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Zinc supplementation restores PU.1 and Nrf2 nuclear binding in alveolar macrophages and improves redox balance and bacterial clearance in the lungs of alcohol-fed rats

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Abstract

Background—Chronic alcohol abuse causes oxidative stress, impairs alveolar macrophage immune function, and increases the risk of pneumonia and acute lung injury. Recently we determined that chronic alcohol ingestion in rats decreases zinc levels and macrophage function in the alveolar space; provocative findings in that zinc is essential for normal immune and antioxidant defenses. Alveolar macrophage immune function depends on stimulation by GM-CSF, which signals via the transcription factor PU.1. In parallel, the antioxidant response element signals via the transcription factor Nrf2. However, the role of zinc bioavailability on these signaling pathways within the alveolar space is unknown.

Methods—To determine the efficacy of dietary zinc supplementation on lung bacterial clearance and oxidative stress, we tested three different groups of rats: control-fed, alcohol-fed, and alcohol-fed with zinc supplementation. Rats were then inoculated with intratracheal *Klebsiella pneumoniae* and lung bacterial clearance was determined 24 hrs later. Isolated alveolar macrophages were isolated from uninfected animals and evaluated for oxidative stress and signaling through PU.1 and Nrf2.

Results—Alcohol-fed rats had a 5-fold decrease in lung bacterial clearance compared to control-fed rats. Dietary zinc supplementation of alcohol-fed rats normalized bacterial clearance and mitigated oxidative stress in the alveolar space, as reflected by the relative balance of the thiol redox pair cysteine and cystine, and increased nuclear binding of both PU.1 and Nrf2 in alveolar macrophages from alcohol-fed rats.

Conclusions—Dietary zinc supplementation prevents alcohol-induced alveolar macrophage immune dysfunction and oxidative stress in a relevant experimental model, suggesting that such a strategy could decrease the risk of pneumonia and lung injury in individuals with alcohol use disorders.

Keywords

rat; macrophages; GM-CSF; oxidative stress; lung; bacterial infections; zinc

INTRODUCTION

Alcohol is the most widely used drug in the world, and its abuse poses a tremendous burden to society. Alcoholics are vulnerable to multiple health problems including dysfunction of the heart, liver, brain, and the immune system (Lieber, 1995;Cook, 1998). The lung is often overlooked as a target of alcohol abuse but is commonly affected, and several reviews have summarized the detriments of alcoholism in the lung including an increased susceptibility to pneumonia and acute lung injury (Joshi and Guidot, 2007;Guidot and Hart, 2005). Despite this awareness, the causative factors remain incompletely understood. Investigating these underlying mechanisms will hopefully identify novel therapies and limit the enormous morbidity and mortality associated with pulmonary disease in individuals with alcohol use disorders.

There are numerous descriptions of alcohol-induced dysfunction of the immune system (von et al., 2002;Szabo and Mandrekar, 2009;Szabo et al., 2007). Specifically within the respiratory system, individuals with alcohol use disorders are prone to aspiration, have decreased mucociliary clearance, and have impaired host immunity in the airways (Happel and Nelson, 2005;Joshi and Guidot, 2007). We have previously shown that chronic alcohol ingestion has detrimental effects on alveolar macrophage immune function and alveolar epithelial barrier function in experimental animals (Guidot et al., 2000;Joshi et al., 2005). These findings build on prior reports that resident alveolar macrophages in alcoholics are impaired not only in their ability to phagocytose bacteria, but also in their release of cytokines, chemokines, and other factors responsible for microbial killing (D'Souza et al., 1995;D'Souza et al., 1996;Mason et al., 2000;Standiford and Danforth, 1997;Zhang et al., 1999). Recent studies from our alcohol research group implicate a defect in signaling by granulocyte/monocyte colony-stimulating factor (GM-CSF), which is integral to macrophage maturation, differentiation and function. We determined that chronic alcohol ingestion decreases GM-CSF receptor expression and signaling capacity through its master transcription factor, PU.1, in both the alveolar macrophage and the alveolar epithelium (Joshi et al., 2006;Joshi et al., 2005). Importantly, treatment with recombinant GM-CSF via the airway rapidly (within 48 hrs) reverses alcohol-induced defects in alveolar epithelial barrier function, and GM-CSF treatment *in vitro* of alveolar epithelial cells and macrophages isolated from alcohol-fed rats restores signaling through PU.1 and normalizes barrier and immune function in these cells, respectively (Joshi et al., 2006;Joshi et al., 2005). Taken together, these experimental studies suggest that even otherwise "healthy" alcoholics may have diminished GM-CSF-dependent priming of the alveolar macrophage pool that leaves them poorly equipped to respond to infectious and inflammatory stimuli.

Another important consequence of chronic alcohol consumption is oxidative stress, which has been defined as an imbalance between production and neutralization of reactive oxygen species (ROS). This pathologic buildup of ROS is thought to play an important role in the development of the alcoholic lung phenotype (Guidot and Hart, 2005). In animal models, chronic alcohol ingestion decreases glutathione (GSH), an important cellular antioxidant in the alveolar space (Bechara et al., 2005;Brown et al., 2001;Holguin et al., 1998). Synthesis of GSH depends on cysteine (Cys), and there is recent evidence that Cys and its oxidized counterpart cystine (Cyss) function as an important extracellular redox pair, while GSH and its oxidized form, glutathione disulfide (GSSG), operate more at the intracellular level (Iyer et al., 2009). Experimental models have shown that many of the detrimental effects of chronic alcohol consumption can be reversed with correction of this imbalanced redox state (Garcia-Ruiz et al., 1995;Zhou et al., 2005). Antioxidant defenses, of which Cys and GSH are just two components, are vital for cellular protection against oxidative stress. When faced with such stresses, cells respond by activating the antioxidant response element (ARE), a genetic program that is regulated by its major transcription factor, nuclear factor

erythroid 2-related factor 2 (Nrf2). The ARE/Nrf2 pathway thereby serves as a broad-based defense system against diverse oxidative stresses. Importantly, several studies suggest that it plays an important role in counteracting the oxidative stress created by chronic alcohol use (Kim et al., 2009; Dong et al., 2008; Cederbaum, 2006). However, we have shown that chronic alcohol ingestion causes profound oxidative stress within the lower airways of experimental animals and in otherwise healthy human subjects (Holguin et al., 1998; Moss et al., 2000). The mechanisms responsible for alcohol-induced oxidative stress are poorly understood, and the failure to mount an appropriate anti-oxidant response within the alveolar space suggests that chronic alcohol ingestion may interfere with the ARE/Nrf2 pathway in this unique microenvironment.

Finally, chronic alcohol abuse also induces many nutritional derangements, including zinc deficiency. Zinc is a trace element that is essential for normal host immune responses, protein metabolism, the function of more than 300 zinc metalloenzymes, and membrane integrity (Tudor et al., 2005; Zalewski, 2006; Zalewski et al., 2005). Immune functions appear to be particularly dependent on zinc bioavailability, and zinc deficiency has been implicated in many aspects of T and B lymphocyte functions as well in innate immunity. Zinc also is critical for the synthesis and function of multiple antioxidants, and zinc deficiency impairs an individual's defense against oxidative stress (Tudor et al., 2005). Although zinc deficiency has been most extensively studied in the liver, recent reviews have summarized important facets of zinc metabolism in pulmonary disorders including asthma (Truong-Tran et al., 2001; Zalewski, 2006; Zalewski et al., 2005). Based on these observations, we recently began investigating the potential role of zinc deficiency in the experimental alcoholic lung. We determined that chronic alcohol ingestion alters the expression of key zinc transporters in the gut and lung epithelia, and decreases zinc levels in the alveolar space and within the alveolar macrophages (Joshi et al., 2008). Remarkably, treating alveolar macrophages from alcohol-fed rats with zinc *in vitro* improved PU.1 nuclear binding and restored bacterial phagocytic capacity within 4 hours. In this current study, we extended those initial observations to ask two new questions: first, does dietary zinc supplementation restore alveolar macrophage function *in vivo*, and thereby produce a biologically significant benefit such as improving lung bacterial clearance? In parallel, does dietary zinc supplementation improve the redox balance within the alveolar space by increasing signaling through the ARE and its transcription factor, Nrf2? If so, then dietary zinc supplementation could be a simple and novel treatment for the alcoholic lung phenotype in humans, as zinc deficiency could represent a previously unrecognized mechanism that mediates alcohol-induced oxidative stress and immune dysfunction within the alveolar space.

MATERIALS AND METHODS

Rat model of chronic alcohol ingestion

Adult male Sprague-Dawley rats (initial weight ~250-350 g; Charles River Laboratory, Wilmington, MA) were fed the Lieber-DeCarli liquid diet (Research Diets, New Brunswick, NJ) containing alcohol (ethanol; 36% of total calories) or an isocaloric carbohydrate substitution. Alcohol-fed rats received 1/3 strength alcohol diet (12% of total calories) for one week, 2/3 strength alcohol diet (24% of total calories) for one week, followed by four weeks of the full strength alcohol diet, for a total of six weeks of alcohol ingestion. In some of the alcohol-fed rats, zinc acetate (100 mg/L) was added to the diet. We previously determined that this dose normalizes alveolar epithelial and macrophage function in alcohol-fed rats and correlates on mg/kg basis with the recommended dosing of dietary supplementation for zinc deficiency in humans (Joshi et al., 2008). All work was performed with the approval of the Atlanta VAMC Institutional Care and Use of Animals Committee.

Experimental model of *Klebsiella pneumoniae*

Klebsiella pneumoniae (Schroeter) Trevisan from ATCC catalog # 43816 (Manassas, VA), was stored in 1 ml aliquots at -80°C in trypticase soy broth obtained from Remel catalog # R112731 (Lenexa, KS) with 10% glycerol. For each experiment, the frozen culture was thawed and grown in 100 ml fresh trypticase soy broth at 37°C for 18 hours with a 150 rpm shaker, a process which allowed bacteria to reach log-phase growth. Bacteria were then centrifuged at 4500 rpm (3645 xg) to create pellet and washed twice with sterile phosphate-buffered saline (PBS). The bacteria were then re-suspended in a total of 10 ml of PBS, which made a concentration of 2×10^{10} colony-forming units (CFU) per ml. This value was determined by performing serial dilutions. This stock solution was used to make a final concentration of 10^5 CFU/ml for inoculation, and a total volume of 100 μl was inoculated in the airway of each rat using the method described below. The bacteria were plated on MacConkey agar (Remel catalog # R01550) to confirm their presence and concentration.

Determination of lung bacterial clearance *in vivo*

Rats were placed in an anesthesia chamber with isoflurane and then transferred to an inoculation apparatus, which allowed for continuous general anesthesia during procedure. An otoscope was used as a light source and a special tip was used for visualization of the vocal cords in a method similar to direct laryngoscopy. Once the cords were visualized, 50 μl of 1% bupivacaine was injected for local anesthesia, followed by passage of a catheter through the vocal cords into the trachea and advanced to distal lung tissue. An inoculating syringe was used to deliver 100 μl of the inoculum prepared as described above. Rats were sacrificed at 24 hours and the lung bacterial clearance was quantified by determining the residual for colony-forming units (CFU) of bacteria. Briefly, the entire infected lung was collected and placed in 20 ml of sterile PBS. All inoculated animals only exhibited evidence of gross infection on one side (presumably the site of inoculation), and only that lung that was collected for plating. The unaffected lung did not show bacteria when plated in preliminary experiments and therefore was not routinely collected. The lung was homogenized using a tissue homogenizer. Serial dilutions of the lung homogenate were performed and then plated on MacConkey agar to determine CFUs of *Klebsiella pneumoniae* per lung. Plates were incubated overnight at 37°C and CFUs were counted manually.

Bronchoalveolar lavage and isolation of alveolar macrophages

Pentobarbital (100 mg/kg IP) was used for anesthesia prior to sacrifice. This was followed by the placement of a tracheotomy tube, and the lungs were first lavaged with 5 ml of sterile PBS, and the returned fluid was used for Cys/Cyss determination. For the isolation of alveolar macrophages, lungs were then lavaged with 10 ml of sterile PBS four times. The recovered sample was centrifuged at 1500 rpm (405 xg) for 7 minutes and the cell pellet was re-suspended for cell count determination using a hemocytometer. Following cell count determination, a smear was made to evaluate for cell types, which showed >90% macrophages. The macrophages were used for various experiments including electromobility shift assay, overnight *in vitro* studies, as well as for RNA extraction followed by RT-PCR and quantitative PCR to determine gene expression.

Determination of thiol pair levels and oxidant stress analysis

GSH and Cys, as well as their respective oxidized forms, GSSG and Cyss, were determined in the lavage fluid and isolated alveolar macrophages by HPLC analysis. Methods for determination of thiol pairs have been previously described. (Brown et al., 2001; Holguin et al., 1998). In short, 500 μl of BAL fluid from each sample was immediately acidified in cold perchloric acid (5% total), which contained the internal standard γ -glutamyl-glutamate (5

μM; final concentration). The samples were placed on dry ice and then stored at -70°C until ready for analysis by the Brown laboratory. To control for dilution by the lavage procedure, the GSH, GSSG, Cys, and Cyss concentrations in extracellular lung fluid were normalized via the urea method. Blood collection was necessary for quantification of thiol pairs. Approximately 3-5 ml of blood was collected by cardiac puncture from each animal and centrifuged at 10,000 rpm (9300 xg) to expose the plasma, which was used for internal controls required in GSH/GSSG and Cys/CySS redox determinations.

Determination of metallothionein and Nrf2 expression by RNA isolation and quantitative PCR

Total RNA was extracted from alveolar macrophages using Qiagen RNeasy Mini Kit (Valencia, CA) and treated with Qiagen DNase I to eliminate contaminating genomic DNA. cDNA was synthesized using reverse transcription with 1 μg of total RNA in total volume of 10 μl per reaction in Bio-Rad iScript cDNA synthesis kit (Hercules, CA). Quantitative PCR was carried on the Bio-Rad iCycler. Amplification was performed in Bio-Rad iQ SYBR green supermix containing specific primers and with denaturing at 95°C for 20 seconds, annealing at 58°C for 20 seconds, and extension at 20 seconds. Samples were run in triplicate. QuantumRNA class II 18S primers were purchased from Ambion (Austin, TX). PCR amplicons from all species were normalized for the amount of 18S in the same cDNA sample. Dilution curves showed that the real-time PCR efficiency was greater than 95% for all genes analyzed. Real-time SYBR green dissociation curves showed one species of amplicon for each primer set. All primers were designed in our laboratory and were obtained from Invitrogen.

Determination of nuclear binding of PU.1 and Nrf2 by electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) for PU.1 and Nrf2 nuclear binding was performed to determine nuclear binding in alveolar epithelial cells and macrophages. This procedure has been previously described. (Joshi et al., 2006; Joshi et al., 2005) In brief, macrophages were first isolated as described above, and nuclear protein extracts were prepared. Double-stranded PU.1 consensus oligonucleotide (5' TGA AAG AGG AAC TTG GT) or Nrf2 consensus oligonucleotide (5' TGG GGA ACC TGT GCT GAG TCA CTG GAG) was radiolabeled with ^{32}P gamma ATP using T4 polynucleotide kinase enzyme. Nuclear protein (5 μg) was incubated with radiolabeled PU.1 or Nrf2 for 30 min at room temperature. When applicable, 2 μg of an anti-PU.1 or an anti-Nrf2 antibody was added to determine if there was a supershift assay (reflective of PU.1 and Nrf2 binding to the same oligonucleotide). After binding, protein-DNA complexes were electrophoresed on a native 4.5% polyacrylamide gel using 1x Tris-glycine buffer, and dried under vacuum prior to x-ray film exposure.

RESULTS

Dietary zinc supplementation restored lung bacterial clearance in alcohol-fed rats

We previously published that dietary zinc supplementation can reverse zinc deficiency in the alveolar space of alcohol-fed rats (Joshi et al., 2008). Here, we determined that pneumonia severity was associated with this alveolar zinc deficiency in the setting of chronic alcohol ingestion. Bacterial clearance was employed as a surrogate marker of pneumonia severity and was significantly impaired in alcohol-fed rats that exhibit alveolar zinc deficiency. As shown in Figure 1, alcohol-fed animals had a five-fold greater ($P<0.05$) lung burden of bacteria than control-fed rats 24 hrs following intratracheal inoculation with *Klebsiella pneumoniae*. In contrast, alcohol-fed rats whose diets were supplemented with zinc acetate (100 mg/L) cleared bacteria from their lungs as well as control-fed rats ($P>0.05$).

Zinc treatment *in vitro* restored nuclear binding of PU.1 in alveolar macrophages

We next explored possible mechanisms that could explain how dietary zinc supplementation preserved lung bacterial clearance in alcohol-fed rats. We had previous work determined that chronic alcohol ingestion down-regulates GM-CSF receptor expression signaling via its master transcription factor, PU.1. Therefore, we next cultured alveolar macrophages from control-fed and alcohol-fed rats overnight with or without zinc acetate (20 μ M) *in vitro*, and then performed electromobility shift assays on nuclear preparations to determine if zinc could improve nuclear binding of PU.1 (which is required to mediate GM-CSF signaling and induce alveolar macrophage immune functions including bacterial phagocytosis). As shown in Figure 2, panel A and consistent with our previously published findings, nuclear binding of PU.1 was decreased in macrophages isolated from alcohol-fed rats compared to macrophages from control-fed rats (Lane 3 vs. Lane 1). In contrast, zinc treatment *in vitro* completely restored PU.1 nuclear binding in macrophages from alcohol-fed rats (Lane 4 vs. Lane 3) but had no effect on macrophages from control-fed rats (Lane 2 vs. Lane 1).

Similarly, zinc treatment *in vitro* restored nuclear binding of Nrf2 in alveolar macrophages

We next examined Nrf2 nuclear binding by electrophoretic mobility shift assays in these macrophages, as Nrf2 is the master transcription factor that activates the antioxidant response element (ARE). As shown in Figure 2, panel B, nuclear binding of Nrf2 was also decreased in macrophages isolated from alcohol-fed rats compared to macrophages from control-fed rats (Lane 3 vs. Lane 1). In contrast, zinc treatment *in vitro* completely restored PU.1 nuclear binding in macrophages from alcohol-fed rats (Lane 4 vs. Lane 3). Interestingly, and as opposed to PU.1 binding in cells from control-fed rats where zinc treatment had no effect, zinc treatment appeared to increase Nrf2 nuclear binding in alveolar macrophages from control-fed rats (Lane 2 vs. Lane 1).

In parallel, dietary zinc supplementation *in vivo* improved both PU.1 and Nrf2 nuclear binding in alveolar macrophages from alcohol-fed rats

We next extended these findings of zinc treatment *in vitro* on PU.1 and Nrf2 nuclear binding in alveolar macrophages from alcohol-fed rats. As dietary zinc supplementation preserved lung bacterial clearance in alcohol-fed rats *in vivo*, we reasoned that PU.1 and/or Nrf2 nuclear binding should also be increased in the alveolar macrophage *in vivo* if activation of GM-CSF signaling and/or the ARE were a plausible explanation for the efficacy of zinc supplementation.

Therefore, we next isolated alveolar macrophages from control-fed rats as well as from alcohol-fed rats whose diets were supplemented with zinc acetate (100 mg/L) and performed electrophoretic mobility shift assays for PU.1 and Nrf2. As shown in Figure 3, both PU.1 (panel A) and Nrf2 (panel B) nuclear binding were decreased in alveolar macrophages from alcohol-fed rats compared to control-fed rats (Lane 2 vs. Lane 1 in each panel). However, consistent with the effects of zinc treatment *in vitro* in Figure 2, dietary zinc supplementation restored both PU.1 (panel A) and Nrf2 (panel B) nuclear binding in alveolar macrophages from alcohol-fed rats (Lane 3 vs. Lane 2 in each panel).

Dietary zinc supplementation *in vivo* restored gene expression of metallothionein and Nrf2 in macrophages from alcohol-fed rats

The results shown in Figure 2 and in Figure 3 suggest that zinc treatment can increase GM-CSF-dependent signaling (via PU.1 nuclear binding) and activation of the ARE (via Nrf2 nuclear binding) in the alveolar macrophages of alcohol-fed rats. We had already published that zinc treatment increases the expression of the GM-CSF receptor and normalizes bacterial phagocytic function in alveolar macrophages from alcohol-fed rats (Joshi et al.,

2008). As both of these functions are dependent on GM-CSF signaling, and therefore on PU.1 nuclear binding, we decided to examine whether or not Nrf2-dependent functions were likewise augmented by zinc treatment. Metallothionein is a zinc storage protein that also possesses antioxidant properties and is important in the synthesis of thiol antioxidants, and whose expression is part of the ARE and therefore mediated by Nrf2. Therefore, we quantified metallothionein gene expression as a surrogate marker of Nrf2-mediated activation of the ARE to correlate with the Nrf2 binding studies in Figures 2 and 3. In parallel, we quantified Nrf2 gene expression to determine if the salutary effects of zinc treatment on Nrf2 nuclear binding could be explained at least in part through a direct induction of Nrf2. As shown in Figure 4, panel A, chronic alcohol ingestion significantly ($P < 0.05$) decreased metallothionein gene expression in alveolar macrophages by more than 50% compared to alveolar macrophages from control-fed rats. In contrast, dietary zinc supplementation significantly increased ($P < 0.05$) metallothionein gene expression in the alveolar macrophages of alcohol-fed rats. In fact, dietary zinc supplementation actually increased ($P < 0.05$) alveolar macrophage metallothionein gene expression ~2-fold compared to control macrophages. In parallel, and as shown in Figure 4, panel B, chronic alcohol ingestion significantly decreased ($P < 0.05$) the gene expression Nrf2 itself in alveolar macrophages when compared to Nrf2 expression in alveolar macrophages from control-fed rats. In contrast, and consistent with the induction of metallothionein in panel A, dietary zinc supplementation increased Nrf2 gene expression in the alveolar macrophages of alcohol-fed rats to the same level of expression ($P > 0.05$) as in alveolar macrophages from control-fed rats.

Dietary zinc supplementation also restored redox balance in the Cys/CySS system in the airways of alcohol-fed rats *in vivo*

We have previously shown that chronic alcohol ingestion causes profound oxidative stress within the airways of experimental animals as well as in otherwise healthy humans with significant alcohol use disorders (Holguin et al., 1998; Moss et al., 2000). As activation of the ARE through Nrf2 is critical to mount a healthy antioxidant response, our findings thus far in this study suggested that alcohol-induced zinc deficiency could be responsible at least in part for the airway oxidative stress we had first discovered in the alcoholic lung. Therefore, we next determined the effects of dietary zinc on the status of the important extracellular thiol pair, cysteine (Cys) and cystine (Cyss) in the airways of alcohol-fed rats. As shown in Figure 5, chronic alcohol ingestion significantly decreased ($P < 0.05$) the relative ratio of cysteine to cystine (Cys/Cyss) in the lung lavage fluid of alcohol-fed rats as compared to the Cys/Cyss ratio in the lung lavage fluid of control-fed rats. In contrast, and consistent with its ability to induce Nrf2 and metallothionein gene expression, dietary zinc supplementation normalized ($P > 0.05$) the Cys/Cyss ratio in the lung lavage fluid of alcohol-fed rats when compared to the lung lavage fluid of control-fed rats.

PU.1 and Nrf2 appear to cooperatively bind DNA in a zinc-dependent fashion

Alcohol has independent effects on the GM-CSF/PU.1 pathway and the ARE/Nrf2 pathway. Our previous studies have always examined these pathways as separate entities, and it is not currently known whether there is any interaction or crosstalk between these two important systems. Therefore, we explored the possibility of cooperative interaction by performing electrophoretic mobility shift assays (EMSA), but with the addition of antibodies to Nrf2 and PU.1 to look for a supershift (see Methods). First, we examined the effect of zinc treatment *in vitro* on alveolar macrophages. As shown in the representative gel in Figure 6, zinc treatment appeared to increase interactive binding of DNA by PU.1 and Nrf2, as indicated by a supershift of the PU.1 band in the presence of the antibody to Nrf2 (in panel A) and the supershift of the Nrf2 band in the presence of the antibody to PU.1 in alcohol-fed rats only (panel B). Next, we extended these findings to alveolar macrophages that were

freshly isolated from alcohol-fed rats whose diets were supplemented with zinc *in vivo* and looked for a comparable supershift in the Nrf2 band when the anti-PU.1 antibody was added. The supershift assay in Figure 7 is qualitatively similar to panel A in Figure 6, providing evidence that dietary zinc treatment promotes cooperative binding of PU.1 and Nrf2 in the alveolar macrophages of alcohol-fed rats *in vivo* and therefore that the findings in Figure 6 are not restricted to zinc treatment of cells *in vitro*. Interestingly, as shown in Figure 7 there did not appear to be any significant interactive binding by Nrf2 and PU.1 in the alveolar macrophages of control-fed rats, suggesting that under normal conditions the ARE and GM-CSF signaling pathways may be less interdependent than during stresses such as chronic alcohol ingestion.

DISCUSSION

In this study we determined that dietary zinc supplementation improved lung bacterial clearance in alcohol-fed rats. Although there are many potential explanations for these salutary effects, it is intriguing that dietary zinc appeared to restore signaling through two important pathways in the alveolar macrophage that protect the lung against infection and oxidative stress. Specifically, it improved nuclear binding of PU.1, the master transcription factor for GM-CSF signaling that is crucial for alveolar macrophage maturation, differentiation, and immune functions including bacterial phagocytosis. In parallel, zinc supplementation restored nuclear binding of Nrf2, the transcription factor required to activate the antioxidant response element (ARE) and therefore critically involved in antioxidant defenses. Concomitant with increasing Nrf2 nuclear binding, zinc supplementation reversed the alcohol-induced oxidative stress, as reflected by restoring balance in the cysteine and cystine redox pair within the alveolar space. Taken together, these findings add to our previous study (Joshi et al., 2008) and further implicate zinc deficiency in the oxidative stress and immune dysfunction seen in the alcoholic lung. Translation of these experimental findings to the clinical setting could offer a potentially safe, effective, and simple therapeutic option for the millions of individuals with alcohol use disorders who are at increased risk of pneumonia and other serious lung diseases.

Zinc is an important micronutrient that plays a key role in the immune response, and there is clear evidence that alcoholism has detrimental effects on zinc homeostasis (Rodriguez-Moreno et al., 1997; Mills et al., 1983). Studies in zinc-deficient children exhibit a vulnerability to pneumonia (Bhandari et al., 1996), and there is clinical evidence that zinc supplementation can be effective in reducing the incidence of pneumonia in certain pediatric populations (Bhandari et al., 2002). In animal models, zinc deficiency has been shown to increase susceptibility and severity to pneumonia as well as decrease the effectiveness of vaccination to organisms such as pneumococcus (Strand et al., 2003). Taken together, these studies illustrate that zinc has important immune functions in the lung. However, zinc deficiency in the lung has not been well studied in the setting of alcohol abuse. Recently, we determined that chronic alcohol ingestion causes zinc deficiency within the alveolar space (Joshi et al., 2008). In this study we extended those findings to an experimental model of bacterial pneumonia *in vivo* and, as expected, alcohol-fed rats showed an increased lung bacterial burden at 24 hours when inoculated with *Klebsiella pneumoniae*, a common pulmonary pathogen in alcoholics. More importantly, dietary zinc supplementation restored bacterial clearance to levels seen in control-fed rats. The mechanisms for these protective effects are still speculative as we did not directly measure alveolar macrophage host defense in this *in vivo* pneumonia model. Extrapolating from our *in vitro* model (Joshi et al., 2008), however, it seems plausible that dietary zinc is augmenting alveolar macrophage immune function. Normal host defenses against lung bacterial infection include both innate and acquired immune responses. The innate response depends on processes such as mucociliary clearance as well as alveolar macrophage recognition and removal of foreign particles and

organisms that reach the distal airways and alveolar space, and bacterial clearance models have long been used as a surrogate measure of host innate immune response (Karaolis et al., 2007; Lehner et al., 2001). Both phagocytosis and intracellular killing of ingested organisms are essential functions of the alveolar macrophage that are impaired by chronic alcohol ingestion. Moreover, experimental models have shown alcohol to impair mucociliary clearance while recent evidence may suggest that zinc may enhance it (Woodworth et al., 2010). Although not tested in our study, this may represent another potential mechanism by which zinc supplementation improves bacterial clearance in alcohol-fed rats. It is reasonable to postulate that zinc deficiency, as a consequence of alcoholism, contributes to innate immune dysfunction, and zinc supplementation could confer protection to this vulnerable population.

The salutary effects of zinc supplementation on alveolar macrophage immune function can plausibly be linked to restoration of GM-CSF signaling through its master transcription factor PU.1. Specifically, we determined previously that GM-CSF signaling appears to be targeted in the alcoholic lung (Joshi et al., 2006; Joshi et al., 2005; Pelaez et al., 2004), and that dietary zinc supplementation in alcohol-fed rats restored phagocytic function to the alveolar macrophage *in vitro* (Joshi et al., 2008). These findings are consistent with earlier recognition that GM-CSF signaling is absolutely required for normal alveolar macrophage maturation and function (Shibata et al., 2001; Trapnell and Whitsett, 2002), and that its receptor-triggered signaling is mediated by its master transcription factor, PU.1. GM-CSF was first discovered in mouse lung extracts and found to be primarily secreted by alveolar epithelial type II cells (Shibata et al., 2001; Trapnell and Whitsett, 2002). The chief function of GM-CSF in facilitating the terminal differentiation of circulating monocytes into mature alveolar macrophages was discovered after development of the GM-CSF knockout mouse, which had normal bone marrow maturation, but abnormal alveolar macrophage maturation and a phenotype that resembles pulmonary alveolar proteinosis (PAP) (Huffman et al., 1996). Consistent with the GM-CSF knockout mouse, later studies from PAP patients showed a significant decrease in PU.1 expression in their alveolar macrophages, which can be restored with exogenous GM-CSF administration (Bonfield et al., 2003). Our experiments demonstrated decreased nuclear binding of PU.1 in macrophages from alcohol-fed animals. This finding follows prior work demonstrating a down-regulation of GM-CSF receptor expression with chronic alcohol ingestion (Joshi et al., 2006; Joshi et al., 2005). Our findings in this current study complement previous studies and show that zinc supplementation, both *in vitro* and *in vivo*, restored nuclear binding of PU.1 in alveolar macrophages (Joshi et al., 2008). Therefore, a plausible conclusion is that increased PU.1 nuclear binding reflects reactivation of GM-CSF signaling and ultimately, restoration of phagocytic function.

A novel finding in this study is that there appear to be remarkably parallel zinc-dependent effects on Nrf2, the master transcription factor for the antioxidant response element (ARE). Similar to its effects on PU.1 and the GM-CSF pathway, zinc supplementation restored Nrf2 nuclear binding in the alveolar macrophage, and this was reflected in improvement of the redox balance in cysteine and cystine within the alveolar space. This is important since one of the major cellular consequences of alcoholism is increased oxidative stress, which has many implications and contributes significantly to the overall alcoholic lung phenotype (Guidot and Hart, 2005; Joshi and Guidot, 2007). Further, despite the oxidative stress, the alcoholic lung appears to respond inadequately in activating its antioxidant defenses. The ARE is a gene program that is a critical defense against oxidative stress through transcription of antioxidant genes (Nguyen et al., 2003). Nrf2 plays an important role in transcriptional activation of ARE-driven genes similar to the role of PU.1 in the GM-CSF pathway. In parallel to the GM-CSF pathway, our studies provide evidence that alcohol interferes with the ARE signaling pathway as reflected by decreased Nrf2 nuclear binding

and antioxidant gene expression in alveolar macrophages from alcohol-fed rats. In fact, it is possible that the alcohol-induced lung oxidative stress that we have identified and characterized in this population is caused by zinc deficiency and impaired antioxidant defenses rather than a primary increase in oxidant production. Interestingly, there is also evidence that zinc depletion alone creates a state of oxidative stress in the lungs and airways (Zalewski, 2006). These studies emphasize the importance of zinc itself in the antioxidant response. In any case, zinc supplementation—either *in vitro* or *in vivo*—restored Nrf2 nuclear binding. To further investigate Nrf2 induction of ARE activation, we also evaluated the ARE-inducible gene metallothionein, which did show decreased expression with alcohol and restoration with zinc supplementation. Indeed, dietary zinc supplementation in alcohol-fed rats decreased oxidative stress and restored redox balance in the lungs. Taken together, these findings highlight the role of dietary zinc in mitigating the effects of alcohol-induced oxidative stress. Clinically, zinc supplementation may prove to be a relatively simple means of restoring redox balance in the airways of individuals with alcohol use disorders, many of whom are in treatment for their addiction but nevertheless are at increased risk for pneumonia and acute lung injury.

In this study we also provide novel preliminary evidence that PU.1 and Nrf2 cooperatively bind DNA in a zinc-dependent fashion, as reflected by supershift assays presented in this study. Although this evidence is not conclusive that the GM-CSF and ARE signaling pathways are coordinately regulated, such a possibility is certainly intriguing and suggests that zinc bioavailability is crucial to regulate both of these related defense systems within the lung (and perhaps, in other tissues) during significant inflammatory and/or oxidative stresses. This would seem reasonable from a teleological perspective for immune and antioxidant defense to be coordinately regulated in the alveolar space, where microbial invasion and oxidative stress are constant threats. These findings are interesting but raise many more questions and highlight some of the limitations of this particular study. First, we were not able to directly test the effects of PU.1 and Nrf2 signaling *in vivo* in our *Klebsiella* pneumonia model. Therefore, we can only speculate that restoration of PU.1 and Nrf2 signaling through zinc supplementation is mechanistic in improving bacterial clearance. Additionally, we did not detect any zinc-dependent interactions between PU.1 and Nrf2 by the supershift assay in alveolar macrophages from control-fed rats. Perhaps these pathways become interdependent (and this interdependence is zinc-dependent) in response to acute or chronic stresses such as alcohol abuse. Whether or not these two pathways are interdependent or simply are activated in parallel, they are clearly critical for defense against inflammatory stresses and evidence is emerging from these experimental studies that alcohol interferes with both pathways by decreasing zinc bioavailability within the alveolar space.

In summary, we determined that zinc supplementation of alcohol-fed rats improves PU.1 and Nrf2 nuclear binding in the alveolar macrophage, reverses alcohol-induced redox imbalance, and enhances lung bacterial clearance in an experimental pneumonia model. These findings provide compelling evidence that zinc deficiency interferes with both GM-CSF-dependent immune function and ARE-mediated antioxidant defenses, and thereby contribute to the alcoholic lung phenotype. Clinically, alcohol abusers are more susceptible to pneumonia and are more likely to develop acute lung injury in response to pneumonia and/or other acute insults. It is intriguing to postulate that zinc deficiency plays an important role in the defective immune and antioxidant defenses in this population. Even more exciting is the possibility that dietary zinc supplementation could be a simple and safe intervention that could improve the health of these vulnerable individuals.

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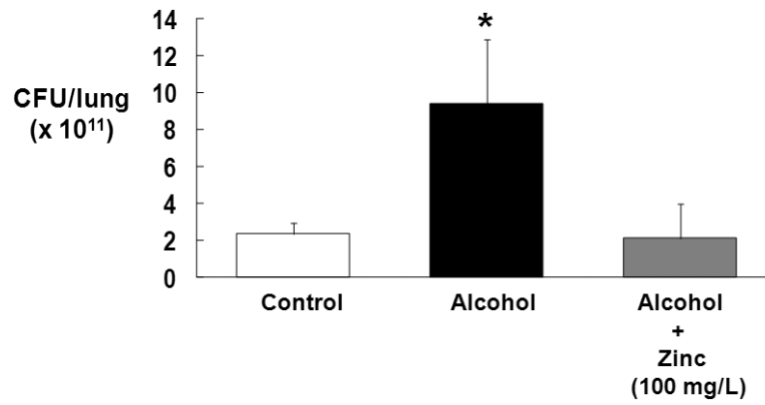


Figure 1. Dietary zinc supplementation preserves lung bacterial clearance of *Klebsiella pneumoniae* in alcohol-fed rats

Rats were fed the Lieber-DeCarli isocaloric liquid diet \pm alcohol \pm zinc acetate (100 mg/L) for 6 wks, and then challenged with an inoculum of *Klebsiella pneumoniae* (10^4 CFU) intratracheally. Rats were sacrificed 24 hrs post-inoculation and lung bacterial burden (in CFUs) determined as described in the Methods. * $P < .05$ compared to control-fed rats

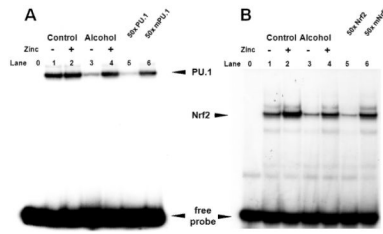


Figure 2. Nuclear binding of PU.1 and Nrf2 is decreased in alveolar macrophages from alcohol-fed rats and is restored with zinc treatment *in vitro*

Alveolar macrophages were isolated from control-fed and alcohol-fed rats (6 wks) and incubated overnight \pm zinc acetate (20 μ M) *in vitro*; nuclear extracts were then isolated and analyzed by electrophoretic mobility shift assay (EMSA). Representative gels are shown illustrating nuclear binding of PU.1 (panel A) and Nrf2 (panel B). In each panel, Lane 0 was loaded with free labeled oligonucleotide (probe) only; Lanes 1-2 are control \pm zinc; Lanes 3-4 are alcohol \pm zinc; Lane 5 is the unlabeled oligonucleotide (50x) and Lane 6 is the mutated oligonucleotide (50x) to confirm specific binding of PU.1 and Nrf2, respectively

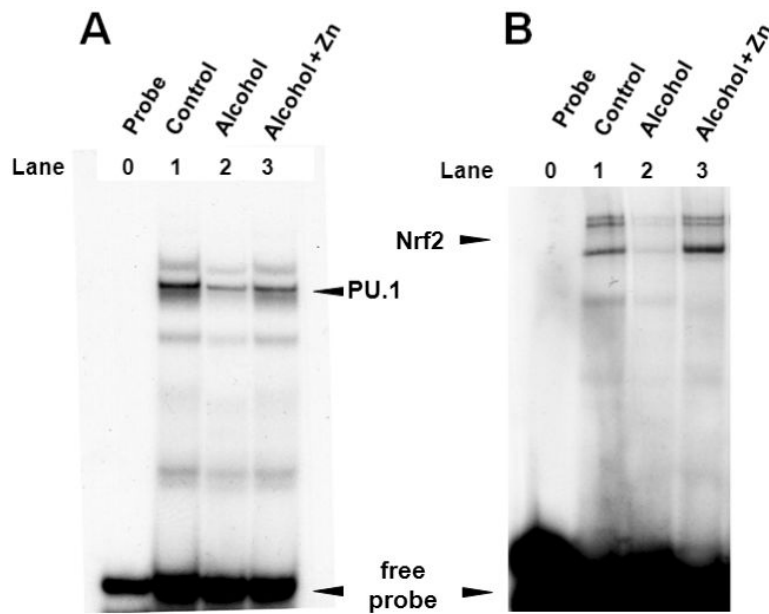


Figure 3. In parallel, dietary zinc supplementation *in vivo* zinc improves PU.1 and Nrf2 nuclear binding in alveolar macrophages from alcohol-fed rats

Alveolar macrophages were isolated from control-fed rats and from alcohol-fed rats \pm dietary supplementation with zinc acetate (100 mg/L) for 6 wks; nuclear extracts were then isolated and analyzed by electrophoretic mobility shift assay (EMSA). Representative gels are shown illustrating nuclear binding of PU.1 (panel A) and Nrf2 (panel B). In each panel, Lane 0 was loaded with free labeled oligonucleotide (probe) only; Lane 1 is control; Lane 2 is alcohol-fed; Lane 3 is alcohol-fed + zinc.

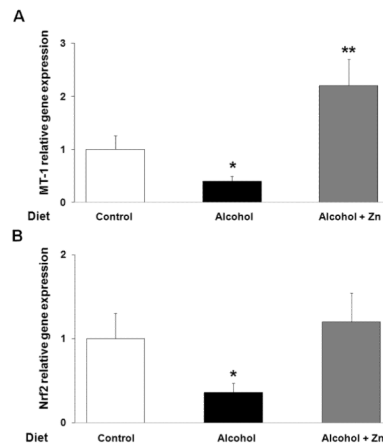


Figure 4. Dietary zinc supplementation *in vivo* restores gene expression of metallothionein and Nrf2 in macrophages from alcohol-fed rats

Relative gene expression of metallothionein-1 (MT-1, panel A) and Nrf2 (panel B) as determined by quantitative PCR (see Methods) in alveolar macrophages isolated from control-fed rats and from alcohol-fed rats \pm dietary supplementation with zinc acetate (100 mg/L) for 6 wks. * $P < 0.05$ decreased compared to expression in macrophages from control-fed rats ** $P < 0.05$ increased compared to expression in macrophages from control-fed rats

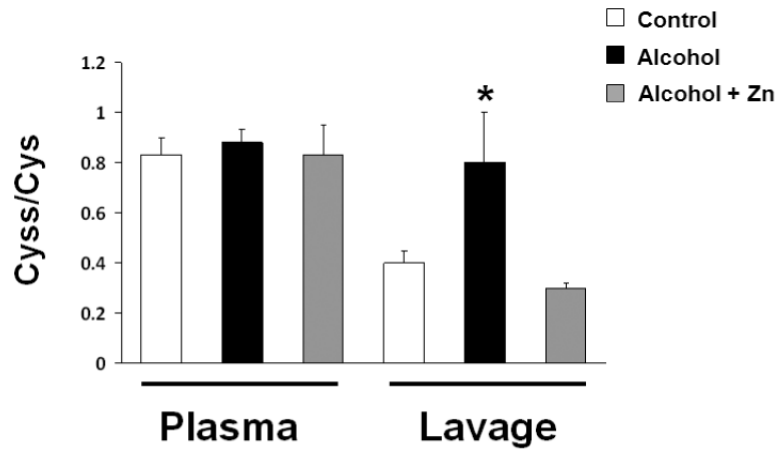


Figure 5. Zinc supplementation *in vivo* restores the cystine/cysteine (Cyss/Cys) redox balance in the alveolar space of alcohol-fed rats

Comparison of the ratios of cystine (Cyss, the oxidized form of cysteine) and cysteine (Cys) (both determined by HPLC as described in the Methods) in the plasma and in the lung lavage fluid (reflecting the alveolar space) in control-fed rats and in alcohol-fed rats \pm dietary supplementation with zinc acetate (100 mg/L) for 6 wks. * $P < 0.05$ compared to control-fed rats

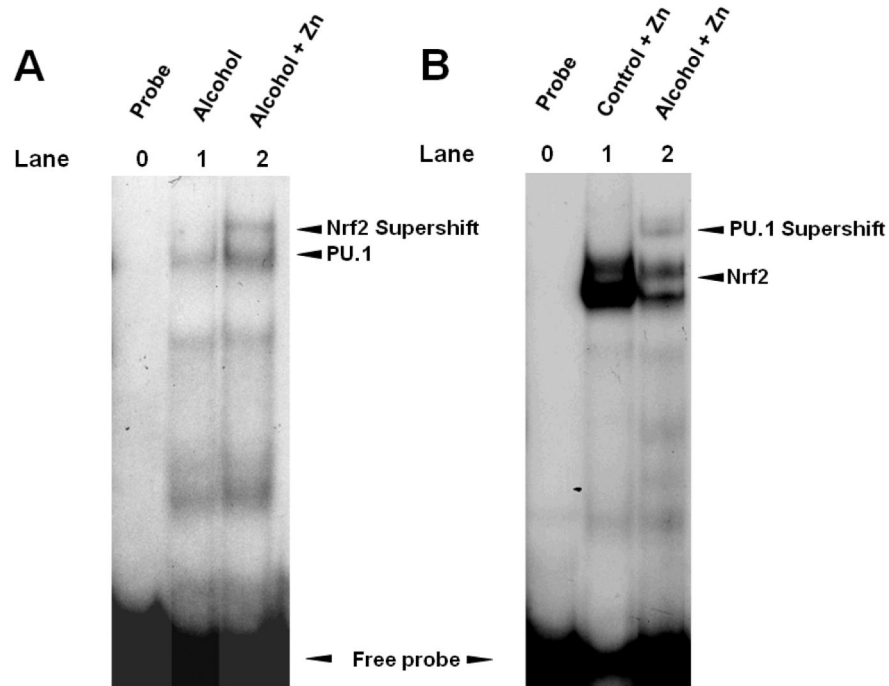


Figure 6. Evidence of zinc-dependent cooperative binding of DNA by PU.1 and Nrf2 *in vitro*
 Representative supershift assays using an antibody to Nrf2 (panel A) and an antibody to PU.1 (panel B) demonstrating that Nrf2 and PU.1 appear to interact in binding DNA in a zinc-dependent manner. In panel A, an electrophoretic mobility shift assay (EMSA) was performed on nuclear preps of alveolar macrophages isolated from alcohol-fed rats and then incubated overnight without zinc (Lane 1) or with 20 μ M zinc (Lane 2) but to which an antibody to Nrf2 was added. In panel B, EMSA was performed on nuclear preps of alveolar macrophages from control-fed rats (Lane 1) and alcohol-fed rats (Lane 2). These macrophages were treated with zinc acetate, 20 μ M, *in vitro*.

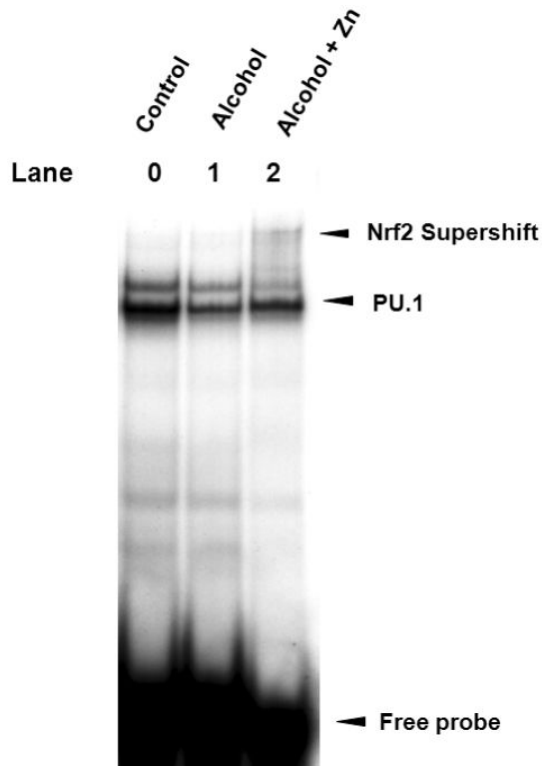


Figure 7. Evidence of zinc-dependent interactive binding of DNA by PU.1 and Nrf2 *in vivo*
 Representative supershift assay showing that dietary zinc supplementation *in vivo* also appears to induce interactive binding of DNA between Nrf2 and PU.1 in alveolar macrophages from alcohol-fed rats. Alveolar macrophages were isolated from control-fed rats, and from alcohol-fed rats \pm dietary supplementation with zinc acetate (100 mg/L) for 6 wks; nuclear extracts were then isolated and analyzed by electrophoretic mobility shift assay (EMSA) for PU.1 nuclear binding as well as for PU.1 and Nrf2 interactions by adding an antibody to Nrf2.