Title: β2-agonists enhance asthma-relevant inflammatory mediators in human airway epithelial cells

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Introduction

Inhaled β_2-agonists are frequently used therapies in asthma. They mediate their protective bronchodilator actions by inducing intracellular cyclic adenosine monophosphate (cAMP) in smooth muscle cells (SMCs). Although β_2-agonists have undoubted beneficial effects, safety concerns have been repeatedly raised regarding their use in asthma. Regular SABAs used in stable asthma results in worse asthma control than as required use(1, 2), suggesting that use when not needed to relax contracted smooth muscle is actually harmful. Overuse of SABAs in the absence of inhaled corticosteroid (ICS) in asthma exacerbations has been repeatedly associated with increased risk of hospitalisation or mortality.(3) LABA use (likely in the absence of ICS) has also been linked to increased asthma mortality(4), although recent studies emphatically confirm their use with ICS is safe and beneficial.(5, 6) A recent National Review of Asthma Deaths identified 40% of deaths were prescribed too many SABA inhalers and in five deaths, LABAs were prescribed without ICS.(7) The mechanisms underpinning these safety concerns have not been elucidated.

We have previously reported that IL-6, a pro-inflammatory cytokine(8, 9), is induced in bronchial epithelial cells (BECs) by salmeterol, but that induction disappeared when co-administered with ICS.(8) Importantly in relation to overuse of β_2-agonists in the context of asthma exacerbations, IL-6 induction by rhinovirus (RV) was further augmented by β_2-agonists.(8) Promoter studies revealed that β_2-agonists augmentation of RV-induced IL-6 occurred via a cAMP response element (CRE) in the IL-6 promoter and thus the adverse effect (induction of IL-6 in BECs) occurs via exactly the same mechanism (induction of cAMP), as beneficial effects in SMCs.(8) Another disease-relevant CRE-regulated gene, brain-derived neurotropic factor

(BDNF), has been shown to be increased in serum following treatment with salmeterol monotherapy.(10)

Previous work reports that hundreds of genes are regulated via CREs in their promoters (and are therefore potentially inducible by β_2-agonists).(11) These studies suggest that β_2-agonists could induce many genes implicated in asthma pathogenesis and this may provide a biological mechanism through which adverse effects occur. Herein, we demonstrate that β_2-agonists induce several mediators relevant to asthma pathogenesis in BECs.

Methods

BEAS-2B BECs were treated with salmeterol, formoterol +/- fluticasone propionate at clinically relevant concentrations, or medium control, with or without infection with rhinovirus (RV)-16, as previously described.(8) Supernatants and cell lysates were collected at 24 hours post-stimulation.

BDNF, IL-6 and IL-11 proteins in cell supernatants were measured by ELISA according to manufacturer's instructions(R&D Systems). RNA-Seq libraries were prepared using the mRNA isolation protocol and NEBNext-Ultra kits (New England Biolabs) following manufacturer's instructions. Libraries were quantitated by Qubit (Invitrogen), and run on a MiSeq instrument with paired-end 75bp reads using v3 chemistry (Illumina). Significant fold changes were calculated by Cuffdiff RNA-Seq analysis software using Storeys Q-test to calculate statistical significance having corrected for the false discovery rate.

Results

Having previously observed that IL-6 is induced by salmeterol alone, and that salmeterol augmented RV-16 induction of IL-6 in airway epithelial cells,(8) we initially re-confirmed these observations in these independent experiments and showed that the same inductions occurred with another commonly used LABA formoterol (figure 1A).

We then determined whether BDNF is similarly upregulated in airway cells, given previous data reporting its induction by salmeterol in blood cells.(10) Salmeterol or formoterol alone induced BDNF protein release and both LABAs also strongly augmented virus-induced BDNF protein release (fig 1B). We also observed that pre-treatment with concomitant fluticasone completely suppressed the induction of BDNF protein by salmeterol (figure 1B).

We carried out RNA-seq to further explore genes upregulated in BECs by salmeterol, alone or in combination with RV. We found that 7 genes were significantly up-regulated by salmeterol alone, compared to media. When compared to RV alone, addition of salmeterol to RV-infected cells further up-regulated 6 of the 7 genes and one additional further gene (*Growth Arrest Specific 1*) (Figure 2A).

We next carried out confirmatory analysis to measure IL-11, the only secreted protein identified. Similar to our observations with BDNF, salmeterol alone significantly induced IL-11 protein in BEC supernatants and enhanced its production in response to RV infection (figure 2B).

Discussion

We demonstrate that IL-6, BDNF and IL-11, mediators that have previously been shown to be important in asthma pathogenesis and/or severity (10, 12, 13) are induced in BECs by the commonly used β_2-agonists salmeterol and formoterol. Our study extends previous work demonstrating that the pro-inflammatory cytokine IL-6 is upregulated by β_2-agonists. Importantly in relation to overuse of β_2-agonists in the context of an asthma exacerbation, induction of all of these mediators by RV was potentiated by β_2-agonists. Previous studies have reported that IL-11 enhances mucus production and Th2 inflammation, and correlates with asthma severity and lung function impairment.(13) BDNF has also been shown to correlate with airway hyperresponsiveness and asthma severity and can be induced in airway epithelium by IL-13.(10)

The RNA-seq, examining the effects of salmeterol alone and salmeterol with rhinovirus infection, demonstrated 8 significantly upregulated genes of interest. However, importantly there were additional non-significant (by the rigorous Storey's q test) increases in targets of interest, including *IL6*. Whilst the RNA-seq found a lack of significant induction of *IL6* for salmeterol relative to media control, this was a result of very low expression levels in the material analysed. A non-significant twofold-induction was shown for salmeterol with rhinovirus infection versus rhinovirus control which is in keeping with our ELISA data (figure 1A and 1B).

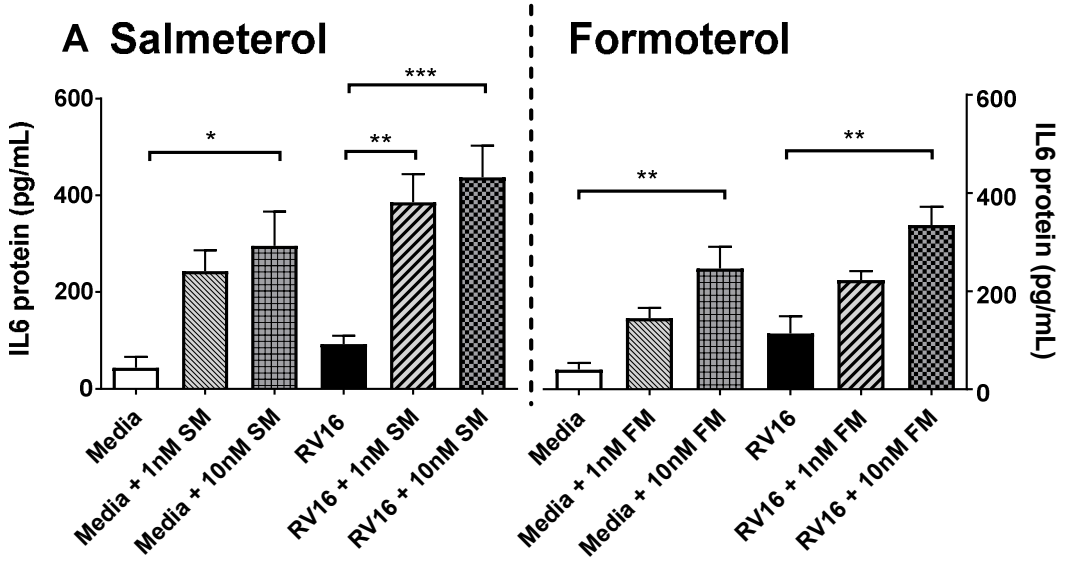
Our data suggest that β_2-agonists, while causing immediate bronchodilatory symptomatic relief through relaxation of contracted airway smooth muscle, have the potential to increase adverse effects such as airway obstruction and

hyperresponsiveness through enhancement of mucosal inflammation, via induction of inflammatory mediators in BECs.(12) Such induction of inflammation would be likely to be most robust at times of use of β_2-agonists in the absence of ICS, such as during virus-induced exacerbations. This leads to speculation that overuse of β_2-agonists could potentially enhance inflammation, mucus secretion and exacerbation severity.

Interestingly our RNA-seq demonstrated that salmeterol enhances cAMP-specific 3',5'-cyclic phosphodiesterase 4B (PDE4B), a key protein responsible for cAMP degradation, this induction may therefore reflect activation of regulatory pathways by beta-agonists.

A limitation of our studies is that they were performed in a transformed bronchial epithelial cell line, thus further *in vitro* studies in primary bronchial epithelial cells as well as other lung cell populations and *in vivo* studies are needed to confirm these preliminary findings and determine whether other mediators relevant to asthma are also induced by β_2-agonists and to determine the mechanistic and clinical relevance of these effects.

Figure 1A and 1B:



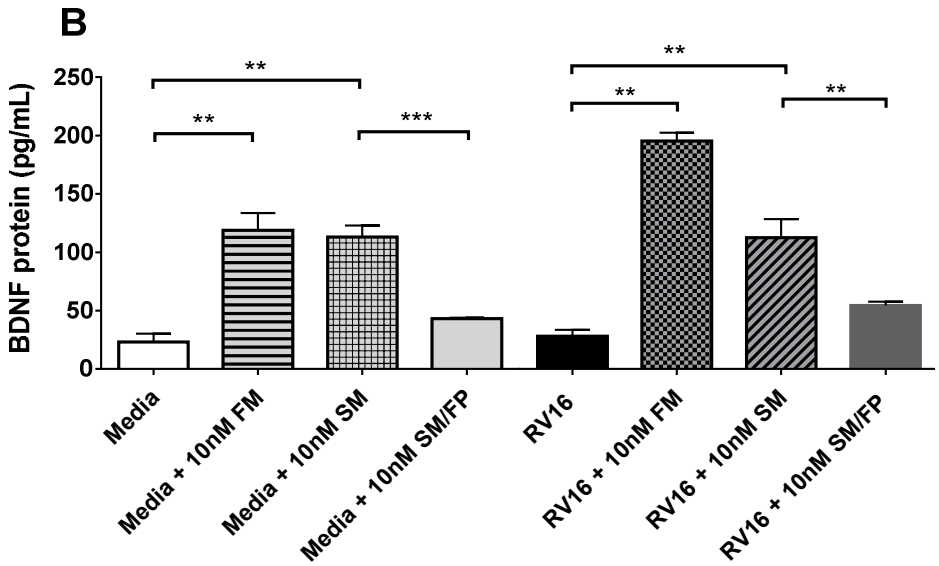


Figure 1: Beta-2 agonists enhance production of inflammatory mediators relevant to asthma in human airway epithelial cells. BEAS-2B cells were treated with salmeterol (SM), formoterol (FM) at 1 and 10nM concentrations or with SM alone or SM combined with fluticasone propionate (FP) both at 10nM concentrations prior to infection with rhinovirus-16 or incubation with medium control. IL-6, and BDNF proteins were measured in cell supernatants at 24 hours post-stimulation by ELISA.

Data presented as mean(±SEM) for n= 3-7 independent experiments. One way ANOVA with Bonferroni's post-test was used to analyse data. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Figure 2A and 2B:

A

Gene	Protein name	Promotor CRE[12]	Conserved CRE[12]	Fold induction by salmeterol relative to media (q value)	Fold induction by salmeterol + RV relative to RV alone (q value)	Function
BMPER	BMP binding endothelial regulator	Half site	Yes: H_-1085_723	3.21 (0.048)	1.64 (NS)	Fibrosis and angiogenesis.
IL11	Interleukin-11	Half site	Yes: H_-2494_790 H_-2512_808	3.20 (0.039)	2.67 (0.035)	Increased in severe asthma, correlates with disease severity and remodelling.
PDE4D	3',5'-cyclic phosphodiesterase 4D	Half site	Yes: HT_-1179_210	4.27 (0.007)	2.74 (0.007)	Degrades cyclic AMP.
SFRP1	Secreted frizzled-related protein 1	Half site	No	2.46 (0.019)	2.43 (0.025)	Associated with lung inflammation, Wnt increased in peripheral blood in asthma.
SMOC1	SPARC related modular calcium binding 1	Half site	Yes: HT_-83_43	2.94 (0.007)	3.45 (0.007)	Airway wall remodelling.
SMOX	Spermine oxidase	Full + half sites	No	4.06 (0.007)	2.56 (0.007)	Promotes oxidation of polyamines.
TFPI2	Tissue factor pathway inhibitor 2	Half site	No	4.05 (0.007)	4.44 (0.007)	Implicated in inflammation.
GAS1	Growth arrest-specific 1	Half site	No	1.78 (NS)	2.57 (0.019)	Tumour suppressor gene.

B

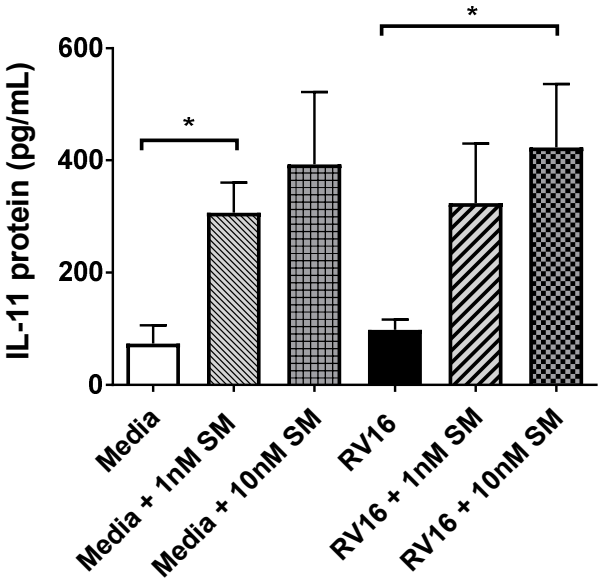


Figure 2A: Genes upregulated by salmeterol, as assessed by RNAseq analysis. BEAS-2B cells were treated with salmeterol at 10nM concentration prior to infection with rhinovirus-16 or incubation with medium control. RNA was extracted from cell lysates at 24 hours post-stimulation. (NS = not significant)

Full Site/Half Site: All occurrences of full site (TGACGTCA) or half site(TGACG/CGTCA) CREs in -5Kb-1Kb region of the transcription start site (TSS). (Computational prediction of functional CREs on promoters of human RefSeq genes: http://natural.salk.edu/CREB/ (accessed 15/5/17))

Conserved CRE: All the conserved full and half site are shown in this column since they are the most likely functional CREs. These CREs are shown similar to the format used by Full site/Half site, with the addition of the distance to closest downstream TATA box, shown as the last number.(11)

Figure 2B: BEAS-2B cells were treated with salmeterol (SM), 1 and 10nM concentrations prior to infection with rhinovirus-16 or incubation with medium control. IL-11 proteins were measured in cell supernatants at 24 hours post-stimulation by ELISA. Data presented as mean(±SEM) for n= 3-5 independent experiments. One way ANOVA with Bonferroni's post-test was used to analyse data. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

References

1. Drazen JM, Israel E, Boushey HA, Chinchilli VM, Fahy JV, Fish JE, et al. Comparison of regularly scheduled with as-needed use of albuterol in mild asthma. Asthma Clinical Research Network. The New England journal of medicine. 1996;335(12):841-7.
2. Salpeter SR, Ormiston TM, Salpeter EE. Meta-analysis: respiratory tolerance to regular beta2-agonist use in patients with asthma. Annals of internal medicine. 2004;140(10):802-13.
3. Anderson HR, Ayres JG, Sturdy PM, Bland JM, Butland BK, Peckitt C, et al. Bronchodilator treatment and deaths from asthma: case-control study. BMJ (Clinical research ed). 2005;330(7483):117.
4. Hasford J, Virchow JC. Excess mortality in patients with asthma on long-acting beta2-agonists. The European respiratory journal. 2006;28(5):900-2.
5. Peters SP, Bleecker ER, Canonica GW, Park YB, Ramirez R, Hollis S, et al. Serious Asthma Events with Budesonide plus Formoterol vs. Budesonide Alone. New England Journal of Medicine. 2016;375(9):850-60.
6. Stempel DA, Raphiou IH, Kral KM, Yeakey AM, Emmett AH, Prazma CM, et al. Serious Asthma Events with Fluticasone plus Salmeterol versus Fluticasone Alone. The New England journal of medicine. 2016;374(19):1822-30.
7. Levy ML. The national review of asthma deaths: what did we learn and what needs to change? Breathe. 2015;11(1):14-24.
8. Edwards MR, Haas J, Panettieri RA, Jr., Johnson M, Johnston SL. Corticosteroids and beta2 agonists differentially regulate rhinovirus-induced interleukin-6 via distinct Cis-acting elements. The Journal of biological chemistry. 2007;282(21):15366-75.
9. Yokoyama A, Kohno N, Fujino S, Hamada H, Inoue Y, Fujioka S, et al. Circulating interleukin-6 levels in patients with bronchial asthma. Am J Respir Crit Care Med. 1995;151(5):1354-8.
10. Lommatzsch M, Lindner Y, Edner A, Bratke K, Kuepper M, Virchow JC. Adverse effects of salmeterol in asthma: a neuronal perspective. Thorax. 2009;64(9):763-9.
11. Zhang X, Odom DT, Koo SH, Conkright MD, Canettieri G, Best J, et al. Genome-wide analysis of cAMP-response element binding protein occupancy,

phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci U S A. 2005;102(12):4459-64.

12. Johnston SL, Edwards MR. Mechanisms of adverse effects of {beta}-agonists in asthma. Thorax. 2009;64(9):739-41.

13. Minshall E, Chakir J, Laviolette M, Molet S, Zhu Z, Olivenstein R, et al. IL-11 expression is increased in severe asthma: Association with epithelial cells and eosinophils. Journal of Allergy and Clinical Immunology. 2000;105(2, Part 1):232-8.