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Chronic Ethanol Ingestion Increases Nitric Oxide Production in the Lung

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Abstract

Purpose: Chronic ethanol (EtOH) ingestion increases the incidence of the Acute Respiratory Distress Syndrome (ARDS). The mechanisms underlying EtOH-induced susceptibility to lung injury continue to be defined. This study examines the hypothesis that EtOH increases endothelial nitric oxide synthase (eNOS) expression and activity in the lungs of a rat model of chronic EtOH ingestion.

Methods: Male Sprague Dawley rats were fed liquid diets containing EtOH (36% of calories) or Maltose-dextrin as an isocaloric substitution for EtOH (Control) for 6 weeks. Selected animals were also treated with the angiotensin converting enzyme (ACE) inhibitor, lisinopril (3 mg/L in diet) for 6 weeks. At study completion, animals were sacrificed, and lung tissue was collected for assays of nitric oxide (NO) metabolism or pulmonary microvascular endothelial cells (MVEC) were isolated for analysis of NO release.

Results: Compared to the Control diet, chronic EtOH ingestion increased lung H₂O₂ production, eNOS expression and activity, lung cGMP content and levels of protein nitration and oxidation. MVEC from animals with chronic EtOH ingestion released greater amounts of NO. EtOH-induced increases in lung H₂O₂ production, eNOS expression and activity, cGMP content, protein nitration and oxidation, and MVEC NO production were all attenuated by treatment with lisinopril.

Conclusions: Chronic EtOH ingestion stimulates ACE-dependent increases in NO production in the lung. These novel findings indicate that chronic EtOH ingestion increases reactive species production in the lung parenchyma and provide new insights into mechanisms by which EtOH causes phenotypic alterations in the lung and its response to inflammatory stimuli.

Keywords

alcohol; nitric oxide; nitric oxide synthase

Introduction

The Acute Respiratory Distress Syndrome (ARDS) is a severe form of lung injury characterized by noncardiogenic pulmonary edema in response to diverse clinical scenarios such as sepsis, trauma, or aspiration that cause systemic activation of proinflammatory cascades. Despite extensive investigation ARDS is associated with a high mortality rate, due in part to the lack of effective therapies. Therefore, identifying mechanisms that predispose the lung to acute

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injury has the potential to further our understanding of ARDS pathophysiology and facilitate the design of treatment strategies capable of limiting the development and/or severity of ARDS.

Chronic alcohol abuse is now established as a predisposing factor that increases the incidence of ARDS through mechanisms that continue to be defined (Moss et al., 1996; Moss et al., 2003). Numerous studies, reviewed elsewhere (Guidot and Hart, 2005), have employed a rat model of chronic EtOH ingestion to demonstrate the ability of EtOH to enhance susceptibility to experimental lung injury through a variety of defects in alveolar epithelial cell function. Although the precise cellular and biochemical causes of EtOH-induced susceptibility to lung injury have not been defined, considerable evidence indicates that EtOH-induced oxidative stress plays a critical role and that many EtOH-induced pulmonary derangements can be prevented or attenuated by providing experimental animals dietary precursors of the antioxidant, glutathione. We recently reported that oxidative stress in the lung related to chronic EtOH ingestion was related in part to renin angiotensin system (RAS)-mediated increases in NADPH oxidase expression and superoxide production (Polikandriotis et al., 2006). In addition, we also demonstrated that chronic EtOH stimulation increased eNOS expression and activity in pulmonary vascular endothelial cells *in vitro* (Polikandriotis et al., 2005). Based on these *in vitro* findings, the current study was designed to examine the impact of chronic EtOH ingestion on NO production in the lung *in vivo*. Our results provide novel evidence that chronic EtOH ingestion increases H₂O₂ production, eNOS expression and NOS activity in the lung that contribute to protein nitration and oxidation. Attenuation of these EtOH-induced alterations in NO metabolism in the lung with lisinopril, coupled with previous evidence that lisinopril inhibited EtOH-induced increases in NADPH oxidase expression and superoxide production in the lung, suggests that chronic EtOH ingestion stimulates the RAS to promote subclinical alterations in the production of reactive oxygen and nitrogen species in the lung that contribute to susceptibility to lung injury.

Materials and Methods

Materials

Type III, mouse eNOS monoclonal antibodies (Lot # 03804) were from BD Transduction Laboratories. Mouse hsp90 antibodies (Lot #C1203) and goat actin antibodies (Lot #L3004) were purchased from Santa Cruz Biotechnology, Inc. All Western blot gradient (4-12%) gels and 4X NuPAGE Lithium Dodecyl Sulfate (LDS) Sample Buffer (40 % glycerol, 423 mM Tris HCL, 8 % LDS, 2.1 mM EDTA, 0.075 % Serva Blue G250, 0.025 % Phenol Red), which was diluted to a 1X solution with 50 mM DTT, were purchased from Invitrogen. Supersignal chemiluminescence substrate, which detects horseradish peroxidase on immunoblots, and the bicinchoninic acid (BCA) reagent to quantify proteins were acquired from Pierce. GammaBind Plus sepharose beads with IgG receptors were from Amersham Pharmacia.

Animal model of chronic EtOH ingestion

Male Sprague-Dawley rats (7-8 weeks old, approximately 150 grams) were fed the liquid Lieber-DeCarli diet (Lieber and DeCarli, 1982) containing EtOH (36% of calories) for 6-weeks. Control animals were pair-fed an isocaloric control diet without EtOH (substitution of Maltose-Dextrin for EtOH) for 6 weeks as previously reported (Polikandriotis et al., 2006). This EtOH feeding regimen produced a blood alcohol level of 0.08% and did not produce significant weight loss as we have previously reported (Bechara et al., 2005). This regimen was employed because it worsens sepsis-mediated lung injury and dysfunction in the rat (Velasquez et al., 2002) and thereby recapitulates evidence from human studies that chronic EtOH ingestion increases the incidence and severity of acute lung injury and ARDS (Moss et al., 1996; Moss et al., 2003). All studies were reviewed and approved by the Institutional Animal Care and Use Committee at the Atlanta VA Medical Center. During the first two weeks

of this dietary regimen, the EtOH-fed rats were gradually acclimated to EtOH, receiving 12% of their total calories as EtOH for one week, then 24% of their total calories as EtOH for one week, and then 36% of their total calories as EtOH for four weeks. Selected animals were also treated with lisinopril (3 mg/L, dosing recommended by the manufacturer, Merck) in the Control or EtOH diets. The diets are otherwise identical in protein, lipid, and essential nutrient composition.

Isolation of pulmonary microvascular endothelial cells from Control and EtOH-fed rats

Pulmonary microvascular endothelial cells (MVEC) were isolated from the lungs of rats fed Control or EtOH diets as previously reported (Chen et al., 1995). Briefly, rat lungs were perfused *in situ* via the pulmonary artery to remove red blood cells. The lungs were then taken from the chest, and the peripheral 3-5 mm of each lung was removed by trimming with scissors under sterile conditions. By collecting only the periphery of the lung, microvascular rather than macrovascular endothelial cells were obtained. The trimmed sections were then cut into approximately 1×1×1.5 mm sections and cultured on plastic for 60 h in MCDB 131 media containing 15% serum, 10 ng/ml epithelial growth factor, 2 mM L-glutamine, 1 mg/ml hydrocortisone, and antibiotic/antimycotic solution. After 60 h, the lung tissue fragments were removed from the dish, and half the media was replaced with fresh media. This technique generated approximately 1×10^6 cells per gram of tissue with > 95% purity as confirmed by: 1) typical cobblestone morphology (light microscopy), 2) staining of >90% of cells with the FITC-conjugated lectin, *Bandeiraea simplicifolia* I isolectin B4 which binds primarily to microvascular rather than macrovascular endothelial cells (Hansen-Smith et al., 1988), and 3) staining of >90% of cells for angiotensin-converting enzyme (ACE), von Willebrand factor (Factor VIII), or DiI-Ac-low-density lipoprotein (data not shown). The cells were cultured for 7 days *in vitro*. The release of nitrite (NO_2^-), a metabolite of NO, into culture media was measured by chemiluminescence as an index of NO production (Hart et al., 2005). Results are expressed as nmol NO_2^- released into culture media / 1×10^6 MVEC.

Immunofluorescence microscopy

Following sacrifice, lungs were rinsed with PBS and then perfused with OCT. Lungs were then embedded in OCT, frozen at -80°C , and sectioned (5 μm). Slides were then thawed at room temperature for 20 min, fixed in acetone for 15 min and air dried for 10 min, followed by two 5-minute washes in PBS. After blocking with 1% gelatin in PBS for 60 minutes, slides were incubated with mouse anti-eNOS antibodies (1:50; BD Transduction) for 1 hour at room temperature, washed twice with PBS, and then incubated with rabbit anti-Factor VIII antibodies (1:50, DAKO) over night at 4°C . Following 2 washes in PBS, slides were incubated with a goat anti-mouse fluorescein-conjugated secondary antibody (1:50; Jackson ImmunoResearch Inc) for 30 minutes, followed by washing with PBS, and incubation with goat anti-rabbit Rhodamine Red-conjugated secondary antibody (1:50; Jackson ImmunoResearch Inc) for 30 minutes. Slides were then washed with PBS twice and mounted with fluoromount (Vectashield) before viewing at 40X magnification and collecting images.

Analysis of H_2O_2 production

H_2O_2 production in lung tissue was determined by measuring conversion of Amplex Red® reagent to resorufin by H_2O_2 following protocols provided by the manufacturer (Molecular Probes, Eugene, OR). Amplex Red is a fluorogenic substrate that reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin. Briefly, 200 μM Amplex Red reagent and 1 U/ml horseradish peroxidase were added to lung samples from Control or EtOH-fed rats or to H_2O_2 standard solutions in 50 mM sodium phosphate buffer (pH 7.4). The samples and standards were then incubated for 30 min in 24-well plates in the dark at room temperature. The fluorescence intensity of samples and standards was measured in a plate reader ($\lambda_{\text{ex}} = 560$

nm, $\lambda_{em} = 590$ nm). After subtraction of background fluorescence, H_2O_2 concentrations in lung tissue were based on comparisons to known concentrations of H_2O_2 and normalized to the wet weight of each sample.

Analysis of lung NOS activity and cGMP levels

Total NOS activity was quantified by measuring conversion of [^3H]-L-arginine to [^3H]-L-citrulline as we have previously reported (Gupta et al., 1998) using a Nitric Oxide Synthase Assay Kit (Calbiochem) according to the manufacturer's instructions. Because soluble guanylate cyclase constitutes an important physiological target of NO that increases cell and tissue levels of cGMP, lung tissue cGMP levels were determined using a cGMP ELISA kit (Amersham) according to the manufacturer's instructions.

Analysis of lung eNOS protein levels

At sacrifice, lungs were perfused blood-free, and sections of peripheral lung tissue were placed in lysis buffer (20 mM Tris pH 7.4, 2.5 mM EDTA, 1% TritonX-100, 1% deoxycholic acid, 0.1% SDS, 100 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4) containing protease inhibitors. Tissue homogenates were prepared, sonicated, and centrifuged at 15,000g for 10 minutes. The supernatants were collected, protein concentrations were determined, and eNOS antibodies (20 μ g) were added to 500 μ g protein homogenate and incubated overnight at 4°C on a rocking platform. Samples were then incubated with sepharose beads at 4°C for 3 hours. Beads were then collected by centrifugation, washed, dissolved in sample buffer at 95°C and subjected to SDS-PAGE, transferred to PVDF membranes and immunoblotted with the same primary antibody in TBS-T (10 mM TrisHCl, 150 mM NaCl, 0.1% Tween, pH 7.4) containing 5% powdered non-fat dry milk. In selected studies, eNOS immunoprecipitates were also probed with antibodies to the molecular chaperone protein, heat shock protein 90 (hsp90). Bands were identified by chemiluminescence and quantified by laser densitometry.

Analysis of protein nitration and oxidation

Total protein nitration was quantified with the BIOXYTECH® nitrotyrosine E1A ELISA according to the manufacturer's instructions (Oxis Research). Lung protein carbonyl groups, an index of protein oxidation, were detected with the OxyBlot™ oxidized protein detection kit (Chemicon International) following protocols provided by the manufacturer.

Statistical Analysis

Overall treatment effects were examined by analysis of variance (ANOVA). Post hoc analysis to detect differences between specific groups was accomplished with the Student Neuman Keuls test. The level of statistical significance was taken as $p < 0.05$.

Results

Chronic EtOH ingestion increased RAS-dependent lung H_2O_2 generation and eNOS expression

We previously reported that chronic EtOH ingestion in the rat leads to oxidative stress in the lung characterized by reductions in glutathione levels in parenchymal lung cells and in BAL fluid (Brown et al., 2001a; Brown et al., 2001b; Guidot and Brown, 2000; Guidot et al., 2000; Holguin et al., 1998; Moss et al., 2000) and ACE-dependent increases in superoxide production in the lung (Polikandriotis et al., 2006). To further characterize potential mechanisms of oxidative stress in the lung caused by chronic EtOH ingestion, H_2O_2 production was measured. H_2O_2 is the product of superoxide dismutation, a reactive oxygen species previously demonstrated to be increased in the lung by chronic EtOH ingestion (Polikandriotis et al., 2006). Lung tissue from Control or EtOH-fed rats treated with or without the ACE

inhibitor, lisinopril, was collected for measurement of H₂O₂ production. As shown in Figure 1, chronic EtOH ingestion increased H₂O₂ production in rat lung, an effect attenuated by treatment with lisinopril.

We previously reported that stimulation with clinically relevant concentrations of EtOH for 72 h increased eNOS expression and activity in pulmonary vascular endothelial cells *in vitro* (Polikandriotis et al., 2005). Previous studies have shown that H₂O₂ increases eNOS expression and activity in endothelial cells *in vitro* (Drummond et al., 2000). Therefore, to determine if EtOH-induced increases in lung H₂O₂ production were associated with increased eNOS expression in the lung, whole lung homogenates were immunoprecipitated with a specific eNOS antibody, and the immunoprecipitated proteins were subjected to immunoblotting with eNOS and hsp90. Hsp90 associates with eNOS to enhance its activity (Fontana et al., 2002; Gratton et al., 2000; Takahashi and Mendelsohn, 2003). As shown in the representative blot (Figure 2a) and the densitometric analysis (Figure 2c), chronic EtOH ingestion increased eNOS protein levels in the rat lung. Neither inducible NOS nor neuronal NOS protein was detected in these lung homogenates (data not shown). In addition, Figure 2 also demonstrates that chronic EtOH ingestion did not alter the association of eNOS and hsp90 (Fig. 2d) and did not alter overall lung hsp90 or actin protein levels (Figure 2b). Averaged densitometric analyses of the results from these studies emphasize that: a) EtOH increases eNOS protein levels, and b) including lisinopril in the diet of EtOH-fed animals prevented EtOH-induced increases in eNOS expression in the lung. Collectively the findings in Figure 2 illustrate that: 1) chronic EtOH ingestion increases lung eNOS expression, 2) lisinopril attenuates EtOH-induced increases in lung eNOS expression, and 3) the interaction of eNOS and hsp90 do not appear to be altered by EtOH.

Chronic EtOH ingestion increased pulmonary MVEC NO production and enhanced endothelial eNOS expression in the intact lung

Because chronic EtOH ingestion increased levels of eNOS, which is expressed most robustly in vascular endothelium, MVEC were isolated from peripheral lung slices of Control and EtOH-fed rats treated with or without lisinopril. After 7 days in culture, the MVEC culture media were replaced with fresh media, and MVEC were incubated for an additional 16 h. The culture media were collected for analysis of the NO metabolite, NO₂⁻, by chemiluminescence as we have previously reported (Gupta et al., 1998). As illustrated in Figure 3, MVEC isolated from EtOH-fed animals released significantly more NO₂⁻ into culture media than Control MVEC consistent with EtOH-induced increases in eNOS expression in whole lung homogenate. Treating animals with the ACE inhibitor, lisinopril, completely attenuated the increased NO release from lung MVEC. As illustrated in Figure 3b, immunofluorescence microscopy of lungs from Control and EtOH-fed animals provides additional evidence that the majority of EtOH-mediated increases in eNOS expression in the lung co-localize with the endothelial marker, Factor VIII. These findings indicate that chronic EtOH ingestion increases endothelial eNOS expression and NO production and that the EtOH-induced enhanced MVEC NO release is preserved *in vitro* for up to 7-days in the absence of EtOH.

Chronic EtOH ingestion increased NOS activity and cGMP levels in lung tissue

To confirm that increased lung eNOS expression in chronic EtOH-treated rats resulted in increased NOS activity, lung homogenates were subjected to NOS activity assays (conversion of radiolabeled L-arginine to L-citrulline). As illustrated in Figure 4a, NOS activity was increased in the lung following chronic EtOH ingestion, an effect that was attenuated by including lisinopril in the diet. These NOS activity assays measure *ex vivo* NOS activity in a tissue homogenate using an assay that employs optimized concentrations of enzyme, substrate, and cofactors. To better examine *in vivo* NO bioavailability in the lung, cGMP levels in lung tissue were measured as a surrogate marker because NO stimulates guanylate cyclase to

increase cGMP levels. As shown in Figure 4b, chronic EtOH ingestion increased lung cGMP levels, an effect attenuated by including lisinopril in the diet of EtOH-fed animals.

Chronic EtOH ingestion increased protein nitration and oxidation

Increased production of superoxide and NO can promote the formation of peroxynitrite, a potent oxidant. Because chronic EtOH ingestion increased the production of superoxide (Polikandriotis et al., 2006) and NO (current study) in the lung, we examined lung proteins for evidence of oxidation and nitration. Compared to Control diets, chronic EtOH ingestion increased the levels of protein nitration approximately 40%, an effect attenuated by lisinopril (Figure 5a). Similarly, the accumulation of oxidized proteins in whole lung homogenates was examined using the OxyBlot™ assay, which derivatizes carbonyl groups to a 2,4-dinitrophenylhydrazine (DNP) moiety that is detected using anti-DNP antibodies. Chronic EtOH ingestion increased levels of oxidized proteins to a small but significant extent, an effect attenuated by lisinopril.

Discussion

Previous reports demonstrated that chronic EtOH ingestion in rats renders the lung intrinsically susceptible to endotoxin-dependent edematous injury, supporting the observation that alcohol abuse predisposes the lung to injury (Holguin et al., 1998). EtOH ingestion markedly decreased the levels of the antioxidant glutathione in lung tissue and in BAL fluid and increased superoxide generation in lung tissue (Brown et al., 2001a; Brown et al., 2001b; Guidot and Brown, 2000; Guidot et al., 2000; Holguin et al., 1998; Polikandriotis et al., 2006). Chronic EtOH exposure also increased eNOS expression and activity *in vitro* in pulmonary vascular endothelial cells (Polikandriotis et al., 2005). Collectively these reports demonstrated that chronic EtOH ingestion leads to previously unrecognized increases in the production of reactive species in the lung and pulmonary cells. The current study extends these observations to demonstrate that EtOH also increases NO production in the lung, and to our knowledge, provides the first demonstration that chronic EtOH ingestion alters eNOS expression and activity in the lung.

Chronic EtOH ingestion increased H₂O₂ production as well as eNOS expression and activity in the lung, effects attenuated by *in vivo* treatment with the ACE inhibitor, lisinopril. An identical protocol of chronic EtOH ingestion in this rat model increased NADPH oxidase expression and superoxide generation in the lung through a renin-angiotensin system pathway that was inhibited by lisinopril (Polikandriotis et al., 2006). H₂O₂ is the product of superoxide dismutation catalyzed by one of several forms of superoxide dismutase. Previous work demonstrated that angiotensin II stimulated H₂O₂ production and increased the expression and activity of eNOS in vascular endothelial cells (Drummond et al., 2000). These findings indicate that: 1) chronic EtOH ingestion activates the RAS, thereby increasing lung NADPH oxidase expression and superoxide generation, and 2) the increased superoxide production increases H₂O₂ formation and stimulates eNOS expression. Consistent with this postulate, RAS inhibition with lisinopril prevented not only EtOH-stimulated NADPH oxidase expression and superoxide production (Polikandriotis et al., 2006) but also downstream H₂O₂ production (Figure 1) and consequent increases in eNOS expression and activity (Figures 2-4). Although our studies do not define precisely all the cellular compartments in the lung where EtOH might increase eNOS expression, the results in Figure 3 demonstrate that the microvascular endothelium constitutes at least one source of enhanced NO production. Furthermore, the immunofluorescence imaging in Figure 3 supports endothelial localization of EtOH-mediated increases in eNOS expression. These studies do not, however, exclude the potential contribution of other lung cells to the EtOH-mediated increases in eNOS expression and NO production.

Oxidation of the eNOS cofactor, tetrahydrobiopterin, can lead to eNOS uncoupling and eNOS-mediated production of superoxide rather than NO (Kuzkaya et al., 2003). Such eNOS uncoupling could thereby contribute to oxidative stress in the lung during chronic EtOH-ingestion. The findings in the current study, however, suggest that chronic EtOH ingestion increases not only eNOS expression but also NO production. For example, chronic EtOH ingestion increased lung cGMP levels as well as NO production by pulmonary MVEC. Furthermore, the association of eNOS with hsp90 facilitates enzyme coupling (Pritchard et al., 2001), and hsp90 has been reported to increase eNOS activity by: (1) recruiting Akt, the serine protein kinase B, to phosphorylate eNOS at ser¹¹⁷⁷ which increases electron flux from the reductase to the oxygenase domain of eNOS and increases enzyme activity, (2) facilitating the displacement of eNOS from inhibitory interactions with caveolin, and (3) increasing the affinity of eNOS for calmodulin (Fontana et al., 2002; Gratton et al., 2000; Takahashi and Mendelsohn, 2003). The co-immunoprecipitation studies indicate that chronic EtOH ingestion did not alter eNOS-hsp90 association. These findings indicate that despite significant EtOH-mediated increases in eNOS expression, the increased levels of eNOS remain associated with hsp90 and support increased NO production even in the face of considerable oxidative stress in the lung following chronic EtOH ingestion. However, the findings in Figure 5 emphasize that at least a small component of lung NO production contributes to protein nitration. These findings indicate that even in the absence of additional inflammatory insults, chronic EtOH ingestion is sufficient to cause peroxynitrite formation. Although the magnitude of EtOH-induced increases in protein oxidation and nitration was small, it must be emphasized that the lungs of rats following chronic EtOH ingestion are morphologically and functionally normal, and the animals exhibit no obvious clinical derangements or evidence of lung injury or edema (Guidot and Hart, 2005).

Identifying the mechanisms of altered NO production in the lung following chronic EtOH ingestion is critical because understanding these processes may provide potential avenues for intervention. However, additional studies will be required to determine whether the increase in eNOS expression and NOS activity contributes to oxidative stress in the lung following chronic EtOH ingestion, or represents an adaptive response to these stresses. Adaptive changes in NO synthesis could confer a beneficial or protective role to counter the deleterious effects of EtOH-mediated superoxide production and oxidative stress. In support of this concept, decreased constitutive NO production enhanced, whereas arginine-stimulated NO production attenuated, EtOH-mediated hepatic injury in the rat (Nanji et al., 2001).

Despite the potential salutary tissue effects of EtOH-induced increases in lung NO production in the face of EtOH ingestion alone, we speculate that these same increases in eNOS expression and activity may be deleterious during subsequent inflammatory insults that promote additional increases in lung oxidative stress. For example, increases in NO production in the lung following chronic EtOH ingestion could contribute to “priming” the lung for exaggerated responses to inflammatory insults that cause oxidative stress, eNOS uncoupling, reductions in bioavailable NO and formation of peroxynitrite. In fact, we have previously shown that lisinopril attenuates EtOH-induced glutathione depletion, transforming growth factor beta activation, and edematous lung injury caused by endotoxin in the rat lung (Bechara et al., 2005). Thus, we believe that inhibiting RAS-mediated, EtOH-induced alterations in lung reactive oxygen and nitrogen species with lisinopril reverses, at least in part, the priming effect of chronic EtOH ingestion on the lung. We postulate that these subclinical modifications in the production of reactive nitrogen and oxygen species in the lung contribute to the enhanced susceptibility of these animals to lung injury when challenged by a second inflammatory stimulus.

In summary, the present study provides novel evidence that chronic EtOH ingestion increased lung eNOS expression, NOS activity, and protein oxidation and nitration through mechanisms

that are inhibited by treatment with an ACE inhibitor. These findings provide new insights into derangements in pulmonary biology caused by chronic EtOH ingestion and may facilitate the identification of new targets for the prevention or treatment of lung injury that develops in the face of chronic alcohol ingestion.

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Figure 1. EtOH-stimulated increases in lung H_2O_2 production are attenuated by lisinopril

Male Sprague Dawley rats were fed liquid Control or EtOH diets $\pm$ lisinopril (3 mg / L) for 6-weeks. H_2O_2 production in lung tissue from these animals was determined as described in *Methods*. Each bar represents the mean $\pm$ SEM lung tissue H_2O_2 production per mg protein as % Control. n = 8. *p<0.05 versus all other groups.

Figure 2. Chronic EtOH ingestion increased eNOS protein levels in the lung

After 6-weeks feeding with Control or EtOH liquid diets $\pm$ lisinopril (lis, 3 mg / L), male Sprague-Dawley rats were sacrificed, and peripheral lung tissue was collected, homogenized, and subjected to immunoprecipitation (IP) with eNOS-specific antibodies as described in *Methods*. These immunoprecipitates were then immunoblotted (IB) with antibodies to eNOS and hsp90. A representative immunoblot is presented in **A**. In **B**, a representative immunoblot (IB) is presented wherein whole lung homogenates were subjected to Western blotting with antibodies to hsp90 and actin. In **C**, each bar represents the mean $\pm$ SEM densitometry of eNOS immunoprecipitated from equivalent amounts of lung homogenate expressed as % Control. In **D**, each bar represents the mean $\pm$ SEM densitometric ratio of hsp90 to eNOS as % Control. $n = 5$. * $p < 0.05$ versus all other groups.

Figure 3. Chronic EtOH ingestion increased NO production by isolated pulmonary MVEC and increased eNOS levels in lung endothelial cells

After 6-weeks on Control or EtOH diets, $\pm$ lisinopril (lis, 3 mg / L), rats were sacrificed, pulmonary MVEC were isolated as described in *Methods*. In the bar graph in panel A, NO production by isolated MVEC was measured as release of nitrite (NO_2^-) into culture medium in nmol per 1×10^6 MVEC. $n = 4$. * $p < 0.005$ versus Control, ** $p < 0.005$ versus EtOH. In panel B, representative images from frozen lung sections of Control and EtOH-fed mice are presented. Sections were treated with primary anti-eNOS antibodies detected with secondary FITC-labeled antibodies (green signal, middle column) and with primary anti-Factor VIII antibodies detected with rhodamine red-labeled secondary antibodies (red signal, right column). Phase contrast images of these sections are also presented (left column). Magnification = 40X.

Figure 4. Chronic EtOH ingestion increased lung NOS activity and cGMP content, effects attenuated by lisinopril

Following treatment with Control or EtOH diets $\pm$ lisinopril for 6-weeks, rats were sacrificed and lung tissues were collected for assays of NOS activity or cGMP levels as described in *Methods*. In **A**, each bar represents mean $\pm$ SEM lung NOS activity in cpm L-citrulline formed / mg lung protein. $n = 3-4$. In **B**, each bar represents the mean $\pm$ SEM lung cGMP content in fmol / mg protein. $n = 3-4$ * $p < 0.05$ versus all other groups.

Figure 5. Chronic EtOH-induced increases in lung protein oxidation and nitration were attenuated by lisinopril treatment

Rats were fed Control or EtOH diets $\pm$ lisinopril for 6-weeks. After sacrifice, whole lung homogenates were prepared and subjected to assays of protein nitration and oxidation as described in *Methods*. In **A**, each bar represents the mean $\pm$ SEM levels of protein nitration / mg protein as % Control. $n = 5-7$. In **B**, each bar represents the mean $\pm$ SEM densitometric intensity of 2,4-dinitrophenyl hydrazone (DNP)-derivatized carbonyl groups, an index of protein oxidation, as % Control. $n = 6-8$ * $p < 0.05$ versus all other groups.