Immunobiology 224 (2019) 490–496

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

# Airway smooth muscle cells are insensitive to the anti-proliferative effects of corticosteroids: The novel role of insulin growth factor binding Protein-1 in asthma

Hong Bui[a], Yassine Amrani[b], Brian Deeney[c], Reynold A. Panettieri[c], Omar Tliba[d,*]

[a] Penn Health System, Microbiology laboratories, University of Pennsylvania, Philadelphia, PA, USA
[b] Institute for Lung Health, Department of Infection, Inflammation and Immunity, University of Leicester, United Kingdom
[c] Rutgers Institute of Translational Medicine and Sciences, Rutgers School of Medicine, New Brunswick, NJ, USA
[d] Department of Biomedical Sciences, College of Veterinary Medicine, Long Island University, Brookville, NY, USA

ARTICLE INFO

*Keywords:*
Asthma
Glucocorticoid insensitivity
Airway smooth muscle
Proliferation
Growth
Airway remodeling

ABSTRACT

Airway remodeling in asthma manifests, in part, as enhanced airway smooth muscle (ASM) mass, due to myocyte proliferation. While the anti-proliferative effects of glucocorticoid (GC) were investigated in normal ASM cells (NASMC), little is known about such effects in ASM cells derived from asthma subjects (AASMC). We posit that GC differentially modulates mitogen-induced proliferation of AASMC and NASMC. Cells were cultured, starved, then treated with Epidermal growth factor (EGF) (10 ng/ml) and Platelet-derived growth factor (PDGF) (10 ng/ml) for 24 h and/or fluticasone propionate (FP) (100 nM) added 2 h before. Cell counts and flow cytometry analyses showed that FP failed to decrease the cell number of and DNA synthesis in AASMC irrespective of mitogens used. We also examine the ability of Insulin Growth Factor Binding Protein-1 (IGFBP-1), a steroid-inducible gene that deters cell growth in other cell types, to inhibit proliferation of AASMC where FP failed. We found that FP increased IGFBP1 mRNA and protein levels. Interestingly, the addition of IGFBP1 (1 μg/ml) to FP completely inhibited the proliferation of AASMC irrespective to the mitogens used. Further investigation of different signaling molecules involved in ASM growth and GC receptor functions (Protein kinase B (PKB/AKT), Mitogen-activated protein kinases (MAPKs), Focal Adhesion Kinase (FAK)) showed that IGFBP-1 selectively decreased mitogen-induced p38 phosphorylation in AASMC. Collectively, our results show the insensitivity of AASMC to the anti-proliferative effects of GC, and demonstrate the ability of IGFBP1 to modulate AASMC growth representing, hence, a promising strategy to control ASM growth in subjects with GC insensitive asthma.

## 1. Introduction

Asthma is a chronic airway inflammatory disease characterized by shortness of breath, chest tightness, coughing, and wheezing (Panettieri, 2016). While inhaled glucocorticoids (GC) are the most commonly used treatment in asthma, some patients with severe asthma are insensitive to GC therapy (Marwick et al., 2010). Consequently, such patients contribute disproportionately to the health care costs and morbidity associated with asthma (Moore and Peters, 2006). Hence, significant research efforts aiming at defining the mechanism(s) underlying GC insensitivity, more importantly, strategies that reestablish GC responsiveness are needed.

Studies showed that patients with severe asthma benefited from bronchial thermoplasty, a therapy that attenuates bronchoconstriction by reducing airway smooth muscle (ASM) mass; this finding and that of increased ASM mass in bronchial biopsies obtained from patients with asthma (Bentley et al., 2009; Bentley and Hershenson, 2008) supports the notion that ASM is a critical player in the pathogenesis of asthma (Wahidi and Kraft, 2012; Panettieri, 2015). Mitogens such as Platelet-Derived Growth Factor (PDGF) and Epidermal Growth Factor (EGF) modulate ASM growth (Stamatiou et al., 2012; Vlahos et al., 2003). The molecular mechanisms modulating ASM cell proliferation involve numerous signaling pathways. For example, to promote cell growth, PDGF and EGF bind to receptors with intrinsic tyrosine kinase activity (RTK) (reviewed in (Tliba and Panettieri, 2009)) and promote the activation of serine-threonine kinases AKT and mitogen-activated protein kinases (MAPKs). Ultimately, via the phosphorylation of other intermediate molecules, the upregulation of cyclin D1 facilitates ASM cells transition through the cell cycle (Tliba and Panettieri, 2009). Interestingly, the ability of some mitogens to promote growth of normal ASM cells is

* Corresponding author at: Department of Biomedical Sciences, College of Veterinary Medicine, Long Island University, Brookville, NY, USA.
*E-mail address:* omar.tliba@liu.edu (O. Tliba).

https://doi.org/10.1016/j.imbio.2019.05.006
Received 29 March 2019; Received in revised form 20 May 2019; Accepted 21 May 2019
Available online 23 May 2019

sensitive to the anti-proliferative effects of GC, although differentially with thrombin being more sensitive to GC than EGF (Vlahos et al., 2003; Stewart et al., 1995; Dekkers et al., 2012). Yet, little is known about the anti-proliferative effects of GC on ASM cells derived from asthma subjects.

GC acts through different molecular mechanisms of action. The two most well-described are transrepression and transactivation (van der Velden, 1998). When GC binds to its receptor in the cytoplasm, the GC receptor (GR) is released from heat shock protein (HSP)-90 complex to translocate to the nuclear compartment of the cell. Transrepression occurs when the GR binds to the cis-element of inflammatory transcription factors such as activating protein-1 (AP-1) and nuclear factor κB (NF-κB) to inhibit the expression of pro-inflammatory genes. Transactivation occurs when the GR binds to the GC response element (GRE) on the promoter of GC inducible genes, such as MAPK phosphatase 1 (MKP-1) (Quante et al., 2008), glucocorticoid-induced leucine zipper (GILZ) (Eddleston et al., 2007), and insulin-like growth factor binding protein-1 (IGFBP-1) (Lee et al., 1997; Suh and Rechler, 1997; Suh et al., 1995, 1996) to induce their transcription. Importantly, while the role of MKP-1 and GILZ in mediating GC effects has been extensively investigated, the role of IGFBP-1 in mediating GC effects has been poorly investigated.

IGFBP1 has IGF1-dependent and -independent activities. IGF1-dependent activity of IGFBP1 involves its binding to IGF1 and subsequently modulates IGF1 cellular actions (Kajimura et al., 2005). IGF1-independent activities involve IGFBP-1′s RGD (Arg-Gly-Asp) motif present in the C-terminal domain. RGD is a ligand of integrin $\alpha_5\beta_1$ that acts independently of IGF receptors (Jones et al., 1993). Several studies investigated the role of IGFBP1 in modulating cell growth. For instance, IGFBP-1 decreases the growth of breast cancer cells in response to estrogen and serum (Van den Berg et al., 1997) and the thymidine uptake in rat aortic smooth muscle cells (Motani et al., 1995). Evidence suggests that IGFBP-1 downregulates the expression of key proteins associated with cell growth such as ERK and AKT in fibroblasts (Prokop et al., 2013). However, the expression and the role of IGFBP-1 in modulating ASM cell responses remains unknown.

Because increased ASM mass in asthma is a critical component of asthma pathogenesis, therapeutic strategies aiming at reducing ASM cell growth will likely improve the therapeutic outcome. The current study demonstrates that anti-proliferative effects of GC in ASM cells is impaired in cells derived from asthma subjects but restored in the presence of IGFBP1, a steroid inducible gene. Thus, targeting IGFBP1 represents a promising strategy to deter GC-insensitive features in asthma.

## 2. Materials and methods

### 2.1. Cell culture

Primary human bronchial ASM cells derived from asthma (AASMC) and non-asthma (NASMC) subjects were bought from Lonza (Walkersville, MD). Briefly, Human Bronchial Smooth Muscle Cells (BMSC) were isolated from the major bronchial tissue of normal or mild asthmatic donors and used from passage 2 onward. All cells were tested negative for mycoplasma, bacteria, yeast, and fungi. HIV-1, hepatitis B and hepatitis C were not detected in all donors. Cells were then cultured as we previously described (Bouazza et al., 2014, 2012). Cells between passages 3 and 7 were used.

### 2.2. Cell count

ASM cells growth-arrested in serum free media for 48 h were treated with EGF 10 ng/ml and PDGF 10 ng/ml for 24 h in the presence or absence of Fluticasone Propionate (FP, 100 nM) added two hours prior treatments with growth factors. IGFBP-1 (1 μg/ml) was administered one hour before FP to assess its potential modulatory effect on the anti-proliferative effects of FP. After that, cells were trypsinized, pelleted by centrifugation, and suspended in a 15 ml conical tubes. Next, 10 μl of suspended cells were mixed with 10 μl of Trypan Blue stain (Life Technologies, Frederick, MD) and loaded onto chamber slides. The Countess II automated cell counter (Life Technologies) was used to perform cell count of viable cells. Of note, IGFBP-1 used at 1 μg/ml did not affect the viability of ASM cell viability as assessed by the MTT assay (data not shown).

### 2.3. Bromodeoxyuridine (BrdU) incorporation

ASM cells were treated as above. To measure DNA synthesis, BrdU (GE Healthcare, Mickleton, NJ) was added to culture media for 18 h before cells were trypsinized and pelleted by centrifugation. To denature DNA, cells were exposed to 2 N Hydrochloric Acid (HCl) for 30 min in 37 °C water bath. Next, cells were neutralized with 0.1 M Sodium Tetraborate and washed with Immunofluorescence Antibody Assay (IFA) buffer supplemented with HEPES Saline, Fetal Bovine Serum (FBS) (Hyclone Laboratories, Logan, Utah), and Sodium Azide (Sigma-Aldrich, St. Louis, MO). After that, cells were incubated with anti-BrdU fluorescence conjugated secondary antibody (BD Biosciences, San Jose, CA). Tween 20/IFA buffer at 0.5% was then used to wash the cells before they were pelleted by centrifugation and re-suspended in solution of Propidium Iodide and RNase (Sigma) for Flow Cytometric analysis on FACScalibur (BD Biosciences).

### 2.4. Immunoblot analysis

Growth-arrested ASM cells were exposed to FP (100 nM), then total proteins were extracted. Immunoblot analyses were conducted as we reported previously (Bouazza et al., 2014, 2012) using IGFBP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For normalization purposes, an anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology) was used to stain the corresponding stripped nitrocellulose membrane.

In parallel experiments, growth-arrested AASMC were treated with PDGF 10 ng/ml for 24 h in and/or FP (100 nM) and/or of IGFBP-1 (1 μg/ml) added 2 and 3 h before, respectively, then total proteins were extracted. Immunoblot analyses were conducted as we reported previously (Bouazza et al., 2014, 2012) using several phosphorylated antibodies against focal adhesion kinase (FAK), AKT, p38, ERK, c-Jun N-terminal kinase (JNK) (Cell signaling Technologies, Danvers, MA). For normalization purposes, anti-total FAK, AKT, p38, ERK1/2, or JNK (Santa Cruz Biotechnology) antibodies were used to stain the corresponding stripped nitrocellulose membrane. A quantification of the intensity of the western blot band signals was then conducted as we previously reported using ImageJ image processing software (National Institutes of Health, Bethesda, MD) (Bouazza et al., 2014, 2012).

### 2.5. Real-time PCR

Growth-arrested ASM cells were exposed to FP (100 nM) for different time points, then mRNA was extracted. Real-time RT-PCR experiments were conducted as we formerly reported (Bouazza et al., 2014, 2012) using predesigned primers for IGFBP-1 (Forward sequence CCAAGGCACAGGAGAGACATCA and Reverse AGGGTAGACGCACCAG CAGA) (Sigma) and previously published primers for β-actin (Sigma) (Bouazza et al., 2014, 2012). The data were analyzed using the comparative cycle threshold method as we previously reported (Bouazza et al., 2014, 2012).

### 2.6. Materials and reagents

Tissue culture reagents were purchased from Lonza. Human recombinant IGFBP-1 protein was obtained from R&D systems (Minneapolis, MN). IGFBP-1 antibody, EGF, and FP were provided by

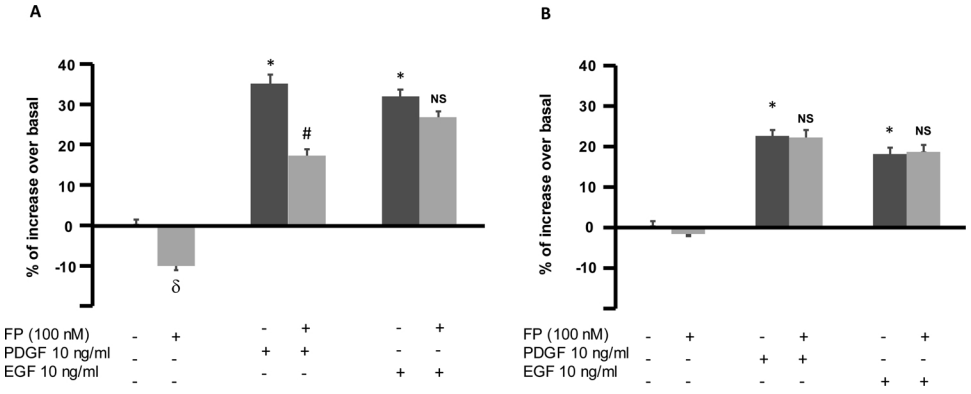


**Fig. 1.** Mitogen-induced increases in ASM cell number is differentially modulated by GC. (A) NASMC and (B) AASMC were exposed to PDGF (10 ng/ml) or EGF (10 ng/ml) for 24 h and/or FP (100 nM) added 2 h before. Cell count was measured as described in material and methods section. Results are presented as % of increase over basal. * $P < 0.05$ when compared to cells treated with diluent, δ $P < 0.05$ when compared to cells treated with diluent, # $P < 0.05$ when compared to cells exposed to mitogens, NS: not significant when compared to cells exposed to mitogens. Each set of experiments was performed in triplicate with a minimum of three different human ASM cell lines.

Sigma. PDGF was obtained from Millipore (Billerica, MA).

### 2.7. Statistics analysis

Intragroup differences were analyzed using ANOVA (Bonferroni-Dunn test) or *t*-test A P value < 0.05 was considered significant. Each set of experiments was performed in triplicate with a minimum of three different human ASM cell lines with "n" corresponding to the number of donors.

## 3. Results

### 3.1. Mitogens and FP differentially modulate ASM proliferation

First, we sought to examine GC effects on the ability of mitogens to increase NASMC number. As shown in Fig. 1A, PDGF and EGF significantly increased ASM cell number by 35% ± 3.2% and 32% ± 2.9% over basal, respectively. Interestingly, while FP significantly decreased by 50% PDGF-induced increase in ASM cell number, EGF-induced mitogenesis was unaffected. In AASMC (Fig. 1B), PDGF and EGF significantly increased ASM cell number by 25% ± 2.1% and 20% ± 1.8% over basal, respectively. Surprisingly, FP had little effect on AASMC number irrespective of the mitogens used. Collectively, these findings indicate that *mitogens and GC differentially modulate ASM proliferation and that* the ability of PDGF to promote ASM hyperplasia is insensitive to GC in cells obtained from patients with asthma.

### 3.2. Mitogens and FP differentially modulate ASM DNA synthesis

Next, we sought to examine GC effects on the ability of different mitogens to increase DNA synthesis in NASMC using BrdU incorporation assays. Flow cytometry analysis showed that PDGF and EGF significantly increased BrdU incorporation (Fig. 2A) by 28% ± 2.5% and by 25% ± 2.1% over basal, respectively. While the addition of FP significantly decreased by 44% PDGF ability to increase DNA synthesis, it had no significant effect on EGF-induced DNA synthesis. We also examined the effect of GC on the ability of different mitogens to increase DNA synthesis in AASMC. As shown in Fig. 2B, the addition of mitogens significantly increased DNA synthesis in AASMC (PDGF by 37% ± 3.1% and EGF by 32% ± 2.7%). Surprisingly, not only FP did not decrease but rather significantly increased DNA synthesis in AASMC irrespective of the mitogen used (PDGF by + 30% and EGF by + 35%). Collectively, these findings indicate that the failure of FP to suppress PDGF-induced increase cell number (Fig. 1B) may derive from its ability to increase DNA synthesis (Fig. 2B).

### 3.3. IGFBP1 is induced by GC in ASM cells

Since ASM growth is insensitive to GCs in AASMC (Figs. 1B and 2 B), we sought to explore strategies to reduce ASM cell growth. Previous studies reported that IGFBP-1, a GC-inducible gene in other cell types (Lee et al., 1997; Suh and Rechler, 1997; Suh et al., 1995, 1996), modulates cell proliferation in a cell-specific manner (van der Velden, 1998; Kajimura et al., 2005; Jones et al., 1993; Motani et al., 1995). As shown in Fig. 3A and B, FP significantly increased, in a time-dependent manner, the proteins and mRNA expression levels of IGFBP-1. Together, these findings indicate that IGFBP-1 is also a GC-inducible gene in ASM cells.

### 3.4. In combination, IGFBP1 and FP inhibit the proliferation of GC-resistant AASMC

In the following experiments, IGFBP1 was used at 1 μg/ml, a concentration shown to significantly interfere with the growth of aortic

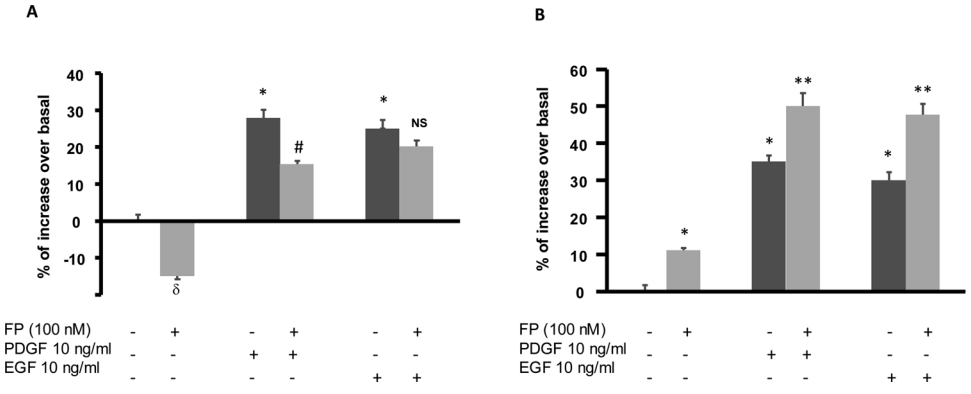


**Fig. 2.** Fluticasone failed to inhibit mitogen-induced increase in BrdU incorporation in AASMC. (A) NASMC and (B) AASMC were exposed to PDGF (10 ng/ml) or EGF (10 ng/ml) for 24 h and/or FP (100 nM) added 2 h before. BrdU was added for 18 h and its incorporation was measured as described in the material and methods section. Results are presented as % of increase over basal. * $P < 0.05$ when compared to cells treated with diluent, ** $P < 0.01$ when compared to cells treated with diluent, δ $P < 0.05$ when compared to cells treated with diluent, # $P < 0.05$ when compared to cells exposed to mitogens, NS: not significant when compared to cells exposed to mitogens. Each set of experiments was performed in triplicate with a minimum of three different human ASM cell lines.

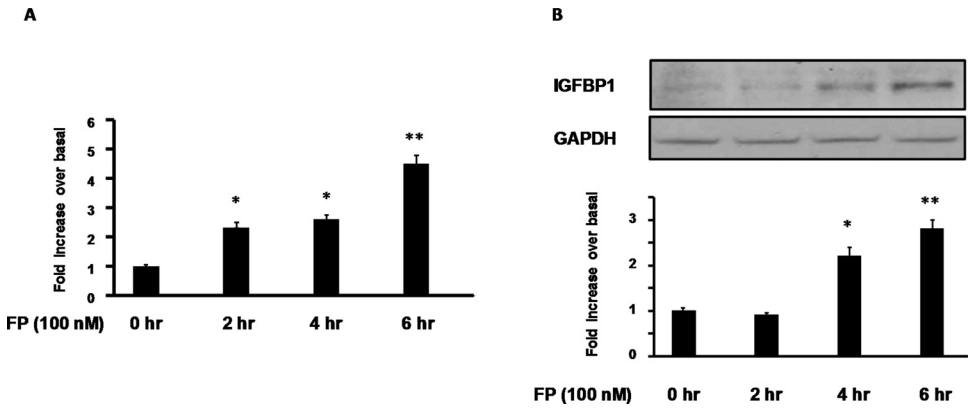


**Fig. 3.** Fluticasone increased the expression of IGFBP1 in ASM cells. Growth-arrested ASM cells were exposed to FP (100 nM) for different time points. (A) Total mRNA was extracted and IGFBP1 mRNA expression was assessed by real-time PCR. IGFBP1 mRNA levels were then normalized to that of GAPDH and are presented as fold increase over basal. Data are representative of three separate experiments. (B) Whole cell lysates were extracted and immunoblotted with anti-IGFBP1 and GAPDH specific antibodies. Results are representative of three separate blots *(top)*. Scanning densitometry of three representative immunoblots with each condition normalized over the area density of the corresponding GAPDH content. The results are expressed as the fold increase over basal values *(bottom)*. * $P < 0.05$ when compared to cells treated with diluent, ** $P < 0.01$ when compared to cells treated with diluent.

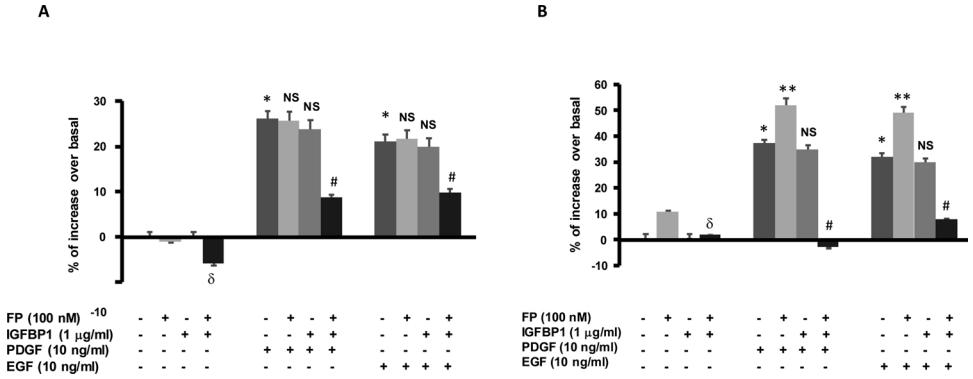


**Fig. 4.** The addition of IGFBP1 to glucocorticoid inhibited mitogen-induced proliferation of AASMC. (A) AASMC were exposed to PDGF (10 ng/ml) or EGF (10 ng/ml) for 24 h and/or FP (100 nM) and/or IGFBP1 (1 μg/ml) added 2 h and 3 h before, respectively. Cell count was assessed as described in material and methods section. Cell count was compared with that of obtained from cells treated with diluent alone (basal). Results are presented as % of increase over basal. (B) AASMC were treated as above and BrdU was added for 18 h and its incorporation was measured as described in the material and methods section. DNA synthesis was compared with that of obtained from cells treated with diluent alone (basal). Results are presented as % of increase over basal where basal. * $P < 0.05$ when compared to cells treated with diluent, ** $P < 0.01$ when compared to cells treated with diluent, δ $P < 0.05$ when compared to cells treated with diluent, # $P < 0.05$ when compared to cells exposed to mitogens, NS: not significant when compared to cells exposed to mitogens.

smooth muscle cells (Motani et al., 1995). Interestingly, while FP or IGFBP1 alone had no effect on a mitogens-induced increase in AASMC number, combining both IGFBP-1 to FP significantly decreased PDGF and EGF ability to increase ASM cell number by 67% and 53%, respectively (Fig. 4A). Similarly, when DNA synthesis was measured by BrdU incorporation assay, while FP or IGFBP1 alone had no effect on a mitogens-induced increase in DNA synthesis, the combination of IGFBP-1 with FP abrogated mitogens' ability to increase DNA synthesis in AASMC (Fig. 4B). Collectively, these findings demonstrate the ability of exogenous IGFBP-1 to restore the inhibitory effect of fluticasone in AASMC.

*IGFBP1 does not affect FAK phosphorylation in AASMC.* Since IGFBP1 modulation of cancer cells' growth involves FAK inhibition (Gleeson et al., 2001; Irwin and Giudice, 1998), we next assessed the phosphorylation of FAK in AASMC by immunoblot analysis. As shown in Fig. 5, no basal phosphorylation of FAK was detected in AASMC. While the addition of PDGF significantly increased FAK phosphorylation, the addition of FP and IGFBP1 either alone or in combination had no effect on FAK phosphorylation. These findings strongly suggest that IGFBP1 modulates AASMC growth in FAK-independent manner.

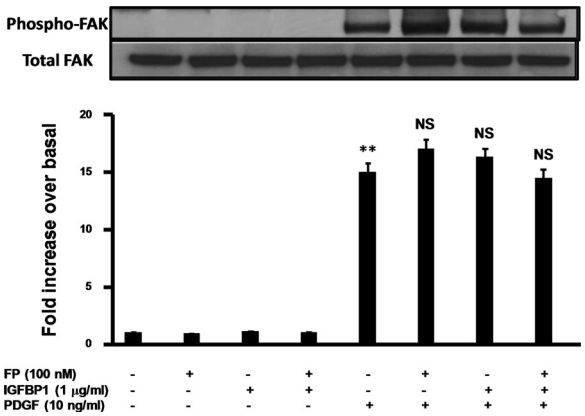


**Fig. 5.** The addition of IGFBP1 to glucocorticoid did not affect mitogen-induced FAK phosphorylation in AASMC. *Top,* AASMC were exposed to PDGF (10 ng/ml) for 24 h and/or FP (100 nM) and/or IGFBP1 (1 μg/ml) added 2 h and 3 h before, respectively. The whole cell lysate was then extracted and immunoblotted with anti-phospho-FAK and total FAK antibodies. Results are representative of three separate blots. *Bottom*, scanning densitometry of three representative immunoblots with each condition normalized over the area density of the corresponding total FAK content. The results are expressed as the fold increase over basal values. ** $P < 0.01$ when compared to cells treated with diluent, NS: not significant when compared to cells exposed to mitogens.

### 3.5. IGFBP1 had little effect on the phosphorylation of kinases commonly associated with AASMC growth

Different kinases have been associated with ASM growth including ERK1/2 and PI3K/AKT pathways (Ammit and Panettieri, 1985; Burgess et al., 2008; Hirst et al., 2004; Orsini et al., 1999). As shown in Fig. 6A, phosphorylation of AKT was not detected in untreated AASMC but significantly increased by PDGF, a response not affected by the addition of FP and IGFBP1 either alone or in combination. Similarly, low basal phosphorylation of ERK1/2 was detected in AASMC (Fig. 6B). While the addition of PDGF significantly increased ERK1/2 phosphorylation, the addition of FP and IGFBP1 either alone or in combination did not affect

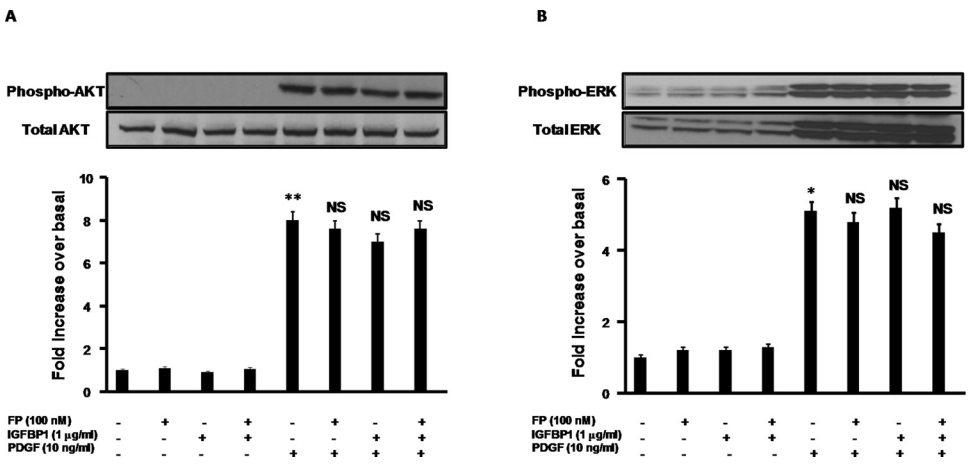


**Fig. 6.** The addition of IGFBP1 to fluticasone did not affect mitogen-induced AKT and ERK1/2 phosphorylation in AASMC. *Top,* AASMC were exposed to PDGF (10 ng/ml) for 24 h and/or FP (100 nM) and/or IGFBP1 (1 μg/ml) added 2 h and 3 h before, respectively. Whole cell lysate was then extracted and immunoblotted with antibodies against phospho-AKT and total AKT (A) or phospho-ERK1/2 and total ERK1/2 (B). Results are representative of three separate blots. *Bottom*, scanning densitometry of three representative immunoblots with each condition normalized over the area density of the corresponding total AKT (A) or ERK1/2 (B) content. The results are expressed as the fold increase over basal values. * $P < 0.05$ when compared to cells treated with diluent, ** $P < 0.01$ when compared to cells treated with diluent, NS: not significant when compared to cells exposed to mitogens.

ERK1/2 phosphorylation (Fig. 6B). Together, these data suggest that IGFBP1 modulates AASMC growth in AKT/PI3K and ERK1/2-independent manner.

### 3.6. IGFBP1 differentially affects the phosphorylation of kinases commonly associated with GR function in AASMC

Since IGFBP1 did not affect the phosphorylation of kinases commonly associated with ASM growth, we next sought determine whether IGFBP1 enhances GC actions by inhibiting kinases known to negatively regulate GR functions such JNK (Adcock et al., 1995; Rogatsky et al., 1998) and p38 (Bouazza et al., 2014). As shown in Fig. 7A, no basal phosphorylation of JNK was detected in AASMC. While the addition of PDGF, significantly increased JNK phosphorylation, the addition of FP and IGFBP1 either alone or in combination did not affect JNK phosphorylation. Interestingly, while low phosphorylation of p38 was detected in AASMC, the addition of IGFBP1 abrogated basal p38 phosphorylation irrespective of FP treatment (Fig. 7B, lane 3 and 4 versus lane 1). Similarly, IGFBP1 either alone (Fig. 7B, lane 7 versus lane 5) or in combination with FP (Fig. 7B, lane 8 versus lane 5), significantly decreased PDGF-induced p38 phosphorylation (by 76% and 94%, respectively). Collectively, these findings indicate that IGFBP1 differentially affects the phosphorylation of kinases commonly associated with GR function in AASMC.

## 4. Discussion

Evidence suggests ASM growth as a critical contributing factor in airway remodeling and narrowing in asthma (Bentley et al., 2009; Bentley and Hershenson, 2008). In the present study, we showed that mitogen-induced cell growth in AASMC was insensitive to GC actions. Interestingly, the addition of exogenous IGFBP1 to fluticasone restored its ability to inhibit cell growth suggesting a new novel therapeutic strategy to treat the main GC insensitive feature contributing to airway remodeling.

In our study, we used both normal and asthmatic cells to introduce the new concept that specific differences with regard to response to growth factors are dependent on health status of the subjects. Indeed, when EGF was used as a stimulus, responses of ASM cells were insensitive to GC effects irrespective of ASM cells origin. However, when PDGF was used as a stimulus, the responses of ASM cells were sensitive to GC only in ASM from non-asthmatic subjects but not in ASM cells from asthmatic subjects. Hence, PDGF stimulation was used in the rest of study as a model to examine the differences in steroid responsiveness between ASM cells from healthy (sensitive) versus asthmatics (insensitive) and to explore ways to restore steroid responsiveness in PDGF-treated cells from asthmatics. In the case of EGF, we believe that EGF is interfering somehow with GR signaling thus explaining why GC failed to modulate responses of ASM cells from both asthmatic and non-

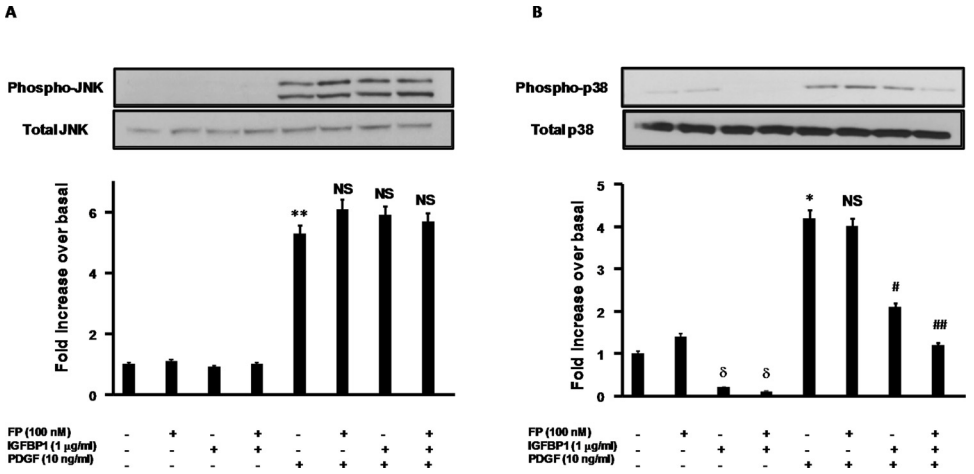


**Fig. 7.** The addition of IGFBP1 to glucocorticoid differentially modulate mitogen-induced JNK and p38 phosphorylation in AASMC. *Top,* AASMC were exposed to PDGF (10 ng/ml) for 24 h and/or FP (100 nM) and/or IGFBP1 (1 μg/ml) added 2 h and 3 h before, respectively. Whole cell lysate was then extracted and immunoblotted with antibodies against phospho-JNK and total JNK (A) or phospho-p38 and total p38 (B). Results are representative of three separate blots. *Bottom*, scanning densitometry of three representative immunoblots with each condition normalized over the area density of the corresponding total JNK (A) or p38 (B) content. The results are expressed as the fold increase over basal values. * $P < 0.05$ when compared to cells treated with diluent, ** $P < 0.01$ when compared to cells treated with diluent, δ $P < 0.05$ when compared to cells treated with diluent, # $P < 0.05$ when compared to cells exposed to mitogens, NS: not significant when compared to cells exposed to mitogens.

asthmatic subjects. Whether this is because the cells from non-asthmatic subjects are abnormal or because EGF is naturally insensitive to GC warrants further investigation.

Steroid responsiveness in asthma varies according to the stage of the disease. For instance, mild persistent asthma are typically sensitive to the steroid anti-inflammatory actions unlike severe persistent asthma that is often insensitive (Chung, 2014). Steroid responsiveness in asthma is usually assessed by examining changes in immunological/inflammatory markers (Chung, 2014) but does not apply to non-immunological parameters such as remodeling features which are often insensitive to steroid therapy even in mild asthmatics. We here show that the proliferation of ASM cells derived from mild asthmatics were insensitive to fluticasone when compared to cells derived from healthy subjects. Perry and colleagues (2014) also showed cell growth induced by another mitogen (TGFβ) was also insensitive to dexamethasone in ASM cells derived from mild asthmatics (Perry et al., 2014). Interestingly, using the same cells, the authors showed that TGFβ-induced cytokine secretion was still sensitive to steroid actions (Perry et al., 2014).

Interestingly, we found that GC enhanced PDGF-induced DNA synthesis in AASMC (Fig. 2B). Misior and colleagues (2008) also found that GC dramatically increase growth of healthy ASM cells treated with EGF in the presence of cytokines (IL1β and/or TNFα) (Misior et al., 2008) or treated with bFGF and cytokine combination (Vlahos and Stewart, 1999). The underlying mechanisms affecting the anti-mitogenic action of GC action in ASM cells remain unknown, but could be due to a decrease expression of CCAAT/enhancer binding protein (C/EBP) alpha previously suggested by others (Borger et al., 2009). Evidence from cancer cell lines suggest the implications of kinases such as GSK3β which can phosphorylate GR at key residues and directly impairs GC actions (Galliher-Beckley et al., 2008). Whether the activation of such kinases in AASMC interferes with GC anti-proliferative functions merits further investigation.

Several earlier reports showed IGFBP1 is a GC-target/inducible gene in different cell types, in part due to several GRE sites present in the IGFBP1 promoter (Lee et al., 1997; Suh and Rechler, 1997; Suh et al., 1995, 1996). In addition to being increased *in vitro* by GC in U2OS/A549 cell lines (Rogatsky et al., 2003) or hepatocytes (Lee et al., 1997), increased systemic levels can also be detected in the bloodstream of infants (Dost et al., 2007) or in the serum of rats following oral dexamethasone treatment (Luo et al., 1990). Our report is the first to show that GC rapidly enhanced the expression of IGFBP1 in ASM cells (within 4 h), as a result of increased gene transcription (Fig. 3**).** We also showed that while exogenous IGFBP1 alone had little effect on AASMC proliferation, treating cells with both IGFBP1 and FP restored the anti-mitogenic action of FP as evidenced by the profound inhibition of both cell number and BrDU incorporation (Fig. 4).

Although previous reports showed IGFBP1 presented anti-proliferative or mitogenic actions, these effects were highly dependent on the cellular context (Matilainen et al., 2005). In endometrial stromal cells and chick embryo fibroblasts, IGFBP1 inhibited EGF and serum-induced DNA synthesis (Cavaille et al., 2006; Liu et al., 1991) while in breast cancer cell lines and arterial smooth muscle cells, IGFBP1 suppressed cell proliferation (Van den Berg et al., 1997; Motani et al., 1995). Conversely, IGFBP1 also demonstrated pro-mitogenic actions as IGFBP1 knockdown was associated with a reduced prostatic cell proliferation (Gray et al., 2011). Similarly, hepatic IGFBP1 acts as a pro-survival factor by protecting the liver from apoptosis (Leu and George, 2007). Together these findings suggest that IGFBP1 effects on growth is cell-specific and exhibits strong anti-proliferative effects in steroid-insensitive ASM cells when combined with GC via mechanisms that remain to be investigated.

Evidence from cancer cell lines suggest that IGFBP-1 binds directly to the cell surface α5β1 integrins (Gleeson et al., 2001; Irwin and Giudice, 1998) and inhibit the phosphorylation of FAK and cell growth (Perks et al., 1999). Interestingly, in ASM cells, fibronectin has been show to increase PDGF effects on cell growth through α5β1 integrins (Nguyen et al., 2005). However, we here show that IGFBP1 did not affect PDGF-induced FAK phosphorylation in AASMC irrespective of GC treatment (Fig. 5) suggesting that IGFBP1 modulates ASM growth in a FAK-independent manner.

We therefore focused on other signaling pathways essential in the control of human ASM cell growth such as ERK1/2 (Orsini et al., 1999). In contrast to what reported in skin fibroblasts where IGFBP1 clearly inhibited ERK1/2 phosphorylation (Prokop et al., 2013), no effect of IGFBP1 on ERK1/2 phosphorylation was seen in PDGF-treated AASMC in the presence or absence of GC (Fig. 6B). Interestingly, the mechanisms underlying the effects of mitogens on ASM growth differ in AASMC versus NASMC (Burgess et al., 2008). While in NASMC, both PI3K/AKT and ERK1/2 kinases equally mediate mitogens effects on growth (Ammit and Panettieri, 1985; Hirst et al., 2004), in AASMC, however, it was shown that PI3K/AKT predominated over ERK1/2 pathways in mediating mitogenic signals (Burgess et al., 2008). Despite a report showing that IGFBP1 can inhibit AKT phosphorylation in fibroblasts (Prokop et al., 2013), we failed to see any inhibitory effect of IGFBP1 on AKT activation (downstream of PI3K) irrespective of GC treatment (Fig. 6A) supporting the novel observation that IGFBP1 can enhance the inhibitory action of GC in ASM by involving both ERK1/2- and AKT-independent mechanisms.

Different kinases negatively regulate GR function. The activation of JNK, for instance, interferes with GR nuclear translocation (Rogatsky et al., 1998) and high levels of JNK phosphorylation occurs in steroid resistant inflammatory cells (Adcock et al., 1995). Similarly, the activation of p38 also impairs GR function and promotes steroid insensitivity not only in immune cells (Goleva et al., 2009; Li et al., 2015) but also in ASM cells (Bouazza et al., 2014). We previously showed that p38 inhibition enhanced GR-mediated transactivation activities in ASM cells (Bouazza et al., 2014). While the addition of IGFBP1 did not affect mitogen-induced JNK phosphorylation irrespective of GC treatment (Fig. 7A), it dramatically inhibited mitogen-induced p38 phosphorylation in the presence of GC (Fig. 7B). Since the role of p38 in PDGF and EGF-mediated ASM cells growth is minimal (Orsini et al., 1999) and p38 negatively regulates GR function in ASM cells (Bouazza et al., 2014), our result suggests that IGFBP1 indirectly increases GR function by inhibiting p38 activation; consequently the enhanced anti-proliferative effects of GC in the presence of IGFBP1 (Fig. 4) may require the inhibition of p38 activation (Fig. 7B), a hypothesis we are currently exploring.

In conclusion, our study demonstrates that ASM cells obtained from mild asthmatics are insensitive to the anti-proliferative actions of GC. Our study also demonstrates the anti-proliferative action of GC can be restored in the presence of IGFBP1 via FAK/AKT/ERK-independent but p38-dependent mechanisms. Further understanding of IGFBP1 signaling may prompt novel insights into the design of novel therapies aimed at treating GC resistant remodeling features in asthma.

## Funding

This research study was funded by NIH grants R01HL111541 (O. Tliba) and 2P012HL114471 (R.A.P.).

## Conflict of interest

None

## References

Adcock, I.M., Lane, S.J., Brown, C.R., Lee, T.H., Barnes, P.J., 1995. Abnormal glucocorticoid receptor-activator protein 1 interaction in steroid-resistant asthma. J. Exp. Med. 182, 1951–1958.

Ammit, A.J., Panettieri Jr., R.A., 1985. Invited review: the circle of life: cell cycle regulation in airway smooth muscle. J. Appl. Physiol. 2001 (91), 1431–1437.

Bentley, J.K., Hershenson, M.B., 2008. Airway smooth muscle growth in asthma: proliferation, hypertrophy, and migration. Proc. Am. Thorac. Soc. 5, 89–96.

Bentley, J.K., Deng, H., Linn, M.J., Lei, J., Dokshin, G.A., Fingar, D.C., Bitar, K.N., Henderson Jr., W.R., Hershenson, M.B., 2009. Airway smooth muscle hyperplasia and hypertrophy correlate with glycogen synthase kinase-3(beta) phosphorylation in a mouse model of asthma. Am. J. Physiol. Lung Cell Mol. Physiol. 296, L176–184.
Borger, P., Miglino, N., Baraket, M., Black, J.L., Tamm, M., Roth, M., 2009. Impaired translation of CCAAT/enhancer binding protein alpha mRNA in bronchial smooth muscle cells of asthmatic patients. J. Allergy Clin. Immunol. 123, 639–645.
Bouazza, B., Krytska, K., Debba-Pavard, M., Amrani, Y., Honkanen, R.E., Tran, J., Tliba, O., 2012. Cytokines alter glucocorticoid receptor phosphorylation in airway cells: role of phosphatases. Am. J. Respir. Cell Mol. Biol. 47, 464–473.
Bouazza, B., Debba-Pavard, M., Amrani, Y., Isaacs, L., O'Connell, D., Ahamed, S., Formella, D., Tliba, O., 2014. Basal p38 mitogen-activated protein kinase regulates unliganded glucocorticoid receptor function in airway smooth muscle cells. Am. J. Respir. Cell Mol. Biol. 50, 301–315.
Burgess, J.K., Lee, J.H., Ge, Q., Ramsay, E.E., Poniris, M.H., Parmentier, J., Roth, M., Johnson, P.R., Hunt, N.H., Black, J.L., Ammit, A.J., 2008. Dual ERK and phosphatidylinositol 3-kinase pathways control airway smooth muscle proliferation: differences in asthma. J. Cell. Physiol. 216, 673–679.
Cavaille, F., Neau, E., Vouters, M., Bry-Gauillard, H., Colombel, A., Milliez, J., Le Bouc, Y., 2006. IGFBP-1 inhibits EGF mitogenic activity in cultured endometrial stromal cells. Biochem. Biophys. Res. Commun. 345, 754–760.
Chung, K.F., 2014. Defining phenotypes in asthma: a step towards personalized medicine. Drugs. 74, 719–728.
Dekkers, B.G., Pehlic, A., Mariani, R., Bos, I.S., Meurs, H., Zaagsma, J., 2012. Glucocorticosteroids and beta(2)-adrenoceptor agonists synergize to inhibit airway smooth muscle remodeling. J. Pharmacol. Exp. Ther. 342, 780–787.
Dost, A., Kauf, E., Schlenvoigt, D., Schramm, D., Zintl, F., Hubler, A., 2007. Effect of dexamethasone on the IGFBP-1 regulation in premature infants during the first weeks of life. Biologics 1, 449–453.
Eddleston, J., Herschbach, J., Wagelie-Steffen, A.L., Christiansen, S.C., Zuraw, B.L., 2007. The anti-inflammatory effect of glucocorticoids is mediated by glucocorticoid-induced leucine zipper in epithelial cells. J. Allergy Clin. Immunol. 119, 115–122.
Galliher-Beckley, A.J., Williams, J.G., Collins, J.B., Cidlowski, J.A., 2008. Glycogen synthase kinase 3beta-mediated serine phosphorylation of the human glucocorticoid receptor redirects gene expression profiles. Mol. Cell. Biol. 28, 7309–7322.
Gleeson, L.M., Chakraborty, C., McKinnon, T., Lala, P.K., 2001. Insulin-like growth factor-binding protein 1 stimulates human trophoblast migration by signaling through alpha 5 beta 1 integrin via mitogen-activated protein Kinase pathway. J. Clin. Endocrinol. Metab. 86, 2484–2493.
Goleva, E., Li, L.B., Leung, D.Y., 2009. IFN-gamma reverses IL-2- and IL-4-mediated T-cell steroid resistance. Am. J. Respir. Cell Mol. Biol. 40, 223–230.
Gray, A., Aronson, W.J., Barnard, R.J., Mehta, H., Wan, J., Said, J., Cohen, P., Galet, C., 2011. Global Igfbp1 deletion does not affect prostate cancer development in a c-Myc transgenic mouse model. J. Endocrinol. 211, 297–304.
Hirst, S.J., Martin, J.G., Bonacci, J.V., Chan, V., Fixman, E.D., Hamid, Q.A., Herszberg, B., Lavoie, J.P., McVicker, C.G., Moir, L.M., et al., 2004. Proliferative aspects of airway smooth muscle. J. Allergy Clin. Immunol. 114, S2–17.
Irwin, J.C., Giudice, L.C., 1998. Insulin-like growth factor binding protein-1 binds to placental cytotrophoblast alpha5beta1 integrin and inhibits cytotrophoblast invasion into decidualized endometrial stromal cultures. Growth Horm. IGF Res. 8, 21–31.
Jones, J.I., Gockerman, A., Busby Jr., W.H., Wright, G., Clemmons, D.R., 1993. Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the alpha 5 beta 1 integrin by means of its Arg-Gly-Asp sequence. Proc. Natl. Acad. Sci. U S A. 90, 10553–10557.
Kajimura, S., Aida, K., Duan, C., 2005. Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation. Proc. Natl. Acad. Sci. U S A. 102, 1240–1245.
Lee, P.D., Giudice, L.C., Conover, C.A., Powell, D.R., 1997. Insulin-like growth factor binding protein-1: recent findings and new directions. Proc. Soc. Exp. Biol. Med. 216, 319–357.
Leu, J.I., George, D.L., 2007. Hepatic IGFBP1 is a prosurvival factor that binds to BAK, protects the liver from apoptosis, and antagonizes the proapoptotic actions of p53 at mitochondria. Genes Dev. 21, 3095–3109.
Li, L.B., Leung, D.Y., Goleva, E., 2015. Activated p38 MAPK in peripheral blood monocytes of steroid resistant asthmatics. PLoS One 10, e0141909.
Liu, L., Brinkman, A., Blat, C., Harel, L., 1991. IGFBP-1, an insulin like growth factor binding protein, is a cell growth inhibitor. Biochem. Biophys. Res. Commun. 174, 673–679.
Luo, J., Reid, R.E., Murphy, L.J., 1990. Dexamethasone increases hepatic insulin-like growth factor binding protein-1 (IGFBP-1) mRNA and serum IGFBP-1 concentrations in the rat. Endocrinology 127, 1456–1462.
Marwick, J.A., Adcock, I.M., Chung, K.F., 2010. Overcoming reduced glucocorticoid sensitivity in airway disease: molecular mechanisms and therapeutic approaches. Drugs 70, 929–948.
Matilainen, M., Malinen, M., Saavalainen, K., Carlberg, C., 2005. Regulation of multiple insulin-like growth factor binding protein genes by 1alpha,25-dihydroxyvitamin D3. Nucleic Acids Res. 33, 5521–5532.
Misior, A.M., Yan, H., Pascual, R.M., Deshpande, D.A., Panettieri, R.A., Penn, R.B., 2008. Mitogenic effects of cytokines on smooth muscle are critically dependent on protein kinase A and are unmasked by steroids and cyclooxygenase inhibitors. Mol. Pharmacol. 73, 566–574.
Moore, W.C., Peters, S.P., 2006. Severe asthma: an overview. J. Allergy Clin. Immunol. 117, 487–494 quiz 495.
Motani, A., Rutherford, C., Anggard, E.E., Ferns, G.A., 1995. Insulin-like growth factor binding protein-1 inhibits arterial smooth muscle cell proliferation in vitro but does not reduce the neointimal response to balloon catheter injury. Atherosclerosis 118, 57–66.
Nguyen, T.T., Ward, J.P., Hirst, S.J., 2005. beta1-Integrins mediate enhancement of airway smooth muscle proliferation by collagen and fibronectin. Am. J. Respir. Crit. Care Med. 171, 217–223.
Orsini, M.J., Krymskaya, V.P., Eszterhas, A.J., Benovic, J.L., Panettieri Jr., R.A., Penn, R.B., 1999. MAPK superfamily activation in human airway smooth muscle: mitogenesis requires prolonged p42/p44 activation. Am. J. Physiol. 277, L479–488.
Panettieri Jr., R.A., 2015. Bronchial thermoplasty: targeting structural cells in severe persistent asthma. Ann. Am. Thorac. Soc. 12, 1593–1594.
Panettieri Jr., R.A., 2016. Neutrophilic and pauci-immune phenotypes in severe asthma. Immunol. Allergy Clin. North Am. 36, 569–579.
Perks, C.M., Newcomb, P.V., Norman, M.R., Holly, J.M., 1999. Effect of insulin-like growth factor binding protein-1 on integrin signalling and the induction of apoptosis in human breast cancer cells. J. Mol. Endocrinol. 22, 141–150.
Perry, M.M., Baker, J.E., Gibeon, D.S., Adcock, I.M., Chung, K.F., 2014. Airway smooth muscle hyperproliferation is regulated by microRNA-221 in severe asthma. Am. J. Respir. Cell Mol. Biol. 50, 7–17.
Prokop, I., Kononczuk, J., Surazynski, A., Palka, J., 2013. Cross-talk between integrin receptor and insulin-like growth factor receptor in regulation of collagen biosynthesis in cultured fibroblasts. Adv. Med. Sci. 58, 292–297.
Quante, T., Ng, Y.C., Ramsay, E.E., Henness, S., Allen, J.C., Parmentier, J., Ge, Q., Ammit, A.J., 2008. Corticosteroids reduce IL-6 in ASM cells via up-regulation of MKP-1. Am. J. Respir. Cell Mol. Biol. 39, 208–217.
Rogatsky, I., Logan, S.K., Garabedian, M.J., 1998. Antagonism of glucocorticoid receptor transcriptional activation by the c-Jun N-terminal kinase. Proc. Natl. Acad. Sci. U S A. 95, 2050–2055.
Rogatsky, I., Wang, J.C., Derynck, M.K., Nonaka, D.F., Khodabakhsh, D.B., Haqq, C.M., Darimont, B.D., Garabedian, M.J., Yamamoto, K.R., 2003. Target-specific utilization of transcriptional regulatory surfaces by the glucocorticoid receptor. Proc Natl Acad Sci U S A. 100, 13845–13850.
Stamatiou, R., Paraskeva, E., Gourgoulianis, K., Molyvdas, P.A., Hatziefthimiou, A., 2012. Cytokines and growth factors promote airway smooth muscle cell proliferation. ISRN Inflamm. 2012, 731472.
Stewart, A.G., Fernandes, D., Tomlinson, P.R., 1995. The effect of glucocorticoids on proliferation of human cultured airway smooth muscle. Br. J. Pharmacol. 116, 3219–3226.
Suh, D.S., Rechler, M.M., 1997. Hepatocyte nuclear factor 1 and the glucocorticoid receptor synergistically activate transcription of the rat insulin-like growth factor binding protein-1 gene. Mol. Endocrinol. 11, 1822–1831.
Suh, D.S., Zhou, Y., Ooi, G.T., Rechler, M.M., 1995. Dexamethasone stimulation of rat insulin-like growth factor binding protein-1 (IGFBP-1) promoter activity involves the interaction of multiple transcription factors. Prog. Growth Factor Res. 6, 131–140.
Suh, D.S., Zhou, Y., Ooi, G.T., Rechler, M.M., 1996. Dexamethasone stimulation of rat insulin-like growth factor binding protein-1 promoter activity involves multiple cis-elements. Mol. Endocrinol. 10, 1227–1237.
Tliba, O., Panettieri Jr., R.A., 2009. Noncontractile functions of airway smooth muscle cells in asthma. Annu. Rev. Physiol. 71, 509–535.
Van den Berg, C.L., Cox, G.N., Stroh, C.A., Hilsenbeck, S.G., Weng, C.N., McDermott, M.J., Pratt, D., Osborne, C.K., Coronado-Heinsohn, E.B., Yee, D., 1997. Polyethylene glycol conjugated insulin-like growth factor binding protein-1 (IGFBP-1) inhibits growth of breast cancer in athymic mice. Eur. J. Cancer 33, 1108–1113.
van der Velden, V.H., 1998. Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma. Mediators Inflamm. 7, 229–237.
Vlahos, R., Stewart, A.G., 1999. Interleukin-1alpha and tumour necrosis factor-alpha modulate airway smooth muscle DNA synthesis by induction of cyclo-oxygenase-2: inhibition by dexamethasone and fluticasone propionate. Br. J. Pharmacol. 126, 1315–1324.
Vlahos, R., Lee, K.S., Guida, E., Fernandes, D.J., Wilson, J.W., Stewart, A.G., 2003. Differential inhibition of thrombin- and EGF-stimulated human cultured airway smooth muscle proliferation by glucocorticoids. Pulm. Pharmacol. Ther. 16, 171–180.
Wahidi, M.M., Kraft, M., 2012. Bronchial thermoplasty for severe asthma. Am. J. Respir. Crit. Care Med. 185, 709–714.