Angiotensin II mediates glutathione depletion, transforming growth factor β₁ expression, and epithelial barrier dysfunction in the alcoholic rat lung

Rabih I. Bechara¹,²
Andres Pelaez¹,²
Andres Palacio¹,²
Pratibha C. Joshi¹,²
C. Michael Hart¹,²
Lou Ann S. Brown³
Robert Raynor¹,²
David M. Guidot¹,²

Atlanta Veterans Affairs Medical Center Pulmonary Section¹, Decatur, GA, USA, and the Division of Pulmonary, Allergy & Critical Care Medicine², and the Department of Pediatrics³, Emory University School of Medicine, Atlanta, Georgia, USA.

Address all correspondence to: David M. Guidot, MD
Atlanta VAMC (151-P)
1670 Clairmont Road
Decatur, GA  30033
(404) 321-6111 ext. 6935
FAX (404) 728-7750
dguidot@emory.edu

Running title: Angiotensin II and ethanol-mediated lung injury
ABSTRACT

Alcohol abuse markedly increases the risk of sepsis-mediated acute lung injury. In a rat model, ethanol ingestion alone (in the absence of any other stress) causes pulmonary glutathione depletion, increased expression of transforming growth factor beta$_1$ (TGF$\beta_1$), and alveolar epithelial barrier dysfunction, even though the lung appears grossly normal. However, during endotoxemia ethanol-fed rats release more activated TGF$\beta_1$ into the alveolar space where it can exacerbate epithelial barrier dysfunction and lung edema. Ethanol ingestion activates the renin-angiotensin system, and angiotensin II is capable of inducing oxidative stress and TGF$\beta_1$ expression. We determined that lisinopril, an angiotensin converting enzyme inhibitor that decreases angiotensin II formation, limited lung glutathione depletion, and treatment with either lisinopril or losartan, a selective angiotensin II type 1 (AT$_1$) receptor blocker, normalized TGF$\beta_1$ expression. The glutathione precursor, procysteine, also prevented TGF$\beta_1$ expression, suggesting that TGF$\beta_1$ may be induced indirectly by angiotensin II-mediated oxidative stress and glutathione depletion. Importantly, lisinopril treatment normalized barrier function in alveolar epithelial cell monolayers from ethanol-fed rats, and treatment with either lisinopril or losartan normalized alveolar epithelial barrier function in ethanol-fed rats in vivo, as reflected by lung liquid clearance of an intratracheal saline challenge, even during endotoxemia. In parallel, lisinopril treatment limited TGF$\beta_1$ protein release into the alveolar space during endotoxemia. Taken together, these results suggest that angiotensin II mediates oxidative stress and the consequent TGF$\beta_1$ expression and alveolar epithelial barrier dysfunction that characterize the alcoholic lung.

Key words: ARDS, epithelium, angiotensin converting enzyme (ACE), alcohol abuse
INTRODUCTION

Chronic alcohol abuse, in addition to its well-known toxicities in the liver, brain, and other organs, is now recognized as a co-morbid variable that independently increases the incidence and severity of acute lung injury. Specifically, alcoholics are 2-4 times more likely to develop the Acute Respiratory Distress Syndrome (ARDS) in response to sepsis, trauma, or other acute inflammatory insults (25;27), and when they develop ARDS they have a higher incidence of extra-pulmonary organ failure (27). ARDS is a common and devastating disease process estimated to afflict approximately 75,000-150,000 individuals per year in the United States alone (19;37). In the two epidemiological studies linking alcohol abuse and ARDS (25;27), ~50% of all individuals with ARDS were alcoholics, indicating that alcohol abuse causes tens of thousands of cases of ARDS per year. A cardinal feature of ARDS is alveolar epithelial cell dysfunction, including disruption of the alveolar epithelial barrier and flooding of the alveolar space with proteinaceous fluid rich in cytokines. Although incremental improvements in supportive care have improved survival in selected individuals (1), the overall mortality from ARDS remains unacceptably high at 40-60% (37), and there are no effective pharmacological therapies to complement our current supportive care. The recently identified association between alcohol abuse and ARDS has prompted laboratory investigations that have generated new insights into the pathophysiology of acute lung injury, particularly in this highly vulnerable subgroup.

To study the fundamental mechanisms underlying the epidemiological association between alcohol abuse and ARDS, we developed a rat model of ethanol-mediated susceptibility to acute lung injury and determined that chronic ethanol ingestion produces multiple defects in alveolar epithelial function. For example, alveolar epithelial type II cells isolated from ethanol-
fed rats formed more permeable monolayers (16), and had abnormal surfactant synthesis and secretion (17) in vitro. In parallel, chronic ethanol ingestion in rats impaired alveolar epithelial barrier function in vivo as reflected by decreased alveolar liquid clearance and increased protein flux (16), and increased sepsis-mediated acute lung injury in vivo (33). Another remarkable and previously unrecognized finding was that chronic ethanol ingestion produces significant oxidative stress, as reflected by markedly decreased glutathione levels within the alveolar epithelial lining fluid and in alveolar epithelial cells (5;6;16;17;22). The critical role of glutathione depletion in ethanol-induced lung dysfunction is supported by multiple studies in which glutathione supplementation of the ethanol diet restores alveolar epithelial function in vitro and in vivo (15-17) and decreases sepsis-mediated surfactant dysfunction and acute lung injury in vivo (33). The potential clinical relevance of these findings in our rat model is reflected by our observation that otherwise healthy human alcoholic subjects have comparably decreased levels of glutathione in their lung lavage fluid (26).

More recently, we determined that transforming growth factor beta1 (TGFβ1) is induced by chronic ethanol ingestion and could mediate alveolar epithelial barrier dysfunction in the alcoholic lung during endotoxemia (4). Specifically, chronic ethanol ingestion increased gene and protein expression for TGFβ1 in the rat lung (4). The majority of the TGFβ1 protein in the alcoholic lung tissue is in the latent form but is released as activated TGFβ1 into the alveolar airspace during endotoxemia, and the lavage fluid from these rats induces permeability in naive alveolar epithelial monolayer via TGFβ1-specific effects (4). Taken together these studies indicate that increased expression of TGFβ1 protein in the chronic alcoholic lung leads to increased release of activated TGFβ1 into the alveolar airspace during acute inflammation where it can further impair epithelial barrier function and promote alveolar protein leak. However,
chronic low-level activation of TGFβ₁ protein in the alcoholic lung could also contribute to the
chronic alveolar epithelial barrier dysfunction that we have identified in both the experimental
rat model (16) as well as in otherwise healthy alcoholic human subjects (8). Therefore,
glutathione depletion and excessive TGFβ₁ expression are important features of a chronic
alcoholic phenotype characterized by alveolar epithelial dysfunction, as well as an acute
phenotype that is highly susceptible to acute edematous injury during sepsis. Now the question
is, how does chronic ethanol ingestion create this susceptible phenotype?

Chronic ethanol ingestion increases plasma levels of angiotensin II in rodents (40) and in
humans (21;30), and it has been postulated that activation of the renin-angiotensin system may
explain the association between alcohol abuse and hypertension in humans (39). One potential
mechanism is direct conversion of angiotensinogen to angiotensin I by metabolites of ethanol
(32), thereby bypassing renin which is the usual regulatory step in the renin-angiotensin system.
Although clearly important for normal homeostatic functions such as salt and water balance and
vascular tone, angiotensin II is implicated in diverse pathophysiological conditions including
vascular and myocardial injury (10;31), in part due to specific activation of apoptosis pathways
(18). The relevance of these pathways to the alcoholic lung is suggested by reports that alveolar
epithelial type II cells have receptors for angiotensin II (11). Angiotensin II induces apoptosis in
human and rat type II cell apoptosis in vitro (34-36) and causes alveolar epithelial cell injury in
rabbits in vivo (13). Relevant to our recent study on ethanol-induced TGFβ₁ expression in the
alcoholic rat lung (4), angiotensin II induces TGFβ expression and lung collagen deposition in
the lungs of bleomycin-treated rats (23), and is a potent inducer of TGFβ₁ expression in other
tissues such as the kidney (14;20;28).
Taken together, these observations suggest that the renin-angiotensin system, through angiotensin II, could play a causative role in mediating the glutathione depletion and excessive TGFβ1 expression that we have identified as being potentially important in ethanol-induced susceptibility to acute lung injury. In this current study we examined the role of angiotensin II in ethanol-mediated glutathione depletion, TGFβ1 expression, and alveolar epithelial barrier function in a model of chronic ethanol ingestion in rats.
MATERIALS AND METHODS

Ethanol feeding. Male Sprague-Dawley rats (200-250 gm; Charles River Laboratory, Wilmington, MA) were fed the Lieber-DeCarli liquid diet (Research Diets, New Brunswick, NJ) containing either ethanol or an isocaloric substitution with Maltin-Dextrin (control diet) for 6 wks as we have published previously (5;6;15;33). Ethanol was added gradually, starting with ethanol as 18% of total calories for 1 wk, then 27% of total calories for 1 week, and then finally 36% of total calories (full-strength) for 4 wks. In some cases the glutathione precursor, procysteine (Sigma), was added to the ethanol diet at a concentration of 0.35% (33). In other cases, either the angiotensin converting enzyme inhibitor, lisinopril (AstraZeneca, Wilmington, DE) at a final concentration of 3 mg/L (7), or the angiotensin II type 1 receptor blocker, losartan (Merck Research Laboratories, Rahway, NJ) at a final concentration of 200 mg/L (42), was added to the ethanol diet. Although rats in all groups appeared healthy, ethanol-fed rats had modestly decreased body weights (~5-10%) compared to control-fed rats at the end of the 6 wk dietary protocol, regardless of whether or not they received lisinopril or losartan. However, body sizes, as reflected by length from nose to tail or width from paw to paw when outstretched, were not affected by the ethanol diet. In parallel, lung sizes, as reflected by baseline dry and wet weights, were not affected by the ethanol diet.

Determination of lung lavage glutathione concentrations. We used a variation of the High Performance Liquid Chromatography (HPLC) method presented by Martin and White (24), as we have published in this model previously (15;17) as well in alcoholic human subjects (8;26). GSH levels were normalized to the levels of secretory IgA in the lavage fluid as we have
reported previously (8). In our experience ethanol ingestion does not affect secretory IgA levels in the lung lavage fluid, and there is less variability with this correction than with the urea dilution method we used in our original study in the rat model (17).

**Induction of endotoxemia in vivo.** In selected experiments, rats were given endotoxin (*S. typhimurium* lipopolysaccharide, 2 mg/kg intraperitoneally) 2 hours prior to either determination of lung liquid clearance in vivo, or determination of TGFβ₁ and total protein concentrations in lung lavage fluid.

**Lung tissue preparation for determination of TGFβ₁.** In selected experiments, rats were anesthetized with pentobarbital (60 mg/kg intraperitoneally), and a tracheostomy cannula was placed and secured with a 2-0 ligature. The chest cavity was opened, and 100 units of heparin were injected into the right ventricle and allowed to circulate for one minute. A perfusion catheter was placed in the pulmonary outflow tract and, after transection of the left atrium, the lungs were perfused blood-free with saline, excised free from other tissues, and stored at -70°C for later analysis of TGFβ₁ protein levels by ELISA.

**ELISA for determination of TGFβ₁ protein levels.** For the tissue TGFβ₁ assays, frozen rat lung tissue (0.5 g) was combined with 2 ml of cold acid-ethanol (93% ethanol + 2% concentrated HCL), 85 μg/ml PMSF, and 5 μg/ml pepstatin A. This mixture was then homogenized 1-2 minutes with a polytron homogenizer. The samples were extracted overnight at 4°C by gentle rocking followed by centrifugation at 10,000 x g for 10 min. The pellets were then discarded, and the supernatants were dialyzed against 4 mM HCL using 3500 molecular weight cut-off
dialysis tubing. Samples were again centrifuged at 13,000 x g for 10 min, and the supernatants were stored at -70°C until assayed. This preparation by necessity activates any latent TGFβ1 in the tissue, because the commercial ELISA kit employed only detects the active form of TGFβ1; therefore the measured levels represent total TGFβ1 in the tissue (latent + active). Levels of TGFβ1 in the lung lavage fluid and the prepared lung tissue were determined with a commercial ELISA kit (R&D Systems, Minneapolis, MN). Absorbance was read at 450 nm quantitated against a standard curve. The amount of TGFβ1 in the lung lavage fluid and lung tissue was then expressed per mg of protein in each sample. For the lung lavage fluid TGFβ1 assays, the lavage supernatants were not acidified prior to performing the ELISA, as we had previously determined that TGFβ1 protein levels in the lavage fluid as determined by the commercial assay were not affected by acidification, indicating that all of the TGFβ1 protein released into the alveolar space was in the free or active form (4).

**Determination of lung liquid clearance in vivo.** As published previously (29), following induction of anesthesia a tracheostomy cannula was placed, a saline challenge (2cc) was given intratracheally, and the rats were mechanically ventilated with a Harvard rodent ventilator (tidal volume of 7 cc/kg at a rate of 60/min) for 30 min. The lungs were then removed *en bloc* and the right lung was isolated and its bronchus tied with a suture; the bronchus was then cut distal to the suture and the right lung weight was determined at baseline (wet weight) and then after desiccation overnight at 70°C (dry weight). The ratio of the wet weight to the dry weight was calculated and expressed (wet:dry) for each experimental determination and used as a marker of lung liquid clearance. Specifically, lung liquid clearance was inversely proportional to the
Determination of alveolar epithelial lining fluid protein. After the right lung was isolated and its bronchus ligated as described above, saline was instilled into the left lung (5 cc via the tracheostomy tube x 3). The recovered lavage fluid (12 ± 1 cc in all cases) was centrifuged at 1500 x g for 10 min, and the supernatant was stored at –80°C for subsequent determinations of total protein and TGFβ1 protein. Total protein levels in the lung lavage fluid were performed as we have published previously (29) using a bicinchoninic acid (BCA) assay.

Alveolar epithelial cell isolation and formation of monolayers in vitro. As reported previously (5;6;16;17), alveolar epithelial type II cells were isolated from control-fed, ethanol-fed rats, and ethanol-fed rats whose diets had been supplemented with either lisinopril or losartan. Cells were resuspended at a density of 1 x 10^6 cells per ml of Dulbecco’s Modified Eagle’s Medium containing 10% serum, and 3 x 10^6 cells (3 ml) were plated on a 35 mm diameter permeable micro porous membrane (Transwell, Corning; 1 μm pore) and cultured for a total of 8 days at 37°C in 90% air/10% CO2.

Determination of alveolar epithelial barrier function in vitro. The barrier function of the cell monolayers after 8 days in culture was determined as published previously (4) by adding ^3H-inulin and ^14C-sucrose (100,000 dpm) to the media covering the basolateral surfaces of the cultured cells. At multiple time intervals (15, 30, 60, or 120 min), the media covering the apical surfaces of the monolayers were removed and the radioactivity determined. Leak was defined as
the fraction of the initial radioactivity placed on the basolateral surface that appeared on the apical surface of the monolayer after 120 min (2 hrs).

**Statistical Analysis.** Values shown represent the mean ± the standard error of the mean. Values were compared by analysis of variance and corrected by Student-Newman-Keuls test for differences between groups. A p value of <0.05 was considered significant.
RESULTS

Effects of the ACE inhibitor, lisinopril, on ethanol-induced glutathione depletion within the alveolar microenvironment. We have shown that chronic ethanol ingestion induces oxidative stress within the alveolar compartment in rats and in humans, as reflected by decreased levels of the antioxidant, glutathione (GSH), in lung lavage fluid (17;26). To evaluate the potential role of the renin-angiotensin system in mediating this oxidative stress, we determined the levels of GSH in the alveolar epithelial lining fluid of control-fed rats, ethanol-fed rats, and ethanol-fed rats whose diets were supplemented with the ACE inhibitor, lisinopril. As shown in Figure 1, chronic ethanol ingestion markedly decreased (P<0.05) levels of GSH in the lung lavage fluid compared to control-fed rats, and to a relative degree comparable to our findings in previous studies in this rat model (17) and in otherwise healthy alcoholic human subjects (26). In contrast, ethanol-fed rats that also received lisinopril in their diets had increased (P<0.05) levels of GSH in the lung lavage fluid when compared to untreated, ethanol-fed rats (Figure 1). Although the levels of GSH were still slightly decreased (P<0.05) in lisinopril-treated, ethanol-fed rats when compared to control-fed rats (Figure 1), approximately 80% of the ethanol-induced GSH depletion was prevented by lisinopril treatment.

Effects of angiotensin II inhibition or glutathione supplementation on lung tissue expression of TGFβ1 protein in ethanol-fed rats. Previously we determined that chronic ethanol ingestion approximately doubles the expression of TGFβ1 protein in the lung (4). However, the mechanism(s) by which ethanol induces TGFβ1 expression was not addressed in that study. We hypothesized that TGFβ1 expression was a consequence of angiotensin II-
induced oxidative stress in the alcoholic lung. Therefore, in these experiments we tested the effects of angiotensin II inhibitors and independently, glutathione supplementation, on TGFβ₁ expression in the lungs of ethanol-fed rats. As shown in Figure 2, TGFβ₁ protein levels were increased (P<0.05) in the lungs of ethanol-fed rats as we have shown previously (4). In contrast, concomitant dietary treatment with the ACE inhibitor, lisinopril, or the angiotensin II receptor blocker, losartan, decreased TGFβ₁ protein levels in the lungs of ethanol-fed rats such that they were not different (P>0.05) than levels in control-fed rat lungs (Figure 2). In parallel, glutathione supplementation with procysteine in ethanol-fed rats also decreased lung TGFβ₁ expression, as reflected by TGFβ₁ protein levels that were also not different (P>0.05) than those in control-fed rats (Figure 2). Taken together, the results shown in Figures 1 and 2 suggest that the renin-angiotensin system mediates lung oxidative stress during chronic ethanol ingestion, which in turn induces TGFβ₁ expression (see Discussion).

**Effects of angiotensin II inhibition on alveolar epithelial barrier formation in vitro.** The ability of the lung to maintain a normal air-liquid interface within the alveolar space requires the dynamic maintenance of a tight epithelial barrier. Our previous studies have shown that chronic ethanol ingestion impairs the ability of the alveolar epithelial cells to establish tight monolayers when grown in culture, and that this permeability defect is also mediated by oxidative stress and glutathione depletion (16;29). Therefore, we predicted that lisinopril treatment would improve or even normalize alveolar epithelial barrier formation in ethanol-fed rats. To test this, we evaluated the permeability of alveolar epithelial monolayers derived from rats fed the control diet, the ethanol diet, or the ethanol diet supplemented with lisinopril. As we have shown previously (16), alveolar epithelial monolayers derived from ethanol-fed rats were more
permeable (P<0.05), as reflected by $^{14}$C-sucrose clearance in 2 hrs, than alveolar epithelial monolayers derived from control-fed rats (Figure 3). In contrast, alveolar epithelial monolayers derived from ethanol-fed rats that were treated with lisinopril had the same permeability (P>0.05) as monolayers derived from control-fed rats (Figure 3).

**Effects of angiotensin II inhibition on alveolar epithelial barrier function *in vivo.*** As angiotensin II appeared to mediate the oxidative stress and TGFβ1 expression that characterize the alcoholic lung, including the ability of alveolar epithelial cells from ethanol-fed rats to form tight monolayers *in vitro*, we next examined the effects of angiotensin II inhibition on alveolar epithelial function in ethanol-fed rats *in vivo*. First, we determined that the baseline wet:dry ratios in control-fed and ethanol-fed rats that were not challenged with saline were identical (4.7±0.7 vs. 4.7±0.7). However, consistent with our previously published findings (16;29) ethanol-fed rats had decreased (P<0.05) lung liquid clearance *in vivo* compared to control-fed rats, as reflected by increased lung tissue wet:dry ratios (Figure 4). In contrast, dietary treatment with either lisinopril or losartan improved lung liquid clearance in ethanol-fed rats *in vivo* to the same capacity (P>0.05) as control-fed rats (Figure 4).

**Effects of angiotensin II inhibition on alveolar epithelial barrier function during endotoxemia *in vivo.*** As angiotensin II inhibition with either lisinopril or losartan treatment normalized TGFβ1 expression and liquid clearance in the lungs of ethanol-fed rats, we predicted that it would improve lung liquid clearance and decrease protein leak in ethanol-fed rats during endotoxemia. We first determined that lung liquid clearance was even more impaired (P<0.05) in endotoxemic, ethanol-fed rats relative to lung liquid clearance in endotoxemic, control-fed rats
(Figure 5), whereas endotoxemia had no significant effect (P>0.05) on the ability of control-fed rats to clear a lung liquid challenge (wet:dry ratios of 6.8±0.4 for control-fed rats in Figure 5 vs. 6.7±0.2 for control-fed rats in Figure 4). Importantly, treatment with either lisinopril or losartan improved (P<0.05) lung liquid clearance in ethanol-fed rats even following endotoxemia, such that their liquid clearance was the same (P>0.05) as endotoxemic, control-fed rats (Figure 5).

Effects of angiotensin II inhibition on the release of TGFβ1 and total protein accumulation in the alveolar airspace during endotoxemia in vivo. Previously we determined that chronic ethanol ingestion induces TGFβ1 expression in lung tissue and that, during endotoxemia, markedly increases the release of activated TGFβ1 into the alveolar space where it promotes epithelial permeability (4). Therefore, as angiotensin II appears to mediate glutathione depletion (Figure 1) and consequent TGFβ1 expression (Figure 2), we predicted that lisinopril treatment would decrease the release of TGFβ1 into the airspace of ethanol-fed rats during endotoxemia. For these experiments, rats were made endotoxemic but were not subjected to a saline challenge. As shown in Figure 6, panel A, ethanol-fed rats released more than three times as much TGFβ1 protein into the alveolar space during endotoxemia as control-fed rats (P<0.05). In contrast, rats fed the ethanol diet supplemented with lisinopril released the same (P>0.05) amount of TGFβ1 protein into the alveolar space during endotoxemia as control-fed rats (Figure 6, panel A). As TGFβ1 protein levels in the lavage fluids were corrected for total protein levels, we needed to ensure that the observed differences shown in panel A were relatively specific for TGFβ1 and not simply a reflection of total protein accumulation in the airspace. Therefore, we also determined total protein concentrations in the lung lavage fluids of these endotoxemic rats. Lung lavage fluid recovery was not significantly different among the treatment groups (not shown), so protein
concentrations were expressed per volume of lavage fluid. As shown in Figure 6, panel B, total protein levels in the lavage fluids of endotoxemic, ethanol-fed rats were increased (P<0.05) compared to endotoxemic, control-fed rats. However, this increase in total protein was modest (~30%) and therefore could not account for the >300% increase in TGFβ1 protein levels shown in panel A. In parallel with its effects on TGFβ1 protein, lisinopril treatment decreased (P<0.05) total protein levels in the alveolar space in endotoxemic, ethanol-fed rats (Figure 6, panel B). Overall, the results in Figure 6 are consistent with the results in Figure 2, and suggest that angiotensin II not only induces the expression of TGFβ1 protein in the alcoholic lung, it ultimately leads to changes in the lung that promote the release of TGFβ1 protein into the alveolar airspace during an acute inflammatory stress such as endotoxemia.
DISCUSSION (paragraphs have been re-ordered in addition to text changes)

Our previous studies implicated glutathione depletion and increased TGFβ1 expression in ethanol-induced alveolar epithelial dysfunction, and this current study unifies these pathophysiological pathways by revealing a common mechanism by which ethanol ingestion produces oxidative stress and induces TGFβ1 expression within the lung. Specifically, we determined that dietary treatment with the angiotensin converting enzyme (ACE) inhibitor, lisinopril, which blocks ACE-dependent formation of angiotensin II, almost completely preserved glutathione levels within the alveolar space of ethanol-fed rats and prevented excess TGFβ1 expression in the lung tissue. In parallel, losartan, a specific angiotensin II type 1 receptor blocker, also normalized ethanol-induced TGFβ1 expression, further evidence that this induction is mediated by angiotensin II and not by some other product of ACE activity. In addition, dietary treatment with the glutathione precursor, procysteine, normalized TGFβ1 expression in the lung tissue, suggesting that angiotensin II-induced glutathione depletion is required for angiotensin II-mediated induction of TGFβ1. Further, ACE inhibition decreased the release of TGFβ1 into the alveolar space during endotoxemia, which could reflect either less TGFβ1 in the lung tissue or some other barrier-protective effect of ACE inhibition in the alcoholic lung. Finally and perhaps most importantly, treatment with either lisinopril or losartan preserved alveolar epithelial barrier function in the alcoholic lung both at baseline and following endotoxemia, as reflected by lung liquid clearance of an intratracheal saline challenge. Taken together, this study provides new evidence that angiotensin II mediates glutathione depletion, increased TGFβ1 expression, and alveolar epithelial barrier dysfunction in the alcoholic lung.

Our surrogate marker of alveolar epithelial barrier function in vivo, namely lung liquid clearance of an intratracheal saline challenge, is an integrated function that depends on active
sodium transport as well as a relatively impermeable epithelium that prevents back leak of the reabsorbed fluid. Our previous work suggests that the primary defect in the alcoholic lung is increased permeability of the epithelium, whereas active sodium transport may actually be increased as a compensatory response (16). Therefore, we have used lung liquid clearance as an index of alveolar epithelial barrier function with or without endotoxemia in vivo in a more recent study (29). This is a relatively simple and reproducible index of epithelial barrier function that correlates with our previous studies in which alveolar fluid clearance and protein flux in vivo were measured with radiolabeled albumin (16). We cannot exclude the possibility that ethanol ingestion also perturbed lung lymphatic drainage and/or cardiovascular function in response to the intratracheal saline challenge, and that angiotensin II inhibition somehow reversed such defects. To our knowledge these potential mechanisms have not been examined in comparable models. However, the evidence from this study and our previous work strongly argues that ethanol-mediated susceptibility to acute edematous injury involves alveolar epithelial dysfunction.

Alcohol abuse increases the risk of developing the acute respiratory distress syndrome (ARDS) more than 3-fold during septic shock (27). This epidemiological association, which was first identified less than a decade ago (25), established alcohol abuse as the first co-morbid factor identified (and to date, the only factor) that independently increases the risk of ARDS. To study the mechanisms underlying this association, we developed a rat model of ethanol-induced susceptibility to acute lung injury (17;33) and determined that within 4-6 weeks of chronic ethanol ingestion, the lung shows signs of significant oxidative stress as reflected by profound depletion of the antioxidant, glutathione, within the alveolar epithelium and associated lining fluid (5;17). In parallel, alveolar epithelial function, as reflected by barrier function and active
fluid transport, is impaired by chronic ethanol ingestion (16). Importantly, dietary supplementation with glutathione precursors prevents glutathione depletion as well as alveolar epithelial dysfunction in ethanol-fed rats (15-17). Recently, we determined that ethanol-induced glutathione depletion is associated with a two-fold increase in the expression of transforming growth factor $\beta_1$ (TGF$\beta_1$) in the lung tissue (4). However, it remains predominantly (but not completely) in a latent or inactive form until an acute stress, such as endotoxemia, releases and activates TGF$\beta_1$ within the alveolar space where it is capable of inducing an acute permeability defect in the alveolar epithelium (4). In this study, lisinopril prevented the exaggerated release of activated TGF$\beta_1$ that is associated with (and may contribute to) impaired alveolar epithelial barrier function in the alcoholic lung during endotoxemia. Taken together, these findings argue that ethanol-induced oxidative stress and TGF$\beta_1$ expression are mediated not by ethanol directly, but rather indirectly through the actions of angiotensin II. Further, when combined with our previous studies, our current findings suggest the following sequence:

**ethanol** $\rightarrow$ $\uparrow$ **angiotensin II** $\rightarrow$ **oxidative stress and $\downarrow$ glutathione** $\rightarrow$ $\uparrow$ **TGF$\beta_1$ expression**

[and that, during acute inflammatory stresses such as endotoxemia]

**sepsis** $\rightarrow$ $\uparrow$ **TGF$\beta_1$ activation and release** $\rightarrow$ $\uparrow$ **alveolar epithelial dysfunction**

If this scheme is correct, it still leaves open the question as to whether TGF$\beta_1$ contributes to the alveolar epithelial defects that characterize the chronic alcoholic lung in addition to mediating acute epithelial barrier disruption during sepsis. Although we did not detect TGF$\beta_1$ in the lung lavage fluid of ethanol-fed rats in the absence of endotoxemia (4), we cannot exclude the possibility that low-level but chronic activation of TGF$\beta_1$ in the adjoining matrix space
interferes with the normally tight alveolar epithelium. Alternatively, angiotensin II could perturb alveolar epithelial barrier function by a mechanism(s) that is independent of TGFβ1, and that the consequences of the excess TGFβ1 expression are only relevant in the context of sepsis or other acute inflammatory stresses that release and activate TGFβ1 to pathophysiological levels. Regardless, this scheme provides a framework to design future studies and is consistent with other evidence that the pathophysiological consequences of chronic ethanol ingestion could be mediated at least in part by the renin-angiotensin system. Angiotensin II is a pluripotent vasoactive peptide that is increased in the lungs of individuals with ARDS (38). It is formed by the sequential conversion of angiotensinogen to angiotensin I and then to angiotensin II, the latter conversion catalyzed primarily, though not exclusively, by the angiotensin converting enzyme (ACE). Chronic ethanol ingestion increases plasma levels of angiotensin II in rats (40), and it has been postulated that activation of the renin-angiotensin system may explain the association between alcohol abuse and hypertension in humans (39;40). Although a mechanism is not known, it has been shown that acetaldehyde, the primary metabolite of ethanol, can convert angiotensinogen to angiotensin I in rat plasma in vitro (32). The biological effects of angiotensin II depend on its interaction with specific angiotensin II receptors, and at least seven subtypes have been identified. Of the angiotensin II receptors, the type 1 receptor has been best characterized. The majority of the well-known effects of angiotensin II, such as vasoconstriction, sodium retention, and tissue hypertrophy and hyperplasia, are mediated via the type 1 receptor (2). Type 1 receptor blockers, including losartan (used in this experimental study), are now in widespread clinical use in the treatment of cardiovascular diseases (9). Cellular responses to angiotensin II are remarkably diverse and include activation of the NADPH oxidase complex and generation of reactive oxygen species (41). As noted earlier, angiotensin II
induces apoptosis in human and rat type II cell apoptosis in vitro (34-36) and causes alveolar epithelial cell injury in rabbits in vivo (13). It is important to emphasize that most studies, including ours, rely on the use of ACE inhibitors and/or angiotensin II receptor blockers to indirectly evaluate the pathophysiological effects of angiotensin II. This is in part due to the fact that it is relatively difficult to measure angiotensin II levels, particularly in relevant microenvironments such as the pulmonary interstitium. Further, the actions of angiotensin II depend not only on ambient levels, but also on the relative expression of its receptor subtypes in target tissues. For example, we determined previously that chronic ethanol ingestion shifts the expression of angiotensin II receptors from predominantly AT₁ to AT₂ on the membranes of alveolar epithelial type II cells, and that this is associated with a pro-apoptotic phenotype in these cells (3). Interestingly, there is evolving evidence that some of the beneficial effects of ACE inhibitors can be attributed to alterations in ACE signaling that are independent of angiotensin II formation per se (12). However, we used both an ACE inhibitor and, in the experiments in which lung tissue TGFβ₁ expression and lung liquid clearance were determined, an AT₁ receptor inhibitor as well. As the effects of lisinopril and losartan were comparable, these findings argue that ethanol-mediated lung dysfunction is yet another circumstance in which angiotensin II plays a pathophysiological role.

In summary, we report that ethanol-induced glutathione depletion and TGFβ₁ expression in the lungs of ethanol-fed rats are mediated by the renin-angiotensin system, most likely by the actions of angiotensin II. The functional consequences of this angiotensin II activity include decreased alveolar epithelial barrier function that is exacerbated during an acute inflammatory stress such as endotoxemia. Therefore, although the metabolic consequences of chronic ethanol ingestion are quite complex, ethanol-induced amplification of the renin-angiotensin system
appears to be the major cause of the alveolar epithelial oxidative stress and TGFβ1-mediated barrier disruption that characterize the alcoholic lung. These observations provide a previously unrecognized mechanism by which chronic ethanol ingestion renders the lung susceptible to acute edematous injury. The potential impact of these findings on our understanding of the pathophysiology of acute lung injury in the setting of alcohol abuse is enormous. Pharmacological blockade of the actions of angiotensin II in other conditions, such as congestive heart failure and glomerular diseases, has dramatically improved survival in many individuals. Clearly, additional studies are necessary before we can place alcohol-mediated susceptibility to acute lung injury on the list of serious human diseases for which inhibition of the renin-angiotensin system is a therapeutic target.
ACKNOWLEDGMENTS

The authors wish to thank Michael Wong and Frank Harris for their technical assistance with this manuscript.

GRANT SUPPORT

This work was supported by an Alcohol Center Grant from the National Institute on Alcohol Abuse and Alcoholism (P50 AA013757).
REFERENCES


FIGURE LEGENDS

Figure 1. The effect of ACE inhibition on lung glutathione levels in ethanol-fed rats.
Alveolar epithelial lining fluid levels of glutathione (GSH) as determined by HPLC and corrected for the levels of secretory IgA in the lungs of rats fed either a control liquid diet, an ethanol-containing liquid diet, or an ethanol-containing liquid diet supplemented with the angiotensin converting enzyme inhibitor lisinopril. Each value represents the mean ± SEM of 12 or more determinations. * P<0.05 decreased compared to control. ** P<0.05 increased compared to ethanol.

Figure 2. The effects of ACE inhibition, angiotensin II blockade, or glutathione supplementation, on TGFβ1 protein expression in the lungs of ethanol-fed rats.
Lung tissue expression of transforming growth factor β1 protein, as determined by ELISA and expressed per mg of tissue protein, in lungs from rats fed either a control liquid diet, an ethanol-containing liquid diet, or an ethanol-containing liquid diet supplemented with either the ACE inhibitor, lisinopril, the angiotensin II type 2 receptor blocker, losartan, or the glutathione precursor, procysteine. Each value represents the mean ± SEM of 6 or more determinations. * P<0.05 increased compared to control.

Figure 3. The effect of ACE inhibition in ethanol-fed rats on alveolar epithelial barrier function in vitro.
Alveolar epithelial permeability, as reflected by the percentage of 14C-sucrose and 3H-inulin leak in 2 hrs (determined independently), in alveolar epithelial monolayers derived from rats fed
either a control liquid diet, an ethanol-containing liquid diet, or an ethanol-containing liquid diet supplemented with the ACE inhibitor, lisinopril. Each value represents the mean ± SEM of the permeability in monolayers derived from five or more rats.

* P<0.05 increased compared to monolayers from control-fed rats.

**Figure 4. The effects of ACE inhibition or angiotensin II blockade on lung liquid clearance in vivo in ethanol-fed rats.** Lung liquid clearance, as reflected by lung tissue wet:dry ratios 30 minutes following intratracheal challenge with 2 cc of saline, in rats fed either a control liquid diet, an ethanol-containing liquid diet, or an ethanol-containing liquid diet supplemented with either the ACE inhibitor, lisinopril, or the angiotensin II type 2 receptor blocker, losartan.

As we have established previously (29), the baseline wet:dry ratios in unchallenged rat lungs in this model is ~4.5. Each value represents the mean ± SEM of six or more determinations.

* P<0.05 increased compared to control.

**Figure 5. The effects of ACE inhibition or angiotensin II blockade on lung liquid clearance during endotoxemia in vivo in ethanol-fed rats.** Lung liquid clearance, as reflected by lung tissue wet:dry ratios 30 minutes following intratracheal challenge with 2 cc of saline (as in Figure 4), after 2 hrs of endotoxemia (induced with *S. typhimurium* lipopolysaccharide (LPS), 2 mg/kg intraperitoneally), in rats fed either a control liquid diet, an ethanol-containing liquid diet, or an ethanol-containing liquid diet supplemented with either the ACE inhibitor, lisinopril, or the angiotensin II type 2 receptor blocker, losartan. Each value represents the mean ± SEM of six or more determinations.

* P<0.05 increased compared to control.
Figure 6. The effects of ACE inhibition on TGFβ₁ protein release and total protein accumulation in the alveolar space during endotoxemia in ethanol-fed rats.

Panel A. Lung lavage fluid levels of TGFβ₁ protein, as determined by ELISA and expressed per mg of total protein, after 2 hrs of endotoxemia (induced with *S. typhimurium* lipopolysaccharide (LPS), 2 mg/kg intraperitoneally), in rats fed either a control liquid diet, an ethanol-containing liquid diet, or an ethanol-containing liquid diet supplemented with the ACE inhibitor, lisinopril.

Panel B. Lung lavage fluid levels of total protein, expressed as micrograms per ml, in same experiments shown in panel A.

Each value represents the mean +/- SEM of 6 or more determinations.

* P<0.05 increased compared to control.
Figure 1

alveolar epithelial lining fluid GSH (nmol/µg IgA)

Diet control ethanol ethanol + lisinopril

Figure 1
Figure 2

Lung tissue TGFβ₁ (ng/mg protein)

Diet control ethanol

+ lisinopril + losartan + procysteine
Figure 3

Alveolar epithelial permeability *in vitro* (% flux in 2 hrs)

<table>
<thead>
<tr>
<th>Diet</th>
<th>control</th>
<th>ethanol</th>
<th>ethanol + lisinopril</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>25</td>
<td>15</td>
</tr>
</tbody>
</table>

* Indicates significant difference.

$^{3}$H-inulin

$^{14}$C-sucrose
lung edema post saline challenge (wet:dry ratio)

Diet

control
ethanol

Figure 4
Figure 5

Lung edema post saline challenge after 2 hrs of endotoxemia (wet:dry ratio)

Diet: control, ethanol, lizinpril, losartan
Lung lavage fluid levels of TGFβ₁ after 2 hrs of endotoxemia (ng/mg protein)

A

Lung lavage fluid total protein levels after 2 hrs of endotoxemia (µg/ml)

B

Diet control ethanol ethanol + lisinopril

Figure 6