concentrations of fluticasone (100 pM), fenoterol (10 nM) or the combination of both for 1 hour after which the cells were stimulated in the absence or presence of PDGF (10 ng/ml) for 18 hours. Treatment with PDGF significantly ($P<0.001$) decreased mRNA expression of p57$^\text{KIP2}$, whereas a trend ($P=0.06$) towards a decreased expression was observed for p21$^\text{WAF1/CIP1}$ (Figure 4). Whereas fenoterol and fluticasone alone were without effect, the combination of the two drugs significantly ($P<0.05$) inhibited the PDGF-induced decrease in expression of the two cell cycle inhibitors. In addition, the combination of fenoterol and fluticasone significantly ($P<0.05$) increased basal mRNA expression of p21$^\text{WAF1/CIP1}$, but not of p57$^\text{KIP2}$. Treatment with fenoterol or fluticasone did not change basal p57$^\text{KIP2}$ or p21$^\text{WAF1/CIP1}$ mRNA expression by themselves.
Fenoterol and fluticasone synergize to inhibit the induction of a hypercontractile phenotype by insulin.

To investigate whether fluticasone and fenoterol also synergize to inhibit the induction of a hypercontractile ASM phenotype, BTSM strips were incubated with insulin (1 µM) in the absence and presence of fenoterol (10 nM), fluticasone (100 pM) or the combination of both. In line with previous studies (Gosens et al., 2003; Dekkers et al., 2009), insulin increased $E_{\text{max}}$ to methacholine compared to controls (Figure 5). Treatment with fenoterol alone did not affect the insulin-induced increase in $E_{\text{max}}$. Surprisingly, treatment with fluticasone (100 pM), in the absence of fenoterol, already fully inhibited the insulin-induced hypercontractile phenotype ($p<0.01$). No additional effect of combined treatment with fenoterol was observed. After combined treatment, a small but significant decrease in sensitivity to methacholine was observed compared to control ($p<0.05$, Table 5 of the online data supplement). No effects on sensitivity to methacholine were observed for other treatments. Similar effects were observed for KCl-induced contractions (Table 5 of the online data supplement).

To assess whether β2-agonists and glucocorticosteroids could synergize to inhibit the induction of a hypercontractile BTSM phenotype, the concentration of fluticasone was lowered to 3 pM. At this concentration, the insulin-induced hypercontractile phenotype was unaffected (Figure 5B). Combined treatment with fenoterol (10 nM), however, fully inhibited the induction of a hypercontractile phenotype ($p<0.01$). Similar effects were observed for KCl-induced contractions (Table 5 of the online data supplement). The sensitivity to either contractile stimulus was unaffected by all treatments.
Discussion

In the present study, we demonstrate that glucocorticosteroids and β₂-agonists synergize to inhibit ASM phenotype switching. The induction of a proliferative, hypocontractile ASM phenotype by PDGF and monomeric collagen type I was inhibited by the glucocorticosteroids fluticasone, budesonide and dexamethasone. The effects of collagen type I, however, were less susceptible to glucocorticosteroid action. The induction of a hypercontractile phenotype by insulin was inhibited by fluticasone as well.

At low concentrations, fenoterol and fluticasone synergized to prevent ASM phenotype switching in response to both mitogens, which was associated with an inhibition of the reduction of transcription of the cell cycle inhibitors p57\(^{kip2}\) and p21\(^{waf1/cip1}\) in response to PDGF. Remarkably, at even lower concentrations, fluticasone synergized with fenoterol to inhibit the induction of a hypercontractile ASM phenotype by insulin.

ASM accumulation is considered to be a major contributor to airway hyperresponsiveness and lung function decline in asthma (Lambert et al., 1993; Oliver et al., 2007). Exposure of ASM tissue and cells to mitogens \textit{in vitro} induces a switch from a (normo)contractile to a hypocontractile phenotype, which is associated with increased proliferation (Gosens et al., 2002; Dekkers et al., 2007). In addition, exposure of intact ASM tissue to growth factors or extracellular matrix proteins, such as monomeric collagen type I and fibronectin, results in a decreased contractility as a result of reduced contractile protein expression in BTSM tissue (Dekkers et al., 2007; Roscioni et al., 2011a). Recently, we also showed that ASM phenotype switching in response to mitogens not only has a functional impact on ASM tissue from bovine origin, but also from human origin (Dekkers et al., 2012; Roscioni et al., 2011b).

ASM proliferation is inhibited by glucocorticosteroids (Stewart et al., 1995; Roth et al., 2002; Fernandes et al., 1999). In the present study, the glucocorticosteroids...
Dexamethasone, budesonide and fluticasone all inhibited both PDGF-induced hypomotility of intact BTSM strips and BTSM cell proliferation, indicating that these glucocorticosteroids also inhibited growth factor-induced ASM phenotype switching. Similarly, hypomotility and proliferation induced by monomeric collagen type I were inhibited, although these effects were less susceptible to glucocorticosteroids, as indicated by the reduced effects of lower concentrations of dexamethasone (100 nM) and fluticasone (100 pM). This finding corresponds to previous observations, showing that bFGF-induced proliferation of human ASM cells cultured on collagen type I was resistant to dexamethasone (100 nM) and fluticasone (1 nM) (Bonacci et al., 2003b; Bonacci and Stewart, 2006). These impaired anti-mitogenic effects of glucocorticoids appear to be independent of the responsiveness to specific glucocorticoid signalling mechanisms, as production of pro-inflammatory cytokines by the ASM cells cultured on collagen type I was still sensitive to glucocorticoid treatment (Bonacci et al., 2003b). Previous studies in bovine ASM indicate that dexamethasone did inhibit bFGF-induced proliferation on collagen type I (Bonacci et al., 2003a), suggesting that ASM cells of bovine origin may remain more sensitive to glucocorticosteroid treatment.

In addition to their anti-mitogenic effects, the glucocorticosteroids also prevented PDGF- and collagen type I-induced hypomotility, without effects on maximal contraction or sensitivity in response to methacholine and KCl in vehicle-pretreated strips, suggesting that these drugs do not affect contractile responsiveness per se. In addition, the induction of a hypercontractile phenotype by insulin was also fully inhibited by fluticasone, which is in line with previous findings showing that glucocorticosteroids inhibit TGF-β-induced sm-α-actin accumulation in ASM cells (Goldsmith et al., 2007). Collectively, these findings suggest that glucocorticoids inhibit switching towards both a hypomotile and a hypercontractile phenotype - both may be present in asthmatics (Ebina et al., 1993; Leguillette et al., 2008) - and support the maintenance of a
normocontractile ASM phenotype, as present in non-asthmatics. In support of the maintenance of such a phenotype in vivo, budesonide has been shown to inhibit ASM hyperplasia and hypercontractility in a guinea pig model of chronic asthma (Bos et al., 2007). Collectively, these findings may explain the beneficial effects of long term treatment with glucocorticosteroids on airway hyperresponsiveness in asthmatic patients (Haahtela, 1998).

β2-Adrenoceptor agonists are the primary treatment for the relief of bronchospasm in asthma and COPD (Deshpande and Penn, 2006). In addition, combined treatment of glucocorticosteroids with β2-agonists results in more effective therapeutic management of asthma and COPD than monotherapy (Giembycz et al., 2008; Barnes, 2002). In human lung, glucocorticoids have been shown to increase transcription of the β2-adrenoceptor gene (Mak et al., 1995). Furthermore, in ASM cells, glucocorticosteroids and β2-agonists synergize to accelerate nuclear translocation of the glucocorticoid receptor and C/EBPα, resulting in the synergistic enhancement of the promotor activity of the gene encoding the cell cycle inhibitor p21WAF1/CIP1 and subsequent inhibition of proliferation (Roth et al., 2002). In addition, β2-agonists synergistically enhance glucocorticosteroid response element-dependent transcription of other genes as well, amplifying the transcription of multiple anti-inflammatory genes, including the cyclin-dependent kinase inhibitor p57KIP2 (Kaur et al., 2008), which has been shown to be involved in the anti-proliferative effects of glucocorticosteroids in HeLa cells (Samuelsson et al., 1999). In our current study, we clearly show that combined treatment with low concentrations of fenoterol and fluticasone synergistically inhibited the induction of a hypocontractile, proliferative phenotype in response to PDGF- and collagen type I and maintained a normocontractile ASM phenotype. This inhibition was associated with a normalization of the PDGF-induced reduction of both cyclin-dependent kinase inhibitors p57KIP2 and p21WAF1/CIP1, indicating that in bovine ASM similar processes are
operative as observed in human ASM (Roth et al., 2002; Kaur et al., 2008). In addition, similar to previous findings (Roth et al., 2002), treatment with both drugs synergistically enhanced basal mRNA expression of p21^{WAF1/CIP1}, whereas no effects were observed on the mRNA expression of p57^{KIP2}. The latter finding is in contrast with previous findings showing an synergistic enhancement of expression of this gene under basal conditions (Kaur et al., 2008), which may be explained by the fact that in those studies high concentrations of dexamethasone and β2-agonists were used, whereas in the current study only very low concentrations of both types of agonists were used.

In addition to increased ASM mass, increased expression of contractile proteins in ASM cells may contribute to increased contractility in asthmatics (Leguillette et al., 2008; Ma et al., 2002). Previously, we have shown that exposure of ASM to insulin results in the induction of a hypercontractile phenotype characterized by increased contractile responses, increased contractile protein expression and reduced proliferation (Gosens et al., 2003; Dekkers et al., 2009; Schaafsma et al., 2007). Our current data indicate that fenoterol and fluticasone not only synergize to inhibit the induction of a hypocontractile phenotype by PDGF and collagen type I, but also the induction of a hypercontractile ASM phenotype by insulin. Moreover, the induction of this phenotype was more sensitive to inhibition by these drugs than the hypocontractile ASM phenotype. This higher sensitivity may be explained by the fact that insulin increases the expression of laminin α2, β1 and γ1 chains, which is required for the increased contractility (Dekkers et al., 2009); previous studies have indicated that the sensitivity to glucocorticosteroids is preserved when ASM cells are cultured on laminin (Bonacci et al., 2003b). On the other hand, PDGF has been shown to increase collagen production by vascular smooth muscle cells (Park et al., 2005). Similarly, PDGF-induced collagen production by ASM cells could explain the reduced sensitivity of this cell to glucocorticosteroid action. In addition, glucocorticosteroids inhibit multiple steps in the insulin signalling cascade (van
Raalte et al., 2009), including phosphoinositol-3 kinase, which is essential for the insulin-induced hypercontractile phenotype (Schaafsma et al., 2007; Dekkers et al., 2009).

Increased ASM mass is likely to be the most important factor in increased airway resistance and airway hyperresponsiveness in asthma (Lambert et al., 1993; Oliver et al., 2007). Our findings may contribute to the increased therapeutic efficacy of combined treatment with β₂-agonists and glucocorticosteroids (Giembycz et al., 2008), by effectively reducing the increase in ASM mass, ASM hypercontractility and development of persistent airway hyperresponsiveness. In addition, airway remodelling in asthmatics is characterized by increased deposition of collagen type I beneath the epithelial basement membrane and surrounding the ASM bundles (Roche et al., 1989; Bai et al., 2000). The monomeric form of collagen type I may contribute to ASM accumulation by increasing proliferation (Dekkers et al., 2007; Bonacci et al., 2003a; Hirst et al., 2000a; Dekkers et al., 2010). Conversely, fibrillar collagen type I does not promote proliferation (Nguyen et al., 2005) or even decreases basal and growth factor-induced proliferation (Schuliga et al., 2010). Degradation of fibrillar collagen type I to its monomeric forms by matrixmetalloproteases may thus enhance ASM proliferation and contribute to ASM hyperplasia (Schuliga et al., 2010; Dekkers et al., 2010). In addition, monomeric collagen type I may also contribute to ASM accumulation by rendering the ASM resistant to the anti-mitogenic actions of glucocorticosteroids (Bonacci et al., 2003b; Bonacci and Stewart, 2006), which may explain poor control of a subgroup of severe asthmatics by glucocorticosteroids (Barnes, 2004). Our current findings, showing that combined treatment with fluticasone and fenoterol synergistically normalizes not only PDGF-, but also collagen type I-induced ASM hypocontractility and proliferation, suggests that, in addition to increasing ant-inflammatory, bronchodilating and anti-proliferative effects, normalizing steroid sensitivity by β₂-agonists contributes to the enhanced asthma control by combination therapy (Giembycz et al., 2008).
In conclusion, our results demonstrate that glucocorticosteroids and \( \beta_2 \)-agonists synergize to prevent the induction of both a proliferative and of a hypercontractile phenotype and maintain a normocontractile ASM phenotype. As ASM accumulation and increased ASM contractility are considered to contribute importantly to airway hyperresponsiveness, these findings may explain the enhanced efficacy of the combination therapy in asthma.
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Authorship contributions

Participated in research design: BGJD, AP, HM, JZ.

Conducted experiments: BGJD, AP, RM, ISTB.

Performed data analysis: BGJD, AP, RM, ISTB, HM, JZ.

Wrote or contributed to the writing of the manuscript: BGJD, AP, HM, JZ.
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Footnotes

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Figure legends

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. Cell numbers were calculated as percentage of Alamar blue conversion by untreated, unstimulated cells (basal, control). 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.

Figure 2: 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. Cell numbers were calculated as percentage of Alamar blue conversion by untreated, unstimulated cells (basal, control). 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.

**Figure 3:** Low concentrations of fluticasone and fenoterol synergistically prevent 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. Cell numbers were calculated as percentage of Alamar blue conversion by untreated, unstimulated cells (basal, control). Data represent means±SEM of 5 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 fluticasone and fenoterol.

**Figure 4:** Low concentrations of fluticasone and fenoterol synergize to prevent the PDGF-induced reduction of the cell cycle inhibitors p57KIP2 and p21WAF1/CIP1. Effects of fenoterol (10 nM), fluticasone (100 pM) or the combination of both on (A) p57KIP2 and (B) p21WAF1/CIP1 mRNA expression after stimulation in the absence and presence of PDGF.
(10 ng/ml). *p<0.05, ***p<0.001 compared to basal control. #p<0.05 compared to PDGF in the absence of fluticasone and fenoterol.

Figure 5: Low concentrations of fenoterol and fluticasone synergistically prevent the induction of a hypercontractile phenotype by insulin (A) Concentration-response curves of methacholine-induced contractions of BTSM strips, pretreated with vehicle (control) or insulin (1 μM), in the absence or presence of fenoterol (10 nM), fluticasone (100 pM), or the combination of both for 8 days. Data represent means±SEM of 4 independent experiments, each performed in duplicate. (B) Concentration-response curves of methacholine-induced contractions of BTSM strips, pretreated with vehicle (control) or insulin (1 μM), in the absence or presence of fenoterol (10 nM), fluticasone (3 pM), or the combination of both for 8 days. Data represent means±SEM of 5 independent experiments, each performed in duplicate. *p<0.05, **p<0.01 compared to vehicle-treated (control). #p<0.01 compared to insulin in the absence of fluticasone and
### Tables

**Table 1:** Primer sequences for p21\textsuperscript{WAF1/CIP1}, p57\textsuperscript{KIP2} mRNA and 18S rRNA used for real-time quantitative PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI Accession</th>
<th>Primer Sequence</th>
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| p21\textsuperscript{WAF1/CIP1} | NM_001098958 | Forward 5' GGG GGT GCC CTA ACC CCC AA 3'  \[
|            |                | Reversed 5' AGG ACC CCC ACC CCA AGA GC 3' |
| p57\textsuperscript{KIP2}      | NM_001077903  | Forward 5' GCC AAG GCG TCG AAC GAG GT 3'  \[
|            |                | Reversed 5' ACA TGG ACG GTC CCA GCC GA 3' |
| 18S rRNA   | AF176811       | Forward 5' AAA CGG CTA CCA CAT CCA AG 3'  \[
|            |                | Reversed 5' TCG CGG AAG GAT TTA AAG TG 3' |
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A

- Control
- Fenoterol 10 nM
- Fluticasone 100 pM
- Fenoterol + Fluticasone

B

- p21WAF1/CIP1 mRNA (% of control)
- Basal
- PDGF

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