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PPAR γ Ligands Regulate NADPH Oxidase, eNOS, and Barrier Function in the Lung Following Chronic Alcohol Ingestion

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Abstract

BACKGROUND—Chronic alcohol ingestion increases the incidence and severity of the Acute Respiratory Distress Syndrome (ARDS), where reactive species contribute to alveolar-capillary barrier dysfunction and non-cardiogenic pulmonary edema. Previous studies demonstrated that chronic alcohol ingestion increased lung NADPH oxidase and endothelial nitric oxide synthase (eNOS) expression and that ligands for the peroxisome proliferator-activated receptor gamma (PPAR γ) reduced NADPH oxidase expression. Therefore, we hypothesized that the PPAR γ ligand, rosiglitazone, would attenuate alcohol-induced NADPH oxidase expression and pulmonary barrier dysfunction.

METHODS—C57Bl/6 mice were treated $\pm$ alcohol in drinking water (20% w/v) for 12 weeks. During the final week of alcohol treatment, mice were gavaged with rosiglitazone (10 mg/kg/day) or vehicle. Selected animals were treated twice with lipopolysaccharide (LPS, 2 mg/kg IP) prior to sacrifice. Pulmonary barrier dysfunction was estimated from protein content of bronchoalveolar lavage (BAL) fluid.

RESULTS—LPS treatment increased BAL protein in alcohol-fed but not control mice, and rosiglitazone attenuated LPS and alcohol-induced pulmonary barrier dysfunction. Alcohol- and LPS-induced increases in lung eNOS, Nox1, and Nox4 expression were attenuated by rosiglitazone. *In vitro*, alcohol (0.10% w/v) increased H₂O₂ production, barrier dysfunction, eNOS, Nox1, and Nox4 expression in human umbilical vein endothelial cell (HUVEC) monolayers, effects also attenuated by rosiglitazone (10 μ M). Alcohol-induced HUVEC barrier dysfunction was attenuated by inhibition of NOS or addition of the eNOS cofactor, tetrahydrobiopterin.

CONCLUSIONS—These results indicate that PPAR γ activation reduced expression of eNOS, Nox1, Nox4, the production of reactive species, and barrier dysfunction caused by chronic alcohol ingestion and suggest that PPAR γ represents a novel therapeutic target for strategies designed to reduce the risk of lung injury in patients with a history of chronic alcohol ingestion.

Keywords

rosiglitazone; Acute Respiratory Distress Syndrome; lung injury

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Introduction

The Acute Respiratory Distress Syndrome (ARDS) is a severe form of acute lung injury characterized by noncardiogenic pulmonary edema following activation of proinflammatory cascades in response to diverse clinical conditions, such as infection, trauma, or aspiration (Ware and Matthay, 2000). A critical component of the pathophysiology of lung injury involves impaired alveolar-capillary barrier function (Ware and Matthay, 2000). This condition, which affects as many as 75,000–100,000 individuals each year in the United States alone, is associated with significant morbidity and mortality due to a lack of specific therapies that directly modulate ARDS pathogenesis (Ware and Matthay, 2000).

Factors that predispose patients to ARDS include chronic alcohol abuse, a comorbid variable known to increase the incidence of ARDS 2–4-fold in critically ill patients (Moss et al., 2003). While chronic alcohol ingestion likely increases ARDS incidence through multiple mechanisms, previous studies in a rat model of chronic alcohol ingestion (using the Lieber DeCarli diet) (Guidot et al., 2000, Moss et al., 2000, Polikandriotis et al., 2006) have indicated that oxidative stress plays a key role in susceptibility to lung injury. In experimental animals, chronic alcohol ingestion enhanced sepsis-induced barrier dysfunction in the lung, a derangement attenuated by treatment with glutathione precursors (Holguin et al., 1998). Chronic alcohol ingestion also altered the production of both reactive oxygen and nitrogen species in the lung through increased expression of NADPH oxidase and endothelial nitric oxide synthase (eNOS), respectively (Polikandriotis et al., 2007, Polikandriotis et al., 2006). These findings have led to the working hypothesis that chronic ethanol ingestion “primes” the lung by increasing the expression of enzymatic sources of reactive species. These alcohol-induced derangements while not sufficient to cause lung injury by themselves, contribute to susceptibility to injury caused by subsequent inflammatory stimuli. Based on this “two hit” model and evidence that oxidative stress promotes endothelial barrier dysfunction *in vitro* and *in vivo* (Lum and Roebuck, 2001), we have sought to identify new interventions that might reduce the priming effect of alcohol on the lung and / or the response of the alcohol-treated lung to inflammatory stimuli.

Treatment with peroxisome proliferator-activated receptor gamma (PPAR γ) ligands represents a novel strategy to regulate NADPH oxidase expression. PPAR γ is a ligand-activated transcription factor that is widely expressed and participates in the regulation of diverse biological processes, including adipocyte differentiation (Barak et al., 1999), lipid metabolism (Schoonjans et al., 1996), smooth muscle cell proliferation (Meredith et al., 2009), and insulin sensitivity (Hevener et al., 2003, Rangwala et al., 2003). Ligands for the PPAR γ receptor include naturally occurring fatty acids and their metabolites as well as synthetic thiazolidinedione (TZD) medications such as pioglitazone and rosiglitazone, which have been used to increase insulin sensitivity in patients with type 2 diabetes (Yki-Jarvinen, 2004). Rosiglitazone reduced vascular NADPH oxidase expression and activity in cultured endothelial cells (Hwang et al., 2005), in the vasculature of diabetic mice (Hwang et al., 2007), and in the lungs of hypoxia-exposed mice (Nisbet et al., 2010). Based on these studies, we hypothesized that PPAR γ ligands would reduce NADPH oxidase expression and oxidative stress in the lung following chronic alcohol ingestion, thereby reducing alcohol-induced susceptibility to barrier dysfunction. We explored this hypothesis by examining the ability of the PPAR γ ligand, rosiglitazone, to attenuate alcohol-induced oxidative stress and barrier dysfunction in endothelial cells *in vitro* and in the mouse lung *in vivo*.

Our results demonstrate that administration of alcohol in the drinking water of mice for 12 weeks increased LPS-induced barrier dysfunction and that treatment with rosiglitazone by gavage over the final week of alcohol administration attenuated barrier dysfunction as well as alcohol-induced increases in the expression of eNOS, Nox1 and Nox4 in the lung. LPS-

induced increases in Nox1, Nox2, Nox4 and eNOS mRNA were also attenuated by rosiglitazone. Similarly, alcohol caused endothelial monolayer barrier dysfunction, H₂O₂ production, and increased eNOS, Nox1, and Nox4 expression *in vitro* which were attenuated by rosiglitazone treatment. Collectively, these results indicate that the PPAR γ receptor may represent a new therapeutic target in strategies designed to reduce oxidative stress and barrier dysfunction in the lung caused by chronic alcohol ingestion.

Materials and Methods

Mouse Model of Chronic Alcohol Ingestion

Male, C57Bl/6 mice aged 8 weeks were given 20% ethanol (EtOH) in their drinking water along with *ad lib* access to standard mouse chow. Mice were acclimated to EtOH by increasing the EtOH concentration in 5% increments from 0% to the target 20% (w/v) over the course of two weeks then maintaining the 20% concentration for an additional ten weeks. This regimen replicates blood alcohol levels following chronic EtOH ingestion in human subjects (Jerrells et al., 2007, Song et al., 2002). In preliminary studies, blood alcohol concentrations were measured with a rapid, high-performance plasma alcohol analyzer (Analox Instruments Ltd., London, UK) according to the manufacturer's protocol. These analyses confirmed that this ethanol regimen produced clinically relevant elevations in blood alcohol concentration ($0.12\% \pm 0.03$, n=24). During the final week of EtOH treatment, all mice were gavaged daily for 7 days with either rosiglitazone (10 mg/kg/day in 100 μ l methylcellulose vehicle) or vehicle alone as previously reported (Hwang et al., 2007, Nisbet et al., 2010). Selected mice were treated with Escherichia coli lipopolysaccharide (Sigma-Aldrich, St. Louis MO, 2 mg/kg IP at 6 and 3 hours prior to sacrifice) as an inflammatory stimulus to promote pulmonary dysfunction. The timing of these studies was based on recent reports showing significant LPS-mediated increases in lung leak in C57Bl/6 mice 6-hours after LPS administration (Rojas et al., 2005). After sacrifice, blood was collected via cardiac puncture, and bronchoalveolar lavage (BAL) performed via tracheotomy. Protein concentration in the BAL fluid was measured using the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford IL), and values were corrected for dilution based on the ratio of blood to BAL urea nitrogen, assayed with a commercially available kit (Pointe Scientific, Inc., Canton MI) as previously reported (Rennard et al., 1986). Lung tissue was collected for subsequent analyses as described below following perfusion of the pulmonary artery with sterile phosphate buffered saline (PBS, Cellgro, Manassas, VA). All animal studies were approved by the Atlanta Veteran's Affairs Medical Center Animal Care and Use Committee.

Human Umbilical Vein Endothelial Cell (HUVEC) Cultures

HUVEC (Clonetics) monolayers were grown at 37°C in endothelial growth medium (Clonetics) containing 10% fetal bovine serum, 10 ng/mL human epidermal growth factor, 1.0 μ g/mL hydrocortisone, 12 μ g/mL bovine brain extract, 50 μ g/mL gentamicin, and 50 ng/mL amphotericin B in a 5% CO₂ atmosphere. Confluent cells were maintained in medium with 2% fetal bovine serum. In some experiments, cells (passage 4–8) were plated on 0.2% gelatin-coated 100 mm or 35 mm plastic tissue culture dishes and treated $\pm$ ethanol (0.10% w/v) and $\pm$ rosiglitazone (10 μ M) for 72 hours as we have previously reported (Hwang et al., 2005, Polikandriotis et al., 2005a, Polikandriotis et al., 2005b). All cell cultures treated with ethanol were maintained in a sealed incubator (Billups-Rothenburg, Del Mar, CA) with a reservoir containing 20 ml standard medium with 0.10% ethanol in order to minimize ethanol evaporation. HUVEC media were replaced every 24 hours. In selected studies, to assess HUVEC monolayer barrier function, cells were plated on 0.2% gelatin-coated 12 mm Transwell Permeable Supports (Costar, Wilkes Barre, PA) with 0.4 μ m polycarbonate membranes and treated with or without EtOH for 72 hours. Selected HUVEC monolayers in transwell supports were treated with rosiglitazone (10 μ M), tetrahydrobiopterin (20 μ M), or

L-N^G-Nitroarginine methyl ester hydrochloride (LNAME, 100 μ M). After 72 hours, all transwells were emptied, 750 μ l Evan's blue dye-associated albumin (0.04 g/ml in standard medium) was added to the upper, luminal chamber, and 1.2 ml standard medium was added to the lower, abluminal chamber. Transmonolayer protein passage was determined at 0.5, 1.5, and 3 hours by withdrawing 100 μ l samples from the stirred abluminal chamber and measuring the spectrophotometric absorbance (595 nm) of Evan's blue dye as we have reported (Knepler et al., 2001). Following sample collection, corresponding quantities of fresh media were added to the abluminal chamber to minimize the development of hydrostatic pressure gradients across the monolayer.

Western Analysis of Lung or HUVEC Homogenates

Lung tissue was homogenized in lysis buffer [20 mM Tris pH 7.4, 2.5 mM EDTA, 100 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate, 1 tablet/10 ml EDTA-free complete protease inhibitor cocktail (Roche, Indianapolis IN), 1 mM β -glycerolphosphate, 2.5 mM Na pyrophosphate] and frozen in liquid nitrogen. The lysate was spun at 28,000 $\times$ g for 15 minutes, the supernatants transferred to new tubes, and protein concentrations were determined using a BCA assay. Equal amounts of sample protein (15–20 μ g/lane) were loaded into wells of a 4–12% bis-tris polyacrylamide gel electrophoresis (PAGE) mini gel. HUVEC were collected in lysis buffer, and lysates were centrifuged at 16,000 $\times$ g for 20 minutes. Supernatants were transferred to new tubes, and protein concentrations were detected using a BCA assay. Sample proteins (40 μ g/lane) were loaded into the wells of 4–12% bis-tris PAGE mini gels. Proteins were separated by electrophoresis and blotted to polyvinylidene fluoride (PVDF) or nitrocellulose membranes. Membranes were incubated with polyclonal antibodies to eNOS (1:1000 dilution; BD Transduction Laboratories), Nox1 (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), Nox2 (1:500 dilution; Santa Cruz Biotechnology), Nox4 (1:2500 dilution; Dr. David Lambeth, Emory University), CDK4 (1:1000 dilution; Santa Cruz Biotechnology), or GAPDH (1:500 dilution; Santa Cruz Biotechnology) in non-fat dry milk (5%) and tris-buffered saline for 8–24 hours at 4°C. Proteins were visualized using a peroxidase-coupled anti-rabbit, anti-goat or anti-mouse IgG in the presence of LumiGlo reagent while exposing in a Biorad Chemidoc XRS/HQ (Hercules, CA). Densitometric analysis was accomplished using Biorad Quantity One (Version 4.5.0) software. The densitometric intensity of each sample was normalized to the density of CDK4 or GAPDH in that sample.

Real Time PCR Analysis of Lung or HUVEC Homogenates

Real-time PCR was performed to quantify mRNA levels of eNOS, Nox1, Nox2 (gp91^{phox}), and Nox4. RNA was extracted from whole lung homogenates or cultured HUVEC monolayers with Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's protocol. mRNA was reverse transcribed to cDNA with the I-Script cDNA synthesis kit (Bio Rad), employing Sybr green for detection. Primers for Nox1 (sequence: F 5'-GTTTTAC CGCTCCAGCAGAA-3', R 5'-GGATGCCATTCCAGGAGA GAG-3'), Nox2 (sequence: F 5'-CCACATACAGGCCCCCTTCAG-3', R 5'-GTTGGGGC TGAATGTCTTCTCTTT-3'), Nox4 (sequence: F 5'-CTGGTCTGACGGGTGTCTGCAT GGTG-3', R 5'-CTCCGCA CAATAAAGGCACAAAGGTCCAG-3'), and eNOS (sequence: F 5'- CCCACACAGAAG GTCTACA-3', R 5'- ACACGGCTGGAGGAAGT G-3') were employed on cDNA derived from lung homogenates or HUVEC monolayers and quantified to starting copy counts through standard curves. Results were normalized to S9 expression (sequence: F 5'-ATCCGCCAACGTCACAT TA-3', R 5'-TCTTCACTCGGCCTGGAC-3') in each sample and confirmed by generation of a standard curve with stock mRNA for each type.

Amplex Red Assay of HUVEC H₂O₂ Production

Detection of HUVEC H₂O₂ production was performed with the Amplex Red assay (Invitrogen, Eugene, OR). Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H₂O₂ in the presence of horseradish peroxidase to generate a highly fluorescent product, resorufin. Confluent HUVEC cultures in 35 mm Petri dishes were washed twice with sterile PBS and incubated for 30 minutes with 1000 µl of Amplex Red (20 µM) and horseradish peroxidase (0.1 U/ml) at 37°C in the dark in a 5% CO₂ incubator. Fluorescence was then measured in triplicate in a fluorescence plate reader (excitation λ 540, emission λ 590). Sample H₂O₂ concentrations were calculated based on standard curves generated with known H₂O₂ concentrations. Cell cultures were then rinsed twice with PBS, scraped into 150 µl of lysis buffer and sonicated. The resulting solution was centrifuged at 16,000 × g, and the supernatant protein concentrations were determined using a BCA assay. H₂O₂ concentrations were normalized to these protein concentrations and expressed relative to average control values.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analyses were accomplished by performing ANOVA (with repeated measures where appropriate) followed by a Tukey posthoc test to detect differences between individual experimental groups. p<0.05 was considered significant. ANOVA and posthoc tests were conducted using GraphPad Prism (ver. 5, La Jolla, CA).

Results

Chronic EtOH Ingestion Increased Pulmonary Barrier Dysfunction Caused by LPS: Attenuation by Rosiglitazone

As illustrated in Figure 1, compared to BAL protein levels of control mice, chronic EtOH ingestion alone caused a small but not statistically significant increase in pulmonary barrier dysfunction. Similarly, treatment with LPS had a small but not statistically significant effect on BAL protein in control mice. However, treatment with LPS significantly increased BAL protein in EtOH-treated mice. Treatment with rosiglitazone had no significant impact on lung barrier function in control animals regardless of LPS treatment. However, rosiglitazone treatment significantly attenuated BAL protein leak in mice that were treated with LPS following chronic EtOH ingestion.

Rosiglitazone Attenuated EtOH-Induced Barrier Dysfunction in Endothelial Monolayers in Vitro

Having shown that chronic EtOH ingestion enhances LPS-induced barrier dysfunction in the mouse lung and that this barrier dysfunction was attenuated by administration of the PPAR γ ligand, rosiglitazone, we examined the ability of rosiglitazone to directly modulate EtOH effects on endothelial barrier function. As shown in Figure 2, EtOH significantly increased the transmonolayer passage of albumin through cultured HUVEC monolayers consistent with impaired endothelial barrier function. Treatment with rosiglitazone had no effect on barrier function of control monolayers but significantly attenuated the observed barrier dysfunction in endothelial monolayers treated with EtOH. Furthermore, treatment with either the non-specific NOS inhibitor, LNAME, or the NOS cofactor, tetrahydrobiopterin, attenuated EtOH-induced barrier dysfunction in HUVEC monolayers.

Rosiglitazone Attenuated EtOH-Induced H₂O₂ Production in Vitro

Because oxidative stress represents a critical mediator of alcohol effects on the lung (Holguin et al., 1998, Moss et al., 2000), we examined the impact of rosiglitazone treatment

on EtOH-induced H₂O₂ production in HUVECs. H₂O₂ production was measured with the Amplex Red assay in HUVEC monolayers treated ± EtOH (0.10% w/v) and ± rosiglitazone (10 μM). As shown in Figure 3, EtOH significantly increased H₂O₂ production, and this increase was attenuated by treatment with rosiglitazone.

Rosiglitazone Attenuated EtOH-induced eNOS Expression in the Lung and in HUVEC monolayers

To further examine the mechanisms by which rosiglitazone treatment attenuates barrier dysfunction and oxidative stress, western blots of lung tissue from mice ± EtOH for 12 weeks, treated with or without rosiglitazone, were examined for changes in the expression of eNOS. As illustrated in Figure 4A, EtOH significantly increased eNOS protein expression in lung tissue, and treatment with rosiglitazone attenuated eNOS expression in mice following chronic EtOH ingestion. Similarly, EtOH stimulation increased eNOS mRNA levels in HUVEC monolayers, and these increases were attenuated by rosiglitazone treatment (Figure 4B).

Rosiglitazone Attenuated EtOH-induced NADPH Oxidase Expression in the Lung and in HUVEC Monolayers

Because we have also reported that chronic EtOH ingestion increased the expression and activity of NADPH oxidases in the rat lung (Polikandriotis et al., 2006), we examined the expression of Nox2 and its homologs, Nox1 and Nox4, in the mouse lung following chronic EtOH ingestion. As shown in Figure 5 (A,B), chronic EtOH ingestion significantly increased the expression of Nox1 and Nox4 protein in the mouse lung. Rosiglitazone attenuated Nox1 and Nox4 expression in EtOH-treated lung but had no significant effect on the expression of NADPH oxidase protein in lungs from control animals. Similar results were found in cultured HUVEC, where EtOH-induced Nox1 and Nox4 protein levels were attenuated by treatment with rosiglitazone (Figure 5, C, D). Nox2 expression was below the detection threshold for all treatments in both lung tissue and cell culture (data not shown). As shown in Figure 6, RT-PCR analysis of lung or HUVEC mRNA levels demonstrated that EtOH increased levels of Nox1 and Nox4 mRNA and that rosiglitazone treatment attenuated these increases both *in vivo* (A,E) and *in vitro* (B, F). Rosiglitazone treatment alone had no significant effect on mRNA expression levels under control conditions. In contrast to the protein results, Nox2 mRNA levels were detectable in lung and HUVEC homogenates (C,D), and although EtOH treatment failed to significantly increase Nox2 mRNA levels, rosiglitazone reduced levels in mouse lung and HUVECs following EtOH treatment.

Rosiglitazone Attenuated LPS-Induced Nox1, Nox2, Nox4, and eNOS mRNA Levels in the Lung

In addition to modulating the responses of the lung to EtOH, we also examined if rosiglitazone directly modulates responses to LPS. As illustrated in Figure 7, treatment with LPS increased the mRNA levels of Nox1, Nox2, Nox4, and eNOS in the mouse lung. Treatment with rosiglitazone had no effect on the mRNA levels of these targets in control animals but significantly attenuated LPS-induced increases in the levels of Nox1, Nox2, Nox4, and eNOS mRNA in the mouse lung.

Discussion

Previous studies demonstrated that chronic alcohol ingestion in rats increased oxidative stress in the lung (Guidot et al., 2000, Polikandriotis et al., 2006) and enhanced susceptibility to endotoxin-mediated lung injury (Holguin et al., 1998). Furthermore, glutathione precursors attenuated EtOH-induced oxidative stress and reduced the effects of EtOH on the lung *in vivo* (Guidot et al., 2000) and on lung cells *in vitro* (Brown et al.,

2007). While the mechanisms of alcohol-induced oxidative stress in the lung remain incompletely defined, chronic EtOH ingestion increased the expression of NADPH oxidase (Polikandriotis et al., 2006) and eNOS (Polikandriotis et al., 2007) in the rat lung. These enzymes produce reactive oxygen and nitrogen species that can contribute to oxidative stress and barrier dysfunction. Because PPAR γ ligands attenuated the expression of NADPH oxidases *in vitro* (Hwang et al., 2005) and *in vivo* (Hwang et al., 2007, Nisbet et al., 2010), the primary objectives of the current study were to determine if treatment with the PPAR γ ligand, rosiglitazone, could attenuate EtOH-induced alterations in the expression of eNOS and NADPH oxidases in the lung.

We employed a mouse model where EtOH was added to the animals' water for 12 weeks. This regimen of EtOH ingestion produced clinically relevant blood alcohol concentrations in mice that were comparable to alcohol concentrations in rats fed the liquid Lieber-DeCarli diet for six weeks (unpublished observations). The current findings indicate that EtOH caused barrier dysfunction *in vitro* in endothelial monolayers, but had no effect on lung barrier function by itself, consistent with clinical observations that chronic alcohol ingestion alone does not cause lung injury. However, the EtOH regimen employed in the current study enhanced lung barrier dysfunction in response to LPS, as reflected by increased protein concentrations in BAL fluid, consistent with previous reports examining rat (Guidot et al., 2000, Holguin et al., 1998) or human (Martin et al., 2005) subjects. These findings are consistent with clinical observations where patients with a history of chronic alcohol abuse demonstrate enhanced susceptibility to lung injury (Moss et al., 2003, Moss et al., 1996). We speculate that while chronic alcohol ingestion alone may "prime" the lung and cause subclinical alterations in lung cell or pulmonary function, it is only following an inflammatory stimulus or "second hit" that susceptibility to acute lung injury becomes manifest.

Previous reports have emphasized that oxidative stress in the lung plays an important role in alcohol-induced susceptibility to lung injury (Guidot and Hart, 2005). While alcohol as well as its metabolites may contribute to derangements in cell and tissue function, our findings indicate that EtOH can directly perturb endothelial barrier function and increase the expression of eNOS and NADPH oxidases in human endothelial cells *in vitro* as well as their production of reactive oxygen species. Comparable EtOH-induced increases in Nox1, Nox4, and eNOS in the mouse lung *in vivo* provide additional evidence that alcohol-induced changes in eNOS and NADPH oxidase expression in the lung may contribute to oxidative stress and susceptibility to lung injury as previously reported in rat models of chronic alcohol ingestion.

NADPH oxidases are membrane-bound enzymes that produce superoxide and hydrogen peroxide (Bedard and Krause, 2007). eNOS produces nitric oxide which, in the presence of superoxide, reacts to produce the oxidant, peroxynitrite (ONOO $^-$) which we have previously reported to cause endothelial barrier dysfunction (Knepler et al., 2001). Hydrogen peroxide can increase the expression and activity of eNOS in endothelial cells (Cai et al., 2003, Cai et al., 2002, Drummond et al., 2000, Thomas et al., 2002), and over expression of eNOS (Bendall et al., 2005, Ozaki et al., 2002) can lead to uncoupling of the enzyme and shift production from NO to superoxide due to insufficient availability of the eNOS cofactor, tetrahydrobiopterin (Bendall et al., 2005). Our *in vitro* studies demonstrated that EtOH-induced endothelial barrier dysfunction was prevented by the NOS inhibitor, LNAME, suggesting that increases in NOS activity play an important role in EtOH-induced endothelial barrier dysfunction. Further, attenuation of EtOH-induced endothelial barrier dysfunction by treatment with tetrahydrobiopterin suggests that eNOS may be uncoupled in this system and generating superoxide or peroxynitrite rather than nitric oxide. Taken together, these findings illustrate that EtOH-induced upregulation of NADPH oxidases and

eNOS can generate reactive species through multiple pathways that contribute to oxidative stress in the lung.

The current report extends our previous findings by demonstrating that the PPAR γ ligand, rosiglitazone, attenuated alcohol-induced barrier dysfunction *in vitro* and LPS-induced lung barrier dysfunction *in vivo*, as well as H₂O₂ production *in vitro* and expression of eNOS, Nox1 and Nox4 *in vitro* and *in vivo*. Although alcohol failed to significantly increase Nox2 expression in the mouse lung, in contrast to our previous observations in the rat lung (Polikandriotis et al., 2006), rosiglitazone treatment significantly reduced Nox2 mRNA levels in the lungs of EtOH-fed mice and in HUVEC monolayers cultured in the presence of alcohol. Collectively, these results provide correlative evidence for the hypothesis that alcohol-induced increases in eNOS and NADPH oxidases play an important role in pulmonary oxidative stress and susceptibility to lung injury.

Previous studies have shown that chronic EtOH ingestion activated the reninangiotensin system (Linkola et al., 1979, Puddey et al., 1987, Wright et al., 1986). Angiotensin II (Ang II), a major effector in this pathway, is increased in patients with ARDS (Wenz et al., 1997) and potentially stimulates NADPH oxidase expression and superoxide production *in vivo* (Griendling et al., 1994, Zafari et al., 1998). We reported that inhibiting the production of Ang II with the angiotensin converting enzyme (ACE) inhibitor, lisinopril, prevented EtOH-induced increases in NADPH oxidase (Polikandriotis et al., 2006) and eNOS expression (Polikandriotis et al., 2007) as well as oxidative stress in the rat lung. However, these potential therapeutic effects of ACE inhibition required that the ACE inhibitor be present for the duration of alcohol ingestion (unpublished observations). Thus we sought to identify interventions that could be introduced following the onset of chronic alcohol ingestion, in a therapeutically relevant paradigm, but retain the ability to attenuate the deleterious effects of alcohol on the lung. The current study examined rosiglitazone because PPAR γ ligands attenuated the expression of NADPH oxidases (Hwang et al., 2005, Hwang et al., 2007, Nisbet et al., 2010) and increased NO production (Bagi et al., 2004, Calnek et al., 2003, Hwang et al., 2005, Polikandriotis et al., 2005a) *in vitro* and *in vivo*. Synthetic thiazolidinedione PPAR γ ligands, including rosiglitazone and pioglitazone, enhance insulin sensitivity and have been employed in the treatment of type 2 diabetes (Yki-Jarvinen, 2004). Although the long-term use of rosiglitazone in diabetic patients has been associated with adverse outcomes (Home et al., 2007, Nissen and Wolski, 2007, Rosen, 2007), it is not clear if similar adverse effects would be observed in short-term applications of this drug in non-diabetic subjects. Further, pioglitazone was not associated with adverse cardiovascular outcomes in diabetic subjects (Dormandy et al., 2005, Erdmann et al., 2007, Lincoff et al., 2007, Wilcox et al., 2007). Taken together, these considerations along with the current findings suggest that targeting PPAR γ with existing pharmacological tools represents an intriguing strategy for reducing adverse effects of alcohol on the lung.

In addition to their effects on alcohol-induced alterations in gene expression in the lung, our results indicate that PPAR γ ligands also attenuated LPS-induced increases in lung NADPH oxidase and eNOS expression. These findings are consistent with previous reports that PPAR γ ligands attenuate inflammatory gene expression in vascular cells and macrophages (Asada et al., 2004, Pascual et al., 2005, Zingarelli et al., 2003). Within the context of the “two hit” model, these findings suggest that PPAR γ ligands can attenuate not only alcohol-induced priming and alterations in gene expression in the lung (the “first hit”) but also responses to the subsequent inflammatory stimulus (the “second hit”). These considerations further support the potential relevance of pursuing PPAR γ as a therapeutic target in strategies to reduce the deleterious effects of alcohol on the lung.

To our knowledge, while this is the first study to examine PPAR γ ligands as a strategy to reduce the effects of chronic alcohol ingestion on the lung, the study has several important limitations. First and foremost, these studies only establish that alterations in eNOS and NADPH oxidase expression caused by alcohol are associated with pulmonary barrier dysfunction. These studies were not designed to demonstrate a causative role for these enzymes in lung barrier dysfunction. However, by demonstrating that chronic alcohol ingestion in mice induces derangements in the lung that are similar to those previously reported in a rat model, the current findings indicate that these alterations are not an artifact of a single experimental alcohol ingestion paradigm or a manifestation of chronic alcohol ingestion limited to a single species. As a result, this model should prove useful for future studies employing knockout and transgenic models examining mechanistic pathways. In addition, although the dosage of rosiglitazone employed in the current study on a mg/kg basis is comparable to that employed in many previous animal studies, it exceeds dosing in human patients. While the higher metabolic rate of rodents may reduce this discrepancy, the lack of pharmacokinetic studies of thiazolidinediones in rodents prevents precise dose modeling in mice. In conclusion, despite these limitations, the current study provides evidence that short term treatment with PPAR γ ligands can attenuate alcohol-induced alterations in the lung in a mouse model and thus might represent a novel and effective strategy for reducing the risk of ARDS in alcoholic patients at risk for lung injury.

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Figure 1. Rosiglitazone Attenuated LPS-Mediated Increases in BAL Protein in EtOH-fed Mice
 Animals were provided control (Con) or ethanol-containing (EtOH, 20% w/v) water for 12 weeks. During the final week of this 12-week regimen, mice were treated with either rosiglitazone (Rosi, 10 mg/kg) or vehicle by gavage daily for 7 days. Selected animals in each group were then treated with two IP injections of E.coli lipopolysaccharide (LPS, 2 mg/kg) at 6 and 3 hours prior to sacrifice. After sacrifice, BAL was performed via tracheostomy in each animal and subjected to protein assays. Each bar represents the mean $\pm$ SEM BAL protein concentration, corrected for dilution, from 6–9 animals in each group. * $p \leq 0.05$ compared to Con + LPS; + $p \leq 0.001$ compared to EtOH + LPS.

Figure 2. Rosiglitazone Attenuated EtOH-Induced Endothelial Barrier Dysfunction *in Vitro*
 HUVEC monolayers were grown to confluence in transwell dishes then incubated in control media (Con) or media with 0.10% w/v ethanol (EtOH) for 72 hours. Selected monolayers were treated with rosiglitazone (Rosi, 10 μ M), LNAME (100 μ M), or tetrahydrobiopterin (20 μ M) as indicated. The transmonolayer clearance of albumin was then measured for 3 hours as described. Each point represents the mean absorbance value for the albumin/Evan's blue dye complex $\pm$ SEM from 19–20 monolayers (A), 9 monolayers (B), or 6 monolayers (C). ** $p < 0.001$ compared to Control; * $p < 0.05$ compared to Control; + $p < 0.05$ compared to EtOH.

Figure 3. Rosiglitazone Attenuated EtOH-Induced H₂O₂ Production in HUVEC Monolayers HUVEC monolayers were incubated in control media (Con) or media with 0.10% w/v ethanol (EtOH) for 72 hours. Selected monolayers were also treated with rosiglitazone (Rosi, 10 μ M) for 72 hours as indicated. Each bar represents the mean $\pm$ SEM H₂O₂ concentration normalized to cell protein from 6 experiments and expressed as % control value. * $p \leq 0.001$ compared to Con; + $p < 0.05$ and ++ $p < 0.01$ compared to EtOH.

Figure 4. Rosiglitazone Attenuated EtOH-induced Increases in Lung eNOS Protein Levels in Mice and mRNA eNOS Levels in HUVEC Culture

A) Selected mice were treated with ethanol (EtOH, 20% w/v) in drinking water for 12 weeks, with or without rosiglitazone (Rosi) by gavage (10 mg/kg) during the 12th week of treatment. Each bar represents the mean densitometric intensity of eNOS protein in the lung relative to CDK4 levels in the same sample $\pm$ SEM expressed as % Control. Blots are representative from 6–9 mice. * $p \leq 0.001$ compared to Con; $^+p \leq 0.01$ compared to EtOH. B) HUVEC monolayers were grown to confluency on 100 mm plastic tissue culture dishes and treated with ethanol (0.10% w/v) for 72 hours $\pm$ rosiglitazone (10 μ M). Monolayers were homogenized, and mRNA was extracted and analyzed via RT-PCR. Results are normalized to 9s levels and expressed as % control for $n=3$. * $p \leq 0.05$ compared to Con; $^+p \leq 0.05$ compared to EtOH.

Figure 5. Rosiglitazone Attenuated EtOH-induced Increases in Nox1 and 4 Protein Levels in Mouse Lung and HUVEC Cultures

Selected mice were treated with ethanol (EtOH, 20% w/v) in drinking water for 12 weeks, with or without rosiglitazone (Rosi) by gavage (10 mg/kg) during the 12th week of treatment. HUVEC monolayers were incubated in control media (Con) or media with 0.10% w/v ethanol (EtOH) for 72 hours. Where indicated, selected monolayers were treated with rosiglitazone (Rosi, 10 μ M). Lung tissue (A,B) or harvested HUVEC cultures (C,D) were homogenized, and protein was extracted and analyzed via western blotting. Each bar represents the mean densitometric intensity of Nox1 or Nox4 relative to GAPDH levels in the same sample $\pm$ SEM expressed as % Control. Blots are representative from n=3–4. * $p \leq 0.05$ compared to Con; + $p \leq 0.05$ compared to EtOH.

Figure 6. Rosiglitazone Attenuated EtOH-induced Increases in Nox1 and 4 mRNA Levels in Mouse Lung and HUVEC Cultures

Selected mice were treated with ethanol (EtOH, 20% w/v) in drinking water for 12 weeks, with or without rosiglitazone (Rosi) by gavage (10 mg/kg) during the 12th week of treatment. HUVEC monolayers were incubated in control media (Con) or media with 0.10% w/v ethanol (EtOH) for 72 hours. Where indicated, selected monolayers were treated with rosiglitazone (Rosi, 10 μ M). Lung tissue or HUVEC monolayers were homogenized, and mRNA was extracted and analyzed via RT-PCR. Results are normalized to 9s levels and expressed as % control for A) Nox1 lung tissue (n=8–9), B) Nox1 HUVEC (n=3), C) Nox2 lung tissue (n=8–11), D) Nox2 HUVEC (n=3), E) Nox4 lung tissue (n=8–11), and F) Nox4 HUVEC (n=3). * $p \leq 0.05$ compared to Con; ⁺ $p \leq 0.05$ compared to EtOH.

Figure 7. Rosiglitazone Attenuated LPS-Mediated Increases in Nox1, Nox2, Nox4 and eNOS mRNA levels in Mouse Lung

C57Bl/6 mice were treated with either rosiglitazone (RSG, 10 mg/kg) or vehicle by gavage daily for 7 days. Selected animals were then treated with two IP injections of *E. coli* lipopolysaccharide (LPS, 2 mg/kg) at 6 and 3 hours prior to sacrifice. After sacrifice, lung tissue was homogenized, and mRNA was extracted and analyzed via RT-PCR. Results are normalized to 9s levels and expressed as fold-change relative to lungs from animals lacking either LPS or RSG treatment. Each bar represents the mean $\pm$ SEM fold-change in Nox1, Nox2, Nox4, or eNOS mRNA levels from 4 animals in each group. * $p \leq 0.05$ compared to all other groups.