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The Relationship Between Airway Antioxidant Levels, Alcohol Use Disorders, and Cigarette Smoking

Ellen L. Burnham, Alicia McNally, Jeanette Gaydos, and Lou Ann S. Brown

Division of Pulmonary Sciences and Critical Care Medicine (ELB, AM, JG), Department of Medicine, University of Colorado School of Medicine, Aurora, Colorado; and Department of Pediatrics (LASB), Emory University School of Medicine, Atlanta, Georgia

Abstract

Background—Alcohol use disorders (AUDs) and cigarette smoking are associated with pulmonary oxidative stress, likely related to antioxidant depletion. Pulmonary oxidative stress may adversely affect innate immunity, leading to increased pneumonia susceptibility and severity, including development of the acute respiratory distress syndrome. In people with AUDs, most of whom smoke, antioxidant therapy can potentially restore immune cell function and attenuate pneumonia development. Challenges to human investigations of antioxidant therapies include an inability to identify pulmonary oxidative stress noninvasively and the optimal route to deliver pulmonary antioxidants. We sought to determine whether bronchoalveolar lavage (BAL) measures of thiol antioxidants from a 50-ml upper airway aliquot approximated those in the alveolar space and to determine whether AUDs and/or smoking affected these relationships.

Methods—Healthy human subjects with and without AUDs, including smokers and nonsmokers, underwent BAL. Samples obtained after the first 50-ml normal saline aliquot were analyzed as representing bronchial airways; subsequent 50-ml aliquots were analyzed as representative of the alveolar space. Reduced and oxidized (GSSG) glutathione, cysteine (Cys), and its oxidized species, cystine, along with mixed disulfides (MDs) were quantified using high-performance liquid chromatography. The percent of total thiols present in their oxidized forms, and thiol redox potentials, were calculated.

Results—Positive correlations between upper and lower BAL fluid thiol species were observed that were most robust for GSSG ($p = 0.85$), Cys ($p = 0.83$), and MDs ($p = 0.69$), but poor for thiol redox potential measures. In contrast to nonsmokers (either with or without AUDs), in subjects with AUDs who smoked, upper BAL fluid %GSSG, Cys, and MD measures were relatively increased compared to lower.

Conclusions—A small volume BAL procedure may be suitable to assess intrapulmonary oxidative stress related to thiol depletion. Factors including AUDs and smoking may disproportionately increase upper airways oxidative stress that could be relevant for therapeutic interventions.

Reprint requests: Ellen L. Burnham, MD, 12700 E. 19th Street, Mail-stop C272, Aurora, CO 80045; Tel.: 303-724-6079; Fax: 303-724-6045; Ellen.burnham@ucdenver.edu.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

Keywords

Glutathione; Oxidative Stress; Alcoholism; Smoking; Bronchoalveolar Lavage

Cells within the lung are susceptible to oxidative injury as a result of constant exposure to oxygen, and regular, intermittent exposure to specific environmental factors (Rahman and MacNee, 2000). Downstream effects of pulmonary oxidative injury are diverse including lipid peroxidation, protein modification, and DNA damage (Moriarty et al., 2003) that may in turn lead to abnormalities in immune modulation, excessive inflammation, and enhanced alveolar-capillary permeability. In humans, the relationship between alcohol use disorders (AUDs), including *alcohol abuse* and *alcohol dependence*, and heightened pulmonary oxidative stress is well established (Burnham et al., 2003; Moss et al., 2000; Yeh et al., 2007). Moreover, the majority of people with AUDs smoke cigarettes (John et al., 2003; McKee et al., 2007) that may further augment pulmonary oxidative stress (Gould et al., 2015; Morrison et al., 1999). AUD-associated oxidative stress has the potential to adversely affect pulmonary innate immunity and enhance susceptibility to pulmonary infections through impairing normal activity of alveolar macrophages (AMs) (Gaydos et al., 2016; O'Halloran et al., 2016) and bronchoalveolar epithelial cells (BAECs) (Bailey et al., 2015), cell types that are critical to pathogen recognition and clearance in the lung. Further, AUD- and smoking-associated oxidative stress may also drive pulmonary inflammation (Albright et al., 2012; Burnham et al., 2013) and contribute to prolonged resolution of pulmonary infections, such as pneumonia. Pneumonia is a major risk factor for the development of the acute respiratory distress syndrome (ARDS), a disorder that is epitomized by pulmonary oxidative stress (Lang et al., 2002; Tasaka et al., 2008) and characterized by the development of noncardiogenic pulmonary edema with respiratory failure. Notably, both AUDs (Moss et al., 1996, 2003) and cigarette smoking (Calfee et al., 2011; Christenson et al., 1996; Iribarren et al., 2000) have been implicated as risk factors for the development of ARDS (Lang et al., 2002; Tasaka et al., 2008). Patients with AUD-associated ARDS have been reported to require prolonged hospitalization and have a mortality of approximately 60% (Clark et al., 2013; Moss et al., 1996, 2003; O'Brien et al., 2007) compared with an average mortality of 29 to 42% in ARDS patients without AUDs (Rubenfeld et al., 2005; Zambon and Vincent, 2008). Whether these differences in morbidity and survival are attributable in part to more severe perturbations of oxidative stress due to alcohol or cigarettes is unknown. However, it seems possible that therapies to mitigate pulmonary oxidative stress may be most beneficial in the setting of AUDs and/or smoking as an adjunct to either reduce the risk for pneumonia, diminish pneumonia severity, or to decrease its likelihood of pneumonia being complicated by ARDS. Nevertheless, the efficacy of normalizing pulmonary oxidative stress has been inadequately examined in the population with AUDs who smoke (Burnham et al., 2012), and targeting oxidative stress in the population with *AUD-associated* ARDS has not been specifically examined (Bernard et al., 1997; Li et al., 2015; Pontes-Arruda et al., 2008; Rice et al., 2011).

The depletion of endogenous pulmonary antioxidants resulting from metabolism of alcohol, and clearance of products related to tobacco combustion is believed to drive pulmonary oxidative stress (Burnham et al., 2003; Gould et al., 2011; Morrison et al., 1999; Moss et al.,

2000). Thiol antioxidants in particular function in this role, including the tripeptide glutathione (L- γ -glutamyl-L-cysteinyl-glycine, abbreviated *GSH*), a ubiquitous intracellular pulmonary antioxidant that is oxidized to GSSG (Li et al., 1994). In comparison with other body compartments, the concentration of GSH in health is relatively high in pulmonary epithelial lining fluid (Gould et al., 2011), the thin layer of liquid that normally lines the alveolar space. The antioxidant and amino acid cysteine (Cys) is also found in the extracellular space and may be oxidized to its disulfide form, CySS. GSH can react with CySS to form mixed disulfides (CySSG, or MDs) and generate Cys (Ashfaq et al., 2008; Rahman et al., 1995). Importantly, Cys availability is thought to be a major rate-limiting factor in GSH production (Cotgreave and Gerdes, 1998). As such, the measurement of pulmonary MDs may uniquely reflect *overall* oxidative stress, as MDs are a combination of the predominant *extracellular* disulfide (CySS) and *intracellular* thiol (GSH). The balance of GSH and GSSG, or Cys and CySS, in tissue compartments can be further assessed by calculating the thiol redox potential (E_h) of the GSH/GSSG or Cys/CySS couple using the Nernst equation that may provide a complementary assessment of the oxidative state in body compartments (Yeh et al., 2007, 2008). These prior investigations demonstrated a relationship between AUDs and an increased percentage of glutathione present in its oxidized form (as GSSG) within the alveolar space that is further augmented by smoking; moreover, thiol redox potential values for GSH/GSSG thiol pairs also favor oxidation in the AUD setting, regardless of cigarette smoking.

Published human data suggest that *systemic* oxidative stress does not accurately reflect *pulmonary* oxidative stress related either to AUDs (Yeh et al., 2007) or smoking (Moriarty et al., 2003; Rahman et al., 2000); moreover, establishing the relative degree of oxidative stress between proximal (i.e., the airways) and distal (i.e., alveolar) sites *within the lung* has not been fully described (Sehlstedt et al., 2010). Although it appears possible that the impact of alcohol consumption on oxidative stress would be similar across the proximal and distal airways as alcohol is generally consumed via ingestion, this has never been examined. Moreover, it is unclear whether cigarette smoking is associated with a disproportionate increase in oxidative stress of the upper airways, given its use via inhalation. Establishing the relative distribution of oxidative stress across the lung may help inform the optimal method to administer exogenous antioxidants (that is, by an oral, intravenous, or inhaled route) that will enhance their efficacy to normalize pulmonary oxidative stress. Further, if antioxidant measures in proximal and distal airways within the same individual are in fact similar, less invasive strategies, such as tracheal aspirate or mini-bronchoalveolar lavage, might be acceptable as methods to assess pulmonary oxidative stress. Characterizing pulmonary oxidative stress could facilitate identification of subjects or patients more likely to benefit from antioxidant replacement in the context of a clinical trial (Homma et al., 2012; Soltan-Sharifi et al., 2007; Zheng et al., 2014) or could be used to assess the response to exogenous antioxidant administration. We therefore measured the absolute quantities of glutathione, Cys, and MDs, the percent of total glutathione or Cys present in its oxidized form, and the thiol redox potential of GSSG and CySS contained in the upper and lower airways of the same individuals. We hypothesized that these measures would be correlated between the upper and lower airways within the same individual. Further, we sought to determine whether AUDs and/or cigarette smoking would influence these measures across

compartments. We postulated that cigarette smoking would be associated with enhanced upper airway oxidative stress that would be further increased in the setting of concomitant AUDs.

MATERIALS AND METHODS

Subject Screening, Recruitment, and Enrollment

Subjects with AUDs were recruited between 2009 and 2012 at the Denver Comprehensive Addictions Rehabilitation and Evaluation Services center, an inpatient detoxification facility affiliated with Denver Health and Hospital System in Denver, CO. Control subjects without AUDs were recruited via approved flyers posted on the University of Colorado's Anschutz Medical Campus in Aurora, CO, and by print and electronic mail advertisements in the Denver metropolitan area. The Colorado multiple institutional review board approved this study. All subjects provided written informed consent before participating.

Subjects with AUDs were eligible to participate whether they met the following criteria at study entry: (i) an Alcohol Use Disorders Identification Test (AUDIT) score of ≥ 8 for men or ≥ 5 for women (Babor et al., 2006; Reinert and Allen, 2002), (ii) alcohol use within the 7 days before testing, and (iii) age ≥ 21 years. To meet eligibility as a control, control subjects' AUDIT values were required to be <8 for men or <5 for women. Screening of potential controls focused on balancing these individuals with AUD subjects in terms of age and smoking history. The ultimate sample sizes of subjects and controls were chosen based on feasibility to recruit comparable numbers of AUD subjects and controls who could be matched on the basis of smoking.

In an effort to minimize potential confounding related to comorbidity, AUD subjects and controls were ineligible to participate in the study whether they met any of the following criteria: (i) prior medical history of liver disease (documented history of cirrhosis, total bilirubin ≥ 2.0 mg/dl, or albumin <3.0), (ii) prior medical history of gastrointestinal bleeding (due to the concern for varices), (iii) prior medical history of heart disease (history of left ventricular ejection fraction $<50\%$, myocardial infarction, or severe valvular dysfunction), (iv) prior medical history of renal disease (end-stage renal disease requiring dialysis or a serum creatinine ≥ 2 mg/dl), (v) prior medical history of lung disease defined as an abnormal chest radiograph or spirometry prior to bronchoscopy (volume in first second of exhalation [FEV₁] or forced vital capacity [FVC] $<75\%$), (vi) concurrent illicit drug use defined as a toxicology screen positive for cocaine, opiates, or methamphetamines, (vii) prior history of diabetes mellitus, (viii) prior history of HIV infection, (ix) failure of the patient to provide informed consent, (x) pregnancy, and (xi) abnormal nutritional status, defined by the calculated nutritional risk index (NRI) with the subject's albumin, current weight, and usual weight values with the following equation: $NRI = 1.519 (\text{albumin in g/l}) + (\text{current weight/usual weight}) \times 100 + 0.417$ (Anonymous 1991). Patients were considered to have a normal nutritional status if the NRI was ≥ 90 . Potential subjects >55 years of age were also excluded to minimize the presence of concomitant but asymptomatic comorbidities.

Clinical Protocol

Eligible AUD subjects and controls were admitted to the University of Colorado Hospital's Clinical and Translational Research Center for bronchoscopy. All bronchoscopy procedures were performed using standard conscious sedation protocols as previously described (Burnham et al., 2012; Hunninghake et al., 1979). Briefly, the bronchoscope was wedged into a subsegment of either the right middle lobe or the lingula, followed by instillation of 50-ml aliquots of sterile, room temperature 0.9% saline. Each aliquot was sequentially instilled and recovered with gentle hand aspiration. The aliquot aspirated after the first 50 ml was collected as representative of the upper airways, denoted as "upper bronchoalveolar lavage (BAL)," and was analyzed separately from subsequent aliquots. All subsequent (second and following) aliquots were combined and used in experiments as representative of the distal airspaces, denoted as "lower BAL." The strategy to separate the first from subsequent BAL aliquots has been employed previously both in healthy individuals (Davis et al., 1982; Rennard et al., 1990) and individuals with airways disease (Bousquet et al., 1990; Van et al., 1992). Further, use of an initial 50-ml aliquot ensured a consistent return volume sufficient to be analyzed. The *percent yield* of the BAL procedure was calculated by dividing the total amount of BAL aspirated by the end of the procedure by the total amount of saline instilled (first and subsequent aliquots), multiplied by 100. BAL samples were transported to the laboratory in sterile 50-ml conical tubes. Whole blood samples were also collected prior to bronchoscopy.

Laboratory Processing

BAL and whole blood were immediately centrifuged ($900 \times g$, 10 minutes) after collection to separate cellular and acellular components. Acellular BAL and plasma samples were preserved immediately after collection in a 5% perchloric acid solution containing iodoacetic acid (6.7 M) and boric acid (0.1 M). The preservation fluid also contained 5 μM of the internal standard γ -glutamyl-glutamate (γ -Glu-Glu). This step was completed within 30 minutes of collection to prevent degradation or oxidation of GSH and Cys.

High-Performance Liquid Chromatography Measurement of Thiols

Thiols and disulfides were measured in acellular BAL and plasma samples using previously described methods (Burnham et al., 2012; Yeh et al., 2007). After subjecting BAL and plasma to the preservation solution (as mentioned), protein was removed. Upper and lower BAL along with plasma samples were derivatized with dansyl chloride and separated on a 10- μm Ultrasil amino column by high-performance liquid chromatography (Waters 2690; Waters Corp., Milford, MA). Fluorescence detection was recorded by 2 detectors (Waters 474 and Gilson model 121 [Gilson, Inc., Middleton, WI]). The quantity, in micromoles, of GSH and GSSG was calculated by quantitating the integrated areas relative to that of γ -Glu-Glu to examine the relationship between these primarily *intracellular* endogenous antioxidants between the upper and lower airway. Cys and cystine (CySS) were also measured in subjects enrolled during the first half of the enrollment period (2009 to 2010) to explore complementary correlations of these predominantly *extracellular* endogenous antioxidants. MDs were measured as an index of *overall oxidative stress* in the upper and lower airways. The concentration of lower BAL thiols after correction for percent yield of

the BAL procedure was also calculated by dividing the micromolar concentration of thiol by percent yield, divided by 100. Concentration of upper airway thiols for percent yield was not performed, but all starting volumes were identical at 50 ml. To assess the relative quantity of *oxidized thiol* species (disulfides) to total thiol (reduced and oxidized) present in a unique compartment, the micromolar quantity of GSSG present in the compartment relative to total micromolar quantity of glutathione (e.g., GSH + GSSG) in that unique compartment was calculated as $[\text{GSSG } \mu\text{M}/(\text{GSSG } \mu\text{M} + \text{GSH } \mu\text{M})] \times 100$. The % CySS was calculated similarly. Finally, the thiol redox potential (E_h) of the GSH/GSSG or Cys/CySS thiol pairs in the BAL was calculated with the Nernst equation, $E_h = E_o + RT/nF \ln [\text{disulfide}]/([\text{thiol } 1] [\text{thiol } 2])$, where the E_o is the standard potential for the redox couple, R is the gas constant, T is the absolute temperature, n is 2 for the number of electrons transferred, and F is Faraday's constant. The standard potential E_o for the 2 GSH/GSSG couple was -264 mV at pH 7.4. The standard potential E_o for the 2 Cys/CySS couple was -250 mV .

Protein and Measurements of BAL

The protein concentration in the samples was analyzed using the Pierce Better Bradford Coomassie assay (Pierce Chemical Co., Rockford, IL) using $25 \mu\text{l}$ upper and lower BAL aliquots for subjects and controls.

Statistics

Distributions of data were examined; nonparametric testing was subsequently chosen based on testing of the variances. Comparisons of continuous data across subgroups of AUD and smoking were conducted using Wilcoxon/Kruskal–Wallis (rank sums) testing, with post hoc tests where applicable. Categorical data were compared across groups using Fisher's exact test. Correlations in data were examined with Spearman's test, calculating the rho (ρ) for these associations. Correlations were examined for thiol analytes both in the whole cohort, as well as after stratifying subjects based on AUD and smoking history. For some correlations, linear regression was also performed to determine the line of best fit in order to examine the relative impact of AUDs and smoking on the upper and lower thiol relationship. A p -value of <0.05 was deemed to be significant.

RESULTS

GSH and GSSG measures, and MD measures were performed in BAL samples from 81 unique individuals, including individuals with AUDs, who either did or did not actively smoke cigarettes, and control subjects, who either did or did not smoke cigarettes (Table 1). AUDIT scores differed significantly across the groups. Among the subjects with AUDs, the median time that had elapsed between last alcoholic beverage consumed and enrollment was 1 day. The 4 subject groups were similar in terms of age and underlying pulmonary function. Among smokers, pack-year history of smoking was similar regardless of alcohol use. There were more men among the AUD group who smoked than in the control smoking group (92% vs. 50%). The percent yield of BAL varied across groups, driven by the relatively lower yield among AUD smokers. Examining the first 3 questions of the AUDIT questionnaire revealed markedly more frequent alcohol consumption, more alcohol consumed per day

drinking, and a greater prevalence of binge alcohol consumption among the AUD subjects (Table 2).

GSH, GSSG, and MD were quantifiable in both upper and lower BAL (Fig. 1). Variations in these measures related to AUD or smoking history were generally parallel across the upper and lower airways, with some exceptions. For example, across-groups differences in GSH were present in upper and lower airways (Fig. 1A); in post hoc analyses, significant differences between AUD smokers' and AUD nonsmokers' upper and lower GSH values were evident ($p = 0.02$). In lower BAL samples, control smokers were also determined to have more elevated GSH than control nonsmokers ($p = 0.05$). Upper and lower GSSG also varied across the 4 subgroups (Fig. 1B); in post hoc analyses, a history of smoking, regardless of AUDs, was determined to be associated with increased GSSG ($p = 0.001$ for upper BAL, $p = 0.0009$ for lower BAL, comparing smokers to nonsmokers, both those with AUDs and in non-AUD subjects). Additionally, AUD nonsmokers also displayed elevated GSSG in lower BAL compared to control nonsmokers ($p = 0.007$). % GSSG in upper BAL (but not lower BAL) differed across the 4 subgroups (Fig. 1C) and appeared to be driven by smoking; in post hoc analyses, control smokers demonstrated higher %GSSG compared with control nonsmokers ($p = 0.02$), while AUD smokers' %GSSG was increased compared with AUD nonsmokers ($p = 0.05$). Redox GSSG did not substantially vary across the 4 groups (Fig. 1D). Upper and lower BAL MD values also varied across the 4 subgroups (Fig. 1E). In post hoc analyses, AUD nonsmokers displayed increased upper and lower MD values compared to control nonsmokers ($p = 0.003$). Smoking controls also tended to have elevations in upper and lower MD ($p = 0.01$). Lower MD values for AUD smokers were also elevated relative to their smoking control counterparts ($p = 0.01$). When absolute values of lower GSH, GSSG, and MD were adjusted for differences in percent yield of the BAL procedure and compared across the 4 subject types, significant between-groups differences previously noted in GSH (upper and lower BAL), GSSG (upper and lower BAL), % GSSG (upper BAL), and MD (upper and lower BAL) persisted ($p = 0.0003$).

Correlations between upper airway BAL and lower airway BAL glutathione (Fig. 2A), GSSG (Fig. 2B), %GSSG (Fig. 2C), thiol redox potential of glutathione (redox GSSG, Fig. 2D), and MD (Fig. 2E) measured in the same individual were subsequently examined. Positive correlations were observed between measures within the upper airways compared to lower airways, with the best linear fit for the relationship between upper and lower GSSG ($R^2 = 0.43$) and % GSSG ($R^2 = 0.36$). The correlation (Pearson's ρ) was strongest for measures of GSSG (Fig. 2B, $\rho = 0.85$, $p < 0.0001$), followed by GSH (Fig. 2A, $\rho = 0.64$, $p < 0.0001$) and % GSSG (Fig. 2C, $\rho = 0.62$, $p < 0.0001$). Correlations between upper and lower redox GSSG were the weakest of those assessed (Fig. 2D, $\rho = 0.49$, $p < 0.0001$). The relationship between upper and lower MD was on the same order as GSH and %GSSG (Fig. 2E, $\rho = 0.69$).

To explore the potential impact of AUDs and active smoking on the relationship of upper and lower airway thiols without the potential confounding of dilutional issues related to bronchoscopy that might particularly affect the quantity of lower BAL thiols, %GSSG was examined by subgroup, based on AUD and smoking histories. Interestingly, among the 13 subjects in control group who neither had AUDs nor smoked, correlations between upper

and lower %GSSG were relatively weak (Fig. 3A). However, among smoking controls *without* AUDs ($n = 14$), robust correlations were observed for %GSSG (Fig. 3B, $\rho = 0.80$, $p = 0.002$). Similarly, among the 18 AUD subjects who did not smoke, positive correlations were observed between upper and lower %GSSG (Fig. 3C, $\rho = 0.65$, $p < 0.003$). In the subgroup of AUD subjects who smoked, correlations between %GSSG (Fig. 3D, $\rho = 0.50$, $p = 0.002$) were more modest. Examining regression lines for %GSSG over the 4 subgroups, the slope of the line was least ($\beta = 0.39 \pm 0.14$, $R^2 = 0.19$) for the AUD smoking group, compared with a relatively higher slope of 0.87 ± 0.21 ($R^2 = 0.54$) in control smokers, and 0.74 ± 0.23 ($R^2 = 0.38$) in AUD nonsmokers. These data suggest the potential for the combination of AUDs and smoking to lead to a more markedly oxidized upper airway environment compared to the lower airway environment in the AUD/smoking subset, compared to either AUDs or smoking in isolation. In comparing upper %GSSG to lower %GSSG across the AUD/smoking subgroup, %GSSG was not, however, found to be significantly elevated in the upper airways (median 62% compared with 60%, $p = 0.07$).

Plasma glutathione species were measured in 48 randomly selected subjects within the cohort of 81. Plasma measures were examined in correlation with upper and lower BAL GSH, GSSG, %GSSG, and redox GSSG values. No correlations between upper or lower BAL glutathione species and their corresponding plasma values were observed ($p > 0.05$, data not shown).

Characteristics of the subgroup of 43 subjects where Cys species were analyzed are illustrated in Table 3. As with the larger cohort, percent yield of BAL varied across the 4 groups ($p < 0.004$), and in post hoc analyses, control nonsmokers had a significantly higher percent yield than the other 3 subject groups ($p = 0.04$).

In this smaller cohort of subjects, in general, the relative quantity (median) of Cys thiols, %CySS, and redox CySS in the upper airways approximated those in the lower airways (Fig. 4), analogous to what was observed in measures of glutathione species. However, the range of Cys and CySS data measures was wider (Fig. 4A,B) in comparison with %CySS or redox CySS. Across-groups' differences in lower BAL Cys measures observed ($p = 0.04$; Fig. 4A). Significant differences across the 4 subgroups were also observed in the upper airways %CySS ($p = 0.04$; Fig. 4C). In post hoc analyses, control smokers' %CySS was significantly higher than control nonsmokers ($p < 0.05$).

Correlations in upper and lower BAL measurements of Cys, its derivative CySS, percent of total Cys as %CySS, and the thiol redox potential of Cys (CySS redox) were examined across the total cohort of 43 subjects. Positive correlations in upper and lower thiol values were observed as for previous glutathione analyses. For the whole cohort, correlations between upper and lower BAL Cys (Fig. 5A, $\rho = 0.83$, $p < 0.0001$) were the most robust, followed by correlations between CySS (Fig. 5B, $\rho = 0.69$, $p < 0.0001$), %CySS (Fig. 5C, $\rho = 0.39$, $p = 0.01$), and CySS thiol redox potential (Fig. 5D, $\rho = 0.54$, $p = 0.0002$).

Given the smaller numbers of subjects with cysteine thiol measures available, further analyses were performed combining *all AUD subjects* ($n = 33$), without regard to smoking, and *all non-AUD controls* ($n = 10$), without regard to smoking, to examine the potential

impact of AUDs on Cys measures (Fig. 6). Among the smaller number of *non-AUD controls*, upper and lower Cys ($\rho = 0.79$, $p = 0.006$; Fig. 6A) remained tightly correlated. Among *AUD subjects*, the relationship between upper and lower BAL Cys also persisted ($\rho = 0.78$, $p < 0.0001$; Fig. 6B). Additional analyses were performed to determine the impact of smoking on cysteine thiols by combining *all smoking subjects* ($n = 31$), without regard to AUD, and *all nonsmokers* ($n = 12$), without regard to AUD, in correlation analyses. Among *nonsmokers*, a modest correlation between upper and lower Cys ($\rho = 0.54$, $p < 0.05$; Fig. 6C) was present, whereas a history of *current smoking* was associated with stronger correlations between upper and lower BAL Cys ($\rho = 0.90$, $p < 0.0001$; Fig. 6D). Notably, most AUD subjects were smokers; given the diminished slope of the regression line observed in Fig. 6B,D, compared to Fig. 6A,B, it remains possible that smoking had a larger impact on upper airways Cys than lower airways. Finally, in subsequent examination of *plasma* cysteine species concentrations in relationship with BAL measures, no single plasma cysteine species was found to correlate strongly with any corresponding upper or lower BAL measure, similar to what was observed with glutathione species in plasma (data not shown).

Total protein values were quantified in the upper and lower BAL in 64 total subjects. Small but significant elevations in protein were observed in lower BAL compared to upper for all subject groups, except control nonsmokers (Fig. 7). Analogous to glutathione and cysteine analyses, positive correlations between upper and lower BAL total protein were determined to be present ($\rho = 0.60$, data not shown). In subgroup analyses, positive associations persisted regardless of AUD or smoking history ($\rho > 0.60$ for all groups); however, among AUD subjects who also smoked, the relationship was not as robust ($\rho = 0.44$) as in the other 3 subgroups.

Given the gender differences in the composition of the 4 subgroups, with more men volunteering who reported AUDs, additional analyses comparing the absolute values of glutathione analytes by gender were performed. Differences in any GSH, GSSG, %GSSG, and redox GSSG were not found to vary significantly between men and women ($p > 0.05$ for all comparisons). Similarly, absolute values of cysteine thiol analytes did not vary by gender ($p > 0.05$). If women and men were further analyzed separately according to smoking or AUD history, gender differences were not apparent.

DISCUSSION

In this cohort of human subjects that included healthy controls and subjects with AUDs who either did or did not smoke cigarettes, endogenous antioxidant thiol species were measureable in upper airways samples obtained after 50-ml saline instillation. The absolute quantities of glutathione, cysteine, and MD thiol species in the upper airways mirrored the quantity present in the lower airways. However, in this cohort, plasma thiol values did not accurately reflect thiol values in upper or lower BAL, confirming that plasma measures are potentially less accurate to assess oxidative stress in the lung (Yeh et al., 2007). One other study assessing antioxidant status (glutathione) between airways and distal alveoli in 19 healthy nonsmoking human adults has been published with results in line with our observations (Sehlstedt et al., 2010). However, this investigation did not specifically examine the influence of smoking or AUDs. In the current investigations, we observed that current

smoking was consistently associated with relative elevations in oxidized thiols in both upper and lower airways. Among subjects who smoked with AUDs, examining *correlations* between the proximal and distal airways highlighted a potential additive effect of smoking and AUDs on oxidative stress affecting the upper airway.

Characterizing pulmonary oxidative stress is important given its potential deleterious impact on pulmonary innate immunity, where it may impair pathogen recognition and clearance by pulmonary cells residing in the proximal (i.e., BAECs) or distal airways (i.e., AMs). Based on observations by our group and others, the relationship of AUDs and smoking on pulmonary oxidative stress deserves particular attention. Preclinical and clinical studies have reported an effect of chronic alcohol exposure to enhance AM nicotinamide adenine dinucleotide phosphate oxidase activity, a main source of reactive oxygen species (Yeligar et al., 2012); to perturb pulmonary mitochondrial and lipid metabolism in the alveolar space (Obianyo et al., 2015; Romero et al., 2014); and to contribute to the formation of adducted surfactant proteins that bind AMs, augmenting pro-inflammatory cytokine production, particularly in the setting of tobacco exposure (McCaskill et al., 2011; Wyatt et al., 2012). Moreover, studies in human BAECs confirmed a relationship between AUDs and smoking with altered Toll-like receptor expression important in Gram (+) pathogen recognition, in parallel with elevated airway pro-inflammatory cytokines (Bailey et al., 2015). As the metabolism of alcohol and exposure to cigarette smoke are both reported to involve utilization of endogenous antioxidants, it follows that antioxidant supplementation may be useful in normalizing these abnormalities. Recently, we demonstrated that AMs from subjects with AUDs that had been stimulated *ex vivo* with lipopolysaccharide displayed enhanced pro-inflammatory cytokine secretion that could be mitigated with *N*-acetylcysteine, a glutathione precursor, suggesting its utility in restoring normal AM function (Gaydos et al., 2016). Additional translational studies will be required to more fully characterize the impact of oxidative stress on pulmonary innate immune cells in order to identify appropriate therapeutic targets. It remains to be determined whether exogenous antioxidant replacement, therapies to limit or attenuate generation of reactive oxygen species, or strategies that promote *de novo* synthesis of endogenous antioxidants would be most useful to minimize oxidative stress.

Along with investigations regarding the specific immunopathologic effects of pulmonary oxidative stress, additional research to establish the relative degree of oxidative stress between the upper and lower airways, both with and without AUDs or smoking, may inform how therapy targeting oxidative stress should be optimally administered (i.e., inhaled vs. systemic). Additionally, investigations to understand which strategies most reliably, and least invasively, identify oxidative stress or endogenous antioxidant depletion would be useful to identify patients who would be most likely to benefit from remediation of oxidative stress. Our data suggest that in general, sampling of the proximal lung via assays in a 50-ml BAL aliquot provides comparable information regarding oxidative stress in the alveolar space. The upper and lower sampling strategy via bronchoscopy we performed was based on the published literature denoting unique characteristics of the first 20- to 60-ml aliquot of BAL compared with subsequent pooled BAL aliquots in studies of healthy controls (Davis et al., 1982; Rennard et al., 1990) and in patients with asthma (Bousquet et al., 1990; Van et al., 1992). Notably, 1 study using digital subtraction radiography further substantiates that an

initial instilled aliquot of up to 60 ml remains proximal to the bronchoscope and therefore primarily reflects substances within the central airways (Kelly et al., 1987). From a practical standpoint, the technique we used was performed with a standard bronchoscope and consistently yielded a sufficient amount of upper airway BAL for analysis. Certainly, sampling of the proximal airways with bronchoscopy is more invasive than blood sampling in human subjects; however, other strategies have been proposed to sample the upper airways. In recent literature, particularly with the advent of microbiome-related investigations, a variety of methods to assess the upper versus lower airways have been employed, suggesting a general lack of consensus regarding the optimal method to assess the 2 compartments. Samples considered to be representative of the upper airway have included exhaled breath condensate, saliva, oral wash, nasal lavage, and tracheal aspirates (Harwood et al., 2011; Hentschel et al., 2014; Hoppe et al., 2015; Morris et al., 2013; Schneibel et al., 2013). Lower airway sampling has included measures in induced and expectorated sputum, as well as BAL and mini-BAL sampling (Boselli et al., 2007; Colucci et al., 2009; Fujitani and Yu, 2006; Hoppe et al., 2015; Meyer et al., 2014; Rogers et al., 2013; Singh et al., 2014; Zemanick et al., 2013). While it remains possible that samples obtained from the first aliquot of BAL may contain a small quantity of substances representing the lower airway, the use of bronchoscopy is a widely available method to assess both bronchial and alveolar compartments of the lung during the same procedure. In light of the myriad methods currently used to measure upper airways substances, additional investigation employing more limited, less invasive sampling of upper airways' endogenous substances as a strategy to measure substances in the distal alveoli seems warranted.

Compared to our prior investigations involving human subjects, active smoking in the current group of subjects was associated with a more robust impact on measures of oxidative stress than were AUDs, particularly in the upper airways. Interestingly, among subjects with AUDs who also smoked, correlations between upper and lower airway %GSSG and MDs were characterized by a smaller change in lower airway values compared to upper airway values (less steep slope), compared with AUD nonsmokers and smokers without AUDs. Reasons for these differences remain to be fully determined. It is possible that chronic alcohol consumption "primes" the upper airway for a further increase in oxidative stress with cigarette smoke exposure. Alternatively, given its proximity to the oropharynx, the dose of cigarette exposure experienced by the upper airway is greater, driving more pronounced oxidative stress in this location compared to lower airways in the same individual. Notably, MD values in upper and lower BAL, reflecting a combination of the predominant extracellular disulfide (CySS) and intracellular thiol (GSH), did display a more consistent association with AUDs, particularly in lower BAL, where values were significantly elevated; additionally, smoking in the absence of alcohol consumption was also associated with elevation of MDs. Collectively, our data suggest that both active smoking and AUDs have the potential to impact thiol antioxidant quantities in plasma and lung compartments; in the absence of overt pulmonary disease, active smoking appears to have the most profound impact on oxidative stress, with more subtle effects observed in relationship with concomitant alcohol use.

In this study, glutathione species were measured as indicators of intracellular oxidative stress, while a subset of subjects' samples underwent Cys species measurements as well to

assess extracellular oxidative stress in parallel to explore whether correlations between upper versus lower airways oxidative stress were unique to intracellular thiol species (e.g., GSH) or to oxidative stress measures more globally. Issues that we identified using BAL to measure thiols included more marked variability in micromolar measurements of individual thiol species (e.g., GSH, CySS) as well as redox measures. Variability we observed may be due in part to differences in the relative percent yield of BAL between subjects, supported by the fact that less variability was observed in measures of %GSSG and %CySS where differences in percent yield are factored out in the calculation. Dilutional factors have been a concern in research involving the performance of tracheal aspirates and BAL (Dargaville et al., 1999), and consistency in BAL performance for research and clinical issues has been the subject of society guidelines (Meyer et al., 2012). Oftentimes the yield of BAL will be corrected for the quantity of protein or urea present in the lavage fluid as a way to standardize measures; however, both AUDs and smoking have the potential to alter alveolar-capillary permeability (Burnham et al., 2003; Li et al., 1994; Moazed et al., 2016) making such correctional strategies imperfect. Moreover, we also noted small but statistically significant differences in total protein between the upper and lower BAL among individuals in conjunction with AUDs and/or smoking in this current cohort. In light of these issues, it seems most prudent to emphasize that measures that are less influenced by dilutional effects of the BAL procedure, such as %GSSG, may be more reliable in assessing oxidative stress in lung. It was encouraging that upper and lower BAL thiol measures were relatively consistent, however, and not orders of magnitude different.

Limitations of these investigations include the modest sample size and therefore power of any inferences that can be made with the data. This is particularly true for analyses of Cys species, which were performed in a smaller cohort of subjects to determine whether alterations in this predominantly extracellular antioxidant were similar to those with glutathione, a predominantly intracellular antioxidant. We acknowledge that sampling was obtained from individuals once and therefore reflect only a snapshot of oxidative stress measures at a single time point. The strength of our data lies in the parallel assessment of thiols across the upper and lower lung compartments within the same individual. As such, we can begin to understand the differences and similarities in oxidative stress across compartments in human organisms as a means to inform therapeutic development. Further, we also attempted to explore whether known modifiers of oxidative stress, alcohol and cigarette smoking, would be associated with further cross-compartment differences. Although subtle differences were observed in association with AUDs and tobacco use, in our cohort of otherwise healthy individuals without overt pulmonary symptoms, the effect was modest. We acknowledge that there were differences in gender across our subgroups; however, gender was not found to be associated with differences in thiol measures in our cohort in subsequent analyses. Finally, it is not clear that oxidative stress measured in BAL fluid reflects oxidative stress in immune cells such as AMs in the lower airway (Rosso et al., 2014) or bronchial airway epithelial cells in the upper airway. Additional investigations will be necessary to determine whether such relationships exist.

In conclusion, measures of thiol antioxidants within the upper airways, obtained from the first aliquot of fluid aspirated during a formal BAL procedure correlate with thiol antioxidant measures in lower airways, obtained from BAL aliquots following the initial

aliquot collection, during the same procedure. AUDs and smoking were associated with subtle but detectable effects on the absolute values of thiols measured and were associated with differences in the strength of certain correlations. An assessment of oxidative stress in the upper airway may provide a window to oxidative stress in more distal portions of the lung; however, measures of upper airways oxidative stress via nonbronchoscopic techniques require further validation. Given marked differences in concentration between plasma and lung antioxidants, strategies to accurately characterize pulmonary oxidative stress may help to identify patients where therapy to normalize pulmonary oxidative stress would be most efficacious and could also help determine the optimal method for drug delivery. Further investigations will be necessary to establish the most useful therapeutic agents to attenuate pulmonary oxidative stress.

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Fig. 1.

Thiol values were measured in upper bronchoalveolar lavage (BAL), obtained from the fluid collected after instilling 50-ml saline, to quantify levels in the bronchial airways. Thiol values were also measured in lower BAL, obtained from subsequent aliquots during BAL, to quantify levels in the alveolar space. Samples were measured in 81 total subjects (control nonsmokers, $n = 13$; control smokers, $n = 14$; alcohol use disorders (AUDs) nonsmokers, $n = 18$; AUD smokers, $n = 36$). High-performance liquid chromatography was used to quantify (A) reduced glutathione (GSH), (B) oxidized glutathione (GSSG), (C) percent of total glutathione present in its oxidized form (%GSSG), (D) redox potential of glutathione, and (E) mixed disulfides (MDs) present in either upper BAL (up-pointing triangles) or lower BAL (down-pointing triangles). Medians with interquartile ranges are presented. Bars indicate $p < 0.05$.

Fig. 2.

Correlations between upper bronchoalveolar lavage (BAL) and lower BAL thiol measures were examined for (A) reduced glutathione (GSH), (B) oxidized glutathione (GSSG), (C) percent of total glutathione present in its oxidized form (%GSSG), (D) redox potential of glutathione, and (E) mixed disulfides (MDs) in 81 total subjects. While a positive relationship was generally observed, the strongest linear relationship was present between upper and lower GSSG ($R^2 = 0.43$) and %GSSG ($R^2 = 0.36$) across the cohort. ρ = Pearson's rho (nonparametric correlation coefficient) presented.

Fig. 3.

Correlations between upper bronchoalveolar lavage (BAL) and lower BAL percent of total glutathione in its oxidized form (%GSSG) were examined for (A) nonsmoking controls, $n = 13$; (B) smoking controls, $n = 14$; (C) nonsmoking subjects with alcohol use disorders (AUDs), $n = 18$; and (D) smoking subjects with AUDs, $n = 36$. A positive relationship was generally observed. ρ = Pearson's rho (nonparametric correlation coefficient) presented.

Fig. 4.

Thiol values were measured in upper bronchoalveolar lavage (BAL), obtained from the fluid collected after instilling 50-mL saline, to quantify levels in the bronchial airways from 43 total subjects (control nonsmokers, $n = 4$; control smokers, $n = 6$; alcohol use disorders (AUD) nonsmokers, $n = 8$; AUD smokers, $n = 25$). Thiol values were also measured in lower BAL, obtained from subsequent aliquots during BAL, to quantify levels in the alveolar space. High-performance liquid chromatography was used to quantify (A) cysteine (Cys), (B) cystine (CySS), (C) percent of total Cys present in its oxidized form (%CySS), and (D) redox potential of Cys, present in either upper BAL (up-pointing triangles) or lower BAL (down-pointing triangles). Medians with interquartile ranges are presented. Bars indicate $p < 0.05$.

Fig. 5.

Correlations between upper bronchoalveolar lavage (BAL) and lower BAL thiol measures were examined for (A) cysteine (Cys), (B) cystine (CySS), (C) percent of total Cys present in its oxidized form (%CySS), and (D) redox potential of Cys in 43 total subjects. A positive relationship was generally observed that was strongest for Cys comparisons between upper and lower compartments. ρ = Pearson's rho (nonparametric correlation coefficient) presented.

Fig. 6.

Correlations between upper bronchoalveolar lavage (BAL) and lower BAL cysteine (Cys) were examined for (A) control subjects without alcohol use disorders (AUDs), both smokers and nonsmokers ($n = 10$), (B) AUD subjects, both smokers and nonsmokers ($n = 33$). In separate analyses, upper and lower BAL Cys was also measured in (C) nonsmoking subjects, both with and without AUDs ($n = 12$), and (D) smoking subjects with and without AUDs ($n = 31$). A positive relationship was generally observed between upper and lower measures; however, the slope of the regression line among the smoking subjects (either with or without AUDs) was diminished compared to corresponding nonsmokers, suggesting a potential impact of smoking driving differences in upper versus lower airways Cys. ρ = Pearson's rho (nonparametric correlation coefficient) presented.

Fig. 7.

Protein values were measured in upper bronchoalveolar lavage (BAL), obtained from the fluid collected after instilling 50-ml saline, to quantify levels in the bronchial airways. Protein values were also measured in lower BAL, obtained from subsequent aliquots during BAL, to quantify levels in the alveolar space. Upper and lower protein values were measured in 64 total subjects: control nonsmokers, $n = 12$; control smokers, $n = 12$; alcohol use disorders (AUD) nonsmokers, $n = 15$; AUD smokers, $n = 25$. Medians (columns) with interquartile ranges (whiskers) are presented. Horizontal floating bars indicate $p < 0.05$.

Table 1
Demographic Characteristics of Subjects with Pulmonary Glutathione Species Measurements

	Control		Control		AUD	AUD	<i>p</i> -Value
	Nonsmokers, <i>n</i> = 13		Smokers, <i>n</i> = 14		Nonsmokers, <i>n</i> = 18	Smokers, <i>n</i> = 36	
Age	40 (38 to 49)		40 (35 to 49)		42 (38 to 45)	48 (41 to 50)	0.17
Gender, % Men	77%		50%		83%	92%	0.01
Pack-year smoking	0		16 (12 to 22)		0	13 (5 to 20)	0.25
AUDIT score	3 (1 to 4)		2 (1 to 4)		28 (24 to 33)	28 (22 to 35)	<0.0001
Time elapsed since last drink, days	n/a		n/a		1 (1 to 3)	1 (1 to 3)	0.70
FEV ₁ , % predicted	99 (94 to 115)		92 (82 to 100)		99 (91 to 109)	98 (93 to 105)	0.18
FEV ₁ /FVC, % predicted	81 (75 to 84)		79 (74 to 85)		81 (74 to 86)	76 (71 to 81)	0.14
Percent yield of BAL procedure ^a	52 (41 to 59)		43 (36 to 53)		47 (41 to 56)	41 (25 to 47)	0.008

AUDIT, Alcohol Use Disorders Identification Test; FEV₁, forced expiratory volume in first second of exhalation; FEV₁/FVC, ratio of FEV₁ to forced vital capacity; BAL, bronchoalveolar lavage.

^aPercent yield calculated as follows: (total quantity of BAL fluid aspirated/total quantity of fluid instilled) × 100.

Continuous data are presented as medians with interquartile ranges. Statistics performed with Wilcoxon/Kruskal-Wallis testing (continuous variables) or Fisher's exact test (categorical variables).

Table 2

Self-Reported Alcohol Consumption According to Alcohol Use Disorders Identification Test^a Questions 1, 2, and 3

Control		Control		AUD		p- Value
Nonsmokers, n = 13		Smokers, n = 14		Nonsmokers, n = 18		
How often do you have a drink containing alcohol?						
Never	2/13 (15)	4/14 (29)	0/18 (0)	0/36 (0)	<0.0001	
Monthly	4/13 (31)	5/14 (36)	0/18 (0)	0/36 (0)		
2 to 4 times/month	5/13 (38)	3/14 (21)	0/18 (0)	1/36 (3)		
2 to 3 times/wk	1/13 (8)	2/14 (14)	5/18 (28)	7/36 (19)		
4 times/wk	1/13 (8)	0/14 (0)	13/18 (72)	28/36 (78)		
How many drinks containing alcohol do you consume on a typical drinking day?						
n/a	2/13 (15)	4/14 (28)	0/18 (0)	0/36 (0)	<0.0001	
1 to 2	7/13 (54)	5/14 (36)	1/18 (6)	0/36 (0)		
3 to 4	4/13 (31)	5/14 (36)	1/18 (6)	3/36 (8)		
5 to 6	0/13 (0)	0/14 (0)	6/18 (33)	8/36 (22)		
7 to 9	0/13 (0)	0/14 (0)	3/18 (16)	7/36 (20)		
10	0/13 (0)	0/14 (0)	7/18 (39)	18/36 (50)		
How often do you have 6 drinks on 1 occasion?						
Never	7/13 (54)	12/14 (86)	1/18 (6)	0/36 (0)	<0.0001	
<Monthly	6/13 (46)	2/14 (14)	0/18 (0)	1/36 (3)		
Monthly	0/13 (0)	0/14 (0)	2/18 (11)	2/36 (5)		
Weekly	0/13 (0)	0/14 (0)	4/18 (22)	13/36 (36)		
Daily/almost daily	0/13 (0)	0/14 (0)	11/18 (61)	20/36 (56)		

^aBabor and colleagues (2006).

Table 3
Demographic Characteristics of Subjects with Pulmonary Cystine Species Measurements

	Control		Control		AUD		<i>p</i> -Value
	Nonsmokers, <i>n</i> = 4	Smokers, <i>n</i> = 6	Nonsmokers, <i>n</i> = 8	Smokers, <i>n</i> = 25	AUD		
Age	43 (40 to 45)	42 (33 to 52)	40 (37 to 46)	48 (44 to 52)			0.17
Gender, % Men	25%	50%	88%	88%			0.01
Pack-year smoking	0	19 (12 to 22)	0	14 (3 to 25)			0.43
AUDIT score	3 (1 to 4)	2 (0 to 4)	28 (25 to 32)	27 (20 to 35)			<0.0001
FEV ₁ , % predicted	102 (96 to 109)	89 (84 to 101)	101 (92 to 106)	99 (95 to 106)			0.22
FEV ₁ /FVC, % predicted	84 (78 to 90)	83 (76 to 86)	78 (71 to 88)	75 (72 to 80)			0.053
Percent yield of BAL procedure ^a	55 (50 to 68)	32 (26 to 50)	45 (43 to 51)	42 (30 to 51)			0.004

AUDIT, Alcohol Use Disorders Identification Test; FEV₁, forced expiratory volume in first second of exhalation; FEV₁/FVC, ratio of FEV₁ to forced vital capacity; BAL, bronchoalveolar lavage.

^aPercent yield calculated as follows: (total quantity of BAL fluid aspirated/total quantity of fluid instilled) × 100.

Continuous data are presented as medians with interquartile ranges. Statistics performed with Wilcoxon/Kruskal–Wallis testing (continuous variables) or Fisher’s exact test (categorical variables).