Published in final edited form as:

Alcohol Clin Exp Res. 2013 June ; 37(6): 914–923. doi:10.1111/acer.12044.

Nicotinic Acetylcholine Receptors are Sensors for Ethanol in Lung Fibroblasts

Jeffrey D. Ritzenthaler, MSc^{1,4}, Susanne Roser-Page, BS³, David M. Guidot, MD³, and Jesse Roman, MD^{1,2,4,5}

¹Division of Pulmonary, Critical Care, and Sleep Disorders, Department of Medicine University of Louisville School of Medicine, Louisville, KY USA

²Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY USA

³Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, Emory University School of Medicine, and Veterans Affairs Medical Center, Atlanta, GA USA

⁴The Louisville Veterans Affairs Medical Center, Louisville, KY USA

Abstract

Background—Chronic ethanol abuse in humans is known to independently increase the incidence of and mortality due to acute lung injury in at-risk individuals. However, the mechanisms by which ethanol affects lung cells remain incompletely elucidated. In earlier work, we reported that ethanol increased the expression in lung fibroblasts of fibronectin, a matrix glycoprotein implicated in lung injury and repair. This effect was blocked by α -bungarotoxin, a neurotoxin that binds certain nicotinic acetylcholine receptors (nAChRs) thereby implicating nAChRs in this process. Here, we examine the identity of these receptors.

Methods—Mouse lung fibroblasts were stimulated with ethanol (60 mM) or acetylcholine (100–500 μ M) and evaluated for the expression of fibronectin and nAChRs. Inhibitors to nAChRs or the antioxidant N-acetyl cysteine were used to assess changes in fibronectin expression. Animals exposed to ethanol for up to 6 weeks were used to evaluate the expression of nAChRs *in vivo*.

Results—First, in ethanol-treated fibroblasts, we observed increased expression of $\alpha 4$ and $\alpha 9$ nAChR subunits. Second, we found that acetylcholine, a natural ligand for nAChRs, mimicked the effects of ethanol. Dihydro- β -erythroidin hydrobromide (D β H), a competitive inhibitor of $\alpha 4$ nAChR, blocked the increase in fibronectin expression and cell proliferation. Furthermore, ethanol-induced fibronectin expression was inhibited in cells silenced for $\alpha 4$ nAChR. However, ethanol-treated cells showed increased α -bungarotoxin binding suggesting that $\alpha 4$ nAChR mediates the effects of ethanol via a ligand-independent pathway. Knowing there are several important cysteine residues near the ligand binding site of $\alpha 4$ nAChRs, we tested the antioxidant N-acetyl cysteine and found that it too blocked the induction of fibronectin expression by ethanol. Also, fibroblasts exposed to oxidant stress showed increased fibronectin expression that was

⁵Address all correspondence and requests for reprint to Jesse Roman, MD, Division of Pulmonary, Critical Care and Sleep Disorders Medicine, Department of Medicine, 550 South Jackson Street (3rd floor), University of Louisville, Louisville, KY 40202, Tel. 502-852-5241, j.roman@louisville.edu.

blocked with α -bungarotoxin. Finally, we showed increased expression of $\alpha 4$ nAChRs in the lung tissue of mice and rats exposed to ethanol suggesting a role for these receptors *in vivo*.

Conclusions—Altogether, our observations suggest that $\alpha 4$ nAChRs serve as sensors for ethanol-induced oxidant stress in lung fibroblasts, thereby revealing a new mechanism by which ethanol may affect lung cells and tissue remodeling, and pointing to nAChRs as potential targets for intervention.

Keywords

Nicotinic; Acetylcholine Receptor; Ethanol; Fibroblasts

INTRODUCTION

Acute Respiratory Distress Syndrome (ARDS) is the most severe form of acute lung injury and has been found to occur 3.7 times more often in people who meet the diagnostic criteria for ethanol use disorders (Moss et al., 1996). Although the exact mechanisms responsible for the detrimental effects of ethanol in lung are unknown, studies performed in rodents show that chronic ethanol exposure (up to 6–8 weeks) is associated with upregulation of proinflammatory cytokines (Bechara et al., 2004; Brown et al., 2001), disruption of normal signaling pathways (Aroor and Shukla, 2004), activation of tissue remodeling (Burnham et al., 2007), and induction of oxidant stress (Guidot and Roman, 2002); all of which contribute to the production of the “alcoholic lung phenotype”. It is this phenotype that appears to render the host highly susceptible to respiratory infections along with other serious lung diseases like acute lung injury (Joshi and Guidot, 2007; Prout et al., 2007). However, to date, the exact cellular mechanisms that allow cells to recognize ethanol remain unclear, yet such knowledge would point to potential targets for intervention in ethanol-related lung dysfunction.

Investigations focusing on the effects of ethanol in neuronal tissue have shown that this agent may act via members of the nicotinic acetylcholine receptor (nAChR) family (Aistrup et al., 1999; Davis and de Fiebre, 2006; Narahashi et al., 1999). NACHRs are part of the *Cys-loop* superfamily of transmitter-gated ion channels that include the GABA_A, strychnine-sensitive glycine, and 5-HT₃ receptors. Thus far, seventeen genes encoding nAChR subunits have been identified in mammals and include the α subtype ($\alpha 1$ – $\alpha 10$), the β subtype ($\beta 1$ – 4), and the δ , ϵ and ψ subunits (Alexander et al., 2004; Millar, 2003; Sargent, 1993). However, little is known about nAChR expression and function outside of the central and peripheral nervous systems.

NACHRs have been demonstrated in immune cells (Davies et al., 1982; Hiemke et al., 1996; Mizuno et al., 1982), keratinocytes (Aztiria et al., 2000), and NIH/3T3 fibroblasts (Aztiria et al., 2000). Evidence for the expression of functional nAChRs in lung cells is also available (Roman et al., 2004). $\alpha 3$, $\alpha 5$, and $\alpha 7$ nAChR subunits have been detected in both human and mouse bronchial epithelial cells, whereas $\alpha 4$ subunits are present in alveolar epithelial cells, $\alpha 7$ subunits are detected in submucosal glands (Zia et al., 1997). $\alpha 3$ and $\alpha 5$ subunits were detected in bronchial epithelial cells and formed channels as demonstrated by patch clamping (Maus et al., 1998). Our group recently reported that $\alpha 7$ nAChR-dependent signals

modulate lung branching morphogenesis in studies of prenatal nicotine exposure (Wongtrakool et al., 2007). $\alpha 7$ and $\alpha 4$ nAChR also appear to mediate the mitogenic effects of nicotine in lung carcinoma cells (Zheng et al., 2007).

Considering the above, we set out to investigate the potential role of nAChRs as mediators of the effects of ethanol in lung. In a prior publication, we reported that ethanol stimulates lung fibroblasts to produce fibronectin, a matrix glycoprotein implicated in tissue injury and repair (Roman et al., 2005). We also reported that ethanol-induced fibronectin is inhibited by α -bungarotoxin, a neurotoxin. Because of the high affinity of α -bungarotoxin towards $\alpha 7$ nAChRs, we tested fibroblasts harvested from $\alpha 7$ nAChR deficient mice. However, these fibroblasts were able to produce fibronectin in response to ethanol thereby implicating other α -bungarotoxin sensitive nAChRs in this process (Roman et al., 2005). Specifically, we found that ethanol-induced fibronectin expression was blocked by an inhibitor of alcohol dehydrogenase suggesting that ethanol metabolism was required (Roman et al., 2005). Herein, we extend those earlier observations to show that $\alpha 4$ nAChR, and perhaps other nAChRs, serve as sensors of ethanol-induced oxidant stress in lung fibroblasts. Furthermore, we show that these nAChRs are expressed in lung and are increased in the setting of chronic ethanol exposure in rodents, thereby suggesting a role *in vivo*.

MATERIALS AND METHODS

Reagents

Mouse nAChRs $\alpha 4$, $\alpha 9$, $\alpha 10$, $\beta 2$, control non-target siRNA, PCR primers (QuantiTect Primer Assays) were purchased from Qiagen (Valencia, CA). Polyclonal antibodies against nAChR subunits $\alpha 4$, $\alpha 9$, $\alpha 10$, and $\beta 2$ were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). All other reagents were purchased from Sigma Chemicals (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

Cell culture and treatment

Primary lung fibroblasts were harvested from wild type or C57BL/6 mice transgenic for the fibronectin-luciferase promoter (Michaelson et al., 2002; Tomic et al., 2005) as previously described (Tomic et al., 2005). Fibroblasts were grown in DMEM (4.5 g/L glucose, 10% FBS) and incubated in a humidified 5% CO₂ incubator at 37°C. Cells were treated with 60 mM ethanol (Roman et al., 2005), a concentration detected in the blood of alcoholics (Henzel et al., 2004). Cell viability was determined by Trypan blue exclusion. No alterations in cell viability were noted under the conditions described.

Silencing of nAChRs and detection of mRNAs by RT-PCR

Primary lung fibroblasts (4×10^4 cells/well) were incubated in DMEM (10% FBS) for 24 h. Fibroblasts were transfected with $\alpha 4$, $\alpha 9$, $\alpha 10$, $\beta 2$ nAChR or control non-target siRNA (150 ng) using HiPerFect Transfection Reagent (Qiagen, Valencia, CA) and incubated at 37°C for 24 h. Fibroblasts were then treated with 60 mM ethanol for an additional 24 hr, harvested and processed for mRNA isolation (Tel-test Inc., Friendswood, TX). The mRNA was reverse-transcribed using SuperScript III (Life Technologies, Gaithersburg, MD). RT reactions were performed using the following primers: mouse $\alpha 4$ nAChR forward (5'

GGGCACCTACAACACC), reverse (5' GGTGATGAGCAGCA GG) primer; $\alpha 9$ nAChR forward (5' AATGTGACCCTGGAGG), reverse (5' CACGTTGGTGC TGGC) primer; $\alpha 10$ nAChR forward (5' CGCTCACCGTCTTCCAG), reverse (5' GGTGGCTGCGGAAGG) primer; $\beta 2$ nAChR forward (5' TCCACTTGTGTTCCCTAGAAGA GC), reverse (5' AGCGCCATAGAGTTGGAGCACC) primer; mouse 18S forward (5' GTGACCAGAG CGAAAGCA), reverse (5' ACCCACGGAATCGAGAAA) primer. PCR protocol was: 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min. for 35 cycles. Negative controls consisted of dH₂O and RNA without RT-PCR. PCR products were resolved on 1% agarose gels and stained with ethidium bromide.

For Real-Time RT-PCR, reactions were set up by adding LightCycler FastStart Master SYBR Green mix (Roche, Indianapolis, IN), template cDNA (500 ng) and forward, reverse primers for mouse $\alpha 4$, $\alpha 9$, $\alpha 10$ and $\beta 2$ nAChR (Qiagen, Valencia, CA). Samples were processed using the Cepheid Smart Cycler: hold 95°C for 120 s; 40 cycles at 95°C for 15 s, 58°C for 30 s, 72°C for 30 s. Results of the log-linear phase of the growth curve were analyzed by the mathematical equation of the second derivative, relative quantification was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Western blot analysis

Ethanol-treated fibroblasts or lung tissue from animals exposed to ethanol were harvested for Western blot analysis as previously described (Roman et al., 2005). Protein concentration was determined by Bradford method (Bradford, 1976). Cell extract (50 μ g protein) was mixed with an equal volume of 2x SDS sample buffer, boiled for 5 min., and resolved on a 5 or 10% SDS-polyacrylamide gel for 1–3 h at 150 v. Separated proteins were transferred onto nitrocellulose using a BioRad Trans Blot semi-dry transfer apparatus for 1 h at 25 mA, blocked with blotto for 1 h at room temperature. Blots were washed, incubated with primary antibody against fibronectin (Sigma, St. Louis, MO) (1:1000 dilution) or $\alpha 4$, $\alpha 9$, $\alpha 10$, $\beta 2$ nAChR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:1000 dilution) for 24 h at 4°C, washed and incubated with secondary goat anti-rabbit IgG (horse radish peroxidase-conjugated, 1:20,000 dilution) for 1.5 h at room temperature. Blots were washed, transferred to ECL solution (Amersham, Arlington Heights, IL) for 1 min., and exposed to X-ray film. Protein bands were quantified by densitometry using a Bio-Rad GS-800 laser densitometer (Hercules, CA).

Fibronectin promoter activity

Fibroblasts (1×10^4 cells/48 well) harvested from transgenic mice expressing the human fibronectin promoter connected to a luciferase reporter gene, pFN(1.2kb)LUC (Michaelson et al., 2002). were pretreated for 2 h with 10 μ M Dihydro- β -erythroidin hydrobromide (Tocris Bioscience, Ellisville, MO), or 10 μ M α -bungarotoxin. Afterwards, cells were exposed to ethanol (60 mM) for 24 h. Fibroblasts were also treated with acetylcholine (100–500 μ M) for 24, 72 or 96 h. In experiments involving N-acetyl cysteine, fibroblasts (1×10^4 cells/48 well) were pretreated with 5 mM N-acetyl cysteine for 2 h prior to exposure to ethanol (60 mM) for 24 h. Oxidant stress was induced by oxidation of the Cysteine/Cystine redox potential; also known as Eh Cys/CySS. The various extracellular thiol/disulfide redox

potentials were established by varying the concentrations of Cys and CySS added to Cys-free DMEM (Sigma-Aldrich, St. Louis, MO) as previously reported (Moriarty et al., 2003).

Afterwards, fibroblasts were harvested, washed, and luciferase activity was quantified using a Labsystems Luminoskan Ascent Plate Luminometer. Results were recorded as relative luciferase units, normalized to total protein as measured by the Bradford method. Each experiment represents 6 separate samples.

Cell viability assay

Wild type or primary lung fibroblasts (1×10^4 cells/ml) were added to 96-well tissue culture plate and incubated at 37°C for 24–72 h. Afterwards, cell numbers were measured using the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI).

Quantification was performed using a Labsystems Luminoskan Ascent Plate Luminometer. Each experiment represents 8 separate samples. Results were recorded in relative luciferase units.

α -bungarotoxin binding assay

The α -bungarotoxin binding assay was performed using the method of Breese et al., (Breese et al., 1997) to detect the presence of cell surface functional nAChRs. Fibroblasts (1×10^5) were incubated with ethanol (60 mM) for 24 h. Media was removed, cells were washed and incubated for 30 min in binding media (complete serum free media, 0.2% BSA) at 37°C and 5% CO₂. Some samples were pretreated with nicotine (2 mM) for 1 h prior to the addition of 5 nM CF488A-labeled α -BGT (Biotium, Hayward, CA). After 3 h, cells were washed, harvested, and CF488A-labeled α -BGT bound to the nAChRs was measured using a Beckman Coulter AD-340 spectrophotometer.

Animal model of chronic ethanol ingestion and lung immunohistochemistry

Ethics Statement. All procedures were approved by the Emory University Institutional Animal Care and Use Committee (protocol 043–2010). The rat model of chronic ethanol ingestion has been described previously (Bechara et al., 2004; Brown et al., 2001; Gauthier et al., 2010a; Guidot and Roman, 2002; Velasquez et al., 2002). Briefly, young adult male Sprague-Dawley rats (200–250 g) were fed the Lieber-DeCarli liquid diet (Research Diets, New Brunswick, NH) containing either ethanol (36% total calories) or the isocaloric carbohydrate substitution with Maltose-Dextrin (control diet) for up to 6 weeks. The mouse model of chronic ethanol ingestion has also been described previously (Gauthier et al., 2010b).

Control and experimental lungs were processed for immunohistochemistry as previously described (Roman et al., 2005). Lung tissue slides were washed, blocked with serum-free protein block solution (Dako, Carpinteria, CA), and stained with antibodies to $\alpha 4$ nAChR (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or control IgG overnight at 4°C using the Vectastain ABC (Vector Laboratories, Inc., Burlingame, CA) method. Slides were counterstained with hematoxylin and coverslipped with mounting medium.

Statistical evaluation

All experiments were repeated 4–6 times. Means plus standard deviation of the mean were calculated for all experimental values. Significance was assessed ANOVA using p values obtained by two-tailed Student's t test.

RESULTS

Ethanol stimulates the expression of nAChRs in lung fibroblasts

Previously, we reported that ethanol stimulates fibronectin expression in lung fibroblasts both *in vivo* and *in vitro* (Roman et al., 2005). In cultured primary lung fibroblasts, ethanol induction of fibronectin was blocked by α -bungarotoxin. Due to the reported specificity of this neurotoxin for $\alpha 7$ nAChRs, we examined the effect of ethanol in primary lung fibroblasts harvested from $\alpha 7$ nAChR null mice. However, ethanol was still able to stimulate fibronectin expression in these cells (Roman et al., 2005). We therefore turned our attention to alternative nAChRs. Considering that nAChR expression is often increased by its ligand (Zia et al., 1997), we began by examining the expression of several nAChRs in lung fibroblasts exposed to ethanol. As depicted in Figure 1A, ethanol stimulated the mRNA expression of the $\alpha 4$ and $\alpha 9$ nAChR subunits, while $\alpha 10$ and $\beta 2$ subunit expression remained relatively unaffected. Interestingly, the $\alpha 4$ protein was also upregulated, but effects on $\alpha 9$ protein were less prominent (Figure 1B).

Ethanol-induced fibronectin expression is mediated via nAChRs in lung fibroblasts

We predicted that if ethanol stimulates fibronectin expression by acting on nAChRs, then acetylcholine should mimic this effect. As depicted in Figure 2A, acetylcholine also stimulated fibronectin expression in a dose-dependent manner in primary lung fibroblasts transfected with the human fibronectin gene promoter fused to a luciferase reporter gene (Tomic et al., 2005). However, acetylcholine is not a specific agonist and, therefore, further work was performed to identify the nAChRs involved.

Because the above data implicated $\alpha 4$ nAChR in ethanol-induced fibronectin expression (Figure 1), we examined the effects of Dihydro- β -erythroidin hydrobromide (D β H), an inhibitor of $\alpha 4$ nAChR. Similar to α -bungarotoxin (Roman et al., 2005), D β H inhibited ethanol-induced fibronectin expression in lung fibroblasts (Figure 2B). Of note, D β H also inhibited the mitogenic effect of ethanol (Figure 2C).

The above data suggested that, in lung fibroblasts, ethanol-induced fibronectin expression and cell proliferation are mediated via D β H-sensitive receptors; the latter suggesting at least a partial role for $\alpha 4$ nAChRs. In order to verify the involvement of these nAChRs, lung fibroblasts were transfected with siRNAs targeting nAChRs. As shown in Figure 3A, siRNA transfection resulted in over 90% inhibition of mRNA and protein expression of the relevant nAChRs. Ethanol-induced fibronectin mRNA (Figure 3B) and protein (Figure 3C) expression were abolished by both $\alpha 4$ and $\alpha 9$ siRNA treatments when compared to a non-specific siRNA. In contrast, reductions in $\alpha 10$ or $\beta 2$ nAChR by siRNA treatment had less of an effect on fibronectin induction and did not block the effects of ethanol on fibronectin protein expression. We also examined the expression of mRNA and protein for other

nAChRs in these siRNA transfected cells and failed to detect a concomitant change in expression of other nAChRs subunits (data not shown).

Ethanol-induced fibronectin is mediated by nAChRs via ligand-independent pathways

The above data suggested that $\alpha 4$, and perhaps other nAChRs expressed on the surface of fibroblasts, mediate the effects of ethanol. However, we previously reported that ethanol metabolism was indispensable for ethanol-induced fibronectin expression. Moreover, we showed that aldehyde, a metabolite of ethanol, mimicked the actions of ethanol in lung fibroblasts (Roman et al., 2005). The latter observations do not fit with a traditional model of ligand-dependent receptor activation in which ethanol binds to surface nAChRs and triggers signal transduction. Instead, the observations suggested that nAChRs might mediate the effects of ethanol indirectly through a ligand-independent mechanism. To begin to test this possibility, we performed ligand binding assays with fluorescent labeled α -bungarotoxin. We predicted that, if ethanol binds nAChRs directly and at the ligand binding site or in close proximity to it, α -bungarotoxin binding would be decreased due to competitive inhibition as we have reported for nicotine and the $\alpha 7$ nAChR (Roman et al., 2004). On the other hand, if ethanol does not bind nAChRs, α -bungarotoxin binding would not be reduced, and perhaps would increase considering that ethanol induces the expression of nAChRs as shown in Figure 1. To test this, we exposed cells silenced for $\alpha 4$ nAChR to ethanol and found that ethanol did not interfere with α -bungarotoxin binding in control cells (Figure 4). Instead, ethanol enhanced α -bungarotoxin binding, which is consistent with its ability to stimulate nAChR expression. Thus, ethanol does not appear to directly bind nAChRs, at least not in a way that competes for α -bungarotoxin binding. Note that silencing of $\alpha 4$ nAChRs decreased α -bungarotoxin binding both at baseline and after ethanol exposure indicating that this receptor, like $\alpha 7$ nAChR, is an α -bungarotoxin-sensitive receptor. The latter is important because it confirms our previous report indicating that ethanol-induced fibronectin is inhibited by α -bungarotoxin (Roman et al., 2005). Nicotine, used as a control, inhibited α -bungarotoxin binding since it binds to both $\alpha 7$ and $\alpha 4$ nAChRs.

Ethanol may induce fibronectin expression through oxidant stress-mediated activation of nAChRs

The experiments described above indicated that ethanol stimulates fibronectin expression through α -bungarotoxin-sensitive receptors, mainly through $\alpha 4$ nAChRs, but perhaps also via $\alpha 9$ nAChRs. These events do not appear to occur through a ligand-dependent process. This idea was considered intriguing since ethanol-induced fibronectin expression is dependent on ethanol metabolism and the generation of aldehyde (Roman et al., 2005). Aldehyde is a strong oxidant and, consequently, we hypothesized that ethanol-induced oxidant stress could trigger nAChR activation. There are several cysteine residues strategically located near the ligand binding site of the receptor that could be the target of oxidant stress (Figure 5A, **insert**). Considering this, we predicted that, if ethanol triggers nAChR activation through the generation of oxidant stress, treatment with a glutathione donor like N-acetyl cysteine (NAC) would inhibit ethanol-induced fibronectin expression. Figure 5A shows that N-acetyl cysteine inhibited fibronectin expression induced by ethanol in lung fibroblasts.

Our hypothesis that ethanol induces nAChR activation through oxidant stress also predicts that oxidant stress should trigger nAChR activation in the absence of ethanol. To test this, lung fibroblasts were exposed to oxidant stress induced by oxidation of the Cysteine/Cystine redox potential; also known as Eh Cys/CySS. In other work, we reported that this type of oxidant stress stimulates fibronectin expression in lung fibroblasts (Ramirez et al., 2007). We reasoned that if our hypothesis was correct, α -bungarotoxin should inhibit this effect considering that it tightly binds nAChRs and locks them in the “off” state. As presented in Figure 5B, we found that oxidation of the Eh Cys/CySS (i.e., Eh -46 mV) induced fibronectin expression, while reduction of the Eh Cys/CySS (i.e., Eh -131 mV) had no effect. Most importantly, α -bungarotoxin inhibited oxidant stress-induced fibronectin expression suggesting that nAChRs mediated the response.

Ethanol stimulates the expression of $\alpha 4$ nAChRs in lung

To explore the relevance of our findings to the situation *in vivo*, we examined lung tissue harvested from mice and rats exposed to ethanol for 6 weeks. As depicted in Figure 6A, $\alpha 4$ nAChR expression was minimal in wild type untreated control lungs examined using immunohistochemistry. However, the lungs of ethanol-treated animals exhibited a dramatic increase in staining for $\alpha 4$ nAChR in peri-airway cells, peribronchial tissue, vascular structures, within the interstitium, and alveolar macrophages. This relationship was confirmed using RT-PCR, Real-time RT-PCR and Western blot to evaluate for $\alpha 4$ nAChR mRNA (Figure 6B) and protein (Figure 6C)

DISCUSSION

Chronic ethanol abuse increases susceptibility to acute lung injury (Moss et al., 1996). In fact, 40% of the over 200,000 cases of acute lung injury diagnosed in this country every year are thought to be linked to alcohol abuse (Guidot and Hart, 2005). Chronic ethanol use is also associated with increased mortality (Guidot and Hart, 2005). In view of its importance, researchers have searched for the mechanisms responsible for this association. In studies performed in rodents, it has been shown that chronic exposure to ethanol (up to 6–8 weeks) is associated with upregulation of proinflammatory cytokines (Bechara et al., 2004; Brown et al., 2001), alterations in surfactant production (Velasquez et al., 2002), and the induction of oxidant stress (Guidot and Roman, 2002; Yeh et al., 2007). Ethanol also results in activation of tissue remodeling in lung as evidenced by increased expression and/or activation of matrix metalloproteinase (Lois et al., 1999), transforming growth factor β (Bechara et al., 2004), and increased fragmentation of collagen (Lois et al., 1999). Together, these changes contribute to the production of what has been termed the “alcoholic lung phenotype”. It is this phenotype that appears to render the host highly susceptible to acute lung injury.

Interestingly, the exact mechanisms by which host lung cells recognize ethanol remain poorly understood. Ethanol is lipid soluble and can transverse cellular membranes. Once inside cells, ethanol is metabolized to acetaldehyde by one of several alcohol dehydrogenases (Crabb et al., 2004; Quertemont and Didone, 2006). Inhibitors of alcohol dehydrogenase function prevent many of the effects of ethanol in liver and lung cells

(Niemela et al., 1998), suggesting an important role for acetaldehyde in ethanol-mediated tissue injury. Acetaldehyde, known to induce oxidant stress, has been proposed as a major contributor to injury in animal models of chronic ethanol exposure (Kopczynska et al., 2001). In addition to the effect of ethanol, metabolites and oxidant stress, others have suggested the existence of sensors for ethanol at the cellular level. The studies presented herein explore the role of nAChRs as sensors for ethanol in lung fibroblasts.

The ability of ethanol to induce fibronectin expression in lung fibroblasts provided a suitable system to study the potential role of nAChRs (Roman et al., 2005). In view of studies implicating fibronectin in lung injury and repair, we set out to investigate how ethanol stimulated fibronectin expression in lung fibroblasts. Earlier reports showed ethanol stimulation of fibroblasts was dependent on ethanol metabolism and resulted in the dose- and time-dependent activation of protein kinase C, Mitogen Activated Protein Kinases, as well as the phosphorylation of the cyclic AMP-responsive element binding protein (CREB), a transcription factor known for its ability to stimulate fibronectin expression (Roman et al., 2005). Importantly, we showed that ethanol-induced fibronectin in lung fibroblasts was inhibited by α -bungarotoxin, a snake toxin known for its specificity towards nAChRs (Arias, 2000). This observation prompted the studies presented here.

The observation that acetylcholine mimics the stimulatory effect of ethanol on fibronectin expression further pointed to nAChRs as sensors for ethanol. Our initial studies directed at identifying the nAChRs involved focused on $\alpha 7$ nAChR, the most common nAChR, but they failed to show a role for this receptor in ethanol-induced fibronectin expression (Roman et al., 2005). This prompted us to search for α -bungarotoxin sensitive nAChRs other than $\alpha 7$ nAChRs and culminated in observations pointing to $\alpha 4$, and perhaps $\alpha 9$, nAChRs as sensors of ethanol in lung fibroblasts. First, we found that ethanol stimulated the expression of both of these receptors, but failed to affect the expression of $\alpha 10$ and $\beta 2$ nAChRs. Furthermore, D β H, an inhibitor of $\alpha 4$ nAChR function (Cheeta et al., 2001), inhibited ethanol-induced fibronectin expression and mitogenesis. Importantly, silencing of $\alpha 4$ and $\alpha 9$ nAChR expression in lung fibroblasts with siRNA inhibited ethanol-induced fibronectin expression. Although there may be the possibility of off-target effects due to the use of siRNA, we have attempted to minimize these effects by the use of minimal concentrations and pooling of multiple siRNAs to the target sequences. These observations, and the finding that ethanol stimulated $\alpha 4$ nAChR protein expression while having a minimal effect on $\alpha 9$, led us to further investigate the role of $\alpha 4$ nAChR.

Ligand binding assays were performed to determine if $\alpha 4$ nAChRs mediate the effects of ethanol through a classic ligand-dependent versus a ligand-independent pathway. We reasoned that ethanol should compete for α -bungarotoxin binding if ethanol activates nAChRs by binding to its ligand binding site as has been observed for nicotine (Roman et al., 2004). In contrast, if ethanol does not bind to nAChRs, it would not compete for α -bungarotoxin binding and may even enhance binding since it stimulates nAChR expression. The latter was found to be true; ethanol enhanced α -bungarotoxin binding. Although it is conceivable that ethanol might bind to a separate site in nAChR that does not affect α -bungarotoxin binding, this is considered less likely. These studies also showed that, in cells silenced for $\alpha 4$ nAChRs, α -bungarotoxin binding decreased further emphasizing that $\alpha 4$

nAChRs are α -bungarotoxin sensitive. To our knowledge, this is the first demonstration of this in the literature. However, it is recognized that the inhibitors tested might affect other nAChRs (e.g. $\alpha 3\beta 4$) and, therefore, other processes might be involved.

We then hypothesized that ethanol might affect nAChRs indirectly through induction of oxidant stress. This is consistent with other reports showing that ethanol-induced susceptibility to lung injury in animals is inhibited by pre-treatment with anti-oxidants such as procysteine and N-acetyl cysteine (Brown et al., 2007; Holguin et al., 1998). Our hypothesis was supported by studies showing that ethanol-induced fibronectin was inhibited by the glutathione donor N-acetyl cysteine. Furthermore, we showed that oxidated Eh Cys/CySS also stimulated fibronectin expression in lung fibroblasts and, importantly, that this effect was inhibited by α -bungarotoxin further pointing to nAChRs as sensors of ethanol-induced oxidant stress. Although these data support our hypothesis, other explanations might be possible. For example, Sun et al. recently showed that NAC promotes survival of neuronal cells through group I metabotropic glutamate receptor activity (Sun et al., 2012). Thus, the reagents used may turn out to affect the processes studied in ways that we cannot anticipate at this time. It is for this reason that we are now focusing on cysteines in nAChRs that might be affected by oxidant stress by manipulating the expression of wildtype and mutant receptors; these studies should shed light into this area.

Finally, we examined for the presence of the $\alpha 4$ nAChR subunit in lung tissue obtained from untreated rats and rats exposed chronically to ethanol. We found very little expression of $\alpha 4$ nAChRs in the lungs of untreated animals, while $\alpha 4$ nAChR expression increased dramatically in the lungs of animals treated with ethanol. Staining for $\alpha 4$ nAChR was most prominent in airway cells, peri-airway cells (fibroblasts and smooth muscle cells), and in vascular structures. The $\alpha 4$ subunit mRNA and protein was dramatically increased in both murine and rat lungs harvested from animals exposed to ethanol.

Together, our data suggest that $\alpha 4$ nAChRs mediate, at least in part, the effects of ethanol in lung fibroblasts. However, the exact identity of these receptors remains unknown since nAChRs are pentameric. In brain cells, $\alpha 4 \beta 2$ heteropentameric nAChRs appear to be important in mediating the effects of ethanol, but the absence of an effect related to $\beta 2$ in our system suggests the involvement of other $\alpha 4$ -containing nAChRs. A heteropentamer composed of both $\alpha 4$ and $\alpha 9$ subunits has not been reported and these subunits failed to co-immunoprecipitate in preliminary experiments (not shown). However, such a putative heteropentamer would explain our siRNA results whereby the alcohol-induced increase in fibronectin could be blocked by targeting either $\alpha 4$ or $\alpha 9$. Thus, at this point in time, the exact identity of the $\alpha 4$ -containing nAChRs responsible for 'sensing' ethanol in lung fibroblasts remains unknown.

In summary, our data suggest that $\alpha 4$ -containing nAChRs serve as sensors for ethanol and we hypothesize that the cellular effects of ethanol are mediated, at least in part, by its metabolism to acetaldehyde which results in oxidant stress. In turn, oxidant stress acts on strategically located cysteines in the nAChR that trigger conformational changes leading to signal transduction and upregulation of fibronectin expression, among other events. This hypothesis explains our observations, especially those showing that acetylcholine and

oxidant stress mimic the effects of ethanol, while α -bungarotoxin and N-acetyl cysteine block them. Although the exact function(s) of nAChRs in lung remain unclear, several studies suggest important roles in lung development, inflammation, and cancer (Sekhon et al., 1999; Tournier and Birembaut, 2011). Interestingly, chronic ethanol exposure has been linked to the pathophysiology of these processes (Bagnardi et al., 2010; Gauthier et al., 2010a; Gauthier et al., 2010b; Lois et al., 1999; Tournier and Birembaut, 2011; Velasquez et al., 2002). It is intriguing to consider that nAChRs may serve as future potential targets for intervention to prevent the devastating effects of ethanol in lung and other tissues.

Acknowledgments

Funding

This work was supported by National Institutes of Health [Grant AA013757] and a Veterans Affairs Grant.

References

- Aistrup GL, Marszalec W, Narahashi T. Ethanol modulation of nicotinic acetylcholine receptor currents in cultured cortical neurons. *Mol Pharmacol*. 1999; 55:39–49. [PubMed: 9882696]
- Alexander SP, Mathie A, Peters JA. Guide to receptors and channels, 1st edition. *Br J Pharmacol*. 2004; 141:S1–126. [PubMed: 15082510]
- Arias HR. Localization of agonist and competitive antagonist binding sites on nicotinic acetylcholine receptors. *Neurochem Int*. 2000; 36:595–645. [PubMed: 10771117]
- Aroor AR, Shukla SD. MAP kinase signaling in diverse effects of ethanol. *Life Sci*. 2004; 74:2339–64. [PubMed: 15027449]
- Aztiria EM, Sogayar MC, Barrantes FJ. Expression of a neuronal nicotinic acetylcholine receptor in insect and mammalian host cell systems. *Neurochem Res*. 2000; 25:171–80. [PubMed: 10685617]
- Bagnardi V, Randi G, Lubin J, Consonni D, Lam TK, Subar AF, Goldstein AM, Wacholder S, Bergen AW, Tucker MA, Decarli A, Caporaso NE, Bertazzi PA, Landi MT. Alcohol consumption and lung cancer risk in the Environment and Genetics in Lung Cancer Etiology (EAGLE) study. *Am J Epidemiol*. 2010; 171:36–44. [PubMed: 19933698]
- Bechara RI, Brown LA, Roman J, Joshi PC, Guidot DM. Transforming growth factor beta1 expression and activation is increased in the alcoholic rat lung. *Am J Respir Crit Care Med*. 2004; 170:188–94. [PubMed: 15105163]
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248–254. [PubMed: 942051]
- Breese CR, Adams C, Logel J, Drebing C, Rollins Y, Barnhart M, Sullivan B, Demasters BK, Freedman R, Leonard S. Comparison of the regional expression of nicotinic acetylcholine receptor alpha7 mRNA and [125I]-alpha-bungarotoxin binding in human postmortem brain. *J Comp Neurol*. 1997; 387:385–398. [PubMed: 9335422]
- Brown LA, Harris FL, Guidot DM. Chronic ethanol ingestion potentiates TNF-alpha-mediated oxidative stress and apoptosis in rat type II cells. *Am J Physiol Lung Cell Mol Physiol*. 2001; 281:L377–386. [PubMed: 11435212]
- Brown LA, Ritzenthaler JD, Guidot DM, Roman J. Alveolar type II cells from ethanol-fed rats produce a fibronectin-enriched extracellular matrix that promotes monocyte activation. *Alcohol*. 2007; 41:317–324. [PubMed: 17889308]
- Burnham EL, Moss M, Ritzenthaler JD, Roman J. Increased fibronectin expression in lung in the setting of chronic alcohol abuse. *Alcohol Clin Exp Res*. 2007; 31:675–683. [PubMed: 17374047]
- Cheeta S, Tucci S, File SE. Antagonism of the anxiolytic effect of nicotine in the dorsal raphe nucleus by dihydro-beta-erythroidine. *Pharmacol Biochem Behav*. 2001; 70:491–496. [PubMed: 11796148]

- Crabb DW, Matsumoto M, Chang D, You M. Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. *Proc Nutr Soc.* 2004; 63:49–63. [PubMed: 15099407]
- Davies BD, Hoss W, Lin JP, Lionetti F. Evidence for a noncholinergic nicotine receptor on human phagocytic leukocytes. *Mol Cell Biochem.* 1982; 44:23–31. [PubMed: 7087960]
- Davis TJ, de Fiebre CM. Alcohol's actions on neuronal nicotinic acetylcholine receptors. *Alcohol Res Health.* 2006; 29:179–185. [PubMed: 17373406]
- Gauthier TW, Kable JA, Burwell L, Coles CD, Brown LA. Maternal alcohol use during pregnancy causes systemic oxidation of the glutathione redox system. *Alcohol Clin Exp Res.* 2010a; 34:123–130. [PubMed: 19860801]
- Gauthier TW, Ping XD, Gabelaia L, Brown LA. Delayed neonatal lung macrophage differentiation in a mouse model of in utero ethanol exposure. *Am J Physiol Lung Cell Mol Physiol.* 2010b; 299:L8–16. [PubMed: 20382747]
- Guidot DM, Hart CM. Alcohol abuse and acute lung injury: epidemiology and pathophysiology of a recently recognized association. *J Investig Med.* 2005; 53:235–245.
- Guidot DM, Roman J. Chronic ethanol ingestion increases susceptibility to acute lung injury: role of oxidative stress and tissue remodeling. *Chest.* 2002; 122:309S–314S. [PubMed: 12475807]
- Henzel K, Thorborg C, Hofmann M, Zimmer G, Leuschner U. Toxicity of ethanol and acetaldehyde in hepatocytes treated with ursodeoxycholic or tauroursodeoxycholic acid. *Biochim Biophys Acta.* 2004; 1644:37–45. [PubMed: 14741743]
- Hiemke C, Stolp M, Reuss S, Wevers A, Reinhardt S, Maelicke A, Schlegel S, Schroder H. Expression of alpha subunit genes of nicotinic acetylcholine receptors in human lymphocytes. *Neurosci Lett.* 1996; 214:171–174. [PubMed: 8878111]
- Holguin F, Moss I, Brown LA, Guidot DM. Chronic ethanol ingestion impairs alveolar type II cell glutathione homeostasis and function and predisposes to endotoxin-mediated acute edematous lung injury in rats. *J Clin Invest.* 1998; 101:761–768. [PubMed: 9466970]
- Joshi PC, Guidot DM. The alcoholic lung: epidemiology, pathophysiology, and potential therapies. *Am J Physiol Lung Cell Mol Physiol.* 2007; 292:L813–823. [PubMed: 17220370]
- Kopczynska E, Torlinski L, Ziolkowski M. The influence of alcohol dependence on oxidative stress parameters. *Postepy Hig Med Dosw.* 2001; 55:95–111. [PubMed: 11355537]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
- Lois M, Brown LA, Moss IM, Roman J, Guidot DM. Ethanol ingestion increases activation of matrix metalloproteinases in rat lungs during acute endotoxemia. *Am J Respir Crit Care Med.* 1999; 160:1354–1360. [PubMed: 10508828]
- Maus AD, Pereira EF, Karachunski PI, Horton RM, Navaneetham D, Macklin K, Cortes WS, Albuquerque EX, Conti-Fine BM. Human and rodent bronchial epithelial cells express functional nicotinic acetylcholine receptors. *Mol Pharmacol.* 1998; 54:779–788. [PubMed: 9804613]
- Michaelson JE, Ritzenthaler JD, Roman J. Regulation of serum-induced fibronectin expression by protein kinases, cytoskeletal integrity, and CREB. *Am J Physiol Lung Cell Mol Physiol.* 2002; 282:L291–301. [PubMed: 11792634]
- Millar NS. Assembly and subunit diversity of nicotinic acetylcholine receptors. *Biochem Soc Trans.* 2003; 31:869–874. [PubMed: 12887324]
- Mizuno Y, Dosch HM, Gelfand EW. Carbamylcholine modulation of E-rosette formation: identification of nicotinic acetylcholine receptors on a subpopulation of human T lymphocytes. *J Clin Immunol.* 1982; 2:303–308. [PubMed: 6982902]
- Moriarty SE, Shah JH, Lynn M, Jiang S, Openo K, Jones DP, Sternberg P. Oxidation of glutathione and cysteine in human plasma associated with smoking. *Free Radic Biol Med.* 2003; 35:1582–1588. [PubMed: 14680681]
- Moss M, Bucher B, Moore FA, Moore EE, Parsons PE. The role of chronic alcohol abuse in the development of acute respiratory distress syndrome in adults. *JAMA.* 1996; 275:50–54. [PubMed: 8531287]
- Narahashi T, Aistrup GL, Marszalec W, Nagata K. Neuronal nicotinic acetylcholine receptors: a new target site of ethanol. *Neurochem Int.* 1999; 35:131–141. [PubMed: 10405997]

- Niemela O, Parkkila S, Pasanen M, Iimuro Y, Bradford B, Thurman RG. Early alcoholic liver injury: formation of protein adducts with acetaldehyde and lipid peroxidation products, and expression of CYP2E1 and CYP3A. *Alcohol Clin Exp Res*. 1998; 22:2118–2124. [PubMed: 9884160]
- Prout M, Martin GS, Drexler K, Brown LA, Guidot DM. Alcohol abuse and acute lung injury: can we target therapy? *Expert Rev Respir Med*. 2007; 1:197–207. [PubMed: 20477184]
- Quertemont E, Didone V. Role of acetaldehyde in mediating the pharmacological and behavioral effects of alcohol. *Alcohol Res Health*. 2006; 29:258–265. [PubMed: 17718404]
- Ramirez A, Ramadan B, Ritzenthaler JD, Rivera HN, Jones DP, Roman J. Extracellular cysteine/cystine redox potential controls lung fibroblast proliferation and matrix expression through upregulation of transforming growth factor-beta. *Am J Physiol Lung Cell Mol Physiol*. 2007; 293:L972–981. [PubMed: 17644756]
- Roman J, Ritzenthaler JD, Bechara R, Brown LA, Guidot D. Ethanol stimulates the expression of fibronectin in lung fibroblasts via kinase-dependent signals that activate CREB. *Am J Physiol Lung Cell Mol Physiol*. 2005; 288:L975–987. [PubMed: 15653713]
- Roman J, Ritzenthaler JD, Gil-Acosta A, Rivera HN, Roser-Page S. Nicotine and fibronectin expression in lung fibroblasts: implications for tobacco-related lung tissue remodeling. *FASEB J*. 2004; 18:1436–1438. [PubMed: 15247149]
- Sargent PB. The diversity of neuronal nicotinic acetylcholine receptors. *Annu Rev Neurosci*. 1993; 16:403–443. [PubMed: 7681637]
- Sekhon HS, Jia Y, Raab R, Kuryatov A, Pankow JF, Whitsett JA, Lindstrom J, Spindel ER. Prenatal nicotine increases pulmonary alpha7 nicotinic receptor expression and alters fetal lung development in monkeys. *J Clin Invest*. 1999; 103:637–647. [PubMed: 10074480]
- Sun L, Gu L, Wang S, Yuan J, Yang H, Zhu J, Zhang H. N-acetylcysteine protects against apoptosis through modulation of group I metabotropic glutamate receptor activity. *PLoS One*. 2012; 7:e32503. [PubMed: 22442667]
- Tomic R, Lassiter CC, Ritzenthaler JD, Rivera HN, Roman J. Anti-tissue remodeling effects of corticosteroids: fluticasone propionate inhibits fibronectin expression in fibroblasts. *Chest*. 2005; 127:257–265. [PubMed: 15653993]
- Tournier JM, Birembaut P. Nicotinic acetylcholine receptors and predisposition to lung cancer. *Curr Opin Oncol*. 2011; 23:83–87. [PubMed: 21045689]
- Velasquez A, Bechara RI, Lewis JF, Malloy J, McCaig L, Brown LA, Guidot DM. Glutathione replacement preserves the functional surfactant phospholipid pool size and decreases sepsis-mediated lung dysfunction in ethanol-fed rats. *Alcohol Clin Exp Res*. 2002; 26:1245–1251. [PubMed: 12198401]
- Wongtrakool C, Roser-Page S, Rivera HN, Roman J. Nicotine alters lung branching morphogenesis through the alpha7 nicotinic acetylcholine receptor. *Am J Physiol Lung Cell Mol Physiol*. 2007; 293:L611–618. [PubMed: 17545491]
- Yeh MY, Burnham EL, Moss M, Brown LA. Chronic alcoholism alters systemic and pulmonary glutathione redox status. *Am J Respir Crit Care Med*. 2007; 176:270–276. [PubMed: 17507544]
- Zheng Y, Ritzenthaler JD, Roman J, Han S. Nicotine stimulates human lung cancer cell growth by inducing fibronectin expression. *Am J Respir Cell Mol Biol*. 2007; 37:681–690. [PubMed: 17600315]
- Zia S, Ndoye A, Nguyen VT, Grando SA. Nicotine enhances expression of the alpha 3, alpha 4, alpha 5, and alpha 7 nicotinic receptors modulating calcium metabolism and regulating adhesion and motility of respiratory epithelial cells. *Res Commun Mol Pathol Pharmacol*. 1997; 97:243–262. [PubMed: 9387186]

Figure 1. Ethanol induces the expression of nAChRs in lung fibroblasts

A. Upper panel: RT-PCR analysis of primary lung fibroblasts (4×10^4 cells/well) in 12-well plates treated with or without ethanol for 24 h. Afterwards, cells were washed, harvested and processed for RT-PCR analysis of nAChR mRNA. PCR products were analyzed on 1% agarose gel stained with ethidium bromide. **Lower panel:** Quantification of nAChR mRNA using Real-Time RT-PCR analysis of cells using a Cepheid Smart Cyclor (Sunnyvale, CA). mRNA values were normalized to 18S and shown as means $\pm$ SD. Note that $\alpha 4$ and $\alpha 9$ nAChR mRNA levels were significantly increased in lung fibroblasts treated with ethanol. *Significant difference from non-treated cells ($n=4$; $p<0.01$). **B. Upper panel:** Primary lung fibroblasts (1×10^6 cells/ml) in 6-well plates treated with or without ethanol for 24 h followed by Western blot analysis for $\alpha 4$, $\alpha 9$, $\alpha 10$ or $\beta 2$ nAChR protein expression. Duplicate blots were analyzed for actin expression and used as loading controls. **Lower panel:** Quantification of protein levels using a Bio-Rad GS-800 laser densitometer (Hercules, CA). Note that only $\alpha 4$ nAChR protein levels were significantly elevated in fibroblasts treated with ethanol. *Significant difference from non-treated cells ($n=4$; $p<0.01$).

Figure 2. Acetylcholine mimics the effect of ethanol; DβH, an α4 nAChR inhibitor, blocks ethanol-induced fibronectin expression

A. Acetylcholine mimics the effect of ethanol on the induction of fibronectin promoter expression. Primary mouse lung fibroblasts isolated from C57BL/6 mice transgenic for the fibronectin-luciferase promoter (4×10^4 cells/well) were added to 48 well plates and cultured in the presence of varying concentrations of acetylcholine (100–500 μM) at 37°C for 24–96 h, harvested, and cell extracts were processed for induction of fibronectin promoter expression by luciferase assay. Quantification was performed using a Labsystems Luminoskan Ascent Plate Luminometer, results were recorded as relative luciferase units, and all samples were normalized for total protein as determined by the Bradford method. *Significant difference from control non-treated cells ($n=8$; $p<0.01$). **B.** Induction of fibronectin promoter activity by ethanol is inhibited by pretreatment with an α7 nAChR inhibitor, α-bungarotoxin (α-BGT) and a competitive inhibitor of α4 nAChR, Dihydro-β-erythroidin hydrobromide (DβH). Primary mouse lung fibroblasts isolated from C57BL/6 mice transgenic for the fibronectin-luciferase promoter (1×10^4 cells/well) were added to 48 well plates and cultured in the presence of physiological concentrations of ethanol (60 mM) at 37°C for 24 h, harvested, and cell extracts were processed for induction of fibronectin promoter expression by luciferase assay. Quantification was performed using a Labsystems Luminoskan Ascent Plate Luminometer, results were recorded as relative luciferase units and all samples were normalized for total protein as determined by the Bradford method. Both α-BGT and DβH were able to abrogate the fibronectin promoter induction by ethanol. *Significant difference from ethanol treated cells ($n=8$; $p<0.01$). **C.** DβH inhibits ethanol-induced fibroblast proliferation. Primary mouse lung fibroblasts (1×10^4 cells/ml) were added to 96-well plates, treated with or without ethanol in the presence or absence of the α4 nAChR inhibitor DβH for 24–72 h. Afterwards, cell proliferation was measured by the Cell Titer-Glo Luminescent Cell Viability. Quantification was performed using a Labsystems

Luminoskan Ascent Plate Luminometer, results were recorded as relative luciferase units. Note that D β H significantly inhibited the increase in cell proliferation at 72 h of ethanol treatment. *Significant difference from control non-treated cells at 72 h (n=8; p<0.01). **Significant difference from ethanol treated cells at 72 h (n=8; p<0.01).

Figure 3. α4 and α9 nAChR siRNAs inhibit ethanol-induced fibronectin expression

A. Western blot and mRNA analysis of the effect of α4, α9, α10 or β2 nAChR or control non-target siRNA knockdown in transfected primary lung fibroblasts. Upper panel shows the ability of the siRNA to eliminate the expression of the nAChRs while the lower panel demonstrates the ability of the siRNA to downregulate mRNA expression. Western blot gels were stripped and reprobed for GAPDH expression to control for loading. The 18S subunit was used to normalize mRNA expression for RT-PCR analysis. **B.** Induction of fibronectin mRNA expression by ethanol is inhibited by knock down of both the α4 and α9 nAChR by siRNA. Primary mouse lung fibroblasts (4×10^4 cells/well) transfected with α4, α9, α10 or β2 nAChR or control non-target siRNA in 12-well plates for 24 h were treated with or without ethanol for an additional 24 h. Afterwards, cells were washed, harvested and processed for RT-PCR analysis of fibronectin mRNA. Relative fibronectin mRNA values were normalized to 18S and shown as means $\pm$ SD. Note that α4 and α9 nAChR siRNA blocked the ethanol induced fibronectin mRNA expression when compared to control cells or cells transfected with control non-target siRNA. Both α10 and β2 nAChR siRNA failed to block the effects of ethanol. *Significant difference from control or non-target siRNA treated cells ($n=4$; $p < 0.01$). **C.** Induction of fibronectin protein expression by ethanol is inhibited by knock down of α4 and α9 nAChR by siRNA. Primary mouse lung fibroblasts (4×10^4 cells/well) transfected with α4, α9, α10 or β2 nAChR or control non-target siRNA in 12-well plates for 24 h were treated with or without ethanol for an additional 24 h. Afterwards, cells were washed, harvested and processed for Western blot analysis of

fibronectin. Identical blots were incubated for β -actin expression and used for gel loading control. Bars in graph are shown as means $\pm$ SD. Note that $\alpha 4$ and $\alpha 9$ nAChR siRNA blocked the ethanol induced fibronectin protein expression when compared to control cells or cells transfected with control non-target siRNA. Both $\alpha 10$ and $\beta 2$ nAChR siRNA failed to block the effects of ethanol. NS, control non-target siRNA.

Figure 4. Ethanol stimulates, while knock-down of $\alpha 4$ nAChR blocks the binding of α -bungarotoxin

Primary lung fibroblasts (1×10^5 cells/ml) transfected with or without control non-target or $\alpha 4$ shRNA were plated into 24-well plates. Cells were then treated with or without ethanol for 24 h. Some samples were pretreated with nicotine (2 mM) for 1 h prior to the addition of 5 nM CF488A-labeled α -BGT (Biotium, Hayward, CA). Cells were incubated an additional 3 h at 37°C and 5% CO₂. Afterwards, the cells were washed twice for 5 min with binding media, washed once for 15 min with TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl), and washed once for 5 min with PBS. CF488A-labeled α -BGT bound to surface nAChRs was quantified using a Beckman Coulter AD-340 spectrophotometer. Values were normalized to total protein and shown as means $\pm$ SD. Nicotine, used as control, inhibited α -bungarotoxin binding. *Significant difference from control cells (n=4; p< 0.01). **Significant difference from ethanol treated control cells at 72 h (n=8; p<0.01). C^{sh}, primary lung fibroblasts transfected with control non-target shRNA; $\alpha 4$ ^{sh}, primary lung fibroblasts transfected with $\alpha 4$ shRNA. Nic, nicotine.

Figure 5. N-acetyl cysteine inhibits ethanol-induced fibronectin expression; α-bungarotoxin inhibits oxidant stress-induced fibronectin expression in lung fibroblasts

A. Primary mouse lung fibroblasts isolated from C57BL/6 mice transgenic for the fibronectin-luciferase promoter (1×10^4 cells/48 well) were pretreated with 5 mM N-acetyl cysteine (NAC) for 2 h prior to exposure to ethanol (60 mM) for 24 h. Cells were harvested, and cell extracts were processed for induction of fibronectin promoter expression by luciferase assay. Quantification was performed using a Labsystems Luminoskan Ascent Plate Luminometer, results were recorded as normalized luciferase units, and all samples were normalized for total protein as determined by the Bradford method. *Significant difference from control non-treated cells ($n=8$; $p<0.01$). **(Insert)** Illustration of the location of several cysteine residues strategically located near the ligand binding site of the α4 nAChR that could be the target of oxidant stress. NAC, N-acetyl cysteine. **B.** Primary mouse lung fibroblasts isolated from C57BL/6 mice transgenic for the fibronectin-luciferase promoter (1×10^4 cells/48 well) were exposed to oxidant stress induced by oxidation of the Cysteine/Cystine redox potential or Eh Cys/CySS. Some cells were pretreated with α-bungarotoxin (α-BGT). Afterwards, fibroblasts were harvested and washed, and luciferase activity was quantified using a Labsystems Luminoskan Ascent Plate Luminometer. For these experiments, results were recorded as normalized luciferase units. Each experiment represents 6 separate samples. Results were normalized to total protein as measured by the Bradford method.

Figure 6. Ethanol increases $\alpha 4$ nAChR expression *in vivo*

A. Immunohistochemical analysis of $\alpha 4$ nAChR expression in the lungs of animals treated with or without ethanol. The lungs from control animals showed little staining for $\alpha 4$ nAChR, while the lungs of ethanol-treated animals exhibited a dramatic increase in staining for α nAChR in peri-airway cells, peribronchial tissue, primary lung fibroblasts, vascular structures, within the interstitium, and alveolar macrophages. **B.** RT-PCR analysis of $\alpha 4$ nAChR mRNA expression in lung of both mice and rats treated with or without ethanol. Upper panel: RT-PCR analysis of nAChR mRNA, PCR products were analyzed on 1% agarose gel stained with ethidium bromide. Lower panel: Quantification of nAChR mRNA using Real-Time RT-PCR analysis of lungs using a Cepheid Smart Cycloer (Sunnyvale, CA). mRNA values were normalized to 18S and shown as means $\pm$ SD. Note that $\alpha 4$ mRNA levels were significantly increased in the lungs of animals treated ethanol. *Significant difference from non-treated cells (n=4; p< 0.01). **C.** Western blot analysis of $\alpha 4$ nAChR protein expression in the lung of both mice and rats treated with or without ethanol.