

## **RAPID PUBLICATION**

### **Increased $\beta$ 2-adrenoceptor phosphorylation in airway smooth muscle in severe asthma: possible role of mast cell-derived growth factors**

Latifa Chachi<sup>1</sup>, Abdulrahman Alzahrani<sup>1,3</sup>, Cynthia Koziol-White<sup>2</sup>, Michael Biddle<sup>1</sup>, Rehab Bagadood<sup>1</sup>, Reynold A. Panettieri, Jr.<sup>2</sup>, Peter Bradding<sup>1\*</sup>, and Yassine Amrani<sup>1\*</sup>

<sup>1</sup>Department of Infection, Immunity and Inflammation, Clinical Sciences, University of Leicester, Glenfield Hospital, Leicester, LE3 9QP, UK

<sup>2</sup>Rutgers Institute for Translational Medicine and Science, Rutgers Biomedical and Health Sciences, Rutgers University, New Brunswick, NJ., USA

<sup>3</sup>Faculty of Applied Medical Sciences, Albaha University, Albaha, Kingdom of Saudi Arabia

#### **Name and Address for Correspondence:**

Dr. Yassine Amrani,  
Institute for Lung Health,  
Leicester Biomedical Research Center Respiratory,  
Clinical Sciences Building,  
Glenfield Hospital, Groby Rd,  
Leicester, LE3 9QP, U.K.  
**Phone:** +44 116 256 3794  
**Fax:** +44 116 2502787  
**e-mail:** ya26@le.ac.uk.

\*Both YA and PB contributed equally to this manuscript

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## Summary

The purpose of the study was to investigate whether growth factors produced by activated human lung mast cells (HLMCs) impair  $\beta_2$ -adrenoceptor ( $\beta_2$ -AR) function in human airway smooth muscle (ASM) cells. Protein array analysis confirmed the presence of various growth factors, including TGF $\beta$ 1, in the supernatants of Fc $\epsilon$ RI-activated HLMCs which when applied to ASM cells impaired albuterol-induced cAMP production, an effect that was prevented following neutralisation of TGF $\beta$ 1. This blunted  $\beta_2$ -AR response was reproduced by treating ASM cells with TGF $\beta$ 1 or FGF2, which induced  $\beta_2$ -AR phosphorylation at tyrosine residues Tyr<sup>141</sup> and Tyr<sup>350</sup>, 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  $\beta_2$ -AR phosphorylation at both Tyr<sup>141</sup> and Tyr<sup>350</sup> and a reduced relaxant response to albuterol. This study shows for the first time that abnormal  $\beta_2$ -AR phosphorylation/function in ASM cells that is *rapidly* induced by HLMC-derived growth factors, is present *constitutively* in cells from severe asthmatics.

## Introduction

$\beta_2$ -adrenoceptor ( $\beta_2$ -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  $\beta_2$ -AR on airway smooth muscle (ASM) and promoting its relaxation. Despite their clinical benefits, the use of  $\beta_2$ -AR agonists in asthma has been associated with loss of  $\beta_2$ -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  $\beta_2$ -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  $\beta_2$ -agonists by phosphorylating  $\beta_2$ -AR on several serine (ser<sup>345/346</sup>) or tyrosine residues (Tyr<sup>350/354/141</sup>), resulting in receptor uncoupling and internalisation [3-6]. In human lung mast cells (HLMCs), we showed that stem cell factor (SCF) can similarly uncouple  $\beta_2$ -AR following receptor phosphorylation at similar Tyr<sup>350</sup> residues [7]. More recently, we observed that  $\beta_2$ -AR responses were suppressed in both mast cells (suppression of Fc $\epsilon$ R1-induced activation) and ASM cells (inhibition of ASM contraction) following cell-cell contact via  $\beta_2$ -AR phosphorylation also on Tyr<sup>350</sup> residues [8]. Whether mast cells also blunt  $\beta_2$ -AR responses in ASM cells via the action of secreted different growth factors remains unknown.

The present study provided the first evidence that abnormal  $\beta_2$ -AR phosphorylation/function can be experimentally induced 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  $\beta_2$ -

AR represents a novel mechanism that could explain the poor clinical efficacy of  $\beta_2$ -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 previously described [9]. The demographics of subjects used in the study are shown in **Fig.3D**.

**Mast cell isolation, culture and stimulation:** Isolation and stimulation of Human Lung Mast Cells (HLMC) were performed as described previously [10].

**Flow cytometry.** Flow cytometry assays were performed as described previously using the Becton Dickinson FACScan (Oxford, U.K.) [9]. The antibodies used were rabbit anti-human  $\beta_2$ -AR (Tyr<sup>141</sup>) antibody, rabbit anti-human  $\beta_2$ -AR (Tyr<sup>350</sup>) antibody (2 $\mu$ g/ml, Santa Cruz Biotechnology), isotype-matched control (rabbit IgG; imminostep, Spain) and secondary anti-rabbit FITC (Cell Signaling Technology).

**Measurement of cAMP production.** ASM cells were stimulated with 10  $\mu$ M albuterol for 10 min in the presence or absence of Fc $\epsilon$ 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 (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 FcεR1-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 PCLS were performed as previously described [11]. The slices were incubated with TGFβ1 or FGF2 at 100 ng/ml for 30 min before responses to isoproterenol were assessed on PCLS pre-contracted with 10<sup>-4</sup>M carbachol. The airway dilation data were expressed as percent 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 ± SEM. Statistical analysis was performed by two-way or one-way ANOVA with Bonferroni's correction for multiple comparisons. Differences were considered significant when P<0.05. Statistical analysis was performed using GraphPad Prism 6 (GraphPad software, USA).

## Result sections

**FcεR1-activated HLMCs produced growth factors and attenuated the response to albuterol in human ASM cells.** **Fig.1A**, Representative protein array membrane showing growth factors) produced in the supernatants of resting and FcεR1-activated HLMCs (IgE(2.5 µg/ml)-anti-IgE(1:1000 dilution)) (**Fig.1A**). Densitometric analyses of the membrane arrays revealed that levels of FGF2, EGF, HB-EGF, HGF, G-SCF, GM-CSF, IGFBP2, IGFBP6, IGFII, SCF, SCF-R, M-CSF, VEGF-A, VEGF-D, VEGF-R3, TGFβ1 were produced by FcεR1-activated HLMCs although only four growth factors (GM-CSF, HGF, IGFII and TGFβ1,  $P < 0.01$ ) reached statistical significance. ASM cells first incubated with supernatants from FcεR1-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 µM albuterol for 10 min ( $n = 3$  ASM cell lines,  $P < 0.01$ ) (**Fig.1B**). The inhibitory effect of a 10-min incubation with FcεR1-activated HLMC supernatants on albuterol responses was almost completely prevented by incubating supernatants of FcεR1-activated HLMCs with 10 µg/ml neutralising TGFβ1 antibody (**Fig.1C**).

**TGFβ1 and FGF2 induced β2-AR phosphorylation on key tyrosine residues and attenuated β2-AR responsiveness.** Interestingly, only pre-treatment of human ASM cells with either TGFβ1 or FGF2 at 10 ng/ml for 10 min significantly increased β2-AR phosphorylation on both Tyr<sup>141</sup> (**Fig.2A**) and Tyr<sup>350</sup> (**Fig.2B**) while VEGF or NGF had no effect despite having functional receptors in ASM cells [12-15]. **Fig.2C** shows that TGFβ1 and FGF2 also reduced cAMP responses to albuterol by more than 85% and 72%, respectively ( $n = 3$ ). **Fig.2D** shows that isoproterenol-evoked bronchodilatation assessed

using human PCLS was inhibited by TGF $\beta$ 1 or FGF2 (30 min, 100 ng/ml) with a reduction of the maximum % bronchodilation from 57.44 $\pm$ 4.11 to 34.29 $\pm$ 5.37 and 32.4 $\pm$ 2.39 (n=6 slices, P<0.05), respectively. Both growth factors had no effect on isoproterenol EC<sub>50</sub> value (LogEC<sub>50</sub>= -6.27 $\pm$ 0.2099).

**ASM cells from severe asthmatics display an increased basal  $\beta$ 2-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  $\beta$ 2-AR at both Tyr<sup>141</sup> (**Fig.3A**, GMFI= 31.13 $\pm$ 2.54, P=0.0045, n=7) and Tyr<sup>350</sup> (**Fig.3B**, GMFI=31.22 $\pm$ 2.40, P=0.0224, n=7) when compared to levels seen in healthy subjects (GMFI=10.03 $\pm$ 3.05 and 13.76 $\pm$ 4.23, n=7). Gel contraction assays also revealed that albuterol failed to abrogate the spontaneous contraction of severe asthmatic ASM cells while reducing by more than 47% the response seen in healthy ASM cells (**Fig.3C**, P<0.05, n=3). **Fig.3D** shows the demographics of the subjects used in the studies. Data are shown as Means  $\pm$  SEM.

## 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  $\beta$ 2-AR dysfunction in human ASM cells could be *experimentally* induced by exposing human ASM cells with supernatants from activated HLMCs, which produced a number of growth factors although only four GM-CSF, HGF, IGFII and TGF $\beta$ 1 reached statistical significance. The observation that HLMC effect was largely prevented by the presence of neutralising TGF $\beta$ 1 antibody, or mimicked by the pre-treatment of ASM cells with exogenous TGF $\beta$ 1 (or FGF2), identified TGF $\beta$ 1 as a novel modulator of  $\beta$ 2-AR function. We believe that the central mechanism by which TGF $\beta$ 1 impaired  $\beta$ 2-AR function in ASM cells results from its capacity to rapidly phosphorylate the receptor at two key tyrosine residues, Tyr<sup>141</sup> and Tyr<sup>350</sup> which was seen within 10 min. This hypothesis is supported by our recent finding that  $\beta$ 2-AR phosphorylation at Tyr<sup>350</sup> in HLMCs was essential in driving the loss of  $\beta$ 2-AR responses when exposed to another mast cell mediator SCF [7] or following adhesion of HLMCs to ASM cells [8]. Whether TGF $\beta$ 1 interferes with  $\beta$ 2-AR responses by acting at a level downstream to  $\beta$ 2-AR remains a possibility, although a previous study also performed in human ASM cells showed that TGF $\beta$ 1 inhibited isoproterenol-induced cAMP production by decreasing receptor number without altering receptor coupling ( $G_s$ /adenylyl cyclase) [16]. Our combined observations suggest that the inhibition of  $\beta$ 2-AR responses by TGF $\beta$ 1 results from different changes occurring mainly at the receptor level. Interestingly, while FGF2 also induced  $\beta$ 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  $\beta_2$ -AR phosphorylation by RTK ligands is highly stimuli- and cell-specific. Indeed, depending on the cell types,  $\beta_2$ -AR can be phosphorylated at Tyr<sup>350/354</sup>, Tyr<sup>364</sup>, Ser<sup>346</sup> or Ser<sup>346</sup> by insulin or Tyr<sup>132</sup> and Tyr<sup>141</sup> by Insulin-like growth factor-1 [4, 6, 18]. It is noteworthy to mention that, irrespective of the type of growth factors,  $\beta_2$ -AR phosphorylation on tyrosine residues is a key trigger of receptor dysfunction [6, 18]. Our PCLS model, widely used to study ASM contractile/relaxant responses [19], confirmed that either TGF $\beta$ 1 and FGF2 (although used at much higher concentrations, possibly due to tissue complexity of the PCLS) dramatically reduced  $\beta_2$ -AR-evoked bronchorelaxant responses. 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 TNF $\alpha$ , IL-1 $\beta$ , IL-13 or IL-5, both TGF $\beta$ 1 or FGF2 can also drive  $\beta_2$ -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  $\beta_2$ -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  $\beta_2$ -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 Tyr<sup>141</sup> and Tyr<sup>350</sup> residues. We propose that targeting the mechanisms leading to abnormal  $\beta_2$ -AR phosphorylation have the potential to enhance  $\beta_2$ -agonist

activity in patients with asthma, and perhaps reduce the unwanted adverse effects sometimes evident with their chronic use [21].

**Author contributions:** LC performed most of the experiments, generated, analysed and interpreted the data. AZ, RB, and MB helped with the experiments and data analysis. RAP and CK-W performed the PCLS studies and contributed to data analysis/interpretation. YA and PB conceived the project, designed the experiments, analysed the data, and YA wrote the paper.

## Figure legends

### **Figure 1: Supernatants of FcεR1-activated HLMCs, which contained different growth factors, suppressed albuterol-induced cAMP production in 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 3 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 FcεRI-activated HLMCs (MC) containing neutralising anti-TGFβ1 antibody (10 μg/ml) restored the albuterol (10 μM)-dependent cAMP response. \*p=0.033 (n=4). Statistical analysis was performed using two-way ANOVA.

### **Figure 2: TGFβ and FGF2 increased β2-AR phosphorylation on tyrosine residues**

**and reduced β2-AR responsiveness:** (A-B) Means ± SEM of the % positive p-β2-AR on Tyr<sup>141</sup> (left panel) and Tyr<sup>350</sup> (right panel) staining over basal assessed by flow cytometry (with representative histograms) in human healthy ASM cells (n=5) treated with 10 ng/ml FGF2 or TGFβ for 10 min, \*P<0.05 vs resting conditions. (C). ASM cells pre-treated with TGFβ or FGF2 (10 min) were incubated with albuterol (10 μM) for 15 min before accumulation of cAMP was assessed by enzyme immunoassay. Data are expressed as % of albuterol-induced cAMP response performed in n=3 healthy subjects and analysed using one sample T-test. \*\*\*P<0.001 versus albuterol. (D) Human PCLS

pre-contracted with  $10^{-4}$ M carbachol were incubated with control diluent, FGF2 or TGF $\beta$  for 30 min prior isoproterenol ( $10^{-8}$  to  $10^{-4}$  M). Each concentration-response curve is expressed as Means  $\pm$  SEM performed in n=6 PCLS isolated from 2 donors. \*P<0.01 vs control PCLS. Statistical analysis was performed using one-way ANOVA on the maximum bronchodilatory responses.

**Figure 3: ASM cells from patients with severe asthma displayed increased  $\beta$ 2-AR phosphorylation and reduced bronchorelaxant responses.** (A,B) Phosphorylation of  $\beta$ 2-AR at Tyr<sup>350</sup> or Tyr<sup>141</sup> 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  $\pm$  SEM of experiments performed in ASM cells derived from n=7 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 n=3 healthy and severe asthmatic subjects. \*P<0.01 vs DMSO controls. Statistical analysis was performed using one-way (panels A/B) or two-way ANOVA (panel C). (D) Demographics of subjects used in the study. Bold indicates significance compared to healthy subjects.

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