EFFECT OF CIGARETTE SMOKE EXPOSURE ON BRONCHIAL HYPERRESPONSIVENESS IN MICE AND RESPONSE TO A CORTICOSTEROID AND/OR A β2-ADRENERGIC AGONIST

AND

EFFECT OF CIGARETTE SMOKE EXTRACT ON CYTOKINES RELEASE FROM U937 CELLS

By

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A THESIS

Submitted to the faculty of the Graduate School of the Creighton University in Partial Fulfillment of the Requirements for the degree of Master of Science in the Department of Pharmacology.

Omaha, NE
Aug, 2012
Abstract

The number of people affected with chronic inflammatory diseases of airways such as asthma and chronic obstructive pulmonary disease (COPD) are growing globally. Asthma is characterized by inflammation driven mainly by eosinophils, reversible airflow obstruction and significant airway hyperresponsiveness to airway stimuli like methacholine (MCh). It is increasingly prevalent in the US (24.6 million in 2009), particularly among children and some minority groups. Three hundred million people worldwide have been reported to have asthma with 250,000 annual deaths attributed to it. COPD is a pulmonary disease with irreversible airway obstruction and inflammation driven by neutrophils and macrophages. In the US in 2005, an estimated 126,000 US deaths were attributed to COPD.

Corticosteroids and/or β2 adrenergic agents are the main line of therapy for both asthma and COPD. However, some people with asthma, and the majority with COPD show diminished response to corticosteroid therapy. Various factors such as an increase in number of glucocorticoid receptors β, increase in activator protein-1 (AP-1), and increase in Th-2 cytokines (IL-4 and IL-13), and diminished histone deacetylase-2 activity are attributed to corticosteroid resistance. Since smoking is the primary risk factor of COPD and most of the patients with COPD show poor response to corticosteroids, oxidative stress should play an important role in causing diminished response to corticosteroids. In addition, various clinical studies have reported that people with asthma who are active smokers show less improvement in lung function with corticosteroids compared to non-smokers.
Based upon these rationales, we wanted to develop a model of smoking and COPD in mice and to study response to inhaled corticosteroid (ICS) and/or long acting beta agonists (LABA) as measured by bronchial hyperresponsiveness (BHR) to MCh and airway inflammation. We designed a 4 weeks smoke exposure study and administered inhaled fluticasone propionate (FP) and/or salmeterol to determine and compare their efficacy in smoke exposed versus non-smoke exposed mice. We also investigated the effect of cigarette smoke extract (CSE) on the contractility of isolated mouse trachea in vitro. Since, alveolar macrophages, and inflammatory proteins released by them, play an important role in causing exacerbations of asthma and worsening COPD, we studied the effect of CSE on cytokines (e.g. IL-6, IL-8 and IL-10) release from macrophage like cells (U937 cell line).

We found that cigarette smoke exposure in mice increased BHR to MCh. Also, the BHR in smoke exposed mice treated with FP and/or salmeterol was significantly higher than BHR in non-smoked mice treated with the same drugs. The drugs did not protect against BHR as demonstrated by Penh and provocative concentration of MCh (PC200). The measured cytokines (IL-1β, TNF-α, IL-13 and GM-CSF) were higher in the bronchial alveolar lavage (BAL) fluid of smoke exposed mice compared to non-smoked mice although the difference was not significant. Further, none of the drugs reduced the level of cytokines in BAL fluid. We found a non-significant increase in MCh induced contraction in CSE pretreated (24 h) isolated mouse tracheas compared to vehicle pretreated tracheas. We also found that there was a significant difference in contractility of the lower portion of trachea compared to the upper portion of trachea.
Treatment of U937 cells with cigarette smoke extract (CSE) showed a differential effect on cytokines release. We found that CSE treatment reduced the release of the inflammatory cytokine, IL-6, from vehicle treated as well as lipopolysaccharide (LPS) stimulated cells. In contrast, CSE treatment induced IL-8 release from these cells in a dose dependent manner. LPS and CSE synergistically stimulated IL-8 release from the cells. Treatment with CSE reduced the release of IL-10, an anti-inflammatory cytokine, from vehicle as well as LPS treated cells. Effect of IL-13 on release of IL-6, 8 and 10 was also evaluated.

In conclusion, cigarette smoke exposure induced BHR to MCh. FP and/or salmeterol did not protect against BHR to MCh in cigarette smoke exposed mice whereas albuterol did protect. Cigarette smoke exposure caused diminished response to a corticosteroids and a long acting β2 adrenergic agonist compared to non-smoked mice. CSE treatment of U937 cells caused inhibition of IL-6 and IL-10 release whereas it caused induction of IL-8 release.
Acknowledgements

I would like to express my sincere gratitude to my advisor Dr. Margaret A. Scofield for her supervision, guidance and encouragement throughout my study. I am deeply indebted to my mentor as well as co-advisor, Dr. Robert G. Townley for all of his kind supports. Without his constant support, guidance and mentoring, it would not have been possible to finish this degree.

My sincere thanks go to the rest of my committee members Dr. Peter W. Abel and Dr. Yaping Tu for their invaluable advice and comments. They not only guided throughout my research period but also provided the opportunity to use their lab equipment. I am thankful to the lab members of Dr. Yaping Tu and Dr. Peter W. Abel for their support while doing experiments in their lab.

I am thankful to Dr. Xiang Fang for his help in statistical analysis. I would also like to thank Dr. Annemarie Shibata for her help while doing cell culture. I am grateful to the entire faculty and my friends in Department of Pharmacology and Division of Allergy and Immunology at Creighton University School of Medicine for their support.

Lastly, my never ending thanks and love to my family, without whose support, none of this would have been possible. My special thanks go to my best partner, friend and wife for always standing by me and for help in some of the experiments.
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<tr>
<td>BAL</td>
<td>Bronchial alveolar lavage</td>
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<tr>
<td>BHR</td>
<td>Bronchial hyperresponsiveness</td>
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<tr>
<td>CCL</td>
<td>CC-chemokine ligand</td>
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<tr>
<td>CCR</td>
<td>CC-chemokine receptor</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<td>CS</td>
<td>Cigarette smoke</td>
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<td>CSE</td>
<td>Cigarette smoke extract</td>
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<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FP</td>
<td>Fluticasone propionate</td>
</tr>
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<td>FS</td>
<td>Combination of fluticasone propionate and salmeterol</td>
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<tr>
<td>FEV1</td>
<td>Forced expiratory volume in 1 second</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<td>GM-CSF</td>
<td>Granulocyte monocyte colony stimulating factor</td>
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<td>HDAC</td>
<td>Histone deacetylase</td>
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<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>IkB</td>
<td>Inhibitor of kappa B</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IBMX</td>
<td>3-Isobutyl-1-methylxanthine</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LABA</td>
<td>Long acting beta adrenergic agonists</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MCh</td>
<td>Methacholine</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Penh</td>
<td>Enhanced pause</td>
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<tr>
<td>PC&lt;sub&gt;200&lt;/sub&gt;</td>
<td>Provocative concentration of MCh to increase Penh by 200%</td>
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<tr>
<td>PEF</td>
<td>Peak expiratory flow</td>
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<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>Sal</td>
<td>Salmeterol</td>
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<tr>
<td>TSP</td>
<td>Total suspended particle</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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1. Introduction

1.1. Asthma and COPD

Asthma: Asthma is a complex disease of the airway which is characterized by chronic inflammation and variable bronchial obstruction. It is increasingly prevalent in US (24.6 million in 2009), particularly among children and some minority groups. Three hundred million people worldwide have been reported to have asthma with 250,000 annual deaths attributed to it (Labre et al., 2012). In asthma, the airway is obstructed by mucus plugs composed of plasma proteins exuded from airway vessels and mucus glycoproteins secreted from surface epithelial cells. The airway wall is infiltrated with inflammatory cells, mainly eosinophils and T-lymphocytes (Hogg, 1997). The inflammation in asthma not only involves trachea and bronchi but also extends to terminal bronchioles (Hamid et al., 1997). Airway obstruction is mostly reversible in the majority of patients with asthma. Airflow limitation might be caused by (i) reduced driving pressure due to loss of elastic recoil of lung parenchyma and (ii) increased resistance due to airway obstruction (Saetta and Turato, 2001). Direct bronchoscopic examination of airways of asthmatic patients has revealed an increase in number of eosinophil, lymphocytes (mainly CD4+), mast cells and macrophages in bronchiole alveolar lavage (BAL) fluid (Bradding et al., 2006; Larché et al., 2003).

Bronchial hyperresponsiveness (BHR) is an important and essential feature of asthma which is defined as an excessive airway narrowing in response to a variety of
physical and chemical stimuli which have little or no effect in healthy individuals (Brusasco et al., 1998). The degree of inflammation is related to the degree of BHR as measured by histamine or methacholine (MCh) challenge. However, the degree of inflammation, as measured by various inflammatory cells, is not linked to severity of asthma and BHR (Brusasco and Pellegrino, 2003). BHR is caused by increased release of inflammatory mediators like histamine and leukotrienes from mast cells or thickening of the airway wall by reversible (edema) or irreversible (fibrosis) elements. Tobacco smoke causes increased asthma prevalence (Gortmaker et al., 1982) and its severity (Chilmonczyk et al., 1993) and worsens the symptoms of asthma. Also, epidemiologic data have suggested that people with asthma who do smoke have a rapid decline in lung function compared to non-smokers with asthma or smokers without asthma (Lange et al., 1998). These studies suggest that there should be some interaction between cigarette smoke (CS) exposure and inflammation in the airway of asthmatics but precise underlying mechanism is still not clear.

*Chronic obstructive pulmonary disease (COPD):* COPD is a chronic inflammatory disease of the airway which is characterized by irreversible airway limitation. It actually refers to group of slowly progressive diseases (emphysema, chronic bronchitis) that cause airflow blockage and interferes with normal breathing. It is a major and important cause of mortality and morbidity worldwide (Lopez, 2006). The growing burden of COPD is partly due to aging and partly due to continued use of tobacco (Lopez et al., 2006). It is progressive and associated with the inflammatory response to noxious particles mainly from cigarette smoke exposure (Górska et al., 2010). The inflammatory response is
characterized by infiltration of leukocytes, mainly macrophages and neutrophils, and CD8+ lymphocytes as well as an increase in inflammatory mediators such as IL-8 and TNF-α (Saetta et al., 2001; Shapiro, 1999; Keatings et al., 1996). In COPD, oxidative stress due to cigarette smoke is the main etiological factor in the pathogenesis of disease (Rahman and MacNee, 1999). Patients with COPD have emphysema and bronchitis and also have the symptoms similar to that of severe asthmatics. Findings from different studies have suggested overlap of up to 30% in people who have clinically diagnosed of COPD and asthma (Soriano et al., 2003a). The main differences between asthma and COPD are listed in table 1.

<table>
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<td>Reversible airflow limitation</td>
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<td>Bronchial hyperresponsiveness is significant</td>
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<tr>
<td>Intact lung parenchyma</td>
</tr>
<tr>
<td>Mostly steroid responsiveness</td>
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<tr>
<td>Cigarette smoke is a trigger</td>
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<tr>
<td>Eosinophil is the main inflammatory cell</td>
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<td>Develops early in life</td>
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1.2. Glucocorticoid resistance

Corticosteroids are among the most widely used drugs for inflammatory diseases such as asthma around the world. They are still being used as first line of therapy for treatment of asthma (Barnes, 2006). The combination of long acting β2 adrenergic agonists (LABA) (e.g. salmeterol, formeterol etc.) (Donohue, 2000; Soriano et al., 2003b)
with oral inhaled corticosteroids (ICS) (e.g. budesonide, fluticasone, mometasone etc.) is playing an important role in the management of asthma and is the most effective therapy for the patients with moderate to severe asthma and COPD (Pauwels et al., 1997; Calverley et al., 2007). Combined use of LABA and ICS complement each other by controlling bronchoconstriction and inflammation (Miller-Larsson and Selroos, 2006). Leukotriene modifiers (e.g. montelukast), cromones (e.g. sodium chromoglycate), and anti-IgE therapy (e.g. omalizumab) are also used for the treatment of asthma but not COPD (Storms, 2007; Leigh et al., 2002; Molimard et al., 2010; Townley et al., 2010; Pesko, 2009; Menzies-Gow and Fan Chung, 2008; Bang and Plosker, 2004; Kelly, 2007). Macrolide antibiotics and phosphodiesterase (PDE)-4 inhibitors also have proven effective for the treatment of asthma and COPD (Giamarellos-Bourboulis, 2008; Gotfried, 2004; Lipworth, 2005; Barnette, 1999).

Corticosteroids (CS) are considered to be among the most effective anti-inflammatory agents in clinical use. However, small cohort of patients with inflammatory condition like asthma show poor or absent response to even higher doses of corticosteroids. CS-resistant is defined as less than 15% improvement in baseline forced expiratory volume in 1 second (FEV1) after a 14-day course of oral prednisolone (40 mg/d) in patients who demonstrate more than 15% improvements in FEV1 following inhaled β2-agonists (Ito et al., 2006). Schwartz and colleagues first identified corticosteroid resistance in asthma and found poorly controlled asthma with no reduction in blood eosinophils even after large dose of corticosteroid therapy (Schwartz et al., 1968). Various studies have reported diminished response to corticosteroids in patients with asthma and COPD (Carmichael et al., 1981; Hew et al., 2006; Bhavsar et al., 2008).
Various factors have been attributed to the mechanism of diminished response to corticosteroids. It has been reported that the expression of the glucocorticoid receptor (GR)-β is increased in airway T cells of CS-insensitive patients with asthma (Hamid et al., 1999). Another study demonstrated that CS-insensitive asthma is associated with increased GR-β immunoreactive peripheral blood cells compared to CS-sensitive asthmatic patients (Leung et al., 1997). Up-regulation of GR-β isoforms compared to GR-α is thought to be one of the mechanisms of CS-resistance (Goleva et al., 2006; Tliba et al., 2006; Livingston et al., 2004). Combination of inflammatory cytokines like IL-2 and IL-4 also caused diminished response of T cells to CS (Kam et al., 1993). Similarly, interleukin-13, a Th-2 cytokine, decreased the binding affinity of dexamethasone to GR-α in monocytes (Spahn et al., 1996). CS deacetylates the hyperacetylated histones through the recruitment of histone deacetylase (HDAC)-2 to the activated co-activator complex thus suppressing the transcription of inflammatory genes (Barnes et al., 2004). HDAC-2 is markedly reduced in alveolar macrophages, airways and peripheral lung of patients with COPD (Ito et al., 2005), in patients with CS-resistant asthma (Hew et al., 2006) and in airways of smoking asthmatics (Murahidy et al., 2005). Excessive activation of activator protein (AP)-1 is also another factor of glucocorticoid resistance since it binds to GR and inhibits its interaction with glucocorticoid response element (GRE) (Adcock et al., 1995). Other mechanisms responsible for CS resistance might be glucocorticoid receptor modification like nitrosylation by increase nitric oxide production induced by inducible nitric oxide synthase (iNOS) (Galigniana et al., 1999) and phosphorylation by increased p38 mitogen activated protein (MAP) kinase (Irusen et al., 2002). The general mechanism of CS-resistance is shown in figure below (Figure 1).
1.3. Cigarette smoking and effects on asthma/COPD therapy

Active cigarette smoking in Europe and US are 29% and 21% of total population respectively. Approximately, 25% of people with asthma smoke and show decline in lung function as compared to non-smokers. Two types of cigarette smoke are produced when a cigarette is smoked. Mainstream cigarette smoke is inhaled by smokers whereas sidestream cigarette smoke produced at the smoldering end when the smoker is not inhaling is inhaled by other people as passive smoking (Nelson, 2001). Cigarette smoke contains
more than 7000 chemicals of which ~60 are said to have carcinogenic property (Rodgman and Perfetti, 2009). According to WHO estimates, 5.4 million premature deaths worldwide are attributable to tobacco smoking (Research for International Tobacco Control, 2008). Most common tobacco smoke related causes of deaths are cardiovascular disease, COPD and cancers, mainly lung cancer (US Department of Health and Human Services, 2004). Among those chemicals present in cigarette smoke, 1,3-butadiene has the greatest risk of causing cancer; acrolein and acetaldehyde are respiratory irritant; and cyanide arsenic and cresols are the source of cardiovascular risk (Fowles and Dybing, 2003).

Compared to non-smokers, asthmatics who are active smokers have worse asthma control (Althuis et al., 1999), accelerated decline in lung function (Apostol et al., 2002) and increased mortality rate. Beside its effect on asthma, various clinical studies have been reported demonstrating the effect of cigarette smoking on the therapeutic outcomes of CS. Lazarus et al. conducted a placebo-controlled, double blind, double dummy, cross over trial with 44 non-smokers and 39 light smokers (Lazarus et al., 2007). They were assigned randomly to treatment with inhaled beclomethasone and oral montelukast. They reported that subjects with mild asthma who smoke showed lesser response to inhaled corticosteroids, as demonstrated by change in FEV1, while there was improvement in lung function of non-smokers. Another study was conducted to compare the effect of inhaled fluticasone propionate (100 µg daily) in patients with mild asthma who are active smokers (n=17) versus non-smokers (n=21) (Chalmers et al., 2002). There was a significant increase in mean morning peak expiratory flow (PEF) and FEV1 and decrease in sputum eosinophils in non-smokers. No significant changes in any of those parameters
were observed in smokers. Another study was conducted to determine the effect of smoking on therapeutic effectiveness of oral prednisolone (40 mg daily) in patients with chronic stable asthma (Chaudhuri et al., 2003). There was a significant improvement, after 2 weeks of oral prednisolone, in FEV1, morning PEF and asthma control score in asthmatics who never smoked but observed no change in smokers with asthma. Another study tested the effect of inhaled beclomethasone (200 or 400 µg daily) in smokers versus non-smokers and found that the evening PEF was significantly different among those groups (Tomlinson et al., 2005). Treatment of allergic asthmatics with oral budesonide decreased inflammatory markers like eosinophil cationic protein (ECP), eosinophil derived neurotoxin and myeloperoxidase from neutrophil in non-smokers but had no effect in smokers (Pedersen et al., 1996).

These studies show that cigarette smoking has a greater impact on features of asthma as well as on diminished efficacy of therapeutic interventions used to treat asthma. Also, people with COPD have poor responses to corticosteroid treatment and oxidative stress is said to be the main cause. Establishing a model of diminished response to current asthma and COPD treatment is very important. If we establish such a model, it will help to understand the underlying mechanism of the diminished response to therapeutic drugs. In this study, we have tested not only corticosteroid but also LABA and their combination, since the combination of corticosteroid and LABA are shown to have a greater effect than either agent alone. This combination is used as maintenance therapy for mild to severe asthma as well as in COPD. The use of corticosteroid increases the expression of beta adrenergic receptors and LABA increases the nuclear translocation
of glucocorticoid receptors thus complementing each other (Sin and Man, 2006; Chung et al., 2009).
2. **Hypothesis and specific aims**

*Hypothesis*

Cigarette smoke exposure in mice causes an increase in bronchial hyperresponsiveness (BHR) and inflammation as well as reduction in the response to a corticosteroid and/or a β2 adrenergic agonist.

*Specific aims*

(i) To determine the effect of 4-week cigarette smoke exposure on BHR and the response to fluticasone propionate (FP) and/or salmeterol treatment in mice.

(ii) To determine the effect of 4-week cigarette smoke exposure on cytokines (IL-13, IL-1β, TNF-α and GM-CSF) production and total cell counts in bronchial alveolar lavage fluid and the response to fluticasone propionate (FP) and/or salmeterol treatment in mice.

(iii) To determine the effect of cigarette smoke extract pretreatment on contraction by methacholine (MCh) and relaxation by isoproterenol of isolated mouse trachea.
3. Materials and methods

3.1. Materials

3R4F reference cigarettes were purchased from the Tobacco and Health Research Institute, University of Kentucky (Lexington, KY, USA). Methacholine was purchased from Sigma-aldrich (USA). Salmeterol and fluticasone propionate were obtained from GlaxoSmithKline (USA). Mouse ELISA kits were purchased from eBioscience (USA).

Animals: C57Bl/6j mice were purchased from Harlan Laboratories (Madison, WI, USA). They were housed and maintained in the Creighton University Animal Resource Facility. Animals were provided with standard chow and water ad libitum except while in the smoking chamber, and maintained on a 12 h/light dark cycle. All animal handling and experimental procedures were in accordance with established federal and institutional guidelines and were approved by the Creighton University Institutional Animal Care and Use Committee (IACUC).

3.2. Experimental methods

3.2.1. Cigarette smoke exposure in mice

C57Bl/6j mice (female, 6-8 weeks of age) were divided in 2 groups (smoked and non-smoked) with 40 mice in each group. Each group was further divided into 4 subgroups with 10 mice in each sub-group for control, salmeterol, fluticasone propionate (FP) and combination of FP and salmeterol (FS). Smoke exposure was conducted for 3 h a day, 5 days a week for 4 weeks using a TE-10 smoking device (Teague Enterprises, CA, USA) to expose mice to cigarette smoke. The TE-10 device is a microprocessor controlled cigarette smoking machine and produces either side-stream or mainstream
Smoke (or both) from cigarettes. Smoke-exposed mice were first placed in plastic cage (5 mice in each cage) with wired top. Their placement was rotated in two chambers in every exposure to maximize the same exposure to all groups. Both chambers (264 L [0.264 m$^3$] per chamber) were attached to the smoke apparatus, mixing chamber, and air pump. Mainstream and side-stream smoke were mixed and passed to the chambers. The flow through the chamber remained constant with the chamber pressure set at 0.2 in. of water. Five Kentucky 3R4F reference cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) were constantly burning at all times and puffed every 2 secs to create an average total suspended particle (TSP) level of 101 ± 31 mg/m$^3$. Non-smoke exposed mice (sham treated) were exposed to room air only. Figure 2 shows the experimental design of the study.

![Figure 2. Experimental design of 4 weeks cigarette smoke exposure in mice. CS, cigarette smoke; Penh, enhanced pause.](image-url)
3.2.2. Measurement of bronchial hyperresponsiveness

Responsiveness to methacholine (MCh) as a measure of BHR was assessed in unanesthetized, non-invasive, unrestrained mice by whole body plethysmography using the software and hardware provided by Buxco electronics (New York, USA) as described in previous publications by our lab (Horiba et al., 2011; Townley et al., 2009; Townley, 2007). This system utilizes ten body plethysmographs thus allowing evaluation of basal levels and BHR to MCh in 10 mice simultaneously. Ten mice were placed within 10 small volume (600 ml) plexiglas chambers that allowed for free movement. Alterations in chamber pressure, as a function of mouse breathing patterns were continuously monitored. Mice were exposed to aerosolized 1 mL physiologic buffered saline for 1 minute and baseline Penh, as a parameter to measure pulmonary function, was recorded for 3 minute. Penh is a unit-less measurement of enhanced pause, which reflects the changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration and represents the amount of bronchial constriction. Subsequently, increasing concentrations of MCh in
phosphate buffer saline (3.125, 6.25, 12.5 and 25 mg/mL) were aerosolized for 1 minute using a ultrasonic nebulizer (De Vilbiss, Somerest, Pennsylvania) connected to an aerosol driver and pump apparatus (Buxco) where Penh was recorded for 3 minutes followed by a 2 minute recovery period. The same method was used for all groups (n=10). The percent change over baseline Penh (enhanced pause) and PC_{200} (provocative concentration of MCh to increase the baseline Penh by 200%) were calculated to evaluate the BHR. The higher the value of Penh, the higher will be the BHR to MCh. In contrast to Penh, the higher the value of PC_{200} (mg/mL) the lower will be the bronchial sensitivity to MCh.

3.2.3. Administration of FP and/or salmeterol

On the last 3 consecutive days of cigarette smoke exposure, each group was administered PBS or other drugs (salmeterol, FP and their combination, FS). Mice were placed in 6 L plexiglas chamber and salmeterol (100 µg/mL in PBS), fluticasone propionate (100 µg/mL in PBS) and their combinations (FS, 100 µg/mL in PBS each) were nebulized using an ultrasonic nebulizer (Ultraneb 2000, Devilbiss, Somerset, USA) for 10 min at a flow rate of 8 L/min before smoking. Control groups were nebulized with PBS only. A similar type of drug treatment was also carried out for non-smoke exposed groups. Penh was recorded after 6 h of last smoke exposure.

3.2.4. BAL fluid collection and total cells count

Mice were euthanized using CO₂ and then BAL was collected after 24 h of last smoke exposure by inserting a needle through the trachea. PBS (1 mL) was injected and
trachea was washed twice before collection. It was repeated three times. Percent recovery of PBS injected was more than 85%. Total cell counts were performed using coulter counter. Then, the BAL fluid was centrifuged and the supernatant was collected and stored in freezer at -80°C for cytokine assay.

3.2.5. Measurement of cytokines in BAL fluid

IL-13, IL-1β, TNF-α and GM-CSF were measured by using ELISA kits according to the manufacturer’s instructions. Briefly, the ELISA plate was coated with the capture antibody and left overnight at 4°C. Then, after washing, it was blocked with assay diluent for 1 h and washed again. After incubation with the detection antibody, the plate was washed and Avidin-HRP was added. It was washed out and substrate was added. After 15 minutes, it was stopped with the stop solution (2N H₂SO₄) and the optical density (O.D.) was measured by a microplate reader at 450 nm. Duplicate samples from each mouse were assayed. Optical density readings were converted to the respective cytokine concentrations (pg/mL) using a standard curve.

3.2.6. Contraction and relaxation of isolated mouse trachea

Preparation of aqueous cigarette smoke extract (CSE)

Research grade cigarettes (3R4F) were obtained from Kentucky Tobacco Research and Development Center at University of Kentucky (Lexington, KY). Cigarette smoke extract was prepared as described by other researchers with a slight modification (Yang et al., 2007). Cigarette smoke extract was prepared by bubbling smoke, using a 60 mL syringe, from one cigarette into 10 mL of culture medium (DMEM) supplemented
with 10% fetal bovine serum (FBS) at a rate of 30-40 secs per puff and 6 puffs per cigarette. The pH of CSE was adjusted to 7.4 and filtered through a 0.22-µM filter (Millipore). This CSE is considered as 100% and kept at -20°C for future studies.

*Isolation and culture of mouse trachea*

Mice (C57Bl/6j; 40-42 weeks) were euthanized by asphyxiation with CO₂. Whole trachea was rapidly removed and placed into dish with Krebs solution. They were dissected free from adherent tissues and cartilages. Each trachea was divided into two equal pieces and placed into wells of a 24-well culture plate with 900 µl Dulbecco’s modified Eagle’s medium (DMEM; 4500 mgL⁻¹ D-glucose, 110 mgL⁻¹ sodium pyruvate, 584 mgL⁻¹ L-glutamine) supplemented with 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin. One trachea was treated with 100 µl of CSE (10.0%) and another was treated with 100 µl of DMEM. Under cultured conditions, tracheal tissues were incubated at 37°C in humidified 5.0% CO₂ in air for 24 h.

*Isometric force measurement*

Tracheal smooth muscle reactivity was analyzed in temperature-controlled (37°C) myographs containing a 10 mL Krebs-Henseleit buffer solution composed of (mM) Na⁺ 143, K⁺ 5.9, Ca²⁺ 1.5, Mg²⁺ 2.5, Cl⁻ 128, H₂PO₄⁻ 1.2, SO₄²⁻ 1.2, HCO₃⁻ 25 and dextrose 11.1 in each double jacketed organ bath chamber. The solution was continuously supplied with 5% CO₂ and 95% O₂ maintaining a pH of 7.4. The tracheal segments were mounted on two L-shaped metal prongs. One of the prongs was connected to a force displacement transducer for continuous recording of isometric tension by chart software. Another
prong was connected to a plexiglas rod allowing adjustment of the distance between two parallel prongs.

Mounted tracheas were allowed to equilibrate for 1 hour with repeated wash out every 15 minutes. After each wash out, tension was adjusted to 300 mg of resting tension. Basal contraction was recorded for 3 minutes with 60 mM KCl. After thirty minutes, a dose response to MCh was carried out. After every drug treatment, it was washed with 3 rapid washouts followed by regular washing every 15 minutes. The tracheal tissue was allowed to equilibrate for 30 minutes before carrying out the dose response curve for another drug.

3.2.7. Statistical analysis

All statistical comparisons were carried out using ANOVA. Turkey multiple comparison method was used to adjust the P values for pair-wise comparison. The values were reported as mean±SEM. For all statistical analyses, P value <0.05 is considered to be statistically significant.
4. Results and discussion

4.1. Results

Our aim was to find out the effect of cigarette smoke exposure on BHR to MCh and response to FP and/or salmeterol. Penh and $PC_{200}$ values were calculated to compare BHR among groups.

4.1.1. Effect of cigarette smoke exposure on BHR and response to FP and/or salmeterol

_Comparing the Penh in CS-exposed versus non-smoke exposed mice_

The smoke exposed mice showed a significant increase in percent change over baseline Penh at 25 mg/mL of methacholine (MCh) compared to non-smoke exposed mice ($p<0.0001$) as shown in figure 4. The percent change over baseline Penh for non-smoke exposed mice was $325.7\pm54.1$ (n=10) while that for smoke exposed mice was $632\pm107.0$ (n=9) at 25 mg/mL of MCh. The percent change over baseline Penh at 12.5 mg/mL of MCh was $160.5\pm30.2$ (n=10) for non-smoke exposed mice while it was $227\pm52.7$ (n=9) for smoke exposed group. Similarly, percent change over baseline Penh at 6.25 and 3.125 mg/mL of MCh for non-smoke exposed group was $76.8\pm23.0$ and $35.7\pm14.6$ (n=10) while for smoke exposed mice it was $111.8\pm11.6$ and $71.9\pm11.2$ (n=9) respectively.
Figure 4. Comparing the effect of smoking on bronchial hyperresponsiveness (BHR) to methacholine (MCh) in smoke exposed mice versus non-smoke exposed mice as measured by Penh. Closed upright triangles (▲) represent non-smoke exposed mice and closed square (■) represent smoke exposed controlled mice at 3.125 mg/mL, 6.5 mg/mL, 12.5 mg/mL and 25 mg/mL of MCh. Data shown represent mean±SEM (n=8-10). ***p˂0.001 compared to percent change of Penh at 25 mg/mL of MCh of non-smoked mice. CS, cigarette smoke exposed mice; NS, non-smoked mice.

Comparing the effects of FP on BHR in CS-exposed versus non-smoke exposed mice

Percent change over baseline Penh in fluticasone treated smoke exposed mice at 25 mg/mL of MCh was significantly higher compared to fluticasone treated non-smoke exposed mice (p= 0.02) as shown in figure 5. The percent change was 458.3±123.9 (n=10) in fluticasone treated smoke exposed mice versus 274.6±50.7 (n=10) in fluticasone treated non-smoke exposed mice at 25 mg/mL of MCh. At 12.5 mg/mL of MCh, percent change in fluticasone treated smoke exposed mice was 182.1±37.7 (n=10) versus 148.5±38.8 (n=10) in fluticasone treated non-smoke exposed mice and the difference was not significant. Similarly, the percent change over baseline Penh at 6.25 and 3.125 mg/mL of MCh for fluticasone treated non-smoke exposed group was
112.5±16.6 and 71.5±10.6 (n=10) while for fluticasone treated smoked mice was 109.2±18.8 and 70.5±16.0 (n=10) respectively.

**Figure 5.** Comparison of effect of fluticasone propionate on BHR to MCh in smoke exposed mice versus non-smoke exposed mice. FP (100 µg/mL) was administered by nebulization on the last three days of smoking to both cigarette smoke exposed and non-smoke exposed groups and Penh was measured by whole body plethysmography. Closed squares (■) represent smoke exposed control mice, solid upright triangles (▲) represent non-smoke exposed mice, solid inverted triangles (▼) represent FP treated smoke exposed mice and solid diamonds (♦) represent FP treated non-smoke exposed mice at 3.125 mg/mL, 6.5 mg/mL, 12.5 mg/mL and 25 mg/mL of MCh. Dotted lines represent smoked versus non-smoke exposed group as shown in figure 4. Data shown represent mean±SEM (n=8-10). *P<0.05 compared to FP treated non-smoke exposed mice at 25.0 mg/mL of MCh. CS, cigarette smoke exposed; NS, non-smoke exposed; FP-CS, fluticasone propionate treated cigarette smoke exposed; FP-NS, fluticasone propionate treated non-smoke exposed mice.

*Comparing the effects of salmeterol on BHR in CS-exposed versus non-smoke exposed mice*

Salmeterol increased the percent change over baseline Penh significantly in smoke exposed mice at 25 mg/mL of MCh compared to the effect of salmeterol in non-smoke exposed mice (p=0.007) as shown in figure 6. The percent change was 515.6±80.8 (n=10) in salmeterol treated smoke exposed mice versus 293.9±64.0 (n=10) in salmeterol
treated non-smoke exposed mice at 25 mg/mL of MCh. At 12.5 mg/mL of MCh, percent change in salmeterol treated smoke exposed mice was 368.5±96.4 (n=10) and was significantly higher than salmeterol treated non-smoke exposed mice (164.8±29.3; n=10; p=0.02). Similarly, the percent change over baseline Penh at 6.25 and 3.125 mg/mL of MCh for salmeterol treated non-smoke exposed group was 129.7±43.1 and 74.2±2 (n=10) while for salmeterol treated smoked mice was 132.7±18.9 and 89.9±11.1 (n=10) respectively. We found no significant difference between salmeterol treated smoke exposed mice versus the smoke exposed control mice at all concentrations of MCh. Also, there was no significant difference between salmeterol treated non-smoke exposed mice and non-smoke exposed control mice.

Figure 6. Comparison of effect of salmeterol treatment on BHR to MCh in smoke exposed mice versus non-smoke exposed mice. Salmeterol (100 µg/mL) was administered by nebulization on the last three days of smoking to both cigarette smoke exposed and non-smoke exposed groups and Penh was measured by whole body plethysmography. Closed squares (■) represent smoke exposed control mice, solid upright triangles (▲) represents non-smoke exposed mice, solid inverted triangles (▼) represent salmeterol treated smoke exposed mice and solid diamonds (♦) represent salmeterol treated non-smoke exposed mice at 3.125 mg/mL, 6.5 mg/mL, 12.5 mg/mL and 25 mg/mL of MCh. Dotted lines represent smoke exposed versus non-smoke exposed group as shown in figure 4. Data shown represent mean±SEM (n=8-10). *P<0.05 and **P<0.01 compared to salmeterol treated non-smoked mice at
12.5 mg/mL and 25.0 mg/mL MCh respectively. CS, cigarette smoke exposed; NS, non-smoke exposed; Sal-CS, salmeterol treated cigarette smoke exposed; Sal-NS, salmeterol treated non-smoke exposed group.

Comparing the effect of FS treatment on CS-exposed versus non-smoke exposed mice

The combination of fluticasone propionate and salmeterol (FS) treated smoke exposed groups had a significantly higher percent change over baseline Penh both at 25 mg/mL and 12.5 mg/mL of MCh compared to the FS-treated non-smoke exposed mice (p=0.0004 and 0.007 respectively) as shown in figure 7. The percent change was 465.8±60.3 (n=10) in FS treated smoke exposed mice versus 271.1±43.0 (n=9) in FS treated non-smoke exposed mice at 25 mg/mL of MCh. Similarly, at 12.5 mg/mL of MCh, the percent change in FS treated smoke exposed mice was 301.3 (n=10) versus 116.9±16.0 (n=9) in FS treated non-smoke exposed mice. The percent change for smoke exposed control mice at 25 mg/mL was 632.5±107.0 (n=10) whereas FS treated smoke exposed group was 465.8±60.3 (n=10) and was not significantly different (p=0.62).

Similarly, the percent change over baseline Penh at 6.25 and 3.125 mg/mL of MCh for FS treated non-smoke exposed group was 117.3±13.9 and 92.4±16.1 (n=9) while for FS treated smoke exposed mice the percent change was 150.7±18.8 and 65.5±14.5 (n=10) respectively.
Figure 7. Comparison of effect of combination of FP and salmeterol treatment on BHR to MCh in smoke exposed mice versus non-smoke exposed mice. FS (100 µg/mL each) was administered by nebulization on the last three days of smoking to both cigarette smoke exposed and non-smoke exposed groups and Penh was measured by whole body plethysmography. Closed squares (■) represent smoked control mice, solid upright triangles (▲) represents non-smoke exposed mice, solid inverted triangles (▼) represent FS treated smoke exposed mice and solid diamonds (♦) represent FS treated non-smoke exposed mice at 3.125 mg/mL, 6.5 mg/mL, 12.5 mg/mL and 25.0 mg/mL of MCh. Dotted lines represent smoke exposed versus non-smoke exposed group as shown in figure 4. Data shown represent mean±SEM (n=8-10). ***P<0.001 compared to percent change over baseline Penh of FS treated non-smoke exposed mice at 12.5 mg/mL and 25.0 mg/mL of MCh. CS, cigarette smoke exposed; NS, non-smoke exposed; FS-CS, combination of FP and salmeterol treated cigarette smoke exposed; FS-NS, combination of FP and salmeterol treated non-smoke exposed group.

From all these above data, we found that FP and/or salmeterol treatment was not able to protect against the BHR induced by cigarette smoke exposure. The percent change from baseline Penh, at maximum dose of MCh (25 mg/mL), PBS, FP and/or salmeterol treated smoke exposed mice and non-smoke exposed mice are given in table 2.
Table 2. The percent change from baseline Penh of smoke exposed and non-smoke exposed groups with/without drug treatment at 25 mg/mL MCh challenge.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Smoke exposed group</th>
<th>Non-smoked group</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>632.5±107.0</td>
<td>325.8±54.1</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>FP</td>
<td>458.4±124.0</td>
<td>274.6±50.7</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>515.6±80.9</td>
<td>294.0±64.0</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>FS</td>
<td>465.9±60.4</td>
<td>271.1±43.0</td>
<td>P&lt;0.001</td>
</tr>
</tbody>
</table>

FP, Fluticasone propionate; FS, combination of fluticasone propionate and salmeterol. Data expressed as mean of % change from baseline Penh at 25 mg/mL MCh±S.E.M.

**Effect of FP, salmeterol, and FS treatment on the PC_{200} in smoked and non-smoke exposed mice**

The provocative concentration to MCh (mg/mL) that produces 200% increase in Penh over baseline Penh (PC_{200}) was calculated for all the groups to define BHR. The lower the dose of MCh that produces the PC_{200}, the higher is the degree of BHR. The PC_{200} in smoke exposed control mice was 10.7±1.2 mg/mL (n=9) versus 16.2±2.2 mg/mL (n=9) in non-smoke exposed mice (P=0.05). Salmeterol treated smoke exposed mice had a PC_{200} of 9.7±1.3 mg/mL (n=8) versus salmeterol treated non-smoke exposed mice had a PC_{200} of 14.1±1.9 mg/mL (n=8) but there was no significant difference (p=0.14).

Similarly, FP treated smoke exposed mice had a PC_{200} of 11.0±1.6 mg/mL (n=7) versus FP treated non-smoke exposed mice which had a PC_{200} of 17.6±2.9 mg/mL (n=8) and the difference was significant (p=0.03). FS treated smoke exposed mice had a PC_{200} of 10.7±1.2 mg/mL (n=10) whereas FS treated non-smoke exposed mice had a PC_{200} of 21.8±3.2 mg/mL (n=7) and the difference was significant (p=0.0003). However, comparing the PC_{200} of FS treated non-smoke exposed group versus non-smoke exposed control group, there was no significant difference (p=0.9). As shown in figure 8, the
smoke exposed group with or without drug treatment had a lower PC\textsubscript{200} and therefore a greater BHR as compared to non-smoke exposed control and drug treated non-smoke exposed groups.

Figure 8. Sensitivity to MCh of different groups as demonstrated by change in PC\textsubscript{200}. PC\textsubscript{200} (mg/mL) represents the provocative concentration of MCh (mg/mL) resulting in a 200% increase in Penh (PC\textsubscript{200}) from baseline. Data shown represent mean±SEM (n=8-10) at 95% confidence interval. *P<0.05 compared to FP treated non-smoke exposed mice; ***P<0.001 compared to FS treated non-smoke exposed mice. CS, cigarette smoke exposed; NS, non-smoke exposed; Sal, salmeterol; FP, fluticasone propionate; FS, combination of fluticasone propionate and salmeterol.

**Comparing the effect of albuterol treatment on Penh of smoke exposed versus non-smoke exposed mice**

Pretreatment with albuterol protected against the BHR to methacholine in both smoke exposed and non-smoke exposed groups as shown in figure 9. The percent change over baseline Penh for non-smoke exposed mice after albuterol treatment was 194.6±32.9 (n=10) while that for albuterol pretreated smoke exposed mice was 118.8±22.0 (n=10) at 25 mg/mL of MCh. The percent change over baseline Penh at 12.5 mg/mL of MCh was
175.7±32.7 (n=10) for albuterol pretreated non-smoke exposed mice while it was 136.3±16.0 (n=9) for albuterol pretreated smoke exposed group.

Figure 9. Effect of albuterol pretreatment on BHR to methacholine (MCh) in smoke exposed versus non-smoke exposed mice. Albuterol (300 µg/mL) was administered by nebulization to both cigarette smoke exposed and non-smoke exposed groups and Penh was recorded using whole-body plethysmography. Closed squares (■) represent smoke exposed control mice, solid upright triangles (▲) represents non-smoke exposed mice, solid inverted triangles (▼) represent albuterol treated smoke exposed mice and solid diamonds (♦) represent albuterol treated non-smoke exposed mice at 3.125 mg/mL, 6.5 mg/mL, 12.5 mg/mL and 25.0 mg/mL of MCh. Dotted lines represent smoke exposed versus non-smoke exposed group as shown in figure 4. Data shown represent mean±SEM (n=8-10) at 95% confidence interval.*P<0.05 compared to Alb treated non-smoke exposed mice; **P<0.01 compared to albuterol treated smoke exposed mice at 25.0 mg/mL MCh. CS, cigarette smoke exposed; NS, non-smoke exposed; Alb-CS, albuterol treated cigarette smoke exposed; Alb-NS, albuterol treated non-smoke exposed group.

Another aim of this study was to determine the effect of cigarette smoke exposure on inflammatory mediators release in BAL fluid after treatment with FP and/or salmeterol. BAL fluid cytokine levels and total cell counts were used as a measure of the anti-inflammatory effect of FP and/or salmeterol after cigarette smoke exposure.
4.1.2. Effect of cigarette smoke exposure on BAL fluid cytokines

**IL-1β in BAL fluid**

IL-1β level was not significantly higher in smoke exposed control mice as compared to non-smoke exposed mice (Figure 10). It was 15.0±1.3 pg/mL (n=6) in smoke exposed mice whereas 13.1±2.3 pg/mL (n=7) in non-smoke exposed mice (p=0.5). FS treated smoke exposed group had IL-1β level of (21.0±1.6 pg/mL; n=4) in BAL fluid but was not significantly different than non-smoke exposed control groups (p=0.06). FP treated smoke expose exposed group had IL-1β level of 19.5±1.9 pg/mL (n=4). The difference between salmeterol treated smoke exposed group (19.2±4.2 pg/mL; n=6) versus non-smoke exposed control group did not reach significance (p=0.1).

![Graph of IL-1β in BAL fluid](image_url)

**Figure 10.** Comparison of the effect of salmeterol, FP and FS on IL-1β in BAL fluid of smoke exposed mice and non-smoke exposed mice. BAL fluid from salmeterol (100 µg/mL), fluticasone propionate (100 µg/mL) and combination of FP and salmeterol (100 µg/mL, each) treated smoked mice was collected and IL-1β (pg/mL) in BAL fluid of these drug treated smoke exposed mice was compared to that of non-smoke exposed group. Data shown represent mean±SEM (n=4-7). NS, non-smoke
exposed; CS, cigarette smoke exposed; Sal-CS, salmeterol treated cigarette smoke exposed; FP-CS, fluticasone propionate treated cigarette smoke exposed; FS-CS, combination of fluticasone propionate and salmeterol treated cigarette smoke exposed group.

**TNF-α in BAL fluid**

The TNF-α level was higher in smoke exposed (31.7±3.0 pg/mL; n=6) control mice as compared to non-smoke exposed mice (23.6±4.2 pg/mL; n=7) but did not reach significance (p=0.38) (Figure 11). The FS treated smoke exposed group (50.1±1.8 pg/mL; n=4) had significantly higher levels of TNF-α in BAL fluid compared to non-smoke exposed control groups (p=0.02). The FP treated smoke exposed group had a TNF-α level of 42.5±2.3 pg/mL (n=4). There was a significant difference between salmeterol treated smoke exposed group (49.4±12.7 pg/mL) versus non-smoke exposed control group (p=0.01).
Figure 11. Comparison of the effect of salmeterol, FP and FS on TNF-α in BAL fluid of smoke exposed mice and non-smoke exposed mice. BAL fluid from salmeterol (100 µg/mL), fluticasone propionate (100 µg/mL) and combination of FP and salmeterol (100 µg/mL, each) treated smoke exposed mice was collected and TNF-α (pg/mL) in BAL fluid of these drug treated smoke exposed mice was compared to that of non-smoke exposed group. Data shown represent mean±SEM (n=4-7). FS and FP treated smoke exposed mice had significantly higher levels of TNF-α compared to non-smoke exposed (*P<0.05). NS, non-smoke exposed; CS, cigarette smoke exposed; Sal-CS, salmeterol treated cigarette smoke exposed; FP-CS, fluticasone propionate treated cigarette smoke exposed; FS-CS, combination of fluticasone propionate and salmeterol treated cigarette smoke exposed group.

**IL-13 in BAL fluid**

The level of IL-13 was not significantly higher in smoke exposed control mice as compared to non-smoke exposed mice as shown in figure 12. It was 11.0±1.4 pg/mL (n=6) in smoke exposed mice but 9.9±1.5 (n=7) pg/mL in non-smoke exposed mice (p=0.6). The FP treated smoke exposed group had IL-13 level of 13.9±2.4 pg/mL (n=4) whereas FS treated smoke exposed group had 13.6±1.2 pg/mL (n=4). The difference
between the salmeterol treated smoke exposed group (14.2 ±2.2 pg/mL, n=5) versus the non-smoke exposed control group (9.9±1.5; n=7) did not reach significance (p=0.1).

![IL-13 in BAL fluid](image)

**Figure 12.** Comparison of the effect of salmeterol, FP and FS on IL-13 in BAL fluid of smoke exposed mice with that of non-smoked mice. BAL fluid from salmeterol (100 µg/mL), fluticasone propionate (100 µg/mL) and combination of FP and salmeterol (100 µg/mL, each) treated smoke exposed mice was collected and IL-13 (pg/mL) in BAL fluid of these drug treated smoke exposed mice was compared to that of non-smoke exposed group. Data shown represent mean±SEM (n=4-7). NS, non-smoke exposed; CS, cigarette smoke exposed; Sal-CS, salmeterol treated cigarette smoke exposed; FP-CS, fluticasone propionate treated cigarette smoke exposed; FS-CS, combination of fluticasone propionate and salmeterol treated cigarette smoke exposed group.

**GM-CSF in BAL fluid**

The GM-CSF level was higher in smoke exposed control mice as compared to non-smoke exposed mice as shown in figure 13, but did not reach significance (p=0.23). It was 3.0±0.4 pg/mL (n=6) in smoke exposed mice whereas 2.1±0.4 pg/mL (n=7) in non-smoke exposed mice. The FP and FS treated smoke exposed group had significantly higher levels of GM-CSF in BAL fluid compared to non-smoke exposed control groups (p=0.03 and 0.004) respectively. The FP treated smoke exposed group had a GM-CSF
level of 4.0±0.4 pg/mL (n=4) whereas the FS treated smoke exposed groups had 4.9±0.7 pg/mL (n=4). There was a higher level of GM-CSF in the salmeterol treated smoke exposed group (3.98±0.9 pg/mL; n=4) compared to the non-smoke exposed control group (p=0.04). There was a significant difference between the FS treated smoke exposed group versus the smoke exposed control group (p=0.04).

![Figure 13. Comparison of the effect of salmeterol, FP and FS on GM-CSF in BAL fluid of smoke exposed mice with that of non-smoke exposed mice. BAL fluid from salmeterol (100 µg/mL), fluticasone propionate (100 µg/mL) and combination of fluticasone and salmeterol (100 µg/mL, each) treated smoke exposed mice was collected and GM-CSF (pg/mL) in BAL fluid of these drug treated smoke exposed mice was compared to that of non-smoke exposed group. Data shown represent mean±SEM (n=4-7). FP, salmeterol and FS treated smoke exposed mice had significantly higher levels of GM-CSF compared to non-smoke exposed group (**P<0.01, *P<0.05). NS, non-smoke exposed; CS, cigarette smoke exposed; Sal-CS, salmeterol treated cigarette smoke exposed; FP-CS, fluticasone propionate treated cigarette smoke exposed; FS-CS, combination of fluticasone propionate and salmeterol treated cigarette smoke exposed group.](image-url)

4.1.3. Effect of cigarette smoke exposure on total cells in BAL fluid

The total cell counts in BAL fluid (x10^5) were higher in the smoked control and drug treated smoke exposed mice compared to the non-smoke exposed group but the
difference was not significant. All smoke exposed groups had higher cell counts than the non-smoke exposed control but did not reach significance (Figure 14). Total cell counts for smoke exposed mice were $(8.7 \pm 3.2) \times 10^5$ (n=5) whereas for non-smoke exposed mice, it were $(6.6 \pm 3.3) \times 10^5$ (n=5). Total cell counts in BAL fluid from salmeterol, FP and FS treated groups were $(12.1 \pm 0.8) \times 10^5$ (n=5), $(8.2 \pm 2.6) \times 10^5$ (n=4), and $(5.9 \pm 2.9) \times 10^5$ (n=4), respectively.

![Total cells count in BAL fluid](image)

**Figure 14.** Comparison of the effect of salmeterol, FP and FS on total cells count in BAL fluid of smoke exposed mice with that of non-smoke exposed mice. BAL fluid from salmeterol (100 µg/mL), fluticasone propionate (100 µg/mL) and combination of FP and salmeterol (100 µg/mL, each) treated smoke exposed mice was collected and total cells count ($x10^5$) was performed using coulter counter. Data shown represent mean±SEM (n=4-5). NS, non-smoke exposed; CS, cigarette smoke exposed; Sal-CS, salmeterol treated cigarette smoke exposed; FP-CS, fluticasone propionate treated cigarette smoke exposed; FS-CS, combination of fluticasone propionate and salmeterol treated cigarette smoke exposed group.

Another aim was to determine and compare the effect of CSE pre-treatment on tracheal smooth muscle contractile response to MCh and relaxation response to isoproterenol.
4.1.4. Effect of CSE pretreatment on contraction and relaxation of isolated mouse trachea

Comparison of MCh induced contraction of CSE versus vehicle pretreated tracheas

A cumulative dose response curve for MCh was obtained by using increasing concentrations of MCh (1x10⁻⁴ M to 1x10⁻⁸ M) and the change in tension (mg) was recorded. Both the CSE and vehicle pretreated tracheas showed similar contractile responses as shown in figure 15. At a cumulative dose of 1x10⁻⁴ M MCh, the force of contraction of CSE pretreated trachea was 580.7±77.8 mg (n=7) and that of vehicle only treated trachea was 444.0±103.0 mg (n=7) but the difference was not significant.

![Contraction of isolated mice tracheas](image)

Figure 15. The cumulative concentration-response curves to MCh obtained from CSE and vehicle pretreated cultured mouse tracheal segments. Tissues were cultured with 10.0% CSE or vehicle for 24 h before measuring the tension (mg). Each data represent the mean tension (mg) of all segments tested with error bars representing S.E.M. (n=7).
Comparison of isoproterenol induced relaxation of CSE versus vehicle pretreated tracheas

A cumulative dose response curve of isoproterenol was obtained by using increasing concentrations of isoproterenol (1x10^4 M to 1x10^-8 M) and the change in tension (mg) was recorded. Both CSE pretreated and vehicle treated tracheas showed a similar contractile response as shown in figure 16. First the optimal contraction of tracheal segments was induced by MCh (1x10^-6 M). Then the relaxation effect of cumulative doses of isoproterenol on MCh induced contraction was recorded. The maximal contraction induced by MCh (1x10^4 M) on CSE and the vehicle pretreated cultured tracheal segments was 354.4±60.0 mg (n=8) and 273.0±66.2 mg (n=8), respectively. A cumulative dose of 1x10^-4 M isoproterenol caused the relaxation of CSE and vehicle pretreated trachea and brought down the tension to 168.0±67.0 mg (n=8) and 124.0±67.5 mg (n=8), respectively. There was no significant difference between isoproterenol induced relaxation of CSE pretreated and vehicle pretreated control at all concentrations of isoproterenol.
Figure 16. The cumulative concentration-response curves to isoproterenol obtained on CSE and vehicle pretreated cultured mouse tracheal segments. Tissues were cultured with 10.0% CSE or vehicle for 24 h before measuring the tension (mg). First optimal contraction was induced by MCh (1x10^-6 M) and relaxation effect of cumulative dose of isoproterenol was measured. Each data represent the mean tension (mg) of all segments tested with error bars representing S.E.M. (n=8).

Relaxation of isolated mouse trachea with various muscle relaxants

A sustained contraction of both 10.0% CSE and vehicle pretreated cultured tracheal segments was obtained by using MCh (1x10^-6 M) which induced about 60-70% of maximal contraction before studying the relaxation effect of each drug. Tissues were allowed to equilibrate for 30 minutes (2 regular washes) before recording the relaxation effect of each drug. The relative percentage contraction of each drug was calculated assuming the contraction induced by MCh (1x10^-6 M) before adding each drug as 100% as shown in figure 17. Table 3 shows the relative percent contraction of different drugs.
Table 3. Percentage contraction of isolated mouse trachea with different relaxant agents.

<table>
<thead>
<tr>
<th>Name of Drugs</th>
<th>% Contraction (MCh)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CSE</td>
</tr>
<tr>
<td>IBMX (1x10^-7 M)</td>
<td>41.3±10.4</td>
</tr>
<tr>
<td>IBMX (1x10^-6 M)</td>
<td>9.0±3.3</td>
</tr>
<tr>
<td>Niflumic acid (1x10^-4 M)</td>
<td>62.4±6.6</td>
</tr>
<tr>
<td>Furosemide (1x10^-4 M)</td>
<td>80.5±5.5</td>
</tr>
<tr>
<td>Nif. Acid and Furo. (1x10^-4 M)</td>
<td>55.2±3.7</td>
</tr>
<tr>
<td>Albuterol (1x10^-6 M)</td>
<td>95.9±8.1</td>
</tr>
<tr>
<td>Rolipram (1x10^-4 M)</td>
<td>7.0±4.0</td>
</tr>
</tbody>
</table>

Relaxation of CSE and vehicle pretreated cultured trachea and data expressed as a mean of % contraction of MCh (1x10^-6 M) pre-contracted trachea±S.E.M. IBMX, 3-isobutyl-1-methyl-xanthine; Nif. Acid, Niflumic acid; Furo., Furosemide.

Figure 17. A graph of different drugs as compared to contraction induced by MCh on CSE and vehicle pretreated cultured mouse tracheal segments. Tissues were cultured with 10.0% CSE or vehicle for 24 h before testing. Optimal contraction was induced by MCh (1x10^-6 M) and relaxation effect of each drug was measured. Each data represent the relaxation (percentage of MCh induced pre-contraction) of all segments tested with error bars representing S.E.M. (n=4-8). IBMX, 3-isobutyl-1-methyl-xanthine; Nif. Acid, Niflumic acid; Furo., Furosemide.
**Effect of repeated challenge of MCh on contraction of mouse trachea**

We measured the effect of methacholine \((1 \times 10^{-6} \text{ M})\) on the contractile response of CSE and the vehicle pretreated cultured tracheal segments after repeated washout and challenge. We found that the contractile potency was maintained even after the 8\textsuperscript{th} MCh challenge as shown in figure 18.

![Differential contraction after repeated washout and challenge](image)

Figure 18. A dose-response graph to MCh after repeated washout and challenge with MCh. Each data represent the contraction (percentage of first MCh \((1 \times 10^{-6} \text{ M})\) induced contraction) of all segments tested with error bars representing S.E.M. \((n=4-10)\).

**Comparison of MCh induced contraction of upper versus lower trachea**

The differential contractile response of upper versus lower tracheal segments to the cumulative concentration of MCh was also studied. We found that tracheal segment connected to the larynx responded to less MCh as compared to the segment connected to bronchi as shown in figure 19. In other words, the lower portion of the trachea contracted more compared to upper portion. The contraction of the upper portion at a cumulative
dose (1x10^{-4} M) of MCh was 410.0±61.0 mg (n=11) whereas that of the lower portion of the trachea was 609.0±76.0 mg (n=9) and the difference was highly significant.

Figure 19. The cumulative concentration-response curves to MCh obtained from lower versus upper tracheal segments. Each trachea was divided into two portions (upper and lower). Each data represent the mean tension (mg) of all segments tested with error bars representing S.E.M. (n=9-11). **p<0.01 and *p<0.05 compared to tension in upper tracheas induced by 1x10^{-5} M and 3x10^{-5} M MCh respectively.

**Comparison of isoproterenol induced relaxation of upper versus lower trachea**

The differential relaxation response of upper versus lower tracheal segments to a cumulative concentration of isoproterenol on MCh (1x10^{-6} M) pre-contracted tissues was also studied. We did not observe any difference on isoproterenol induced relaxation of the segment connected to the larynx and the segment connected to bronchi as shown in figure 20. The pre-contraction of upper and lower trachea with a cumulative dose of MCh (1x10^{-4} M) was 312.0±55.3 mg (n=7) and 377.7±62.0 mg (n=9), respectively. After
treatment with cumulative dose of isoproterenol ($1 \times 10^{-6}$ M), the contraction for the upper and the lower trachea was dropped down to $136.0 \pm 43.5$ mg ($n=7$) and $175.1 \pm 73.0$ mg ($n=9$), respectively.

Figure 20. The cumulative concentration effect curves to isoproterenol obtained from lower versus upper tracheal segments. Each trachea was divided into two portions (upper and lower). Each data represent the mean tension (mg) of all segments tested with error bars representing S.E.M. ($n=9-11$).

4.2. Discussion

In this study, we investigated the effect of acute cigarette smoke exposure on BHR to MCh, airway inflammation and the efficacy of fluticasone propionate and/or salmeterol. The harmful effect of long term smoking is well known and included respiratory disorders leading to COPD, cardiovascular diseases, different cancers (Lange et al., 1998; Bartal, 2001). Generally, chronic tobacco smoke exposure may be used to
generate features of COPD such as emphysema and airway remodeling. However, models showing the effect of acute or sub-acute exposure of tobacco smoke may be useful for testing potential anti-inflammatory candidate compounds. As mentioned before in introduction section, the patients with severe asthma, COPD and asthmatics who are active smokers show diminished response to anti-inflammatory drugs and solving this problem is an unmet need.

In our current study, after 4 weeks of tobacco smoke exposure, we found an increase in BHR to a cumulative dose of MCh (25 mg/mL) in mice exposed to cigarette smoke compared to non-smoked mice as revealed by change in Penh. Cigarette smoke exposed mice were more sensitive to MCh than sham control mice as demonstrated by PC_{200} values. FP, salmeterol and FS treatments were not able to protect against the cigarette smoke induced BHR to MCh in smoke exposed mice. However, we found that FP, salmeterol and FS treated non-smoke exposed mice were significantly protected compared to FP, salmeterol and FS treated smoke exposed mice as demonstrated by significantly lower BHR. In other words, there was diminished effect of these drugs in tobacco smoke exposed groups. Also, from PC_{200} calculation, we found that combination of FP and salmeterol treatment of a non-smoke exposed group was more protective than the non-smoke exposed group treated with only FP or salmeterol which is consistent with previous findings (Townley et al., 2009). Albuterol, which is a full agonist of β2 adrenergic receptor, protected against MCh induced BHR in both the cigarette smoke exposed group and the non-smoke exposed control group.

The airway inflammation was studied by measuring inflammatory cytokines released in bronchial alveolar lavage (BAL) fluid. There was a non-significant increase in
the level of measured pro-inflammatory cytokines (IL-13, GM-CSF, IL-1β and TNF-α).

We would normally expect that FP, a corticosteroid, an anti-inflammatory drug, decreases the inflammatory cytokines such as IL-13, GM-CSF, IL-1β and TNF-α. However, we found elevated level of TNF-α and GM-CSF in drug treated smoke exposed groups. Our lab previously had reported elevated levels of IL-13 after smoking in a larger number of mice (Bastola et al., 2010) but we could not see a significant increase in IL-13 level. Total cell counts did not show any difference among groups.

Various studies of cigarette smoke have been conducted in mice and rats to elucidate the effects of cigarette smoke exposure on pathological features of airway diseases like asthma and COPD. Moerloose et al. have published the effect of cigarette smoke exposure in OVA-sensitized BALB/c mice. They investigated the sensitivity, to carbachol, of 2 weeks of cigarette smoke exposure with/without OVA-sensitization and found that the airway hyperresponsiveness was greater in the smoke exposed mice which were sensitized compared to the other groups (Moerloose et al., 2005). TNF-α plays an important role in cigarette smoke induced emphysema. A smoking done on TNF-α KO and WT mice indicated that 70% of emphysema, probably driving neutrophil influx (Churg et al., 2002), is mediated by TNF-α associated inflammatory cells influx and matrix breakdown and rest 30% is driven by TNF-α independent direct matrix attack by MMP (Churg et al., 2004). Macrophages elastase is also required for the induction of emphysema by cigarette smoking in mice (Hautamaki et al., 1997). Macrophage elastase KO mice did not develop emphysema even after chronic cigarette smoke exposure.

Reduced corticosteroid insensitivity due to oxidative stress might be related to activation of phosphoinositide-3-kinase (PI3K)-δ (Mercado et al., 2011). In cigarette
smoke induced COPD model of mice, low dose theophylline enhanced anti-inflammatory activity of dexamethasone with concomitant restoration of HDAC-2 activity (To et al., 2010) suggesting the potential role of low dose theophylline and other PI3K-δ specific inhibitors in the treatment of oxidative stress induced corticosteroid insensitivity in several diseases such as severe asthma, COPD and other inflammatory diseases (Marwick et al., 2009).

Although various studies have been performed to investigate the effect of cigarette smoking on clinical efficacy of corticosteroids and/or β2-adrenergic agonists in human, we are not aware of any animal studies conducted to see if cigarette smoke exposure effects on the therapeutic efficacy of those drugs in reducing BHR and inflammation. As described previously in introduction section, various clinical trials have reported about diminished response to corticosteroids in smokers compared to non-smokers. The non-smokers, but not the smokers, showed improvement on morning PEF, FEV1 and mean concentration of MCh causing 20% fall in FEV1 (PC20) and decrease in eosinophils count in sputum after inhaled FP treatment (100 µg daily) (Chalmers et al., 2002). Another clinical trial designed to investigate the long-term effect of inhaled budesonide (400 or 1600 µg daily) or theophylline (600 mg) on blood markers of inflammatory cells such as eosinophilic cationic protein, myeloperoxidase in smoker and non-smoker patients with asthma. They found that inhaled budesonide was effective in non-smokers but not in smokers (Pedersen et al., 1996). Prednisolone (40 mg, daily) was effective in improving pre-bronchodilator FEV1, morning PEF and asthma control score in never smokers but not in smokers. Ex-smokers showed trend for improvement in each of above parameters (Chaudhuri et al., 2003). All these study show that cigarette smoking
has adverse effect on efficacy on asthma therapy. In consistent to these findings, we also observed the reduced efficacy of FP and/or salmeterol in cigarette smoking mice as revealed by BHR and PC200.

We also performed a study to find out the effect of aqueous cigarette smoke extract (CSE) on contraction and relaxation strength of isolated mouse trachea. We did not observe any significant difference in CSE or vehicle pretreated isolated mouse tracheas as demonstrated by the force of contraction although there was a trend of increased contraction to MCh in CSE pretreated tracheas compared to vehicle pretreatment. The relaxation effect of a cumulative dose of isoproterenol on MCh (1x10^{-6} M) pre-contracted trachea was also not different. We also studied the effect of different drugs on relaxation of isolated tracheas. There was no significant difference on relaxation of CSE and vehicle pretreated tracheas. We found that IBMX, which is a non-selective phosphodiesterase (PDE) inhibitor, and rolipram, a PDE4 inhibitor, reduced the contraction induced by MCh (1x10^{-6} M) in both CSE treated and vehicle only treated tracheas. We also tried to see the effect of niflumic acid, calcium activated chloride channel (CaCC) antagonist and/or furosemide, sodium-potassium-chloride co-transporter (NKCC) on relaxation effect in MCh (1x10^{-6} M) induced pre-contraction. They both inhibited MCh induced contraction in CSE treated and vehicle alone treated tracheas. We found that simultaneous blockade of CaCC and NKCC with niflumic acid and furosemide respectively had more of a relaxation effect than either drug alone. Authors have demonstrated that furosemide is effective in inhibiting allergen induced BHR (Wang et al., 2011). Niflumic acid decreased asthma features induced by IL-13 (Nakano et al., 2006) and cigarette smoke induced mucin synthesis (Hegab et al., 2007). Since NKCC
and CaCC are expressed in airway smooth muscle cells, simultaneous blockade of these transporters may significantly reduce the contraction induced by contractile stimuli like ACh and MCh (Iwamoto et al., 2003; Huang et al., 2009).

The relaxation effect of albuterol, a β2 adrenergic agonist, on MCh pre-contracted trachea was surprising. We did not observe any effect of albuterol although the isoproterenol (both β1 and β2 agonist) had approximately 50.0% relaxation on MCh induced contraction. In our in vivo study, we found that albuterol was very protective against BHR to MCh in both smoked and non-smoked mice. Albuterol relaxes guinea pig and human airways essentially 100.0%. Thus further experiments are needed to find out if mouse trachea and possibly bronchi express predominantly β1 adrenergic receptors compared to β2 adrenergic receptors. This finding could be important in interpreting studies in mice since mouse model is used extensively. It has previously been reported that β-adrenoceptor-mediated relaxation of isolated mouse trachea is mainly through activation of β1-adrenoceptor (Henry and Goldie, 1990). We also tried to compare the contractile effect of MCh after repeated washouts and challenge. We found that the contraction was maintained even after the 8th wash out and MCh challenge. Also, we investigated the differential contraction/relaxation of upper portion of trachea versus lower portion of trachea. We found higher contraction to a cumulative dose of MCh of the lower portion as compared to the upper portion and the difference was highly significant (p<0.01) at 1x10^{-4} M MCh. There was no difference on isoproterenol induced relaxation of the upper versus lower trachea after pre-contraction with MCh.
5. Conclusion

Smoking increased the degree of BHR as measured by the response to methacholine compared to non-smoking mice. Salmeterol, FP and FS treated smoked mice had a higher increase in BHR than salmeterol, FP and FS treated non-smoked mice. FP did not significantly decrease the BHR in smoked mice. Therefore FP and/or salmeterol did not protect significantly the BHR to MCh induced by cigarette smoke exposure. In contrast, albuterol pretreatment protected against the BHR to MCh in both cigarette smoke exposed and non-smoked group. There was a non-significant increase in pro-inflammatory cytokines (IL-13, TNF-α, IL-1β and GM-CSF) in BAL fluid after cigarette smoke exposure compared to non-smoked control mice and FP and/or salmeterol were not able to decrease the level of cytokines. Total cells in BAL fluid of smoked control, drug treated smoked mice and non-smoked mice were not statistically different.

Pretreatment of isolated mouse trachea with 10.0% CSE increased the contraction due to MCh but was not statistically significant compared to the MCh induced contraction of vehicle pretreated mice tracheas. CSE pretreatment did not cause any difference on contraction and relaxation of isolated mouse trachea.

In conclusion, cigarette smoke exposure caused BHR in mice. FP and/or salmeterol showed diminished response in reducing BHR in smoked mice compared to non-smoked mice. From the BAL fluid cytokines and total cell count, we could not make any conclusion regarding inflammation. Further studies are needed to explain the mechanism of diminished response of these drugs after cigarette smoke exposure.
Chapter II. Effect of cigarette smoke extract on cytokines release from U937 cells

1. Introduction

1.1. Role of monocytes/macrophages on asthma and COPD

Macrophages are long lived effector cells in lungs which are produced by differentiation of monocytes. Circulating monocytes migrate to the lung in response to chemoattractants such as CCL2 and CXCL1 which act on CC chemokine receptor (CCR)-2 and CXCR2 (Traves et al., 2004). An early response to inhaled toxicants is the recruitment of macrophages into the lungs where they undergo phagocytosis. Normally, toxic particulate laden macrophages exit the lung via mucociliary escalator or lymphatic system. But, in smokers there is continuous sequestration and accumulation of alveolar macrophages in the lungs. Thus, macrophages in people with COPD and smokers are elevated many folds (Tetley, 2002). Another reason for the elevated number of macrophages in the lungs might be increased proliferation and survival. One analysis of macrophage numbers in the parenchyma of patients with emphysema showed a 25-fold increase in the numbers of macrophages in tissue and alveolar space compared with normal smokers (Retamales et al., 2001). Studies have shown a strong correlation between the number of macrophages and severity of emphysema (Di Stefano et al., 1998). This might be due to an increased influx of monocytes from blood to lung tissue.

It has been reported that monocyte chemotactic protein-1 (MCP-1), a monocyte selective chemokine which attracts monocytes to lung, was increased in sputum and BAL fluid of patients with COPD indicating its role in macrophage migration (Traves et al., 2002; Alam et al., 1996). Also, people with asthma have elevated level of macrophages but their numbers are far greater in COPD. There is increasing evidence that lung
macrophages mediate inflammation in COPD through the release of chemokines that attract neutrophils, monocytes and T-cells and release serine proteases like matrix metalloproteinases (MMP)-9 (Barnes, 2004). Matrix metalloproteinases inhibit α1-antitrypsin, a proteinase inhibitor, and lead to emphysema like feature. Although, neutrophil influx is required for cigarette smoke induced connective tissue matrix breakdown, it appears to be dependent on the presence of macrophage derived MMP-12 suggesting that both macrophages and neutrophils are required for cigarette smoke induced emphysema (Churg et al., 2002). Macrophages obtained from patients with COPD had elevated levels of proteases such as cathepsins K, L and S as well as MMP-1, MMP-2, MMP-9 and MMP-12 (Wallace et al., 2008).

1.2. U937 cells

U937 cells are a human monocytic cell line derived by Sundstrom and Nilsson in 1974 from malignant cells obtained from the pleural effusion of a patient with histiocytic lymphoma. These cells, after treatment with phorbol 12-myristate 13-acetate (PMA) or 12-O-tetradecanoylphorbol-13-acetate (TPA) differentiate into macrophage like cells (Hass et al., 1989; Koren et al., 1979). This cell line normally grows in suspension culture and has a smooth surface but after differentiation with PMA or TPA, they become adherent to each other and to the surface of vessel like macrophages. U937 cells are widely being used for the study of monocyte/macrophage associated effector functions. They are relatively easy to grow and maintain and can be used for multiple passage compared to primary cells. This is the main reason for choosing these cells for this study. Lipopolysaccharides (LPS) obtained from outer membrane of gram negative bacteria acts
like endotoxin and elicits strong immune response in animals. It acts through toll like receptor 4 (TLR-4) and promotes the release of pro-inflammatory cytokines from varieties of cells specially macrophages and B-cells.

1.3. Role of cytokines on asthma and COPD

Activation and migration of inflammatory cells is regulated by small extracellular signaling proteins, termed cytokines, which are secreted by different structural cells such as epithelial, endothelial, smooth muscle as well as inflammatory cells. They can act in long distance signaling (endocrine), short distance signaling (paracrine) or on the same cells from which they were secreted (autocrine) (Chung, 2001). There are various cytokines related with asthma and COPD such as tumor necrosis factor (TNF)-α, interferon (INF)-γ, Interleukin (IL)-1β and IL-6 (De Boer, 2002). Chemotactic cytokines are known as chemokines (e.g. IL-8). Studies in CD4+ T cells have revealed two polarized functional subsets termed T-helper type 1 (Th1) and Th type 2 (Th2). Th1 are characterized by the predominant secretion of IL-2, IFN and TNF whereas Th2 mainly secrete IL-4, IL-5, IL-9 and IL-13 (Mosmann and Coffman, 1989). In asthma, Th2-derived cytokines such as IL-4, IL-5 and IL-13 are highly dominant and hence asthma results from imbalance of Th2-derived cytokines (Robinson et al., 1992). IL-1, IL-6, IL-8, TNF-α, GM-CSF, IL-11, IL-16 and IL-17 are pro-inflammatory cytokines whereas IL-10 and IL-18 are inhibitory cytokines (Chung, 2001).

IL-6 is an inflammatory cytokine secreted from monocytes/macrophages, T-cells, B-cells, fibroblasts and airway epithelial cells and acts as a T-cell and B-cell growth factor and increases IgE secretion. It plays an important role in the transition from
neutrophils to mononuclear-cell infiltrate which is a hallmark of inflammation (Kaplanski et al., 2003; Hurst et al., 2001).

IL-8, (a CXC chemokine), is a pro-inflammatory chemo-attractant cytokine secreted by monocytes/macrophages, airway epithelial cells, airway smooth muscle cells, neutrophils and eosinophils. It is a chemo-attractant and activator of neutrophils. It also induces histamine and cysteinyl leukotrienes from basophils. IL-8 is an important cytokine initiating and amplifying inflammatory responses in COPD and pathogenesis of emphysema (Gadgil and Duncan, 2008). Different studies have reported elevated level of IL-8 in BAL fluid and sputum of patients with COPD. It has also been shown that its level is correlated with an increase in the number of neutrophils and disease severity in COPD (Gorska et al., 2008; Pesci et al., 1998; Simpson et al., 2009).

IL-10 is an inhibitory cytokine released from monocytes/macrophages, Th2 cells and CD8+T-cells. It decreases eosinophil survival and levels of Th1 and Th2 cells. It also decreases monocytes/macrophages activation and decreases BHR. IL-10 level is inversely related to the severity of allergic disease and asthma (Lim et al., 1998; Borish et al., 1996; Berkman et al., 1995; Driscoll et al., 1998; Grünig et al., 1997). It is secreted by monocyte and macrophage and its release is induced by Th1 cytokines like TNF-α (Wanidworanun and Strober, 1993). It has been reported that production by airway immune cells is reduced in patients with asthma (Borish et al., 1996; Calhoun et al., 1996). This indicates that reduction in IL-10 level may allow the amplification of pro-inflammatory pathway leading to development of asthma.
The main objective of this study is to find out the effect of cigarette smoke extract (CSE) treatment on the release of pro-inflammatory and anti-inflammatory cytokines from macrophages *in vitro* using U937 cells.
2. Hypothesis and specific aims

_Hypothesis_

Treatment with cigarette smoke extract (CSE) contributes to lung inflammation in pulmonary diseases like asthma and COPD by inducing the release of inflammatory cytokines like IL-6, IL-8 and decreasing the release of anti-inflammatory cytokine like IL-10 from lung macrophages.

_Specific aims_

(i) To determine the effect of different concentrations of CSE on the release of various inflammatory cytokines (IL-6, IL-8) from U937 cells in presence and absence of LPS.
(ii) To determine the effect of different concentrations of CSE on the release of the anti-inflammatory cytokine (IL-10) from U937 cells in the presence and absence of LPS.
3. Materials and methods

3.1. Cells and reagents

Unless otherwise stated, all reagents used were obtained from Sigma. The human monocytic cell line U937 was purchased from American Type Culture Collection (Catalog # CRL-1593.2; Manassas, VA, USA). To differentiate into macrophage like cells, the U937 cells were treated with phorbol 12-myristate 13-acetate (PMA) from Ascent Scientific (MA, USA). Fetal bovine serum and penicillin-streptomycin were purchased from Atlanta Biological (GA, USA). Hyclone RPMI 1640 medium, supplemented with +2.05 mM L-Glutamine, was purchased from Thermo-Scientific (Hyclone Laboratories, UT, USA).

3.2. Experimental methods

3.2.1. Culture of U937 cells

The human monocytic cell line U937 was cultured on RPMI 1640 medium supplemented with 10.0% fetal bovine albumin, 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C with 5.0% CO₂ in a humidified incubator. Cultures were subcultured by total medium replacement by centrifugation with subsequent resuspension at 2x10⁵ viable cells/mL. Cultures were maintained at a cell concentration between 1x10⁵ and 2x10⁶ cells/ml. To convert these cells to macrophage like cells, PMA (50 ng/mL) was added and the cells were incubated for 24 h at 37°C with 5.0% CO₂. Medium with PMA was removed and replaced with fresh complete medium and further incubated for another 48 h. Then, adherent cells were scrapped off with policeman rubber spatula and used for the subsequent experiments.
3.2.2. Cigarette smoke extract preparation

Cigarette smoke extract (CSE) was prepared as described previously (chapter I, section 3.2.6). In this case, we used 5 mL of RPMI 1640 medium per cigarette. Freshly prepared cigarette smoke extract was used for each experiment.

3.2.3. Cigarette smoke extract and other drugs treatment

PMA treated cells were centrifuged and re-suspended in fresh complete RPMI medium and added to a 24-well tissue culture plate (1x10^6 cells/mL). Cells were stressed with various concentrations of CSE (2.5%, 5.0%, 10.0% and 20.0%) and IL-13 and incubated at 37°C overnight. LPS was then added and incubated for 24 h. Cells supernatants were collected after centrifugation and kept in freezer (-80°C) for cytokine analysis by ELISA.

3.2.4. Measurement of cytokines in cell supernatants

Frozen supernatants were analyzed for cytokines (IL-6, IL-8 and IL-10) using commercially available enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, Biolegend and R&D systems, USA) according to manufacturer’s protocol.
4. Results and discussion

4.1. Results

The results obtained from CSE pretreated vehicle alone or LPS stimulated cells on inflammatory and anti-inflammatory cytokines are presented below.

4.1.1. Effect of CSE on IL-6 release

*Effect of CSE pretreatment on IL-6 release from vehicle treated cells*

U937 cells were differentiated with PMA (50 ng/mL) and used for subsequent experiments. Cells were first treated with different concentrations of CSE (2.5%, 5.0%, 10.0% and 20.0%) and incubated for 24 h. Supernatants were collected after centrifugation and IL-6 was measured. Surprisingly, IL-6 was inhibited by all the concentrations of CSE as shown in figures 21 and 22. IL-6 released by cells without any CSE was 119.6±32.7 pg/mL (n=8) whereas after addition of CSE 2.5%, 5.0%, 10.0% and 20.0%, IL-6 release was decreased to 32.6±12.7 pg/mL, 18.6±5.5 pg/mL, 14.5±4.5 pg/mL and 9.3±2.3 pg/mL (n=5-6), respectively (Figure 21). Treatment with CSE (all concentrations) significantly lowered the IL-6 release compared to vehicle alone treated control cells.
Figure 21. Effect of cigarette smoke extract (CSE) pretreatment on IL-6 release from vehicle treated U937 cells. PMA differentiated U937 cells were treated with different concentrations of CSE and incubated for 24 h. Cell supernatants were collected after centrifugation and IL-6 was measured by a human IL-6 ELISA kit from eBioscience (n=5-6). *P˂0.05 and *P˂0.01 compared to vehicle treated control cells.

**Effect of CSE pretreatment on IL-6 release from LPS stimulated cells**

IL-6 released by LPS stimulated cells without any CSE was 917.0±86.7 pg/mL (n=8) whereas after addition of CSE 2.5%, 5.0%, 10.0% and 20.0%, IL-6 release was decreased to 658.3±89.5 pg/mL, 467.6±141.0 pg/mL, 225.3±109.0 pg/mL and 17.66±7.5 pg/mL (n=6-7), respectively (Figure 22). Treatment with CSE (5.0%, 10.0% and 20.0%) significantly lowered (p <0.05) the IL-6 release from LPS stimulated cells.
Figure 22. Effect of cigarette smoke extract (CSE) pretreatment on IL-6 release from LPS stimulated U937 cells. PMA differentiated U937 cells were pre-treated with different concentrations of CSE and incubated for 24 h with LPS (1 µg/mL) stimulation. Cell supernatants were collected after centrifugation and IL-6 was measured by a human IL-6 ELISA kit from eBioscience (n=6-7). **P<0.01 and ***P<0.001 and compared to LPS alone treated cells.

Effect of IL-13 pretreatment on IL-6 release from vehicle treated and LPS stimulated cells

Effect of IL-13 on IL-6 release from U937 cells was also studied (Figure 23). The levels of IL-6 release in IL-13 pretreated cells in vehicle treated or LPS stimulated cells were 39.3±13.5 pg/mL and 486.1±108.0 pg/mL respectively.
Figure 23. Effect of IL-13 pretreatment on IL-6 release from vehicle treated and LPS stimulated U937 cells. PMA differentiated U937 cells were pre-treated with IL-13 (10 ng/mL) and incubated for 24 h in presence or absence of LPS (1 µg/mL). Cell supernatants were collected after centrifugation and IL-6 was measured by a human IL-6 ELISA kit from eBioscience (n=4-7). ***<0.001 compared to LPS alone treated cells.

4.1.2. Effect of CSE on IL-8 release

Effect of CSE pretreatment on IL-8 release from vehicle treated cells

Cell supernatants, obtained after treatment of differentiated U937 cells with different concentrations of CSE (2.5%, 5.0 %, 10.0% and 20.0%) with/without LPS, were analyzed for IL-8 release and shown in figures 24 and 25. IL-8 released by cells without any CSE pretreatment was 15,190.0±2,826.2 pg/mL (n=11). After the addition of CSE 2.5%, 5.0%, 10.0% and 20.0%, IL-8 release was 29,665.0±7,128.1 pg/mL,
30,584.0±3635.8 pg/mL, 24,563.4±4,889.4 pg/mL and 20,264.0 ±6329.7 pg/mL (n=5-7), respectively (Figure 24). There was a significant increase in IL-8 release after treatment with 2.5% and 5.0% CSE (p=0.04 and 0.008 respectively) compared to IL-8 release from vehicle treated cells only.

**Effect of CSE on IL-8 release**

![Graph showing IL-8 release](image)

Figure 24. Effect of cigarette smoke extract (CSE) pretreatment on IL-8 release from vehicle treated U937 cell. PMA differentiated U937 cells were treated with different concentrations of CSE and incubated for 24 h. Cell supernatants were collected after centrifugation and IL-8 (pg/mL) was measured by a human IL-8 ELISA kit from R&D research (n=5-7). *p<0.05 compared to vehicle treated control cells.

**Effect of CSE pretreatment on IL-8 release from LPS stimulated cells**

IL-8 released by LPS stimulated cells without any CSE was 30,395.5±4,470.9 pg/mL (n=12) whereas after addition of CSE 2.5%, 5.0%, 10.0% and 20.0%, IL-8 release from LPS stimulated cells was 45,257.4±7,087.8 pg/mL, 35,564.6±5,143.8 pg/mL.
pg/mL, 26,249.7±3,352.1 pg/mL and 18,160.8±8043.1 pg/mL (n=5-7), respectively (Figure 25). At high concentration of CSE (20.0%), IL-8 release was non-significantly lower compared to IL-8 release at lower concentration of CSE (2.5%) in both vehicle and LPS treated cells.

Figure 25. Effect of cigarette smoke extract (CSE) pretreatment on IL-8 release from LPS stimulated U937 cells. PMA differentiated U937 cells were pre-treated with different concentrations of CSE and incubated for 24 h with LPS (1 µg/mL) stimulation. Cell supernatants were collected after centrifugation and IL-8 was measured by a human IL-8 ELISA kit from R&D research (n=5-7). *<0.05 compared to LPS alone treated cells.
Effect of IL-13 pretreatment on IL-8 release from vehicle treated and LPS stimulated cells

Effect of IL-13 on IL-8 release from U937 cells was also studied (Figure 26). The levels of IL-8 release in IL-13 pretreated cells in vehicle treated or LPS stimulated cells were 13,715.7±2063.3 pg/mL and 34,909.0±2,063.4 pg/mL respectively.

Figure 26. Effect of IL-13 pretreatment on IL-8 release from vehicle treated and LPS stimulated U937 cells. PMA differentiated U937 cells were pre-treated with different concentrations of IL-13 (10 ng/mL) and incubated for 24 h in presence or absence of LPS (1 µg/mL). Cell supernatants were collected after centrifugation and IL-8 was measured by a human IL-8 ELISA kit from R&D research (n=5-6).
4.1.3. Effect of CSE on IL-10 release

Effect of CSE pretreatment on IL-10 release from vehicle treated cells

Cell supernatants obtained after treatment of differentiated U937 cells with different concentrations of CSE (2.5%, 5.0%, 10.0% and 20.0%) with/without LPS were used to study IL-10 release and shown in figures 27 and 28. IL-10 released by cells without any CSE was 557.0±125.1 pg/mL (n=8) whereas after addition of CSE 2.5%, 5.0%, 10.0% and 20.0%, IL-10 release was 532.5±136.4 pg/mL, 121.3±57.4 pg/mL, 85.9±36.8 pg/mL and 100.3±70.5 pg/mL (n=5-6), respectively (Figure 27).

Figure 27. Effect of cigarette smoke extract (CSE) pretreatment on IL-10 release from vehicle treated U937 cell. PMA differentiated U937 cells were treated with different concentrations of CSE and incubated for 24 h. Cell supernatants were collected after centrifugation and IL-10 (pg/mL) was measured by a human IL-10 ELISA kit from R&D research (n=5-6).*<0.01 compared to vehicle treated control cells.
**Effect of CSE pretreatment on IL-10 release from LPS stimulated cells**

IL-10 released by LPS stimulated cells without any CSE was 2418.3±743.3 pg/mL (n=6) whereas after addition of CSE 2.5%, 5.0%, 10.0% and 20.0%, IL-10 release was 1990.4±907.6 pg/mL, 1438.9±527.4 pg/mL, 775.6±541.8 pg/mL and 34.5±15.4 pg/mL (n=5-6), respectively (Figure 28).

With the exception of treatment of cells with 2.5% CSE, there was a significant decrease in IL-10 release from (except treated control cells. 2.5%, 5.0% and 10.0% CSE treatment showed a non-significant decrease in IL-10 release whereas 20.0% CSE significantly decreased IL-10 release from LPS stimulated cells.

![Effect of CSE on IL-10 release](image)

Figure 28. Effect of cigarette smoke extract (CSE) pretreatment on IL-10 release from LPS stimulated U937 cells. PMA differentiated U937 cells were pre-treated with different concentrations of CSE and incubated for 24 h with LPS (1 µg/mL) stimulation. Cell supernatants were collected after centrifugation and IL-10 was measured by a human IL-10 ELISA kit from R&D research (n=5-6). *<0.05 compared to LPS alone treated cells.
**Effect of IL-13 pretreatment on IL-10 release from vehicle treated and LPS stimulated cells**

Effect of IL-13 on IL-10 release from U937 cells was also studied (Figure 29).

The levels of IL-10 release in IL-13 pretreated cells in vehicle treated or LPS stimulated cells were 577.2±323.5 pg/mL and 3396.6±5361.1 pg/mL respectively.

![Effect of IL-13 on IL-10 release](image)

**Figure 29. Effect of IL-13 pretreatment on IL-10 release from vehicle treated and LPS stimulated U937 cells.** PMA differentiated U937 cells were pre-treated with IL-13 (10 ng/mL) and incubated for 24 h in presence or absence of LPS (1 µg/mL). Cell supernatants were collected after centrifugation and IL-10 was measured by a human IL-10 ELISA kit from R&D research (n=5-6).

4.2. Discussion

Macrophages are key inflammatory cells in COPD. Cigarette smoke, which is a major etiological factor in this condition, contains high concentration of free radicals and oxidants. Activation of immune cells by these free radicals leads to production of
inflammatory cytokines like IL-8. Tobacco smoke is associated with the inflammation of the lung and one of the clinically defined events of cigarette smoke induced inflammation is the infiltration of macrophages (Yanbaeva et al., 2007; Van der Vaart et al., 2004; Engels, 2008; Thacker, 2006; Laberge and El Bassam, 2004).

This study was designed to establish the role of CSE on the release of inflammatory (IL-6 and IL-8) and anti-inflammatory cytokines (IL-10) from macrophage like cells in vitro. We demonstrated that CSE treatment (24 h) decreased the release IL-6 and IL-10 from both vehicle treated and LPS stimulated cells in dose dependent manner. We also demonstrated that CSE increased the release of IL-8 in both control and LPS stimulated cells. CSE and LPS demonstrated synergistic effects in terms of IL-8 release. The differential effect of CSE treatment on IL-8 and IL-10 was consistent with our hypothesis. Surprisingly, the release of IL-6, which is a pro-inflammatory cytokine, was also inhibited by CSE. The effect of IL-13, which is commonly referred to as an inflammatory cytokine and sometimes as anti-inflammatory, was also monitored. Although IL-13 treatment did not significantly affect IL-8 and IL-10 release, we found inhibitory effects on IL-6 release demonstrating its anti-inflammatory activity. Treatment of cells with LPS significantly increased the release of all of the measured cytokines.

Kent et al. have reported on the transcriptional regulation of inflammatory signaling pathways by different concentrations of CSE on macrophages from patients with COPD (Kent et al., 2008). They found from the polymerase chain reaction (PCR) and microarray analysis that there was an up regulation of the inflammatory gene for cytokine like IL-8 at low CSE (10.0%) concentration (down-regulation at 25.0%) and down regulation of genes encoding cytokines like IL-1β, IL-6, IL-10 and IL-18 after
treatment with CSE which was consistent with our findings. They also reported in another study that combined treatment of CSE and dexamethasone caused greater inhibition on mRNA level of inflammatory cytokines such as IL-1β, IL-6, GM-CSF release from COPD macrophages compared to inhibition by either agent alone (Kent et al., 2010). It is also reported that, in THP-1 macrophages, treatment with CSE down-regulated the protein expression of IL-1β and IL-6 as demonstrated by suspension microarray and PCR analysis (Nordskog et al., 2005; Birrell et al., 2008). Studies have shown that BAL fluid from smokers contains lower levels of IL-6 and increased levels of IL-8 (McCrea et al., 1994). Also, alveolar macrophages obtained from smokers secreted less IL-6 compared to macrophages from non-smokers (Soliman and Twigg, 1992). Alveolar macrophages obtained from guinea pig and human subjects, exposed with cigarette smoke (gas phase), secreted significantly reduced levels of IL-6 and TNF-α (Dubar et al., 1993). Also, murine macrophages exposed to cigarette smoke exposure produced reduced levels of IL-6 and TNF-α (Gaschler et al., 2008).

IL-6 has long been considered as pro-inflammatory cytokine induced by LPS along with TNF-α. However, its anti-inflammatory properties have also been reported. It caused the reduction in number of deaths caused by *staphylococcus aureus* in mice (Barton et al., 1996). Intra-peritoneal delivery of endotoxin resulted in much pronounced level of TNF-α, GM-CSF, MIP-2 and IFN-γ systemically in IL-6 (-/-) mice than in IL-6 (+/+ ) mice revealing its anti-inflammatory role in systemic inflammation (Xing et al., 1998).

Various studies have been published demonstrating the induction of IL-8 release after treatment of monocyte/macrophage like cells (U937, THP-1) and blood derived
monocytes/macrophages with CSE (Yang et al., 2007; Birrell et al., 2008; Walters et al., 2005; Karimi et al., 2006; Yang et al., 2006; Facchinetti et al., 2007). IL-8 is a powerful chemotactic cytokine (chemokine) for neutrophils which plays a key role in pulmonary inflammation (Strieter and Kunkel, 1994; Guo and Ward, 2002) and its expression is regulated by oxidative stress (DeForge et al., 1993). CSE also induces IL-8 release from human bronchial epithelial cells (Pace et al., 2008). The CSE associated stimulation of IL-8 is through activation of NF-κB (Sarir et al., 2010; Lerner et al., 2009) via inhibitor of κ B kinase (IKK) and reduction in histone deacetylase (HDAC) level (Yang et al., 2006).

IL-13, a cytokine which is elevated after cigarette smoke exposure (Bastola et al., 2010) is associated with increased severity of symptoms of asthma. Our lab previously has reported that IL-13 causes BHR to MCh and also reduces the response to glucocorticoids (Horiba et al., 2011; Townley et al., 2009; Townley, 2007; Townley et al., 2011). Various other literatures also have demonstrated its inflammatory roles in worsening the symptoms of asthma and has been considered to be pro-inflammatory cytokine. Various IL-13 antagonists have been shown to be effective in treatment of asthma and are in the process of clinical development (Mitchell et al., 2010). However, literatures also have demonstrated its anti-inflammatory roles in vitro. IL-13 has been reported as an anti-inflammatory cytokine which inhibited the release of IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12, MIP-1α, GM-CSF, G-CSF by human monocytes activated with LPS (deWaal Malefyt et al., 1993). Consistent with this finding, we also observed in this study that IL-13 inhibited LPS induced IL-6 release in our cells stimulated with LPS or in absence of LPS. Anti-inflammatory activity of IL-13 has also been reported by other
authors as well. Intra-tracheal administration of human recombinant IL-13 dose dependently reduced the number of inflammatory cells like macrophages, eosinophils and neutrophils in BAL fluid of gpTNF-α and antigen induced lung inflammation in guinea pigs (Watson et al., 1999). IL-4, IL-6, IL-10 and IL-13 are all regarded as an anti-inflammatory cytokines and has been reviewed (Opal and DePalo, 2000). IL-4, IL-10 and IL-13 inhibited monocyte chemotactic and activating factor (MCAF) and LPS induced inflammatory cytokines (TNF-α and IL-1β) from human monocytes in vitro (Yano et al., 1995). Kambayashi et al. reported that IL-4 and IL-13 inhibited LPS induced the release of inflammatory cytokines (IL-6 and TNF-α) from murine peritoneal macrophages which is modulated by the enhanced release of IL-10, which is an anti-inflammatory cytokine and the inhibitory effect of IL-4 and IL-13 was blocked by IL-10 neutralizing antibody (Kambayashi et al., 1996). IL-4 and IL-13 reduced the release of β-amyloid or LPS induced IL-6 release from human monocytic cell line (THP-1) in time and dose dependent manner (Szczepanik et al., 2001). IL-13, also protected mice from LPS induced lethal endotoxemia in similar manner to IL-10 and this was correlated with significant inhibition of pro-inflammatory cytokines such as TNFα, IFN-γ and IL-12 (Muchamuel et al., 1997). The inhibitory effect of IL-13 on inflammatory cytokine such as TNF-α is due to suppression of TNF-α induced activation of NF-κB and activator protein (AP)-1. IL-13 inhibited the NF-κB activation induced by TNF-α, LPS, H2O2 in human monocytic cell line (U937) in vitro (Manna and Aggarwal, 1998). In an in vivo model of rat lung injury, IL-13 also demonstrated the suppressive effect on NF-κB and preserved inhibitor of kappa B (IκB) (Lentsch et al., 1997). The recombinant IL-13 inhibited TNF-α, IL-1α, IL-1β, MCP-1, IL-8 mRNA expression dose dependently in
human mesangial cells (HMCs) by blocking NF-κB and JNK/AP-1 activation (Zhu et al., 2010). Recombinant IL-13 treatment to human PBMCs resulted in the inhibition of LPS induced IL-6 release. It also suppressed the mRNA expression of inflammatory cytokines such as IL-6, TNF-α, IL-1β and IL-8 (Minty et al., 1993). Other researchers also have reported the inhibitory effect of IL-13 and IL-4 on TNF-α and IL-10 release from human peripheral blood mono nuclear cells (PBMCs) stimulated with LPS (Luttmann et al., 1999). Most of these previous studies have demonstrated IL-13 as an anti-inflammatory cytokine. *In vivo* animal studies involving IL-13 have demonstrated its adverse effect in asthma and emphysema (Ma et al., 2006). Our lab also reported its effect on BHR in mice and found that IL-13 causes BHR which is sustained for up to 1 week (Horiba et al., 2011; Townley et al., 2009). So, further investigation about the role and mechanism of IL-13 function in asthma and COPD is warranted.
5. Conclusion

This study showed that CSE pretreatment reduced the secretion of pro-inflammatory cytokine (IL-6) and anti-inflammatory cytokine (IL-10) from U937 cells in dose dependent manner. CSE reduced the level of IL-6 and IL-10 from both vehicle treated as well as LPS stimulated cells. However, CSE pretreated cells demonstrated increased level of IL-8 release in both vehicle treated and LPS stimulated cells. Treatment with IL-13 had no effect on IL-8 and IL-10 release in both vehicle treated and LPS stimulated cells. However, it decreased IL-6 release in both LPS stimulated and vehicle treated cells.

In conclusion, CSE increased the release of pro-inflammatory cytokines (e.g. IL-8) and decreased the release of anti-inflammatory cytokines (e.g. IL-10) which is consistent with our hypothesis. However, it also decreased IL-6 release which was opposite to our hypothesis considering this as an inflammatory cytokine.
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