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N-acetylcysteine Improves Group B Streptococcus Clearance in a Rat Model of Chronic Ethanol Ingestion

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Abstract

Background—Sepsis is the most common risk factor associated with acute respiratory distress syndrome (ARDS) and results in a 40–60% mortality rate due to respiratory failure. Furthermore, recent epidemiological studies have demonstrated that a history of alcohol abuse increases the risk of ARDS by 3.6-fold. More recently, *group B streptococcus* (*GBS*) infections in non-pregnant adults have been increasing, particularly in alcoholics where there is an increased risk of lobular invasion and mortality. We have shown in an established rat model that chronic ethanol ingestion impaired macrophage internalization of inactivated infectious particles *in vitro* and enhanced bidirectional protein flux across the alveolar epithelial-endothelial barriers, both of which were attenuated when glutathione precursors were added to the diet. We hypothesized that chronic ethanol ingestion would increase the risk of infection even though *GBS* is less pathogenic but that dietary N-acetylcysteine (NAC), a glutathione precursor, would improve *in vivo* clearance of infectious particles and reduce systemic infection.

Methods—After 6 weeks of ethanol feeding, rats were given *GBS* intratracheally and sacrificed 24 hours later. *GBS* colony-forming units were counted in the lung, liver, spleen and bronchoalveolar lavage fluid. Acute lung injury in response to *GBS* was also assessed.

Results—Chronic ethanol exposure decreased *GBS* clearance from the lung indicating an active lung infection. In addition, increased colonies formed within the liver and spleen indicating that ethanol increased the risk of systemic infection. Ethanol also exacerbated the acute lung injury induced by *GBS*. NAC supplementation normalized *GBS* clearance by the lung, prevented the appearance of *GBS* systemically and attenuated acute lung injury.

Conclusions—These data suggested that chronic alcohol ingestion increased the susceptibility of the lung to bacterial infections from *GBS* as well as systemic infections. Furthermore, dietary NAC improved *in vivo* clearance of *GBS* particles, attenuated acute lung injury and disseminated infection.

Keywords

Acute lung injury; alcohol abuse; sepsis; oxidative stress; glutathione

Introduction

Acute and chronic alcohol abuse are independent risk factors for developing severe community-acquired pneumonia (Fernandez-Sola et al., 1995; Ruiz et al., 1999; Sisson et al., 2005). Within the lung, chronic alcohol consumption suppresses the cough reflex and induces

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paralysis of the mucociliary escalator increasing the possibility of deposition into the alveolar space (Wyatt and Sisson, 2001). If clearance in the conducting airways fail, more pathogen-specific innate immune mechanisms within the alveolar space such as alveolar macrophages and epithelial barrier integrity become important (Green and Kass, 1964; Happel et al., 2004).

Both clinical and animal models demonstrated that chronic alcohol abuse promotes oxidative stress within the lung and the alveolar space via the depletion of the critical antioxidant glutathione (**GSH**) (Brown et al., 2004; Brown et al., 2007; Holguin et al., 1998; Moss et al., 2000; Yeh et al., 2007). Previous studies by this laboratory demonstrated that ethanol-induced alveolar macrophage dysfunction was related to GSH depletion and subsequent chronic oxidant stress within the alveolar space (Brown et al., 2004; Brown et al., 2007). Furthermore, alcohol reduced macrophage killing of internalized bacteria (Brown et al., 2007; Gamble et al., 2006). In addition to compromised macrophage clearance of infectious particles, alcohol consumption damages the barrier integrity of the lung (Guidot et al., 2000; Holguin et al., 1998) increasing the potential for systemic dissemination of infectious particles.

Alcoholics are most commonly at risk for infections from *Streptococcus pneumoniae* or *Klebsiella pneumoniae* (Jong et al., 1995; Joshi and Guidot, 2007; Perlino and Rimland, 1985). However, reports of *Group B Streptococcus* (*GBS*) infections in non-pregnant adults have been on the rise with an increasing global trend. In retrospective studies, *GBS* cases in adults doubled in Spain (Blancas et al., 2004) and Taiwan (Huang et al., 2006). More importantly, in the former study, 11% of these cases had a history of alcohol abuse. In 1999, a prospective study of postmortem bacterial cultures from subjects that abused alcohol observed that *GBS* infections were almost as prevalent as pneumococci, but with twice the lobular invasion and increased mortality (Thomsen and Sogaard, 1999). In addition, the mortality rate of *GBS* positive subjects that abused alcohol was 13% compared to zero mortality in the control group with no underlying conditions (Blancas et al., 2004). Together these studies suggested that *GBS* infections are on the rise, particularly in subjects that abuse alcohol, and are associated with increased mortality.

Given the increasing rate of *GBS* infections in subjects with a history of alcohol abuse, a rat model was used to determine if chronic ethanol consumption increased the risk and severity of respiratory and systemic infections from *GBS*. Since GSH depletion is a central feature in the risk of lung injury, the ability of the glutathione precursor N-acetylcysteine (NAC) to attenuate *GBS*-induced respiratory infection and dissemination were also assessed. In these studies, chronic ethanol ingestion was associated with greater *GBS* infections in the lung, liver and spleen as well as greater acute lung injury. Furthermore, these effects were attenuated when the GSH precursor NAC was added to the ethanol diet.

Materials and Methods

Animals

Sprague Dawley rats (300–400 g, Harlan, St. Louis, MO, n = 5 per group) were fed an isocaloric liquid diet containing ethanol (Lieber DeCarli, Research Diets, New Brunswick, NJ; 36% of total calories) for six weeks as we have previously described (Brown et al., 2007). The control animals were fed the liquid diet without ethanol. Some rats were fed the liquid ethanol diet supplemented with 0.163 mg/ml NAC (Sigma, St. Louis, MO). There was no statistical difference in animal body weights at the end of the feeding period. All animals were used in accordance with the NIH Guidelines (Guide for the Care and Use of Laboratory Animals) with protocols reviewed and approved by the Emory University Institutional Animal Care Committee.

Preparation of Bacteria Inoculum

All work was completed in a BSL2 rated laminar flow hood following aseptic procedures. *Group B Streptococcus* (GBS, ATCC# BAA-22, Manassas, VA) was cultured overnight in sterile Todd Hewitt Broth (Sigma) at 37°C with 5% CO₂. Prior to instillation, the suspension was centrifuged at 8,000g for 15 minutes and the pellet washed twice with 10 ml of sterile phosphate buffered saline (PBS, Cellgro, Mediatech, Herndon, VA). The final bacterial pellet was resuspended in 10 ml PBS for a working inoculum concentration of 2×10^7 colony forming units (CFU) per ml. To verify the inoculum concentration, serial dilutions were cultured overnight on trypticase soy agar plates containing 5% blood (BBL Stacker plate, BD, Franklin Lakes, NJ). Gram staining (Protocol, Fisher Scientific, Fair Lawn, NJ) was performed to ensure pure *streptococci* colonies.

GBS Inoculation

After six weeks on the appropriate diet, rats were anesthetized (ketamine, 30mg/kg and xylazine, 2mg/kg mixture), the neck and upper body cleaned with 70% ethanol, and the trachea exposed via blunt dissection. GBS (0.5 ml; $\sim 1 \times 10^7$ CFU) were then injected into the trachea using a 26G 1/4 needle. The incision was cleaned with 70% ethanol, closed with a surgical staple and again swabbed with 70% ethanol. The animals were allowed to recover in the laminar flow hood for 24 hours. None of the animals exhibited signs of external infection at the wound site or tampering with the incision staples.

Determination of Tissue and Fluid Sample CFUs

After the 24 hour treatment period, the animals were sacrificed with an intraperitoneal overdose of pentobarbital (Sigma). The upper right lobe was ligated and removed for histology. The remaining lobes were lavaged four times with 10 ml of PBS. Lung, liver and spleen samples (~ 100 mg) were homogenized in PBS (9:1 ratio) and plated on blood agar plates (100 μ l). Cell-free BAL fluid samples (100 μ l) were also plated on blood agar plates. After an 18 hour culture period at 37°C and 5% CO₂, the CFUs were determined. The tissue CFUs were normalized to tissue weight. For the lavage fluid, the CFUs were not normalized and presented as that obtained from 100 μ l of fluid.

Lung Histology and Injury

The upper right lobe was excised, fixed in 3.7% paraformaldehyde and then placed in 70% ethanol until further processing. After samples were embedded in paraffin and sectioned, paraffin was removed and sections stained with Hematoxylin and Eosin (H&E, Fisher Scientific). ImageTool (UTHSCSA) was used to assess septal wall thickness. Ten images were obtained from each lung and ten septal thickness measurements were taken from each image. Using a 0 to 4 scale, an observer blinded to the treatment group scored the lung images for infiltration. Other measures of lung injury included assessment of the protein content by a Coomassie blue assay (Bio-Rad) and myeloperoxidase content by an ELISA (Hycult Biotechnology) in the lavage fluid.

Statistics

Each assay was repeated in triplicate. One way analysis of variance (ANOVA, SigmaStat) was used to test for overall statistical differences. Post-hoc analysis via the Student-Neuman-Keuls test was used to determine significance between individual groups. A p-value ≤ 0.050 was considered significant.

Results

Chronic Ethanol Ingestion Decreased GBS Clearance

When compared to the controls, chronic ethanol ingestion increased *GBS* infiltration into the lung tissue by 120-fold (Figure 1A). In the BAL fluid, the *GBS* content was increased by 26-fold in the ethanol-fed rats when compared to the controls (Figure 1B). Chronic ethanol ingestion also significantly increased colonization by 5-fold in the liver (Figure 2A) and 5-fold in the spleen (Figure 2B), when compared to the controls.

Chronic Ethanol Ingestion Increased Acute Lung Injury

As shown in the morphometric analysis, chronic ethanol ingestion increased lung injury in response to *GBS* as assessed by increased thickening of the alveolar septa and consolidation of the alveolar space in some areas (Figure 3). Using a semi-quantitative score from 0 to 4, lung injury increased approximately 40% in the ethanol-fed animals (Figure 4A). Correspondingly, septal wall thickness increased by 2-fold when the lungs from the ethanol-fed group were compared to the control group (Figure 4B). Using protein in the BAL fluid as a marker of leak, the ethanol group had a 3-fold increase when compared to the controls (Figure 5A). Myeloperoxidase protein, a marker of activated neutrophils, was increased 10-fold in the BAL fluid derived from ethanol-fed rats when compared to controls (Figure 5B).

NAC Supplementation Improved GBS Clearance and Attenuated Acute Lung Injury in the Ethanol-fed Rat

In the lung tissue, supplementation of the ethanol diet with NAC ablated the ethanol-induced risk of *GBS* infection (Figure 1A). In the BAL fluid, NAC supplements normalized the CFUs to control values (Figure 1B). NAC supplementation also prevented bacterial colonization in the liver (Figure 2A) and spleen (Figure 2B). When the control and ethanol+NAC groups were compared, there was no statistical difference in CFUs of the BAL fluid or tissues examined (lung, liver or spleen).

NAC supplementation of the ethanol diet also preserved the morphometric characteristics of the lung after *GBS* inoculation (Figure 3C). When compared to the ethanol group, lung sections from the ethanol+NAC group exhibited less overall lung injury as evidenced by normalization of the lung injury score (Figure 4A) and septal thickness (Figure 4B). Total protein (Figure 5A) and myeloperoxidase (Figure 5B) content of the BAL fluid were also normalized by NAC supplementation suggesting decreased neutrophil infiltration into the airspace and loss of endothelial/epithelial barrier integrity.

Discussion

Chronic alcoholism is an independent risk factor for the development of severe community and nosocomially acquired pneumonia. While *GBS* infections are not prevalent in adult populations, *GBS* infections are on the rise in non-pregnant adults, especially those with underlying debilitating diseases such as chronic alcohol abuse. Given the increase in *GBS* infections and a suggested association with alcohol abuse, we assessed *GBS* clearance from the lung in a rat model of chronic ethanol ingestion 24 hours after intratracheal administration of the bacterium. In these studies, chronic ethanol ingestion increased *GBS* colonization of lung tissue and decreased bacterial clearance from the alveolar space when compared to the control group. These results were similar to that observed for clearance of *Streptococcus pneumoniae* (Boé et al., 2001)(Auerbach-Rubin and Ottolenghi-Nightingale, 1971) after acute ethanol intoxication. Therefore, chronic ethanol ingestion increased the risk of *GBS* infection even though this Gram positive microorganism is less pathogenic than *Streptococcus pneumoniae*.

Undoubtedly multiple mechanisms that are involved in bacterial clearance could be altered by chronic ethanol ingestion. For alveolar macrophages, chronic alcohol abuse greatly reduces alveolar and peritoneal macrophage phagocytosis of infectious particles (Bagasra et al., 1988; Brown et al., 2004; Brown et al., 2007; Mufti et al., 1988), primarily due to an alcohol-induced GSH depletion and oxidative stress in the alveolar space. Therefore, ethanol-induced derangements in the capacity of alveolar macrophages to clear infectious particles would contribute to the increased infection by GBS. Ethanol-induced impairment of the mucociliary escalator (Wyatt and Sisson, 2001) could also have contributed to the increased GBS infection. Given that the experiment was terminated 24 h after inoculation, chronic ethanol ingestion may have impaired the capacity of alveolar epithelial cells to prevent infection and replication of GBS, contributing to an increased bacterial load.

Both clinical studies (Burnham et al., 2003; Burnham et al., 2004) and animal studies (Guidot et al., 2000) demonstrated that chronic ethanol ingestion impairs lung endothelial and epithelial barrier integrity. Therefore, it was not surprising that increased colonization of the liver and spleen were also observed within the ethanol-fed rat. In addition, an increased bacterial load in the alveolar space would also contribute to increased dissemination to other organs. Ethanol-induced compromises in the immune function of the other organs would also contribute to the large increases in colonies present in the livers and spleens of the ethanol-fed rats.

Previous studies by this research group have demonstrated in clinical (Burnham et al., 2003; Moss et al., 2003; Moss et al., 1999) and animal studies (Guidot et al., 1999; Guidot et al., 2000; Holguin et al., 1998; Velasquez et al., 2002) that chronic alcohol abuse primes the lung for acute lung injury in response to a secondary insult. In the current study, results from several different markers further suggested that chronic ethanol ingestion increased the risk and degree of acute lung injury. The increases in septal thickness and the lung injury score demonstrated that chronic ethanol ingestion exacerbated the acute lung injury associated with a bacterial infection. The increased myeloperoxidase in the lavage fluid of ethanol-fed rats suggested that the transmigration of neutrophils from the tissue into the alveolar space was significantly increased in the ethanol group. However, increased neutrophil recruitment for bacterial clearance would also contribute to the increased myeloperoxidase content. The increased protein content of the BAL fluid from ethanol-fed rats further supported exacerbation of endothelial/epithelial barrier dysfunction and acute lung injury. These diverse markers demonstrated that chronic ethanol ingestion increased the risk and morbidity of GBS-induced acute lung injury.

In both clinical (Burnham et al., 2003; Burnham et al., 2004) and animal studies (Guidot et al., 1999; Guidot et al., 2000; Holguin et al., 1998; Velasquez et al., 2002), GSH depletion in the alveolar space was central to the increased risk of lung injury associated with chronic ethanol ingestion. In the current study, the GSH precursor NAC prevented the ethanol-induced increases in the GBS infections of the lung, liver and spleen. Additional studies are needed to determine if NAC attenuated the bacterial load by preventing ethanol-induced derangements in mucociliary clearance, alveolar macrophage phagocytosis, infection of alveolar epithelial cells, as well as endothelial and epithelial barrier integrity. Equally important, NAC supplements in the ethanol diet prevented the exacerbation of GBS-induced acute lung injury associated with chronic ethanol ingestion. These findings suggested that the increased sensitivity to GBS-induced alveolar epithelial and endothelial barrier dysfunction and lung injury were directly related to ethanol-induced GSH depletion and oxidant stress. Although NAC supplements prevent ethanol-induced GSH depletion in the alveolar space (Brown et al., 2007), additional studies are needed to determine if the beneficial effects of NAC were dependent on the GSH concentrations in the epithelial lining fluid.

In summary, chronic ethanol consumption decreased the ability to clear *GBS* from the alveolar space and increased the bacterial load of the lung tissue. These effects of ethanol were associated with exacerbation of acute lung injury in response to *GBS* and increased dissemination to the liver and spleen. These studies support the epidemiological observations of increased *GBS* infections in subjects that abused alcohol with increased lobular invasion and mortality (Thomsen and Sogaard, 1999). Dietary NAC supplementation normalized bacterial clearance and dissemination suggesting that this increased sensitivity to infections and tissue injury were secondary to ethanol-induced GSH depletion and subsequent oxidant stress in the lung and potentially the other tissues. Whether short-term supplements with NAC can attenuate the risk of infection, tissue injury and dissemination after the deleterious effects of ethanol have been established remains to be determined.

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Abbreviations

ARDS	Acute Respiratory Distress Syndrome
GSH	Glutathione
<i>GBS</i>	<i>Group B Streptococcus</i>
NAC	N-acetylcysteine
BAL	Bronchoalveolar Lavage
CFU	Colony-Forming Unit

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Figure 1. NAC supplementation prevented the ethanol-induced increases in *GBS* lung infections (A) and BAL fluid (B)

Sprague Dawley rats were fed the Lieber DeCarli liquid diet with or without ethanol (36% of the total calories) or NAC (0.163 mg/ml) for 6 weeks. *GBS* was administered intratracheally to all rats. After 24 hours, the lung tissue from the control, ethanol and ethanol+NAC rats were homogenized and CFUs determined (A). After 24 h, the lungs were lavaged and CFUs of the cell-free fluid determined (B). Bar heights represent mean $\pm$ SEM for 5 animals. ^a $p \leq 0.050$ compared to control and ^b $p \leq 0.050$ compared to ethanol.

Figure 2. NAC supplementation attenuated the increased bacterial colonization of the liver (A) and spleen (B) associated with ethanol ingestion

After 6 weeks on the appropriate diet and intratracheal delivery of *GBS*, liver (A) and spleen (B) tissue were harvested, homogenized and the CFUs quantified. Bar heights represent mean $\pm$ SEM for 5 animals. ^a $p \leq 0.050$ compared to control and ^b $p \leq 0.050$ compared to ethanol.

(A) Control**(B) Ethanol****(C) Ethanol + NAC**

Figure 3. Lung histology sections from control (a), ethanol (b) and ethanol+NAC (c) rats
The upper right lobe from the *GBS*-infected control, ethanol and ethanol+NAC lungs were fixed in 3.7% paraformaldehyde, embedded in paraffin, sectioned and prepared for Hematoxylin & Eosin staining. Figures a, b and c are representative images of lung tissue 24 hours after intratracheal *GBS* injection from control, ethanol and ethanol+NAC, respectively. Images were taken at 4× magnification. Bar equals 1 mm.

Figure 4. NAC supplementation attenuated the increased acute lung injury associated with chronic ethanol ingestion

After the *GBS* inoculation, rats were then sacrificed at 24 h. The upper right lobe was excised, fixed in 3.7% paraformaldehyde, embedded in paraffin and sections stained with Hematoxylin and Eosin. An observer blinded to the treatment groups assigned a lung injury score between 0 and 4 to each image based on the following criteria: the amounts of inflammation and edema formation and septal thickness (A). ImageTool imaging freeware (UTHSCSA) was used to measure septal wall thickness (B). Bar heights represent the mean $\pm$ SEM of 5 animals. ^a $p \leq 0.05$ compared to control and ^b $p \leq 0.05$ compared to the ethanol group.

Figure 5. NAC supplementation attenuated ethanol-induced exacerbation of *GBS*-induced protein leak (A) and neutrophil infiltration (B)

Rats were fed the appropriate diet, inoculated with *GBS* and then sacrificed 24 h later. After the lungs were lavaged, the total protein (A) and myeloperoxidase (B) in the cell-free BAL fluid was assessed. Bar heights represent mean $\pm$ SEM for 5 animals. ^a $p \leq 0.050$ compared to control and ^b $p \leq 0.05$ compared to the ethanol group.