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Chronic Alcohol Ingestion Impairs Rat Alveolar Macrophage Phagocytosis via Disruption of RAGE Signaling

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Abstract

Background—Alcohol significantly impairs antioxidant defenses and innate immune function in the lung and increases matrix metalloproteinase 9 (MMP-9) activity. The receptor for advanced glycation end products (RAGE) is a well-characterized marker of lung injury that is cleaved by MMP-9 into soluble RAGE and has not yet been examined in the alcoholic lung. We hypothesized that chronic alcohol ingestion would impair RAGE signaling via MMP-9 in the alveolar macrophage and thereby impair innate immune function.

Methods—Primary alveolar macrophages were isolated from control-fed or alcohol-fed rats. Real-time polymerase chain reaction (qRT-PCR), Western blotting, and enzyme-linked immunosorbent assays were performed to evaluate RAGE expression. Silencing of MMP-9 ribonucleic acid (RNA) in a rat alveolar macrophage cell line was confirmed by qRT-PCR and immunofluorescence (IF) was utilized to assess the association between alcohol, MMP-9, and RAGE. Phagocytosis was assessed using flow cytometry. Sulforaphane and glutathione were used to assess the relationship between oxidative stress and RAGE.

Results—RAGE messenger RNA expression was significantly increased in the alveolar macrophages of alcohol-fed rats, but IF showed membrane-bound RAGE protein expression was decreased. Lavage fluid demonstrated increased levels of soluble RAGE (sRAGE). Decreasing MMP-9 expression using si-MMP-9 abrogated the effects of alcohol on RAGE protein. Phagocytic

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AUTHOR CONTRIBUTIONS

Conception and Design (BSS, EEE, DMG, XF), Performing Experiments (BSS, EEE, AA), Data Analysis (BSS, EEE), Drafting and Revising of Manuscript (BSS, EEE, DMG, XF)

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function was suppressed by direct RAGE and the impairment was reversed by antioxidant treatment.

Conclusions—Chronic alcohol ingestion reduces RAGE protein expression and increases the amount of sRAGE in alveolar lavage fluid, likely via cleavage by MMP-9. In addition, it impairs phagocytic function. Antioxidants restore membrane-bound RAGE and phagocytic function.

Keywords

alcohol; RAGE; alveolar macrophage; antioxidant; innate immunity

INTRODUCTION

Alcohol is one of the most widely abused substances in the world. Its deleterious effects on the liver are widely known, but its effects on the lung are equally profound. The increased risk of pneumonia in patients suffering from chronic alcohol abuse was observed by physicians over 200 years ago [1]. Modern epidemiologic data confirm this key clinical observation and have quantified the increased risk of pneumonia and acute respiratory distress syndrome conferred by chronic alcohol ingestion [2]. The effects of alcohol in the lung are multifarious and include an increased risk of aspiration in individuals suffering from alcohol use disorders, decreased mucociliary clearance, and impaired airway immunity [3, 4]. Within the alveolar space itself, our group and others have made great strides in characterizing the mechanisms underlying the defects in the “alcoholic lung” in a variety of cell types and functions. In alveolar macrophages, alcohol-related impairment is associated with significant defects in innate immune function, including impaired phagocytosis and reduced bacterial clearance [5]. Alcohol also causes abnormally leaky epithelial barriers which predispose the lung to injury [6] and increased susceptibility to apoptosis [7].

An important marker of lung injury that has not yet been evaluated in the alcoholic lung is the receptor for advanced glycation end products (RAGE), a promiscuous damage-associated molecular pattern receptor and member of the immunoglobulin subgroup that has an important role in inflammatory signaling [8]. RAGE ligands include advanced glycation end products, lipopolysaccharide, S100 proteins, and high mobility group box 1 protein. Binding of these ligands with the receptor results in downstream activation of the NF- κ B pathway [9]. There are 6 RAGE isoforms, but only the full length transmembrane variant is capable of inflammatory signaling. The other major variant is soluble RAGE (sRAGE), which is primarily created by cleavage of full length RAGE by MMP-9. Because it is capable of binding to RAGE ligands, sRAGE is thought to act as a decoy receptor by competing for ligands and preventing them from binding to RAGE and activating the inflammatory cascade. Although sRAGE has been studied as a marker of lung epithelial injury [10], little is known regarding the role of RAGE and sRAGE in alveolar macrophages, and less about its role in alcohol-induced lung impairment. Given the known effects of alcohol on alveolar macrophage function and pulmonary innate immunity, as well as the known role of RAGE as a marker for underlying lung injury, we hypothesized that chronic alcohol ingestion may impair inflammatory signaling in the macrophage via impairment of RAGE.

MATERIALS AND METHODS

Animal model

Adult male Sprague-Dawley rats were fed alcohol as previously described [11]. Briefly, animals were fed the standard Lieber-DeCarli liquid diet (Research Diets, New Brunswick, NJ) containing either alcohol (ethanol, 36% of total calories) or an isocaloric carbohydrate substitution *ad libitum* for a total of 6 weeks. All animal work was performed with the approval of Institutional Use and Care of Animals Committee at Emory University.

Primary alveolar macrophage isolation

Animals were sacrificed with a lethal intraperitoneal dose of Euthazol (Virbac Co., Fort Worth, TX), followed by pulmonary artery perfusion. Bronchoalveolar lavage (BAL) was then performed by intra-tracheal instillation of four 10 mL aliquots of PBS. For sRAGE detection in BAL, the first aliquot was used for analysis. BAL fluid was then centrifuged at 400g, and cells were washed before plating in F12-K (ATCC, Manassas, VA) with penicillin (1 U/mL), streptomycin (1 µg/mL), and amphotericin B (2.5 ng/mL) (Sigma-Aldrich, St. Louis, MO) and 10% fetal bovine serum at 37°C in 5% CO₂. Each independent experiment made use of cells from 3–4 different animals per feeding group which were pooled and plated at a density of 400,000/well on 24-well plates for immunofluorescence (IF) and polymerase chain reaction and at a density of 900,000/well on 6-well plates for Western blotting. Two hours later after plating, non-adherent cells were removed to obtain an enriched population of alveolar macrophages. Macrophages were cultured with or without treatments for a total of 24 hours prior to analysis.

Western blot

Cells pooled from alcohol- and control-fed animals were independently lysed using Laemmli Sample Buffer (Bio-Rad, Hercules, CA) 24 hours after treatment with or without 5 µM sulforaphane (SFN, LKT Laboratories, St. Paul, MN). Protein electrophoresis was then performed, followed by transfer to polyvinylidene difluoride membranes. The membranes were probed with primary antibodies to RAGE (1 µg/ml) (ThermoFisher Scientific, Waltham, MA), and secondary antibodies (1:1000 dilution) from GE Healthcare Life Sciences (Pittsburgh, PA) before capture of immuno-reactive bands with the ChemiDoc XRS (Bio-Rad, Hercules, CA) system as previously described [11]. Immuno-reactive bands representing RAGE were determined using molecular weight and normalized to GAPDH (Sigma-Aldrich).

RNA isolation, Reverse Transcription, Real-Time Polymerase Chain Reaction

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Reverse transcription (RT) and real-time PCR (RT-PCR, Bio-Rad) were performed using primers for RAGE, MMP-9, and 18S (Life Technologies, Grand Island, NY, USA). MMP-9 and RAGE PCR products were normalized to 18S from the same RT sample and analyzed using the delta count method by calculating $2^{(-\Delta\Delta\text{Count})}$. Primer sequences are presented in Table 1.

sRAGE Enzyme-Linked Immunosorbent Assay

The first 5 mL of bronchoalveolar lavage fluid from each rat were saved and analyzed by a rat RAGE DuoSet sandwich enzyme-linked immunosorbent assay (ELISA) Development System (R&D Systems, Minneapolis, MN) as suggested by the manufacturer. Optical densities were obtained using a Synergy H1 microplate reader (BioTek, Winooski, VT) and normalized to total protein.

Determination of membrane-bound and intracellular RAGE

Alveolar macrophages were detached from their plates using Versene solution (ThermoFisher) and attached to microscope slides by cytospin. Immunofluorescence staining was performed using primary antibodies to RAGE (1:100 dilution) and Alexa Fluor-labeled secondary antibodies (ThermoFisher Scientific). For intracellular staining, a subgroup of samples was treated with 0.1% Triton X-100 (Sigma-Aldrich) prior to primary antibody incubation. Images were obtained with a fluorescence microscope and cellSens software (Olympus Life Science, Waltham, MA). Corrected total cell fluorescence (CTCF) was calculated with ImageJ (Fiji, Madison, WI) by assessment of multiple random fields from multiple replicates for a total of 40–100 cells per condition in the following manner: $CTCF = (\text{integrated density} / \text{area}) - (\text{background density} / \text{area})$.

RNA silencing

A set of 3 MMP-9 Stealth silencing RNAs was obtained from ThermoFisher Scientific (MMP-9, oligo ID: RSS330771, RSS330772, and RSS330773). All 3 were tested at 10, 20, and 40 nM, and RSS330773 was found to be most effective (at a concentration of 40 nM) and was therefore chosen for this study. NR8383 cells, a rat alveolar macrophage cell line (ATCC, Manassas, VA) were plated on 2 24-well plates (400,000 cells/well) in OptiMEM (Life Technologies, Grand Island, NY, USA) and transfected with 40 nM of stealth silencing RNA for rat MMP-9 or stealth RNA negative control using the Lipofectamine 3000 transfection reagent (ThermoFisher). One plate was treated with alcohol at 60 mM and the other was left untreated (both were kept in sealed chambers to ensure that the treatment would not evaporate). After 72 hours, the cells were taken for immunofluorescence.

Phagocytosis assay

Cells from alcohol- and control-fed animals were treated with the RAGE inhibitor FPS-ZM1 (MilliporeSigma, Billerica, MA) at a concentration of 10 $\mu\text{g/mL}$ [12], glutathione (GSH) 500 μM [13], or SFN 5 μM [14] for 24 hours before incubation with pHrodo Red *E. coli* particles (ThermoFisher) for 2 hours. Data were acquired with an LSR-II flow cytometer in conjunction with FACSDiva software (BD, Franklin Lakes, NJ). Mean fluorescence intensities (MFI) were acquired with FlowJo (FlowJo LLC, Ashland, OR).

Statistical analyses

Student's t-test was used for single comparisons and analysis of variance (ANOVA) was used for multiple comparisons using Prism (Graphpad, La Jolla, CA). Data are presented as mean $\pm$ standard error of the mean (SEM). Significance was accepted at $p < 0.05$.

RESULTS

Chronic alcohol ingestion decreases membrane-bound RAGE protein in alveolar macrophages by increasing levels of sRAGE in lavage fluid

To determine whether chronic alcohol exposure influences RAGE expression in the alveolar macrophage, rats were fed either a control diet or the alcohol-containing diet for 6 weeks. Animals were then sacrificed and total RNA and protein were isolated from alveolar macrophages obtained by bronchoalveolar lavage. As shown in Figure 1, RAGE mRNA expression was significantly increased in macrophages from alcohol-fed rats ($257\% \pm 71\%$ compared to $100\% \pm 15\%$ for macrophages from control-fed rats, $p < 0.05$). We then determined levels of membrane-bound RAGE protein in alveolar macrophages from control- and alcohol-fed rats by immunofluorescence. Cells from both groups were treated with or without Triton-X for membrane permeabilization. As shown in Figure 2, no difference was noted in RAGE levels between cells with or without cell membrane permeabilization, suggesting that the decrease in membrane-bound RAGE in the setting of alcohol is not due to a change in the intracellular pool of RAGE ($99\% \pm 7.5\%$ vs $100\% \pm 5.5\%$ in rats fed control diet) and that the intracellular RAGE pool is fairly limited under both control and alcohol conditions. Immunofluorescence of macrophages from alcohol-fed rats demonstrated significantly lower levels of RAGE than the controls ($67\% \pm 5.7\%$ vs $100\% \pm 5.5\%$, $p < 0.05$). The comparison between cells from control and alcohol-fed rats suggests that the decreased levels of RAGE protein in the macrophages from alcohol-fed rats cannot be explained by a defect in localization from the cytosol to the cell membrane.

Given that alcohol decreased the amount of membrane-bound RAGE, and based on prior work from our group suggesting an upregulation of MMP-9 in the setting of chronic alcohol ingestion that would be available to cleave RAGE into sRAGE, we next assessed sRAGE levels in BAL fluid from control- and alcohol-fed rats. As shown in Figure 3, sRAGE was significantly higher in BAL fluid from alcohol-fed rats when compared to the BAL fluid from control-fed rats ($163.5\% \pm 14.1\%$ vs $100\% \pm 10.8\%$, $p < 0.05$).

The effects of alcohol treatment on membrane-bound RAGE are abrogated by silencing MMP-9 RNA

Based on our prior work on MMP-9 in the alcohol model and the known link between RAGE cleavage and MMP-9, we hypothesized that silencing MMP-9 RNA would prevent the alcohol-induced decrease in membrane-bound RAGE. We first tested a series of si-RNAs to MMP-9 at various concentrations and selected the si-RNA and concentration that resulted in the most significant knockdown of MMP-9 mRNA expression. As shown in Figure 4A, si-MMP-9 at 40 nM resulted in marked suppression of MMP-9 mRNA expression in NR8383 cells, a relevant rat alveolar macrophage cell line ($3\% \pm 2\%$ vs. $100\% \pm 25.5\%$ for si-Control). The same conditions were then used in the setting of alcohol treatment. 72 hours after transfection with si-MMP-9 and alcohol treatment, cells were analyzed for RAGE protein levels by immunofluorescence. As shown in Figure 4B, si-MMP-9 protected the cells from the effects of alcohol on membrane-bound RAGE expression ($124\% \pm 6\%$ vs. $100\% \pm 7\%$ for untreated cells + si-Control, $p < 0.05$). Interestingly, si-MMP-9 also enhanced

RAGE protein expression in untreated cells ($168\% \pm 9\%$ vs. $100\% \pm 7\%$, $p < .05$) suggesting that RAGE cleavage occurs under basal conditions.

Treatment with the antioxidant SFN reverses the effects of chronic alcohol ingestion on RAGE protein expression in rat alveolar macrophages

We previously determined that chronic alcohol abuse impairs antioxidant defenses, and that glutathione depletion in the airway promotes the activation of MMP-9 [15], the key protein involved in RAGE cleavage to sRAGE. To test the functional consequences of that mechanism in the alcohol model, we sought to prevent the increase in sRAGE caused by chronic alcohol ingestion by enhancing antioxidant defenses with the phytochemical SFN. Alveolar macrophages were isolated from control- and alcohol-fed rats and treated with or without SFN *ex vivo* for 24 hours prior to assessment of RAGE levels by Western blot. As shown in Figure 5, protein expression of RAGE was again significantly decreased in alcohol-fed rats when compared to controls ($80\% \pm 2.8\%$ vs. $100\% \pm 6\%$, $p < 0.05$). In contrast, SFN treatment significantly enhanced RAGE levels in alcohol-fed rat macrophages even beyond levels seen in control-fed rat macrophages ($123\% \pm 4\%$ in alcohol-fed macrophages treated with sulforaphane vs $100\% \pm 6.6\%$ in untreated control-fed macrophages, $p < 0.05$).

Impairment in RAGE signaling mimics the effects of chronic alcohol ingestion on macrophage phagocytic function, but those effects can be abrogated with antioxidant treatment

To determine the functional consequences of RAGE impairment, alveolar macrophages were collected from alcohol- and control-fed rats and incubated with fluorescently labeled *E. coli* particles prior to analysis by flow cytometry. Twenty-four prior to bacterial incubation, macrophages from control-fed rats were treated with or without FPS-ZM1 (a RAGE inhibitor) and macrophages from alcohol-fed rats were treated with either SFN or GSH. As seen in Figure 6, macrophages isolated from alcohol-fed rats and control macrophages treated with FPS-ZM1 both demonstrated lower fluorescence intensity when compared to control-fed rats, suggesting that significantly fewer *E. coli* particles were phagocytosed by these cells ($61\% \pm 6.2\%$ and $45.7\% \pm 7.5$ for alcohol-fed macrophages and FPS-ZM1, respectively, vs $100\% \pm 6.6\%$ for control fed rats, $p < 0.05$). There was no significant difference between phagocytic function of macrophages treated with the inhibitor when compared to cells isolated from alcohol fed rats. Both sulforaphane and GSH reversed the impairment in phagocytic function ($p > 0.05$ relative to control-fed macrophages).

DISCUSSION

This study builds on our prior work on the relationship between antioxidant defenses and innate immunity in the alcoholic lung and illustrates a novel pathway by which impairment of RAGE signaling in the alcoholic airway prevents the macrophage from defending the alveolar space. Given the role of RAGE in inflammatory signaling and its importance as a marker of pulmonary injury, we hypothesized that chronic alcohol ingestion would disrupt RAGE signaling. We first assessed RAGE mRNA expression in the alveolar macrophages of alcohol-fed rats and although it was markedly increased, the expression of membrane-bound

RAGE protein was in fact significantly decreased. The discordant relationship between RAGE mRNA and protein expression led us to consider the possibility that alcohol was increasing cleavage of RAGE into sRAGE. We therefore examined lavage fluid from alcohol-fed rats and identified that sRAGE levels were significantly increased. Given our previous data demonstrating the increase in MMP-9 activity in the setting of alcohol treatment, we next silenced MMP-9 RNA and found that alcohol treatment no longer decreased membrane-bound RAGE. We then assessed the effects of antioxidant therapies on the balance between membrane-bound and soluble RAGE and determined that treatment with the SFN normalized membrane-bound RAGE levels in alveolar macrophages from alcohol-fed rats. Finally, we assessed the functional consequences of chronic alcohol ingestion and direct RAGE inhibition in the alveolar macrophage and found that both impair phagocytic function, and that treatment with either SFN or GSH restores phagocytic function in the setting of alcohol use. Taken together, these findings provide novel evidence that chronic alcohol ingestion disrupts the dynamic regulation between membrane-bound RAGE, which promotes immune responses in airway cells, and sRAGE, which acts as a decoy and if present in excess could potentially decrease airway immune responsiveness to pathogens. Importantly, antioxidant defenses, which are suppressed by alcohol, restore both membrane-bound RAGE levels and phagocytic function, indicating that the proper function of RAGE is dependent on functional redox signaling. These findings are likely of clinical significance as we have identified that mechanisms by which chronic alcohol ingestion causes oxidative stress within the airways and impairs alveolar macrophage immune function in our animal model have translated to individuals living with chronic alcohol abuse [16, 17].

Chronic alcohol ingestion decreases the expression of membrane-bound RAGE in the alveolar macrophage and increases RAGE mRNA expression in these cells. Although we can only speculate at present about the mechanism underlying this discordance, our findings raise the intriguing possibility of a positive feedback loop that responds when levels of membrane-bound RAGE are diminished. Specifically, we identified that membrane-bound RAGE levels are decreased by alcohol ingestion while the levels of soluble RAGE within the alveolar space were increased. Therefore, overall RAGE protein expression may be increased and consistent with the observed increase in RAGE mRNA expression but the balance is shifted markedly toward cleavage of membrane-bound RAGE to the soluble form. Additionally, the comparison of permeabilized cells to those with intact membranes allows us to conclude that intracellular RAGE levels (which are negligible at baseline) are not altered by exposure to chronic alcohol ingestion. This is a key point given prior studies by our group demonstrating that alcohol can alter trafficking of other membrane-bound receptors [18]. Of note, the direct RAGE inhibitor used in this study has been shown to be highly specific in other systems [19] and although we suspect it is specific in our system as well, we did not evaluate it for off-target effects in this study. Therefore, although more investigation is needed to dissect the precise mechanisms, our novel findings suggest that chronic alcohol ingestion shifts the relative balance toward soluble RAGE and this appears to play a role in the long-recognized defects in alveolar macrophage immune function that render individuals living with alcohol abuse susceptible to pneumonia and lung injury.

This pathophysiological shift toward soluble RAGE within the alveolar space by alcohol would not only decrease immune signaling by membrane-bound RAGE in the airway but could decrease the “alert level” within this space. Specifically, sRAGE is thought to act as a decoy receptor for RAGE signaling by binding excess RAGE ligands and blocking aberrant and/or over-exuberant inflammatory responses. However, if sRAGE levels are increased at the expense of membrane-bound RAGE at baseline in the alcoholic lung, this would leave the airways relatively hypo-responsive to pathogens that enter the distal airways. As membrane-bound RAGE is cleaved primarily by MMP-9, these findings are consistent with our prior study in this same model in which we determined that chronic alcohol ingestion increases the activation of MMP-9 within the alveolar space via glutathione depletion [15]. This observation is strongly supported by the salutary effects of si-MMP-9 on membrane-bound RAGE protein in the setting of alcohol treatment. Taken together, the data presented here raise the intriguing possibility that the balance between the immune signaling functions of membrane-bound RAGE and the decoy or immune dampening effects of sRAGE are dependent on antioxidant defenses via MMP9. Our use of 2 different antioxidants (SFN and GSH) in this study also supports that possibility.

It is important to note that the cellular sources of the increased levels of sRAGE in the alveolar space of alcohol-fed rats certainly include more than the alveolar macrophage alone. Airway epithelial cells express RAGE and the increase in sRAGE observed in the lavage fluid of individuals with acute lung injury has been touted as a biomarker of alveolar epithelial injury [20]. We did not analyze the effects of chronic alcohol ingestion on the expression of membrane-bound RAGE in the alveolar epithelium and this will be an important complementary area of investigation in future studies. Here, though, our focus was on the novel action of RAGE in macrophage innate immune function. To that end, we believe it is particularly important that, in parallel to restoring membrane-bound RAGE protein levels, SFN treatment restored phagocytic function.

In summary, this study strongly suggests that RAGE plays an important role in macrophage phagocytic function and inflammatory signaling and that chronic alcohol ingestion impairs RAGE signaling by shifting the balance from the membrane-bound form that activates innate immune responses to the soluble form that limits cellular responses. Our results also suggest that improving antioxidant defenses restores both membrane-bound RAGE expression and phagocytic function in the alcoholic macrophage. As noted above, this hypothesis still requires translation to the clinical setting. However, these experimental results are novel and provocative as they may elucidate a previously unrecognized function for RAGE signaling in the alveolar macrophage and how it can be impaired by stresses such as alcohol abuse. Further, considering the already-recognized expression of RAGE in the alveolar epithelium and its role as a biomarker of epithelial injury, our data suggest a mechanism through which chronic alcohol ingestion could increase sRAGE and prevent necessary crosstalk between the epithelium and the macrophage. Similarly, the data presented here further support our underlying contention that antioxidant defenses and innate immunity are inextricably linked in the lung microenvironment. If this proves to be true, then pharmacologic enhancement of antioxidant defenses in the right clinical context could improve both antioxidant and immune defenses within the lung and decrease the risk of pneumonia and lung injury in vulnerable individuals.

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Figure 1. Receptor for advanced glycation end products messenger RNA expression is increased in alveolar macrophages from alcohol-fed rats

Alveolar macrophages were obtained from rats fed a control diet or alcohol-containing for 6 weeks. Receptor for advanced glycation end products (RAGE) messenger RNA (mRNA) expression was quantified by real time polymerase chain reaction. Cells obtained from alcohol-fed rats had significantly higher levels of RAGE mRNA compared to control-fed rats. Data presented as mean $\pm$ standard error of the mean, $n = 4$ wells/condition (pooled from 3 rats per feeding group). * $p < 0.05$.

Figure 2. Receptor for Advanced Glycation End Products protein expression is decreased in the alveolar macrophages of alcohol-fed rats

Alveolar macrophages from control-fed and alcohol-fed rats were cytopspined on to slides and stained for receptor for advanced glycation end products (RAGE) with a fluorescent-labeled antibody. Prior to staining, samples were incubated with or without Triton-X 100 to permeabilize the membrane. There was no difference between samples from control-fed rats with and without membrane permeabilization, suggesting that intracellular RAGE protein expression has a negligible contribution to total cellular RAGE. Alcohol decreased RAGE protein significantly when compared to control-fed samples non-permeabilized samples, and membrane permeabilization again demonstrated a negligible intracellular RAGE pool. Data presented as mean $\pm$ SEM, $n = 6$ wells/condition (pooled from 4 rats per feeding group).

* $p < 0.05$ compared to untreated cells from control-fed rats.

Figure 3. Chronic alcohol exposure is associated with increased levels of soluble receptor for advanced glycation end products in bronchoalveolar lavage fluid

The first 5mL of BAL fluid from control or alcohol-fed rats were assayed for soluble receptor for advanced glycation end products (sRAGE) using a sandwich enzyme-linked immunosorbent assay. Levels of sRAGE were significantly increased in bronchoalveolar lavage fluid from alcohol-fed rats. Data presented as mean $\pm$ standard error of the mean, $n = 4$ aliquots/condition (each taken from an independent animal). * $p < 0.05$.

Figure 4.A

Figure 4.B

Figure 4. Silencing Matrix Metalloproteinase 9 RNA abrogates the effects of alcohol on membrane-bound receptor for advanced glycation end products

(A) NR8383 cells were transfected with either 40 nM si-Control or 40 nM si-Matrix Metalloproteinase 9 (si-MMP-9) and MMP-9 mRNA expression was assessed by real-time polymerase chain reaction. Relative to si-Control, si-MMP-9 resulted in a significant decrease in MMP-9 mRNA expression. Data presented as mean $\pm$ standard error of the mean (SEM), $n = 4$ wells/condition. $*p < .05$. (B) NR8383 cells were transfected with either si-Control or si-MMP-9 and treated $\pm$ 60 mM alcohol for 72 hours. Membrane-bound receptor for advanced glycation end products (RAGE) was then assessed by immunofluorescence. Si-MMP-9 prevented the alcohol-induced decrease in membrane-bound RAGE and enhanced RAGE protein expression in untreated cells. Data presented as mean $\pm$ SEM, $n = 4$ wells/condition, $*p < .05$.

Figure 5. Sulforaphane treatment negates the effects of chronic alcohol exposure on cellular receptor for advanced glycation end products

Alveolar macrophages isolated from either control or alcohol-fed rats were treated for 24 hours with sulforaphane *ex vivo* before assessment of protein expression by Western Blot. Sulforaphane treatment was administered in each well independently, and four wells were tested for each condition (each one run on its own lane). As before, alveolar macrophages from alcohol-fed rats demonstrated a decrease in cellular receptor for advanced glycation end products (RAGE) expression, but expression was enhanced to supra-normal levels with sulforaphane treatment. Data presented as mean $\pm$ standard error of the mean, $n = 4$ wells/condition (pooled from 3 rats per feeding group). All four wells run for each condition were used for statistical analysis. * $p < 0.05$ decreased compared to control. ** $p < 0.05$ increased compared to control. Bands pictured above each condition are taken from one lane and are representative of the four lanes run for each condition.

Figure 6. Exposure to an inhibitor of the receptor for advanced glycation end products and chronic alcohol ingestion both impair phagocytosis in alveolar macrophages and phagocytic function is restored by antioxidant treatments

Alveolar macrophages were isolated from control-fed and alcohol-fed rats. After plating, macrophages from control-fed rats were treated with or without FPS-ZM1 (a RAGE inhibitor) for 24h and macrophages from alcohol-fed rats were treated with or without sulforaphane (SFN) or glutathione (GSH). Cells were then incubated for 2 hours with fluorescently labeled *E. coli* particles. Signal intensity was measured by flow cytometry. Control cells treated with FPS-ZM1 demonstrated impaired phagocytic function compared to controls. Cells from alcohol-fed animals exhibited a lower phagocytic capacity at baseline compared to controls, which was restored to baseline levels with SFN or GSH treatment. Data presented as mean $\pm$ standard error of the mean, $n = 4$ wells/condition (pooled from 3 rats per feeding group). * $p < 0.05$.

Table 1

Target Gene	Forward (5'-3')	Reverse (5'-3')
Receptor for Advanced Glycation End Products	GAGCCTGAAGGTGGAACAGT	CATCACCGGTTTCTGTGACCC
Matrix Metalloproteinase-9	AGCCGACGTCACTGTAAGT	AACAGGCTGTACCCCTTGGTC