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Early View

Research letter

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Please cite this article as: Rutting S, Xenaki D, Reddy KD, *et al*. Airway smooth muscle cells from severe asthma patients with fixed airflow obstruction are responsive to steroid and bronchodilator treatment *in vitro*. *ERJ Open Res* 2021; in press (https://doi.org/10.1183/23120541.00117-2021).

Airway smooth muscle cells from severe asthma patients with fixed airflow obstruction are responsive to steroid and bronchodilator treatment *in vitro*

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To the Editor:

Asthma is characterized by recurrent symptoms associated with variable airflow obstruction and airway hyperresponsiveness, all of which are improved with combination inhaled corticosteroids (ICS)/long-acting β-agonist (LABA) treatment in mild-to-moderate asthma[1]. A proportion of patients however develop fixed airflow obstruction (FAO), despite optimized treatment. FAO is prevalent in up to 60% of patients with severe asthma, and is associated with more rapid decline in lung function and increased symptoms[2]. The underlying mechanisms of FAO in asthma are poorly understood; therefore, development of novel treatment strategies remain a challenge.

Airway smooth muscle cells (ASMCs) are the major effector cells of bronchoconstriction in asthma and also contribute to the inflammatory process by secreting pro-inflammatory cytokines and chemokines, and are therefore a major target of both β_2-agonist and ICS treatment[3]. Although several studies have suggested that steroid signalling[4] or β_2 adrenoceptor signalling may be abnormally regulated in severe asthma[5], it remains unknown whether impaired airway smooth muscle corticosteroid and/or β_2 agonist response may contribute to the development of FAO. The aim of this study was therefore to investigate whether primary human ASMCs obtained from severe asthma patients with FAO differ in their response to β_2-agonists and corticosteroids compared to asthma patients without FAO and healthy controls. We hypothesized that ASMCs from asthma patients with FAO are less responsive to corticosteroid and β_2-agonist treatment than from patients without FAO.

Human ASMCs were obtained from bronchial biopsies of severe asthma patients with FAO (*n*=6, mean FEV_1 percent predicted ±SD: 56±16%, mean FEV_1/FVC: 0.48±0.06), who were clinically unresponsive to high dose ICS/LABA treatment[6], mild asthmatic patients with reversible airflow obstruction (*n*=6, mean FEV_1 percent predicted: 79.8±10%, mean FEV_1/FVC:0.77±0.10) who had clinically improved after ICS/LABA treatment[7] and from healthy volunteers without any respiratory disease (*n*=4). Patient demographics are summarized in Table 1. FAO was defined as less than 200ml and 12% improvement in spirometry post-bronchodilator (400mcg inhaled salbutamol) and a post-bronchodilator FEV_1/FVC ratio below the lower limit of normal. The ASMCs were isolated and grown in culture as previously described[8]. All experiments were carried out using ASMCs with passage number 4 or 5. Ethical approval for this study was granted by the Ethics Review Committees of Sydney South West Area Health Service (RPA, X02-0137) and Sydney Local Health District (CRGH, HREC/14/CRGH/75).

To assess β_2-adrenoreceptor (β_2AR) signalling, the cells were stimulated with the β_2 agonist, salbutamol (Sigma-Aldrich, NSW, Australia) and levels of intracellular cAMP and phosphorylated vasodilator stimulated phosphoprotein (pVASP) were measured as markers of β_2AR activation. Activation of β_2AR leads to activation of adenylyl cyclase and subsequent cAMP formation. cAMP phosphorylates protein kinase A (PKA), which phosphorylates multiple proteins including VASP, leading to reductions in intracellular calcium, smooth muscle relaxation, and bronchodilation. Maximal cAMP levels (in presence of a phosphodiesterase (PDE) inhibitor) were measured using an ELISA kit according to the manufacturer's instructions (Cayman Chemical Company, USA). In cells with no PDE inhibition, VASP phosphorylation was assessed by Western Blotting as previously described[9], using mouse anti-VASP/pVASP (1:1500; BD Biosciences, San Jose, CA) and mouse anti-GAPDH (loading control) (1:5000; MilliporeSigma) antibodies.

To assess whether ASMCs from severe asthmatics with FAO are intrinsically more resistant to corticosteroids compared to those from mild asthmatics and healthy controls, cells were treated with increasing concentrations of fluticasone (0.1, 1, 10 nM; Sigma-Aldrich) for 1h before stimulation with Tumor Necrosis Factor-α (TNFα) (10 ng/ml) for 24h. TNFα is a multipotent proinflammatory mediator, mainly produced by macrophages, and plays a critical role in the immunoregulation of asthma by enhancing inflammation and airway hyperresponsiveness. Release of Interleukin (IL)-6 and CXCL8, two pro-inflammatory chemo-attractant cytokines that have been implicated in the pathogenesis of severe asthma[10] were measured in the supernatant using sandwich ELISA (BD Biosciences) according to the manufacturer's instructions.

We observed similar salbutamol-induced cAMP release from ASMCs derived from healthy individuals and patients with mild asthma and severe asthma with FAO (*Figure 1A*). There was a trend for greater cAMP production as asthma severity increased. Consistent with this, there was a similar increase in VASP phosphorylation in response to salbutamol in the three groups (*Figure 1B and 1C*).

Furthermore, fluticasone partially reduced IL-6 and CXCL8 release induced by TNFα *(Figure 1D and 1E*). At the highest concentration of 10 nM there was an inhibition of approximately 80% of TNFα-induced IL-6 release and 40% of TNFα-induced CXCL-8 release. Again, there were no differences between ASMCs from healthy volunteers or mild versus severe asthmatics.

To our knowledge, our study is the first that has addressed the question of whether airway smooth muscle cells from severe asthmatic patients with FAO are intrinsically more resistant to β_2-agonists and/or steroids compared to cells from responsive asthmatics and healthy subjects. We have previously found both maximal cAMP production and ASM-derived cytokine inhibition with corticosteroids to be similar in mild asthma in comparison to non-asthmatic individuals[11, 12] but hypothesized that these would be abnormal in patients with FAO, since clinically these patients are unresponsive to these treatments[6]. We found that ASMCs derived from severe asthmatic patients with FAO respond to β2-agonists and corticosteroids *in vitro*, and at a level similar as mild asthmatics who are treatment responsive[7] and therefore reject our hypothesis.

These findings suggest that FAO in asthma is unlikely due to intrinsic dysfunction in β_2-adrenoceptor or corticosteroid signalling in airway smooth muscle. Instead, patients with FAO may be refractory to ICS/LABA treatment related to other factors, such as the presence of airway fibrosis limiting the ability of the airways to dilate in response to bronchodilators. We further assessed the relationship between reticular basement membrane (RBM) thickness and FEV_1/FVC ratio. We found that greater RBM thickness was related to increased airflow obstruction (decreased FEV_1/FVC ratio) (*Figure 1F*), thereby supporting the notion of greater airway fibrosis occurring in patients with FAO. Other factors that could contribute to fixed airflow obstruction in asthma, include increased smooth muscle layer thickness, which was previously shown to be related to the clinical severity of asthma [13] or changes in the extracellular matrix and its regulators within the airway smooth muscle that may affect airway smooth muscle function [14]. Furthermore, parenchymal changes in patients with

asthma and FAO resulting in loss of elastic recoil may also affect the airways ability to dilate because of alteration to the airway-parenchymal interdependence [6].

A potential limitation to the present study is that the ASMCs were derived from bronchial biopsies taken from the large airways only. Although both the small and large airways have been implicated in the pathogenesis of asthma and several studies have reported that structural and functional changes occur in both the small and large airways, it is important to note that the small airways may be more implicated in severe disease [15]. Another potential limitation of our study is that we only measured the biochemical pathway downstream of the β_2 receptor. It is possible that either relaxation or contraction are affected independently of the ability to produce cAMP.

Our findings have important implications for the treatment of patients with FAO, and in particular given our observation of no intrinsic β_2AR desensitisation or corticosteroid resistance, our data does not support the use of high dose treatments in these patients if these are used in an attempt to overcome intrinsic abnormalities. Treatment should therefore be titrated according to clinical outcomes. Our data indicate that novel pharmacological interventions targeting β_2 receptor or corticosteroid signalling are unlikely to be effective in asthma with FAO and there is a need for treatments that effectively target the underlying causes of FAO and treatment resistance. Unfortunately, mechanisms resulting in FAO in asthma remain poorly understood[2], and until these are discovered FAO will remain difficult to treat.

Disclosures: The authors have no conflicts of interest to disclose

Support statement: This study was supported by a bridging grant from the University of Sydney, a Philanthropic grant from the Berg Family Foundation and the Lung Foundation Australia Ludwig Engel grant-in-aid.

Figure Legends

Figure 1. Human airway smooth muscle cells (ASMCs) derived from healthy volunteers (*n*=4), mild asthma patients *(n*=6) and severe asthma patients with fixed airflow obstruction (*n*=6) were treated with β_2-agonist salbutamol (A, B, C). cAMP (pmol/ml) was measured by ELISA in presence of a PDE inhibitor, IBMX 0.5mM (A). VASP phosphorylation (pVASP) was measured using Western Blotting (B, C) and is expressed a ratio of total VASP. ASMCs were also treated with increasing concentrations of fluticasone for 1h before stimulation with Tumor Necrosis Factor-α (TNFα) (10 ng/ml) for 24h. IL-6 and CXCL8 release was measured using ELISA and data is presented as percent inhibition of TNFα-induced IL-6 (D) and CXCL8 (E) release. Reticular basement membrane (RBM) thickness was measured from endobronchial biopsies of the airway wall of asthma patients (*n*=8) with a range of airflow obstruction as measured by FEV_1/FVC ratio. RBM thickness constituted the measurement of the length of a straight line drawn transversely across the basement membrane perpendicular to the mucosal surface. Six measurements were taken per biopsy using Image J, of which the average was taken. Increasing RBM thickness was related to decreasing FEV_1/FVC ratio, Spearman's rank correlation coefficient =-0.82, *p*=0.02

Table 1 Patient demographics

	Severe asthma	Mild asthma
Gender (F/M)	2/4	1/5
Age (years)	66.2 ± 9.4	23.2 ± 3.9
BMI (kg/m^2)	28.4 ± 10.1	24.2 ± 5.5
Smoking history		
- non-smokers (n)	5	6
- ex-smokers (n)	1	-
Atopic (n)	3/6	6/6
FEV_1 (L)	1.74 ± 0.73	3.43 ± 0.70
FEV_1 (% predicted)	56.0 ± 16	79.8 ± 10
FVC (L)	3.38 ± 1.29	4.49 ± 0.87
FVC (% predicted)	86.2 ± 20.2	88.9 ± 4.3
FEV_1/FVC	0.48 ± 0.06	0.77 ± 0.10
FEV_1/FVC (% predicted)	65.7 ± 9.6	90.0 ± 10.8

Data are expressed as mean ± SD. Atopy was assessed by skin prick test. FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity. The one ex-smoker in the severe asthma group had smoking history of 1 pack-year.

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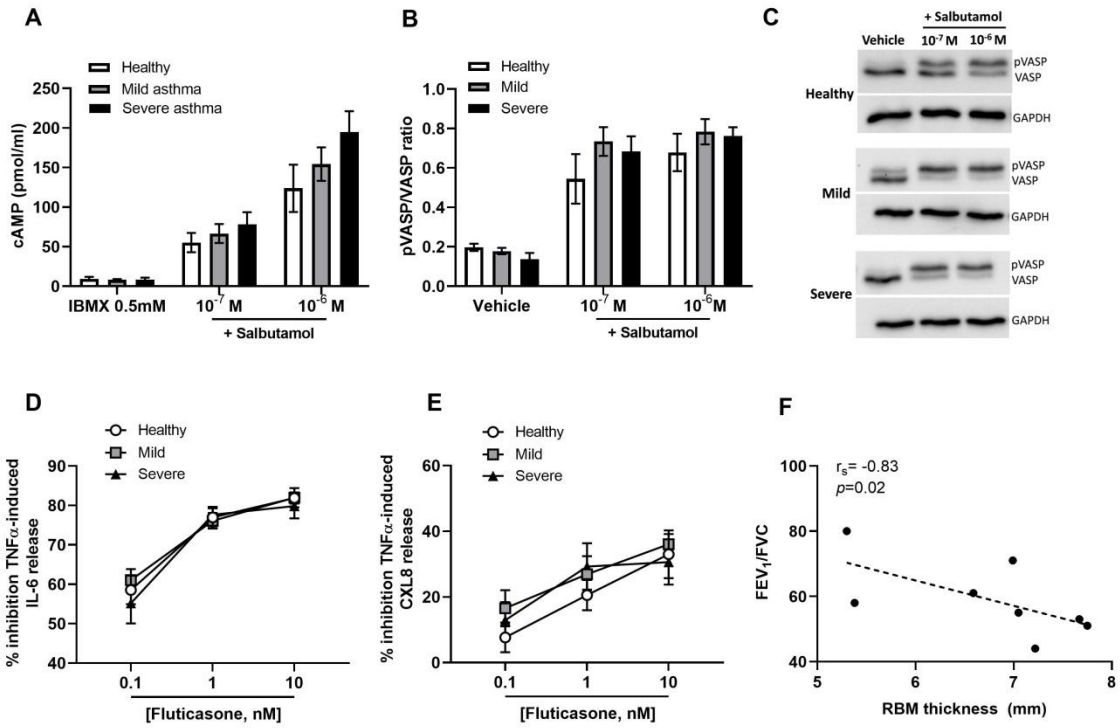
A
Healthy
Mild asthma
Severe asthma
cAMP (pmol/ml)
250
200
150
100
50
0
IBMX 0.5mM
10-7 M
10-6 M
+ Salbutamol
B
Healthy
Mild
Severe
pVASP/VASP ratio
1.0
0.8
0.6
0.4
0.2
0.0
Vehicle
10-7 M
10-6M
+ Salbutamol
C
+ Salbutamol
Vehicle
10-7 M
10-6 M
Healthy
Mild
Severe
pVASP
VASP
GAPDH
D
Healthy
Mild
Severe
% inhibition TNFα-induced IL-6 release
90
80
70
60
50
40
0.1
1
10
[Fluticasone, nM]
E
Healthy
Mild
Severe
% inhibition TNFα-induced CXL8 release
60
40
20
0
0.1
1
10
[Fluticasone, nM]
F
rs= -0.83
p=0.02
FEV1/FVC
100
80
60
40
5
6
7
8
RBM thickness (mm)