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Targeting of Receptors for Advanced Glycation End-Products (RAGE) Diminishes Acute Secondhand Smoke-Induced Inflammation in Mice

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ABSTRACT

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The receptor for advanced glycation end-products (RAGE) has increasingly been demonstrated to be an important modulator of inflammation in cases of pulmonary disease. Published reports involving tobacco smoke exposure have demonstrated increased expression of RAGE, its participation in pro-inflammatory signaling and its role in irreversible pulmonary remodeling. The current research evaluated for the first time the *in vivo* effects of short-term tobacco smoke exposure in RAGE null and control mice compared to identical animals exposed to room air only. Quantitative real time PCR, immunoblotting, and immunohistochemistry revealed elevated RAGE expression in controls after four weeks of exposure and an anticipated absence of RAGE expression in RAGE null mice regardless of smoke exposure. Inflammatory cell behaviors were confirmed by measuring active Ras, NF-κB, and cytokine synthesis and secretion. Furthermore, bronchoalveolar lavage fluid (BALF) was procured from RAGE null and control animals after exposure for the assessment of total protein in order to indirectly measure vascular permeability, inflammatory cells and chemoattractant molecules involved in the inflammatory response. As a general theme, inflammation induced by tobacco smoke exposure was influenced by the availability of RAGE. These data reveal captivating information suggesting a role for RAGE signaling in lungs exposed to tobacco smoke. Furthermore, research may demonstrate RAGE signaling as an important therapeutic target capable of ameliorating cell level inflammation in those coping with exposure.

Keywords: RAGE, lung, tobacco smoke

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CHAPTER 1: A Brief Introduction

Receptor for Advanced Glycation End-Products (RAGE)

RAGE is a member of the immunoglobulin superfamily of cell surface receptors expressed in many cell types including endothelial and vascular smooth muscle cells, fibroblasts, neurons and macrophages/monocytes. RAGE contains an extracellular ligand-engaging V-region-like domain and two C-region-like domains, a single transmembrane domain, and a short, highly charged cytoplasmic domain essential for signal transduction. RAGE expression is most abundant in the lungs where it was initially isolated, and identified to be selectively localized to the basolateral membranes of well-differentiated alveolar type I (ATI) cells. RAGE interacts with a spectrum of ligands, and is named for its ability to bind advanced glycation end-products (AGEs) that accumulate in hyperglycemia and oxidant stress. As a pattern recognition receptor (PRR), RAGE also binds S100/calgranulins, amyloid-β-peptide, and HMGB-1 to influence gene expression via activated signal transduction pathways (Stern, 2002). RAGE activation tends to be ligand-specific in that it generates programmed outputs controlled by various transcription factors according to specific ligand input (Sukkar, 2012), (Figure 1.1). As a dynamic cell-surface receptor, RAGE utilizes diffuse signaling pathways to influence the progression of many inflammatory conditions. In fact, recent genome-wide association studies have linked RAGE polymorphisms with pathophysiology of the lung and several additional targeted organs.

Further complicating the dynamics of RAGE activation is the presence of several isoforms. In addition to full-length membrane-bound RAGE (mRAGE), proteolytic cleavage and differential splicing lead to the derivation of soluble RAGE (sRAGE) that lacks transmembrane

and cytosolic domains. Alternative splicing can also result in endogenous secretory RAGE (esRAGE). Both sRAGE and esRAGE are capable of binding ligands, presenting the possibility that they act as a means of sequestering otherwise deleterious ligand accumulation (Buckley, 2010), (Figure 1.2).

Downstream Targets of RAGE Influenced by Tobacco Smoke

Our laboratory has demonstrated that cigarette smoke acts through Ras to induce the nuclear translocation and activation of NF-κB, leading to an increase in pro-inflammatory cytokines such as TNFα, IL-1β, and IL-8 in rat alveolar epithelial cells and human macrophages (Kasteler, 2008). These effects were successfully counteracted by pre-treatment with siRNA for RAGE that blocks its expression (Reynolds, 2010). NF-κB controls several genes involved with inflammation. The inflammatory effects associated with diabetes, Alzheimers Disease, and cancer are orchestrated to a large degree by NF-κB and the signaling axis implicates RAGE at the point of cellular activation. These diseases create conditions necessary for the derivation and elaboration of several RAGE ligands (AGEs in diabetes, amyloid-β in Alzheimer's), which results in a positive feedback cycle, leading to chronic inflammation (Yan, 2009; Yan, 1996; Sparvero 2009). RAGE exacerbates NF-κB-mediated inflammatory loops due to the perpetual elaboration of exogenous ligands released following various forms of stress (ischemic, immune/inflammatory stimuli, physical stress), and the synthesis of new RAGE ligands. Furthermore, NF-κB activation causes *de novo* synthesis of NF-κBp65 leading to elevated signaling potency (Sparvero 2009, Schmidt, 2001).

Assessment of RAGE Biology in Tobacco Smoke-Exposed Mice

We sought to assess the biology of RAGE because of its clear implication in the inflammatory response and its effects in relation to smoke exposure. We tested the hypothesis that RAGE, at least in part, mediates the inflammatory phenotype observed in lungs involuntarily exposed to tobacco smoke. What follows is a description of RAGE in the context of smoke exposure and the first study that involves precious RAGE knock out animals and normal controls exposed to secondhand smoke. Our discoveries provide an important first step in the identification of molecular targets potentially effective in anti-inflammatory therapeutic modalities for those that are unable or unwilling to eradicate smoke exposure.

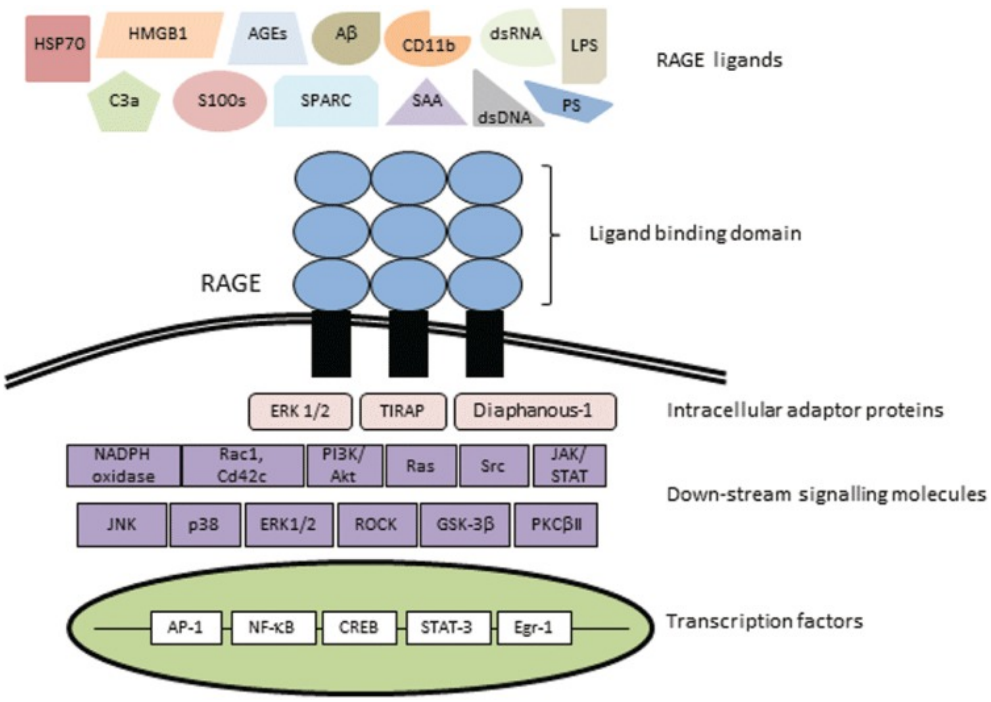

Figure 1.1: Schematic Depicting RAGE, RAGE Ligands and Down-Stream Signalling Molecules. RAGE is a pattern-recognition receptor involved in mediating cellular responses to soluble and cell-associated molecules involved in the host response to tissue injury, infection and inflammation. RAGE ligands identified to date include HMGB1, SAA, Aβ, C3a, HSP70, the injury-related glycoprotein SPARC, several members of the S100 protein family, the β2-integrin Mac-1 (CD11b), phosphatidylserine (PS), double-stranded DNA (dsDNA), double stranded RNA (dsRNA) and LPS. The RAGE cytoplasmic domain interacts with a number of intracellular adaptor proteins including diaphanous-1, ERK1/2 and TIRAP. Ligation of RAGE induces the activation of multiple signaling pathways that may vary depending on the ligand, cell and tissue microenvironment. Signaling molecules activated down-stream of RAGE include NADPH oxidase, Ras, Src kinase, Ras- ERK1/2, SAPK/JNK and p38 MAPK pathways, PI3K/Akt, small GTPase Cdc42/Rac, ROCK, PKC βII and GSK-3β, resulting in the activation of a number of transcription factors including NF-κB, AP-1, CREB protein, STAT3 and egr-1. Image regenerated from Sukkar et al. (2012).

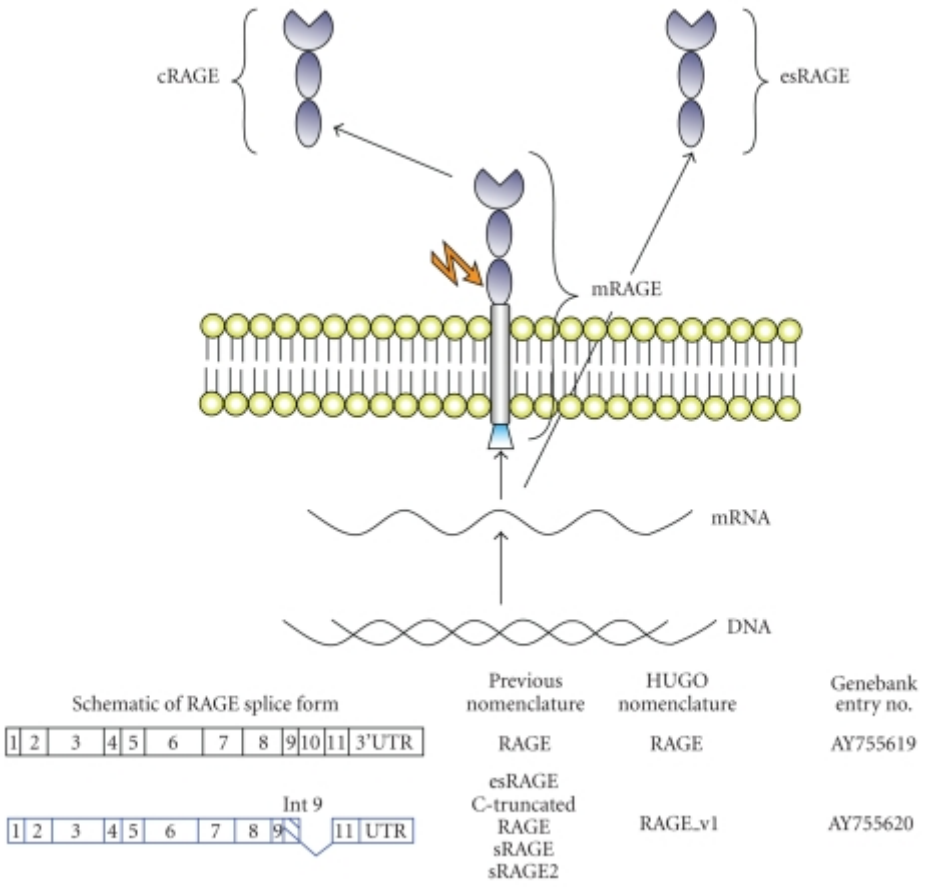

Figure 1.2: Schematic Representation of RAGE and the Generation of Some of its Isoforms Commonly Found in the Lung. In addition to its full-length form (mRAGE), RAGE also exists in a soluble form (sRAGE) which lacks the transmembrane and cytosolic domains found in mRAGE. Production of sRAGE isoforms is via either proteolytic cleavage, which gives rise to cleaved RAGE (cRAGE) or alternative splicing at exon 9 resulting in a C-truncated form termed endogenous secretory RAGE (esRAGE). Image regenerated from Buckley et al. (2010).

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CHAPTER 2: RAGE and Tobacco Smoke: Insights Into Modeling Chronic Obstructive Pulmonary Disease

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Abstract

Chronic obstructive pulmonary disease (COPD) is a progressive condition characterized by chronic airway inflammation and airspace remodeling, leading to airflow limitation that is not completely reversible. Smoking is the leading risk factor for compromised lung function stemming from COPD pathogenesis. First- and second-hand cigarette smoke contain thousands of constituents, including several carcinogens and cytotoxic chemicals that orchestrate chronic lung inflammation and destructive alveolar remodeling. Receptors for advanced glycation end-products (RAGE) are multi-ligand cell surface receptors primarily expressed by diverse lung cells. RAGE expression increases following cigarette smoke exposure and expression is elevated in the lungs of patients with COPD. RAGE is responsible in part for inducing pro-inflammatory signaling pathways that culminate in expression and secretion of several cytokines, chemokines, enzymes, and other mediators. In the current review, new transgenic mouse models that conditionally over-express RAGE in pulmonary epithelium are discussed. When RAGE is over-expressed throughout embryogenesis, apoptosis in the peripheral lung causes severe lung hypoplasia. Interestingly, apoptosis in RAGE transgenic mice occurs via conserved apoptotic pathways also known to function in advanced stages of COPD. RAGE over-expression in the adult lung models features of COPD including pronounced inflammation and loss of parenchymal tissue. Understanding the biological contributions of RAGE during cigarette smoke-induced inflammation may provide critically important insight into the pathology of COPD.

Keywords: RAGE, COPD, tobacco, mouse model, lung
Running title: RAGE and COPD

Introduction

Chronic obstructive pulmonary disease (COPD) is defined by airflow obstruction that is not fully reversible (Carp and Janoff, 1978). In particular, COPD involves chronic airway inflammation and pulmonary emphysema, which is defined anatomically via pathology samples as an abnormal permanent enlargement of airspaces distal to the terminal bronchioles accompanied by destruction of their walls without obvious fibrosis (Pauwels et al., 2001). COPD morbidity and mortality continue to rise as physician diagnoses of COPD increased from approximately 7 million in 1980 to approximately 13.1 million in 2004 (Adams and Barnes, 2006). COPD was responsible for 8 million outpatient visits, 1.5 million emergency room visits, and 672,000 hospitalizations in the U.S. in 2006 (US Department of Health and Human Services, 2009) and compared to 1980, deaths in 2007 increased 74% to over 124,000 people (American Lung Association COPD Fact Sheet, 2011). While as recent as 2010 the cost associated with COPD was $49.9 billion (Dalal et al., 2010), the precise pathobiochemical basis of COPD exacerbated by voluntary or involuntary tobacco smoke exposure remains enigmatic.

Cigarette smoking is currently the most considerable risk factor for the development of COPD, consisting of emphysema and chronic obstructive bronchitis (Anderson et al., 1964; Fletcher and Peto, 1977; Thun et al., 2000; Hogg, 2004). Notwithstanding, only one quarter of cigarette smokers develop clinically detectible airflow limitation and other symptoms of COPD, suggesting an important role for genetic susceptibility (Sethi and Rochester, 2000; Stockley et al., 2009). Although most people that develop COPD currently smoke cigarettes or have smoked in the past, COPD also develops in individuals that have never smoked (Higgins, 1991). This harmful outcome is due in part to exposure to second-hand smoke (Janson, 2004; Wakefield et

al., 2005; Eisner et al., 2006). Furthermore, because some former smokers still live with active smokers and are observed to develop COPD later in life, passive smoke exposure is likely to contribute to disease progression.

First- and second-hand smokers diagnosed with moderate COPD have altered expression of several genes, including transcription factors, growth factors, and extracellular matrix proteins (Ning et al., 2004). These and other gene products likely function to stimulate the recruitment of inflammatory cells, cytokine secretion, cell death, and elevated protease production observed after prolonged cigarette smoke exposure (Carp and Janoff, 1978; Wright and Churg, 1990; Kuschner et al., 1996; Hautamaki et al., 1997; Sopori, 2002). As such, it is critical to examine how genes influence disease presentation so that precise mechanisms through which passive and active cigarette smoke contribute to COPD/emphysema can be identified.

General Mechanisms of COPD Pathogenesis

Numerous reviews that address COPD pathogenesis, its impact, and plausible therapies have been composed (Bridevaux and Rochat, 2011; Budinger and Mutlu, 2011; Caramori et al., 2011; Lugade et al., 2011; Rooney and Sethi, 2011). The intent of the current work is to concisely provide a foundational summary of conserved COPD modalities and discuss the plausible influence of receptors for advanced glycation end-products (RAGE) signaling. The prevailing pathogenic concept states that COPD is associated with chronic inflammation, imbalances between proteases/antiproteases, oxidative stress, and an elevated apoptotic index. Inflammation arising predominantly from neutrophilic contributions has been proposed due to enhanced neutrophil abundance in bronchoalveolar lavage (BAL) and sputum from COPD

patients (Thompson et al., 1989; Stanescu et al., 1996; O'Donnell et al., 2004). Levels of chemoattractants that recruit neutrophils and other potent inflammatory mediators are also elevated in COPD, including leukotriene B4 (Beeh et al., 2003), CXCL2 and 8 (Keatings et al., 1996; Tanino et al., 2002; Beeh et al., 2003), CXCL1 (Keatings et al., 1996), CXCL5 (Tanino et al., 2002), IFN-γ (Hodge et al., 2007), IL-1β (Thacker, 2006; Churg et al., 2009), and TNF-α (Barnes and Karin, 1997). Matrix metalloproteinases (MMPs) produced by macrophages and neutrophils are also misregulated in COPD (Shapiro, 1994). In particular, levels of MMP-1, MMP-2, MMP-7, MMP-9, and MMP-12 are all up-regulated in pulmonary tissue, BAL, and/or sputum of patients with COPD (Shapiro et al., 1993; Hautamaki et al., 1997; Ohnishi et al., 1998; Pratico et al., 1998; Shaykhiev et al., 2009), however because smoke exposed MMP-9 knock out mice are protected from emphysema, MMP-9 may require cooperation with other proteases during adverse lung remodeling (Atkinson et al., 2011) The chemical assessment of tobacco smoke reveals that it contains high levels of reactive oxygen species (ROS) that are in excess of intrinsic antioxidant defense mechanisms (Pauwels et al., 2001; Barnes et al., 2003). Generated in the airways, oxidants lead to cell dysfunction and/or death and also influence inflammatory signaling and protease augmentation via NF-κB-mediated mechanisms (Moodie et al., 2004). During the last decade, enhanced apoptosis stemming from diverse signaling pathways has also been implicated in alveolar septal cell loss observed in COPD patients (Kasahara et al., 2000, 2001; Tuder et al., 2003; Petrache et al., 2006). As a programmed event of removing unwanted cells and debris, apoptosis occurs via extrinsic signaling processes (Degterev et al., 2003), and intrinsic mitochondria or endoplasmic reticulum-mediated processes (Darmon et al., 1995; Slee

et al., 1999). In summary, COPD is characterized by progressive destruction of the distal lung and small airway obstruction resulting from chronic inflammation and elevated cell death.

Constituents of Tobacco Smoke

Tobacco smoke is a toxic and carcinogenic mixture of more than 5000 chemicals (Talhout et al., 2011). Of these, around 400 have been quantified, at least 200 are toxic to humans and/or experimental animals, and over 50 have been identified as known, probable, or possible human carcinogens (Kirsti, 2004). Studies indicate that compared with mainstream smoke collected under standard FTC/ISO smoking parameters, sidestream smoke has higher levels of PAHs (Grimmer et al., 1987; Evans et al., 1993), nitrosamines (Brunnemann et al., 1977, 1980; Hoffmann et al., 1979a; Ruhl et al., 1980), aza-arenes (Dong et al., 1978; Grimmer et al., 1987), aromatic amines (Patrianakos and Hoffmann, 1979), carbon monoxide (Hoffmann et al., 1979b; Rickert et al., 1984), nicotine (Rickert et al., 1984; Pakhale and Maru, 1998), ammonia (Brunnemann and Hoffmann, 1975), pyridine (Johnson et al., 1973; Brunnemann and Hoffmann, 1978), and the gas phase components 1,3-butadiene, acrolein, isoprene, benzene, and toluene (Brunnemann et al., 1990). In addition to these deleterious compounds, other factors such as the type of tobacco, the chemicals added to the tobacco, the way the tobacco product is smoked, and, for cigarettes and cigars, the material in which the tobacco is wrapped can also affect second-hand smoke chemical composition (International Agency for Research on Cancer, 2002; National Toxicology Program, 2005; US Department of Health and Human Services, 2006).

Cigarette smoke is also an important exogenous source of reactive glycation products capable of promoting formation of AGEs, advanced glycation end-products, which are

irreversibly glycated proteins that efficiently bind RAGE (Cerami et al., 1997). Studies have shown that both aqueous extracts of tobacco and cigarette smoke contain glycotoxins, highly reactive glycation products that can rapidly induce AGE formation on proteins in vitro and in vivo (Nicholl and Bucala, 1998; Nicholl et al., 1998). These activities can be eliminated by passing the samples through a dry packed column of aminoguanidine, a potent and specific inhibitor of AGE formation. Additional studies have shown that serum AGEs and apolipoprotein B-linked AGE levels are significantly elevated in cigarette smokers relative to non-smokers and AGEs or immunochemically related molecules are present at higher levels in the tissues of smokers compared to non-smokers, regardless of the presence of diabetes (Nicholl et al., 1998).

Receptor for Advanced Glycation End Products

RAGE are cell-surface receptors of the immunoglobulin superfamily expressed in many cell types including endothelial and vascular smooth muscle cells, fibroblasts, macrophages/monocytes, and epithelium (Brett et al., 1993). RAGE expression is most abundant in the lung, from which it was initially isolated, and is selectively localized to well-differentiated alveolar type I (ATI) epithelial cells (Schmidt, 2001). Identification in respiratory epithelium (Dahlin et al., 2004; Koslowski et al., 2004) and studies that document RAGE-mediated adherence to collagen IV (Demling et al., 2006) have led to the implication of RAGE in important developmental processes such as the spreading, thinning, and adherence that characterize the transitioning of ATII cells to squamous ATI cells. RAGE was first described as a progression factor in cellular responses induced by AGEs that accumulate in hyperglycemia and oxidant stress. Subsequent studies have distinguished RAGE as a pattern recognition receptor

that also binds S100/calgranulins, amyloid-β-peptide, and HMGB-1 (or amphoterin), to influence gene expression via divergent signal transduction pathways (Reddy et al., 2006; Hudson et al., 2008; Kim et al., 2008; Toure et al., 2008). Because RAGE expression can also increase when ligands accumulate (Schmidt, 2001), RAGE-ligand interaction may contribute to chronic pathological states where ligands are common including diabetic complications, neurodegenerative disorders, atherosclerosis, and inflammation (Hofmann et al., 1999; Taguchi et al., 2000). Specifically, a host of pro-inflammatory responses such as those coordinated by MAP kinases (ERK, JNK, and p38), NF-κB, ROS, and other pro-inflammatory mediators such as TNF and IL-1 (Bianchi et al., 2010) result from RAGE-ligand interactions (Figure 2.1). In contrast to short-lived cellular activation mediated by LPS, engagement of RAGE by its ligands results in prolonged inflammation (Lin et al., 2009). If left unchecked, such chronic inflammation results in severe tissue injury.

The full length membrane bound form of RAGE (mRAGE) contains an extracellular variable V-region-like immunoglobulin domain crucial for ligand binding and two constant C-region-like immunoglobulin domains, a single-pass hydrophobic transmembrane domain and a short, 43 amino acid, highly charged cytoplasmic domain essential for intracellular signaling (Buckley and Ehrhardt, 2010). The cytoplasmic domain of RAGE contains four possible phosphorylation sites, S391, S399, S400, and T401, of which only S391 is conserved among humans, mice, guinea pigs, rats, rabbits, dogs, and cats (Sakaguchi et al., 2011). Replacement of S391 to alanine was sufficient to abrogate PKCζ-dependent phosphorylation and subsequent signal transduction in vitro (Sakaguchi et al., 2011). Although not explicitly stated, RAGE behaves similarly to a receptor tyrosine kinase (RTK) cell surface receptor, requiring

homodimerization to effectively potentiate intracellular signaling cascades (Zong et al., 2010). Distinct alternative isoforms also exist for the receptor due to differential splicing variants of the RAGE message. Dominant negative RAGE (dn-RAGE) is a membrane anchored splice variant of RAGE capable of ligand binding but lacking the intracellular domain necessary for signal transduction. Endogenous secreted RAGE (esRAGE) is generated via alternative splicing at exon 9 yielding the same V and C-regions of the full length-RAGE but lacks both the hydrophobic transmembrane and the intracellular domains (Buckley and Ehrhardt, 2010). Additionally, full-length RAGE can be cleaved by MMPs to render sRAGE, a non-splice variant of RAGE closely resembling esRAGE in structure and function (Yamakawa et al., 2011). These altered variants of RAGE incapable of transducing signals are thought to function as decoy receptors that prevent the interaction of mRAGE with its ligands.

The pro-inflammatory role of RAGE in cardiovascular diseases is well documented (Yan et al., 2009). Furthermore, several studies strongly suggest that RAGE signaling is a key regulator of inflammation in major pulmonary diseases. A study demonstrated that abrogation of RAGE signaling (using RAGE null mice) attenuated pulmonary ischemia and reperfusion injury associated with decreased NF-κB activation and IL-8 production (Sternberg et al., 2008). Another important role for RAGE signaling in lung disease shows that RAGE-deficient mice under hyperoxic conditions survived longer than wild type controls and the mice had less airway cellularity and diminished alveolar damage compared to wild type controls (Reynolds et al., 2010). RAGE has been implicated in the fibrotic process in a number of tissues, including the peritoneum, kidney, and liver (Li et al., 2004; De Vriese et al., 2006; Xia et al., 2008), where it has been shown to promote fibrosis. In the lung, evidence continues to accumulate suggesting

an important role for RAGE in pulmonary fibrosis, yet conflicting data portray RAGE as having both protective and destabilizing functions. Acute lung injury (ALI) and a more severe condition known as acute respiratory distress syndrome (ARDS) are characterized by deterioration of the alveolar-capillary barrier and impaired alveolar fluid clearance (Lucas et al., 2009). ALI and ARDS are associated with damage to ATI cells, a population of cells with significant RAGE expression, and several different animal models of ALI express increased RAGE levels in BALF (Uchida et al., 2006; Su et al., 2007, 2009; Zhang et al., 2008). A published study from our laboratory considered the effects of smoke exposure on RAGE expression both in lung cells and mice (Reynolds et al., 2008). The research revealed that RAGE and its ligands were up-regulated in lung epithelial cells cultured with cigarette smoke extract (CSE) and that mice exposed to cigarette smoke for 6 months had elevated RAGE expression in pulmonary epithelium (Reynolds et al., 2008). While the full extent of RAGE function in smoke-induced COPD has not been sufficiently examined, these studies demonstrate that RAGE may play a role in COPD pathogenesis.

Contributions of RAGE to COPD Progression

RAGE and two of its ligands S100A12 and HMGB-1 were up-regulated in a rat alveolar type I-like cell line (R3/1), a human alveolar type 1I-like epithelial cell line (A549), and a macrophage-like murine cell line (RAW 264.7) following exposure to CSE (Reynolds et al., 2008). S100A12 is a calcium-binding pro-inflammatory modulator and HMGB-1 is a non-histone nuclear protein that acts as a potent pro-inflammatory mediator when secreted. In human lungs with smoke-related lesions, widespread RAGE expression has been documented in bronchiolar epithelia, small respiratory airways, reactive ATI cells, and alveolar macrophages

(AMs; Morbini et al., 2006). The same study identified elevated S100A12 in polymorphonuclear granulocytes and in extracellular fluid and the number and intensity of carboxymethyl-lysine positive cells (cells that stain for AGEs) were measurably enhanced in epithelial and inflammatory cells of the lungs of smokers (Morbini et al., 2006).

Another factor highly expressed in the lungs of smokers with COPD is early growth response gene 1 (Egr-1), a zinc finger-containing, hypoxia-inducible transcription factor (Ning et al., 2004). Egr-1 expression significantly increased in lung cell lines following CSE exposure in vitro and it activated the RAGE promoter (Reynolds et al., 2006, 2008). Because the RAGE gene also contains NF-κB and SP-1 promoter response elements (Li and Schmidt, 1997) and is transcriptionally regulated by cis-acting Egr-1 (Reynolds et al., 2006), a possible auto-inflammatory loop may be triggered suggesting cooperation between Egr-1 and RAGE in chronic smoke-related inflammatory disease states. More recently, it was discovered that Ras, a small GTPase that functions as a molecular switch in the control of diverse signaling cascades, was induced in R3/1 cells following exposure to CSE, resulting in up-regulation of NF-κB-mediated secretion of TNF-α, IL-1β, and IL-8 (Figure 2.1; Reynolds et al., 2011a).

Our lab has recently expanded research into the biology of smoke-exposed primary mouse AMs also known to express RAGE. Studies document that low levels of RAGE are expressed by mouse primary macrophages during normal conditions and that RAGE overexpression by these primary macrophages is associated with inflammation and the coordination of lung damage (Morbini et al., 2006). Our studies indicate that acute exposure of mice to CSE via nasal instillation resulted in diminished BAL cellularity and fewer AMs in RAGE null mice compared to controls. Additionally, AMs isolated from wild type mice exposed to CSE

significantly increased RAGE expression (Robinson et al., 2012). This recently published work also demonstrated for the first time that RAGE null AMs exposed to CSE experienced reduced Ras and p38 MAPK activation, less NF-κB translocation, and diminished expression of TNF-α and IL-1β when compared to CSE exposed wild type AMs (Figure 2.1). Evidence suggests that primary AMs coordinate CSE-induced inflammation, at least in part, via RAGE-mediated mechanisms and that cooperation with alveolar epithelium in coordinated inflammatory responses is likely.

Use of RAGE Transgenic Mice in Modeling Characteristics of COPD

Several animal models that seek to recapitulate various aspects of COPD have been presented within the past decade. These models include mouse IL-1β over-expressers (Lappalainen et al., 2005), rat VEGF signaling nulls (VEGF or VEGFR2 blockers: Kasahara et al., 2000), intratracheal administration of active caspase-3 (Aoshiba et al., 2003) and several others that aim to elucidate inflammatory and other destructive mechanisms during smoke-less and smoke-exposed disease progression (Petrache et al., 2005; Giordano et al., 2008; Kang et al., 2012). The vast majority of these models present emphysema-like anatomical characteristics and inflammatory indexes in the presence of room-air and notable exacerbation in the presence of cigarette smoke. Although RAGE has been shown to be a marker for many inflammatory diseases including COPD, a genetic mouse model for COPD had not been previously examined.

We generated a bi-transgenic in vivo mouse model that utilizes two transgenes to conditionally up-regulate RAGE (Figure 2.2). One transgenic mouse line employs surfactant protein C (SP-C) to drive expression of rtTA (reverse tetracycline transactivator) and another

transgenic line contains binding sites for a complex between rtTA and doxycycline (dox; Reynolds et al., 2011b). Although COPD is an adult lung disease, we initially sought to characterize RAGE bi-transgenic mice during development with the realization that aspects of COPD may be detected during organogenesis. Our model was thought to compliment research that centers on bronchopulmonary dysplasia (BPD), an embryonic disease highly correlated with emphysema in terms of oxidative stress, pulmonary inflammation, increased apoptosis, protease/antiprotease imbalance and altered microvasculature (Hargitai et al., 2001; Danan et al., 2002; Saugstad, 2003; Ekekezie et al., 2004; Speer, 2006). While COPD is characterized by sustained inflammation and alveolar destruction, remarkably similar mechanisms are implicated in the altered branching and impaired alveolarization observed in BPD (Bourbon et al., 2009).

Embryonic RAGE Bi-Transgenic Mice Have Perturbed Distal Epithelium

Complete perinatal lethality was observed when dox was supplied to RAGE bi-transgenic mice throughout embryogenesis. At embryonic day (E) 18.5, pulmonary tissues were severely hypoplastic and only minimal respiratory surface area near the visceral pleura remained. Several immunohistochemical and flow cytometric experiments demonstrated diminished abundance of differentiated distal lung cell types, most notably ATI and ATII cells (Reynolds et al., 2011b).

Altered cellular differentiation has not sufficiently been characterized in the distal lung of COPD patients; however, new research has emerged demonstrating that human ciliated cells can respond to cigarette smoke by promoting GDF15, a factor capable of driving Muc5A expression in goblet cells (Wu et al., 2011). RAGE and RAGE ligands have been implicated in

altered cellular differentiation of several cell types including smooth muscle cells, skeletal myocytes and developing neural tissue (Suga et al., 2011; Kim et al., 2012; Riuzzi et al., 2012). Thyroid transcription factor 1 (TTF-1; also known as Nkx2.1) is a key regulator of pulmonary development and present in distal lung epithelium that can negatively regulate RAGE expression (Reynolds et al., 2008) and SP-1 positively regulates the active promoter region of TTF-1 in surfactant producing cells (Das et al., 2011). Because NF-κB (a crucial intermediate of RAGE signaling) can interfere with SP-1 binding (Benjamin et al., 2010), RAGE may play a role in inhibited surfactant synthesis observed when ATII cells are abnormally regulated.

Embryonic RAGE Bi-Transgenic Mice Have Abnormal Distal Pulmonary Endothelial Cell Growth

In addition to the decreased cellularity of the lungs, RAGE over-production disturbed capillary growth and maintenance through the inhibition of FoxM1 (a critical transcription factor necessary for endothelial expansion) and PECAM (a marker for endothelial cells) expression (Geyer et al., 2011). Endothelial cell apoptosis has been observed in COPD patients using TUNEL, immunohistochemistry and DNA ligation techniques that coincided with the reduction of endothelial markers including VEGF and VEGFR2 (Kasahara et al., 2001). Additionally Dinh-Xuan et al. and Peinado et al. both showed that resected lung samples from COPD patients had extensive endothelial dysfunction, which they proposed to contribute to hypertension (Dinh-Xuan et al., 1991; Peinado et al., 1998). It is hypothesized that vascular tone in the lung can be regulated by direct stimulation of the vascular compartment by cigarette smoke and indirect stimulation stemming from smoke-exposed epithelial cells. Our discoveries relating to pulmonary endothelium in the RAGE bi-transgenic mouse correlate with numerous studies that demonstrate RAGE signaling in cases of depressed endothelial function and

increased barrier disruption (Sun et al., 2009; Pollreisz et al., 2010; Wolfson et al., 2011; Chen et al., 2012; Huang et al., 2012).

Embryonic RAGE Bi-Transgenic Mice Have Extracellular Matrix Abnormalities

We also demonstrated that MMP-9 secretion is increased, coincident with diminished collagen IV (a principle component of the alveolar basement membrane) deposition and production (Bukey et al., 2011). COPD is characterized by an increase in several MMPs including MMP-1, 2, 9, and 12 (Ohnishi et al., 1998; Geraghty et al., 2011). Other research groups have also demonstrated AGE-RAGE dependent mechanisms in MMP-9 production (Ishibashi et al., 2010; Zhang et al., 2010; Zhu et al., 2012). While not yet evaluated in our embryonic RAGE bi-transgenic mouse model, MMPs 1 and 2 have been implicated as RAGE targets (Kamioka et al., 2011; Du et al., 2012; Yu et al., 2012). Interestingly, MMP-1 has been shown to be up-regulated not only in the lungs of COPD patients but in osteoarthritis as well, a chronic inflammatory disease affecting articular cartilage (Steenvoorden et al., 2006). Ongoing research seeks to test hypotheses related to matrix-targeting protease imbalances such as those that involve α1-antitrypsin.

Embryonic RAGE Bi-Transgenic Mice Have Elevated Parenchymal Cell Apoptosis

Thorough evaluations of apoptosis were performed in order to ascertain causes for the hypoplastic lung phenotype in the embryonic RAGE bi-transgenic mouse. RAGE over-expressing lungs detrimentally declined during the canalicular phase, a period identified by terminal bronchiole branching, initial alveolarization, and microvascular organization. The abrupt loss of tissue was observed in tandem with a significant increase in pro-apoptotic Fas ligand (FasL), a

decrease in the anti-apoptotic factor Bcl-2, elevated cleaved active caspase-3 (a critical mediator of cell death), and quantifiable apoptosis by TUNEL assessment (Stogsdill et al., 2012). Electron microscopy also confirmed apoptosis via the detection of numerous bleb-like structures within cells that were physically separated from the underlying basement membrane. Importantly, cellular proliferation was not changed, suggesting there was no feedback mechanism to compensate for elevated cell death. Evidence is mounting that demonstrates active apoptosis of epithelial and endothelial cells in human COPD patients (Segura-Valdez et al., 2000; Kasahara et al., 2001; Majo et al., 2001; Yokohori et al., 2004; Hodge et al., 2005; Imai et al., 2005). Lending support for FasL-mediated apoptosis observed in RAGE bi-transgenic mice was research by Mahali et al. that demonstrated FasL elaboration is a direct product of AGE-RAGE ligation (Mahali et al., 2011). Furthermore, RAGE and its ligands have been shown to promote apoptosis in other tissue types, including myocytes (Tsoporis et al., 2010), endothelial cells (Chen et al., 2010), neuronal cells (Kim et al., 2011), epithelial cells (Jin et al., 2011), and pancreatic β-cells (Lee et al., 2010). Our studies have shown for the first time that increased expression of RAGE using transgenic mouse technology directly activates apoptosis in lung parenchyma. In fact, sustained RAGE expression during development is capable of modeling disorders characterized by cell loss including BPD. Furthermore, these data reveal important RAGE-mediated mechanisms that control cell quantity possibly introduced at the initiation of smoke-induced COPD pathogenesis.

Adult RAGE Over-Expression Yields an Emphysematous Lung

Conditional up-regulation of RAGE for 2 to 3 months in the adult bi-transgenic mouse lung lead to incremental dilation of alveolar spaces, assessed by standard H&E staining

(Stogsdill et al., 2011). Quantification of the mean chord length of the airspace revealed progressive dilation of alveolar spaces as RAGE over-expression persisted (unpublished data). The adult RAGE bi-transgenic mice had increased MMP-9 and decreased elastin expression consistent with other COPD models. Furthermore, RAGE bi-transgenic mice manifested significant inflammation measured by elevated BALF protein, leukocyte infiltration, and secreted cytokines (MIP-2, IFN-γ; Stogsdill et al., 2011). These data support the concept that innovative transgenic mice that over-express RAGE may model pulmonary inflammation and alveolar destabilization independent of tobacco smoke. Furthermore, it validates RAGE signaling as a target pathway in the prevention or attenuation of smoke-related inflammatory lung diseases.

Conclusions

Despite the progression in the field of RAGE biology in the context of lung disease, the full extent of RAGE localization, the molecular mechanisms that control its expression and its downstream effects should remain topics of focused investigation. While a great deal is known about COPD, relatively little is known about factors that perpetuate inflammation or modalities that sustain them. Our research has shown that mechanisms of COPD progression including chronic inflammation, imbalances involving proteases, oxidative stress, and elevated apoptosis may be mediated by RAGE. Several endogenous (S100/calgranulins, HMGB-1, AGEs) and exogenous ligands (cigarette smoke) may be responsible for the sustained activation of RAGE leading to disease progression (Figure 2.1). As such, it remains possible that targeting RAGE may, at least in part, provide successful opportunities in the therapeutic alleviation of debilitating inflammatory lung disease exacerbated by tobacco smoke.

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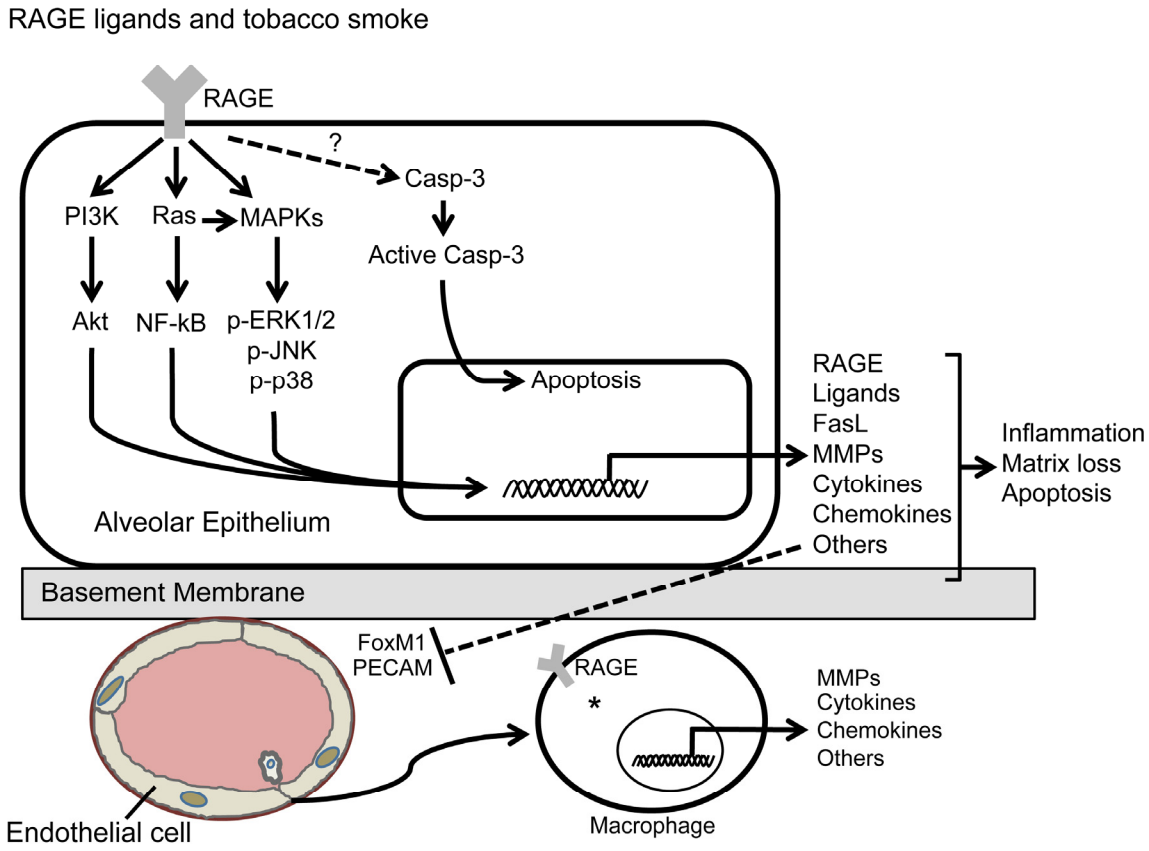

Figure 2.1: RAGE Signaling Pathways. Deleterious effects characteristic of COPD are elicited via several pro-inflammatory signaling pathways observed in RAGE-expressing alveolar epithelial cells and resident alveolar macrophages (*). Direct stimulation of RAGE by tobacco smoke, *de novo* AGE generation in a tobacco smoke environment, or genetic up-regulation of RAGE in the lungs of conditional bi-transgenic mice results in characteristics of COPD including inflammation, matrix destabilization, and apoptosis.

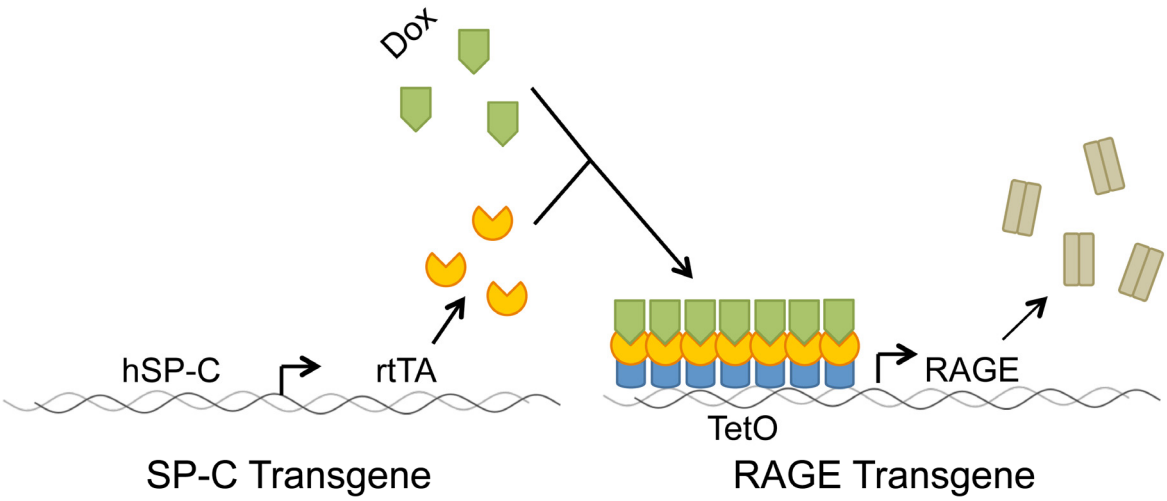

Figure 2.2: RAGE Over-Expression. Full length RAGE was over expressed in alveolar type (AT) II cells by obtaining progeny from two transgenic lines of mice. The reverse tetracycline transactivator (rtTA) was produced under the control of the human surfactant protein C (hSP-C) promoter in distal respiratory epithelium. Following the administration of doxycycline (dox), a dox-rtTA complex activates the TetO promoter, thereby expressing RAGE.

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CHAPTER 3: Targeting of Receptors for Advanced Glycation End-Products (RAGE) Diminishes Acute Secondhand Smoke-Induced Inflammation in Mice

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Abstract

The receptor for advanced glycation end-products (RAGE) has increasingly been demonstrated to be an important modulator of inflammation in cases of pulmonary disease. Published reports involving tobacco smoke exposure have demonstrated increased expression of RAGE, its participation in pro-inflammatory signaling and its role in irreversible pulmonary remodeling. The current research evaluated the *in vivo* effects of short-term secondhand smoke exposure in RAGE knock out and control mice compared to identical animals exposed to room air only. Quantitative PCR, immunoblotting, and immunohistochemistry revealed elevated RAGE expression in controls after four weeks of exposure and an anticipated absence of RAGE expression in RAGE knock out mice regardless of smoke exposure. Inflammatory cell behaviors were assessed by measuring Ras activation, NF-κB activity, and cytokine elaboration. Furthermore, bronchoalveolar lavage fluid (BALF) was procured from RAGE knock out and control animals prior to assessment of inflammatory cells and molecules. As a general theme, inflammation induced by tobacco smoke exposure was influenced by the availability of RAGE. These data reveal captivating information suggesting a role for RAGE signaling in lungs exposed to tobacco smoke and further research is necessary in order to fully explain roles for receptors such as RAGE in cells coping with exposure.

Keywords: RAGE, tobacco, lung,

Running Title: RAGE protects from tobacco-induced inflammation

Introduction

Current trends indicate that by 2030, tobacco use will kill an estimated 9 million people annually (Xie, 2008). The link between tobacco smoke and COPD is well established. Several factors contribute to the progression of COPD, including chronic inflammation, oxidative stress, protease/antiprotease imbalance, and apoptosis (MacNee, 2005). In cases of COPD, chronic inflammation results from tobacco smoke exposure in 80-90% of cases and oxidative stress resultant from smoking delineates COPD pathogenesis (Moussa, 2014). While it has been observed, the relationship between secondhand smoke (SHS) and COPD has only recently been contemplated (Leberl, 2013). COPD presents a significant burden internationally, costing $49 billion in the United States alone. It is currently the third most prevalent cause of death in the United States and the fourth worldwide (Pauwels 2001, Minino 2010), and is projected to become the third leading cause of death worldwide by 2020 (Vijayan, 2013). A recent review of 114 publications containing 155 studies concluded that animals exposed to SHS and mainstream exposure noted that it was highly evident that SHS has many of the same mechanisms and detrimental effects that mainstream smoke does, as indicated by increase expression cytokine/chemokine levels including TNF-α and IL-8 (Leberl, 2013).

Of particular interest to our lab is the participation of RAGE in pulmonary inflammation. As a cell-surface membrane protein of the immunoglobulin superfamily, RAGE plays an important role in intracellular signaling, including developmental tasks including cell spreading and adherence (Winden, 2013). Following organogenesis, RAGE is localized to the basolateral membrane of differentiated human alveolar epithelial cells, where it influences interactions with the extracellular matrix and may contribute to structural and apoptotic changes associated

with maintenance of alveoli (Demling, 2006, Stogsdill, 2012). RAGE also has a well-known inflammatory role in cardiovascular and pulmonary diseases, among others (Robinson, 2012). Due to its importance in several inflammatory conditions, RAGE abrogation has been suggested as a possible therapeutic intervention for COPD (Chen, 2013).

While RAGE has been for the focus of several studies, even specifically in the context of tobacco smoke exposure, the current undertaking is the first study to our knowledge that presents data detailing inflammation in the context of RAGE availability after acute SHS exposure. This work confirms *in vitro* studies from our laboratory showing inflammatory abrogation after siRNA knockdown of RAGE in human cells (Reynolds, 2010). General assessments included histological evaluations and the characterization of bronchoalveolar lavage fluid. To assess molecular markers of inflammation, immunoblotting, quantitative PCR, and IHC analysis of RAGE were conducted.

Evaluating SHS and not primary smoking provides additional impact in the context of respiratory disease. Many studies have proposed a correlation between tobacco smoke exposure (mainstream or secondhand), and Acute eosinophilic pneumonia (AEP) (Philit, 2002; Uchiyama, 2008; Shorr, 2004). A recent study from Kyung Chung et al. demonstrated the development of AEP after only 4 weeks of secondhand smoke exposure (Kyung Chung, 2014). To increase the realism of involuntary smoke exposure, we used nose-only exposure of SHS (InExpose System, Scireq). Traditionally, tobacco smoke studies have utilized the particulate fraction of cigarette smoke, and not the complete aerosol. More than 6000 chemicals have been identified in tobacco smoke aerosol and recent emphasis on direct exposure to the full

makeup of primary and SHS (Okuwa, 2010; Aufderheide, 2003; Scian, 2009; Phillips, 2005) presents a more realistic approach for comparison to human tobacco use (Thorne, 2014).

Materials and Methods

Mice: Wild type mice of C57BI/6 background were obtained from Jackson Laboratories (Bar Harbor, ME). RAGE knock out mice were generated on a C57BL/6 background. Mice were housed in a conventional animal facility, supplied with food and water *ad libitum*, and kept on a 12-hour light—dark cycle.

Cigarette Smoke Exposure: Mice were exposed to SHS generated from 3R4F research cigarettes from Kentucky Tobacco Research and Development Center, University of Kentucky in nose-only exposure system (InExpose System, Scireq). Mice were individually placed in soft restraints and connected to an exposure tower, wherein a puff of smoke generated every minute results in 10 seconds of CS exposure followed by 50 seconds of fresh air. 5 mice in each group were exposed to smoke from two cigarettes over 10 minutes, then allowed to breathe room air for 10 minutes, then exposed to smoke from one cigarette over an additional 10 minutes. This procedure was repeated daily for 4 weeks and compared to similar groups of mice (n=5 per group) restrained and exposed to room air for the same duration over the same period of time. The SHS challenge was determined to be at an acceptable level of particulate density concentration according to previously published reports (Rinaldi et al., 2012; Vlahos et al., 2010). Animal use was in accordance with IACUC protocols approved by Brigham Young University.

Anesthesia: Avertin was freshly prepared before the mouse sacrifices by dissolving 10g of tribromoethyl alcohol into tertiary amyl alcohol. This solution was vortexed to dissolve all crystals, then portioned into aliquots of .25ml in 15ml tubes stored at 4°C and protected from light. 9.75ml of ddH20 are added to an aliquot as needed for surgeries. The resultant 2.5% avertin solution is used as needed for the surgeries, and not used more than 10 days from dilution. The avertin was administered via a 29.5 gauge needle and syringe into the lower abdomen. About .30 ml was given to each mouse. Full anesthesia was ensured prior to surgical exsanguination.

Bronchoalveolar Lavage: Immediately after euthanasia, the trachea was exposed and cannulated with a 20-gauge catheter. PBS was lavaged according to the weight of the mouse prior to surgery, and removed. Lavage fluid was then centrifuged at 4°C, following which total cells were counted via hematocytometer. An aliquot of cells was used for a differential count using a Wright stain.

Histology and Immunohistochemistry: After euthanasia, one lung from animals exposed to smoke or room air was inflation-fixed at 25 cm of water pressure with 4% paraformaldehyde in PBS for 1 minute, processed, and sectioned to 5 microns. The other lung was split in two and frozen in liquid nitrogen for RNA and protein isolation. Sections were stained with antibodies against RAGE and the appropriate biotinylated secondary antibodies (Winden et al., 2013).

Protein and RNA Characterization: Total protein from whole lung was obtained after tissue homogenization with RIPA buffer supplemented with protease inhibitors (Fisher Scientific). Following centrifugation, the supernatant was analyzed with BCA Protein Assay Kit (Thermo

Scientific) for quantification. RNA was isolated using TRIZOL reagent (Invitrogen). Optical density was employed to determine RNA concentration following isolation. Immunoblotting for RAGE protein and quantitative PCR for RAGE mRNA were completed as already outlined (Stogsdill 2013). Concentrations of IL-8 were obtained using a molecule-specific ELISA kit used as directed in the manufacturer's instructions (RnD Systems, Minneapolis, MN).

Results

Mice Exposed to Acute SHS Up-Regulate RAGE Expression

Mice were exposed to 4 weeks of SHS as outlined and control mice exposed to room air were maintained for comparison. Quantitative RT-PCR demonstrated that acute SHS lead to a significant increase in the transcription of RAGE mRNA in wild type mice when compared to room air exposed controls (Figure 3.1A). As expected, RAGE was not detected in RAGE knock out mice regardless of smoke exposure (Figure 3.1A). Immunoblotting was completed In order to correlate RAGE protein expression with mRNA levels. Blotting for RAGE revealed that SHS was sufficient to increase RAGE levels when compared to room air controls (Figure 3.1B). As was the case with the mRNA assessments, no RAGE protein was detected in RAGE knock out animals (not shown). We next completed a qualitative evaluation of RAGE expression in the SHS-exposed lung. Immunohistochemistry revealed that RAGE expression increased in the lung parenchyma following SHS exposure (Figure 3.2) when compared to basal RAGE expression observed in the room air-exposed wild type animals (Figure 3.2).

RAGE Mediates the Activation of SHS-Induced Ras and NF-κB

In order to identify downstream molecules that participate in RAGE signaling during short-term inflammatory response to SHS, quantitative assessments of active Ras and NF-κB were evaluated. Lungs from SHS-exposed wild type mice presented significantly increased levels of active Ras when compared to room air controls (Figure 3.3). However, Ras activation was not significantly different in RAGE knock out animals notwithstanding smoke exposure. Furthermore, Ras activity was significantly decreased in SHS-exposed RAGE knock out animals compared to SHS-exposed wild type animals (Figure 3.3).

NF-κB is a central intermediate in RAGE signaling that bridges the gulf between intracellular signal transduction and a programmed nuclear response. We observed that NF-κB activity was significantly increased in the lungs of wild type animals exposed to SHS when compared to room air controls (Figure 3.4). We also discovered that NF-κB activity was significantly decreased in SHS-exposed lungs that lack RAGE expression when compared to RAGE-expressing lungs following SHS exposure (Figure 3.4). These results reveal that SHS exposure correlated with increased levels of these important inflammatory perpetuation markers, while protection from activation was afforded by absence of RAGE.

RAGE Ablation Diminishes SHS-Induced Pulmonary Inflammation

To further characterize SHS-mediated inflammatory responses, markers of pulmonary inflammation were assessed in bronchoalveolar lavage fluid (BALF) obtained from each experimental group. Because cells respond to stresses by secreting into and altering BALF, its classification is an excellent measure to evaluate organ-level responses. BALF from wild type mice exposed to SHS had significantly more protein compared to room air controls (Figure

3.5A), suggesting elevated vascular permeability coincident with extravasation observed in inflammation. Conversely, protein levels were not different in RAGE knock out lungs in either the presence or absence of SHS exposure (Figure 3.5A). BALF also showed a SHS-mediated increase in total BALF cells (Figure 3.5B). While total cells were elevated in RAGE knock out lungs with and without SHS, there was no SHS-mediated increase when compared to controls (Figure 3.5B). An evaluation of the cells observed in BALF led to the discovery that polymorphonuclear cells (PMNs) were significantly increased in wild type BALF after SHS exposure; however, RAGE knock out lungs did not induce PMN extravasation during SHS exposure (Figure 3.5C). Lastly, IL-8, a potent chemoattractant that functions as a pro-inflammatory cytokine, was quantified in BALF. There was a significant increase in secreted IL-8 in wild type BALF after SHS exposure when compared to BALF from room air exposed controls (Figure 3.6). RAGE abrogation in knock out lungs was sufficient to significantly inhibit SHS-induced IL-8 secretion (Figure 3.6).

Discussion

Though often overlooked, the interaction between lungs and the atmospheric air accounts for a significant portion of the exogenous materials humans encounter. Historical records indicate inhaled agents for medicinal purposes have been in use for more than 4000 years (Rau, 2005). Under normal circumstances, the lung uses cytokines, resident macrophages, and other typical immune response factors to respond to exogenous pathogens (Laskin, 2009). In the case of tobacco smoke exposure, persistence of these inflammatory mediators may account for the exaggerated inflammatory response that leads to the COPD phenotype (Robinson, 2012). Our discovery that RAGE is increased in the lungs of mice

exposed to SHS demonstrates that this effective modulator of inflammation functions when lungs are involuntarily exposed to side stream smoke. In fact, the observation that RAGE is elevated in the lungs of SHS-exposed mice opens a new area of RAGE research; further implicating RAGE as a dynamic, multi-functional mediator of lung disease.

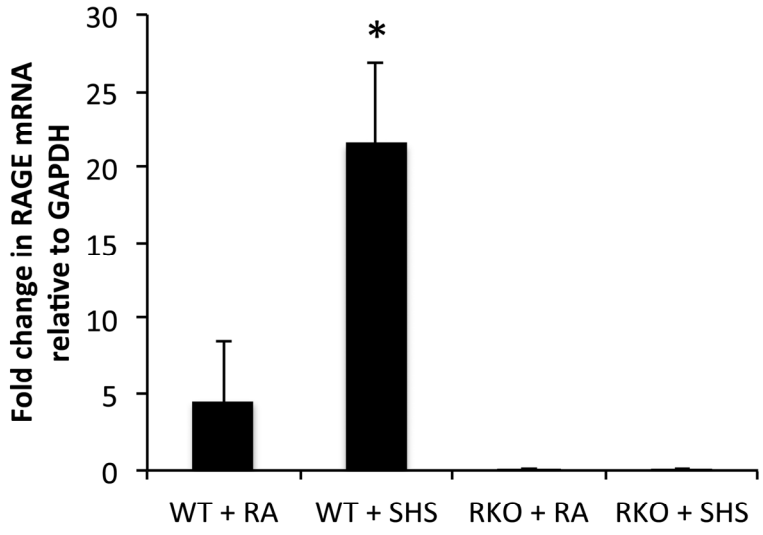
To our knowledge this is the first study to investigate the inflammatory impact of the absence of RAGE via RAGE knock out animals in the context of SHS exposure. Our lab has provided unequivocal evidence for RAGE-mediated inflammation due to tobacco smoke (Reynolds, 2010; Reynolds, 2006; Reynolds, 2011; Robinson, 2012, Winden, 2012). That inflammation was seen after only 4 weeks of acute SHS exposure confirms the harmfulness of SHS as an agent that exacerbates lung disease complications. Secondly, protection in RAGE knock out animals from enhanced activation of pro-inflammatory signaling intermediates such as Ras and NF-κB further clarify a central role or RAGE in inflammation. However, additional research is still necessary because of the incomplete protection from SHS exposure in RAGE null animals that indicates other parallel pathways through which inflammatory signals are conveyed.

Relevance of Research

This research is medically relevant because it provides a clear snapshot of the potential initial triggers of inflammation that can lead to prolonged COPD diagnoses. It is well understood that smoking is harmful to health, however debate continues regarding the status of SHS and disease progression. This research helps mechanistically clarify the initiation of inflammation, and indicates how genetic factors may contribute to individual susceptibility to COPD. Physicians have begun to recognize the importance of cytokines in COPD patients as

evidenced by current studies on the use of blocking antibodies for TNFα and IL-8 and planned studies employing antibodies against IL-17, IL-18, IL-1β, and TSLP (Caramori, 2014). Though these studies have yet to unlock the key to reversing COPD, the prominent reduction of the inflammatory response in RAGE knock out animals indicates the possibility of finding successful therapeutic targets (cytokines, receptors, transcription factors, or intermediates) in the inflammatory cascade.

A



B

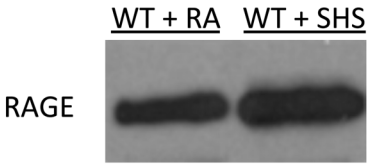


Figure 3.1: RAGE Quantification. A, There was a significant increase in the expression of RAGE mRNA by wild type mice exposed to secondhand smoke (SHS) compared to animals exposed to room air (RA). Transcripts were normalized to GAPDH and representative data from experiments performed in triplicate are shown. *$p \leq 0.05$. B, Immunoblotting revealed that RAGE was increased in WT + SHS animals compared to WT + RA.

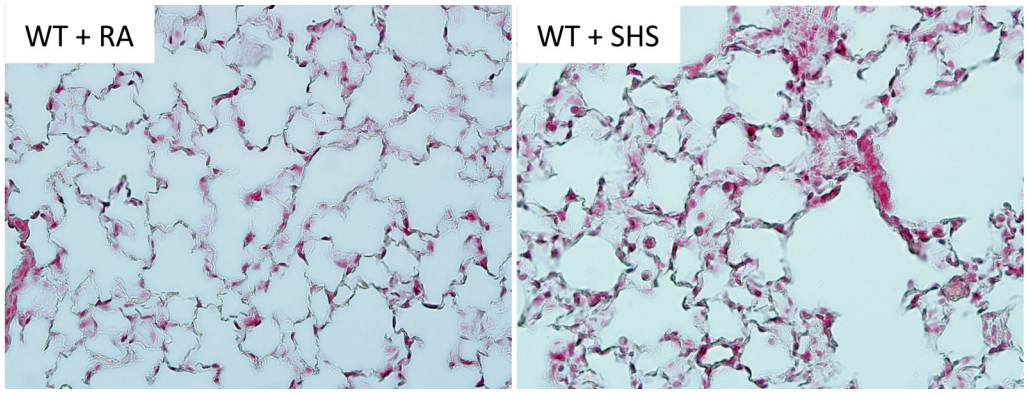


Figure 3.2: RAGE Immunohistochemistry. Immunohistochemical staining for RAGE demonstrated increased expression in the lung parenchyma following SHS exposure for 4 weeks compared to RA controls.

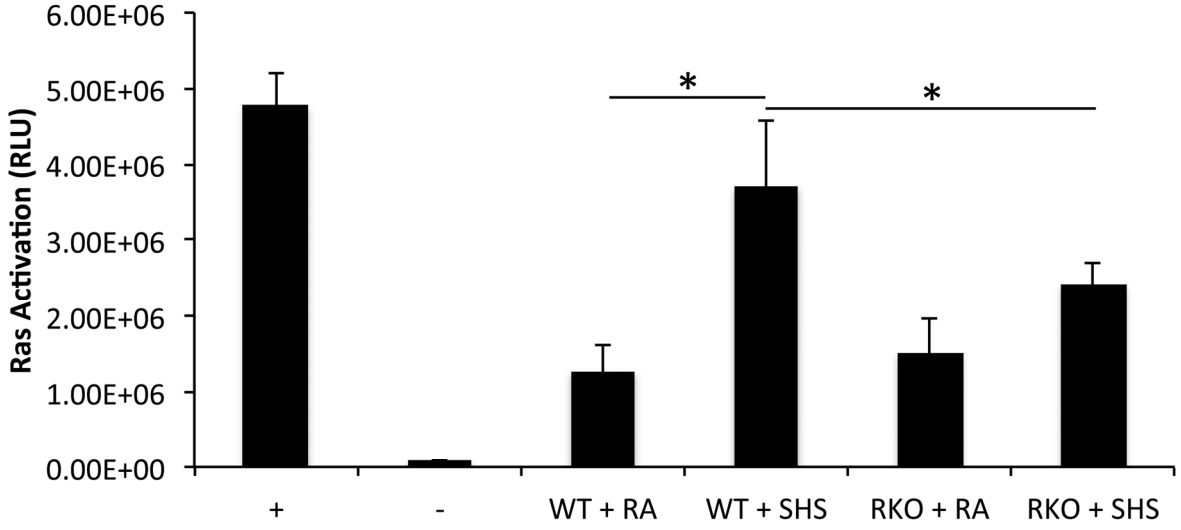


Figure 3.3: Ras Elisa. Active Ras was significantly elevated in wild type animals following SHS exposure compared to RA controls and active Ras was significantly decreased in RKO + SHS compared to WT + SHS. Assessments of Ras activity included positive (+) and negative controls (-). Data are representative of triplicate experiments and *$p \leq 0.05$.

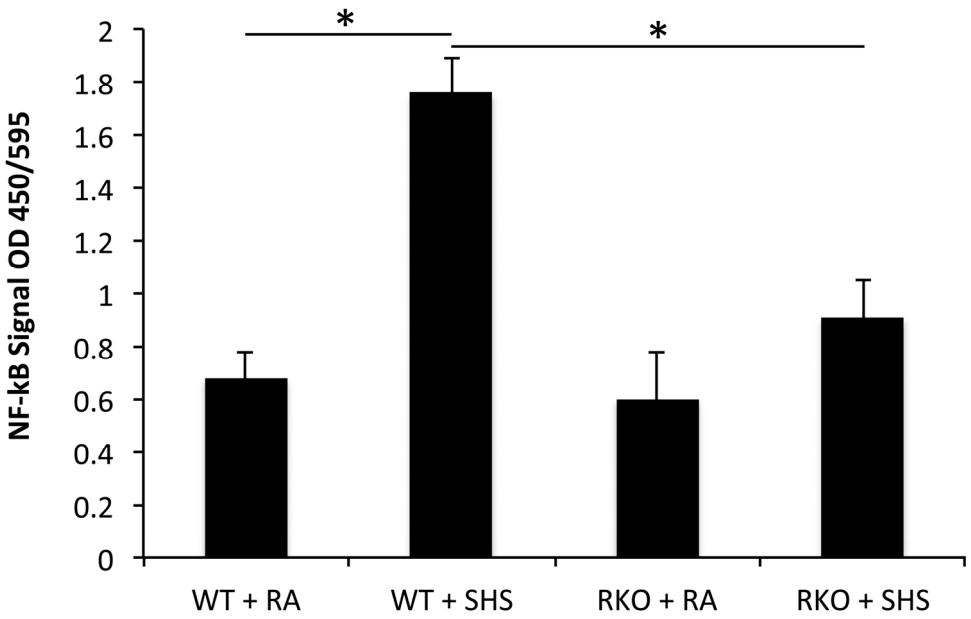


Figure 3.4: NF-κB Elisa. Active NF-κB was significantly increased after exposure of WT mice to SHS. NF-κB was still elevated in RKO + SHS; however, WT + SHS mice had significantly more active NF-κB when compared to RKO + SHS mice. Data are representative of triplicate experiments and *$p \leq 0.05$.

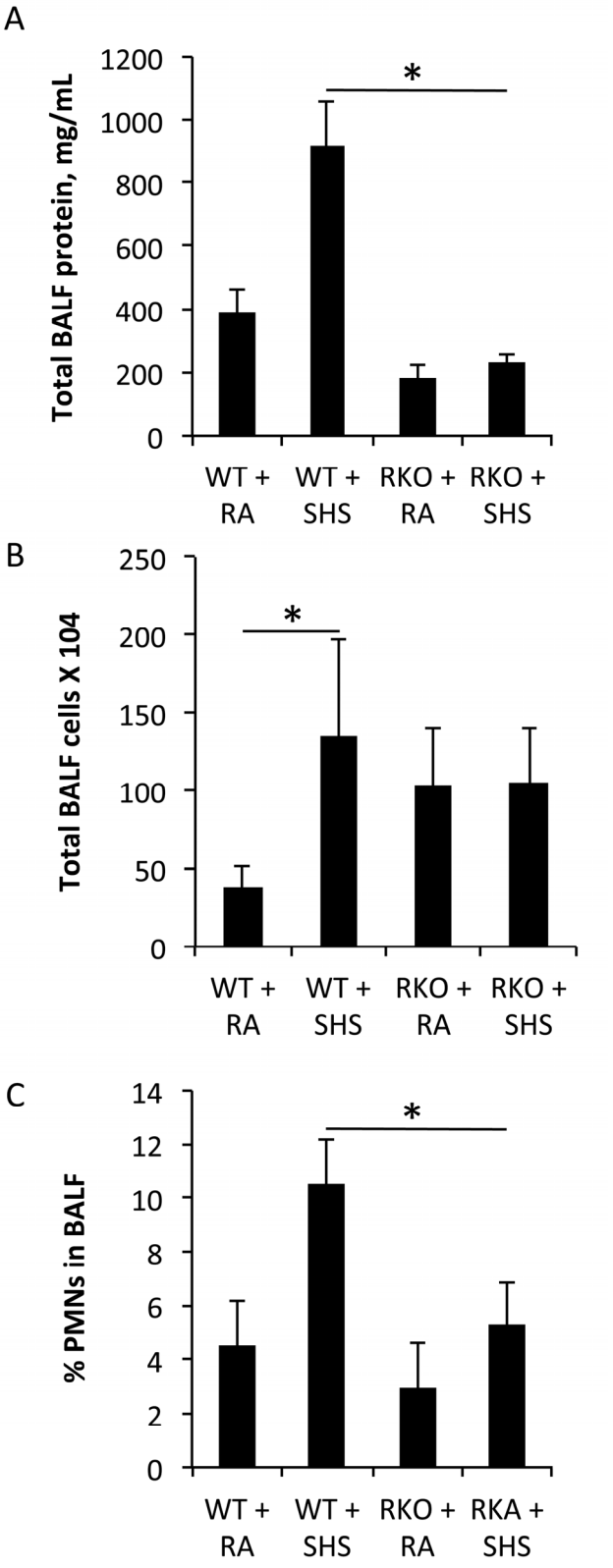


Figure 3.5: BALF Total Protein and Cells. A, Total BALF protein was assayed using the BCA technique to demonstrate vascular permeability. Protein was significantly elevated in WT + SHS animals compared to WT + RA. There was no change in RKO animals. Data are representative of triplicate experiments and $*p \leq 0.05$. B, Total BALF cells were significantly increased in WT + SHS animals compared to WT + RA. There was no change in total cells in the RKO animals. Data are representative of triplicate experiments and $*p \leq 0.05$. C, The percentage of PMNs was significantly higher in WT + SHS compared to WT + RA animals. There was no change in % PMNs in RKO + SHS mice. Data are representative of triplicate experiments and $*p \leq 0.05$.

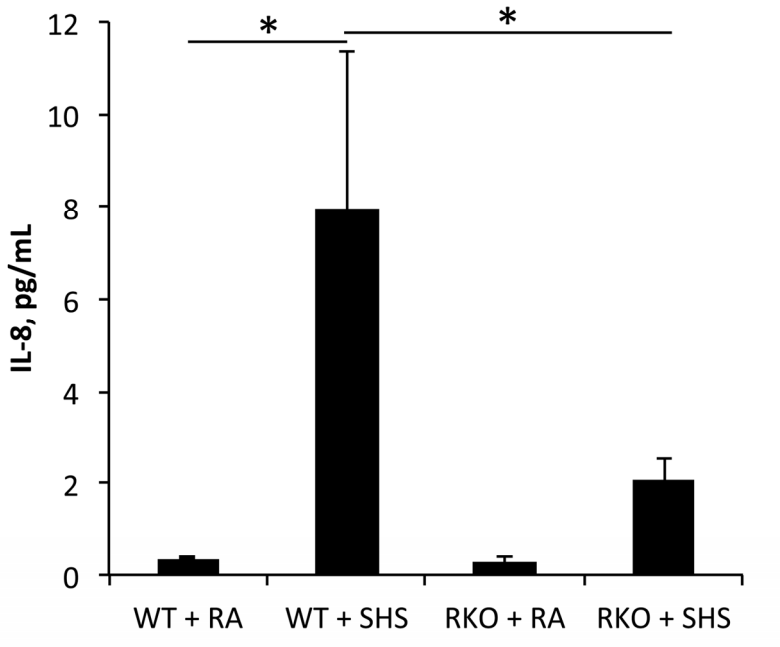


Figure 3.6: IL-8 Elisa. Secreted IL-8 was assessed by ELISA and there was a significant increase in BALF from WT + SHS compared to WT + RA. RKO + SHS was significantly lower compared to WT + SHS. Data are representative of triplicate experiments and $*p \leq 0.05$.

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Educational Background

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Brigham Young University, Department of Physiology and Developmental Biology. Bachelor of Science Degree, 2012.

Professional Organizations, Awards and Service

- American Physiological Society
- Operating Room Volunteer at UVRMC, Provo, UT
- LDS mission to Raleigh North Carolina
- BYU Academic Scholarship
- Eagle Scout

Professional Experience

- BYU Department of Physiology and Developmental Biology 2013–Current
  Provo, Utah
  Research Assistant: Responsibilities: Researched the molecular mechanism of tobacco smoke induced inflammation via experimentation on mouse models.

- Trusty Tree Care 2013–Current
  Provo, Utah
  Residential Tree Technician: Responsibilities: Prune and remove residential trees, grind stumps, ensure jobsite safety.

- Orrock Mendenhall Professional Physical Therapy 2012
  Alpine, Utah
  Physical Therapy Aide: Responsibilities: Assist patients through strengthening and flexibility exercises. Administer electrical stimulation, ultrasound, and massage, ensure patient safety.

- Ecofirst Pest Control 2011
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  Route Manager: Responsibilities: Outside sales.

Research Publications

Peer-Reviewed Publications

1. Stogsdill M.P., Stogsdill J.A., Bodine B.G., Fredrickson A.C., Sefcik T.L., Wood T.T., Kasteler S.K., and Reynolds P.R. 2013. Conditional RAGE over expression in the adult murine lung causes airspace enlargement and induces inflammation. *Am J Resp Cell Mol Biol* 49(1):128-134.
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Accepted Publications

1. Jimenez, F.R., Lewis J.B., Belgique S.T., Wood T.T. and Reynolds P.R. Developmental lung expression and transcriptional regulation of Claudin-6 by TTF-1, Gata-6, and FoxA2. *Respir Res.* Accepted.

Abstracts

1. Jimenez F.R., Lewis J.B., Belgique S.T., Wood T.T. and Reynolds P.R. 2014. Pulmonary expression and regulation of Cldn6 by tobacco smoke. *FASEB J* 28:834.3.
2. Wood T.T., Winden D.R., Barton D.B., Betteridge B.C., Marlor D.R., Wright A.J., Jones C.M., Chavarria M., Rogers G.D., and Reynolds P.R. 2014. Targeted mice reveal a role for RAGE in an early inflammatory response to tobacco smoke. *FASEB J* 28:834.4.
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References

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