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Fluticasone propionate and Salmeterol combination induces SOCS-3 expression in airway epithelial cells ☆

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ABSTRACT

Fluticasone propionate (FP) and Salmeterol (SAL) are commonly used in combination therapy for patients with Chronic obstructive pulmonary disease (COPD). Clinical studies show that FP/SAL used in combination therapy was found to inhibit airway inflammation in COPD patients. However, the mechanisms associated with FP/SAL induced anti-inflammatory effects were not clear. We have evaluated the effect of FP/SAL and tobacco smoke (TS) on SOCS-3 and interleukine-6 expression in bronchial airway epithelial cells (BAEpCs). Human BAEpCs were exposed to TS and subsequently treated with FP or SAL alone or in combinations in the presence and absence of mitogen activated protein kinase (MAPK) inhibitors for either *Erk1/Erk2*, or *p38* or *PI3* kinase. In BAEpCs, TS induced IL-6 expression via ERK1/ERK2 MAPK pathway and FP/SAL inhibited TS mediated IL-6 expression. TS down regulated the SOCS-3 expression via activation of *Erk1/Erk2*, and *p38* MAPK signaling. When TS exposed BAEpCs were treated with FP/SAL SOCS-3 expression was restored. FP/SAL combinations induced significantly higher expression of SOCS-3 in BAEpCs when compared to individual drug. Pretreatment with Ly294002 a *PI3* MAPK inhibitor significantly attenuated FP/SAL induced SOCS-3 expression in BAEpCs. Furthermore, FP/SAL blunted TS induced phosphorylation of *Erk1/Erk2* and *p38* MAPK in BAEpCs. Our study suggests that TS inhibits SOCS-3, combination of FP/SAL has a profound synergistic effect on SOCS-3 induction in BAEpCs and it is dependent on *PI3* kinase signaling pathway. SOCS-3 may represent a potential biomarker for understanding the efficacy and a novel anti-inflammatory mechanism of FP/SAL combination therapy in the treatment of COPD.

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1. Introduction

Chronic-obstructive pulmonary disease (COPD) is a complex disease often caused by tobacco smoking, and is currently the fourth leading cause of death worldwide [1]. COPD is characterized by chronic inflammation involving the airways, parenchyma and pulmonary vasculature resulting in remodeling of the airways and destruction of respiratory bronchioles, alveoli and pulmonary capillary bed [2]. Cigarette smoking has been shown to be the single most important risk factor for developing COPD [3]. Inhaled corticosteroid Fluticasone propionate (FP) and long acting β2-adrenergic receptor agonist Salmeterol (SAL) are commonly prescribed for COPD patients in the United States. Combination therapy has been shown to reduce the frequency of exacerbations, mortality and improve overall quality of life for patients with COPD [4–7].

Airway epithelium serves as an important anatomical barrier to the environment. BAEpCs are the first cells to respond to smoking and in the event of inhalation of drugs. In COPD, airway inflammation is associated with epithelial cell damage, enlarged sub-mucosal mucus-secreting glands, and an increase in the amount of smooth muscle and connective tissue in the airway wall. Earlier studies have shown that glucocorticoids inhibit the expression of adhesion molecules and inflammatory cytokine production in various cell types. For example glucocorticoid inhibited the expression of cytokines GM-CSF, IL-8 and IL-6 by epithelial cells and fibroblasts [8–10]. Traditionally β-2 agonists are considered as bronchial smooth muscle relaxants. Recently their anti-inflammatory properties have been described [11–13]. The combination of glucocorticoid with β-2 agonists has shown enhanced anti-inflammatory effects in clinical studies as well as in cell culture model based studies [7,12,14–16]. Initiation of signal transduction by cytokines or their natural ligands and activation of signaling pathways in response to variety of cytokines and hormones have been described [17]. However the mechanisms which inhibit the signal transduction pathways are unclear.

Interleukine-6 (IL-6) is secreted during the process of tissue injury due to infection and exposure to noxious material. IL-6 modulates inflammatory processes via activation of signal transducer and activator of transcription (STATs) [18]. Although the cytokines that induce IL-6

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are well defined, the mechanisms which attenuate these signaling pathways are less clear. Recently, suppressors of cytokine signaling (SOCS) family of cytoplasmic proteins were reported to complete the negative feedback loop to attenuate signal transduction from cytokines. SOCS proteins inhibit components of cytokine signaling cascade via direct binding or by preventing access to the signaling complex by acting through JAK/STAT pathway [12,19–21]. SOCS family of inhibitors is comprised of at least eight members of SH2-domain-containing proteins, which are named suppressors of cytokine signaling 1–7 (SOCS 1–7), and cytokine-inducible SH2 (CIS)-containing proteins [22]. In a recent study using SCOS3 knock-out mice Croker et al. have demonstrated that SOCS-3 is an essential negative regulator of IL-6, and in the absence of SOCS-3, IL6 promotes inflammation [23]. However, previously IL-6 was found to induce SOCS3 expression in hepatocytes during acute inflammation [49]. TS exposure induces IL-6 expression in mononuclear phagocytes [24].

Recent studies in COPD suggest that treatment with the combination of a long acting β2-agonist (SAL), and low dose of inhaled corticosteroid (FP) improve lung function, and controlled symptoms of exacerbations and inflammation when compared to responses induced by either drug alone [25–28]. However, the effects of FP/SAL on BAEpCs inflammatory responses are not clear. In the present study we determined the effect of TS on BAEpC expression of SOCS-3 and the association of MAPK signaling pathways such as *Erk1/Erk2*, *p38*, and *PI3* kinases has been elucidated. In addition, we also studied the effects of FP/SAL in combination and separately on SOCS-3 and IL-6 expression in BAEpCs.

2. Materials and methods

2.1. Reagents

Anti-SOCS-3 antibody (Santa Cruz Biotechnology, CA), *Erk1/Erk2* and *p38* antibodies were purchased from Cell Signaling (Beverly, CA); anti-PI3 kinase antibodies (Santa Cruz, CA) Fluticasone propionate and Salmeterol hydroxynaphthoate received from GlaxoSmithKline, Durham, DL, UK. The MAPK inhibitors PD98059 or SB203580 or Ly294002 were purchased from Cell Signaling (Beverly, CA). All the other reagents were purchased from Sigma-Aldrich unless otherwise indicated.

2.2. Cell line and cell culture

Primary cultures of BAEpCs were obtained from Cell Applications (San Diego, CA), and were cultured in BEGM (Clonetics Corp. CA) as reported primary cultures of human bronchial airway epithelial cells (BAEpCs) were obtained from Cell Applications (San Diego, CA), and were cultured in BEGM (Clonetics Corp. CA) as reported earlier [29]. In brief, cell culture dishes were incubated at 37 °C in a humidified atmosphere of 5% CO_2. When the cells reached confluence, the cells were trypsinized and plated on to vitrogen-coated dishes. Third-passage cultures were stored in liquid nitrogen following trypsinization for later use. Cells of passages 3–6 were used in these experiments. Cells were routinely checked with anti-human cytokeratin antibody and anti-vimentin antibody for purity. The cultured BAEpCs were keratin positive and vimentin negative.

2.3. Preparation of FP/SAL and BAEpCs treatment

FP was dissolved in dimethylsulfoxide (DMSO) and SAL was dissolved in ethanol at a concentration of 100 mM, and 50 mM stock concentrations respectively and stored at −20 °C. BAEpCs were stimulated with different concentrations of FP (10 nm–1000 nmol) or SAL (0.10–100 nmol) alone for 4 h, 16 h, and 24 h or left untreated. After determining the effective concentration of the FP and SAL each separately on SOCS-3 expression, cells were stimulated with FP (10 nM–50 nM) + SAL (1 nM–10 nM) combination for 16 h. BAEpC cultures were exposed to TS followed by FP/SAL treatment [30]. The inhibitors for *Erk1/Erk2* (PD98059), *p38* (SB203580), and *PI3* (Ly294002) Mitogen-activated protein kinases were included in some of the cultures prior to TS exposure or with FP/SAL treatment.

2.4. BAEpCs exposure to TS

BAEpCs were exposed to mainstream TS derived directly from burning 2R4F reference cigarettes (Tobacco and Health Research Institute of the University of Kentucky) as reported earlier [30]. Briefly, the inlet of a modular incubator chamber (Billups-Rothenberg, CA) was connected to a cigarette holder in a fume hood. To mimic *in vivo* alveolar lung fluid, the monolayers of cultured BAEpCs on the bottom of a 60-mm culture dish were covered by 4 ml medium. The culture dishes in the chamber were exposed to TS generated by burning 3 2R4F cigarettes at a rate of one cigarette per 5 min for 15 min in a blow-by system. BAEpCs in a parallel chamber were treated under identical conditions but without TS (in presence of un-lighted cigarette) and used as controls. Then, the BAEpC cultures were returned to normal culture conditions, *i.e.* into a CO_2 incubator (95% room air–5% CO_2 at 37 °C), for recovery for 45 min to mimic the intervals between smoking of cigarettes in normal smokers. In this exposure protocol, one exposure unit (EU) is defined as a 15-min TS exposure plus a 45-min recovery (a total of 1 h). Exposure of lung cells to TS for 0–12 EU (0–18 cigarettes in 0–12 h) would be relevant to the clinical parameter of 0–1 pack smoked per day. To evaluate the effect of increasing dose of the TS on BAEpCs we have increased the number of exposure units up to 4 and the expression of SOSC-3 was evaluated. BAEpCs viability was tested by Trypan blue dye exclusion and there was no significant cell death noticed in TS exposed BAEpCs.

2.5. Western blot analysis

The total BAEpCs lysates were prepared after TS exposure and appropriate treatment with varied concentrations of drugs individually or in combination. BAEPCs were lysed at 4 °C in 0.1 ml lysis buffer. Insoluble material was removed by centrifugation as reported earlier [29].

2.6. Immunofluorescence microscopy

To evaluate the SOCS-3 expression *in situ*, the cells were labeled with SOCS-3 specific antibodies and analyzed by confocal laser scanning microscopy (Zeiss LSM 510; Axiovert 100 M, Zeiss, Thornwood, NY) as reported earlier [31].

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

BAEpCs were exposed to TS with and without FP or SAL alone or in combination for an indicated period of time and RNA was extracted. SOCS-3 and IL-6 mRNA expression was evaluated by RT-PCR using specific primers as reported earlier [31].

2.8. Statistical analysis

The statistical analyses were performed by using SigmaStat 3.5 (SYSTAT Software, Inc. San Jose, CA). Results were expressed as mean ± SEM. Comparisons were made using one way ANOVA followed by Student-Newman–Keuls test for multiple comparisons. Differences were considered significant when p values were <0.05.

3. Results

3.1. FP and SAL induce low levels of SOCS-3 expression in BAEpCs

BAEpCs were cultured in the presence of various concentrations of either FP alone or SAL alone for 4 h, 16 h and 24 h and the SOCS-3 expression was measured by quantitative-PCR analysis. We also tested BAEpC