Ethanol stimulates the expression of fibronectin in lung fibroblasts via kinase-dependent signals that activate CREB

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Ethanol stimulates the expression of fibronectin in lung fibroblasts via kinase-dependent signals that activate CREB. Am J Physiol Lung Cell Mol Physiol 288:L975–L987, 2005. First published January 21, 2005; doi:10.1152/ajplung.00003.2004.—Ethanol renders the lung susceptible to acute lung injury in the setting of insults such as sepsis. The mechanisms mediating this effect are unknown, but activation of tissue remodeling is considered key to this process. We found that chronic ethanol ingestion in rats increased the expression of fibronectin, a matrix glycoprotein implicated in acute lung injury. In cultured NIH/3T3 cells and in primary rat and mouse lung fibroblasts, ethanol induced fibronectin mRNA and protein expression in a dose- and time-dependent fashion. The effect of ethanol was prevented by inhibitors of protein kinase C and mitogen-activated protein kinases and was associated with the phosphorylation and increased DNA binding of the transcription factor cAMP response element binding protein, followed by increased transcription of the fibronectin gene. Fibroblasts were found to express α7 nicotinic acetylcholine receptor (nAChR), and ethanol induction of fibronectin was abolished by α7-nAChR and methyllycaconitine, inhibitors of α7 nAChRs. However, ethanol was able to induce fibronectin mRNA and protein in primary lung fibroblasts isolated from α7 nAChR knockout mice. The ethanol-induced fibronectin response was dependent on ethanol metabolism since 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, abolished the effect and acetaldehyde induced it. These observations suggest that ethanol or ethanol metabolites stimulate lung fibroblasts to produce fibronectin by inducing specific signals transmitted via nAChRs independent of the α7 subunit, and this might represent a mechanism by which ethanol renders the lung susceptible to acute lung injury.

The acute respiratory distress syndrome (ARDS) is a devastating disease that affects ~75,000–150,000 individuals per year in the United States (1). The most common at-risk diagnoses associated with the development of ARDS are sepsis, trauma, and the aspiration of gastric contents. The mechanisms that lead to the development of this syndrome in some patients and not others are unknown, but a recent discovery points to alcohol abuse as an important predisposing factor. This association was first identified by the work of Moss and colleagues (33) who demonstrated that chronic alcohol abuse in humans independently increases the incidence of ARDS in at-risk patients and is associated with increased mortality related to multiorgan failure.

Ethanol also predisposes rats to edematous lung injury elicited by endotoxemia or sepsis, thereby mimicking the human condition (25). The use of this model has greatly improved our understanding of the cellular mechanisms responsible for the effects of ethanol in the lung. The data available to date indicate that chronic (6–8 wk) ingestion of ethanol in these animals results in decreased levels of glutathione, an important antioxidant in the lung (25, 52). This defect is associated with alterations in epithelial cell permeability (21), decreased alveolar liquid clearance (21), decreased cell viability (9), and decreased surfactant production (22). Alterations in glutathione metabolism have also been confirmed in humans that abuse alcohol (34).

Studies performed in rats chronically fed with ethanol also revealed activation of tissue remodeling in the lung. In particular, ethanol induced activation of matrix-degrading enzymes of the matrix metalloproteinase family (29) and increased the production of the profibrotic factor transforming growth factor-β1 (4). These studies suggest that activation of tissue remodeling, with subsequent alterations in extracellular matrix expression, deposition, and degradation, might represent another mechanism by which ethanol can affect the lung and render it susceptible to acute lung injury (42).

More recently, we found that chronic ethanol ingestion also increases the expression of fibronectin in the lung. This multidomain cell-adhesive glycoprotein is increased in acute lung injury, and its production is elicited experimentally by agents associated with this illness (e.g., paraquat) (27, 43). Although the exact role of fibronectin in lung is unknown, its ability to promote matrix deposition and coagulation, and to induce the migration and activation of inflammatory cells in vitro, among other functions, suggests that fibronectin is not only a sensitive marker of injury but that it is a key player in the pathogenesis of acute lung injury (44). Accordingly, this report explores the intracellular mechanisms that mediate ethanol-induced fibronectin expression in fibroblasts in order to gain insight into the pathways involved in activation of tissue remodeling in the lungs of experimental animals exposed to ethanol chronically.

MATERIALS AND METHODS

Experimental reagents. α-Bungarotoxin was purchased from Amersham Biosciences (Piscataway, NJ). The anti-cAMP response element-binding protein (CREB) antibody, anti-phospho-CREB antibody, and mitogen enhanced kinase-1 (MEK-1) inhibitor PD-98059 were purchased from Cell Signaling Technology (Beverly, MA). Ethanol, N°°, 2′-O-dibutyl adenosine 3′,5′-cyclic monophosphate, calphostin C, 4-methylpyrazole, and methyllycaconitine (MLA) were purchased from Sigma Chemical (St. Louis, MO). All other reagents...
Detection of mRNAs by RT-PCR. Fibroblasts were exposed to ethanol (0–100 mM) and tested at 2, 6, 8, and 24 h for various mRNAs using an RT-PCR bioluminescence assay. The procedure for bioluminescent detection of mRNA was performed as previously described (38, 41). Amplification of PCR products was achieved using 5′-biotinylated (forward) primers; the 3′ primers were not modified, and the PCR products ranged in size from 300 to 350 bp. Cycled curve studies were performed to ensure that for the amounts of cDNA being amplified, the reaction had not reached plateau of the amplification curve at a constant number of cycles for any primer pair. Negative controls consisted of deionized H2O and RNA without RT-PCR products, and standardization was made to the housekeeping gene β-actin or hypoxanthine phosphoribosyltransferase (HPRT). The biotinylated primer–PCR product was captured on streptavidin-coated plates (Boehringer Mannheim) and probed with digoxigenin (DIG)-labeled probes. The oligos were DIG labeled using DIG Oligonucleotide Tailing kit plates (Boehringer Mannheim). Anti-digoxigenin antibody labeled with the bioluminescent molecule aequorin (Aquaporin; SealLine Sciences, Bogart, GA) was added, and luminescence was measured using a LabSystems Luminoskan Ascent Plate Luminometer after triggering with calcium. Because of its semiquantitative nature, the relative amounts of a specific mRNA were compared with one another within the same experiment. All products were verified by agarose gel electrophoresis to ensure that the predicted mRNA species was being examined.

Primers and probes for RT-PCR reactions were based on GenBank published sequences and are as follows: rat fibronectin forward primer (AGAGCATACCTCTCCAGAG), rat fibronectin reverse primer (CTGTCATACGGTGGGGA), rat fibronectin probe (TCTATACACCTT GCAACCA); rat HPRT forward primer (GGATAGGGA GAGATGGGGA), rat HPRT reverse primer (CAGGCAA GCTTGCACCTT), rat HPRT probe (GCTTGACACGAAAAAGCCA); mouse fibronectin forward primer (CTTGTGACACTCGCGTAG), mouse fibronectin reverse primer (CAGCTTTCAACAGACCTAGC), mouse fibronectin probe (ACAGAGTCCAATACCATC), mouse β-actin forward primer (ATGGTAGAGATGATGCT), mouse β-actin reverse primer (AGATGAGTATGCTGAAAG), mouse β-actin primer (GATGGCTACGATACTGT); mouse nAChR α7 forward primer (GTAACCGATTTGGCGTTC), mouse nAChR α7 reverse primer (CCGGAGCTTGTGGTCAA), mouse nAChR α7 probe (GGTGTGGCGACACTG).

**125I-α-bungarotoxin-binding and competition assay.** The α-bungarotoxin (α-BGT) binding assay was performed using the method of Breese et al. (7, 8) to detect nAChRs on the surface of fibroblasts. Fibroblasts (1 × 10^6) incubated with or without ethanol (60 mM) for 16 h at 37°C/5% CO2. Control cells were incubated with binding buffer (TBS + 0.2% BSA). The cells were rinsed twice in binding buffer at 37°C for 5 min, followed by three washes in TBS for 15 min and one wash in PBS for 5 min. Afterward, 125I radioactivity bound to the functional nAChRs contained in the samples was quantified by a gamma counter.

The α-BGT competition assay was performed on primary mouse lung fibroblasts (1 × 10^6) incubated with or without ethanol (60 mM) in the presence or absence α-BGT (0.5–2.5 nM) or a second specific α7nAChR inhibitor, ML-1331 (0.5–1.0 nM), for 8 h at 37°C and 5% CO2. Afterwards, cells were harvested and mRNA levels for fibronectin and β-actin were determined as described above.

**Examination of fibronectin gene transcription.** To evaluate for fibronectin gene transcription, the pFN(1.2kb)LUC promoter construct was introduced into murine NIH/3T3 fibroblasts via electroporation to create stable transfectants (32). pFN(1.2kb)LUC contains ~1,200 bp of the 5′ flanking region of the human fibronectin gene isolated from the human fibrosarcoma cell line HT-1080. This construct includes 69 bp of exon 1, a CAAT site located at −150 bp, and the sequence ATATAA at −69 bp. Endogenous levels of CREB activated by phosphorylation at Ser133 were detected using an antibody specific for CREB phosphorylated at Ser133. CREB phosphorylation at Ser133 was detected using an antibody specific for CREB phosphorylated at Ser133. CREB phosphorylation at Ser133 was detected using an antibody specific for CREB phosphorylated at Ser133.
three cAMP response elements (CREs) located at −415, −270, and −170 bp, and an SP-1 site at −102 bp from the transcription start site. The promoter was subcloned into the Simul site of pGL3 Basic Luciferase Reporter Vector (Promega, Madison, WI) (32).

The stably transfected NIH/3T3 fibroblasts were maintained in DMEM with 4.5 g/l of glucose supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic solution (100 U/ml penicillin G sodium, 100 U/ml streptomycin, 0.25 µg/ml amphotericin B) and incubated in a humidified 5% CO2 incubator at 37°C. The cells were harvested by trypsinization with 2.5% trypsin and 5.3 mM EDTA (Sigma), washed with PBS, counted, and plated at 1.5 × 10^5 cells/ml in 12-well tissue culture dishes in 10% FBS. Concurrently, cells were treated with ethanol (0–160 mM) for various periods of time. Afterwards, the cells were tested for luciferase activity. For this, the cells were harvested by cell scraper, washed with PBS, resuspended in 100 µl of cell lysis buffer (Promega), and sonicated, and a 10-µl aliquot was tested by adding 50 µl of Luciferase Assay Reagent (Promega). Light intensity was measured using a Labsystems Luminoskan Ascent Plate Lumimeter. Results were recorded as normalized light units and adjusted for total protein content that was measured using the Bradford method (6).

Electrophoretic DNA mobility shift assay. Fibroblasts (3 × 10^5) were seeded onto 150-mm² tissue culture flasks and incubated in 10% FBS for 24 h with and without concurrent treatment with ethanol at the doses described above. Cells were washed with ice-cold PBS, and nuclear binding proteins were extracted by a published method (18). Protein concentration was determined by the Bradford method using Bio-Rad protein assay reagent (6). Double-stranded CREB consensus oligonucleotide (5′ AGAGATTGCCTGTGGTCAGAGAGCTAG) was labeled with biotin-N4-CTP using terminal deoxynucleotidyl transferase enzyme. Nuclear protein (5 µg) was incubated with biotin-labeled double-stranded CREB for 20 min at room temperature as described previously (32). For competition reactions, non-biotin-labeled consensus and mutated CREB double-stranded oligonucleotides (5′ AGAGATTGCGCTGTGGTCAGAGAGCTAG) were added to the reaction mixture at 50× molar concentration. DNA-protein complexes were separated on 6% native polyacrylamide gel (20:1 acrylamide/bis ratio) in low ionic strength buffer (22.25 mM Tris borate, 22.25 mM sodium, 0.1% SDS) for 2–3 h at 4°C at 10 V/cm. DNA and DNA-protein complexes were transferred to nylon membrane using a Bio-Rad Trans Blot semidry transfer apparatus for 1 h and 25 V and cross-linked using the Fb-UXL-1000 UV Crosslinker (Fisher Scientific, Pittsburgh, PA). DNA and DNA-protein complexes were detected using Streptavidin-Horseradish Peroxidase Conjugate and Lightshift Chemiluminescent Substrate according to the manufacturer’s instructions (Pierce Biotechnology, Rockford, IL). The membrane was exposed to X-ray film for 1 min.

Screening for LPS. Experimental reagents were reconstituted in LPS-free water (Sigma). All treatment materials and culture media were screened with a limulus-based endotoxin assay with a sensitivity of 0.06 ng/ml (EndoTect-Schwarz/Mann Biotech, Cleveland, OH) as described (39, 40). Reagents were found to remain endotoxin free throughout all experiments.

Animal model of chronic ethanol ingestion and lung immunohistochemistry. The animal model of chronic ethanol ingestion has been described previously (9, 21, 22, 25, 29, 52). Briefly, young adult male Sprague-Dawley rats (200–250 g) were fed the Lieber-DeCarli liquid diet (Research Diets, New Brunswick, NJ) containing either ethanol (36% total calories) or the isocaloric carbohydrate substitution with Maltin-Dextrin (control diet). The diets were otherwise identical in protein, lipid, and essential nutrient composition. This is a standard experimental diet in ethanol ingestion models, and we have used it extensively. During the first 2 wk of the dietary regimen, the ethanol-fed rats were gradually acclimated to the ethanol, receiving 12% of their total calories as ethanol (1/3 strength) for 1 wk, then 24% of their total calories as ethanol (2/3 strength) for 1 wk, and then full-strength diet (36% of total calories as ethanol) for 4 wk, for a total of 6 wk of ethanol ingestion. Afterwards, the animals were killed, followed by isolation of the lungs for RNA isolation (see above) and immunohistochemistry. Control and experimental lungs were processed and submitted to immunohistochemistry with an anti-fibronectin antibody as previously described (39).

Statistical evaluation. Means ± SD of the mean were calculated for all experimental values. Significance was assessed by ANOVA followed by Student’s t-test. All experiments were repeated four to eight times.

RESULTS

Ethanol increases fibronectin expression and deposition in rat lungs. To examine the effects of ethanol on fibronectin expression in lung, rats were fed the Lieber-DeCarli isocaloric liquid diet that contains 36% of total calories provided as ethanol. This diet was previously shown to increase endotoxin-mediated acute edematous injury in rat lungs isolated and perfused ex vivo (25). After 6 wk, the lungs of control rats and rats fed with ethanol diets were harvested and processed for the detection of fibronectin mRNA (by RT-PCR) and protein (by immunohistochemistry). The lungs of rats fed with ethanol showed increased fibronectin mRNA content compared with pair-fed control animals (Fig. 1A). As expected, immunohistochemical analysis of control lungs showed fibronectin deposition in vascular airway structures and within alveolar septae (Fig. 1B).

Because of the increased deposition of fibronectin around fibroblasts in the lungs of ethanol-exposed rats, we explored the effects of ethanol in cultured lung fibroblasts with the intention of developing an in vitro model of ethanol-induced fibronectin expression. Using RT-PCR, we found that ethanol induced the expression of endogenous fibronectin mRNA in primary rat lung fibroblasts (Fig. 2A). This coincided with an increase in fibronectin protein production as determined by Western blotting (Fig. 2B). These observations identify the lung fibroblast as a potential target for ethanol in the lung.

The data presented above also suggested that the effects of ethanol on fibronectin expression occur at the level of gene transcription. To test this, we used NIH/3T3 fibroblasts that were considered a suitable model for our studies because they respond to ethanol in a similar fashion as primary lung fibroblasts. This is demonstrated in Fig. 3A where NIH/3T3 cells treated with ethanol (60 mM) showed increased accumulation of endogenous fibronectin mRNA as determined by RT-PCR. The increase in fibronectin mRNA was detectable as early as 8 h after ethanol stimulation. As expected, the ethanol-induced response was associated with a subsequent increase in fibronectin protein production as determined by Western blotting that was highest at 48 h (Fig. 3B).

When the transcription of the gene was tested in NIH/3T3 cells stably transfected with a construct containing the human fibronectin promoter fused to the luciferase reporter gene pFN(1.2kb)LUC, as previously reported (32), we found that...
ethanol increased the transcription of pFN(1.2kb)LUC compared with nontreated control fibroblasts (Fig. 4, A–C). As before, the effects of ethanol were both time dependent (peaking around 8 h) and dose dependent (optimal at 60 mM). At the concentrations used, ethanol had no effect on cell viability (not shown).

**Fig. 2.** Ethanol induces the synthesis of fibronectin in rat lung fibroblasts. A: induction of fibronectin mRNA. Rat lung fibroblasts were cultured in the presence of physiological concentrations of ethanol (60 mM) for 24 h and harvested, and the cell extracts were processed for RT-PCR analysis of fibronectin mRNA. Relative fibronectin mRNA values are shown as means ± SD. Note that ethanol induced fibronectin mRNA accumulation compared with nontreated control cells. *Significant difference from control cells (n = 6; P < 0.05). B: induction of fibronectin protein. Fibroblasts were cultured as described above for up to 48 h. Afterwards, the cell extracts were tested for fibronectin protein by Western blotting. The data are depicted as densitometric units obtained from analysis of a representative Western blot. Ethanol induced fibronectin production at both 24 and 48 h (n = 5).

**Ethanol induction of fibronectin gene transcription is dependent on protein kinase activity and the induction of CREB.** The stimulation of fibronectin by serum and other agents has been shown to be preceded by protein kinase activation and phosphorylation of the transcription factor CREB, followed by CREB binding to CREs in the fibronectin promoter (32). To...
test whether this pathway also mediates the ethanol-induced fibronectin response, we performed the following experiments. First, we tested the effects of protein kinase inhibitors on the ethanol-induced response. As depicted in Fig. 5A, a protein kinase C inhibitor, calphostin C (24), abolished the constitutive expression of fibronectin as well as expression induced by ethanol. PD-98059, an inhibitor of MEK-1 (2), which is upstream of Erk-1 and Erk-2 in the mitogen-activated protein kinase pathway, also inhibited the ethanol-induced fibronectin response (Fig. 5B). The inhibitory effects of these agents at the concentrations used were not associated with increased cell death (not shown).

Second, we tested the effects of ethanol on CREB. The exposure of fibroblasts to 60 mM ethanol induced the phosphorylation of CREB in a time-dependent fashion (Fig. 6A). This event was associated with increased binding of CREB to DNA as determined by EMSA (Fig. 6B). Note that a competing oligonucleotide abolished the binding of CREB, whereas the mutated oligonucleotide did not affect CREB binding.

**Ethanol induction of fibronectin gene transcription is dependent on specific promoter elements.** The above studies suggested a role for specific promoter elements within the fibronectin gene, namely the CREs, in the ethanol-induced effect. To confirm this role, we tested fibroblasts transfected...
Calphostin C (ACC) inhibited the ethanol-stimulated expression of the gene (pFN(1.2kb)LUC) over control as before. The stimulatory effect lacked all three CREs. Ethanol stimulated the transcription of CREs, or we tested cells transfected with pFN(0.2kb)LUC that sequences present in pFN(1.2kb)LUC but contain the three transfected with pFN(0.5kb)LUC that lack most of the 5' sequences proximal to the CREs. Ethanol stimulated the transcription of pFN(1.2kb)LUC over control as before. The stimulatory effect of ethanol was unaffected when the deletion construct pFN(0.5kb)LUC was tested, indicating that 5' sequences proximal to the CREs are not needed for optimal stimulation. In contrast, little stimulation was noted for ethanol when all CREs were lacking.

These data suggest, but do not prove, a role for CREs in the ethanol-induced response. To strengthen the association between CREs and the response tested, we measured fibronectin expression in cells exposed to 60 mM ethanol after transfection with a competing consensus CRE oligonucleotide (Fig. 7A). The consensus CRE oligonucleotide greatly diminished the ethanol-induced fibronectin response, whereas the control-mutated CRE oligonucleotide had no effect.

Ethanol stimulates fibronectin gene transcription via nAChR-dependent signals. Ethanol has been shown to act on nAChRs in neuronal cells (16). Because nAChRs have been detected in NIH/3T3 fibroblasts and monkey lung fibroblasts (49), among other nonneuronal cells, we examined the role of these receptors in our system. First, we demonstrated that NIH/3T3 cells express mRNA coding for α7 nAChRs (16). Consistent with the expression of nAChRs, we found binding sites for α-BGT on the surface of these receptors in our system. First, we demonstrated that NIH/3T3 cells express mRNA coding for α7 nAChRs (16). Consistent with the expression of nAChRs, we found binding sites for α-BGT on the surface of these receptors in our system. First, we demonstrated that NIH/3T3 cells express mRNA coding for α7 nAChRs (16). Consistent with the expression of nAChRs, we found binding sites for α-BGT on the surface of these receptors in our system.

Together, these observations indicated that fibroblasts express α7 mRNA and have nAChR protein on their surface. To confirm a role for nAChRs in the ethanol-induced fibronectin response, we pretreated transfected fibroblasts with α-BGT before exposing them to ethanol. As shown in Fig. 8B, α-BGT completely prevented the expression of the fibronectin in response to ethanol (P < 0.001).

![Image](http://ajplung.physiology.org/DownloadedFrom/10.2203.36-04-17/L980)
To further define the specificity of the nAChR binding, studies with both α-BGT and MLA, two separate α7 nAChR competitors, were tested on primary lung fibroblasts. As shown in Fig. 8C, pretreatment of fibroblasts with unlabeled α-BGT (0.5–2.5 nM) or with MLA (0.25–1 μM) resulted in inhibition of fibronectin mRNA expression in response to ethanol.

Ethanol-induced fibronectin response is dependent on ethanol metabolism. In hepatic cells, ethanol induction of procollagen can be inhibited by 4-methylpyrazole, a blocker of alcohol dehydrogenase (17). This suggests that the ability of ethanol to stimulate matrix gene expression is dependent on its metabolism and conversion into aldehyde. To test this possibility in our system, NIH/3T3 transfected fibroblasts were pretreated with 4-methylpyrazole before stimulation with ethanol. As depicted in Fig. 9A, this treatment inhibited the induction of fibronectin by ethanol. In contrast, 4-methylpyrazole did not affect the induction of fibronectin by nicotine, another ligand for α7 nAChRs (Fig. 9B). Consistent with the need for ethanol metabolism, we found that acetaldehyde mimicked its ability to induce fibronectin expression (Fig. 9A, inset).

Ethanol induction of fibronectin expression in primary mouse lung fibroblasts. To determine whether similar results could be obtained in fibroblasts that are more relevant to lung and the in vivo model of ethanol ingestion, primary lung fibroblasts were isolated from transgenic mice that contain the fibronectin gene promoter. Together, these studies suggest that ethanol induces fibronectin gene transcription by acting on α-BGT-sensitive nAChRs and by inducing the phosphorylation and nuclear translocation of CREB, a key transcription factor capable of initiating fibronectin gene expression (32).

Finally, as demonstrated in Fig. 10G, the phosphorylation of CREB by ethanol increased as soon as 2 h after treatment and remained slightly elevated as long as 24 h.

To determine the role of the α7 nAChR in ethanol induction of the fibronectin gene, primary lung fibroblasts were isolated and tested for their ability to produce fibronectin after ethanol stimulation. As shown in Fig. 11A, fibroblasts deficient in α7 nAChR were still able to produce more fibronectin mRNA when treated with ethanol for 12–48 h as determined by RT-PCR. Western blot data demonstrated that ethanol was also able to increase the secretion of fibronectin protein after 48 h of stimulation (Fig. 11B). Together, these data generated in fibroblasts deficient in α7 nAChRs show that α7 nAChRs are not required for induction of fibronectin expression in the setting of ethanol stimulation.

DISCUSSION

Mechanisms of ethanol induction of fibronectin in fibroblasts. This report demonstrates for the first time that chronic ethanol ingestion in animals results in increased expression of fibronectin mRNA and deposition of its protein in the lung. In addition, it identifies the lung fibroblast, among other cells, as a target for ethanol. Studies performed with cultured fibroblasts suggest that ethanol induces fibronectin gene transcription by acting on α-BGT-sensitive nAChRs and by inducing the phosphorylation and nuclear translocation of CREB, a key transcription factor capable of initiating fibronectin gene expression (32). Finally, the report demonstrates that ethanol induction of fibronectin is dependent on ethanol metabolism, protein kinase activity, and specific transcriptional elements within the fibronectin gene promoter. Together, these studies suggest that ethanol can directly affect lung fibroblasts and induce their expression of fibronectin. We speculate that this may result in alterations in the composition of the lung matrix and that this...
represents yet another potential mechanism by which ethanol renders the host susceptible to acute lung injury.

For years, it has been known that ethanol induces tissue remodeling in the liver where it can cause fibrosis and cirrhosis (48), but its effects on the lung have been poorly recognized until recently. In the liver, both ethanol and its metabolite acetaldehyde are considered to be fibrogenic and have been shown to induce the expression of collagen (10). Ethanol induces the production of collagen and the expression of procollagen mRNA in fibroblasts and in primary cultures of liver stellate cells (19, 20, 50). Similar to our observations with fibronectin, the ethanol-induced collagen response in liver cells was found to be maximal with doses of ethanol between 50 and 100 mM, optimal after 24-h exposure, dependent on protein synthesis, and appeared to occur at the level of gene transcription (19). In that system, as in ours, the response was abolished by an inhibitor of ethanol metabolism to acetaldehyde, 4-methylpyrazole, suggesting that ethanol metabolism was needed to observe its effects on collagen expression. Of interest, the induction of collagen by ethanol was detected in liver stellate cells, but not in primary cultures of hepatocytes, suggesting that not all cells of an organ respond to ethanol equally. Consistent with a role for ethanol metabolism, we found that acetaldehyde mimics the stimulatory effect of ethanol in our system.

Ethanol-induced fibronectin expression has also been demonstrated in the liver. Increased total and cellular fibronectin protein production was detected in the liver of rats exposed to ethanol in their diet for 8–12 wk (20). However, the intracellular pathways responsible for its induction and how they relate to fibronectin induction in pulmonary cells are unclear. Our studies show that ethanol induction of fibronectin is dependent on the activity of protein kinases such as protein kinase C and MEK-1/Erks. This is reminiscent of the work of Svegliati-Baroni and colleagues (50) who demonstrated that the stimulation of fibronectin expression in human hepatic stellate cells is associated with a time-dependent phosphorylation of pp70(S6K) and Erk-1 and -2. In their system, the stimulatory effect of ethanol was also inhibited by calphostin C and PD-98059.
This study demonstrates the presence of nAChR expression in lung fibroblasts and that α7 nAChRs play a role in the ethanol induction of fibronectin via specific intracellular signals. These observations are consistent with most other studies available to date in neuronal cells showing that ethanol acts mainly via nAChRs.

Role of nAChRs in ethanol-induced fibronectin expression. This study demonstrates the presence of α7 nAChRs in primary lung fibroblasts and that α-BGT-sensitive nAChRs play a role in the ethanol induction of fibronectin via specific intracellular signals. These observations are consistent with most other studies available to date in neuronal cells showing that ethanol acts mainly via nAChRs. α7 nAChRs are a family of multimeric acetylcholine-triggered cation channel proteins that form the predominant excitatory neurotransmitter receptors on muscles and nerves in the peripheral nervous system. They are also expressed in lower amounts throughout the central nervous system. At least 13 genes that code for nAChRs have been identified to date, four β-subunits and nine α-subunits. In each of these receptors, the various subunits assemble into pentamers in a homomeric or heteromeric fashion. The most abundant homomeric form is (α7)5. This is the receptor that our data suggest that α7 nAChRs might play a role.

NIH/3T3 fibroblasts (3). Evidence for the expression of functional nAChRs in lung cells is also available. α7 nAChR subunits have been detected in both human and mouse bronchial epithelial cells and in submucosal glands (30). α7 has also been reported in small cell lung cancer (SCLC) and SCLC cell lines, and the growth of these cells can be inhibited by α-BGT, an antagonist of α7 receptors (11). Others have demonstrated in primates that nicotine, by binding to specific nAChRs, can affect lung development (49). When Sekhon et al. (49) examined for nAChRs in control animals, they detected α7 predominantly in fibroblasts surrounding the walls of airways and vessels, among other cell types. The expression of this receptor increased dramatically in animals exposed to nicotine. This was associated with increased collagen deposition surrounding the cartilaginous large airways and vessels. Overall, our observations and those described above suggest that lung cells (in particular fibroblasts) express functional nAChRs and that, by binding to these receptors, ethanol (and other ligands such as nicotine) can affect tissue remodeling in lung. Fibroblasts are not the only cells that recognize ethanol, and this explains the diffuse nature of the staining for fibronectin in the lungs of ethanol-treated rats.

In view of the above, we focused our attention on the possibility that α7 nAChRs mediated the stimulatory effect of ethanol. Unexpectedly, we found that in primary lung fibroblasts harvested from mice lacking α7 nAChRs, ethanol still stimulated the expression of the fibronectin gene. This suggests that α7 nAChRs are not required for ethanol to stimulate fibronectin expression in lung fibroblasts and that other α-BGT-sensitive nAChRs (perhaps α3β2, α4β2, α8, or α9/10 nAChRs) might play a role. In addition, this finding suggests that α7 nAChRs are not required for ethanol to stimulate fibronectin expression in lung fibroblasts and that other α-BGT-sensitive nAChRs (perhaps α3β2, α4β2, α8, or α9/10 nAChRs) might play a role. In addition, this finding suggests that α7 nAChRs are not required for ethanol to stimulate fibronectin expression in lung fibroblasts and that other α-BGT-sensitive nAChRs (perhaps α3β2, α4β2, α8, or α9/10 nAChRs) might play a role.
Fig. 10. Ethanol stimulation of fibronectin in primary lung fibroblasts. A: induction of fibronectin mRNA. Primary lung fibroblasts were treated with ethanol (60 mM) for 24 h, and the cell extracts were processed for RT-PCR analysis of fibronectin mRNA. Relative fibronectin mRNA values are shown as means ± SD. Note that ethanol induced fibronectin mRNA accumulation by 16 h compared with nontreated control cells. *Significant difference from time 0 control fibroblasts, P < 0.05. 
B: induction of fibronectin protein. Primary lung fibroblasts were cultured as described above for up to 48 h. Afterwards, the cell extracts were tested for fibronectin protein by Western blotting. The data are depicted as densitometric units obtained from analysis of a representative Western blot gel. 
C: dose-dependent stimulation of fibronectin gene transcription. Primary lung fibroblasts were treated with ethanol, and cells were harvested for the detection of fibronectin gene transcription. Data are presented as means ± SD (n = 6). Note that ethanol significantly induced the transcription of the fibronectin gene at a dose of 60 mM. *Significant difference from time 0 control fibroblasts, P < 0.001. 
D: ethanol stimulates fibronectin expression in a time-dependent manner. Primary lung fibroblasts were treated with ethanol (60 mM) for 0–48 h, and cells were harvested and processed to assess fibronectin gene transcription as described above. Data are presented as means ± SD (n = 6). Note that ethanol doubled the transcription of the fibronectin gene at 8 h compared with time 0 control cells (P < 0.001). 
E: protein kinase C-dependent production of fibronectin. Primary lung fibroblasts were treated with ethanol (60 mM) for 16 h in the presence and absence of the protein kinase C inhibitor calphostin C. Data are presented as means ± SD. Note that ACC inhibited the ethanol-stimulated expression of the gene (n = 6). The inactive reagent had no effect (not shown). *Significant difference from ethanol-treated fibroblasts, P < 0.001. 
F: mitogen-activated protein kinase-dependent production of fibronectin. Primary lung fibroblasts were cultured as before in the presence of ethanol (60 mM) with or without the MEK-1 inhibitor PD-98059 (50 μM) for 16 h, and fibronectin gene transcription was measured. Data are presented as means ± SD. Note that the MEK-1 inhibitor prevented the stimulatory effect of ethanol, but it did not block the constitutive expression of the gene (n = 6). *Significant difference from ethanol-treated fibroblasts, P < 0.001. 
G: induction of CREB phosphorylation. Primary lung fibroblasts were treated with ethanol (60 mM) and processed for Western blotting with an antibody to phosphorylated CREB (n = 5). In this representative Western blot, note that ethanol induced the phosphorylation of CREB and ATF-1, most noticeably by 2–4 h.
also raises the possibility of parallel pathways that are both nAChR dependent and independent.

Another interesting observation relates to the ability of 4-methylpyrazole to inhibit the effect of ethanol on fibronectin expression but not that of nicotine. In the case of ethanol, it appears that signal transduction requires alcohol metabolism and acetaldehyde production. This and other observations suggest that even though both ethanol and nicotine can stimulate nAChRs, they trigger different intracellular signals. This, together with differences in cell recognition and metabolism, might explain why nicotine and ethanol abuse are associated with the development of different clinical entities.

**Implications for our understanding of acute lung injury.** We have shown that ethanol stimulates fibronectin expression in lung fibroblasts both in vitro and in vivo. It is important to point out that the level of ethanol used in the in vitro studies, 60 mM, is physiologically relevant because it translates to a blood alcohol level of ~0.1 g/dl, which is within the range one might find in a moderate to heavy drinker (24). In the rats fed with the Lieber-DeCarli liquid diet containing ethanol (36% total calories), the blood alcohol concentrations averaged ~117 ± 7.9 mg/dl (37). Despite the above, one should be careful when extrapolating our findings to the situation in vivo because the mechanisms involved in the effects observed might differ in view of the differences in time of exposure, metabolism, etc. Independent of the mechanisms involved, the observation that ethanol can induce fibronectin expression in the lung is an important one because fibronectin deposition in the lung is an important one because fibronectin deposition is increased in many, if not all, forms of clinical and experimental acute lung injury, and it has been implicated in the pathogenesis of this illness (27, 43, 44). Its exaggerated deposition under these circumstances has considerable effects on lung structure. For example, fibronectin promotes collagen deposition in connective tissue (31). In doing so, the newly deposited fibronectin-containing matrices provide a scaffold for the migration of epithelial cells across denuded basement membranes and the organization of immune cells and fibroblasts in extravascular spaces (13). Fibronectin also affects many cellular functions. It has been shown to promote the adhesion, migration, proliferation, and differentiation of many lung cell types including epithelial and endothelial cells and fibroblasts (27, 43). With regard to immune cells, fibronectin has been shown to be chemotactic to monocytes and endothelial cells, among other cells (44), and to stimulate their expression of proinflammatory cytokines that, in turn, could amplify the inflammatory and repair responses of the lung after injury (5, 37, 45, 46).

The biological effects of fibronectin are possible because of its ability to interact with specific cell surface integrin receptors capable of signal transduction (12). The activation of the integrin fibronectin receptor α5β1 elicits the activation of intracellular signals including increased cAMP levels, calcium fluxes, and the activation of protein kinases. These events lead to the induction of potent transcription factors including activator protein-1 and nuclear factor-κB (37, 45, 46) that control the transcription of many genes including the proinflammatory cytokines interleukin-1β and tumor necrosis factor-α and vascular cell adhesion molecule-1.

In view of the above, it is postulated that the exaggerated deposition of fibronectin in the lungs of ethanol-treated animals alters the composition of the lung extracellular matrix. In turn, the newly deposited fibronectin-containing matrix primes lung resident and incoming cells to respond to injurious agents in an exaggerated manner. In doing so, fibronectin promotes the development of an aggressive uncontrolled tissue remodeling and inflammatory response that leads to tissue destruction rather than repair after injury. Further delineation of the factors and conditions that regulate ethanol-induced fibronectin expression, and the receptor and signaling events involved, is required before a full understanding of the true consequences this process has in the lung is possible.

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