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Fluticasone, but not salmeterol, reduces cigarette smoke-induced production of interleukin-8 in human airway smooth muscle

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Running title: Fluticasone & cigarette smoke-induced IL-8

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Abstract (235 words)
Cigarette smoke is the leading risk factor for the development of chronic obstructive pulmonary disease. We have recently shown that cigarette smoke extract synergises with TNFα in the induction of IL-8 from human airway smooth muscle cells. We have investigated the effect of fluticasone propionate, a corticosteroid, and salmeterol, a β2-adrenergic receptor agonist, on cigarette smoke extract-induced IL-8 production by human airway smooth muscle cells.

Human airway smooth muscle cells in primary culture were exposed to cigarette smoke extract and/or TNFα (1 ng ml⁻¹) with and without pretreatment with fluticasone (10⁻¹³ – 10⁻⁸ M) and/or salmeterol (10⁻¹¹ – 10⁻⁶ M). IL-8 was analysed by ELISA. Fluticasone dose-dependently inhibited IL-8 release induced by cigarette smoke extract, TNFα or combined cigarette smoke extract and TNFα. However, while IL-8 release in the presence of cigarette smoke extract alone was completely inhibited by fluticasone, IL-8 production induced by cigarette smoke extract and TNFα was only partially reduced. Salmeterol alone had no effect on cigarette smoke extract and/or TNFα-induced IL-8 production from human airway smooth muscle cells. Combined fluticasone and salmeterol did not cause further inhibitory effects compared to fluticasone alone.

Fluticasone but not salmeterol is effective in reducing cigarette smoke extract-induced IL-8 production in human airway smooth muscle cells. The reduced inhibition of cigarette smoke extract- and TNFα-induced IL-8 release by fluticasone may explain why corticosteroids are less effective in chronic obstructive pulmonary disease where increased amounts of TNFα are present.
**Keywords:** airway smooth muscle, chronic obstructive pulmonary disease, cigarette smoke, interleukin-8, glucocorticosteroids, β_{2}-agonists
Introduction

Chronic obstructive pulmonary disease is a major public health problem that is currently ranking as the fourth leading cause of death in the world [1]. It is characterised by progressive and largely irreversible airflow limitation associated with symptoms such as cough, sputum production, and dyspnoea, and by a chronic inflammatory response which may represent a response to noxious particles and gases present in tobacco smoke. Thus, cigarette smoke represents a powerful inflammatory insult to the lungs which may be the most important pathological mechanism leading to chronic obstructive pulmonary disease. Increased numbers of inflammatory cells are found even in the lungs of asymptomatic smokers [2, 3]. Cigarette smoke activates lung macrophages as well as resident lung cells such as epithelial cells and fibroblasts to release inflammatory mediators including tumour necrosis factor (TNF-) α and the neutrophil chemokine, interleukin (IL-) 8 [4-6]. These mediators, together with proteases and oxidants produced by activated neutrophils and macrophages, are capable of sustaining inflammation and damaging lung architecture [7].

Human airway smooth muscle cells represent an important structural component of the airway wall. In addition to their traditionally accepted role as contractile cells, human airway smooth muscle cells are a rich source of chemokines including IL-8, eotaxin and RANTES, [8, 9]. Chemokines play an integral role in the coordination and persistence of airway inflammation by enhancing leukocyte migration into inflamed airways. Cytokines such as IL-1β, TNFα, IL-17 and transforming growth factor (TGF) β are capable of activating airway smooth muscle synthetic capacity [10, 11, 12]. We recently found that cigarette smoke extracts activate human airway smooth muscle cells to release IL-8, an effect that was
significantly enhanced when cigarette smoke extract was combined with TNFα [13] and that a similar phenomenon occurs in human monocytes and macrophages [6]. In contrast, the TNFα-induced production of eotaxin and RANTES by human airway smooth muscle cells was inhibited by cigarette smoke. The synergy between cigarette smoke and TNFα in the induction of IL-8 release from human airway smooth muscle cells is of clinical significance because inflammatory cytokines, such as TNFα, are present in the lungs of chronic obstructive pulmonary disease patients [14].

Currently, the established pharmacotherapy of chronic obstructive pulmonary disease consists of inhaled bronchodilators, including β2-agonists and anticholinergics and corticosteroids [1]. The reported beneficial effects of inhaled corticosteroids in the treatment of chronic obstructive pulmonary disease have been somewhat variable. Although recent studies indicate that regular treatment with inhaled corticosteroids does not influence the rate of decline of airflow obstruction, other studies have shown a reduction in the rate of exacerbations particularly in severe patients and a significant improvement in health status [15]. More effective symptom control and reduction in exacerbation rates is achieved by a combination of inhaled corticosteroids and long-acting β2-agonist treatment [16, 17]. In addition, patients on combined fluticasone propionate and salmeterol therapy show a survival advantage over patients using each of these drugs alone [18] and current guidelines recommend treatment with both drugs for patients with moderate to severe chronic obstructive pulmonary disease [1].

Previous studies have reported that there may be additive or synergistic effects of the combination of corticosteroids and β2-agonists on human airway smooth muscle cells in the inhibition of the release of IL-8 by TNFα [19]. For better applicability of these data to chronic obstructive pulmonary disease, we examined the potential mechanism by which corticosteroids and the combination of corticosteroids
and β2-agonists may be beneficial in patients with chronic obstructive pulmonary disease by studying whether fluticasone propionate and salmeterol alone or in combination can modulate cigarette smoke extract-induced IL-8 release.

Methods

Materials

Cigarettes (Phillip Morris, UK) were bought from a London high street supermarket. Tissue culture reagents were obtained from Sigma (Poole, UK). Cell culture plasticware was purchased from Falcon Labware (Becton Dickinson, Oxford, UK). Recombinant human TNFα and matched antibody pairs for IL-8 enzyme-linked immunosorbent assays (ELISA) were purchased from R&D Systems (DuoSet, Abingdon, UK). All other chemical reagents were obtained from Sigma (Poole, UK).

Isolation and culture of human airway smooth muscle cells

Human airway smooth muscle was obtained from lobar or main bronchus from patients undergoing lung resection for carcinoma of the bronchus. This study was approved by the Ethics Committee and, informed and signed consent was obtained from each patient. The smooth muscle was dissected out under sterile conditions and placed in culture as previously described [20]. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum supplemented with sodium pyruvate (1mM), L-glutamine (2mM), non-essential amino acids (1:100), penicillin (100 U ml⁻¹)/streptomycin (100 µg/ml) and amphotericin B (1.5 µg ml⁻¹) in a humidified atmosphere at 37°C in air/CO₂ (95:5 % vol/vol). At confluence, human airway smooth muscle cell cultures exhibited a typical hill-and-valley appearance.
Immunofluorescence techniques for calponin, smooth muscle $\alpha$-actin and myosin heavy chain revealed that more than 95% of the cells displayed the characteristics of smooth muscle cells in culture. Human airway smooth muscle cells at passages 3-7 from 8 different donors were used.

**Cigarette Smoke Extract**

In order to prepare cigarette smoke extract, four cigarettes (filters removed) were combusted through a modified 60 ml syringe apparatus and the smoke passed through 100 mls of Dulbecco’s modified Eagle’s medium. Each cigarette yielded 5 draws of 60 ml of the syringe, with each individual draw taking approximately 10 seconds to complete. This solution represents ‘100%’ strength. Smoked medium was then passed through a 0.25 $\mu$m filter in order to sterilise the solution. Smoked medium was diluted to the required strength in Dulbecco’s modified Eagle’s medium and placed upon the cells immediately afterwards.

**Cell treatment**

Prior to the experiments, confluent cells were growth-arrested by fetal calf serum deprivation for 24h in Dulbecco’s modified Eagle’s medium supplemented with sodium pyruvate (1 mM), L-glutamine (2 mM), non-essential amino acids (1:100), penicillin (100 U/ml)/ streptomycin (100 $\mu$g/ml), amphotericin B (1.5 $\mu$g/ml), insulin (1 $\mu$M), transferrin (5 $\mu$g/ml), ascorbic acid (100 $\mu$M) and bovine serum albumin (0.1 %). Cells were then exposed to cigarette smoke extract (10 %) in the presence and absence of TNF$\alpha$ (1 ng/ml). In additional experiments cells were pretreated with fluticasone propionate ($10^{-13}$ – $10^{-8}$ M) and/or salmeterol
(10^{-11} – 10^{-6} M) for 2 hours before adding cigarette smoke extract or cigarette smoke extract and TNFα.

**Cytokine assay**

Cell supernatants were harvested 24 hours after stimulation and stored at -70°C until assayed for IL-8. Cytokine levels were determined by using specific sandwich enzyme-linked immunosorbent assays (ELISA) according to the manufacturers’ instructions.

**Data analysis**

Data are presented as mean ± SEM. Data were compared using one-way analysis of variance (ANOVA) followed by Bonferroni’s t test post hoc to determine statistical differences. A p value <0.05 was considered significant. SigmaStat software (Jandel Scientific, Germany) was used for statistical analysis.
Results

Effect of fluticasone propionate and salmeterol on cigarette smoke induced IL-8 release

As shown previously [13], cigarette smoke extract (10%) and TNFα (1ng/ml) alone induced IL-8 release from human airway smooth muscle cells (41.1 ± 10.9 pg/ml and 400.12 ± 80.7 pg/ml, respectively), an effect that was significantly enhanced when cigarette smoke extract and TNFα were combined (743.12 ± 66.0 pg/ml). Fluticasone propionate inhibited cigarette smoke extract-induced IL-8 release from human airway smooth muscle cells in a dose dependent manner (IC50 (-log M): 11.2 ± 0.7) with significant effect at 10^-10 to 10^-8 M (Figure 1). Complete inhibition of IL-8 release from human airway smooth muscle cells by fluticasone propionate was observed at 10^-9 M.

Salmeterol alone at a concentration of 10^-11 – 10^-6 M had no effect on cigarette smoke extract-induced IL-8 production from human airway smooth muscle cells (data not shown). There was no enhanced inhibitory effect of fluticasone propionate on cigarette smoke extract-induced IL-8 release when combined with salmeterol (Figure 1).

Effect of fluticasone propionate and salmeterol on TNFα and cigarette smoke induced IL-8 release

IL-8 release from human airway smooth muscle cells induced by either TNFα alone or by the combination of TNFα and cigarette smoke extract, was dose-dependently inhibited by fluticasone propionate (IC50 (-log M): 10.7 ± 0.6; 10.7 ± 0.8, respectively) with maximum inhibition (65.3 ± 3.2% and 75.5 ± 4.3%, respectively) observed at the highest concentration used (10^-8 M, Figure 2 and 3). In contrast to
complete inhibition of cigarette smoke extract-induced IL-8 release by fluticasone propionate at $10^{-9}$ M, IL-8 production in the presence of TNFα and cigarette smoke extract was reduced by $62.1 \pm 3.5\%$ (p<0.001), associated with a right-shift of the concentration-response curve (Figure 4). The combination of fluticasone propionate and salmeterol showed no enhanced inhibitory effect compared to fluticasone propionate alone.
Discussion

We recently showed that cigarette smoke extract induces IL-8 release from human airway smooth muscle cells and synergises with TNFα in enhancing IL-8 production [13]. In the present study, fluticasone propionate inhibited IL-8 release induced by cigarette smoke extract, TNFα or the combination of cigarette smoke extract and TNFα in a dose-dependent manner. However, while IL-8 release in the presence of cigarette smoke extract alone was abolished by fluticasone propionate at $10^{-9}$ M, IL-8 production induced by cigarette smoke extract and TNFα was reduced by $62.1 \pm 3.5\%$ only, indicating reduced efficacy of fluticasone propionate in the presence of TNFα. In contrast, salmeterol had no effect on cigarette smoke extract and/or TNFα induced IL-8 production from human airway smooth muscle cells. The combination of fluticasone propionate and salmeterol did not result in enhanced inhibitory effect compared to fluticasone propionate alone. Our data show that TNFα alone causes some degree of steroid resistance, similar to cigarette smoke extract and IL-1β in monocytes [6] that was not reversed by the addition of salmeterol.

Neutrophils may mediate many of the aspects of lung damage seen in chronic obstructive pulmonary disease because they release various substances such as proteases, cytokines and oxidants that have deleterious effects on the airways and lung parenchyma [21, 22]. Elevated levels of IL-8, a potent neutrophil chemoattractant, were found in the bronchoalveolar lavage fluid of smokers compared to nonsmokers and expression of IL-8 in lungs of patients with chronic obstructive pulmonary disease increases with progression of disease [5, 23]. Accumulation of neutrophils in the lungs of patients with chronic obstructive pulmonary disease may therefore be linked to increased amounts of IL-8 in the lungs of smokers and patients with chronic obstructive pulmonary disease.
Glucocorticosteroids and long-acting β2-agonists remain central to the management of patients with chronic obstructive pulmonary disease [16]. Human airway smooth muscle cells express both β2-adrenergic and glucocorticoid receptors and therefore represent interesting target cells for treatment with corticosteroids and β2-agonists in inflammatory lung diseases. Previous studies have shown that corticosteroids are effective in inhibiting cytokine release from human airway smooth muscle cells [10, 24]. Dose-dependent inhibition of TNFα-induced IL-8 release by fluticasone propionate (10⁻⁸ - 10⁻⁶ M) has been shown previously [19]. In the present study, we used a wider range of concentrations to investigate the effect of fluticasone propionate on TNFα-induced IL-8 release and show that significant inhibitory effects occurred at a fluticasone propionate concentration as low as 10⁻¹⁰ M. In addition, fluticasone propionate was very potent in inhibiting cigarette smoke extract-induced IL-8 production from human airway smooth muscle cells with complete inhibition at concentrations as low as 10⁻⁹ M. The mechanisms involved in the cigarette smoke extract-induced cytokine release are not clear. However, oxidative stress-mediated activation of transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which regulate expression of various inflammatory genes including IL-8, may be involved [25]. Corticosteroids, after forming a complex with its cytoplasmic receptor, directly suppress the transcription of pro-inflammatory genes by binding to the promoter-enhancer regions of target genes. In addition, corticosteroids reduce the inflammatory effects of activator protein-1 and nuclear factor-κB by binding with these transcription factors and increasing the expression of the nuclear factor-κB inhibitor, IκBα [26]. Both direct suppression of gene transcription (by transactivation) and interaction with transcription factors (by transrepression) may
therefore contribute to inhibition of cigarette smoke extract-induced IL-8 release by fluticasone propionate.

Glucocorticosteroids achieve significant control of clinical symptoms in chronic obstructive pulmonary disease. However, corticosteroids are less efficient in reducing airway inflammation in chronic obstructive pulmonary disease than in asthma and the mechanisms of this reduced effectiveness in chronic obstructive pulmonary disease as compared to asthma is not known [27]. Oxidative stress may decrease the efficacy of corticosteroids in chronic obstructive pulmonary disease by reducing glucocorticoid receptor nuclear translocation and glucocorticoid receptor α expression [27]. In line with this hypothesis, a recent study reported impaired efficacy of inhaled and oral corticosteroids in smoking asthmatics compared to non-smoking asthmatics [28, 29]. Cigarette smoke extract may indeed induce oxidative stress in human airway smooth muscle cells and the release of IL-8 induced by cigarette smoke extract is blocked by the antioxidant, glutathione [13]. Culpitt et al [30] showed that dexamethasone inhibited IL-8 production by alveolar macrophages from smokers without evidence of chronic obstructive pulmonary disease but failed to inhibit IL-8 release from alveolar macrophages from patients with chronic obstructive pulmonary disease. Interestingly, we observed potent inhibition of cigarette smoke extract-induced IL-8 release by fluticasone propionate while the presence of TNFα significantly reduced the inhibitory effect of fluticasone propionate. Therefore, TNFα may induce resistance to the anti-inflammatory actions of corticosteroids and may contribute to steroid resistance in chronic obstructive pulmonary disease where increased amounts of TNFα are present [31]. Activation of mitogen-activated protein (MAP) kinases, such as p38 and c-Jun N-terminal kinase (JNK), may be linked to TNFα-induced steroid resistance. Mitogen-activated protein kinases, are important
mediators of TNFα-induced gene activation in human airway smooth muscle cells [32]. Szatmary et al [33] showed suppression of GR function by activated JNK and p38 MAP kinase indicating a potential mechanism behind reduced efficacy of steroids in chronic obstructive pulmonary disease. Activation of p38 MAP kinase has also been reported as underlying the reduced affinity of glucocorticoid receptor for its ligand induced by combination of IL-2 and IL-4 in peripheral blood mononuclear cells [34].

β2-agonists bind to the G-protein-coupled seven-transmembrane β2-adrenoreceptor to activate adenylyl cyclase resulting in the formation of intracellular cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A which in turn leads to phosphorylation of the transcription factor, cAMP response element binding element. Interaction of cAMP response element binding element with its binding protein may then modulate gene transcription [35]. In human airway smooth muscle cells, induction as well as suppression of gene expression by β2-agonists has been reported [35]. Pang et al [18] reported induction of IL-8 release by salmeterol at a concentration of 10^-7 M with further increase at 10^-6 M; however, they saw no effect of salmeterol on TNFα-induced IL-8 release. In line with clinical trials demonstrating additive effects of combined corticosteroid and β2-agonist treatment [36], combination of salmeterol and fluticasone propionate enhanced inhibition of IL-8 release from human airway smooth muscle cells compared to fluticasone propionate alone [18]. In a study by Ammit et al, TNFα-induced RANTES release from human airway smooth muscle cells was inhibited by dexamethasone with further additive inhibition when salmeterol was added to dexamethasone [37]. We saw no effect of salmeterol alone at 10^-11 – 10^-6 M on TNFα-induced IL-8 release over a 24 hour time
period. In addition, inhibition of TNFα-induced IL-8 release by fluticasone propionate was not enhanced when combined with salmeterol. We obtained similar data when examining effects on combined cigarette smoke extract and TNFα effects.

We have shown that fluticasone propionate but not salmeterol is effective in reducing smoking-induced IL-8 release from human airway smooth muscle cells. The increased presence of pro-inflammatory cytokines such as TNFα in airway inflammation in chronic obstructive pulmonary disease may impair the anti-inflammatory actions of corticosteroids in chronic obstructive pulmonary disease.

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Competing interests
KFC has been renumerated for participation at Advisory Board meetings of GSK, Astra-Zeneca and Novartis, and has received unrestricted research grant from GSK. MJ is an employee of GSK. Other authors have no competing interests.
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**Figure legends**

Figure 1: Effect of increasing concentrations of fluticasone propionate (10^{-13} - 10^{-8} M; □) alone and in combination with salmeterol (10^{-7} M; ■) on cigarette smoke-induced IL-8 release from human airway smooth muscle cells. Data show the percentage of IL-8 release from cells treated with cigarette smoke alone (mean ± SEM, n=4). *p<0.05, **p<0.01, ***p<0.001 compared to IL-8 release from cells in the presence of cigarette smoke alone.

Figure 2: Effect of increasing concentrations of fluticasone propionate (10^{-13} - 10^{-8} M; ○) alone and in combination with salmeterol (10^{-7} M; ●) on TNFα-induced IL-8 release from human airway smooth muscle cells. Data show the percentage of IL-8 release from cells treated with TNFα alone (mean ± SEM, n=4). *p<0.05, ***p<0.001 compared to IL-8 release from cells in the presence of TNFα alone.

Figure 3: Effect of increasing concentrations of fluticasone propionate (10^{-13} - 10^{-8} M; ○) alone and in combination with salmeterol (10^{-7} M; ●) on IL-8 release from human airway smooth muscle cells in the presence of cigarette smoke extract and TNFα. Data show the percentage of IL-8 release from cells co-stimulated with cigarette smoke (10%) and TNFα (mean ± SEM, n=4). *p<0.05, **p<0.01, ***p<0.001 compared to IL-8 release from cells in the presence of cigarette smoke and TNFα.

Figure 4: Comparison of the effect of increasing concentrations of fluticasone propionate (10^{-13} - 10^{-8} M) on IL-8 release from human airway smooth muscle cells in the presence of cigarette smoke alone (■) and when co-stimulated with cigarette
smoke and TNFα (○). Data show the percentage of IL-8 release from cells treated with cigarette smoke (10%) alone or in combination with TNFα (mean ± SEM, n=4). ***p<0.001 compared to IL-8 release from cells in the presence of cigarette smoke alone.
Figure 1

![Graph showing IL-8 release (% control) vs Fluticasone [M].]

- CSE (10%)
- CSE (10%)
  + salmeterol (10^{-7} M)

IL-8 release (% control)

Fluticasone [M]
Figure 2

![Graph showing IL-8 release (% Control) against Fluticasone [M]. The graph compares TNFα (1 ng/ml) with and without Salmeterol (10^-7 M).](image-url)
Figure 3

IL-8 (% control) vs. Fluticasone [M]

- TNFα (1 ng/ml) + CSE (10%)
- CSE (10%)
  + TNFα (1 ng/ml)
  + Salmeterol (10^-7 M)

Significance levels:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
Figure 4

![Graph showing IL-8 release (% Control) against Fluticasone [M]. The graph compares the effects of CSE (10%) and TNFα (1ng/ml) + CSE (10%) on IL-8 release.](image)