



The University of
Nottingham

UNITED KINGDOM • CHINA • MALAYSIA

Cigarette Smoke Extract Induces Inflammatory Cytokine and Chemokine Production in Human Bronchial Epithelial Cells

Mohammed Abdulrahman M Alshehri

Division of Respiratory Medicine

Thesis submitted to the University of Nottingham
for the degree of Master of Science by Research
in Respiratory Medicine

Supervisor: Prof. Linhua Pang

February 2018

Abstract

Chronic obstructive pulmonary disease (COPD) is a common inflammatory lung disease. It has been known that cigarette smoke (CS) is a major cause of COPD and can induce alteration of the expression of many genes, including inflammatory genes. The receptor for advanced glycation end products (RAGE) is a 35 kDa pattern recognition receptor and its signalling pathways are involved in inflammatory responses. Soluble form of RAGE (sRAGE) is recognised as a decoy receptor of RAGE. However, the role of RAGE in mediating CS-induced inflammatory cytokine and chemokine production, contributing to airway inflammation in COPD, is poorly understood. Inhaled corticosteroids are the main anti-inflammatory therapies for COPD, but their effectiveness is controversial. This underlines the needs for new anti-inflammatory drugs. Cordycepin and simvastatin are drugs with potential anti-inflammatory effects, but their effects on CS-induced inflammatory cytokine and chemokine production have not been explored. This study aimed to investigate whether cigarette smoke extract (CSE) could induce inflammatory cytokine and chemokine production in human bronchial epithelial cells, to assess the role of RAGE in this process, and to explore the potential effects of cordycepin and simvastatin on CSE-induced cytokine and chemokine production.

Confluent BEAS-2B human bronchial epithelial cells were treated with CSE (1%, 2.5%, and 5%) for 24 hours. The concentration of a group of

cytokines and chemokines implicated in COPD, such as CXCL5 (ENA-78), CCL11 (eotaxin), CXCL1(GRO- α), CXCL10 (IP-10), CXCL8 (interleukin-8, IL-8), and IL-6 in the culture medium was measured by ELISA. sRAGE secretion and RAGE expression were analyzed by ELISA and Western blotting, respectively. The effect of the specific RAGE inhibitor (FPS-ZM1) (0.5 -1.5 mM) on CSE-induced cytokine/chemokine production was explored to assess the role of RAGE. Experiments were also conducted to investigate the effects of cordycepin (5-25 μ M) and simvastatin (0.625-5 μ M) on CSE-induced cytokine/chemokine production and the possible involvement of RAGE and RAGE ligand high mobility group box 1 (HMGB-1). The cytotoxicity of CSE (1-5%) and FPS-ZM1 (0.5-1.5 mM) was assessed by MTT cell viability assay.

We found that CXCL1, CXCL5, CXCL10 and CCL11 could not be detected in both untreated and CSE-treated cells, but the production of IL-8 and IL-6 was markedly increased by CSE. sRAGE secretion was undetectable in both untreated and CSE-treated cells. Interestingly, RAGE was highly expressed in untreated cells and was not further increased by CSE treatment. CSE-induced IL-8 and IL-6 production was partially inhibited by FPS-ZM1, but RAGE expression was not affected. CSE-induced IL-8 production was also partially inhibited by both cordycepin and simvastatin, but CSE-induced IL-6 production was enhanced by cordycepin and was not affected by simvastatin.

Furthermore, RAGE and HMGB-1 expression was not affected by cordycepin and simvastatin. Our results suggest cigarette smoke may contribute to the pathogenesis of COPD by promoting IL-6 and IL-8 production from human bronchial epithelial cells. This process may be partially mediated by RAGE. Cordycepin and simvastatin can reduce CSE-induced IL-8 production, unlikely via inhibition of RAGE pathway, and may have therapeutic potential in neutrophil-driven airway inflammation in COPD.

Acknowledgement

I would like to give my sincere appreciation and warm thanks to Prof. Linhua Pang for giving me the opportunity to study the MSc in Respiratory Medicine and also for his patience, advice, and encouragement to make me grow as a research scientist. My thesis would not have been done without your wisdom, guidance, support, and help. Prof. Linhua, you have been extremely supportive through the past year and I am grateful for that. I would also thank Dr. Oliver Brand for his valuable guidance and help with my laboratory skills. Also, I would like to thank Dr. Alice for her support and help with my laboratory skills.

I would also like to express my thanks to the great people of the Division of Respiratory Medicine for their support throughout my study journey. Special thanks to the PhD student Abdullah Alqarni for his help and advices.

Appreciative thanks to my sponsor King Fahad Medical City in Saudi Arabia/Riyadh for giving me the opportunity to continue my education to serve my country (Saudi Arabia). Also, warm thanks to Saudi Arabian Cultural Bureau in London for their help and support during my study, especially for honouring me with the excellence award.

Mom and Dad, thank you for your support and prayers, your sacrifices meant a lot for me, without you I would not be here, and I hope to achieve the things that make you happy.

ABBREVIATIONS

A

AGEs: Advanced glycation end products

B

BALF: Bronchoalveolar lavage fluid

BCA: Bicinchoninic Acid protein Assay

BSA: Bovine Serum Albumin

C

cAMP: Cyclic adenosine monophosphate

CS: Cigarette smoke

CSE: Cigarette smoke extract

D

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl sulfoxide

E

ELISA: Enzyme-linked immunosorbent assay

F

FEV1: Forced expiratory volume-1

FVC: Forced volume capacity

G

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GOLD: Chronic Obstructive Lung Disease

H

HASMC: Human airway smooth muscle cells

HDL: High-density lipoprotein

HMGB-1: High mobility group box 1

HUVEC: Human umbilical vein endothelial cells

J

JNK: c-Jun N-terminal kinase

L

LABA: Long-acting beta-2 agonist

LDL: Low-density lipoprotein

M

mRAGE: Membrane-bound RAGE

N

NF- κ B: Nuclear factor kappa B

NHBE: Normal human bronchial cells

P

PMA: Phorbol myristate acetate

R

RAGE: Receptor of advanced glycation end products

S

sRAGE: Soluble RAGE

T

TNF α : Tumour necrosis factor alpha

T

TRL4: Toll-like receptor 4

W

WD-HBEC: Well-differentiated human bronchial epithelial cells

Table of Contents

Abstract	2
Acknowledgement	5
ABBREVIATIONS	6
Table of Contents.....	8
Chapter 1. Introduction	11
1.1. Chronic Obstructive Pulmonary Disease (COPD)	12
1.1.1. COPD Definition	12
1.1.2. COPD Prevalence, Morbidity, Mortality and Impact	13
1.1.3. Association of COPD with Cigarette Smoke	14
1.1.4. Airway Inflammation and Anti-inflammatory Treatments in COPD	15
1.2. RAGE (Receptor of Advanced Glycation End Products).....	17
1.2.1. Structure	17
1.2.2. Expression.....	18
1.2.3. Ligands	18
1.2.4. Signalling Pathways	19
1.3. Cigarette Smoke and Airway Inflammation.....	21
1.4. Alternative Anti-inflammatory Drugs	23
1.5. Summary.....	26
1.6 Hypothesis and Aims	27
1.6.1 Hypothesis.....	27
1.6.2 Aims	27
Chapter 2. Materials and Methods	28
2.1. Tables of Materials.....	29
2.1.1. Drugs.....	29
2.1.2. ELISA Kits.....	30
2.1.3. ELISA Buffers and Reagents	30
2.1.4. Materials for Cell Culture and MTT Cell Viability Assay	31
2.1.5. Antibodies for Western Blotting.....	32
2.1.6. Materials for Western Blot	32
2.1.7. Western Blot Buffers and Reagents	32
2.2. CSE Preparation.....	35

2.3.	Drug Preparation.....	35
2.4.	Cell Culture	36
2.4.1.	Cell Sub-Culture.....	36
2.4.2.	Cell Counting.....	37
2.4.3.	Cell Treatment	37
2.5.	Protein Expression Analysis	38
2.5.1.	Western Blot Analysis	38
2.5.2.	Cell Lysate Preparation	38
2.5.3.	Bicinchoninic Acid Protein Assay	39
2.5.4.	SDS-PAGE	39
2.5.5.	Protein Transfer	40
2.5.6.	Blocking and Antibody Incubation	40
2.5.7.	Protein Detection	41
2.6.	Enzyme-Linked Immunosorbent Assay (ELISA)	42
2.6.1.	Plate Preparation	42
2.6.2.	Assay Procedure.....	42
2.7.	Cell Viability Assay	43
2.8.	Statistical Analysis	44
	Chapter 3. Results	45
3.1	Effect of CSE on cytokine/chemokine production	46
3.2	Effect of CSE on sRAGE secretion and RAGE expression	49
3.3	Effect of FPS-ZM1 on CSE-induced IL-8 and IL-6 production	52
3.4	Effect of FPS-ZM1 on RAGE expression	54
3.5	Effect of cordycepin on CSE-induced IL-8 and IL-6 production	56
3.6	Effect of cordycepin on the expression of RAGE and its ligand HMGB-1	58
3.7	Effect of simvastatin on CSE-induced IL-8 and IL-6 production.....	60
3.8	Effect of simvastatin on RAGE expression.....	62
3.9	Effect of CSE and FPS-ZM1 on cell viability	64
	Chapter 4. Discussion	66
4.1.	CSE and Cytokine/Chemokine Production	68
4.2.	CSE and sRAGE Secretion and RAGE Expression	71
4.3.	RAGE Inhibitor and CSE-induced IL-8 and IL-6 production	72
4.4.	Effect of Cordycepin on CSE-Induced IL-8 and IL-6 Production and RAGE/HMGB-1 Expression.....	74
4.5.	Effect of Simvastatin on CSE-Induced IL-8 and IL-6 Production and RAGE/HMGB-1 expression	76
	Chapter 5. Future Directions and Conclusions	78

5.1.	Future Directions	79
5.2.	Conclusions	81
	References.....	82

Chapter 1. Introduction

1.1. Chronic Obstructive Pulmonary Disease (COPD)

1.1.1. COPD Definition

COPD has been defined by the World Health Organization (WHO) as *'a lung disease characterized by chronic obstruction of lung airflow that interferes with normal breathing and is not fully reversible. The more familiar terms "chronic bronchitis" and "emphysema" are no longer used, but are now included within the COPD diagnosis'* (1). Cigarette smoke is the main cause of COPD (1).

Parenchymal damage (emphysema) and narrowing of the small airway (bronchitis) or loss of its elasticity are the causes of the airflow limitation. Although they do not necessarily have to occur together, they usually evolve over time (2).

Spirometry testing methods are needed to assess the pulmonary function in patients with COPD. Decreases in FEV1 and its ratio to FVC (FEV1/FVC) results from airway obstruction (3). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) has published the Global strategy for prevention, diagnosis and management of COPD report for 2018, and it has classified the presence of the airflow obstruction using the spirometry in COPD patients post-bronchodilator FEV1/FVC <70% predicted and the severity into four GOLDs (stages) based on post-bronchodilator FEV1. GOLD-1 mild, $FEV1 \leq 80\%$ predicted; GOLD-2 moderate, $50\% \leq FEV1 < 80\%$ predicted; GOLD-3 severe, $30\% \leq FEV1 < 50\%$ predicted; GOLD-4 very severe and a $FEV1 < 30\%$ predicted (3).

1.1.2. COPD Prevalence, Morbidity, Mortality and Impact

COPD is the second most common respiratory disease in the United Kingdom (UK) (4). In UK there are 1.2 million people diagnosed with COPD in 2011(4). However, COPD remains underestimated with its true prevalence across the world (5).

In 2012, the number of people diagnosed with COPD in the UK per 100,000, by age was 596 (41–50 years), 2228 (51–60), 5385 (61–70), 8151 (71–80), and 7,999 (81 and over). The number of males and females diagnosed with COPD in the UK per 100,000 was 2022 (males) and 1821 (female) in 2012 (4).

COPD is one of most common causes of death worldwide (6). Globally, 3.2 million people died from COPD in 2015 (7). UK is ranked 12th among the top 20 countries for COPD mortality rates (4). A recent study (2016) estimated that, in 2011, the number of deaths among COPD patients in England was 99,200 (range 92,500–128,500) and it is projected to increase to 129,400 (range 126,400–133,400) by 2030. In Scotland, the number of deaths among COPD patients was 9,700 (range 9,000–12,300) in 2011 and it is projected to increase to 13,900 (range 13,400–14,500) in 2030 (8).

COPD has a direct impact on healthcare costs in the UK. In one study, it was estimated that the direct costs on healthcare in England and Scotland for people with moderate and severe COPD, including maintenance costs, are £1.50 billion and £159 million, respectively, in

2011. However, these numbers are expected to increase to £2.32 billion and £207 million by 2030 (8).

Furthermore, COPD has many impacts on patients' daily life activities (e.g. work limitations, health complaints and psychosocial issues). The national health institute survey estimated that in the USA, 48% of COPD patients aged between 20 and 70 years have limitations in their ability to work (9). Another study showed that COPD patients are most likely to have paid sick leave because of fatigue (10).

1.1.3. Association of COPD with Cigarette Smoke

The US Department of Health and Human Services (DHHS) has linked for 50 years the biologic plausibility between cigarette smoking and COPD. By evidence, tobacco cigarette smoking is the major cause of COPD in both men and women. Inhaled cigarette smoke induces all elements of COPD phenotypes (11). Other tobacco smoking types, e.g. waterpipe (hookah), increases the prevalence of COPD (12). It was estimated that lifelong smokers have 50% probability of developing COPD (13). A study reported that among patients with COPD, the median smoking pack per year index was 26.7 (14). In fact, smoking duration has a direct impact on COPD prevalence. A study reported that among 4135 adults aged ≥ 45 years with a history of tobacco smoking of 1–9 years, 10–19 years, 20–29 years and ≥ 30 years, 6.8%, 9.1%, 11.6% and 25.6%, respectively, developed COPD, suggesting that longer smoking duration is likely to increase the prevalence of COPD. A lower prevalence of COPD was seen in former smokers who have less than 10 years of

tobacco history than current smokers (11, 15). The Global Burden of Disease (GBD) research showed that smoking prevalence over the past 30 years was decreased among the general population. However, the number of smokers has actually increased because of the population growth (16). A study proposed that the prevalence of COPD is higher among smokers who are 40 years and older (17). Furthermore, according to the Institute for Health Metrics and Evaluation (IHME), in the UK, the numbers of male and female smokers are estimated to be, on average, 5.5 million (5.0–6.0) and 5.1 million (4.6-5.6) in 2015, respectively (18). The number of cigarette smokers is expected to rise by 2030 (19).

1.1.4. Airway Inflammation and Anti-inflammatory Treatments in COPD

COPD is an inflammatory disease involving many inflammatory, neutrophils in particular, as well as structural cells and a large number of inflammatory cytokines and chemokines (20, 21). Cytokines are small secreted proteins released by cells and play a key role in acute and chronic inflammation via complex communications and interactions between cells (22). CXCL8 (IL-8), CXCL1 (GRO- α), and CXCL5 (ENA-78) are neutrophil chemoattractants that enact in neutrophilic inflammation of COPD (20, 23). It has been shown that Interleukin 6 (IL-6) can contribute to leukocytosis by promoting IL-8 production and recruitment of neutrophils and its level is increased in the sputum of patients with COPD (20). IL-6 has also been regarded as a player in the pathogenesis of COPD by modulating pulmonary function, but not

necessarily a parameter for exacerbated inflammation (24). Moreover, CXCL10 (interferon-inducible protein-10, IP-10), a chemoattractant for mast cells, and CCL11 (eotaxin), a chemoattractant for eosinophils, have also been implicated in COPD (25, 26). These cytokines and chemokines can be produced in many cells, including bronchial epithelial cells, and play a critical role in continuous airway tissue damage in COPD. (22, 27).

Anti-inflammatory drugs, e.g. inhaled corticosteroids, and bronchodilators, e.g. long-acting beta-2 agonist (LABA), are commonly used to treat the COPD symptoms (28). Inhaled corticosteroids are wide spectrum anti-inflammatory drugs that reduce inflammation by inhibiting the activation of inflammatory genes in many diseases and can temporarily suppress the airway inflammation in COPD; however, their effectiveness in COPD remains controversial (29). COPD patients are also at increased risk of side effects over time because of using inhaled corticosteroids. Short-term corticosteroid use may be associated with hypertension, electrolyte abnormalities, neuropsychological effects and pancreatitis. On the other hand, long-term corticosteroid use may be associated with hepatic, gastrointestinal, ophthalmologic effects, pneumonia, tuberculosis, and increased risk of diabetes (30-35). Thus, there is a need for new alternative anti-inflammatory drugs in COPD in terms of complementing inhaled corticosteroids to treat the symptoms with reduced risk of side effects.

1.2. RAGE (Receptor of Advanced Glycation End Products)

1.2.1. Structure

RAGE is a 35 kDa protein member belonging to the immunoglobulin superfamily of cell surface receptors. In 1992, RAGE was discovered as a binding receptor for advanced glycation end products (AGEs), known as non-enzymatic oxidative products derived from either modified lipids, nucleic acids or proteins (36, 37). AGEs are recognized as a marker of oxidative ageing and stress (38). AGEs are mostly formed at a slow rate, but under inflammatory state and oxidative stress, e.g. cigarette smoke, the formation of endogenous AGEs is accelerated (39, 40). RAGE exists in two forms: membrane-bound RAGE (mRAGE) and soluble RAGE (sRAGE) (41). mRAGE is composed of immunoglobulin domains (V1, C1, C2), transmembrane domain, and cytosolic tail, which are the primary requirements for interceding RAGE signaling pathways intracellularly. V1 domain identifies ligands before they interact with it, many ligands bind with V1, while some binds to V1/C1 or V1/C2 (42) (Figure 1.1). On the other hand, sRAGE has only an extracellular domain (43). It is recognised as a decoy receptor, and cannot transduce signals after ligand binding (43).

1.2.2. Expression

RAGE is highly expressed in the lung, whereas it is either not or less expressed in other tissues (44, 45). Studies showed that RAGE is expressed in many cells, including bronchial epithelial cells (46). The expression of RAGE is upregulated on different cell types in pathological inflammatory states such as COPD (47). RAGE expression is also up-regulated by the binding of its own ligands (48)(49)(49)(49)(49)(50). Evidence from clinical studies showed that RAGE expression is increased in patients with COPD (47).

1.2.3. Ligands

RAGE has the ability to bind a variety of endogenous ligands (49, 50). RAGE ligand expression is increased in patients with COPD (47). High mobility group box 1 (HMGB1), S100 proteins, AGEs are the common ligands that bind to RAGE. HMGB1 is identified as a partner binding ligand with RAGE and can take part in developing embryos for outgrowth neurite (51). HMGB1 can be secreted by bronchial epithelial cells (52). S100 proteins are small calcium-binding proteins that can activate RAGE to initiate inflammatory responses in a variety of tissues, and they are highly expressed under basal conditions (53, 54).

1.2.4. Signalling Pathways

RAGE has various functions and plays an essential role in the amplification of cellular inflammatory responses. RAGE is found on the cell surface of numerous cells, and most of its ligands are produced by many cells (55). RAGE ligands such as HMGB-1 activate RAGE, leading to inflammatory gene expression through multiple signaling pathways. With ligand-binding, signals are transduced through the transmembrane domain to cytosolic tail, resulting in the activation of intercellular signaling pathways and transcriptional factors such as nuclear factor kappa B (NF- κ B), and leading to the expression of inflammatory genes (Figure 1.1) (42).

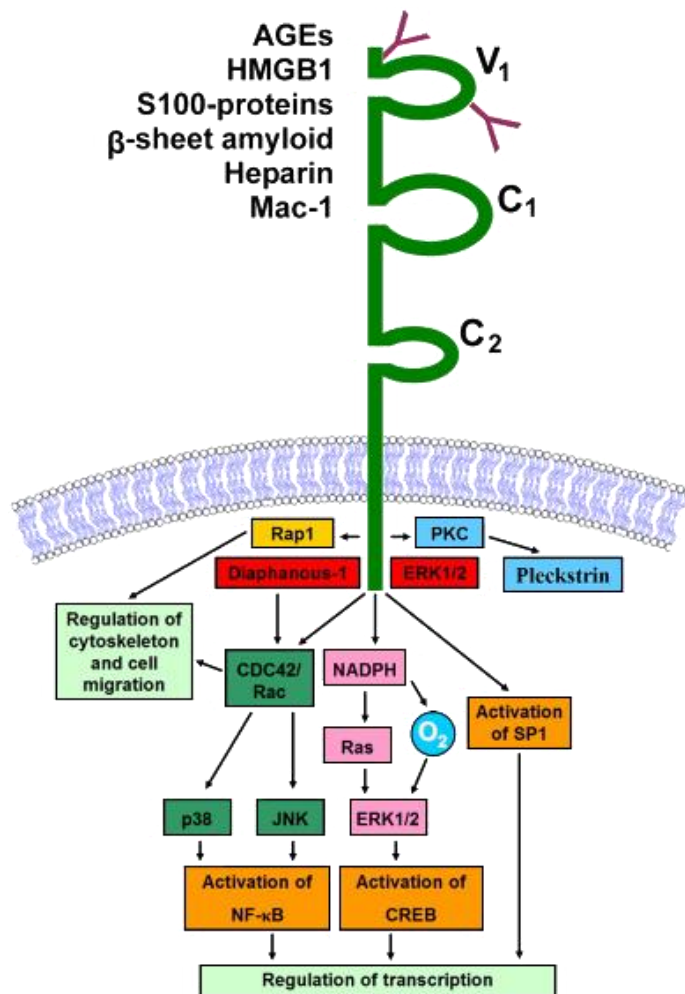


Figure 1.1. Signalling pathways induced by ligand binding to RAGE. RAGE is a multi-ligand receptor consisting of three extracellular domains (V₁, C₁ and C₂), the transmembrane domain and the cytosolic tail required for RAGE-mediated intracellular signalling. The extracellular domains bind RAGE ligands such as HMGB1. Signals are then transduced intracellularly through transmembrane domain and cytosolic tail, leading to the activation of p38, JNK (c-Jun N-terminal kinase) and ERK1/2 mitogen-activated protein kinases, and consequently the expression of inflammatory genes through the transcription factors NF-κB, CREB and SP1. Adopted from Rauvala H, Rouhiainen A. *Biochim Biophys Acta*. 2010 Jan-Feb;1799(1-2):164-70.

1.3. Cigarette Smoke and Airway Inflammation

Cigarette smoke (CS) is an important cause of airway inflammation. Studies showed that CS and CS extract (CSE) induce inflammatory gene expression in different experimental designs.

In vivo human studies showed IL-6, IL-8 and IL-5 levels in blood were higher in patients with COPD caused by tobacco smoke compared with COPD caused by biomass smoke (56).

In vivo animal studies found that CS increased IL-6, IL-8 and IL-1 β in bronchoalveolar lavage fluid (BALF) of mice (57, 58).

In vitro experiments demonstrated that CSE induced IL-5, IL-6 and IP-10 in macrophage cell line isolated from mouse thymus (Ana-1) (59). In addition, it also increased IL-6, IL-8 in human bronchial epithelial cells (BEAS-2B) (58).

There is increasing evidence that RAGE may play a crucial role in inflammation induced by CS. For instance, in human lungs from smokers, RAGE was highly expressed in small airways, bronchiole, alveolar type 1 cells and alveolar macrophages (60). RAGE was not only highly expressed in COPD-smokers alveoli and bronchioles, but also widely expressed in smokers without COPD (61). Furthermore, the expression of IL-6 and IL-1 β induced by CSE in gingival epithelial cells (ca9-22) was shown to be partially mediated by RAGE (62). Moreover, the expression of TNF α and IL-1 β by alveolar macrophages from RAGE knockout mice was significantly reduced compared with wild type controls following

exposure to CSE ex vivo (63). However, whether CSE-induced cytokines/chemokine production is mediated via RAGE in human bronchial epithelial cells has not been explored (63).

1.4. Alternative Anti-inflammatory Drugs

Exploring new alternative anti-inflammatory drugs is important to complement the anti-inflammatory therapy with ICS in COPD. Recent studies have found many potential benefits of using alternative anti-inflammatory drugs, such as simvastatin and cordycepin.

Simvastatin is a lipid-lowering agent from a group of drugs called HMG CoA reductase inhibitors, or “statins”. The main function of Simvastatin is to reduce the level of bad cholesterol that is associated with low-density lipoprotein (LDL) in the blood and increase the level of good cholesterol that is associated with high-density lipoprotein (HDL) (64). However, studies have also reported that simvastatin has anti-inflammatory effects (65). Simvastatin reduced IL-6 production in human umbilical vein endothelial cells (HUVEC) stimulated with phorbol myristate acetate (PMA) (66) and reduced IL-6 in BALF of rats with cecal ligation and puncture, which induced acute lung injury (67). It also increased the ability of roflumilast N-oxide to inhibit CSE-induced epithelial to mesenchymal transition in well-differentiated human bronchial epithelial cells (WD-HBEC) (68). Furthermore, statins were shown to increase the sRAGE levels in human embryonic kidney cells (69), increase serum sRAGE levels in patients with angina pectoris (70) and decrease RAGE expression in human retinal pigment epithelium cells (71). These observations suggest that simvastatin, as a statin, may have an effect on CSE-induced and RAGE-mediated inflammatory

response in bronchial epithelial cells, which has not been investigated so far.

Cordycepin (3'deoxyadenosine) is a purine nucleoside antimetabolite isolated from the caterpillar fungus *Cordyceps militaris*. This fungus is highly prized in Tibetan and Chinese traditional medicine and used for a large number of afflictions, including kidney, heart, and lung conditions' (72). Studies found that cordycepin has anti-inflammatory, anti-hepatic fibrosis and anti-anoxia effects (73, 74). Cordycepin was shown to inhibit IL-6, IL-8, IL-1 β , tumour necrosis factor (TNF α) expression in human mast cells stimulated with PMA and calcium ionophore (75) and to alleviate airway hyperreactivity in a murine model of asthma by attenuating the inflammatory process with reduced intercellular cell adhesion molecule-1, eotaxin, IL-4, IL-5 and IL-13 and decreased eosinophils and macrophages in BALF (76). It was also discovered that *Cordyceps sinensis*, with cordycepin as a main bioactive component, inhibited CSE-induced oxidative stress and senescence in human bronchial epithelial cells (77). The mechanisms of its anti-inflammatory effects are not well understood. One study from our research group showed that cordycepin specifically inhibited the mRNA expression of inflammatory genes (CCL2, CXCL8 and CXCL1) by reducing mRNA polyadenylation and cleavage in human airway smooth muscle cells (72). But studies also revealed that RAGE was upregulated through oxidative stress-dependent activation of p38 and Akt signalling pathways (78) and that cordycepin inhibited p38 and JNK signalling

pathways (79). Therefore, cordycepin may be able to inhibit possible RAGE up-regulation by CSE-induced oxidative stress. However, whether cordycepin has any effect on CSE-induced inflammatory cytokine/chemokine production in human bronchial epithelial cells remains to be determined.

1.5. Summary

COPD is an inflammatory lung disease characterized by chronic airflow obstruction resulted from persistent inflammation and is among the most common causes of death worldwide. CS is the major risk factor for COPD and can induce alteration of many gene expression, contributing to airway inflammation. Inhaled corticosteroids are wide spectrum of anti-inflammatory drugs that can inhibit the inflammation in COPD, resulting in reduced exacerbations. Studies showed that inhaled corticosteroids can have many side effects, and there is a need for new alternative anti-inflammatory drugs to complement inhaled corticosteroids. RAGE play a key role in mediating inflammatory gene expression. Studies demonstrated that RAGE was highly expressed and the production of inflammatory cytokines/chemokines was increased in patients with COPD. But the role of RAGE in mediating CSE-induced cytokine/chemokine production in human bronchial epithelial cells has not been understood. Simvastatin and cordycepin have anti-inflammatory properties with their inhibition of the production of inflammatory cytokines and chemokines. However, their effects on CSE-induced inflammatory cytokine/chemokine production in human bronchial epithelial cells have not been explored.

1.6. Hypothesis and Aims

1.6.1 Hypothesis

We hypothesize that cigarette smoke extract (CSE) can induce inflammatory cytokine/chemokine production in human bronchial epithelial cells and this process is partially mediated by RAGE.

1.6.2 Aims

- To explore the effect of CSE on the production of cytokines and chemokines that have been implicated in the airway inflammation of COPD, including CXCL5 (ENA-78), CCL11 (eotaxin), CXCL1(GRO- α), CXCL10 (IP-10), CXCL8 (IL-8), and IL-6.
- To investigate the role of RAGE in mediating CSE-induced cytokine/chemokine production by using the RAGE specific inhibitor (FPS-ZM1) and analysing the protein expression of RAGE and sRAGE.
- To assess the effect of cordycepin and simvastatin on CSE-induced cytokine/chemokine production and the possible involvement of RAGE.

Chapter 2. Materials and Methods

2.1. Tables of Materials

2.1.1. Drugs

FPS-ZM1	Merck Millipore • Mass: 25 mg • Molecular weight: 327.9 • To make original stock of 40 Molar, dissolved in 1.9061 mL DMSO
Cordycepin	Sigma-Aldrich • Mass: 25 mg • Molecular weight: 251.24 • To make original stock of 40 Molar, dissolved in 2.4877 mL DMSO
Simvastatin	Sigma-Aldrich • Mass: 25 mg • Molecular weight: 418.57 • To make original stock of 40 Molar, dissolved in 1.4932 mL DMSO

2.1.2. ELISA Kits

Human CXCL1/GRO alpha ELISA DuoSet kit	DY275 , R&D SYSTEMS
Human CXCL5/ENA-78 ELISA DuoSet kit	DY254 , R&D SYSTEMS
Human IL-8/CXCL8 ELISA DuoSet kit	DY208 , R&D SYSTEMS
Human IL-6 ELISA DuoSet kit	DY206 , R&D SYSTEMS
Human CCL11/Eotaxin ELISA DuoSet kit	DY320 , R&D SYSTEMS
Human CXCL10/IP-10 ELISA DuoSet kit	DY266 , R&D SYSTEMS
Human RAGE Quantikine ELISA Kit	DRG00 , R&D SYSTEMS

2.1.3. ELISA Buffers and Reagents

Bovine Serum Albumin (BSA)	Thermo Fisher Scientific
Phosphate-buffered saline (PBS)	Sigma-Aldrich
Tris-buffered saline (TBS)	• 20 mM trizma base • 150 mM NaCl
Tween 20	Sigma-Aldrich
Wash buffer	• 0.05% Tween 20 in PBS • pH 7.2-7.4
Block buffer (also serves as Reagent diluent-1 for IL-6, CCL11, CXCL10, CXCL5, and CXCL1)	• 1% BSA in PBS • pH 7.2-7.4 • Filtered by 0.2 µm pore size syringe filter

Reagent diluent-2 For IL-8	0.1% BSA - TBS 0.05% Tween 20 - pH 7.2-7.4 Filtered by 0.2 µm pore size syringe filter
Substrate solution	1:1 mixture of Color Reagent A(H ₂ O ₂) and Color Reagent B (Tetramethylbenzidine)

2.1.4. Materials for Cell Culture and MTT Cell

Viability Assay

Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich
Amphotericin B	Sigma-Aldrich
L-glutamine	Sigma-Aldrich
Penicillin/Streptomycin	Sigma-Aldrich
Foetal Bovine Serum (FBS)	Thermo Fisher Scientific
Trypsin-EDTA	Sigma-Aldrich
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich
Thiazolyl Blue Tetrazolium Bromide	Sigma-Aldrich

2.1.5. Antibodies for Western Blotting

RAGE (A-9): Mouse anti-human monoclonal antibody	Santa Cruz biotechnology
HMG-1 (alternative name for HMGB-1)(J2E1): Mouse anti-human monoclonal antibody	Santa Cruz biotechnology
GAPDH: Mouse anti-human monoclonal antibody	Santa Cruz biotechnology
Rabbit anti-mouse IgG/HRP secondary antibody	Dako Group

2.1.6. Materials for Western Blot

Hyperfilm ECL	Amersham BioSciences
Polyvinylidene difluoride (PVDF) membrane	Bio-Rad Laboratories

2.1.7. Western Blot Buffers and Reagents

	Buffer Contents
RIPA buffer	• 50 mM tris-HCL, pH 8.0 • 0.5% sodium deoxycholate • 150 mM sodium chloride • 0.1% sodium dodecyl sulphate (SDS) Once the RIPA buffer is ready the following additives are added just before use: • 2 mM phenylmethylsulfonyl fluoride • 1 mM sodium orthovanadate • 1 mM protease inhibitor cocktail

10x TBST buffer	• 0.2 M tris base • 87.6 g NaCl (Sodium chloride) • 10 mL tween 20 • dH₂O was added to make final volume of 1000 mL • pH to be between 7.4 - 7.6 • To make 1X TBST: 100 mL of 10X TBST + 900 mL dH₂O
Blocking buffer	1X TBST + 5% non-fat dry milk (Santa Cruz Biotechnology)
NuPAGE™ 2-(N-morpholino) ethanesulfonic acid (MES) SDS Running Buffer (20X) For (Pre casted Bis- Tris gels)	Thermo Fisher Scientific • 50 mM tris base • 0.1% SDS • 1 mM EDTA • pH 7.3 To make 1X: 50 mL MES buffer + 950 mL dH ₂ O
Pre-casted gels: Bolt™ 10% Bis-Tris plus gels 10 wells (For Mini Gel Tank)	Thermo Fisher Scientific • Separation range 3.5 to 160 kDa with MES buffer • Capacity up to 60 µL per well
10x transfer buffer	• 1.91 M glycine • 0.2 M tris base To make 1X transfer buffer: 200 mL of 10x transfer buffer + 400 mL menthanol + 1400 mL dH ₂ O
4x lamellae buffer	• 62.5 mM tris HCL pH 6.8 (In 100 mL of dH₂O) • 7 mL dH₂O • 4 mL 100% glycerol • 0.01% bromophenol blue • 1% SDS
Rainbow molecular weight marker	Amersham BioSciences

ECL™ Western blotting detection reagent	Amersham BioSciences.
Carestream® Kodak® autoradiography GBX developer/replenisher	Thermo Fisher Scientific Diluted in water ratio of 1:5
Carestream® Kodak® autoradiography GBX fixer/replenisher	Thermo Fisher Scientific Diluted in water ratio of 1:5

2.2. CSE Preparation

Fresh aqueous CSE was used for all experiments. CSE was generated from 3R4F research-grade cigarettes (Kentucky Tobacco Research and Development Centre, University of Kentucky, USA). Smoke from each cigarette contains 9.4 mg tar, 0.7 mg nicotine and 12.0 mg carbon monoxide. CSE was obtained as described before (80) by bubbling the smoke (air for control) from two cigarettes (one cigarette/min) into 80 mL glass bottle containing 20 mL of medium. After that, the medium was filtered by using 0.2 μ m pore filters and pH was adjusted to 7.4. To ensure the desired concentration and reproducibility, the absorbance (optical density) set at 320 nm with a spectrophotometer (CE 7500, Cecil Instruments) was used to measure CSE strength (1.5=100%). After the preparation, CSE was used immediately. Three CSE concentrations (1%, 2.5%, and 5%) were first used. However, 5% CSE was later excluded because of cell toxicity shown on MTT cell viability assay and 2.5% CSE was used for the rest of the experiments.

2.3. Drug Preparation

Following the manufactures' instructions, FPS-ZM1 (a RAGE specific inhibitor), cordycepin, and simvastatin original stocks were dissolved in DMSO (refer to section 2.1.1 for details). Three final concentrations of each drug were used in the experiments: 0.5 mM, 1.0 mM, and 1.5 mM for FPS-ZM1, 5 μ M, 10 μ M, and 25 μ M for cordycepin,

and 0.625 μ M, 1.25 μ M, and 2.5 μ M for simvastatin. To minimise the effect of DMSO on cells, 2 μ L of the drug solution (DMSO only for control) were added to 1 mL medium with a final DMSO concentration of 0.2%.

2.4. Cell Culture

BEAS-2B human bronchial epithelial cells were provided by Professor Ian Sayers's group (Division of Respiratory Medicine, University of Nottingham, UK). The cells were originally obtained from autopsy of non-cancerous subjects and were immortalized by infection with a replication-defective SV40 adenovirus 12 hybrid (81). The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FBS, 0.02% antibiotics (penicillin/streptomycin solution), 0.02% L-glutamine and 0.01% amphotericin B, at 37°C, with 21% O₂ and 5% CO₂, in a humidified incubator. The medium was changed every 48 hours until the cells were close to 100% confluence. The cells were routinely checked for bacterial contaminations under the microscope and for mycoplasma infection by MycoAlert™ mycoplasma detection kit (Lonza).

2.4.1. Cell Sub-Culture

Cells were received at passage 14 and seeded in T-225 flasks. The medium was changed every two days. After reaching confluence, cells were washed with 10 mL of PBS and then incubated with 10 mL of

trypsin-EDTA solution at 37°C for 3 minutes. Cell detachment from the flask was verified under the microscope. Following detachment, 10 mL of medium was added to neutralise trypsin, and the cells were collected, centrifuged for at 1100 rpm 5 min and resuspended in 10 mL of fresh medium. The cells were counted then prepared for either seeding the next passage or freezing (in FBS + 10% DMSO) at 1×10^6 cells per cryogenic tube (1.0 mL). The tubes were then placed in Mr. Frosty Freezing Container (Thermo Fisher Scientific), which contains 100% of isopropyl alcohol. After that, Mr. Frosty was placed in -80°C freezer overnight to achieve a rate of cooling very close to -1°C/minute, the optimal rate for cell preservation. The frozen cells were then transferred to liquid nitrogen for storage.

2.4.2. Cell Counting

Cells were counted using a haemocytometer. A 10 µL droplet of the cells suspension was placed onto the counting grid. Cell numbers were determined in each of the four haemocytometer squares, and the average was taken and multiplied by the dilution factor and 10^6 .

2.4.3. Cell Treatment

The cells were used at passage 15-18. The cells were cultured in 12-well plates at a density of 0.083×10^6 cells per well in DMEM which was changed every 48 hours. After reaching confluence on the fifth day, cells were growth-arrested with fresh low serum (0.5% FBS) medium (DMEM + 0.5% FBS and other supplements) for 24 hours. Cells were

then treated with CSE in fresh low serum medium and incubated for 24 hours. To test the effect of drugs (FPS-ZM1, cordycepin, and simvastatin), cells were pre-treated with the drugs for 2 hours before being incubated with CSE for 24 hours. At the completion of the experiments, medium was collected for analysis of cytokine/chemokine concentration and total cell lysate was collected for analysis of protein expression by Western blotting.

2.5. Protein Expression Analysis

2.5.1. Western Blot Analysis

The concept of this analytical method is to detect specific proteins in cell lysates by isolating the proteins based on their molecular weight as they run through a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was used to assess expression levels of RAGE and HMGB-1.

2.5.2. Cell Lysate Preparation

After cell treatment, culture medium was removed, and PBS was used to wash the cells. After that, 75 μ L of radioimmunoprecipitation assay (RIPA) buffer (a lysis buffer) and other supplements (see section 2.1.7) was added to each well. Cell scrapers were used to collect the lysates. The cell lysates were then centrifuged at 13000 rpm and 4°C for 10 min. A BCA assay kit was used to quantify the protein concentration

in accordance with the manufacturer's instructions (see section 2.2.1). Samples were then diluted (1:3) in 4x lamellae buffer and boiled for 10 minutes to denature the protein before loading.

2.5.3. Bicinchoninic Acid Protein Assay

A bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) was used to measure the total protein concentration in a solution. In a 96-well plate, 5 μ L of sample was added in duplicate along with bovine serum albumin (BSA) standards (0 μ g/mL-2000 μ g/mL dilution range), which were prepared according to the manufacturer's instructions. Next, BCA reagent A:B (1:50) was prepared, and 95 μ L was added to each well and then incubated at 37°C for 30 minutes. Following incubation, a FLUOstar Omega micro-plate reader (BMG LABTECH) was used to measure the absorbance at 562 nm. Protein concentration was determined relative to the standards and calculated as μ g/mL using Omega analysis software.

2.5.4. SDS-PAGE

Following the manufacturer's instructions, pre-cast gels (Bolt™ 10-well, 10% Bis-Tris Plus Gels; see section 2.1.7 for details) were used and placed in the Mini Gel Tank (Thermo Fisher Scientific) and then soaked with 1x running buffer diluted in dH₂O (NuPAGE™ MES SDS Running

Buffer 20x; see section 2.1.7). Next, 10 µL of a rainbow molecular weight marker and 25 µg of protein samples were loaded onto the gels. Finally, the electrophoresis was conducted at 200 V constant voltage for 35 minutes.

2.5.5. Protein Transfer

Following electrophoresis, immune-blot PVDF membrane was prepared by soaking in 100% methanol for 30 seconds. A sandwich of filter papers, sponges, PVDF membrane and gels was then soaked in 1x transfer buffer (see section 2.1.7) and placed between positive and negative electrodes. Transfer buffer was added to the electrophoretic transfer apparatus (Bio-Rad, UK) as recommended, and then run for 40 minutes at 150 V constant voltage.

2.5.6. Blocking and Antibody Incubation

After the transfer process finished, the PVDF membrane was washed in 1x TBST for 2x 5 minutes (see section 2.1.7). Membranes were blocked in blocking buffer (5% non-fat milk diluted in TBST) at room temperature for 1 hour to prevent non-specific binding of antibodies. Following blocking, PVDF membranes were incubated with primary antibodies in blocking buffer at 4°C overnight. The titrated primary antibodies used were: mouse monoclonal IgG1 anti-human RAGE (1:1000), and mouse monoclonal IgG2b anti-human HMG-1 (1:200). The following day, the PVDF membranes were washed in 1x TBST for 3x 5 minutes and then incubated with the secondary antibody in blocking

buffer at room temperature for 1 hour. The titrated secondary antibody used was polyclonal HRP-rabbit anti-mouse IgG (1:2000).

2.5.7. Protein Detection

Following incubation with antibodies, the PVDF membranes were washed in TBST for 3x 5 minutes and then incubated in enhanced chemiluminescence (ECL) reagent for 1 minute. PVDF membranes were then exposed to hyperfilm ECL for 2 seconds to 15 minutes. Following ECL exposure, the films were developed manually by soaking in GBX developer/replenisher reagents. Films were rinsed with water and then placed in GBX fixer/replenisher reagents to make the films light resistant and permanent. Finally, the films were dried and placed against the molecular weight ladder to confirm the detected protein size was correct.

To detect the loading control GAPDH, the membranes were subsequently washed in 1x TBST for 3x 5 minutes and then incubated in blocking buffer with anti-GAPDH mouse monoclonal IgG1 (1:10000) at room temperature for 1 hour. Once the incubation was complete, the membranes were washed for 3x 5 minutes and then incubated in blocking buffer with polyclonal HRP-rabbit anti-mouse IgG (1:2000). The membranes were then developed in the darkroom as described above.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs were used to quantify sRAGE, IL-8, IL-6, CXCL1, CXCL5, CXCL10, and CCL11 levels in cell culture medium samples. DuoSet ELISA Development Systems (R&D systems) were used for analysis of cytokines/chemokines with the reagents and buffers prepared according to the manufacturer's instructions (see section 2.1.3 for details). Human RAGE Quantikine ELISA kit (R&D systems) was used for sRAGE detection.

2.6.1. Plate Preparation

To prepare plates from the DuoSet kits, capture antibodies were diluted to working concentrations in PBS, and then 100 μ L of antibody solution was added to each well of a 96-well microplate. The plates were then sealed and incubated at room temperature overnight.

2.6.2. Assay Procedure

After removal of antibody solution, 400 μ L of wash buffer was used to wash each well 3 times, and the plates were inverted and blotted on paper towels. Following washing, 300 μ L of reagent diluent was added to each well for blocking and incubated at room temperature for 1 hour, and then the washing process was repeated. Next, 100 μ L of diluted standards and samples (all in duplicate) was added to individual wells and incubated at room temperature for 2 hours, followed by the washing

process. Subsequently, 100 μ L of diluted biotinylated goat anti-human detection antibody was added to each well and incubated at room temperature for 2 hours, and then the washing process was repeated. After that, 100 μ L of diluted Streptavidin-HRP was added to each well and incubated in a dark cupboard (to avoid light) at room temperature for 20 minutes, with subsequent washing. Following that, 100 μ L of a 1:1 mixture of A and B substrate solution (see section 2.1.3) was added to each well and incubated in a dark cupboard (to avoid light) at room temperature for 20 minutes, and then 50 μ L of stop solution was added to each well followed with a gentle tap to ensure thorough mixing. A FLUOstar Omega micro-plate reader (BMG LABTECH, UK) was used immediately to determine the optical density of each well at 450 nm, and Omega software used to analyse the data. All data were normalised to total protein of each sample and presented as pg/ μ g protein.

2.7. Cell Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the cytotoxicity of (A) CSE and (B) FPS-ZM1 to cells. After the cells were treated with the respective chemicals for 24 hours, the medium was removed and replaced with 250 μ L 1 mg/mL MTT solution (see section 2.1.4) in warm, serum-free medium, and the cells were then incubated at 37°C for 20 minutes. After incubation, the medium was removed, and the plates were left to dry

overnight. The following day, 250 μ L of DMSO was added to each well and mixed thoroughly using the pipette, and then transferred to a new 96-well plate to measure absorbance at 550 nm using a FLUOstar Omega microplate reader (BMG LABTECH, UK). Viability data were presented relative to the control group at 100%.

2.8. Statistical Analysis

Data were analysed using GraphPad Prism software (version 6). Independent t-test was used to determine the significant mean differences between untreated and treated cells; p value <0.05 was considered as statistically significant.

Chapter 3. Results

3.1 Effect of CSE on Cytokine/Chemokine Production

To investigate whether CSE has an effect on CXCL5, CCL11, CXCL1, CXCL10, IL-8, and IL-6 production, confluent and growth-arrested BEAS-2B cells were treated with CSE (1%, 2.5%, and 5%) in fresh low serum medium for 24 hours. The medium was collected and the concentration of the above cytokines/chemokines was measured by ELISA. We found that CXCL5, CCL11, CXCL1, and CXCL10 were undetectable in the medium from unstimulated cells and that these chemokines were still undetectable after CSE treatment (Figure 3.1). Raw microplate spectrophotometer data of the standards and samples from ELISA were shown in Figure 3.1. In contrast, IL-8 and IL-6 were detectable in unstimulated cells and treatment with CSE increased the production of both IL-8 and IL-6 in a concentration-dependent manner; significant increase was observed with 2.5% CSE for both ($P < 0.01$) and further increase was achieved with 5% CSE for IL-8 ($P < 0.0001$) and IL-6 ($P < 0.001$) (Figure 3.2). The data suggest that CSE can induce IL-8 and IL-6 production from BEAS-2B cells but has no effect on the production of CXCL5, CCL11, CXCL1, and CXCL10.

A		1	2	3	4	5	6	7	8	9	10	11	12
STANDARDS	A	1200.842	389.673	271.144	120.285	60.413	34.59	12.116		<< Y range	<< Y range	<< Y range	<< Y range
	B	>> Y range	419.438	273.804	123.355	64.856	33.302	11.219		<< Y range	<< Y range	<< Y range	<< Y range
SAMPLES	C	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	D	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	E	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	F	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	G	73.01	73.232	61.647	62.842	57.801	62.941	64.836	49.018	<< Y range	<< Y range	<< Y range	<< Y range
	H	71.844	76.069	72.775	62.373	61.568	71.991	63.654	52.837	<< Y range	<< Y range	<< Y range	<< Y range
B		1	2	3	4	5	6	7	8	9	10	11	12
STANDARDS	A	987.104	567.597	286.194	111.558	42.419	7.042	-5.02					
	B	925.064	576.311	306.913	114.444	46.286	8.138	-5.251					
SAMPLES	C	-17.024	-19.621	-20.14	-19.967	-20.429	-19.736	-14.946	-19.621	-17.082	-19.852	-20.314	-26.604
	D	-17.024	-20.775	-18.64	-18.12	-20.891	-21.122	-18.524	-20.891	-19.506	-22.103	-20.602	-27.008
	E	-2.365	-15.754	-20.314	-21.179	-21.641	-19.448	-23.141	-21.237	-23.141	-22.968	-22.16	-35.953
	F	32.262	-20.775	-19.448	-20.718	-20.833	-22.911	-21.237	-21.064	-21.295	-23.026	-22.911	-16.62
	G	109.365	94.302	16.218	10.158	-8.713	-6.52	-14.946	-16.678	-23.661	-21.872	-23.141	-30.24
	H	-6.405	17.834	2.367	-20.66	-26.604	-25.104	-26.2	-23.892	-27.008	-25.508	-25.738	-30.875
C		1	2	3	4	5	6	7	8	9	10	11	12
STANDARDS	A	1976.199	1079.864	568.828	239.11	82.526	28.113	10.305					
	B	1903.528	1111.162	569.458	247.655	82.256	26.314	12.283					
SAMPLES	C	-7.054	-8.493	-8.493	-9.032	-7.953	-8.403	-10.382	-11.461	-12.18	-10.921	-7.324	-8.403
	D	-8.493	-9.122	-12.63	-12.45	-13.709	-12.09	-13.439	-13.17	-11.461	-12.81	-9.212	-9.482
	E	-10.741	-12.81	-9.032	-12.81	-17.397	-13.799	-13.439	-7.683	-14.519	-12.09	-12.99	-5.345
	F	-9.302	-11.101	-7.503	-15.148	-19.735	-16.318	15.881	-14.429	-13.709	-13.439	-11.371	-11.371
D		1	2	3	4	5	6	7	8	9	10	11	12
STANDARDS	A	1855.483	1024.772	475.491	269.132	119.245	51.681	24.041					
	B	2148.801	981.859	500.259	267.192	126.611	63.916	21.585					
SAMPLES	C	<< Y range	<< Y range	<< Y range	47.763	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	D	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	E	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	F	<< Y range	<< Y range	<< Y range	49.084	42.306	21.585	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	G	<< Y range	<< Y range	<< Y range	std range	54.223	std range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	H	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range

Figure 3.1. Effect of CSE on cytokine/chemokine production.

Confluent and growth-arrested BEAS-2B cells were treated with CSE (1%, 2.5%, and 5%) for 24 hours. The medium was collected to measure the cytokine/chemokine by ELISA. (A) CXCL5, (B) CCL11, (C) CXCL1, and (D) CXCL10. Raw absorbance data for standards and samples were presented and highlighted differently. The standards were ranged 15.6-1000 pg/mL for CXCL5, 15.6-1000 pg/mL CCL11, 31.3-2000 pg/mL for CXCL1, and 31.3-2000 pg/mL for CXCL10.

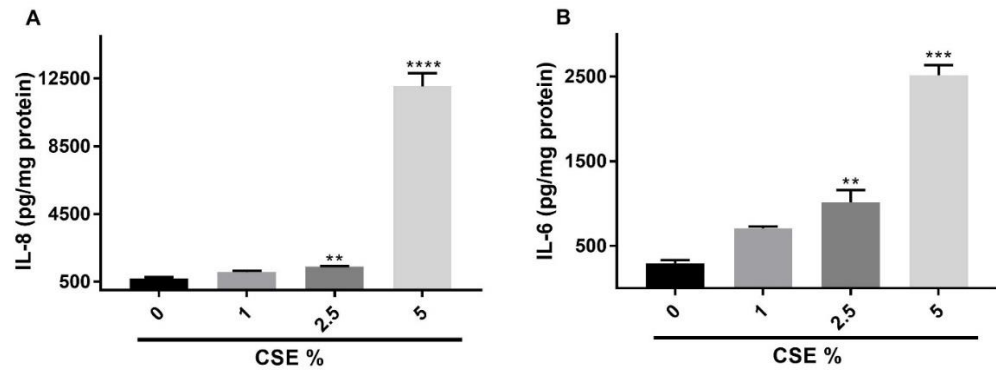


Figure 3.2. Effect of CSE on cytokine/chemokine release. Confluent and growth-arrested BEAS-2B cells were treated with CSE (1%, 2.5%, and 5%) for 24 hours. The medium was collected to measure IL-8 (A) and IL-6 (B) by ELISA. Data were normalised to total protein. Each data point represents mean $\pm$ SEM of three experiments carried out in duplicate. **P < 0.01, ***P < 0.001, and **** P < 0.0001 versus control.

3.2 Effect of CSE on sRAGE Secretion and RAGE Expression

Experiments were then conducted to explore the effect of CSE on sRAGE secretion and RAGE expression. Confluent and growth-arrested BEAS-2B cells were treated with CSE (1%, 2.5%, and 5%) in a fresh low serum medium for 24 hours. The medium was collected to measure sRAGE level using ELISA. We found that sRAGE could not be detected in both untreated and CSE-treated cells (Figure 3.3). The total cell lysate was collected to assess RAGE expression by Western blotting. Interestingly, RAGE was highly expressed in untreated cells and CSE treatment did not further increase its expression (Figure 3.4). The results suggest that CSE has no effect on sRAGE secretion and RAGE expression in BEAS-2B cells.

		1	2	3	4	5	6	7	8	9	10	11	12
STANDARDS	A	4919.277	2651.077	1076.283	641.024	341.035	164.947	39.079					
	B	4884.731	2601.099	1253.457	657.188	345.241	155.906	46.158					
	C	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
SAMPLES	D	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	E	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	F	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	G	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	H												

Figure 3.3. Effect of CSE on sRAGE secretion. Confluent and growth-arrested BEAS-2B cells were treated with CSE (1%, 2.5%, and 5%) for 24 hours. The medium was collected to measure sRAGE secretion using ELISA. Raw absorbance data for standards and samples were presented and highlighted differently. The standards were ranged 78-5000 pg/mL and the sensitivity for the kit is 16.14 pg/mL.

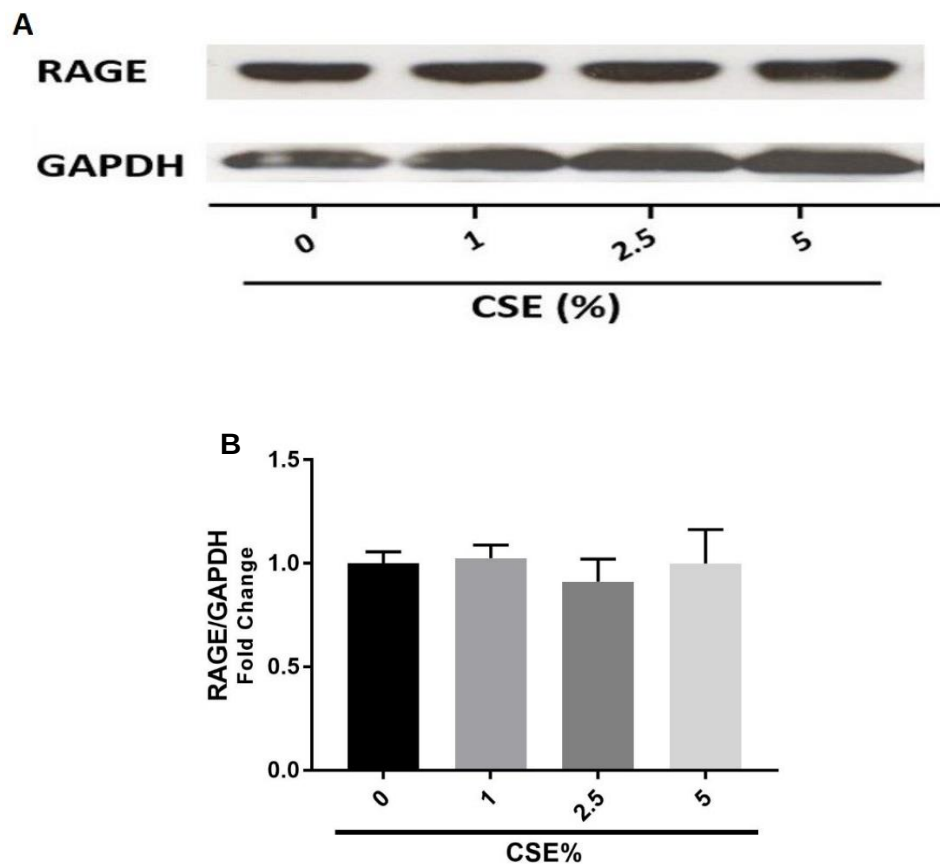


Figure 3. 4. Effect of CSE on RAGE expression. Confluent and growth-arrested BEAS-2B cells were treated with CSE (1%, 2.5%, and 5%) for 24 hours. Total cell lysate was collected, and the expression of RAGE was analysed by Western blotting. (A) A representative of results obtained from three experiments. (B) Optical densitometry analysis of Western blotting bands for (A). Data were normalized with the loading control GAPDH and fold change over control cells was calculated. Each data point represents mean $\pm$ SEM of three experiments.

3.3 Effect of FPS-ZM1 on CSE-Induced IL-8 and IL-6 Production

To prove our hypothesis, experiments were conducted to explore the role of RAGE in mediating CSE-induced IL-8 and IL6 production in BEAS-2B cells by using the RAGE specific inhibitor FPS-ZM1. Confluent and growth-arrested cells were pre-treated with FPS-ZM1 (0.5 mM, 1.0 mM, and 1.5 mM) in fresh low serum medium for 2 hours; following that, CSE (2.5%) was added and the cells were further incubated for 24 hours. 2.5% CSE was chosen because it was not toxic to the cells but 5% CSE was (Figure 3.11). The medium was then collected to measure IL-8 and IL-6 by ELISA. As shown in Figure 3.5, CSE (2.5%) significantly increased IL-8 ($P < 0.05$) and IL-6 ($P < 0.01$) production as compared with control cells, consistent with the findings described in section 3.1. This increase was partially inhibited by FPS-ZM1 in a concentration-dependent manner, with significant inhibition observed for both ($P < 0.05$) at the highest concentration tested (1.5 mM) (Figure 3.5). The findings suggest that RAGE may play a partial role in mediating CSE-induced IL-8 and IL-6 production in BEAS-2B cells.

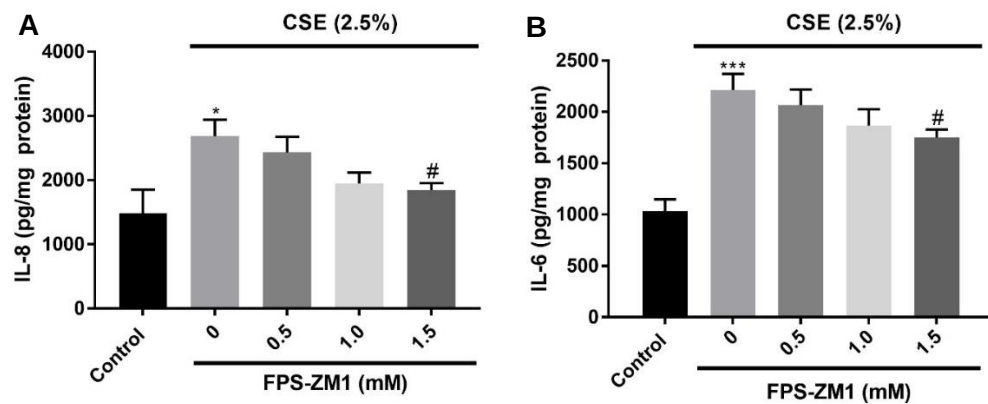


Figure 3.5. Effect of FPS-ZM1 on CSE-induced IL-8 and IL-6 production. Confluent and growth-arrested BEAS-2B cells were pre-treated with FPS-ZM1 (0.5 mM, 1.0 mM, and 1.5 mM) for 2 hours prior to incubation with CSE (2.5%) for 24 hours. The medium was collected to measure IL-8 (A) and IL-6 (B) by ELISA. Data were normalised to total protein. Each data point represents mean $\pm$ SEM of three experiments carried out in duplicate. * $P < 0.05$ and *** $P < 0.001$ versus control, and # $P < 0.05$ versus CSE alone.

3.4 Effect of FPS-ZM1 on RAGE Expression

To investigate the effect of FPS-ZM1 on RAGE expression, total cell lysate was also collected from the same experiment described in section 3.3 and the expression of RAGE was analysed by Western blotting. We found that RAGE was highly expressed in untreated cells as demonstrated earlier in Figure 3.4 and that treatment with CSE (2.5%) and CSE + FPS-ZM1 did not alter its expression (Figure 3.6). The results suggest that FPS-ZM1 has no effect on RAGE expression in BEAS-2B cells.

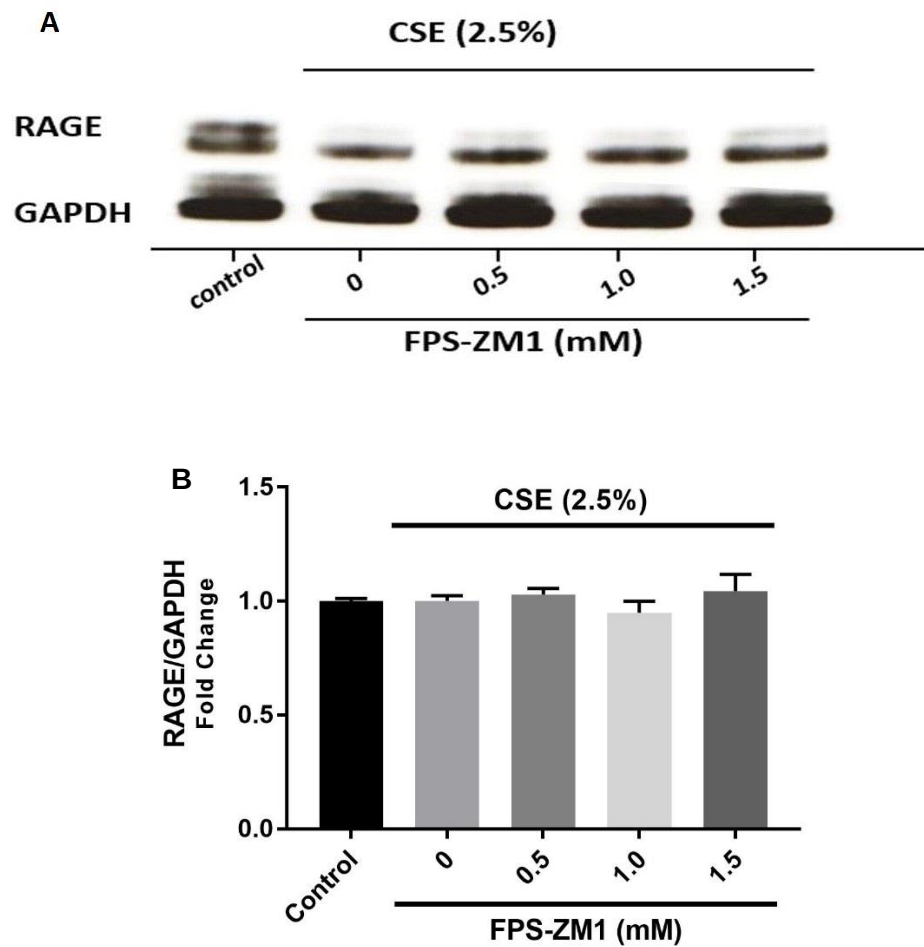


Figure 3.6. Effect of FPS-ZM1 on RAGE expression. Confluent and growth-arrested BEAS-2B cells were pre-treated with FPS-ZM1 (0.5 mM, 1.0 mM, and 1.5 mM) for 2 hours prior to incubation with CSE (2.5%) for 24 hours. Total cell lysate was collected, and the expression of RAGE was analysed by Western blotting. (A) A representative of results obtained from three experiments. (B) Optical densitometry analysis of Western blotting bands for (A). Data were normalized with the loading control GAPDH. Each data point represents mean $\pm$ SEM of three experiments.

3.5 Effect of Cordycepin on CSE-Induced IL-8 and IL-6 Production

To explore the effect of cordycepin on CSE-induced IL-8 and IL-6 production, confluent and growth-arrested BEAS-2B cells were pre-treated with cordycepin (5 μ M, 10 μ M, and 25 μ M) in fresh low serum medium for 2 hours; following that, CSE (2.5%) was added and the cells were further incubated for 24 hours. After the incubation, the medium was collected measure IL-8 and IL-6 by ELISA. As shown in Figure 3.7, IL-8 and IL-6 production was significantly increased by CSE ($P < 0.05$). Treatment with cordycepin partially inhibited CSE-induced IL-8 production in a concentration-dependent manner, with significant inhibition ($P < 0.05$ and $P < 0.001$) achieved at three concentrations (5 μ M , 10 μ M, and 25 μ M) tested (Figure 3.7 A). Surprisingly, cordycepin significantly enhanced CSE-induced IL-6 production at all concentrations tested and in a concentration-dependent manner (Figure 3.7 B). The findings suggest that cordycepin can partially inhibit CSE-induced IL-8 production but enhances CSE-induced IL-6 production in BEAS-2B cells.

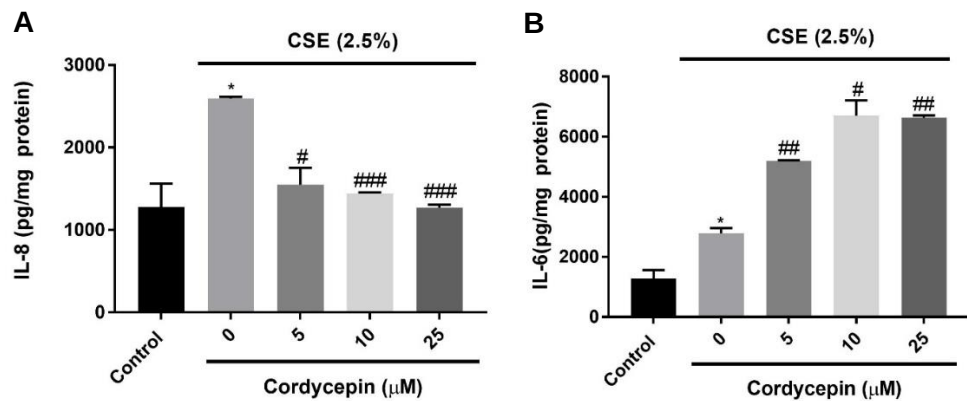


Figure 3.7. Effect of cordycepin on CSE-induced IL-8 and IL-6 production. Confluent and growth-arrested BEAS-2B cells were pre-treated with cordycepin (5 μM, 10 μM, and 25 μM) for 2 hours prior to incubation with CSE (2.5%) for 24 hours. The medium was collected to measure IL-8 (A) and IL-6 (B) by ELISA. Data were normalised to total protein. Each data point represents mean $\pm$ SEM of three experiments carried out in duplicate. *P < 0.05 versus control, and #P < 0.05, #P < 0.01, and ###P < 0.001 versus CSE alone.

3.6 Effect of Cordycepin on the Expression of RAGE and its Ligand HMGB-1

To investigate whether cordycepin would have any effect on the expression of RAGE and HMGB-1, total cell lysate was also collected from the same experiment described in section 3.5 and the expression of RAGE and HMGB-1 was assessed by Western blotting. We found that RAGE was constitutively expressed in untreated cells as shown before and that treatment with CSE and CSE + cordycepin did not affect RAGE expression (Figure 3.8A). Similarly, HMGB-1 was basally expressed in untreated cells and CSE and CSE + cordycepin did not affect HMGB-1 expression (Figure 3.8 B). Our findings suggest that CSE and cordycepin have no effect on RAGE and HMGB-1 expression in BEAS-2B cells and that the inhibition of CSE-induced IL-8 production by cordycepin is unlikely to be mediated by RAGE.

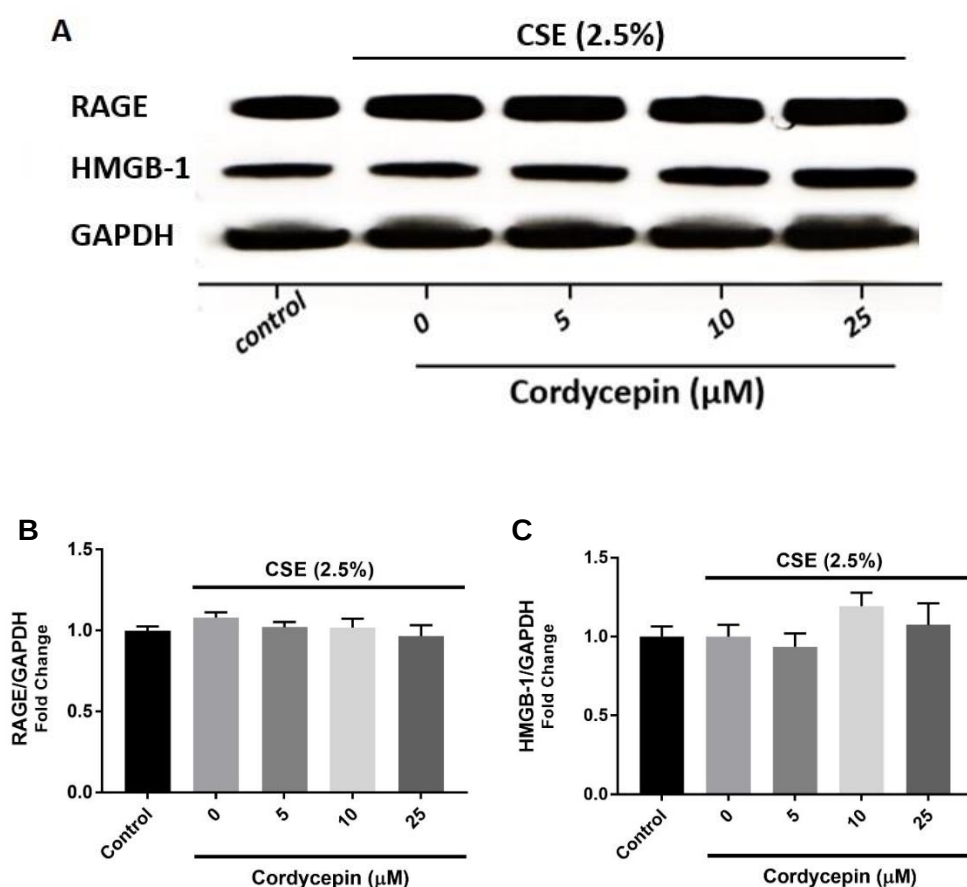


Figure 3.8. Effect of cordycepin on RAGE and HMGB-1 expression.

Confluent and growth-arrested BEAS-2B cells were pre-treated with cordycepin (5 μM , 10 μM , 25 μM) for 2 hours prior to incubation with CSE for 24 hours. Total cell lysate was collected and the expression of RAGE and HMGB-1 (A) was analysed by Western blotting. (A) A representative of results obtained from three experiments. (B and C) Optical densitometry analysis of Western blotting bands for RAGE and HMGB-1. Data were normalized with the loading control GAPDH. Each data point represents mean $\pm$ SEM of three experiments.

3.7 Effect of Simvastatin on CSE-Induced IL-8 and IL-6 Production

To study the effect of simvastatin on CSE-induced IL8 and IL-6 production, Confluent and growth-arrested BEAS-2B cells were pre-treated with simvastatin (0.625 μ M, 1.25 μ M, and 2.5 μ M) in fresh low serum medium for 2 hours; following that, CSE (2.5%) was added and the cells were incubated for a further 24 hours. After that, medium was collected to measure IL-8 and IL-6 by ELISA. As shown previously, CSE significantly increased IL-8 and IL-6 production ($P < 0.05$) (Figure 3.9). Treatment with simvastatin partially inhibited CSE-induced IL-8 production in a concentration-dependent manner with significant effect observed at two concentrations (1.25 μ M and 2.5 μ M) tested ($P < 0.05$) (Figure 3.9A). In contrast, no effect of simvastatin on CSE-induced IL-6 production was observed at all three concentrations tested (Figure 3.9B). The results suggest that simvastatin can partially inhibit CSE-induced IL-8 production but has no effect on CSE-induced IL-6 production in BEAS-2B cells.

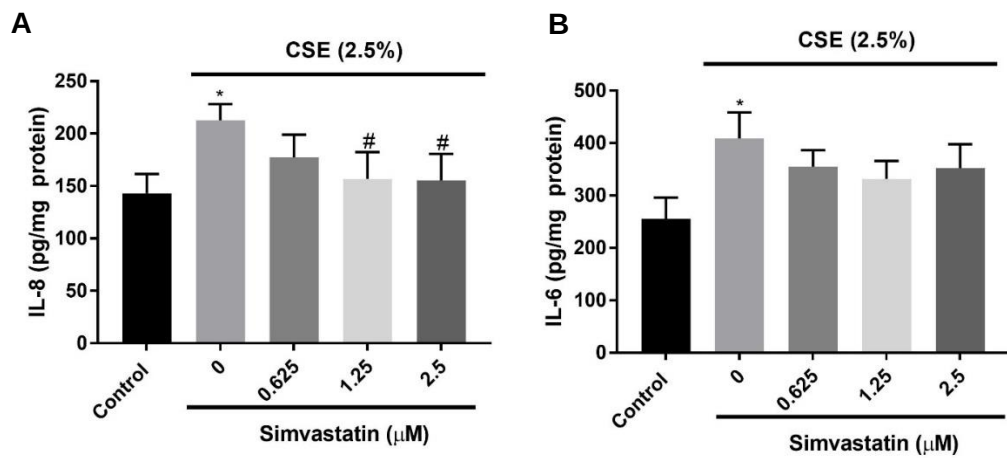


Figure 3.9. Effect of simvastatin on CSE-induced IL-8 and IL-6 production. Confluent and growth-arrested BEAS-2B cells were pre-treated with simvastatin (0.625 μM, 1.25 μM, and 2.5 μM) for 2 hours prior to incubation with CSE (2.5%) for 24 hours. The medium was collected to measure IL-8 (A) and IL-6 (B) by ELISA. Data were normalised to total protein. Each data point represents mean $\pm$ SEM of three experiments carried out in duplicate. *P < 0.05 versus control, and #P < 0.05 versus CSE alone.

3.8 Effect of Simvastatin on RAGE Expression

To explore the effect of simvastatin on RAGE expression, total cell lysate was also collected from the same experiment described in section 3.7 and the expression of RAGE was analysed by Western blotting. As shown in Figure 3.10, treatment with CSE and CSE + simvastatin did not show any effect on the constitutive expression of RAGE. The results suggest that simvastatin has no effect on RAGE expression in BEAS-2B cells and that the inhibition of CSE-induced IL-8 production by simvastatin is unlikely to be mediated by RAGE.

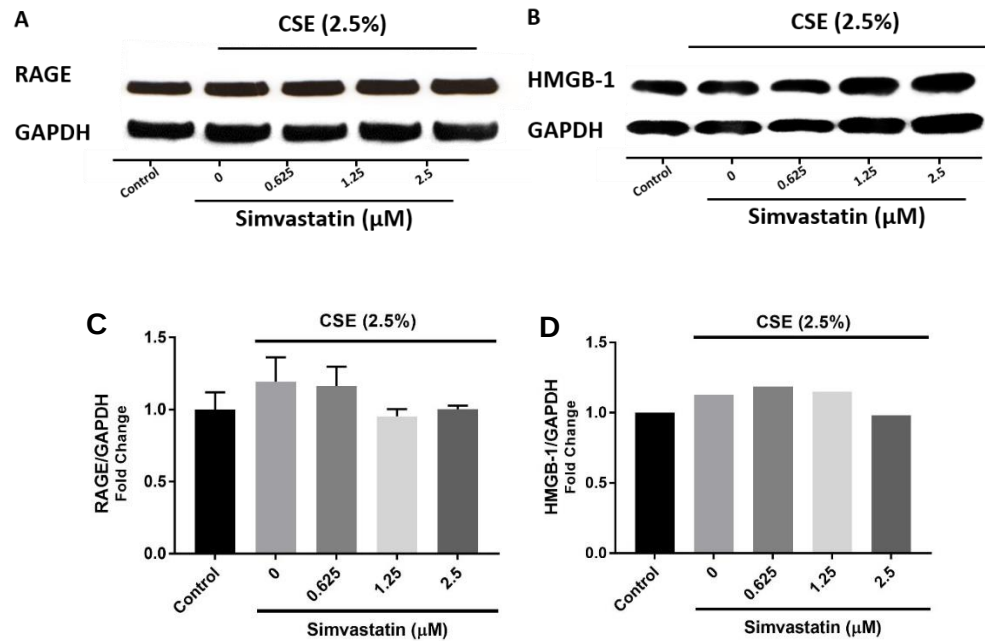


Figure 3.10. Effect of simvastatin on RAGE and HMGB-1 expression.

Confluent and growth-arrested BEAS-2B cells were pre-treated with simvastatin (0.625 μM, 1.25 μM, and 2.5 μM) for 2 hours prior to incubation with CSE for 24 hours. Total cell lysate was collected, and the expression of RAGE was analysed by Western blotting. (A) A representative of results obtained from three experiments and (B) results from one experiment. (C and D) Optical densitometry analysis of Western blotting bands for (A) and (B), respectively. Data were normalized with the loading control GAPDH. (C) Each data point represents mean ± SEM of three experiments, and (D) data from one experiment.

3.9 Effect of CSE and FPS-ZM1 on Cell Viability

MTT assay was used to assess the cytotoxicity of (A) CSE (1%, 2.5%, and 5%) and (B) CSE (2.5%) + FPS-ZM1 (0.5 mM, 1.0 mM, and 1.5 mM) on BEAS-2B cells. The assay was performed after the cells were incubated with CSE or 2.5% CSE + FPS-ZM1 for 24 hours as described in Section 2.7. As shown in Figure 3.11, treatment of the cells with (5%) CSE, but not other lower concentrations of CSE, significantly reduced the cell viability (Figure 3.11 A) ($P < 0.001$ and $P < 0.0001$). The data suggest that 5% CSE is toxic to the cells. 2.5% CSE was therefore used for the study of drug effect. On the other hand, treatment of the cells with 2.5% CSE + FPS-ZM1 (0.5 mM, 1.0 mM, and 1.5 mM) did not show any effect on the cell viability (Figure 3.11 B), suggesting 2.5% CSE and FPS-ZM1 at the concentrations tested are not toxic to BEAS-2B cells.

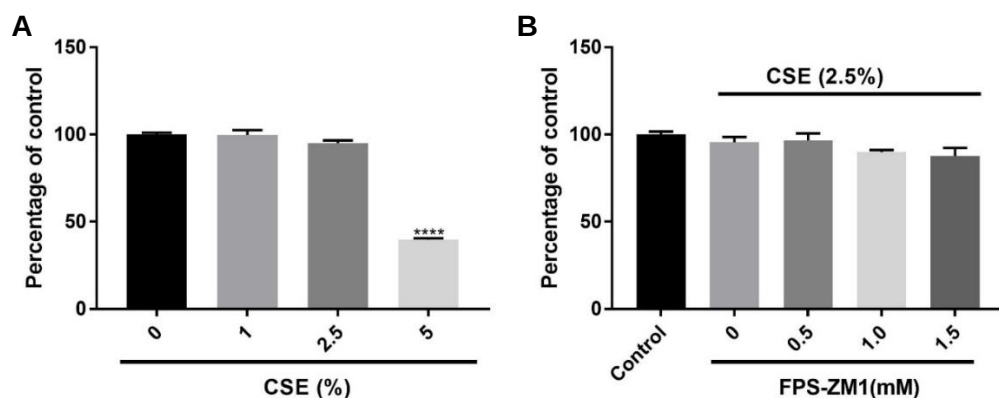


Figure 3.11. Effect of CSE and FPS-ZM1 on cell viability. Confluent and growth arrested BEAS-2B cells were treated with (A) CSE (1%, 2.5%, and 5%) or (B) CSE (2.5%) + FPS-ZM1 (0.5 mM, 1.0 mM, and 1.5 mM) for 24 hours. Cell viability was analysed by MTT cell viability assay. OD value at 590 nm (reflecting cell numbers) from untreated cells was set as 100%. Each data point represents mean $\pm$ SEM of three experiments carried out in duplicate. ****P < 0.0001 versus control.

Chapter 4. Discussion

COPD is a common chronic inflammatory lung disease. It has been known that cigarette smoke (CS) is a major cause of COPD and plays a crucial role in the progression of COPD. Airway epithelial cells are the first defence cells against cigarette smoke and are an important source of pro-inflammatory cytokines and chemokines. However, the effect of cigarette smoke on the inflammatory responses of airway epithelial cells and the underlying mechanisms remain poorly understood. In the present study, by using an *in vitro* experimental model mimicking a situation of a smoker subject, we sought to understand whether CSE can induce inflammatory cytokine/chemokine production in human bronchial epithelial cells and whether the inflammatory responses of the cells are partially mediated by RAGE. We were also interested in exploring the effect of two alternative anti-inflammatory drugs, cordycepin and simvastatin, on CSE-induced cytokine/chemokine production. We demonstrated that CSE increased the production of IL-8 and IL-6 and the effect may be mediated partially by RAGE. We also found that both drugs inhibited CSE-induced IL-8 production. Since neutrophil-driven airway inflammation is critically involved in the pathogenesis of COPD and IL-8 is a potent chemoattractant for neutrophils, our findings suggest cigarette smoke, by inducing inflammatory responses from human bronchial epithelial cells, may play an important role in airway inflammation in COPD and that the two alternative anti-inflammatory drugs, cordycepin and simvastatin, may have therapeutic potential in COPD.

4.1. CSE and Cytokine/Chemokine Production

To explore the effect of CSE on the inflammatory responses from human bronchial epithelial cells, we first assessed the effect of CSE on the production of a group of cytokines and chemokines that have been shown to be implicated in COPD, such as CXCL5, CCL11, CXCL1, CXCL10, IL-8, and IL-6, in BEAS-2B cells. Interestingly, our results showed that CXCL5, CCL11, CXCL1, and CXCL10 were undetectable under basal condition and CSE treatment at the concentration range and incubation time did not stimulate their production. It has been demonstrated that different human airway epithelial cells have different basal level expression patterns of these chemokine. For instance, CXCL5 is basally expressed in normal human bronchial cells (NHBE) (82), CXCL1 is basally expressed in human alveolar (carcinoma) epithelial cells (A549) (83), and CXCL10 is basally expressed in human bronchial epithelial cells (16-HBE) (84). One study using BEAS-2B cells shows big variation of basal CXCL10 release in different experiments, from undetectable to around 250 pg/ml [5]. This is inconsistent with our finding and could be explained by possible differences in cell passages and the media used for cell culture and growth-arrest. CSE has been shown to have different effects on the production of some of these chemokines in different cell types. CSE treatment increases CXCL1 production in human airway smooth muscle cells (HASMCS) [2], CXCL5 and CXCL10 production in mouse macrophages (59), has no effect on the production of CXCL1 and CCL11 in A549 human alveolar epithelial

cells [3] and HASMCs [2], respectively, but decreases constitutive CXCL10 production in 16-HBE cells (84). Although the effect of CSE on the production of these chemokines in BEAS-2B cells has not been explored before, the fact that CSE can induce the production of some of them in other cells, but not in human airway epithelial cells, including BEAS-2B cells in our current study, suggests that CSE alone is not enough to induce their production from human airway epithelial cells to contribute to airway inflammation in COPD. In contrast to the no effect of CSE on the aforementioned chemokines, we found that the production of IL-8 and IL-6 from BEAS-2B cells was significantly increased by CSE treatment. Our findings are consistent with previous studies showing increased IL-8 production by CSE in HASMCs [2] and 16-HBE cells and also appear to be well substantiated by one study that demonstrated increased IL-8 and IL-6 expression by CSE in BEAS-2B cells (85). However, there are a number of significant differences in the experimental design and methods. Although the same research cigarette (3R4F) was used in both studies, 15 cigarettes were used to prepare CSE in 50 mL of medium in the previous study (85), whereas 2 cigarettes were used to prepare CSE in 20 mL of medium in our study. The CSE solutions were regarded as 100% in their case but was adjusted by absorbance at 320 nm in our case ($1.5=100\%$). The cells were then treated with 15% CSE in their study and with up to 5% in our study. Although the two concentrations are not directly comparable, the 15% CSE used by them seemed to be much higher than the CSE concentration we used (initially up to 5%, then 2.5%). Cell treatment with

15% CSE resulted in significant apoptosis (one of their aims) in their study, but cell treatment with 2.5% CSE did not lead to significant cytotoxicity (5% CSE did and was then removed after initial study) in our study. In addition, the protein expression of IL-8 and IL-6 was analysed by the semi-quantitative Western blotting in their study but was analysed by quantitative ELISA in our study. These differences suggest that our observations provide a more accurate account of the true effect of CSE on IL-8 and IL-6 production in BEAS-2B than the previous study [6]. Since studies have suggested that IL-6 is likely a player in the pathogenesis of COPD by modulating pulmonary function but not necessarily a parameter for exacerbated inflammation (24) and that neutrophil accumulation in the airways of patients with COPD driven by an increased release of chemokines such as IL-8 is critically involved in airway inflammation in COPD, our results suggest cigarette smoke may contribute to the pathogenesis of COPD by modulating pulmonary function through IL-6 and modulating neutrophil-driven airway inflammation through IL-8.

4.2. CSE and sRAGE Secretion and RAGE Expression

To find out whether CSE-induced IL-8 and IL-6 production from human bronchial epithelial cells was mediated partly by RAGE, we assessed the effect of CSE on sRAGE secretion and RAGE expression. It is known that sRAGE functions extracellularly as RAGE decoy and its secretion can be measured from the blood, urine, and culture medium. We showed that sRAGE was not detectable in untreated and CSE-treated BEAS-2B cells. These findings are inconsistent with the results from a previous study (61) showing that sRAGE was basally secreted under normal conditions, although at a low level (<30 pg/ml), in 16-HBE cells. This inconsistency may be due to the differences in the cell lines and cell culture methods used. Interestingly, CSE did not affect sRAGE secretion in 16HBE cells (61), thus supporting our findings and suggesting that CSE-induced IL-8 and IL-6 production from human bronchial epithelial cells is not mediated by reduced secretion of sRAGE. We also showed that RAGE was highly expressed in untreated BEAS-2B cells and its expression was not further increased by CSE treatment. This is the first such study in BEAS-2B cells. Our results share similarity with a previous study (61), showing that RAGE was basally expressed in 16-HBE cells; however, in contrast to our results, RAGE expression was significantly increased by CSE (61). This inconsistency could be due to differences in methodology, cell line, and materials. We assessed RAGE expression by Western blot, but the group assessed RAGE expression from the cell lysate by ELISA (61). The cigarettes they used were not described but were most likely different from the research cigarettes we

used. Since our study is the first to explore sRAGE secretion and RAGE expression in BEAS-2B cells, our results suggest CSE has no effect on RAGE expression; however, it is still possible that CSE-induced IL-8 and IL-6 production from these cells may be mediated partly through existing RAGE.

4.3. RAGE Inhibitor and CSE-Induced IL-8 and IL-6 Production

To further investigate the role of RAGE in mediating CSE-induced IL-8 and IL-6 production in BEAS-2B cells, we treated the cells with the RAGE specific blocking agent FPS-ZM1, a tertiary amide compound that acts as a high affinity potent and multimodal blocker of RAGE V domain. FPS-ZM1 inhibits V domain of RAGE from binding RAGE ligands and then prevents downstream multiple signaling pathways that lead to the expression of inflammatory genes and RAGE (86). We found that FPS-ZM1 partially but significantly inhibited CSE-induced IL-8 and IL-6 release at the highest concentration used (1.5 mM), but had no effect on RAGE expression. Our findings are in a good agreement with a previous study (87), showing that FPS-ZM1 inhibited CSE-induced IL-6 production in mouse alveolar type II epithelial cell line MLE-12. Our findings are also supported by the observation that RAGE neutralising antibody reversed CSE-induced IL-1 β and TNF α production from 16-HBE cells [7]. Since there are no previous studies explored the role of RAGE in mediating CSE-induced IL-8 production, our study is the first to suggest that RAGE may also play a role in partly mediating CSE-induced IL-8 production in

human bronchial epithelial cells. As RAGE ligand binding can lead to increased RAGE expression, blocking the binding by FPS-ZM1 may reverse the increased RAGE expression. This was demonstrated by a study showing that FPS-ZM1 downregulated CSE-induced RAGE expression in MLE12 murine lung epithelial type II cell line [9]. However, we did not find either CSE or CSE+FPS-ZM1 had any effect on RAGE expression in our experimental setting. This inconsistency may be due to the very different cell types used. However, caution should be exercised when interpreting our results since it is possible that FPS-ZM1 may have off target effects other than inhibiting RAGE ligand binding at the high concentrations (up to 1.5 mM) we used in our experiments, although they were not toxic to the cells. The highest concentration other people used in *in-vitro* studies was (0.1 mM) (88), which was lower than the concentrations we used. Our observations thus suggest that RAGE may play a role in mediating CSE-induced IL-8 and IL-6 production from human bronchial epithelial cells and that blockade of RAGE from binding RAGE ligands could be targeted for a potential therapeutic approach for treating airway inflammation in COPD. However, further studies using small interfering RNA to knock down the expression of RAGE will be needed to confirm the role of RAGE. It is also possible pattern-recognition receptors other than RAGE may also be involved in CSE-induced inflammatory responses from human bronchial epithelial cells, as it was demonstrated that CSE increased Toll-like receptor 4 expression and led to preferential release of IL-8 from 16-HBE cells [3].

4.4. Effect of Cordycepin on CSE-Induced IL-8 and IL-6 Production and RAGE/HMGB-1 Expression

COPD is known to be an airway inflammatory disease that is not reversible and mostly caused by CS. Current inhaled corticosteroids therapies are poorly effective in improving the symptoms of COPD. This underlines the need to target the inflammatory mediators involved in COPD pathogenesis by new anti-inflammatory therapies. We therefore went on to explore the potential effect of cordycepin, a purine nucleoside antimetabolite isolated from the caterpillar fungus *Cordyceps militaris*, on CSE-induced IL-8 and IL-6 production in BEAS-2B cells. Our results showed that cordycepin partially inhibited CSE-induced IL-8 expression in BEAS-2B cells. It was demonstrated before that cordycepin inhibited cytokines and chemokines such as IL-8, IL-6, IL-1 β , and tumour necrosis factor (TNF α) expression in human mast cells stimulated with PMA and calcium ionophore (75). A study from our research group showed that cordycepin specifically inhibited the mRNA expression of inflammatory genes IL-8, CCL2, and CXCL1 by reducing mRNA polyadenylation and cleavage in human airway smooth muscle cells (72). Our findings are in a good agreement with these previous findings (72, 75) on IL-8 production. In contrast, our findings that showed cordycepin enhanced CSE-induced IL6 production is not consistent with the previous study (75). Previous studies showed that an increase of cAMP level caused an increase in IL-6 production (89), but decreased IL-8 production (90). Interestingly, cordycepin was reported to increase cAMP (91). It is therefore reasonable to speculate that the increase of cAMP may

contribute to both inhibition of CSE-induced IL-8 production and enhancement of CSE-induced IL-6 production by cordycepin in our current study . However, further studies on the effect of cordycepin alone on cAMP, IL-8 and IL-6 production and the effect of cAMP inhibitors and mimetics on CSE-induced IL-8 and IL-6 production in BEAS-2B cells are needed to confirm this explanation.

It has been shown that long term exposure to CS leads to increased inflammation, and impaired lung function and increased expression of the RAGE ligand HMGB-1 in mouse lung (92). In addition, HMGB-1 can induce inflammatory response through activating RAGE/JNK/NF-kB pathway in human bronchial epithelial cells (93). We showed previously that cordycepin inhibited CSE-induced IL-8. We then went to explore the effect of CSE and cordycepin+CSE on RAGE and HMGB-1 expression. Our results showed that HMGB-1, like RAGE, was constitutively expressed in BEAS-2B cells and that the expression of RAGE and HMGB-1 was not affected by CSE alone and cordycepin+CSE. These observations suggest that CSE-induced IL-8 and IL-6 production from human bronchial epithelial cells is unlikely mediated by increased expression of HMGB-1. However, it is possible that CSE may increase the expression of RAGE ligands other than HMGB-1, but this needs to be confirmed by further studies. Our data also suggest that the effect of cordycepin on CSE-induced IL-8 and IL-6 production is unlikely via the inhibition of the RAGE/HMGB-1 axis. As cordycepin has opposing effect on IL-8 and IL-6 production, the net benefit of cordycepin may depend on the roles of IL-8 and IL-6 in the pathogenesis of COPD. Since neutrophil-

driven airway inflammation is a prominent feature of COPD and IL-8 is a potent chemokine for neutrophils, it is likely that cordycepin may still have therapeutic potential in COPD by reducing IL-8 production in response to CS and other stimulants, although the mechanisms of action of cordycepin are unclear.

4.5. Effect of Simvastatin on CSE-Induced IL-8 and IL-6 Production and RAGE/HMGB-1 Expression

Simvastatin is a lipid-lowering drug that has been shown to have several anti-inflammatory effects [67], but its effect on CSE-induced inflammatory responses has not been studied before. We explored for the first time the effect of simvastatin on CSE-induced IL-8 and IL-6 production in BEAS-2B cells. Our results showed that simvastatin partially inhibited CSE-induced IL-8 production but had no effect on the production of IL-6. Our findings are consistent with a previous study (94), showing that simvastatin inhibited cyclic airway reopening forces-induced IL-8 production, but are not consistent with the same study (94), showing that simvastatin inhibited cyclic airway reopening forces-induced IL-6 production in A549 cells. Simvastatin also reduced IL-6 production in human umbilical vein endothelial cells in response to phorbol myristate acetate (66). The differences could be due to different cell lines and treatments. It was reported that simvastatin decreased RAGE expression in human retinal pigment epithelium cells (71) and suppressed vascular inflammation and atherosclerosis in ApoE(-/-) mice by downregulating the HMGB-1-RAGE axis (95). However, no studies so far have

investigated the effect of simvastatin on RAGE and HMGB-1 expression in human bronchial epithelial cells. We reported here that neither CSE nor CSE + simvastatin had any effect on RAGE and HMGB-1 expression, suggesting that the HMGB-1-RAGE axis is not involved in the inhibition of CSE-induced IL-8 production by simvastatin. But further studies are needed to assess the effect of CSE and simvastatin on the production of other RAGE ligands and other possible mechanisms of the anti-inflammatory effect of simvastatin. Our observations suggest that simvastatin, like cordycepin, may have therapeutic potential in COPD by reducing CSE-induced IL-8 production and subsequently the prominent neutrophil-driven airway inflammation.

Chapter 5. Future Directions and Conclusions

5.1. Future Directions

In our current study there are a number of limitations that need to be addressed in future studies.

Firstly, we investigated the role of RAGE in mediating CSE-induced IL-8 and IL-6 production by treating the cells with high concentrations (up to 1.5 mM) of FPS-ZM1, and that would possibly cause effects other than inhibiting RAGE ligand binding. Although the CSE effect on cytokine/chemokine production in our experimental setting may be mediated CSE, it is necessary in future studies to use small interfering RNA to knock down the expression of RAGE to confirm its role.

Secondly, we showed that the treatment of cordycepin inhibited CSE-induced IL-8 production but enhanced IL-6 production in BEAS-2B cells. We propose that cordycepin could increase cAMP level, which then contribute to inhibition of IL-8 and increase of IL-6 production in BEAS-2B cells. It would be interesting in our future work to investigate the effect of cordycepin alone on cAMP, IL-8 and IL-6 production and the effect of cAMP inhibitors and mimetics on CSE-induced IL-8 and IL-6 production.

Thirdly, in addition to HMGB-1, there are a number of other RAGE ligands that may be produced by CSE treatment and contribute to CSE-induced inflammatory responses. We did not have enough time to analyse other RAGE ligands for the current study. Future studies are therefore needed to analyse the release of other RAGE ligands by CSE and drug treatment. The effect of cordycepin and simvastatin on sRAGE secretion should also be conducted.

Finally, we used immortalised human bronchial epithelial cells to study the effect of CSE on the inflammatory genes expression. The responses of immortalised cell lines may be different from those of primary cells. It is necessary in our future investigations to use primary human bronchial epithelial cells from non-smokers, smokers with COPD and smokers without COPD to strengthen and confirm our findings with CSE, as these cells would be more representative of the cellular environment and conditions in COPD.

5.2. Conclusions

COPD is a common chronic inflammatory lung disease. It has been known that cigarette smoke (CS) is a major cause of COPD and plays a crucial role in the progression of COPD. Airway epithelial cells are the first defence cells against cigarette smoke and are an important source of pro-inflammatory cytokines and chemokines. This thesis has highlighted that CSE can induce IL-8 and IL-6 production and that this process may be partially mediated by RAGE. Data of this study have also shown that cordycepin and simvastatin can inhibit CSE-induced IL-8 and this effect is unlikely mediated by RAGE. Since IL-8 is a potent chemoattractant for neutrophils and IL-6 is critically involved in modulating pulmonary function, our findings suggest that cigarette smoke, by inducing inflammatory responses from human bronchial epithelial cells, may contribute to the pathogenesis of COPD by modulating neutrophil-driven airway inflammation through IL-8 and by modulating pulmonary function through IL-6. Our findings also suggest that the two alternative anti-inflammatory drugs, cordycepin and simvastatin, by reducing CSE-induced IL-8 production, may have therapeutic potential in COPD.

References

1. (WHO) WHO. 2017 [Available from: <http://www.who.int/respiratory/copd/definition/en/>.
2. Pauwels RA, Buist AS, Ma P, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: National Heart, Lung, and Blood Institute and World Health Organization Global Initiative for Chronic Obstructive Lung Disease (GOLD): executive summary. *Respiratory care*. 2001;46(8):798-825.
3. (GOLD) TGIFCOLD. 2018 GLOBAL STRATEGY FOR PREVENTION, DIAGNOSIS AND MANAGEMENT OF COPD 2017 [Available from: <http://goldcopd.org/wp-content/uploads/2016/04/GOLD-2018-WMS.pdf>.
4. Foundation BL. Chronic obstructive pulmonary disease (COPD) statistics.
5. Lopez AD, Shibuya K, Rao C, Mathers CD, Hansell AL, Held LS, et al. Chronic obstructive pulmonary disease: current burden and future projections. *Eur Respir J*. 2006;27(2):397-412.
6. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS medicine*. 2006;3(11):e442.
7. Soriano JB, Abajobir AA, Abate KH, Abera SF, Agrawal A, Ahmed MB, et al. Global, regional, and national deaths, prevalence, disability-adjusted life years, and years lived with disability for chronic obstructive pulmonary disease and asthma, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *The Lancet Respiratory Medicine*. 5(9):691-706.
8. McLean S, Hoogendoorn M, Hoogenveen RT, Feenstra TL, Wild S, Simpson CR, et al. Projecting the COPD population and costs in England and Scotland: 2011 to 2030. *Scientific Reports*. 2016;6:31893.
9. Ward MM, Javitz HS, Smith WM, Whan MA. Lost income and work limitations in persons with chronic respiratory disorders. *Journal of clinical epidemiology*. 2002;55(3):260-8.
10. Boot CRL, Vercoulen JHMM, van der Gulden JWJ, Orbon KH, van den Hoogen H, Folgering HTM. Sick leave in patients with obstructive lung disease is related to psychosocial and work variables rather than to FEV1. *Respiratory Medicine*. 2005;99(8):1022-31.
11. (WHO) WHO. Causes of COPD [Available from: <http://www.who.int/respiratory/copd/causes/en/>.
12. Bahtouee M, Maleki N, Nekouee F. The prevalence of chronic obstructive pulmonary disease in hookah smokers. *Chron Respir Dis*. 2017:1479972317709652.
13. Laniado-Laborín R. Smoking and Chronic Obstructive Pulmonary Disease (COPD). *Parallel Epidemics of the 21(st) Century*. *International Journal of Environmental Research and Public Health*. 2009;6(1):209-24.
14. Jaen Diaz JJ, de Castro Mesa C, Gontan Garcia-Salamanca MJ, Lopez de Castro F. [Prevalence of chronic obstructive pulmonary disease and risk factors in smokers and ex-smokers]. *Arch Bronconeumol*. 2003;39(12):554-8.
15. Liu Y, Pleasants RA, Croft JB, Wheaton AG, Heidari K, Malarcher AM, et al. Smoking duration, respiratory symptoms, and COPD in adults aged ≥45 years with a smoking history. *International Journal of Chronic Obstructive Pulmonary Disease*. 2015;10:1409-16.

16. Ng M, Freeman MK, Fleming TD, et al. Smoking prevalence and cigarette consumption in 187 countries, 1980-2012. *JAMA*. 2014;311(2):183-92.
17. Lindberg A, Bjerg-Bäcklund A, Rönmark E, Larsson L-G, Lundbäck B. Prevalence and underdiagnosis of COPD by disease severity and the attributable fraction of smoking: Report from the Obstructive Lung Disease in Northern Sweden Studies. *Respiratory Medicine*. 2006;100(2):264-72.
18. (IHME) IfHMaE. Tobacco Burden visualization 2015 [Available from: <https://vizhub.healthdata.org/tobacco/>].
19. (WHO) Who. Future predictions of tobacco smoke
20. Chung KF. Cytokines in chronic obstructive pulmonary disease. *The European respiratory journal Supplement*. 2001;34:50s-9s.
21. Rovina N, Koutsoukou A, Koulouris NG. Inflammation and immune response in COPD: where do we stand? *Mediators Inflamm*. 2013;2013:413735.
22. Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2014;1843(11):2563-82.
23. Barnes PJ. The Cytokine Network in Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory Cell and Molecular Biology*. 2009;41(6):631-8.
24. Rincon M, Irvin CG. Role of IL-6 in Asthma and Other Inflammatory Pulmonary Diseases. *International Journal of Biological Sciences*. 2012;8(9):1281-90.
25. Bade G, Khan MA, Srivastava AK, Khare P, Solaiappan KK, Guleria R, et al. Serum cytokine profiling and enrichment analysis reveal the involvement of immunological and inflammatory pathways in stable patients with chronic obstructive pulmonary disease. (1178-2005 (Electronic)).
26. Yin T, Zhu Z, Mei Z, Feng J, Zhang W, He Y, et al. Analysis of viral infection and biomarkers in patients with acute exacerbation of chronic obstructive pulmonary disease. *Clin Respir J*. 2017.
27. Buist AS. Similarities and differences between asthma and chronic obstructive pulmonary disease: treatment and early outcomes. *European Respiratory Journal*. 2003;21(39 suppl):30s.
28. Miller-Larsson A, Selroos O. Advances in asthma and COPD treatment: combination therapy with inhaled corticosteroids and long-acting beta 2-agonists. *Current pharmaceutical design*. 2006;12(25):3261-79.
29. Zeng Q. A New Anti-inflammatory Therapy for COPD. *Journal of Pulmonary & Respiratory Medicine*. October 03,2016.
30. Singh S, Loke YK. An overview of the benefits and drawbacks of inhaled corticosteroids in chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis*. 2010;5:189-95.
31. Wang JJ, Rochtchina E, Tan AG, Cumming RG, Leeder SR, Mitchell P. Use of inhaled and oral corticosteroids and the long-term risk of cataract. *Ophthalmology*. 2009;116(4):652-7.
32. Suissa S, Kezouh A, Ernst P. Inhaled corticosteroids and the risks of diabetes onset and progression. *The American journal of medicine*. 2010;123(11):1001-6.
33. Lee CH, Kim K, Hyun MK, Jang EJ, Lee NR, Yim JJ. Use of inhaled corticosteroids and the risk of tuberculosis. *Thorax*. 2013;68(12):1105-13.
34. Buchman AL. Side effects of corticosteroid therapy. *Journal of clinical gastroenterology*. 2001;33(4):289-94.
35. Singh S, Amin AV, Loke YK. Long-term use of inhaled corticosteroids and the risk of pneumonia in chronic obstructive pulmonary disease: a meta-analysis. *Archives of internal medicine*. 2009;169(3):219-29.

36. Neeper M, Schmidt AM, Brett J, Yan SD, Wang F, Pan YC, et al. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *The Journal of biological chemistry*. 1992;267(21):14998-5004.
37. Nowotny K, Jung T, Hohn A, Weber D, Grune T. Advanced glycation end products and oxidative stress in type 2 diabetes mellitus. *Biomolecules*. 2015;5(1):194-222.
38. Nedic O, Rattan SI, Grune T, Trougakos IP. Molecular effects of advanced glycation end products on cell signalling pathways, ageing and pathophysiology. *Free radical research*. 2013;47 Suppl 1:28-38.
39. Schmidt AM, Vianna M, Gerlach M, Brett J, Ryan J, Kao J, et al. Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. *The Journal of biological chemistry*. 1992;267(21):14987-97.
40. Peterszegi G MJ, Ravelojaona V, Robert L. *Pathol Biol* ((Paris). 2006;54:396–404).
41. Yonekura H, Yamamoto Y, Sakurai S, Petrova RG, Abedin MJ, Li H, et al. Novel splice variants of the receptor for advanced glycation end-products expressed in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury. *Biochemical Journal*. 2003;370(3):1097.
42. Srikrishna GH, B. AGER (advanced glycosylation end product-specific receptor). *Atlas Genet Cytogenet Oncol Haematol*. 2011(15(3):239-243).
43. Oczypok EA, Perkins TN, Oury TD. All the “RAGE” in lung disease: The receptor for advanced glycation endproducts (RAGE) is a major mediator of pulmonary inflammatory responses. *Paediatric Respiratory Reviews*. 2017;23(Supplement C):40-9.
44. Brett J, Schmidt AM, Shi Du Y, Yu Shan Z, Weidman E, Pinsky D, et al. Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. *American Journal of Pathology*. 1993;143(6):1699-712.
45. Katsuoka F, Kawakami Y, Arai T, Imuta H, Fujiwara M, Kanma H, et al. Type II Alveolar Epithelial Cells in Lung Express Receptor for Advanced Glycation End Products (RAGE) Gene. *Biochemical and Biophysical Research Communications*. 1997;238(2):512-6.
46. Chen YC, Statt S, Wu R, Chang HT, Liao JW, Wang CN, et al. High mobility group box 1-induced epithelial mesenchymal transition in human airway epithelial cells. *Sci Rep*. 2016;6:18815.
47. Sukkar MB, Ullah MA, Gan WJ, Wark PAB, Chung KF, Hughes JM, et al. RAGE: a new frontier in chronic airways disease. *British Journal of Pharmacology*. 2012;167(6):1161-76.
48. Bierhaus A Fau - Humpert PM, Humpert Pm Fau - Morcos M, Morcos M Fau - Wendt T, Wendt T Fau - Chavakis T, Chavakis T Fau - Arnold B, Arnold B Fau - Stern DM, et al. Understanding RAGE, the receptor for advanced glycation end products. (0946-2716 (Print)).
49. Lin L. RAGE on the Toll Road? *Cellular & molecular immunology*. 2006;3(5):351-8.
50. Srikrishna G, Freeze HH. Endogenous Damage-Associated Molecular Pattern Molecules at the Crossroads of Inflammation and Cancer. *Neoplasia*. 2009;11(7):615-28.
51. Hori O, Brett J, Slattery T, Cao R, Zhang J, Chen JX, et al. The Receptor for Advanced Glycation End Products (RAGE) Is a Cellular Binding Site for Amphotericin: MEDIATION OF NEURITE OUTGROWTH AND CO-EXPRESSION OF RAGE AND

AMPHOTERIN IN THE DEVELOPING NERVOUS SYSTEM. *Journal of Biological Chemistry*. 1995;270(43):25752-61.

52. Wu X, Mi Y, Yang H, Hu A, Zhang Q, Shang C. The activation of HMGB1 as a progression factor on inflammation response in normal human bronchial epithelial cells through RAGE/JNK/NF-kappaB pathway. *Mol Cell Biochem*. 2013;380(1-2):249-57.

53. Leclerc E, Fritz G, Vetter SW, Heizmann CW. Binding of S100 proteins to RAGE: An update. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2009;1793(6):993-1007.

54. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, et al. RAGE Mediates a Novel Proinflammatory Axis: A Central Cell Surface Receptor for S100/Calgranulin Polypeptides. *Cell*. 1999;97(7):889-901.

55. Chuah YK, Basir R Fau - Talib H, Talib H Fau - Tie TH, Tie Th Fau - Nordin N, Nordin N. Receptor for advanced glycation end products and its involvement in inflammatory diseases. (2090-8040 (Print)).

56. Golpe R, Martin-Robles I, Sanjuan-Lopez P, Perez-de-Llano L, Gonzalez-Juanatey C, Lopez-Campos JL, et al. Differences in systemic inflammation between cigarette and biomass smoke-induced COPD. *Int J Chron Obstruct Pulmon Dis*. 2017;12:2639-46.

57. Gaschler GJ, Zavitz CC, Bauer CM, Skrtic M, Lindahl M, Robbins CS, et al. Cigarette smoke exposure attenuates cytokine production by mouse alveolar macrophages. *Am J Respir Cell Mol Biol*. 2008;38(2):218-26.

58. Xue H, Li MX. MicroRNA-150 protects against cigarette smoke-induced lung inflammation and airway epithelial cell apoptosis through repressing p53: MicroRNA-150 in CS-induced lung inflammation. *Human & experimental toxicology*. 2017;960327117741749.

59. Zhao J, Li X, Xie F, Yang Z, Pan X, Zhu M, et al. Immunomodulatory effects of cigarette smoke condensate in mouse macrophage cell line. *International journal of immunopathology and pharmacology*. 2017;30(3):315-21.

60. Morbini P, Villa C, Campo I, Zorretto M, Inghilleri S, Luisetti M. The receptor for advanced glycation end products and its ligands: a new inflammatory pathway in lung disease? *Modern Pathology*. 2006;19:1437.

61. Chen L, Wang T, Guo L, Shen Y, Yang T, Wan C, et al. Overexpression of RAGE Contributes to Cigarette Smoke-Induced Nitric Oxide Generation in COPD. *Lung*. 2014;192(2):267-75.

62. Sanders NT, Dutton DJ, Durrant JW, Lewis JB, Wilcox SH, Winden DR, et al. Cigarette smoke extract (CSE) induces RAGE-mediated inflammation in the Ca9-22 gingival carcinoma epithelial cell line. *Arch Oral Biol*. 2017;80:95-100.

63. Robinson AB, Johnson KD, Bennion BG, Reynolds PR. RAGE signaling by alveolar macrophages influences tobacco smoke-induced inflammation. *Am J Physiol Lung Cell Mol Physiol*. 2012;302(11):L1192-9.

64. Drugs. Simvastatin.

65. Hothersall E, McSharry C, Thomson NC. Potential therapeutic role for statins in respiratory disease. *Thorax*. 2006;61(8):729-34.

66. Inoue I, Goto S-i, Mizotani K, Awata T, Mastunaga T, Kawai S-i, et al. Lipophilic HMG-CoA reductase inhibitor has an anti-inflammatory effect: Reduction of mRNA levels for interleukin-1 β , interleukin-6, cyclooxygenase-2, and p22phox by regulation of peroxisome proliferator-activated receptor α (PPAR α) in primary endothelial cells. *Life Sciences*. 2000;67(8):863-76.

67. Wang Y, Yang W, Zhao X, Zhang R. Experimental Study of the Protective Effect of Simvastatin on Lung Injury in Rats with Sepsis. *Inflammation*. 2017.

68. Milara J, Peiró T, Serrano A, Artigues E, Aparicio J, Tenor H, et al. Simvastatin Increases the Ability of Roflumilast N-oxide to Inhibit Cigarette Smoke-Induced Epithelial to Mesenchymal Transition in Well-differentiated Human Bronchial Epithelial Cells in vitro. COPD: Journal of Chronic Obstructive Pulmonary Disease. 2015;12(3):327-38.
69. Quade-Lyssy P, Kanarek AM, Baierdorfer M, Postina R, Kojro E. Statins stimulate the production of a soluble form of the receptor for advanced glycation end products. J Lipid Res. 2013;54(11):3052-61.
70. Nozue T, Yamagishi S-i, Takeuchi M, Hirano T, Yamamoto S, Tohyama S, et al. Effect of statins on the serum soluble form of receptor for advanced glycation end-products and its association with coronary atherosclerosis in patients with angina pectoris. IJC Metabolic & Endocrine. 2014;4:47-52.
71. Tsujinaka H, Itaya-Hironaka A, Yamauchi A, Sakuramoto-Tsuchida S, Shobatake R, Makino M, et al. Statins decrease vascular epithelial growth factor expression via down-regulation of receptor for advanced glycation end-products. Heliyon. 2017;3(9):e00401.
72. Kondrashov A, Meijer HA, Barthet-Barateig A, Parker HN, Khurshid A, Tessier S, et al. Inhibition of polyadenylation reduces inflammatory gene induction. RNA. 2012;18(12):2236-50.
73. Yue GG-L, Lau CB-S, Fung K-P, Leung P-C, Ko W-H. Effects of Cordyceps sinensis, Cordyceps militaris and their isolated compounds on ion transport in Calu-3 human airway epithelial cells. Journal of Ethnopharmacology. 2008;117(1):92-101.
74. Fei X, Zhang X, Zhang GQ, Bao WP, Zhang YY, Zhang M, et al. Cordycepin inhibits airway remodeling in a rat model of chronic asthma. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2017;88:335-41.
75. Yoo M-s, Jin MH, Lee SY, Lee SH, Kim B, Roh SS, et al. Cordycepin Suppresses Thymic Stromal Lymphopoietin Expression <i>via</i> Blocking Caspase-1 and Receptor-Interacting Protein 2 Signaling Pathways in Mast Cells. Biological and Pharmaceutical Bulletin. 2016;39(1):90-6.
76. Yang X, Li Y, He Y, Li T, Wang W, Zhang J, et al. Cordycepin alleviates airway hyperreactivity in a murine model of asthma by attenuating the inflammatory process. Int Immunopharmacol. 2015;26(2):401-8.
77. Liu A, Wu J, Li A, Bi W, Liu T, Cao L, et al. The inhibitory mechanism of Cordyceps sinensis on cigarette smoke extract-induced senescence in human bronchial epithelial cells. Int J Chron Obstruct Pulmon Dis. 2016;11:1721-31.
78. de Bittencourt Pasquali MA, Gelain DP, Zeidan-Chulia F, Pires AS, Gasparotto J, Terra SR, et al. Vitamin A (retinol) downregulates the receptor for advanced glycation endproducts (RAGE) by oxidant-dependent activation of p38 MAPK and NF-κB in human lung cancer A549 cells. Cellular signalling. 2013;25(4):939-54.
79. Hong T, Cui LK, Wen J, Zhang MH, Fan JM. [Cordycepin protects podocytes from injury mediated by complements complex C5b-9]. Sichuan da xue xue bao Yi xue ban = Journal of Sichuan University Medical science edition. 2015;46(2):173-8, 227.
80. Volpi G, Facchinetti F, Fau - Moretto N, Moretto N, Fau - Civelli M, Civelli M, Fau - Patacchini R, Patacchini R. Cigarette smoke and alpha,beta-unsaturated aldehydes elicit VEGF release through the p38 MAPK pathway in human airway smooth muscle cells and lung fibroblasts. (1476-5381 (Electronic)).
81. Aldrich S. BEAS-2B.
82. Foronjy RF, Salathe MA, Dabo AJ, Baumlin N, Cummins N, Eden E, et al. TLR9 expression is required for the development of cigarette smoke-induced emphysema in mice. Am J Physiol Lung Cell Mol Physiol. 2016;311(1):L154-66.

83. Victoni T, Gicquel T, Bodin A, Daude M, Tenor H, Valenca S, et al. Roflumilast n-oxide associated with PGE2 prevents the neutrophil elastase-induced production of chemokines by epithelial cells. *International immunopharmacology*. 2016;30:1-8.
84. Pace E, Ferraro M, Siena L, Melis M, Montalbano AM, Johnson M, et al. Cigarette smoke increases Toll-like receptor 4 and modifies lipopolysaccharide-mediated responses in airway epithelial cells. *Immunology*. 2008;124(3):401-11.
85. Xue H, Li MX. MicroRNA-150 protects against cigarette smoke-induced lung inflammation and airway epithelial cell apoptosis through repressing p53: MicroRNA-150 in CS-induced lung inflammation. *Human & Experimental Toxicology*. 2017;0960327117741749.
86. Deane R, Singh I, Sagare AP, Bell RD, Ross NT, LaRue B, et al. A multimodal RAGE-specific inhibitor reduces amyloid beta-mediated brain disorder in a mouse model of Alzheimer disease. *The Journal of clinical investigation*. 2012;122(4):1377-92.
87. Lee H, Park JR, Kim WJ, Sundar IK, Rahman I, Park SM, et al. Blockade of RAGE ameliorates elastase-induced emphysema development and progression via RAGE-DAMP signaling. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2017;31(5):2076-89.
88. Migrino RQ, Davies HA, Truran S, Karamanova N, Franco DA, Beach TG, et al. Amyloidogenic medin induces endothelial dysfunction and vascular inflammation through the receptor for advanced glycation endproducts. *Cardiovascular research*. 2017;113(11):1389-402.
89. Reddy PJ, Aksoy MO, Yang Y, Li XX, Ji R, Kelsen SG. Inhibition by salmeterol and cilomilast of fluticasone-enhanced IP-10 release in airway epithelial cells. *Copd*. 2008;5(1):5-11.
90. Wetthey FR, Xue L, Pettipher R. Salbutamol inhibits trypsin-mediated production of CXCL8 by keratinocytes. *Cytokine*. 2006;36(1-2):29-34.
91. Lee D-H, Kim H-H, Cho H-J, yu yb, Kang H-C, Kim J-L, et al. Cordycepin-Enriched WIB801C from Cordyceps militaris Inhibits Collagen-Induced [Ca]²⁺ Mobilization via cAMP-Dependent Phosphorylation of Inositol 1, 4, 5-Trisphosphate Receptor in Human Platelets 2014. 223-31 p.
92. Bezerra FS, Valença SS, Pires KMP, Lanzetti M, Pimenta WA, Schmidt AC, et al. Long-term exposure to cigarette smoke impairs lung function and increases HMGB-1 expression in mice. *Respiratory Physiology & Neurobiology*. 2011:120-6.
93. Wu XS, Mi YS, Yang HS, Hu AS, Zhang QS, Shang CS. The activation of HMGB1 as a progression factor on inflammation response in normal human bronchial epithelial cells through RAGE/JNK/NF-κB pathway. *Molecular and Cellular Biochemistry*. 2013:249-57.
94. Higueta-Castro N, Shukla VC, Mihai C, Ghadiali SN. Simvastatin Treatment Modulates Mechanically-Induced Injury and Inflammation in Respiratory Epithelial Cells. *Annals of biomedical engineering*. 2016;44(12):3632-44.
95. Liu M, Yu Y, Jiang H, Zhang L, Zhang PP, Yu P, et al. Simvastatin suppresses vascular inflammation and atherosclerosis in ApoE(-/-) mice by downregulating the HMGB1-RAGE axis. *Acta pharmacologica Sinica*. 2013;34(6):830-6.