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Acute secondhand smoke-induced pulmonary inflammation is diminished in RAGE knockout mice

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Acute secondhand smoke-induced pulmonary inflammation is diminished in RAGE knockout mice. Am J Physiol Lung Cell Mol Physiol 307: L758–L764, 2014. First published September 26, 2014; doi:10.1152/ajplung.00185.2014.—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 proinflammatory signaling, and its role in irreversible pulmonary remodeling. The current research evaluated the in vivo effects of short-term secondhand smoke (SHS) exposure in RAGE knockout and control mice compared with identical animals exposed to room air only. Quantitative PCR, immunoblotting, and immunohistochemistry revealed elevated RAGE expression in controls after 4 wk of SHS exposure and an anticipated absence of RAGE expression in RAGE knockout mice regardless of smoke exposure. Ras activation, NF-xB activity, and cytokine elaboration were assessed to characterize the molecular basis of SHS-induced inflammation in the mouse lung. Furthermore, bronchoalveolar lavage fluid was procured from RAGE knockout and control animals for the assessment of inflammatory cells and molecules. As a general theme, inflammation coincident with leukocyte recruitment was induced by SHS exposure and significantly influenced by the availability of RAGE. These data reveal captivating information suggesting a role for RAGE signaling in lungs exposed to SHS. However, ongoing research is still warranted to fully explain roles for RAGE and other receptors in cells coping with involuntary smoke exposure for prolonged periods of time.

RAGE; lung; secondhand smoke; inflammation

A LINK BETWEEN TOBACCO SMOKE and chronic obstructive pulmonary disease (COPD) is well established. COPD is currently the third most prevalent cause of death in the United States and the fourth worldwide (18, 23). Furthermore, COPD is projected to become the third leading cause of mortality by 2020 (42). Several factors contribute to the progression of air-space enlargement and airway inflammation observed in COPD, including chronically active inflammatory axes, oxidative stress, protease/antiprotease imbalance, and apoptosis (16). 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 (19). While a relationship between secondhand smoke (SHS) and COPD has recently been documented, confirma
tory studies of such a link has only recently been contemplated (15). A recent review of 114 publications containing 155 studies involving both primary and SHS concluded that SHS functions via many of the same mechanisms implicated in primary smoking and elicits similar detrimental effects as indicated by increased expression of cytokines responsible for acute inflammation including tumor necrosis factor- (TNF-), and IL-8 (15, 36).

Of particular interest to our lab is the participation of the receptor for advanced glycation end-products (RAGE) in pulmonary inflammation. RAGE is a surface membrane protein of the immunoglobulin superfamily expressed by many cell types including endothelial and vascular smooth muscle cells, fibroblasts, macrophages/monocytes, and epithelium (3, 40). RAGE is purported to play an important role in intracellular signaling observed during the coordination of developmental tasks such as cell spreading and adherence during organ formation (46). Following organogenesis, however, RAGE expression persists at the basolateral membrane of differentiated alveolar epithelial cells, where it influences interactions with the extracellular matrix and may contribute to structural and apoptotic changes associated with alveolar epithelial cell maintenance (8, 38). RAGE also has inflammatory roles in cardiovascular and lung diseases including pulmonary fibrosis, asthma, pneumonia, and acute lung injury, among others (7, 10, 33). Of interest to the current investigation, RAGE expression is prominently detected in the alveolar compartments of smokers with COPD (48). Additionally, studies have revealed inflammatory abrogation after siRNA knockdown of RAGE in human lung cells (27) and impaired smoke-induced lung inflammation mediated by primary pulmonary macrophages isolated from RAGE knockout mice (32). Due to its importance in diverse inflammatory conditions, RAGE abrogation has been suggested as a possible therapeutic intervention for COPD (5).

While RAGE has been a focus of several tobacco smoke-related studies, the current undertaking is the first study that presents data detailing lung inflammation in the context of RAGE deficiency after acute SHS exposure. Previous studies using primary cigarette smoke extract (CSE) exist, but no in vivo analyses of lung inflammation involving RAGE-deficient mice exposed to CSE or SHS have been disseminated. Evaluating SHS and not primary smoking provides notable impact in the context of respiratory disease progression. Initially, tobacco
smoke studies utilized the particulate fraction of cigarette smoke, and not the complete aerosol. More than 6,000 chemicals have been identified in tobacco smoke aerosol, and recent emphasis on direct exposure to the full content of primary and SHS (1, 22, 24, 34) presents a more realistic approach for comparison to human tobacco use (41). To translate applicable effects of involuntary smoke exposure, we utilized a nose-only exposure system calibrated to deliver controlled doses of SHS (InExpose System, Scireq). Specifically, we exposed RAGE knockout and wild-type mice to SHS to determine to what extent RAGE is required for SHS-induced pulmonary inflammation.

MATERIALS AND METHODS

Mice. Wild-type mice in a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME). RAGE knockout mice were also generated on a C57BL/6 background. Mice were housed in a conventional animal facility, supplied with food and water ad libitum, and maintained on a 12 h light-dark cycle.

Secondhand cigarette smoke exposure. Mice were exposed to SHS generated from 3R4F research cigarettes from Kentucky Tobacco Research and Development Center, University of Kentucky, via a nose-only exposure system (InExpose System, Scireq, Montreal, Canada). Mice were individually placed in soft restraints and connected to an exposure tower, wherein a computer-controlled puff of smoke generated every minute resulted in 10 s of SHS exposure followed by 50 s of fresh air. Six mice in each group were exposed to SHS from two cigarettes over 10 min, allowed to breathe room air for 10 min, and then exposed to smoke from one cigarette for an additional 10 min. This procedure was repeated 5 days a week for 4 wk and compared with similar groups of mice (n = 6 per group) restrained and exposed to room air 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 (31, 43) and was tolerated without evidence of toxicity. The specific total particulate density concentration was measured weekly and an average of 132.6 mg total particulate matter per m³ in the tower was detected. Furthermore, this nose-only model of smoke exposure yielded chronic blood carboxyhemoglobin levels of ~5%, a value similarly observed in human smokers (47). Animal use was approved by the Institutional Animal Care and Use Committee at Brigham Young University.

Protein and RNA characterization. To assess RAGE expression in mouse lung tissue, we performed quantitative real-time RT-PCR (qPCR) and immunoblotting for RAGE under conditions already described in detail (26). RNA was isolated with Trizol reagent (Invitrogen, Grand Island, NY), and optical density measurement was employed to determine RNA concentration following isolation. Total protein from whole lung was obtained after tissue homogenization with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors (Fisher Scientific, Waltham, MA). Isolated protein was precisely quantified with a BCA Protein Assay kit (Fisher Scientific), and specifically 20 µg of total lung protein was used in blotting. Immunoblotting for RAGE protein using a mouse polyclonal antibody (#AF1179, RnDSystems, Pittsburg, PA) and qPCR for RAGE mRNA were completed as already outlined (39). Band densitometry was performed using Un-Scan-It software (Silk Scientific, Orem, UT) and digitized images of the resulting immunoblot.

Immunohistochemistry. Qualitative assessment of RAGE localization was completed by immunohistochemistry. Lungs from animals exposed to SHS or room air was inflation-fixed at 25 cm of water pressure with 4% paraformaldehyde in PBS for 1 min, processed, and sectioned (25). Sections from four animals per group were stained with antibodies against RAGE (#AF1179, RnDSystems), the appropriate biotinylated secondary antibodies already outlined (26), and sections were counterstained with nuclear fast red. A no primary control involved the staining of sections without the primary antibody.

Ras and NF-kB assessments. Ras Activation ELISA kits (Millipore, Temecula, CA) were used to measure active and inactive Ras. Total lung lysates were quantified by bicinchoninic acid assay then screened for Ras in 20 µg aliquots obtained by homogenization in RIPA buffer with inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA). Ras assessments were compared with EGF-treated HeLa cell lysates included in the kit as a positive control and in replicates performed without lysates as a negative control. Total and active NF-kB levels were assessed through the use of colorimetric high-throughput FACE assays available from Active Motif (Carlsbad, CA) that utilize antibodies specific to total and active phosphorylated proteins as outlined in the manufacturer’s instructions. Experiments involved six animals per group.

Bronchoalveolar lavage fluid analysis. Mice were killed, and bronchoalveolar lavage fluid (BALF) was procured and evaluated as outlined previously (26). In brief, the trachea was exposed and cannulated with a 20-gauge catheter. PBS was lavaged in accordance with the weight of the mouse prior to surgery and removed. Lavage fluid was then centrifuged at 4°C, following which total cells were counted on a hemocytometer. An aliquot of cells was stained with a modified Wright-Giemsa stain (Diff-Quik; Baxter, McGaw Park, IL) and subjected to a blinded manual differential cell count in which 200 cells were counted per slide, and the percent of total cells was determined. Counting was performed in triplicate, and the average was obtained. Concentrations of TNF-α and macrophage inflammatory protein (MIP)-2 were obtained with molecule-specific ELISA kits used as directed in the manufacturer’s instructions (RnD Systems, Minneapolis, MN).

Statistics. Mean values ± SD from at least six animals per group were assessed by one- and two-way analysis of variance (ANOVA). When ANOVA indicated significant differences, student t-tests were used with Bonferroni correction for multiple comparisons. Results are representative and those with P values <0.05 were considered significant.

RESULTS

Mice exposed to acute SHS upregulate RAGE expression. Mice were exposed to 4 wk of SHS as outlined, and control mice exposed to room air were maintained for comparison. At the completion of the exposure, no evidence of histopathological remodeling was detected. qRT-PCR demonstrated that acute SHS lead to a significant increase in the transcription of RAGE mRNA in wild-type mice compared with room air-exposed controls (Fig. 1A). As expected, RAGE was not detected in RAGE knockout mice regardless of smoke exposure (Fig. 1A). We performed immunoblotting to compare RAGE protein expression with mRNA levels. Blotting for RAGE revealed that SHS was sufficient to increase RAGE levels compared with room air controls (Fig. 1B). As was the case with the mRNA assessments, no RAGE protein was detected in RAGE knockout animals (not shown). Band densities demonstrated that SHS exposure coincided with an ~80% increase in RAGE protein expression (Fig. 1C). We next completed a qualitative evaluation of RAGE expression in the SHS-exposed lung. Immunohistochemistry revealed basal RAGE expression in wild-type animals exposed to room air (Fig. 2A) and a qualitative increase in parenchymal RAGE expression following SHS exposure (Fig. 2B). Control sections incubated without primary antibody resulted in no immunostaining (Fig. 2C).

RAGE mediates the activation of SHS-induced Ras and NF-kB. Quantitative assessments of active Ras and NF-kB were evaluated to identify downstream molecular intermedi-
ates that plausibly participate in RAGE signaling during a short-term inflammatory response to SHS in vivo. Ras is a molecular switch already implicated in smoke-induced lung inflammation mediated by RAGE (27). Lungs from SHS-exposed wild-type mice presented significantly increased levels of active Ras compared with room air controls (Fig. 3). However, Ras activation was not significantly different in RAGE knockout animals notwithstanding smoke exposure. Furthermore, Ras activity was significantly decreased in SHS-exposed RAGE knockout animals compared with SHS-exposed wild-type animals (Fig. 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 compared with room air controls (Fig. 4). We also discovered that NF-κB activity was significantly decreased in SHS-exposed lungs that lack RAGE expression compared with RAGE-expressing lungs following SHS exposure (Fig. 4). There was no difference in total NF-κB levels (not shown). These results reveal that SHS exposure coincided with increased levels of these important inflammatory perpetuation markers, while protection from activation was afforded by the absence of RAGE.

RAGE ablation diminishes SHS-induced pulmonary inflammation. Evaluation of lung histology by hematoxylin and eosin staining after 4 wk of SHS exposure (not shown) did not reveal evidence of tissue pathology. Owing to the acute nature of exposure, abnormal inflammatory profiles likely portended histopathological remodeling. Accordingly, we sought to characterize SHS-mediated responses by evaluating markers of pulmonary inflammation in BALF obtained from each experimental group. Because cells respond to stresses by secreting into and altering BALF, its characterization is an excellent measure to evaluate organ-level responses. BALF from wild-type mice exposed to SHS had significantly more protein compared with room air controls (Fig. 5A), suggesting elevated vascular permeability coincident with extravasation observed during inflammation. Conversely, protein levels were not different in RAGE knockout lungs in either the presence or absence of SHS exposure (Fig. 5A). BALF from wild-type

Fig. 1. A: there was a significant increase in the expression of receptor for advanced glycation end-products (RAGE) mRNA by wild-type (WT) mice exposed to secondhand smoke (SHS) compared with animals exposed to room air (RA). Transcripts were not detected in RAGE knockout (RKO) animals. Message was normalized to GAPDH and representative data from experiments performed with 6 mice per group and *P < 0.05. B: immunoblotting revealed that RAGE was increased in WT + SHS animals compared with WT + RA, and a significant increase was demonstrated by densitometry. C: blots are representative of experiments using 6 animals per group, and significance was identified at *P < 0.05.

Fig. 2. Immunohistochemical staining for RAGE demonstrated increased expression in the WT lung parenchyma following SHS exposure (B) for 4 wk compared with RA control animals (A). C: control sections incubated without primary antibody resulted in no immunostaining. Images are representative of experiments involving 4 animals from each group.
mice also showed SHS-mediated increases in total BALF cells (Fig. 5B). While total cells were modestly elevated in RAGE knockout lungs with and without SHS, there was no SHS-mediated increases compared with controls (Fig. 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 knockout lungs did not induce PMN extravasation when quantified at the conclusion of SHS exposure (Fig. 5C). Lastly, MIP-2, a potent chemoattractant homologous to human IL-8, and TNF-α, a cytokine implicated in COPD-related inflammation and emphysematous remodeling (17), were quantified in BALF. There was a significant increase in secreted TNF-α and MIP-2 in wild-type BALF after SHS exposure compared with BALF from room air-exposed controls (Fig. 6). RAGE abrogation in knockout lungs was sufficient to significantly inhibit SHS-induced TNF-α and MIP-2 secretion (Fig. 6).

**DISCUSSION**

Though often overlooked, the interaction between lungs and atmospheric air accounts for a significant portion of the exogenous materials humans encounter. Under normal circumstances, the lung uses an array of cytokines, activated immune cells such as resident macrophages, and other modulators to respond to exogenous pathogens (13). In the case of tobacco smoke exposure, persistence of these inflammatory mediators may account for the exaggerated inflammatory response that leads to a COPD phenotype. Our discovery that RAGE was increased in the lungs of mice exposed to SHS demonstrated that this effective modulator of inflammation functions when lungs are involuntarily exposed to side stream smoke. To our knowledge this is the first study to investigate the inflammatory impact of the absence of RAGE via RAGE knockout animals in the context of SHS exposure. In fact, the observation that RAGE was elevated in the lungs of SHS-exposed mice opens a new area of RAGE research, further implicating RAGE as a dynamic, multifunctional mediator of lung disease.

Our laboratory has provided firm evidence for RAGE-mediated inflammation in tobacco smoke environments (27–30, 32, 46). That inflammation was seen after only 4 wk of acute SHS exposure further confirms the impact of SHS as an agent capable of exacerbating lung disease complications.
RAGE was originally characterized for its ability to bind advanced glycation end-products (AGES) and for its role as a prominent feed-forward receptor involved in inflammation (35). AGES are derived via the nonenzymatic combination of amino groups and reducing sugars (20). While AGES are abundantly formed at locations of inflammation in hyperglycemic conditions and in sites of oxidative stress (44), Maillard chemical reactions that induce AGE formation in the presence of tobacco smoke also provide abundant ligands for RAGE signaling (21). Importantly, AGES derived during smoking and available in SHS may provide a collection of diverse entities capable of triggering inflammatory cascades via interaction with RAGE, a competent pattern recognition receptor. RAGE signaling is accordingly dependent upon a variety of ligands and signaling leads to the activation of several pathways, including Ras-extracellular signal-regulated kinase 1/2, which drives NF-κB activation (11, 45). Our data detailing elevated active Ras expression and enhanced NF-κB activity when RAGE is present link these important signaling intermediates in animals responding to SHS. Data support shifts toward active Ras in the progression of COPD (49); therefore, abrogation of RAGE may assist in alleviating acute predisposing inflammation observed during initial stages of inflammatory disease. Because Ras and other downstream RAGE signaling intermediates are implicated in the pathology of COPD, long-term smoke exposure of mice lacking RAGE should be conducted to further connect RAGE signaling and chronic lung histopathology.

Our research using BALF also demonstrated inflammatory characteristics observed in smoke-induced disease states. Elevated protein content in BALF suggested increased vascular permeability, a finding that coincides with previous research showing less BALF protein in RAGE knockout mice subjected to acute lung injury (26). Our discovery that the induction of PMN diapedesis was decreased in SHS-exposed RAGE knockout mice also reinforces prior research that demonstrated increased PMN admission in the airways and bronchial tissue of smokers diagnosed with COPD (37). Furthermore, this research identifies RAGE signaling as a perpetrator of PMN recruitment in smoke-exposed lungs and that these potent reservoirs of elastolytic enzymes, leukocyte chemoattractants, and other mediators of inflammation and remodeling are partially regulated by RAGE signaling (2, 37).

Molecules involved in the mechanistic control of pulmonary inflammation were also differentially expressed in the lungs of animals exposed to SHS (6). In particular, important proinflammatory effector molecules including MIP-2 and TNF-α were significantly decreased in SHS-exposed mice that lacked the capacity to express RAGE compared with SHS-exposed wild-type controls. MIP-2, also referred to as CXCL2, is the mouse homolog of IL-8 observed in humans. MIP-2 has been characterized as a strong neutrophil chemoattractant in tissue injuries and infections (9), and IL-8 is increased in patients with COPD (48). TNF-α elicits the elaboration of various inflammatory and cytotoxic mediators including IL-1β, IL-6, platelet activating factor, and reactive oxygen species (14). TNF-α also regulates cell proliferation (14) and the expression of ICAM-1, an adhesion molecule expressed by targeted endothelial cells that assists in leukocyte transmigration (12, 17). TNF-α secretion is elevated in experimental models of organ injury and upregulated TNF-α coincides with the release of matrix metalloproteinases connected to emphysematous damage (36). Importantly, TNF-α has been detected in lung cells, BALF, and sputum from COPD patients (4). It is clear that elevated synthesis and secretion of these and other inflammatory molecules may lead to enhanced inflammation and the subsequent chronic pulmonary remodeling observed in lungs exposed to primary and SHS.

In summary, this work provides a snapshot of the potential initial triggers of inflammation that may 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. The present research sought to mechanistically clarify the initiation of inflammation mediated, at least in part, by RAGE signaling and identify genetic factors that may influence individual susceptibility to SHS. Further elucidation of RAGE signaling in the context of prolonged SHS exposure remains an important consideration, however. As such, additional research should be pursued that centers on roles for RAGE during primary and SHS exposure and its likely influence on lung inflammation and remodeling.
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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS


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