RAGE signaling by alveolar macrophages influences tobacco smoke-induced inflammation

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Receptors for advanced glycation end-products (RAGE) are multiligand cell surface receptors of the immunoglobulin family expressed by epithelium and macrophages, and expression increases following exposure to cigarette smoke extract (CSE). The present study sought to characterize the proinflammatory contributions of RAGE expressed by alveolar macrophages (AMs) following CSE exposure. Acute exposure of mice to CSE via nasal instillation revealed diminished bronchoalveolar lavage (BAL) cellularity and fewer AMs in RAGE knockout (KO) mice compared with controls. Primary AMs were obtained from BAL, exposed to CSE in vitro, and analyzed. CSE significantly increased RAGE expression by wild-type AMs. Employing ELISAs, wild-type AMs exposed to CSE had increased levels of active Ras, a small GTPase that perpetuates proinflammatory signaling. Conversely, RAGE KO AMs had less Ras activation compared with wild-type AMs after exposure to CSE. In RAGE KO AMs, assessment of p38 MAPK and NF-kB, important intracellular signaling intermediates induced during an inflammatory response, revealed that CSE-induced inflammation may occur in part via RAGE signaling. Lastly, quantitative RT-PCR revealed that the expression of proinflammatory cytokines including TNF-α and IL-1β were detectably decreased in RAGE KO AMs exposed to CSE compared with CSE-exposed wild-type AMs. These results reveal that primary AMs orchestrate CSE-induced inflammation, at least in part, via RAGE-mediated mechanisms.

precise pathophysiological mechanisms are not well known. Furthermore, a clearer understanding of causative mechanisms may provide avenues for research into lessening the debilitating effects of disease progression.

Inflammation involves interrelated processes that center on the activation of the immune system to defend against infection and/or repair of damaged tissue. The effects of even basic inflammatory responses become amplified and problematic in the lung as a result of the critical nature of its role in gas exchange. A significant outcome of inflammation in the lung includes the influx and accumulation of phagocytic and reactive immune cells at the site of injury (16). Activated macrophages produce proinflammatory cytokines that amplify the immune response via upregulation of adhesion molecules required for leukocyte chemotaxis. Simultaneously, cytokines also help to induce monocyte differentiation into additional macrophages (15). Among others, the primary cytokines responsible for acute inflammation stemming from tobacco smoke exposure include tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (7, 46). In addition to proinflammatory cytokines, macrophages also secrete collagenases and elastases that destroy connective tissue in the lung.

The receptor for advanced glycation endproducts (RAGE) is a member of the immunoglobulin superfamily of cell surface receptors found in various cell types including smooth muscle cells, fibroblasts, macrophages, and epithelium (39). RAGE is most abundant in the lung (2) with sparse expression by alveolar type (AT) II cells and abundant expression on membranes of differentiated ATI cells (43). Although RAGE was first described as a progression factor in cellular responses induced by advanced glycation end-products (AGEs) that accumulate in hyperglycemia and oxidant stress, studies have also indicated that RAGE binds a myriad of other molecules (28) including proinflammatory cytokine-like mediators of the S100/calgranulin family (S100A12 and S100B), amyloid β-fibrils, high-mobility group box 1 (HMGB1), Mac-1 (3, 43), and specific DNA and RNA structures (44).

RAGE expression increases as its ligands accumulate, and RAGE-ligand interactions may contribute to pathological processes including diabetic complications, neurodegenerative disorders, atherosclerosis, and inflammation. We have recently demonstrated that RAGE influences cigarette smoke extract (CSE)-induced inflammation mediated by pulmonary epithelial cells. Specifically CSE induces pulmonary epithelium to increase the expression of RAGE, its ligands, Egr-1 (a transcription factor abundantly expressed in the lungs of patients with COPD), proinflammatory signaling intermediates, and various cytokines (36–38). The possibility that alveolar macrophages (AMs) cooperate with pulmonary epithelium in CSE-induced inflammation via upregulation of RAGE and its signaling pathway has not yet been assessed.
As an intracellular signaling molecule that regulates the fate of target cells, Ras oscillates between active GTP-bound and inactive GDP-bound conformations (11). Ras signaling has been associated with development, cellular proliferation, and differentiation, as a result of signaling through Raf/MAPK, phosphatidylinositol 3-kinase, JNK/p38, and Rho pathways (35). Furthermore, Ras is a key regulator of many features of normal cell growth and malignant transformation as a result of the signaling through MAPKs (12). Published work by our laboratory provides evidence indicating that the role of Ras in inflammation appears to be downstream of RAGE in pulmonary epithelial cells. However, the role of Ras and other intermediate signaling molecules in AMs stimulated by CSE requires further investigation.

In the present study, we tested the hypothesis that AMs exposed to CSE induce RAGE and that signaling involving Ras, p38 MAPK, and proinflammatory cytokine secretion occurs via RAGE-mediated mechanisms. Although RAGE knockout (KO) AMs are purported to be indistinguishable from AMs from wild-type strains of mice (53), we demonstrate that RAGE KO AMs exposed to CSE experience reduced Ras and p38 MAPK activation, less NF-κB translocation, and diminished production of TNF-α and IL-1β compared with CSE-exposed wild-type AMs. Collectively, these data offer new insights into the potential mechanisms whereby RAGE expressed by AMs participates in inflammation following tobacco smoke exposure. Further research may demonstrate that RAGE and its specific downstream effectors are potential targets in the treatment or prevention of tobacco smoke-related pulmonary complications.

MATERIALS AND METHODS

Mice. Wild-type C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). RAGE KO mice that lack RAGE were generated in a C57BL/6 background (48). In line with The Guide for the Care and Use of Laboratory Animals and approved IACUC protocols at Brigham Young University, mice had free access to food and water and were housed in a dedicated animal facility that controlled for light, temperature, and humidity. Bronchoalveolar lavage fluid (BALF) was obtained from RAGE KO and C57Bl/6 control mice as outlined below. Additional mice were nasally instilled with either sterile PBS or 10% CSE (36) as outlined previously (34). Twenty-four hours after nasal instillation, lungs (n = 6 mice per group) were inflated fixed for immunohistochemistry as already described (37) or used to determine total cell quantity and differential cell counts in BALF as already outlined (34).

BALF and cell culture. To obtain BALF, mice were sedated and then exsanguinated to ensure euthanasia as outlined previously (39). BALF was specifically harvested through the instillation and recovery of 7-ml boluses of PBS attached to a catheter for a total of 7 ml. Each 7-ml sample of BALF was centrifuged at 1,000 revolution/min for 10 minutes. The cell pellet was resuspended in warm DMEM, and equal concentrations of ~50,000 cells were plated and exposed to adherent overnight before exposure to CSE (36) or fresh DMEM. Cells were exposed to 10% CSE for 30 min to 4 h depending on the experiment. Following exposure to either CSE or fresh media, cells were washed with two changes of PBS before total RNA or protein was isolated.

Immunocytochemistry and quantitative RT-PCR. Cells obtained from BALF were washed and fixed with 4% paraformaldehyde and stained for Mac-3 (1:50; BD Biosciences, San Jose, CA), an antibody that recognizes the 110-kDa Mac-3 protein expressed by mononuclear phagocytes, and a goat anti-rat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as outlined previously (38). Sections of lung tissue (5 μm each) were similarly immunostained with Mac-3 to qualitatively assess relative AM quantity. Total RNA was isolated from cells using the Qiagen RNeasy Plus Micro kit (QIAGEN, Valencia, CA) in accordance with the provided instructions, and reverse transcription of RNA was performed using the Invitrogen Superscript III First-Strand Synthesis System (Life Technologies, Grand Island, NY) to obtain cDNA for PCR. The following primers were synthesized and HPLC purified by Invitrogen Life Technologies: RAGE (For-ACC GAG TCC GAC TCT ACC and Rev-GTA GCT TCC CTA GAC ACA), TNF-α (For-TGG CTA GTG CTC AGC CTC TTC and Rev-GAG GCC ATT TGG GAA CCT CT), IL-1β (For-TGG AAT GAA AGA CGG CAC ACC and Rev-TCC TTG GGT ATT GCT TGG), and GAPDH (For-TAT GTC GTG GAG TCT ACT GGT and Rev-GAG TGG TCT TAC TGT TCG TGG). cDNA amplification and data analysis were performed using Bio-Rad IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and a Bio-Rad Single Color Real Time PCR detection system (Bio-Rad Laboratories). Primers were used at a concentration of 75 nM each in 25-μl reactions. Cycle parameters were as follows: 3 min at 95°C, and 40 cycles composed of 1 min at 95°C, 15 s at 55°C and 15 s at 72°C. Control wells lacking template or RT were included to identify primer-dimer products and to exclude possible contaminants. Ras activation ELISA. Ras activation ELISA kits (Millipore, Temecula, CA) were used to assess active and inactive Ras (38). Cell lysates were quantified by BCA assay and then screened for Ras in 20-μg aliquots of total cell lysates obtained by homogenizing cells in RIPA buffer with inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA). In vitro experiments were repeated at least three times, each in triplicate.

RESULTS

AMs induce RAGE expression following CSE exposure. Wild-type and RAGE KO mice were exposed to an acute bolus of PBS or CSE, and BALF was assessed. Total cell quantity in BALF was significantly increased in wild-type mice following CSE exposure compared with PBS-instilled controls (Fig. 1A). Even though total cell quantity was also elevated in RAGE KO BALF, total BALF cells were significantly diminished in RAGE KO mice exposed to CSE compared with CSE-exposed wild-type animals (Fig. 1A). The percentage of AMs in WT and RAGE KO BALF following acute CSE exposure trended downward but was not significantly different compared with PBS-exposed mice (Fig. 1B). Lung sections from RAGE KO and wild-type animals were also immunostained for Mac-3 24 h after a single exposure to PBS or CSE by nasal instillation. There was a detectable increase in Mac-3-positive cells in the wild-type lung exposed to CSE (Fig. 1D) compared with age-matched wild-type littermates exposed to PBS vehicle only (Fig. 1C). In addition, qualitative assessment of AMs in CSE-
exposed RAGE KO mice by immunostaining (Fig. 1E) revealed elevated Mac-3 cells, but most histological views appeared similar to wild-type PBS-exposed mice (Fig. 1C). To dissect RAGE-mediated signaling in AMs, total BAL cells from wild-type and RAGE KO mice were harvested and cultured as outlined in MATERIALS AND METHODS. So that a stable population of AMs could be identified, immunohistochemistry was performed using a Mac-3 antibody, and significant immunoreactivity was observed in adherent cells (Fig. 1, F and G).

To assess whether RAGE signaling was involved in primary AMs exposed to CSE, we evaluated the degree to which RAGE was expressed at baseline and whether RAGE expression increases following exposure to CSE. Through experiments involving quantitative RT-PCR, we discovered that there was a significant increase in RAGE expression by primary AMs isolated from wild-type mice following CSE exposure compared with wild-type AMs exposed to fresh media (Fig. 2). RAGE KO AMs were similarly evaluated, and, as anticipated, no RAGE expression was detected in both the CSE- or media-exposed cell populations (Fig. 2).

RAGE KO AMs have significantly diminished CSE-induced Ras activation. Active Ras, an intracellular molecular switch involved in many signal transduction pathways, was recently assessed in alveolar epithelium exposed to tobacco smoke (38). Our research revealed RAGE-mediated mechanisms of elevated Ras activity that precedes inflammatory molecule elaboration in response to smoke exposure. To test the hypothesis that Ras is also activated in primary AMs exposed to CSE, we exposed AMs isolated from wild-type and RAGE KO mice to 10% CSE over the course of 1, 2, and 4 h. Using techniques that immunologically assess active Ras, we discovered a significant increase of active Ras expression in wild-type primary AMs after only 1 h of CSE exposure (Fig. 3). The data related to the time course revealed consistent increases in Ras activity as the duration of CSE exposure progressed. Ras activity was also increased in primary AMs obtained from RAGE KO mice; however, the amount of active Ras was consistently and significantly decreased at each time point assessed (Fig. 3).

RAGE KO AMs have significantly diminished CSE-induced p38 MAPK and NF-κB activation. Because RAGE is elevated in AMs that encounter tobacco smoke and Ras, an important

Fig. 1. Bronchoalveolar lavage fluid (BALF) was procured from receptor for advanced glycation end-products (RAGE) knockout (KO) acutely exposed to cigarette smoke extract (CSE) and assessed. Total BALF cells and the percentage of alveolar macrophages (AMs) were significantly diminished in RAGE KO animals exposed to CSE compared with CSE-exposed controls (A–B). 24 h after a single nasal instillation of PBS (C) or 10% CSE (D), wild-type (WT) mice stained for Mac-3 revealed a marked increase in Mac-3-positive AMs following CSE exposure. RAGE KO mice were similarly administered 10% CSE, and there was a detectable decrease in the number of Mac-3-positive AMs following qualitative immunohistochemical assessment (E). BALF cells were procured, plated, and immunostained with Mac-3 to identify a homogenous AM population. All adherent cells expressed Mac-3 (F), and immunostaining in the absence of primary antibody revealed no immunoreactivity (G). BALF analyses were performed in triplicate and *P ≤ 0.05 (ANOVA). Representative images (x400 original magnification) of n = 3 mice in each group are shown.
cytosolic intermediate that perpetuates inflammation and is activated in part by RAGE, it was important to dissect additional RAGE-mediated signaling targets. A series of experiments was performed to analyze the activity of p38, an important MAPK involved in inflammatory cell signaling (17). Total p38 MAPK was not significantly different when comparing AMs from RAGE KO or wild-type mice (Fig. 4A). However, total p38 MAPK was decreased in both AM populations after 30 minutes of CSE exposure (Fig. 4A). Data revealed that CSE exposure for 30 min resulted in increased phosphorylation and subsequent activation of p38 MAPK in both wild-type and RAGE KO AMs (Fig. 4B). Importantly, p38 MAPK activation in RAGE KO AMs after 60 min of CSE exposure was significantly diminished compared with levels observed in CSE-exposed wild-type AMs (Fig. 4B).

We next assayed the state of NF-κB activation in cells exposed to CSE. As a downstream target of Ras and MAPKs, including p38, NF-κB is phosphorylated and liberated from cytosolic sequestration during an inflammatory response, and it functions as a potent nuclear transcription factor. Total NF-κB was not significantly different when comparing AMs from RAGE KO and wild-type mice (Fig. 5A). Results revealed that the expression of active NF-κB in CSE-exposed RAGE KO AMs was significantly reduced compared with CSE-exposed AMs from wild-type controls (Fig. 5B). Combined, these data suggest that CSE induces the activation of p38 MAPK and NF-κB in AMs at least partially through RAGE signaling pathways.

CSE-induced proinflammatory cytokine secretion is diminished in RAGE KO AMs. To test the hypothesis that CSE-induced RAGE expression leads to proinflammatory cytokine production, we analyzed the levels of TNF-α and IL-1β in AMs with and without CSE exposure. Following exposure to CSE for 2 h, quantitative RT-PCR revealed a significant increase in the expression of both cytokines by primary AMs from wild-type mice (Fig. 6). Production of TNF-α (Fig. 6A) and IL-1β (Fig. 6B) mRNA was significantly decreased in primary AMs from RAGE KO mice exposed to CSE compared with CSE-exposed AMs from wild-type controls. Although AMs from both wild-type and RAGE KO mice increased cytokine mRNA expression following CSE exposure, primary wild-type AMs exposed to CSE had significantly more cytokine expression compared with CSE-exposed RAGE KO AMs. Differential expression of TNF-α and IL-1β after 2 h of CSE exposure (Fig. 6) was also observed in experiments that involved 4 h of CSE exposure (not shown). An assessment of secreted cytokines after 4 h revealed that IL-1β production was similar to the mRNA profile in that a significant decrease was observed in media obtained from CSE-exposed KO AMs compared with CSE-exposed wild-type AMs (Table 1). Interestingly, acute TNF-α secretion after 4 h was not different in any of the groups regardless of CSE exposure (Table 1).

DISCUSSION

AMs and tobacco smoke-induced RAGE expression. Macrophages are dynamic cells capable of remarkably diverse gene expression patterns following signal recognition of tissue-specific events. In particular, phagocytosis and enhancement of the inflammatory response are defining macrophage characteristics in programmed responses to acute and chronic patholog-
In fact, macrophages are significant contributors to the organism’s immune system, with functions that include phagocytic clearance of pathogens and other debris and service as a reservoir for a variety of mediators that regulate inflammation, adaptive immunity, and homeostasis (25). Such modulators of inflammation and homeostasis are normally released to target invading pathogens and enhance wound repair (24). However, regulatory mechanisms that control inflammatory mediators can also become dysfunctional when external stimulation, including exposure to tobacco smoke, persists. Such chronic stimulation of macrophage-mediated immune responses may culminate in irreversible remodeling of resident tissues.

Originally characterized for its ability to bind AGEs, abundant data implicate RAGE as a potent feed-forward receptor involved in inflammation (45). AGEs arise in vivo from the nonenzymatic addition of reducing sugars to amino groups (30). Although AGEs such as carboxymethyllysine are formed more abundantly at sites of inflammation in hyperglycemic renal failure and in areas of localized oxidative stress (50), tobacco-derived AGEs formed via Malliard chemistry also provide abundant ligand for RAGE signaling (29). Importantly, RAGE is a pattern-recognition receptor, so there is a clear likelihood that diverse yet related AGEs derived by a burning cigarette combine to induce proinflammatory RAGE signaling (5).

Our previous research demonstrated that exposure of pulmonary epithelial cells to CSE led to increased expression of RAGE and its ligands, suggesting that epithelial cells respond acutely to CSE by stimulating molecules required in the initial stages of RAGE signaling (37). Importantly, RAW264.7 cells, a murine macrophage cell line, upregulated RAGE expression following exposure to CSE (37). Despite these data, the literature was silent in terms of RAGE biology in murine primary AMs that encounter tobacco smoke. Although not as robustly characterized in macrophages, RAGE has been shown to be the receptor responsible for HMGB1-induced inflammation in rodent macrophages (22). Studies document that low levels of RAGE are expressed by macrophages in normal conditions and that RAGE overexpression is observed in macrophages that elicit inflammation and cause lung damage (28). Morbini et al. (28) confirmed the presence of AGEs in macrophages and observed that AGE and RAGE are both coexpressed by these immune cells. Our discovery that RAGE was markedly increased by primary AMs exposed to tobacco smoke led to the hypothesis that RAGE signaling influences acute responses by macrophages in smoke environs.
Table 1. Production of IL-1β and TNF-α by AMs with or without CSE exposure

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<tr>
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<th>WT AMs</th>
<th>WT AMs + CSE</th>
<th>KO AMs</th>
<th>KO AMs + CSE</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>28.9 ± 9.9</td>
<td>85.86 ± 9.3*</td>
<td>25.38 ± 4.3</td>
<td>61.44 ± 2.9†</td>
</tr>
<tr>
<td>TNF-α</td>
<td>235 ± 5.9</td>
<td>197 ± 19.2</td>
<td>226 ± 18.2</td>
<td>211 ± 21.2</td>
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Data are expressed as average pg/ml ± SD from at least 2 experiments performed in triplicate. *Significant differences between no cigarette smoke extract (CSE) vs. CSE-exposed wild-type (WT) or knockout (KO) cells; †significant differences between WT alveolar macrophages (AMs) + CSE vs. KO AMs + CSE (P ≤ 0.05, AVOVA).

RAGE signaling during tobacco smoke exposure. Activation of RAGE is dependent on a variety of ligands, and RAGE/ligand interaction leads to the activation of diverse signal transduction pathways, including Ras-ERK1/2, Cdc42/Rac, SAPK/JNK, and p38 MAPK pathways (14). These signaling events can culminate in the activation of transcription factors including NF-κB or cAMP response element-binding protein (20, 51). Our laboratory has previously published data that propose the likelihood of a positive feedback loop in which ligand binding led to intracellular signaling that eventually enhanced additional RAGE expression (37). Furthermore, RAGE in CSE-exposed lung epithelial cells has been shown to activate Ras in the initiation of smoke-induced proinflammatory signaling (38). Our data detailing Ras activation in concert with increased RAGE availability links this important signaling cascade in AMs with similar effects observed in pulmonary epithelial cells. Because anomalous Ras expression has been implicated as a biomarker for COPD (26), our research suggests plausible cooperation between pulmonary epithelial cells and resident macrophages in advanced stages of inflammatory disease.

Our data further demonstrated that AMs exposed to CSE trigger an intracellular signaling pathway mediated by MAPKs and NF-κB. Specifically, p38 MAPK activity peaked in AMs from wild-type mice after just 30 min of CSE exposure (Fig. 4). Whereas p38 MAPK activation was also observed in RAGE KO AMs, there was a significant decrease in p38 MAPK activity after 60 min of CSE exposure compared with wild-type AMs (Fig. 4). We also demonstrated that, after 4 h, nuclear translocation of active NF-κB was significantly diminished in smoke-exposed RAGE KO AMs compared with smoke-exposed AMs from control mice (Fig. 5). Although RAGE signaling via p38 MAPK and NF-κB has recently been proposed in diverse tissue types under various conditions (19, 40, 54), such signaling paradigms have not been elucidated in AMs exposed to tobacco smoke. Importantly, the signaling kinetics presented in the current research are similar to those presented by Zhang et al. (53) in that p38 MAPK activation peaked at 30 min and NF-κB activation peaked at 4 h following exposure of RAW234.7 to AGEs.

RAGE-mediated inflammatory cytokine secretion by AMs. The current investigation identified significant upregulation of TNF-α and IL-1β, two proinflammatory cytokines related to COPD pathogenesis (6), by wild-type AMs. A substantial element of our research revealed that these important proinflammatory effector molecules were significantly decreased in CSE-exposed AMs that lacked the capacity to express RAGE.

Conclusions. The current study provides support for a model of smoke-exposed AMs wherein RAGE-mediated signal transduction pathways influence inflammation. Therefore, our findings that smoke increases RAGE expression, activates known intracellular pathways, and causes NF-κB-mediated cytokine elaboration have important implications for elucidating the mechanisms of progressive lung inflammation experienced by both former smokers and those unable or unwilling to quit. Clarifying RAGE signaling in the context of possible parallel pathways remains an important consideration, however. As such, additional research that centers on the important role of RAGE signaling and the resulting inflammatory response should remain a priority.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


