TGFβ1 Mediates Alcohol-Induced Nrf2 Suppression in Lung Fibroblasts

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Background: Chronic alcohol ingestion induces the expression of transforming growth factor beta-1 (TGFβ1), inhibits nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-mediated activation of the antioxidant response element (ARE), depletes alveolar glutathione pools, and potentiates acute lung injury. In this study, we examined the mechanistic relationship between TGFβ1 and Nrf2-ARE signaling in the experimental alcoholic lung.

Methods: Wild-type mice were treated ± alcohol in drinking water for 8 weeks and their lungs were assessed for Nrf2 expression. In parallel, mouse lung fibroblasts were cultured ± alcohol and treated ± sulforaphane (SFP; an activator of Nrf2), ±TGFβ1, ±TGFβ1 neutralizing antibody, and/or ±activin receptor-like kinase 5 inhibitors (to block TGβ1 receptor signaling) and then analyzed for the expression of Nrf2, Kelch-like ECH-associated protein 1 (Keap1) and TGFβ1, Nrf2-ARE activity, and the expression of the Nrf2-ARE-dependent antioxidants glutathione s-transferase theta 2 (GSTT2) and glutamate-cysteine ligase catalytic subunit (GCLC). Finally, silencing RNA (siRNA) of Nrf2 was then performed prior to alcohol exposure and subsequent analysis of TGFβ1 expression.

Results: Alcohol treatment in vivo or in vitro decreased Nrf2 expression in murine whole lung and lung fibroblasts, respectively. In parallel, alcohol exposure in vitro decreased Keap1 gene and protein expression in lung fibroblasts. Furthermore, alcohol exposure increased TGFβ1 expression but decreased Nrf2-ARE activity and expression of the ARE-dependent genes for GSTT2 and GCLC. These effects of alcohol were prevented by treatment with SFP; in contrast, Nrf2 SiRNA expression exacerbated alcohol-induced TGFβ1 expression. Finally, TGFβ1 treatment directly suppressed Nrf2-ARE activity whereas blocking TGFβ1 signaling attenuated alcohol-induced suppression of Nrf2-ARE activity.

Conclusions: Alcohol-induced oxidative stress is mediated by TGFβ1, which suppresses Nrf2-ARE-dependent expression of antioxidant defenses and creates a vicious cycle that feeds back to further increase TGFβ1 expression. These effects of alcohol can be mitigated by activation of Nrf2, suggesting a potential therapy in individuals at risk for lung injury due to alcohol abuse.

Key Words: Nrf2, Antioxidant Response Element, TGFβ1, Glutathione, Lung Fibroblasts, Alcohol.

OUR GROUP RECENTLY reported that chronic alcohol ingestion induces oxidative stress in the lung through suppression of nuclear factor (erythroid-derived 2)-like 2–antioxidant response element (Nrf2-ARE) activity in alveolar epithelial cells (Jensen et al., 2013). This chronic oxidative stress underlies the development of the "alcoholic lung" phenotype, which when evaluated clinically and in experimental models includes near depletion of the glutathione pools in the alveolar space (Holguin et al., 1998; Moss et al., 2000), impaired alveolar epithelial surfactant production (Guidot and Brown, 2000; Holguin et al., 1998) and barrier formation (Burnham et al., 2003; Guidot et al., 2000), and decreased alveolar macrophage innate immune capacity (Joshi et al., 2005). These and other previously unrecognized effects of alcohol on the lung are likely causative in the strong association between alcohol use disorders and serious pulmonary diseases including pneumonia and acute lung injury (Happel and Nelson, 2005; Moss et al., 2003).

Several studies have demonstrated the lung-protective effects of Nrf2. For example, activation of Nrf2 decreases fibroblast proliferation and fibrosis in mouse models of bleomycin-induced lung injury (Liu et al., 2013). Conversely, Nrf2 silencing causes myofibroblast transdifferentiation of primary lung fibroblasts (Artaud-Macari et al., 2013). These and other studies suggest that a decrease in Nrf2-ARE activity promotes chronic fibrotic remodeling (Walters et al.,

2008). Furthermore, conditional deletion of Nrf2 in Clara cells exacerbates epithelial dehiscence and inflammation in response to hyperoxia-induced lung injury (Reddy et al., 2011), and Nrf2 knockout mice are more susceptible to aspiration-mediated pneumonitis (Davidson et al., 2013). Taken together, the experimental evidence to date has elucidated a pivotal role for Nrf2 in regulating the responses to both acute and chronic lung injury.

We had previously identified that alcohol exposure and ingestion is associated with increase in expression and activation of transforming growth factor beta-1 (TGFβ1) leading to impaired alveolar epithelial barrier function and aberrant tissue remodeling (Bechara et al., 2004; Roman et al., 2005). Several studies have suggested that there is an association between Nrf2 and TGFβ1. For example, activation of Nrf2 attenuates TGFβ1-induced liver and lung fibrosis (Nakamura et al., 2011; Oh et al., 2012b). In airway smooth muscle cells, the profile of increased TGFβ1 and diminished Nrf2 is observed in patients with reactive airways (Michaeloudes et al., 2011). We previously showed that alcohol exacerbates fibrotic disrepair following bleomycin-induced acute lung injury in mice and that this was associated with alcohol-induced phenotype alteration in lung fibroblasts (Sueblinvong et al., 2014). Therefore, we hypothesized that the now well-characterized alcohol-induced oxidative stress in the lungs, as well as the associated changes in lung fibroblast function, are mediated by a relative imbalance between Nrf2 and TGFβ1.

MATERIALS AND METHODS

Animals and Alcohol Treatment

Three month old C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were fed with standard chow and water ad libitum. In the alcohol treatment group, mice were started on alcohol with an initial concentration of 5% (v/v) in their drinking water that was increased by 5% every 3 days until a final concentration of 20% was reached. Mice were then continued on 20% alcohol for 8 weeks and then euthanized and their lungs collected for analysis. All studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University and conformed to institutional standards for the humane treatment of laboratory animals.

Cell Culture and Treatment

Primary lung fibroblasts isolated from the lungs of control-fed or alcohol-fed mice were expanded in vitro and harvested for mRNA and protein analysis (passage 4). In parallel, primary lung fibroblasts from control-fed mice and NIH 3T3 mouse lung fibroblasts (ATCC, Manassas, VA) were cultured in DMEM with 4.5 g/l glucose supplemented with 20% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were seeded in 6-well plates (for mRNA and protein expression analyses) or 96-well plates (for Nrf2-ARE activity using a luciferase reporter construct described below). Treatments included alcohol (60 mM), sulforaphane (SFP; 5 μM), recombinant mouse TGFβ1 (5 ng/ml), TGFβ1 neutralizing antibody (2 μg/ml), IgG isotype antibody (2 μg/ml), activin receptor-like kinase 5 (ALK5) inhibitors (SB431542 or A8301, 8 μM) dissolved in dimethyl sulfoxide (DMSO; 1:500 dilution), or with DMSO alone (vehicle control for ALK5 inhibitors).

Messenger RNA Expression Analysis

Messenger RNA mRNA was isolated from whole lung lysate, from primary lung fibroblasts isolated from control-fed and alcohol-fed mice, from primary lung fibroblasts isolated from control-fed mice and then treated with alcohol in vitro, and from NIH 3T3 lung fibroblasts as previously described using RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized and quantitative polymerase chain reaction (PCR) was performed with primers set for 18s, Nrf2, Kelch-like ECH-associated protein 1 (Keap1), glutathione s-transferase theta 2 (GSTT2), glutamate-cysteine ligase catalytic subunit (GCLC), and TGFβ1 using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA); the real-time iCycler sequence detection system (Bio-Rad) was used for the real-time PCR analysis. The level of target mRNA expression was normalized to 18s housekeeping gene levels and relative target mRNA levels were determined according to the comparative cycle threshold method (Applied Biosystems 7900HT Sequence Detection System, User Bulletin No. 2; Foster City, CA) and relative expression values were calculated as previously described (Sueblinvong et al., 2008).

Protein Isolation and Analysis

Total protein from whole lung lysate and lung fibroblasts cell lysate were isolated as previously described (Sueblinvong et al., 2012) and protein from cytosol and nucleus from lung fibroblasts were separated. Equal amounts of protein samples were separated on 4 to 15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membranes. The blots were blocked in blocking buffer (5% nonfat dry milk, 0.1% Tween 20 in Tris-buffered saline, pH 7.4) for 1 hour and then incubated with rabbit anti-mouse Nrf2 (1:500), mouse anti-mouse β-actin (1:1,000), rat anti-mouse TGFβ1 (1:500), rabbit anti-Keap1 (1:1,000), or rabbit anti-mouse histone (for nuclear protein) at 4°C overnight, washed, and incubated for 1 hour at room temperature with an appropriate horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences, Pittsburgh, PA), washed, and visualized via enzyme-linked chemiluminescence using the SuperSignal West Pico kit (Pierce Biotechnology, Rockford, IL).

Analysis of TGFβ1 Protein Expression by Flow Cytometry

Flow cytometry was performed to assess for cell-bound and intracellular TGFβ1 protein expression. After 16 hours of treatment, GolgiPlug (1 μl/ml; BD Bioscience, San Jose, CA) was added and at 24 hours after treatment, cells were harvested, permeabilized, and fixed (using CytoFix/CytoPerm; BD Bioscience) and stained with 1:50 rabbit anti-mouse antibody against TGFβ1 (Abcam, Cambridge, MA) and 1:100 goat anti-rabbit-alexa 488 secondary antibody (Invitrogen, Carlsbad, CA). Cells were fixed with 5% formalin then analyzed for TGFβ1 expression using a FACSCalibur (Becton Dickinson, Mountain View, CA). Flow cytometry data were analyzed using the FlowJo vX.0.7 software (Tree Star, San Carlos, CA). In parallel, conditioned media were collected, cells were separated by centrifugation, proteinase inhibitors were added, and media were frozen prior to analysis for secreted TGFβ1 by ELISA (R&D Systems, Minneapolis, MN).

Nrf2 Silencing RNA Transfection

NIH 3T3 cells were seeded in 6-well plates and transfected the next day with rat Nrf2 silencing RNA (siRNA) vector (Invitrogen) at 5 nM of final siRNA concentration or the same concentration of scrambled control vector (Invitrogen) using HiPerFect (Qiagen) as described in the manufacturer protocol. At 24 hours posttransfection, some groups were exposed to alcohol at 60 mM

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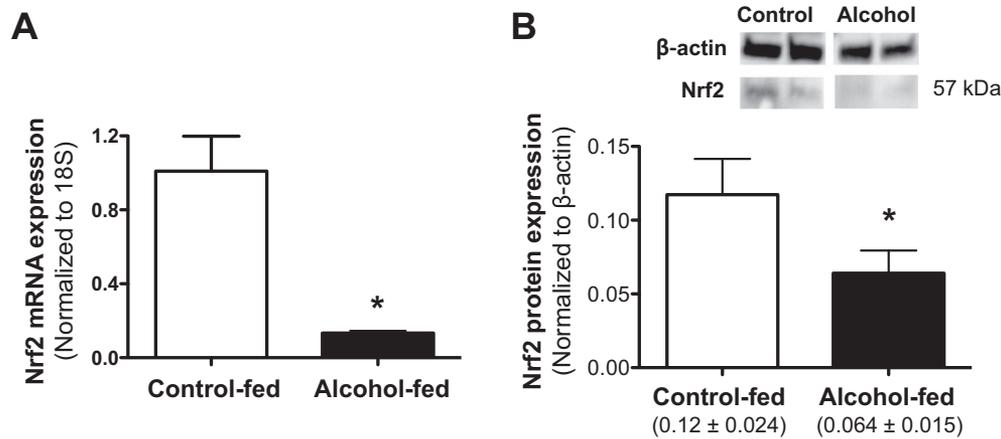
Fig. 1. Chronic alcohol ingestion decreased nuclear factor (erythroid-derived 2)-like 2 (Nrf2) gene and protein expression in the whole lung. Control-fed and alcohol-fed mice were sacrificed after 8 weeks of feeding. The lungs were harvested and analyzed for (**A**) Nrf2 mRNA expression by quantitative polymerase chain reaction and (**B**) Nrf2 protein expression by Western immunoblot analyses (representative gels shown above summary data). $N = 6$ per group. *$p < 0.05$ decreased compared to lungs from control-fed mice.

and all groups were allowed in culture for additional 24 hours. Cells were then harvested for mRNA and TGFβ1 protein expression analysis as described above.

Assessing Nrf2-ARE Activity

To assess for Nrf2-ARE activity, we performed an Nrf2-ARE luciferase activity assay. NIH 3T3 cells were seeded in 96-well plate and transfected with the Cignal ARE reporter using HiPerFect transfection reagent (Qiagen) and were incubated overnight. Transfected cells were then exposed to treatment as described above. A dual-luciferase reporter assay was performed according to the manufacturer protocol (Promega Corp., Madison, WI) and Nrf2-ARE promoter activity values were expressed as ratios of arbitrary units of firefly luciferase/Renilla luciferase activity.

Statistical Analysis

Unpaired *t*-tests or 1-way analysis of variance (ANOVA) were used for comparisons between groups using GraphPad Prism and GraphPad InStat version 4 (La Jolla, CA). Posttest analysis using Bonferroni's method was performed if statistical significant was

reached by 1-way ANOVA. GraphPad Prism and GraphPad InStat version 5 were used to calculate the statistics. Significant differences were accepted at a *p* level of <0.05.

RESULTS

Chronic Alcohol Ingestion Decreased Nrf2 Gene and Protein Expression in the Whole Lung and in the Primary Lung Fibroblasts

As shown in Fig. 1, panel *A*, chronic alcohol ingestion decreased Nrf2 gene expression, as reflected by mRNA levels, by approximately 80% decreased ($p < 0.05$) in the whole lung compared to control-fed mouse lungs. In parallel and as shown in Fig. 1, panel *B*, alcohol-fed mouse lungs showed a decrease of Nrf2 protein expression by approximately 67% ($p < 0.05$) as compared to control-fed mouse lungs. In parallel, lung fibroblasts isolated from control-fed and alcohol-fed mice were expanded ex vivo and likewise analyzed for Nrf2

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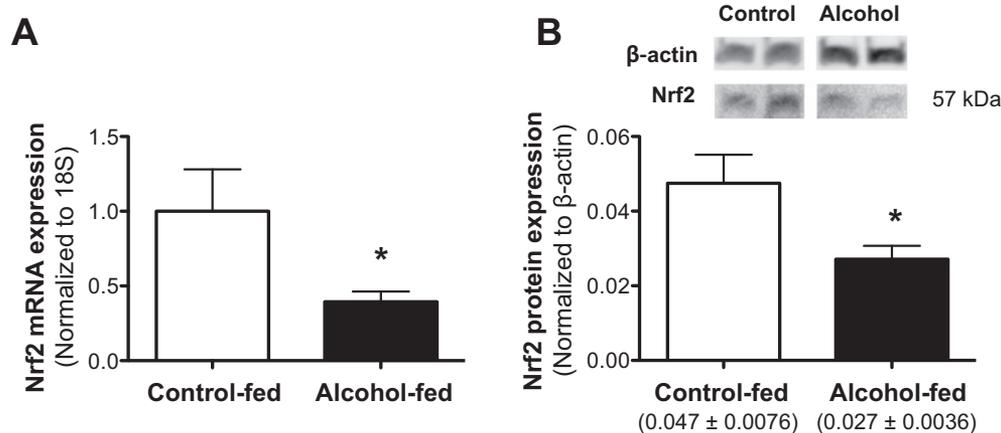
Fig. 2. Chronic alcohol ingestion decreased nuclear factor (erythroid-derived 2)-like 2 (Nrf2) gene and protein expression in the primary lung fibroblasts. Control-fed and alcohol-fed mice were sacrificed after 8 weeks of feeding and lung fibroblasts were isolated. The lung fibroblasts were cultured and expanded and cells were harvested at passage 4 and analyzed for (**A**) Nrf2 mRNA expression by quantitative polymerase chain reaction and (**B**) Nrf2 protein expression by Western immunoblotting analyses (representative gels shown above summary data). $N = 6$ per group. *$p < 0.05$ decreased compared to lungs from control-fed mice.

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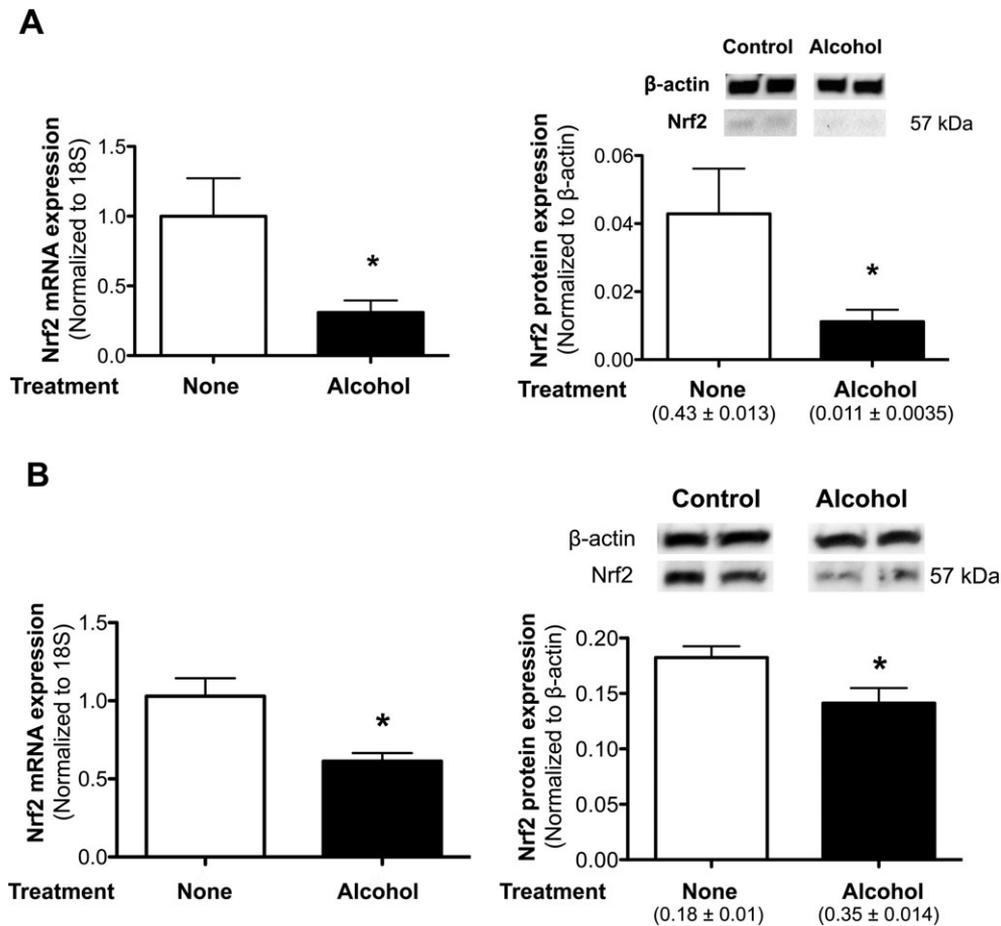
Fig. 3. Alcohol exposure in vitro decreased nuclear factor (erythroid-derived 2)-like 2 (Nrf2) gene and protein expression in primary lung fibroblasts and immortalized lung fibroblasts (NIH 3T3 lung fibroblasts). Primary lung fibroblasts (passage 5 to 8) and NIH 3T3 lung fibroblasts were cultured ± alcohol (60 mM) and mRNA and protein were isolated at 24 and 72 hours respectively. (**A**) Primary lung fibroblasts with in vitro alcohol treatment showed a decrease in Nrf2 gene (as reflected by Nrf2 mRNA levels, normalized to 18s) and Nrf2 protein expression by Western immunoblotting analyses (normalized to β-actin; representative gels shown above summary data). $N = 6$ per group. (**B**) In parallel, NIH 3T3 lung fibroblasts treatment with alcohol in vitro showed a decrease in Nrf2 gene and Nrf2 protein expression by Western immunoblotting analyses (normalized to β-actin). *$p < 0.05$ decreased compared to unexposed cells (None).

gene and protein expression. As shown in Fig. 2, panels *A* and *B* respectively, chronic alcohol ingestion decreased Nrf2 gene expression by approximately 50% ($p < 0.05$) and decreased Nrf2 protein expression by approximately 45% ($p < 0.05$) in the lung fibroblasts isolated from alcohol-fed mice as compared to lung fibroblasts isolated from control-fed mice.

Alcohol Exposure In Vitro Decreased Nrf2 Gene and Protein Expression in Both the Primary Lung Fibroblasts and NIH 3T3 Immortalized Lung Fibroblasts But Increased the Relative Translocation of Nrf2 Protein into the Nuclear Compartment

Consistent with the inhibitory effects of chronic alcohol ingestion in vivo on lung tissue and lung fibroblast Nrf2 expression, alcohol exposure in vitro of primary lung fibroblasts (Fig. 3, panel *A*) and of NIH 3T3 mouse lung fibroblasts (Fig. 3, panel *B*) decreased ($p < 0.05$) both Nrf2 gene and protein expression. However, even though alcohol

decreased total cellular levels of Nrf2 protein, it significantly increased the relative proportion of the intracellular Nrf2 in the nuclear compartment. Specifically, and as shown in Fig. 4, there was approximately twice as much Nrf2 protein ($p < 0.05$) in the nuclear compartment of alcohol-exposed fibroblasts compared to the unexposed fibroblasts (and correspondingly, far less Nrf2 in the cytosolic compartment of alcohol-exposed fibroblasts).

Alcohol Exposure In Vitro Decreased Nrf2-ARE Activity and this Activity Could be Restored by Treatment with SFP

As shown in Fig. 4, alcohol exposure actually increased Nrf2 nuclear translocation in lung fibroblasts. However, Nrf2 protein levels alone do not regulate Nrf2 activity as the protein can be inactivated by oxidative modification. Therefore, we assessed Nrf2 activity, as reflected by its ability to activate the ARE using the Nrf2-ARE reporter construct as well as to induce downstream expression of GCLC (the
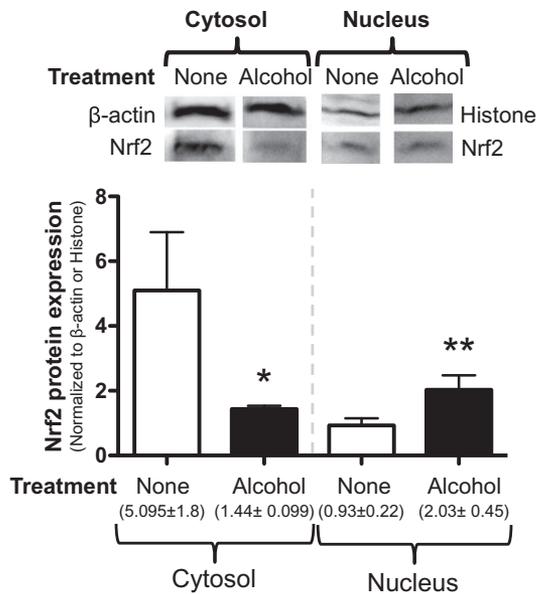
Fig. 4. Alcohol exposure increased the relative translocation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) protein into the nuclear compartment. NIH 3T3 lung fibroblasts were cultured ± alcohol (60 mM) for 72 hours. Total protein were separated into nuclear and cytosolic fractions and analyzed for Nrf2 protein expression by Western immunoblotting analyses (normalized to β-actin in the cytosolic fraction and to histone in the nuclear fraction; representative gels shown above summary data). $N = 8$ per group. *$p < 0.05$ decreased compared to cytosolic fractions from unexposed cells (None). **$p < 0.05$ increased compared to nuclear fractions from unexposed cells (None).

rate-limiting enzyme in glutathione synthesis) and of GSTT2 (another important enzyme in glutathione homeostasis). As shown in Fig. 5, panel *A*, alcohol exposure decreased ($p < 0.05$) Nrf2-ARE activity by approximately 50% compared to unexposed cells. SFP is an isothiocyanate with many biological properties, one of which is activation of the Nrf2-ARE system (Thimmulappa et al., 2002). We determined that cotreatment of alcohol-exposed cells with SFP increased ($p < 0.05$) Nrf2-ARE activity by approximately 60%; in fact, the Nrf2-ARE activity was even higher ($p < 0.05$) than in untreated, unexposed cells. Interestingly, treating unexposed cells with SFP increased ($p < 0.05$) Nrf2-ARE activity by >50%, suggesting that even under "normal" conditions Nrf2 activation can be augmented. Consistent with these findings shown in Fig. 5, panel *A*, alcohol exposure decreased ($p < 0.05$) gene expression of both GSTT2 and GCLC, as reflected by Nrf2 mRNA levels (Fig. 5, panel *B*), but the expression of both of these ARE-responsive genes was restored to that of cells not exposed to alcohol by treatment with SFP.

Alcohol Exposure In Vitro Decreased Keap1 Protein Expression in NIH 3T3 Lung Fibroblasts

As shown in Figs 4 and 5, alcohol exposure decreased Nrf2-ARE activity even though there was a relative
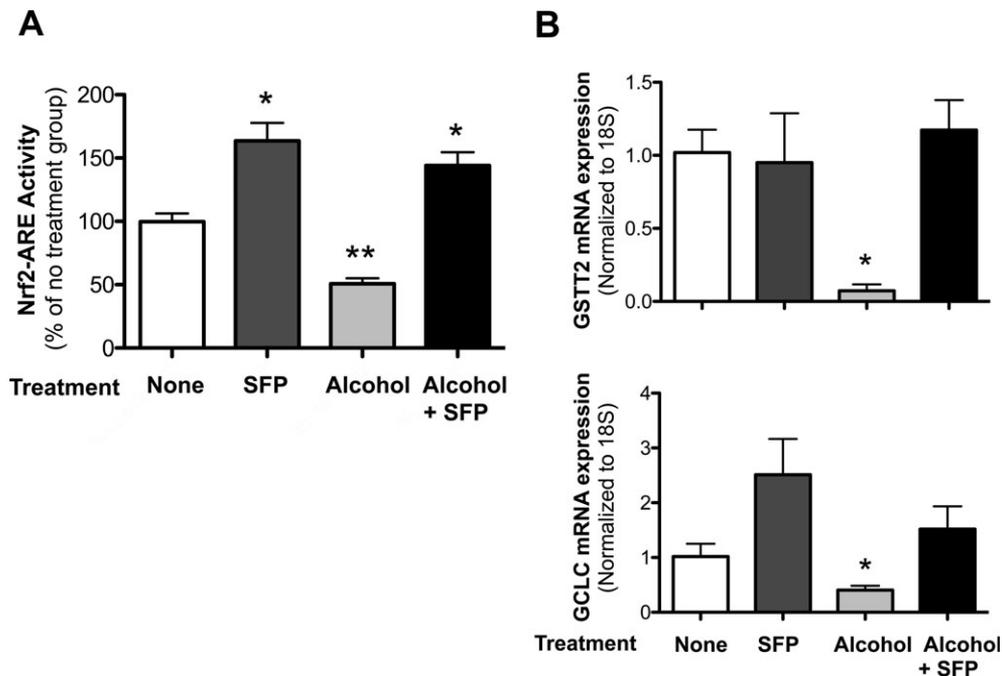
Fig. 5. Alcohol exposure suppressed Nrf2-ARE activity and downstream induction of antioxidant genes, but this suppression was reversed by treatment with the Nrf2 activator, sulforaphane (SFP). (**A**) NIH 3T3 lung fibroblasts were transfected with ARE-luciferase or renilla-luciferase for 24 hours and then cultured ± alcohol (60 mM) ± SFP (5 μM) for 20 hours at which time relative Nrf2-ARE activity (Nrf2 luciferase activity normalized to renilla luciferase activity) was quantified. (**B**) NIH 3T3 lung fibroblasts were cultured ± alcohol (60 mM) ± SFP (5 μM) for 24 hours and then analyzed for GSTT2 and GCLC gene expression by quantitative polymerase chain reaction of mRNA. $N = 8$ per group. *$p < 0.05$ increased compared to unexposed cells (None). **$p < 0.05$ decreased compared to unexposed cells (None). ARE, antioxidant response element; GCLC, glutamate-cysteine ligase catalytic subunit; GSTT2, glutathione s-transferase theta 2; Nrf2, nuclear factor (erythroid-derived 2)-like 2.

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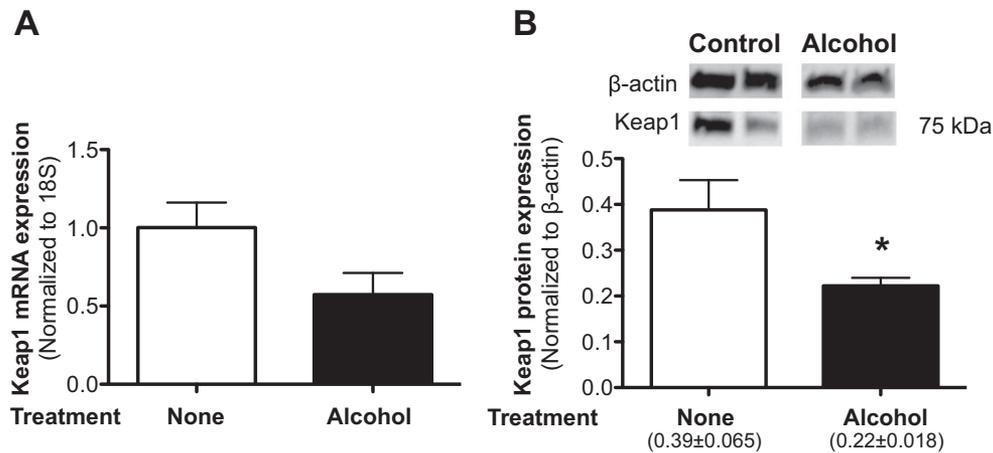
Fig. 6. Alcohol exposure decreased Kelch-like ECH-associated protein 1 (Keap1) protein expression in the NIH 3T3 lung fibroblasts. NIH 3T3 lung fibroblasts were cultured ± alcohol (60 mM) for 24 or 72 hours and cells were harvested for mRNA and protein analysis respectively. (**A**) Keap1 mRNA expression by quantitative polymerase chain reaction and (**B**) Keap1 protein expression by Western immunoblot analyses (representative gels shown above summary data). $N = 6$ per group. *$p < 0.05$ decreased compared to lungs from control-fed mice.

increase in Nrf2 protein translocation to the nuclear compartment. As Nrf2 translocation involves dissociation from Keap1 in the cytosol, we next examined Keap1 expression. Interestingly, Keap1 knockout mice have increased accumulation of Nrf2 in the nuclear compartments of various tissues (Taguchi et al., 2011). As shown in Fig. 6, there was a strong trend ($p = 0.06$) toward decreased Keap1 gene expression in alcohol-treated cells (Fig. 6, panel *A*) and a significant ($p < 0.05$) decrease in Keap1 protein expression, as compared to Keap1 gene and protein expression in untreated cells ($p < 0.05$; Fig. 6, panel *B*). This could explain why the relative translocation of Nrf2 protein to the nucleus is increased by alcohol even though it decreases cellular Nrf2 protein levels. However, a decrease in Keap1 could explain the relative increase in Nrf2 translocation to the nuclear compartment but it does not explain how alcohol decreases Nrf2-ARE activity.

SFP Treatment Attenuated Alcohol-Induced TGFβ1 Expression in Lung Fibroblasts

We previously showed that alcohol ingestion induced TGFβ1 expression in the lung and in lung fibroblasts, and that this was dependent on oxidative stress and glutathione availability (Bechara et al., 2004; Sueblinvong et al., 2014). Other investigators have reported that Nrf2 activation attenuated TGFβ1-induced fibrosis in liver, kidney, and airways (Michaeloudes et al., 2011; Oh et al., 2012a,b). Therefore, we hypothesized that alcohol-mediated inhibition of Nrf2 expression and activity induces TGFβ1 expression, and that the ability of SFP to activate Nrf2 could attenuate this

alcohol-induced TGFβ1 expression. As shown in Fig. 7 and consistent with our previously published studies, alcohol exposure increased ($p < 0.05$) TGFβ1 gene expression (panel *A*) and protein expression (panels *B* and *C*) in lung fibroblasts. Importantly, SFP treatment attenuated ($p < 0.05$) alcohol-induced TGFβ1 expression. In contrast, SFP treatment had no effect on baseline TGFβ1 gene or protein expression in fibroblasts not exposed to alcohol.

siRNA of Nrf2 Expression Exacerbated Alcohol-Induced TGFβ1 Expression in Lung Fibroblasts

As we showed in Fig. 7 that SFP treatment attenuated alcohol-induced TGFβ1 expression, we speculated that decreasing Nrf2 activity would increase TGFβ1 expression and activation. We therefore used an siRNA approach to decrease Nrf2 expression prior to exposing fibroblasts to alcohol. As shown in Fig. 8, panel *A*, Nrf2 gene expression (as reflected by mRNA levels) was decreased by approximately 60% ($p < 0.05$) using the silencing vector (see Materials and Methods). In contrast, the scrambled control vector had no effect on Nrf2 gene expression. We then exposed these fibroblasts to alcohol, as before and in these experiments quantified the percentage of cells that stained positively for TGFβ1 protein by flow cytometry. As shown in Fig. 8, panel *B*, alcohol treatment increased ($p < 0.05$) the percentage of TGFβ1 positive cells as expected. As predicted, prior siRNA of Nrf2 exacerbated this alcohol-induced increase in the percentage of fibroblasts that were positive for TGFβ1 protein expression ($p < 0.05$ higher compared to alcohol-exposed fibroblasts in which Nrf2 was not

Fig. 7. (next page) Alcohol exposure induced transforming growth factor beta-1 (TGFβ1) expression in lung fibroblasts, which was mitigated by sulforaphane (SFP) treatment. NIH 3T3 lung fibroblasts were cultured ± alcohol (60 mM) ± SFP (5 μM) and then analyzed for (**A**) TGFβ1 gene expression (TGFβ1 mRNA levels) and (**B**) protein expression (by flow cytometry) at 6 and 24 hours respectively. The inset above panel **B** shows representative flow cytometry histograms (histogram for SFP-exposed lung fibroblasts data is not shown) and representative flow cytometry density plot. (**C**) In parallel, cells were treated for 48 hours and harvested for TGFβ1 protein analysis by Western immunoblotting. $N = 5$ to 6 per group. *$p < 0.05$ increased compared to unexposed cells (None). **$p < 0.05$ decreased compared to alcohol-exposed cells.

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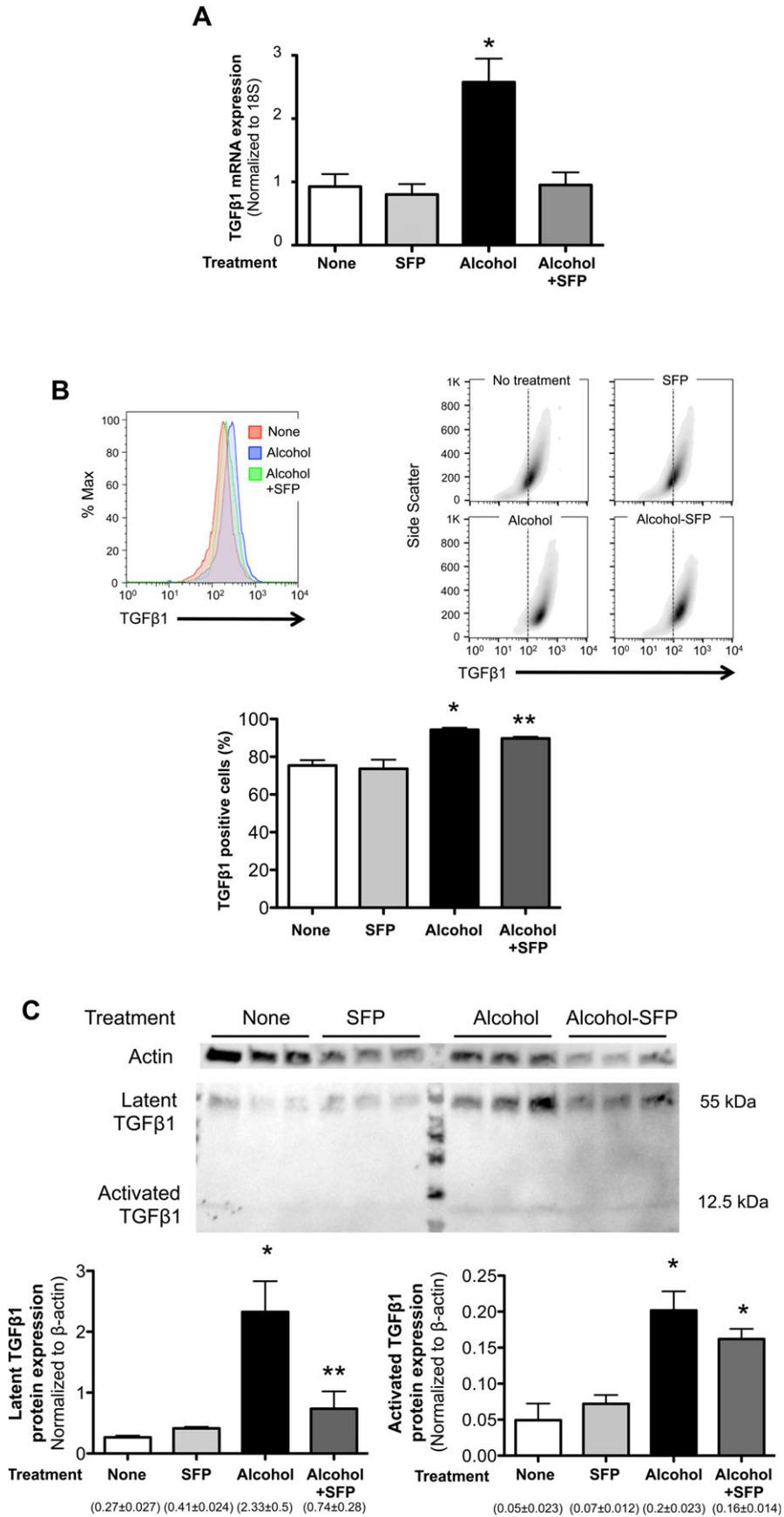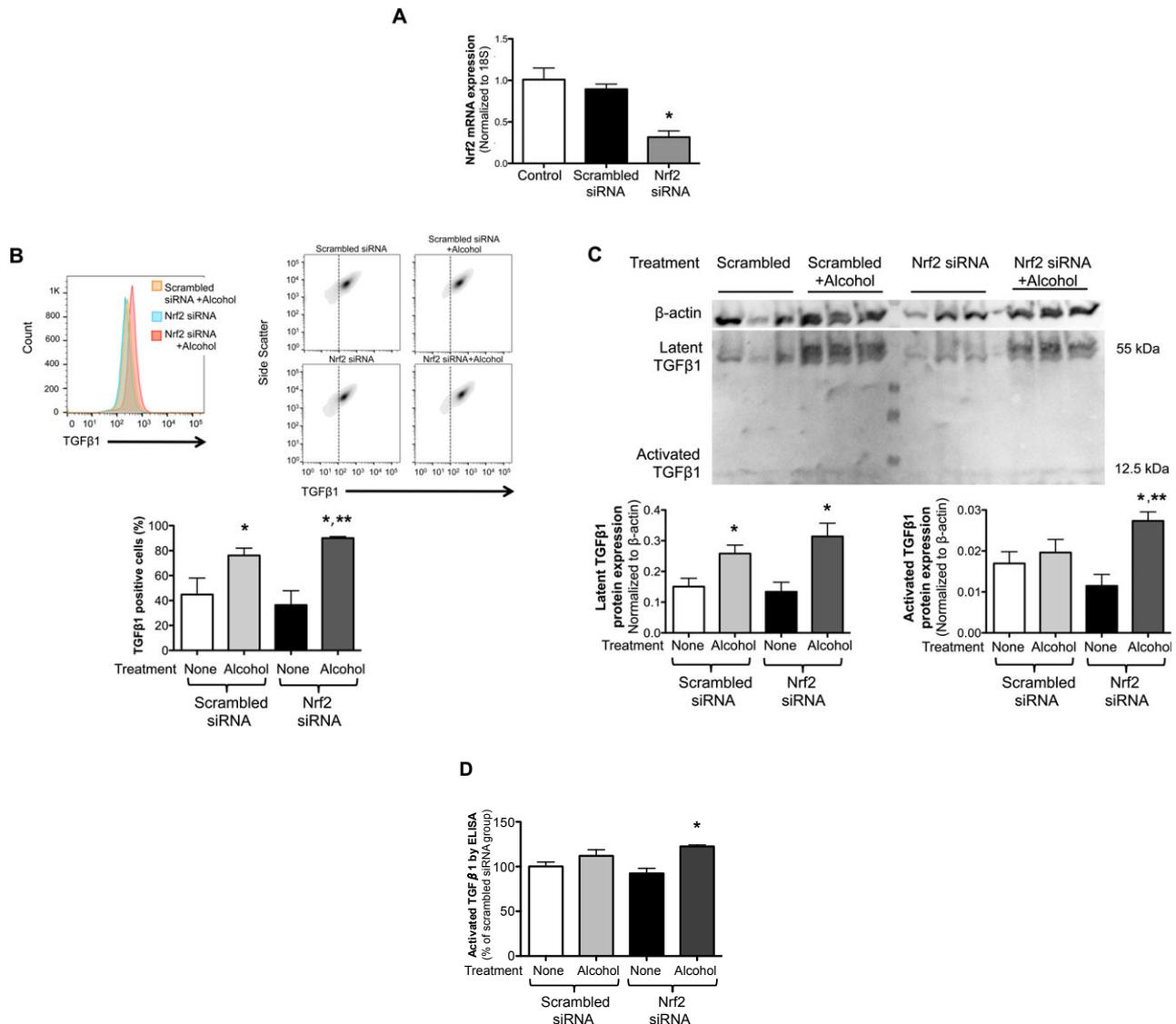
Fig. 8. Silencing RNA (siRNA) of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression amplified alcohol-induced transforming growth factor beta-1 (TGFβ1) expression in lung fibroblasts. (**A**) NIH 3T3 lung fibroblasts were transfected with an Nrf2 siRNA vector or a control scrambled vector, and then relative Nrf2 gene expression (Nrf2 mRNA levels) was assessed (*$p < 0.05$ decreased compared to control). $N = 3$. (**B**) Transfected fibroblasts were then cultured ± alcohol for 24 hours at which time the percentages of fibroblasts in each group that were positive for TGFβ1 protein expression were quantified by flow cytometry. The inset above panel **B** shows representative flow cytometry histograms (histogram for unexposed, scrambled vector-transfected lung fibroblasts data is not shown) and representative flow cytometry density plot. (**C**) In parallel, cells were treated for 48 hours and harvested for TGFβ1 protein analysis by Western immunoblotting. (**D**) Culture media from the above conditions were collected and were analyzed for secreted (activated) TGFβ1 by ELISA. Data are expressed as TGFβ1 expression relative to the unexposed, scrambled vector-transfected lung fibroblasts. $N = 5$ to 6 per group. *$p < 0.05$ increased compared to cells transfected with the scrambled vector (None) and **$p < 0.05$ increased compared to alcohol-exposed cells transfected with the scrambled vector.

silenced). Interestingly, Nrf2 siRNA alone did not increase the percentage of fibroblasts that were positive for TGFβ1 protein, suggesting that low Nrf2 expression/activity by itself does not induce TGFβ1 expression but rather primes lung fibroblasts for more exuberant TGFβ1 expression in response to the stress of alcohol exposure. Representative histograms and density plot of the flow cytometry analyses are shown above (Fig. 8, panel *B*). In parallel, we also showed the increase in TGFβ1 protein (inactive and active TGFβ1) by Western immunoblot (Fig. 8, panel *C*). Moreover and as shown in Fig. 8, panel *D*, fibroblasts in which Nrf2 RNA

was silenced and exposed to alcohol released significantly more ($p < 0.05$) TGFβ1 (indicative of activated TGFβ1) into the culture (as determined by ELISA) than untreated fibroblasts or than fibroblasts treated with Nrf2 siRNA.

Alcohol-Induced Inhibition of Nrf2-ARE Activity is Mediated Through TGFβ1 Signaling

As siRNA of Nrf2 alone did not influence TGFβ1 expression in lung fibroblasts, we speculated that there is a dynamic regulation and relation between Nrf2 activity and

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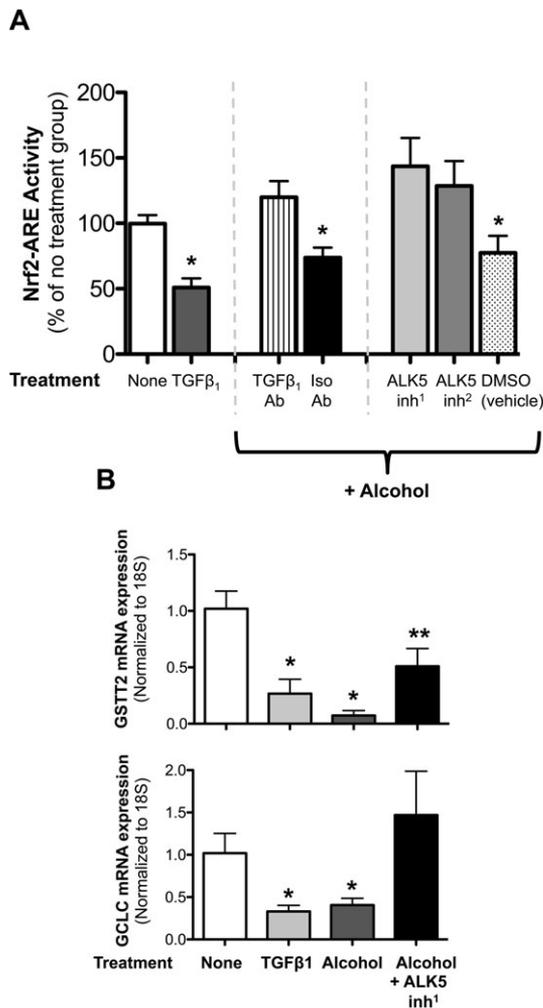
Fig. 9. TGFβ1 treatment independently suppressed Nrf2-ARE activity, and alcohol-suppressed Nrf2-ARE activity was prevented by inhibitors of TGFβ1 signaling. (**A**) NIH 3T3 lung fibroblasts were transfected with ARE-luciferase or renilla-luciferase for 24 hours and then cultured for 20 hours alone or with one of the following: TGFβ1 (5 ng/ml), alcohol (60 mM) and either a TGFβ1 neutralizing antibody (2 μg/ml) or an isotype control antibody (2 μg/ml), or alcohol (60 mM) and 1 of 2 ALK5 inhibitors (ALK5 inh[1] is SB431542 and ALK5 inh[2] is A8301; each at 8 μM) or dimethyl sulfoxide (1:500 dilution) as an ALK5 inhibitor vehicle control. The fibroblasts were then analyzed for relative Nrf2-ARE activity. (**B**) NIH 3T3 lung fibroblasts were cultured for 24 hours either alone, with TGFβ1 (5 ng/ml), with alcohol (60 mM), or with alcohol (60 mM) and an ALK5 inhibitor (ALK5 inh[1] is SB431542). The relative gene expression (mRNA levels) of the ARE-dependent antioxidants GSTT2 and GCLC were then determined. $N = 8$ per group. *$p < 0.05$ decreased compared to unexposed, untreated cells (None). **$p < 0.05$ increased compared to alcohol-treated cells but still decreased compared to unexposed, untreated cells. ALK5, activin receptor-like kinase 5; ARE, antioxidant response element; GCLC, glutamate-cysteine ligase catalytic subunit; GSTT2, glutathione s-transferase theta 2; Nrf2, nuclear factor (erythroid-derived 2)-like 2; TGFβ1, transforming growth factor beta-1.

TGFβ1 expression in the alcohol-induced stress in the lung. Specifically, we sought to determine the signaling relationship between Nrf2 and TGFβ1 during alcohol-induced stress in the lung, and particularly in the lung fibroblast. Therefore, we next determined the effects of TGFβ1 on Nrf2-ARE activity. To do so, we utilized a neutralizing

antibody against TGFβ1 as well as an inhibitor of TGFβ1 receptor 1 signaling through ALK5, which transduces the TGFβ1 signals to the nucleus through phosphorylation of Smad2/3 (Egorova et al., 2011). As shown in Fig. 9, panel *A*, treating lung fibroblasts with TGFβ1 alone decreased ($p < 0.05$) Nrf2-ARE activity by approximately 50%, which was comparable to the degree of inhibition in response to alcohol exposure alone (Fig. 4, panel *A*). We next quantified Nrf2-ARE activity in alcohol-exposed cells treated with either a neutralizing antibody to TGFβ1 or to either of 2 well-characterized inhibitors of ALK5. As shown in Fig. 9, panel *A*, treatment with the TGFβ1 neutralizing antibody or either of the ALK5 inhibitors (SB431542 is "inh[1]" and A8301 is "inh[2]") completely prevented ($p < 0.05$) alcohol-induced suppression of Nrf2-ARE activity; note that neither the isotype control antibody nor DMSO (vehicle for the ALK5 inhibitors) had any protective effects. In parallel and as shown in Fig. 9, panel *A*, treatment with TGFβ1 alone decreased ($p < 0.05$) the gene expression of both GSTT2 and GCLC, comparable to the inhibition of these ARE-responsive antioxidant genes by alcohol alone (Fig. 4, panel *B*). In contrast, treating alcohol-exposed cells with the ALK5 inhibitor SB431542 (inh[1]) partially restored GSTT2 gene expression ($p < 0.05$ compared to alcohol-exposed cells) and completely restored GCLC gene expression.

DISCUSSION

In this study, we determined that chronic alcohol ingestion suppressed Nrf2 expression in the experimental mouse lung in vivo. Rodent models have been instrumental in identifying the previously unrecognized effects of chronic alcohol ingestion in the lung, including profound glutathione depletion, increased expression of TGFβ1, and aberrant fibroblast function (Bechara et al., 2004, 2005; Holguin et al., 1998; Moss et al., 2000; Roman et al., 2005) that almost certainly play causative roles in rendering alcoholics 2 to 4 times more susceptible to acute lung injury in response to critical illnesses such as septic shock and trauma (Moss et al., 1996, 1999). Furthermore, we found that alcohol exposure in vitro decreased Nrf2 expression in lung fibroblasts and increased the expression of TGFβ1. Interestingly, although alcohol exposure increased the relative translocation of Nrf2 protein into the nuclear compartment, it decreased Nrf2-ARE activity and inhibited the expression of the ARE-dependent target genes GSTT2 and GCLC. Interestingly, alcohol exposure in vitro also decreased the protein expression of Keap1, which itself is ARE-dependent and serves to keep Nrf2 in a latent but "ready" state in the cytoplasm as well as maintaining the levels of Nrf2 (Lee et al., 2007). The decrease in Keap1 expression would explain why Nrf2 translocation to the nuclear compartment is increased by alcohol but does not explain how it dampens Nrf2-ARE activity. A potential explanation for this latter effect can be inferred from the observation that alcohol-induced suppression of Nrf2-ARE activity could be restored by treatment with SFP, a

phytochemical that appears to work in part by preserving and/or even augmenting Nrf2 function, through inducer of protein kinases (JNK and ERK) phosphorylation which lead to activation of ARE-dependent gene expression and perhaps by preventing its oxidation through balancing redox potential in the nucleus (Hansen et al., 2004; Keum, 2011). In contrast, whereas SFP mitigated alcohol-induced TGFβ1 expression, Nrf2 siRNA amplified alcohol-induced TGFβ1 expression in lung fibroblasts. Last, inhibition of TGFβ1 signaling restored Nrf2-ARE activity in alcohol-exposed fibroblasts. Taken together, these results implicate TGFβ1 signaling as the proximal mechanism by which alcohol suppresses Nrf2-ARE activity and thereby causes oxidative stress by inhibiting global antioxidant defenses. Furthermore, the decrease in Nrf2-ARE activity promotes even greater TGFβ1 expression, thereby creating a vicious feed-forward cycle of chronic oxidative stress in the alcoholic lung.

These new findings extend our current understanding of the mechanisms by which alcohol use disorders predispose even otherwise healthy individuals to various types of lung infections and injuries. For example, we recently reported that alcohol-fed mice had an exaggerated fibrotic response to bleomycin-induced acute lung injury and that this was associated with increased expression of TGFβ1 (Sueblinvong et al., 2014). We speculated that the increased fibrotic response was due to alcohol-induced chronic oxidative stress, and that the mechanism was impaired Nrf2-ARE signaling required for the programmatic expression of hundreds of genes that code for components of antioxidant defenses. This speculation was based on our previously published data showing a decrease in Nrf2 expression in the alcoholic rat lung epithelium (Jensen et al., 2013). Consistent with our speculation, we found that alcohol decreased overall Nrf2 gene and protein expression in the lungs of alcohol-fed mice and in their lung fibroblasts and inhibited Nrf2-ARE activity via a TGFβ1-dependent mechanism. Taken together, our previous and current findings that alcohol suppresses Nrf2-ARE signaling in these complementary experimental models have helped explain an initial paradox. Specifically, our earliest experimental and clinical studies in this field revealed previously unrecognized and profound (>90%) glutathione depletion in the "alcoholic lung" (Holguin et al., 1998; Moss et al., 2000) despite the absence of any overt pulmonary inflammation or injury. In contrast, another common environmental stress in the airways, namely cigarette smoking, significantly increases airway glutathione levels (Moss et al., 2000). These more recent findings that alcohol inhibits Nrf2-ARE signaling provide a mechanism to explain this phenomenon. Specifically, alcohol appears to render the lung susceptible to oxidative stress and injury by lowering its antioxidant defenses as opposed to overwhelming them by primarily increasing the formation of reactive oxygen species, which in fact would induce Nrf2 expression and activity.

This alcohol-induced suppression of Nrf2-ARE signaling appears to be mediated by TGFβ1. Specifically, TGFβ1 treatment alone decreased Nrf2 expression and Nrf2-ARE activity to the same degree as alcohol exposure. Furthermore, inhibition of TGFβ1 signaling with either a neutralizing antibody to TGFβ1 or with ALK5 inhibitors restored Nrf2-ARE activity in the presence of alcohol. These results provide strong evidence that alcohol-induced inhibition of Nrf2-ARE is mediated through TGFβ1 signaling. Further, the dampening of antioxidant defenses and subsequent oxidative stress appears to drive even greater expression of TGFβ1 in a feed-forward fashion. Specifically, when we suppressed Nrf2 expression in lung fibroblasts with siRNA, which decreased Nrf2 gene expression by approximately 60% in the lung fibroblasts, this manipulation exacerbated alcohol-induced TGFβ1 expression even though Nrf2 siRNA alone had no effect on TGFβ1 protein expression. In contrast, treatment with TGFβ1 alone was able to inhibit Nrf2 expression to a comparable degree as alcohol exposure, providing additional evidence that the induction of TGFβ1 precedes and drives the suppression of Nrf2. Although this overall mechanistic sequence need to be confirmed in other cell types such as lung epithelial cells and macrophages, there is no reason to believe that it is restricted to lung fibroblasts as alcohol also increases TGFβ1 expression and dramatically lowers glutathione levels in the airways.

A discrete molecular mechanism by which alcohol induces TGFβ1 expression and starts this cascade still remains unknown. We previously identified that alcohol induces fibroblasts to express fibronectin via previously unrecognized signaling through nicotinic receptors (Roman
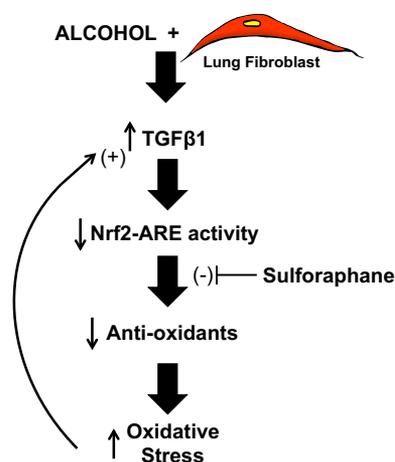
Fig. 10. Proposed pathophysiological sequence by which alcohol alters transforming growth factor beta-1 (TGFβ1) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) homeostasis in the lung. The data shown in this manuscript as well as those from previous studies suggests that alcohol induces the expression of TGFβ1 in the lung, including in lung fibroblasts. TGFβ1 in turn suppresses Nrf2 expression and its ability to activate the antioxidant response element (ARE), which then leads to inadequate production of antioxidants including glutathione and subsequent oxidative stress. The decrease in Nrf2-ARE activity and the oxidative stress creates a vicious cycle that feeds back to further increase TGFβ1 expression.

et al., 2005). Whether or not such a signaling mechanism is also exploited by alcohol to induce TGFβ1 expression, either directly or indirectly, is yet to be determined. Furthermore, it is not yet clear how this feed-forward cycle involving TGFβ1 and Nrf2 is propagated by alcohol. However, this study is consistent with other reports that activation of Nrf2 can attenuate the effects of TGFβ1 (Choi et al., 2009; Churchman et al., 2009; Michaeloudes et al., 2011; Nussler et al., 2010), and the observation that treatment with glutathione or glutathione precursors prevents alcohol-induced TGFβ1 expression in the alveolar macrophage and in the lung tissue suggests that this cycle is driven at least in part by redox imbalances (Bechara et al., 2005; Brown and Brown, 2012).

Nrf2 signaling is a complex process involving multiple steps, starting with the activation of Nrf2 in response to redox changes in the cytoplasm that dissociate it from Keap1, its subsequent translocation into the nucleus where it binds to the ARE sequence in the promoters of hundreds of genes, and ultimately the transcription of various antioxidant and immune response genes (Cho et al., 2006; Hybertson et al., 2011). Therefore, total intracellular Nrf2 protein levels alone do not by themselves reflect its antioxidant signaling capacity. Accordingly, we assessed the relative levels of Nrf2 protein in the cytosolic and nuclear compartments of lung fibroblasts exposed to alcohol. We found that even though alcohol-exposed cells had lower total levels of Nrf2 protein, a significantly higher portion of the intracellular Nrf2 protein was localized to the nuclear compartment. These data suggest that alcohol-induced redox stress within the cytoplasm promotes the dissociation of Nrf2 from Keap1 as expected, but that its ability to activate the ARE once inside the nuclear compartment is impaired. Consistent with this interpretation, we assessed Nrf2-ARE signaling by 2 independent means. First, we used an Nrf2-ARE luciferase reporter construct and determined that alcohol exposure suppressed Nrf2-ARE activity. In parallel, we determined that alcohol inhibited the expression of multiple ARE-dependent antioxidant genes. As further evidence that the defect was in the ability of Nrf2 protein to activate the ARE within the nuclear compartment, ARE activity could be restored by treatment with SFP even in the presence of alcohol. Although the precise mechanism(s) by which SFP activates Nrf2 protein is unknown, these results suggest that the functional state of Nrf2 is somehow reversibly altered by alcohol as it can be "re-activated" with SFP, and not that alcohol interferes with Nrf2 translocation into the nuclear compartment. Furthermore, the suppression of Nrf2-ARE activity likely led to the decrease in Keap1 expression through the lack of negative feedback.

In summary, this study extends our understanding of the mechanisms by which chronic and excessive alcohol ingestion leads to what we have called the "alcoholic lung." This is a vulnerable condition in which an individual who is otherwise apparently healthy has profound glutathione deficiency within their alveolar space (Moss et al., 2000) and is approximately 4 times more likely to develop acute lung injury in response to a severe inflammatory stress, such as sepsis (Moss et al., 2003). Specifically, alcohol induces oxidative stress in the lung at least in part by inducing the aberrant expression of TGFβ1 expression which in turn suppresses Nrf2 expression and interferes with its ability to activate the ARE. The decrease in Nrf2-ARE activity and the oxidative stress creates a vicious cycle that feeds back to further increase TGFβ1 expression (Fig. 10). This study further elucidates a novel mechanism by which alcohol causes oxidative stress and mediates the protein pathophysiological derangements in lung fibroblasts, epithelial cells, and macrophages that together manifest as the "alcoholic lung" phenotype. Taken together, these preclinical studies suggest that targeting the Nrf2-ARE signaling pathway, perhaps with phytochemicals such as SFP that are already being studied in other clinical contexts, could enhance lung health in individuals with alcohol use disorders and thereby decrease their risk for pneumonia and acute lung injury.

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AUTHOR CONTRIBUTIONS

VS and DMG designed the research project; VS, VT, TS, RS, DCN, and STM performed experiments and analyzed data; VS and DMG interpreted the results of the experiments; VS and DMG drafted, edited, and revised the manuscript.

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