

Increased β_2 -adrenoceptor phosphorylation in airway smooth muscle in severe asthma: possible role of mast cell-derived growth factors

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Introduction

β_2 -adrenoceptor (β_2 -AR) agonists represent the most commonly used drugs in the management of pulmonary diseases, including asthma [1]. These drugs provide relief from acute bronchoconstriction by promoting bronchodilation through direct activation of β_2 -AR on airway smooth muscle (ASM) and promoting its relaxation. Despite their clinical benefits, the use of β_2 -AR agonists in asthma has been associated with loss of β_2 -AR function, deleterious effects and/or worsening of the symptoms and asthma deaths [2]. Understanding the potential mechanisms that result in the loss of function and/or deleterious clinical effects in patients treated with β_2 -agonists could offer novel therapeutic alternatives.

Studies performed in different cell types have reported that some, but not all, growth factors can impair cell responsiveness to β_2 -agonists by phosphorylating β_2 -AR on several serine (ser^{345/346}) or tyrosine residues

Summary

The purpose of this study was to investigate whether growth factors produced by activated human lung mast cells (HLMCs) impair β_2 -adrenoceptor (β_2 -AR) function in human airway smooth muscle (ASM) cells. Protein array analysis confirmed the presence of various growth factors, including transforming growth factor (TGF)- β 1, in the supernatants of high-affinity IgE receptor (Fc ϵ RI)-activated HLMCs which, when applied to ASM cells, impaired albuterol-induced cyclic adenosine monophosphate (cAMP) production, an effect that was prevented following neutralization of TGF- β 1. This blunted β_2 -AR response was reproduced by treating ASM cells with TGF- β 1 or fibroblast growth factor (FGF)-2, which induced β_2 -AR phosphorylation at tyrosine residues Tyr¹⁴¹ and Tyr³⁵⁰, and significantly reduced the maximal bronchorelaxant responses to isoproterenol in human precision cut lung slices (PCLS). Finally, ASM cells isolated from severe asthmatics displayed constitutive elevated β_2 -AR phosphorylation at both Tyr¹⁴¹ and Tyr³⁵⁰ and a reduced relaxant response to albuterol. This study shows for the first time that abnormal β_2 -AR phosphorylation/function in ASM cells that is induced rapidly by HLMC-derived growth factors, is present constitutively in cells from severe asthmatics.

Keywords: allergy, inflammation, mast cells, signal transduction.

(Tyr^{350/354/141}), resulting in receptor uncoupling and internalization [3–6]. In human lung mast cells (HLMCs), we showed that stem cell factor (SCF) can similarly uncouple β_2 -AR following receptor phosphorylation at similar Tyr³⁵⁰ residues [7]. More recently, we observed that β_2 -AR responses were suppressed in both mast cells (suppression of Fc ϵ R1-induced activation) and ASM cells (inhibition of ASM contraction) following cell–cell contact via β_2 -AR phosphorylation, on Tyr³⁵⁰ residues [8]. Whether mast cells also blunt β_2 -AR responses in ASM cells via the action of secreted different growth factors remains unknown.

The present study provides the first evidence that abnormal β_2 -AR phosphorylation/function can be induced experimentally by exposing healthy ASM cells to growth factors released from activated mast cells, a feature that was constitutively present in ASM cells from severe asthmatics. Abnormal tyrosine phosphorylation of β_2 -AR

represents a novel mechanism that could explain the poor clinical efficacy of β_2 -AR therapy seen in severe asthma.

Materials and methods

Culture of human airway smooth muscle cells

Primary human ASM cells were obtained from consented healthy subjects and asthmatic patients isolated from endobronchial biopsies as described previously [9]. The demographics of subjects used in the study are shown in Fig. 3d.

Mast cell isolation, culture and stimulation

Isolation and stimulation of HLMC were performed as described previously [10].

Flow cytometry

Flow cytometry assays were performed as described previously using the Becton Dickinson FACScan (Oxford, UK) [9]. The antibodies used were rabbit anti-human β_2 -AR (Tyr¹⁴¹) antibody, rabbit anti-human β_2 -AR (Tyr³⁵⁰) antibody (2 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA), isotype-matched control (rabbit immunoglobulin (Ig)G; Imminostep, Salamanca, Spain) and secondary anti-rabbit fluorescein isothiocyanate (FITC; Cell Signaling Technology, Danvers, MA, USA).

Measurement of cyclic adenosine monophosphate (cAMP) production

ASM cells were stimulated with 10 μ M albuterol for 10 min in the presence or absence of Fc ϵ R1-activated HLMC supernatants (1 : 4 dilution, added for different time-points or 10 min, as indicated) before cell extracts were used for the determination of intracellular cAMP concentration using the cAMP EIA kit from Amersham Biosciences (Little Chalfont, UK) (detection limit curve range > 25 fmol/well) following the manufacturer's protocol.

Collagen gel contraction assay

Spontaneous ASM cells contraction was examined in collagen gels, as described previously [8].

Growth factor array

Supernatants were prepared from Fc ϵ R1-activated HLMCs, as described previously [10], and were used to perform the growth factor array analysis which allows the simultaneous detection of 41 different human growth factors, following the manufacturer's protocol (Abcam, Cambridge, UK).

Human lung slice preparation

The bronchorelaxant responses using the six precision cut lung slices (PCLS) were performed as described previously [11]. The slices were incubated with transforming growth factor (TGF)- β or fibroblast growth factor (FGF)-2 at 100 ng/ml for 30 min before responses to isoproterenol were assessed on PCLS precontracted with 10^{-4} M carbachol. The airway dilation data were expressed as percentage dilation of the maximum carbachol constricted airway luminal area with the baseline value prior to contraction considered as 100%, as described previously [11].

Statistical analysis

All data are presented as mean \pm standard error of the mean (s.e.m.). Statistical analysis was performed by two- or one-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons. Differences were considered significant when $P < 0.05$. Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA).

Results

Fc ϵ R1-activated HLMCs produced growth factors and attenuated the response to albuterol in human ASM cells

Representative protein array membrane showing growth factors) produced in the supernatants of resting and Fc ϵ R1-activated HLMCs [IgE (2.5 μ g/ml)-anti-IgE (1 : 1000 dilution)] (Fig. 1a). Densitometric analyses of the membrane arrays revealed that levels of FGF-2, epidermal growth factor (EGF), heparin-binding (HB)-EGF, hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, insulin-like growth factor binding protein (IGF)-BP2, IGF-BP6, IGF-II, SCF, SCF-R, macrophage CSF (M-CSF), vascular endothelial growth factor (VEGF)-A, VEGF-D, VEGF-R3 and TGF- β 1 were produced by Fc ϵ R1-activated HLMCs, although only four growth factors (GM-CSF, HGF, IGF-II and TGF- β 1, $P < 0.01$) reached statistical significance. ASM cells first incubated with supernatants from Fc ϵ R1-activated HLMCs (compared to that of resting HLMCs) for different time-points (10–30 min) displayed a complete inhibition of cAMP accumulation measured in ASM cells treated with 10 μ M albuterol for 10 min ($n = 3$ ASM cell lines, $P < 0.01$) (Fig. 1b). The inhibitory effect of a 10-min incubation with Fc ϵ R1-activated HLMC supernatants on albuterol responses was almost completely prevented by incubating supernatants of Fc ϵ R1-activated HLMCs with 10 μ g/ml neutralizing TGF- β 1 antibody (Fig. 1c).

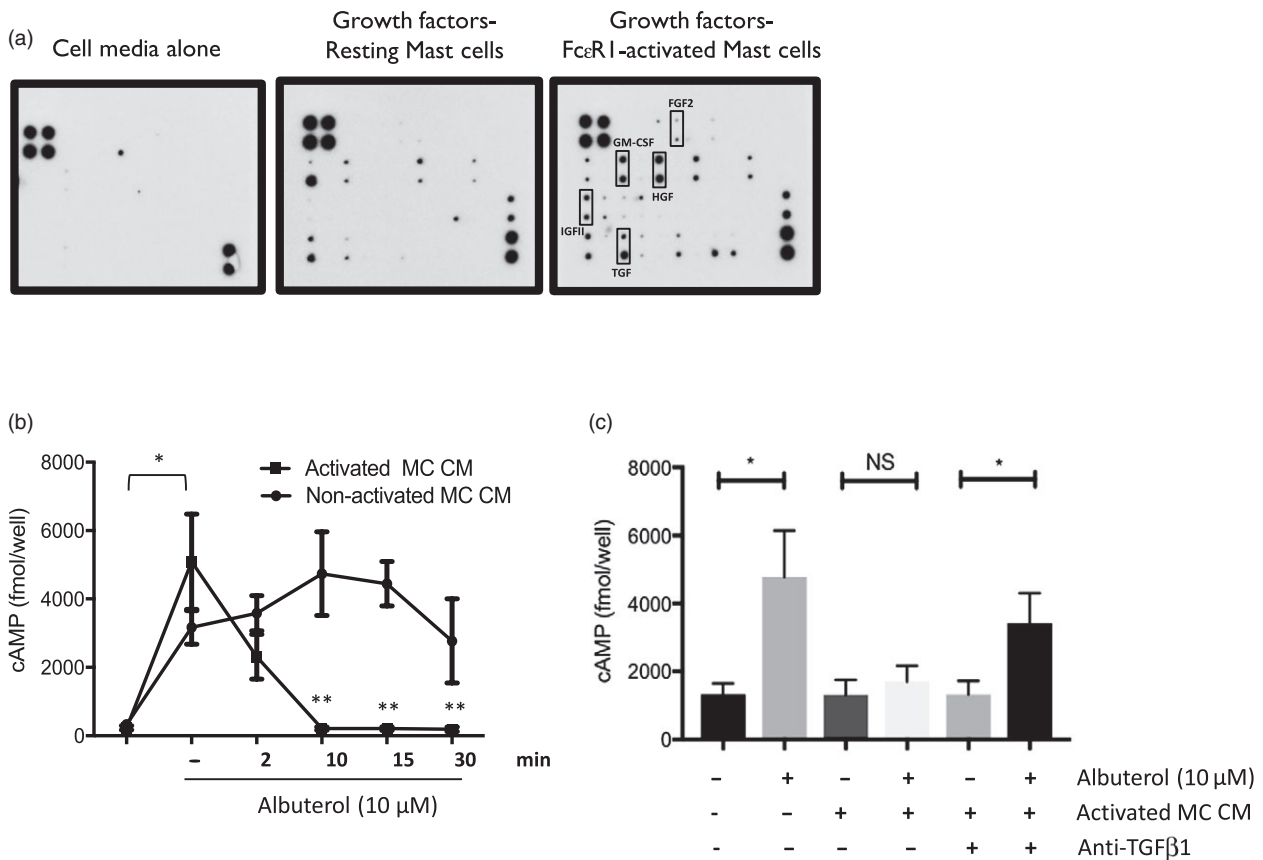


Fig. 1. Supernatants of high-affinity immunoglobulin (Ig)E receptor (FcεR1)-activated human lung mast cells (HLMCs), which contained different growth factors, suppressed albuterol-induced cyclic adenosine monophosphate (cAMP) production in airway smooth muscle (ASM) cells. (a) Representative blot showing the growth factor membrane arrays incubated with HLMC media alone, supernatants from resting HLMCs or FcεR1-activated HLMCs. Representative of three individual donors. (b) ASM cells were treated with supernatants of FcεR1-activated HLMCs (1 : 4 dilution, 30 min) or non-activated HLMCs for 2, 10, 15 and 30 min before cAMP production (fmol/well) in response to a 10-min stimulation with 10 μM albuterol was investigated using enzyme immunoassay ($n = 3$ donors). * $P < 0.05$ versus basal, ** $P < 0.01$ versus cells treated with non-activated HLMCs. (c) Incubating ASM cells for 10 min with the conditioned medium (CM) of FcεR1-activated HLMCs (MC) containing neutralizing anti-transforming growth factor (TGF)-β1 antibody (10 μg/ml) restored the albuterol (10 μM)-dependent cAMP response. * $P = 0.033$ ($n = 4$). Statistical analysis was performed using two-way analysis of variance (ANOVA).

TGF-β1 and FGF-2 induced β2-AR phosphorylation on key tyrosine residues and attenuated β2-AR responsiveness

Interestingly, only pretreatment of human ASM cells with either TGF-β1 or FGF-2 at 10 ng/ml for 10 min increased β2-AR phosphorylation significantly on both Tyr¹⁴¹ (Fig. 2a) and Tyr³⁵⁰ (Fig. 2b), while VEGF or nerve growth factor (NGF) had no effect, despite having functional receptors in ASM cells [12–15]. Figure 2c shows that TGF-β1 and FGF-2 also reduced cAMP responses to albuterol by more than 85 and 72%, respectively ($n = 3$). Figure 2d shows that isoproterenol-evoked bronchodilation assessed using human PCLS was inhibited by TGF-β1 or FGF-2 (30 min, 100 ng/ml), with a reduction of the maximum % bronchodilation from 57.44 ± 4.11 to 34.29 ± 5.37 and 32.4 ± 2.39 ($n = 6$ slices, $P < 0.05$), respectively. Both growth

factors had no effect on isoproterenol EC₅₀ value ($\log EC_{50} = -6.27 \pm 0.2099$).

ASM cells from severe asthmatics display an increased basal β2-AR phosphorylation at tyrosine residues and an impaired relaxant response to albuterol

Our flow cytometry data show that ASM cells derived from severe asthmatics, but not from non-severe asthmatics, displayed a constitutive increased phosphorylation of β2-AR at both Tyr¹⁴¹ (Fig. 3a, geometric mean fluorescence intensity (GMFI) = 31.13 ± 2.54 , $P = 0.0045$, $n = 7$) and Tyr³⁵⁰ (Fig. 3b, GMFI = 31.22 ± 2.40 , $P = 0.0224$, $n = 7$) when compared to levels seen in healthy subjects (GMFI = 10.03 ± 3.05 and 13.76 ± 4.23 , $n = 7$). Gel contraction assays also revealed that albuterol failed to abrogate the spontaneous contraction of severe asthmatic

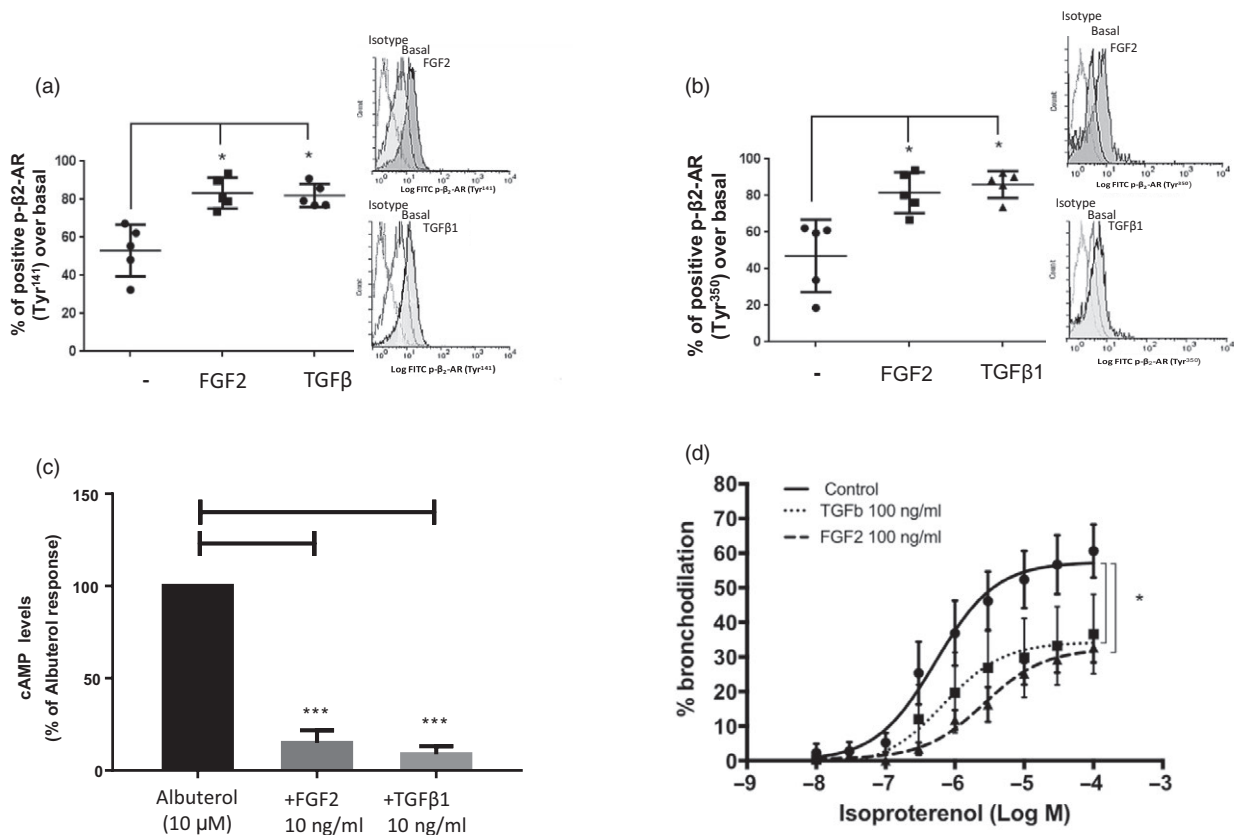


Fig. 2. Transforming growth factor (TGF)-β1 and fibroblast growth factor (FGF)-2 increased β₂-adrenoceptor (β₂-AR) phosphorylation on tyrosine residues and reduced β₂-AR responsiveness. (a,b) Means ± standard error of the mean (s.e.m.) of the % positive p-β₂-AR on Tyr¹⁴¹ and Tyr³⁵⁰ staining over basal assessed by flow cytometry (with representative histograms) in human healthy airway smooth muscle (ASM) cells (*n* = 5) treated with 10 ng/ml FGF-2 or TGF-β for 10 min, **P* < 0.05 versus resting conditions. (c) ASM cells pretreated with TGF-β or FGF-2 (10 min) were incubated with albuterol (10 μM) for 15 min before accumulation of cyclic adenosine monophosphate (cAMP) was assessed by enzyme immunoassay. Data are expressed as % of albuterol-induced cAMP response performed in three healthy subjects and analysed using the one-sample *t*-test. ****P* < 0.001 versus albuterol. (d) Human PCLS precontracted with 10⁻⁴ M carbachol were incubated with control diluent, FGF-2 or TGF-β for 30 min prior isoproterenol (10⁻⁸ to 10⁻⁴ M). Each concentration–response curve is expressed as means ± s.e.m. performed in six precision cut lung slices (PCLS) isolated from two donors. **P* < 0.01 versus control PCLS. Statistical analysis was performed using one-way analysis of variance (ANOVA) on the maximum bronchodilatory responses.

ASM cells while reducing by more than 47% the response seen in healthy ASM cells (Fig. 3c, *P* < 0.05, *n* = 3). Figure 3d shows the demographics of the subjects used in the studies. Data are shown as means ± s.e.m.

Discussion

The possibility that cell–cell interactions between mast cells and ASM cells may play a role in the pathogenesis of asthma is supported by multiple *in-vitro* reports showing the ability of mast cell mediators to induce various pro-asthmatic responses in ASM cells/tissues [2]. We found that β₂-AR dysfunction in human ASM cells could be induced experimentally by exposing human ASM cells with supernatants from activated HLMCs, which produced a number of growth factors, although only four (GM-CSF, HGF, IGF-II and TGF-β1) reached statistical significance. The observation that

HLMC effect was largely prevented by the presence of neutralizing TGF-β1 antibody, or mimicked by the pretreatment of ASM cells with exogenous TGF-β1 (or FGF-2), identified TGF-β1 as a novel modulator of β₂-AR function. We believe that the central mechanism by which TGF-β1 impaired β₂-AR function in ASM cells results from its capacity to phosphorylate the receptor rapidly at two key tyrosine residues, Tyr¹⁴¹ and Tyr³⁵⁰, which was seen within 10 min. This hypothesis is supported by our recent finding that β₂-AR phosphorylation at Tyr³⁵⁰ in HLMCs was essential in driving the loss of β₂-AR responses when exposed to another mast cell mediator SCF [7] or following adhesion of HLMCs to ASM cells [8]. Whether TGF-β1 interferes with β₂-AR responses by acting at a level downstream to β₂-AR remains a possibility, although a previous study also performed in human ASM cells showed that TGF-β1 inhibited isoproterenol-induced cAMP production by decreasing

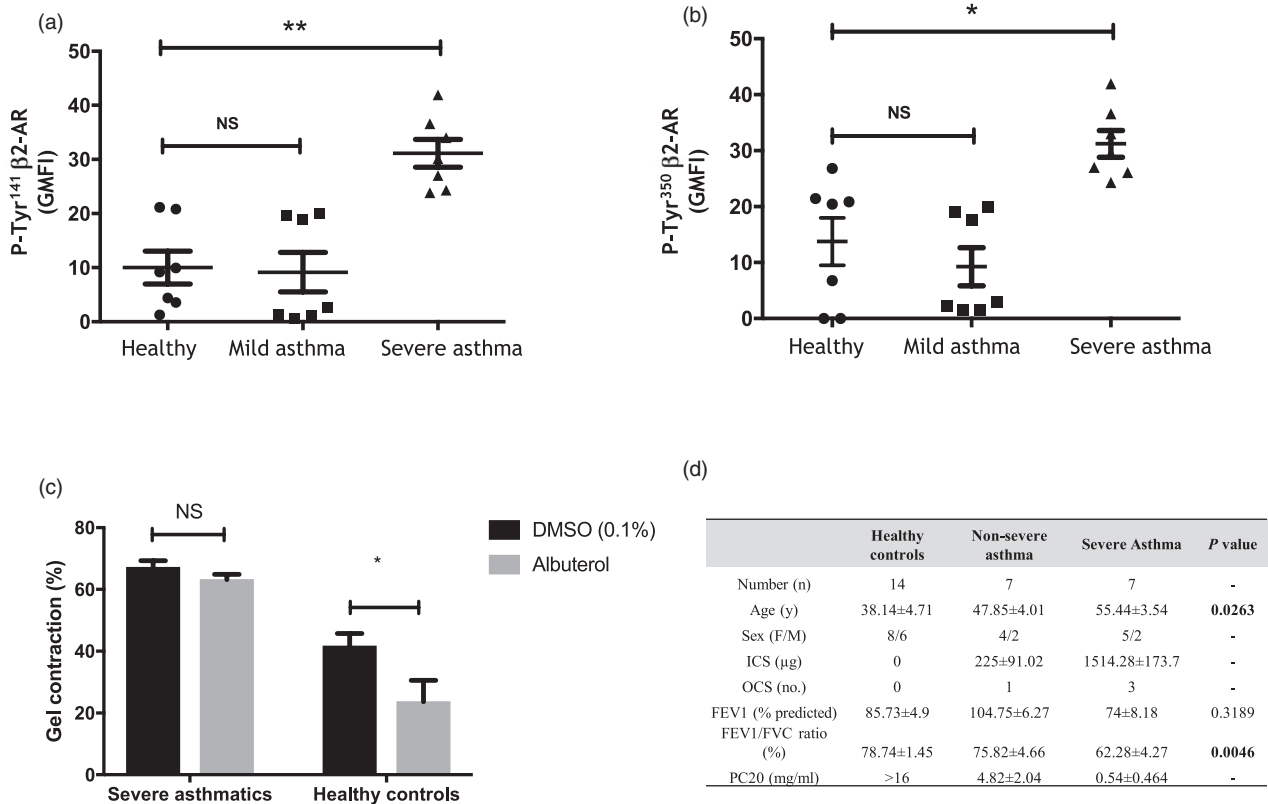


Fig. 3. Airway smooth muscle (ASM) cells from patients with severe asthma displayed increased β_2 -adrenoceptor (β_2 -AR) phosphorylation and reduced bronchorelaxant responses. (a,b) Phosphorylation of β_2 -AR at Tyr³⁵⁰ or Tyr¹⁴¹ was assessed by flow cytometry and expressed as the fold increase in geometric mean fluorescence intensity (GMFI) over the isotype control antibody. Data are presented as means \pm standard error of the mean (s.e.m.) of experiments performed in ASM cells derived from seven subjects. * $P < 0.05$ and ** $P < 0.01$ versus untreated cells. (c) Effect of albuterol on the spontaneous contraction of ASM cells embedded in collagen gel matrices in three healthy and severe asthmatic subjects. * $P < 0.01$ versus dimethylsulphoxide (DMSO) controls. Statistical analysis was performed using one-way (a/b) or two-way analysis of variance (ANOVA) (c). (d) Demographics of subjects used in the study. Bold indicates significance compared to healthy subjects.

receptor number without altering receptor coupling (G_s /adenyl cyclase) [16]. Our combined observations suggest that the inhibition of β_2 -AR responses by TGF- β 1 results from different changes occurring mainly at the receptor level. Interestingly, while FGF-2 also induced β_2 -AR phosphorylation, we found that VEGF and NGF failed to have any effect (data not shown), despite previous studies showing the existence of functional receptors in human ASM cells [12–14,17]. This supports the interesting concept that β_2 -AR phosphorylation by RTK ligands is highly stimuli- and cell-specific. Indeed, depending on the cell types, β_2 -AR can be phosphorylated at Tyr^{350/354}, Tyr³⁶⁴, Ser³⁴⁶ or Ser³⁴⁶ by insulin or Tyr¹³² and Tyr¹⁴¹ by insulin-like growth factor-1 [4,6,18]. It is noteworthy to mention that, irrespective of the type of growth factors, β_2 -AR phosphorylation on tyrosine residues is a key trigger of receptor dysfunction [6,18]. Our PCLS model, used widely to study ASM contractile/relaxant responses [19], confirmed that either TGF- β 1 and FGF-2 (although used at much higher concentrations, due possibly to tissue complexity of the PCLS) reduced β_2 -AR-evoked

bronchorelaxant responses dramatically. Although a different agonist (isoproterenol) was used in PCLS, both albuterol and isoproterenol were shown to have a similar bronchodilatory profile in patients [20]. Our study shows that in addition to tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-13 or IL-5, both TGF- β 1 or FGF-2 can also drive β_2 -AR dysfunction in human ASM cells (reviewed in [21]). We also made the novel finding that ASM cells from severe asthmatics displayed a constitutive tyrosine phosphorylation of β_2 -ARs and blunted response to albuterol in the collagen gel assays. Because ASM cells from severe asthmatics also have a reduced sensitivity to corticosteroids [22–24], our study further supports the existence of the factors in severe asthma that blunt the response to the two main anti-asthma drugs.

Our study shows that β_2 -AR dysfunction in ASM in asthma could result from the transient (induced by growth factors) or permanent (unknown mechanisms) phosphorylation of the receptor at both Tyr¹⁴¹ and Tyr³⁵⁰ residues. We propose that targeting the mechanisms leading to

abnormal β_2 -AR phosphorylation have the potential to enhance β_2 -agonist activity in patients with asthma, and perhaps reduce the unwanted adverse effects sometimes evident with their chronic use [21].

Author contributions

L. C. performed most of the experiments, generated, analysed and interpreted the data. A. Z., R. B. and M. B. helped with the experiments and data analysis. R. A. P. and C. K.-W. performed the P. C. L. S. studies and contributed to data analysis/interpretation. Y. A. and P. B. conceived the project, designed the experiments and analysed the data and Y. A. wrote the paper.

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Disclosures

None.

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