



Alcohol Decreases Organic Dust-Stimulated Airway Epithelial TNF-Alpha Through a Nitric Oxide and Protein Kinase-Mediated Inhibition of TACE

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Background: Farm workers in rural areas consume more alcohol than those who reside in urban areas. Occupational exposures such as agricultural work can pose hazards on the respiratory system. It is established that hog barn dust induces inflammation in the airway, including the release of cytokines such as tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and IL-8. We have shown that alcohol alters airway epithelial innate defense through changes in both nitric oxide (NO) and cAMP-dependent protein kinase A (PKA). Simultaneous exposure to hog barn dust and alcohol decreases inflammatory mediators, TNF- α , IL-6, and IL-8, in mice. Previously, mice exposed to both alcohol and hog barn dust showed a depleted amount of lymphocytes compared to mice exposed only to hog barn dust. Weakening of the innate immune response could lead to enhanced susceptibility to disease. In addition, mice that were co-exposed to hog barn dust and alcohol also experienced increased mortality.

Methods: Because we recently demonstrated that PKA activation inhibits the TNF- α sheddase, TNF- α -converting enzyme (TACE), we hypothesized that an alcohol-mediated PKA pathway blocks TACE activity and prevents the normative inflammatory response to hog barn dust exposure. To delineate these effects, we used PKA pathway inhibitors (adenylyl cyclase [AC], cAMP, and PKA) to modulate the effects of alcohol on dust-stimulated TNF- α release in the bronchial epithelial cell line, BEAS-2B. Alcohol pretreatment blocked TACE activity and TNF- α release in hog barn dust-treated cells.

Results: Alcohol continued to block hog barn dust-mediated TNF- α release in the presence of the particulate AC inhibitor, SQ22,536. The soluble adenylyl cyclase inhibitor, KH7, however, significantly increased the inflammatory response to hog barn dust. phosphodiesterase 4 inhibitors significantly elevated cAMP and enhanced alcohol-mediated inhibition of dust-stimulated TNF- α release. In addition, the NO synthase inhibitor, L-NMMA, also reversed the alcohol-blocking effect on dust-stimulated TNF- α .

Conclusions: These data suggest that alcohol requires a soluble cyclase-generated cAMP-PKA pathway that is dependent upon the action of NO to inhibit TACE and TNF- α release. These findings support our observations that alcohol functions through a dual NO and PKA pathway in bronchial epithelial cells.

Key Words: Alcohol, Inflammation, TNF- α -Converting Enzyme, Airways, Nitric Oxide.

THE RESPIRATORY SYSTEM encounters an onslaught of airborne matter. Agriculture workers, for instance, are exposed to various types of dusts on a daily

basis. Animal husbandry dust, composed of feces, bacteria, and endotoxins, as well as many other components, is extremely complex and can include respirable particles that distribute deep in the airway (Gerald et al., 2014; May et al., 2012). In particular, concentrated animal feeding operations (CAFOs) can harbor higher levels of dust than ambient air due to the lack of normal airflow and poor ventilation (Cormier et al., 2000). Swine CAFOs in particular are known to have higher levels of harmful dusts than those found in other agricultural settings (May et al., 2012). Inhalation of organic dust can cause airway inflammation (Poole and Romberger, 2012) and for farm workers, result in an increased prevalence of chronic bronchitis and other respiratory symptoms including runny nose, watery eyes, and shortness of breath (Alterman et al., 2008). One explanation for the immunological responses to swine production dust is that it causes impaired human macrophage function (Poole et al., 2008). Another explanation is found

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Received for publication May 22, 2015; accepted November 18, 2015.

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DOI: 10.1111/acer.12967

in the increased epithelial cell inflammatory cytokine production observed in mouse models exposed to organic dust (Wyatt et al., 2014). The release of pro-inflammatory cytokines tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and IL-8 is regulated by the intracellular activation of protein kinase C alpha (PKC α) followed by the subsequent downstream activation of PKC ϵ following organic dust exposure (Wyatt et al., 2014).

The effects of hog barn dust on the airways have been studied extensively; however, co-exposure to dust and alcohol is understudied despite mounting evidence that people exposed to CAFO dust also consume alcohol (Stallones and Xiang, 2003). Likewise, the demographics of farmers are changing rapidly and a new population of agricultural workers is emerging (Kandel, 2008). This emerging population often consumes higher levels of alcohol than their nonrural counterparts (Brumby et al., 2013; Stallones and Xiang, 2003).

Alcohol affects a variety of pulmonary functions. Chronic alcohol exposure induces cilia desensitization in vitro and in vivo (Elliott et al., 2007; Wyatt and Sisson, 2001). This leads to ineffective mucociliary clearance as alcohol impacts the airway through the nitric oxide (NO) and protein kinase A (PKA) pathway (Sisson et al., 1999). Alcohol alters airway epithelial innate defense through changes in both NO and the cAMP-dependent PKA (Wyatt et al., 2003, 2013). In addition, alcohol can lead to leaky lung (Brown et al., 2004), a reduction in alveolar macrophages (Brown et al., 2007), and a decrease in glutathione levels (Holguin et al., 1998). With a decrease in immune cells and antioxidants, the airway is primed for microbes to colonize. Without normal innate and adaptive immune processes, infections are at risk for going unresolved.

Previous work in our laboratory has shown that TNF- α , IL-6, and IL-8 cytokines are produced in the lungs following hog barn dust exposure in mice (Poole et al., 2009). Alcohol exposure, however, negatively affects the normal inflammatory response to hog barn dust in mice. Elevations in lung lavage inflammatory cytokines and mononuclear cell aggregates normally observed in histological evaluations as a result of repetitive hog barn dust extract (HDE) exposure are abrogated in mice that were previously fed alcohol (McCaskill et al., 2012). Mice treated with the concurrent exposure of alcohol and HDE had a decrease in weight and experienced 20% mortality. Previously, it was shown that alcohol effects on the inflammatory response can impair the host's ability to clear debris, infectious agents, and microbes (Happel and Nelson, 2005). Recently, we identified a cyclic AMP-mediated inhibition of the TNF- α -converting enzyme (TACE) that can block dust-stimulated TNF- α release (Wyatt et al., 2014). Because alcohol elevates cAMP in airway epithelium (Sisson et al., 1999), we hypothesized that alcohol exposure blocks dust-mediated TNF- α production in airway epithelium through a cAMP-dependent inhibition of TACE activation.

MATERIALS AND METHODS

Cell Culture

BEAS-2B, a human bronchial epithelial transformed cell line (ATCC, Manassas, VA), was treated with or without organic dust extract (5%), ethanol (EtOH; 100 mM) or the following inhibitors: SQ22,536, KH7, H-89, L-NMMA, and Rp-cAMPS (each obtained from Sigma, St. Louis, MO). Cells were exposed to EtOH (24 hours) before being exposed to inhibitors in the presence of EtOH for 1 hour. After treating with inhibitors for 1 hour, cells were treated with dust extract, EtOH, and inhibitors for 6 hours. Human bronchial epithelial cells (HBECs) were cultured as described in Romberger and colleagues (2002).

Organic Dust Extract and Collection

Dust was collected from raised surfaces of hog confinement facilities and an aqueous extract prepared as previously detailed (Dusad et al., 2013; Poole et al., 2015). The dust was made into an aqueous extract (HDE) by vortexing 1 g/ml of dust into Hank's balanced salt solutions for 1 minute and allowed to sit for 1 hour. The solution was then centrifuged for 20 minutes at 2,000 \times g, the supernatant fraction was removed, and this step was repeated for an additional 30 minutes centrifugation. The supernatant fraction was then sterile filtered (0.22 μ m). Aliquots were kept at -20°C until utilized. A 5% dust extract diluted in media was used for cell culture stimulations. Extensive characterization of the components in this extract has been previously reported with regard to endotoxin, muramic acid, ergosterol, fatty acids, and other bacterial components (Boissy et al., 2014; Poole et al., 2010).

Enzyme-Linked Immunosorbent Assay for Human IL-6, IL-8, and TNF- α

BEAS-2B cells were grown to 95% confluence on collagen-coated 6-well tissue culture plates (Falcon, Pittsburgh, PA) in LHC-9/RPMI media and incubated at 37°C, 5% CO₂. TNF- α , IL-6, and IL-8 concentrations were evaluated by antibody detection using a commercially prepared enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). Cytokines were normalized by the corresponding protein concentrations using a Nanodrop spectrophotometer (Thermo Fisher, Pittsburgh, PA).

PKC Activity Assay

BEAS-2B cells were cultured on 60 mm tissue culture dishes (B-D Falcon, Franklin Lakes, NJ) to 95% confluence and pretreated with 100 mM of EtOH 24 hours. The cells were then subjected to a 1-hour pretreatment of inhibitor and EtOH. Following this exposure, cultures were stimulated with dust extract (5%), EtOH, and/or inhibitors. Phorbol-12-myristate-13-acetate (PMA; Sigma) was added to some dishes for 5 to 15 minutes as a positive control. Cell lysis buffer (35 mM Tris-HCl, 0.4 mM EGTA, and 10 mM MgCl) containing a 1:10 dilution of protease inhibitor cocktail (Sigma), 0.2 mM PMSF and 0.1% Triton X-100 was added and cell monolayers in culture dishes frozen in liquid nitrogen. Dishes were scraped, the supernatant fraction containing the cells was collected and sonicated for 5 seconds, and crude cell protein was centrifuged at 10,000 \times g for 30 minutes at 4°C. The cell fractions containing either PKC α or PKC ϵ were measured for activity as previously described (Wyatt et al., 2012).

TACE Activity Assay

TACE activity was measured using a commercially available fluorometric assay (SensoLyte 520 TACE activity assay kit; AnaSpec, Fremont, CA). On 6-well plates, BEAS-2B were grown confluent to

~90% and treated with above-mentioned conditions. Fractions from supernatant fractions were utilized using the supplied TACE substrate solution as per the manufacturer's instructions. To stop the horseradish peroxidase reaction, 2 M H₂SO₄ was added to each plate well and fluorescence recorded at 490/520 nm. Experimental sample readings were extrapolated to a standard curve of recombinant human TACE and results expressed as μ g protein/ml of sample.

Nuclear Factor-Kappa Beta Binding Assay

BEAS-2B cells were treated with media only, EtOH (100 mM), HDE (5%), or both EtOH and HDE. BEAS-2B cells were exposed to EtOH for 24 hours and then EtOH and HDE for 6 hours. Nuclear factor-kappa beta (NF- κ B) binding assay was completed as described (Wyatt et al., 2014).

Statistical Analyses

Each experiment contained 3 technical replicates, and each experiment was conducted 3 separate times. GraphPad Prism software (version 6.0; San Diego, CA) was used to analyze values as well as statistical analyses. One-way analysis of variance (ANOVA) was used to determine statistical significance with a $p < 0.05$ (95% confidence interval).

RESULTS

Alcohol Blocks HDE-Mediated PKC ϵ Activation and IL-8 Release in Bronchial Epithelial Cell Lines

BEAS-2B cells were exposed to 100 mM EtOH for 24 hours only, or EtOH followed by a 6-hour 5% HDE exposure (Fig. 1A). Cells were also exposed to media alone. Supernatant fractions were collected following the 6-hour exposure and analyzed for IL-8 by ELISA. HDE exposure significantly increased IL-8 release as compared to the media control ($p < 0.001$) while EtOH pretreatment significantly decreased HDE-stimulated IL-8 release ($p < 0.001$). EtOH alone had no effect on IL-8 release. In Fig. 1B, PKC ϵ activity

was measured in BEAS-2B cells that were exposed to media or HDE (5%) for 0, 1, 6, and 24 hours in the presence or absence of EtOH. HDE stimulated a significant increase in PKC ϵ activity at 6 hours compared to the EtOH-treated cells ($p < 0.001$). EtOH-exposed cells did not show any increases in PKC ϵ activity when compared to media-only treated cells. These data indicate that EtOH blocks HDE-stimulated IL-8 release and PKC ϵ activity in BEAS-2B cells.

Alcohol Blunts HDE-Stimulated PKC ϵ Activation in Primary Airway Epithelial Cells

To ensure that alcohol is having the same effects in primary airway cells, HBECs were exposed to EtOH (100 mM) for 24 hours, or 24-hour EtOH with a 6-hour posttreatment of 5% HDE. The supernatant fractions were collected and assayed by ELISA for IL-8 release (Fig. 2A). HDE exposure increased IL-8 release and EtOH significantly blocked this HDE-mediated release ($p < 0.001$). The media and EtOH-only controls showed nominal baseline levels of IL-8 release. In Fig. 2B, HBECs were exposed to EtOH (100 mM), 5% HDE, EtOH and HDE or media only for 6 hours. HDE exposure increased PKC ϵ activity while EtOH treatment decreased HDE-stimulated PKC ϵ activity ($p < 0.001$). These data confirm that EtOH and HDE affect IL-8 release and PKC ϵ activity in airway primary cells and cell lines in a similar manner.

Alcohol Does Not Alter HDE-Stimulated PKC α Activation in Bronchial Epithelial Cells

Because HDE activates PKC α upstream of PKC ϵ , we evaluated the effects of EtOH on PKC α . In Fig. 3, PKC α and PKC ϵ activities were observed in BEAS-2B cells that were exposed to 0, 0.1, 1, and 5% HDE over 1 hour with or without EtOH (100 mM). In Fig. 3A, PKC α activity increased

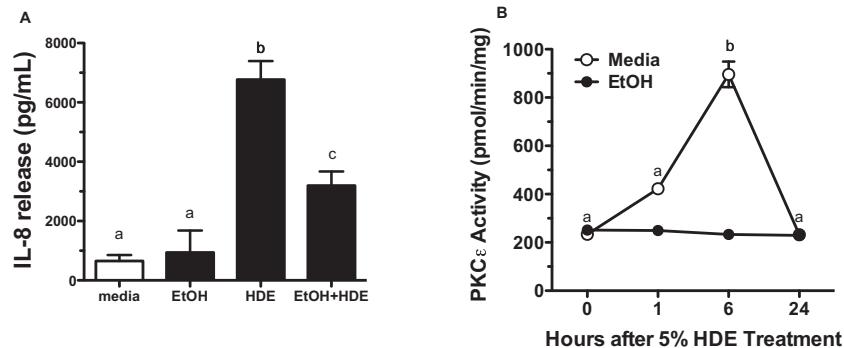


Fig. 1. Alcohol blocks hog barn dust extract (HDE)-stimulated protein kinase C epsilon (PKC ϵ) activation and interleukin-8 (IL-8) release in BEAS-2B. (A) BEAS-2B cells were exposed to media, 5% HDE, ethanol (EtOH; 100 mM) 24 hours and HDE and EtOH (24 hours prior to the 6-hour exposure) for 6 hours. Following treatment, IL-8 release was assayed. HDE-stimulated IL-8 release compared to the controls, b denotes $p < 0.001$. Co-treatment of HDE-EtOH decreased IL-8 release significantly when compared to HDE-exposed cells, c denotes $p < 0.0001$. (B) BEAS-2B cells were exposed to media, 5% HDE and EtOH (100 mM) for 0, 1, 6, and 24 hours. Posttreatment cells were assayed for the PKC ϵ activity. HDE increased the PKC ϵ activity when compared to the controls at 6 hours, b denotes $p < 0.001$. EtOH failed to increase the PKC ϵ activity at any of the time points, $p < 0.001$. a denotes $p < 0.001$ versus media. One-way ANOVA was conducted with a Tukey post hoc test.

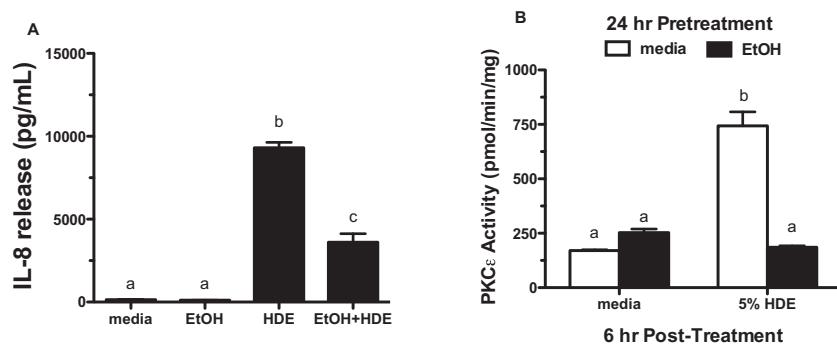


Fig. 2. Alcohol blocks hog barn dust extract (HDE)-stimulated protein kinase C epsilon (PKC ϵ) activation in primary human bronchial epithelial cells (HBECs). **(A)** HBECs were exposed to media, 5% HDE, ethanol (EtOH; 100 μ M) and HDE and EtOH for 6 hours. EtOH treatment occurred 24 hours prior to the 6-hour exposure. Posttreatment interleukin-8 (IL-8) release was assayed. HDE-stimulated IL-8 release when compared to the controls, b signifies $p < 0.001$. The co-treatment of HDE and EtOH decreased IL-8 release significantly when compared to the HDE-exposed cells, c signifies $p < 0.0001$. **(B)** HBECs were exposed to media, 5% HDE and EtOH (100 μ M) for 6 hours. Posttreatment cells were assayed for PKC ϵ activity. HDE increased PKC ϵ activity when compared to the controls, b signifies $p < 0.001$. EtOH decreased PKC ϵ activity significantly, a signifies $p < 0.001$. A 1-way ANOVA was conducted with a Tukey post hoc test.

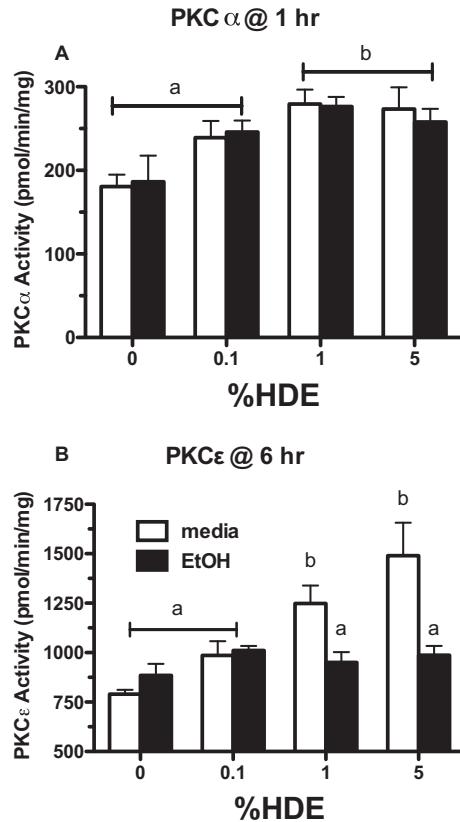


Fig. 3. Alcohol does not block hog barn dust extract (HDE)-stimulated protein kinase C alpha (PKC α) activation in BEAS-2B. **(A)** BEAS-2B cells were exposed to media only, which served as the control, HDE (0.1, 1, 5%) in the presence or absence of ethanol (EtOH; 100 mM) for 6 hours, a denotes $p < 0.05$. Posttreatment, cells were assayed for PKC α activity. HDE (1 and 5%) increased PKC α activity significantly compared to media-only exposed cells, b denotes $p < 0.05$. A co-exposure of HDE and EtOH did not alter PKC α activity, $p > 0.05$. **(B)** BEAS-2B cells were exposed to media, HDE (0.1, 1, 5%) in the presence or absence of EtOH (100 mM) for 6 hours. Posttreatment, cells were assayed for protein kinase C epsilon (PKC ϵ) activity. HDE (1 and 5%) stimulated an increase of PKC ϵ activity when compared to control cells, b denotes $p \leq 0.05$. When cells were exposed to HDE and EtOH PKC ϵ activity was decreased, a signifies $p < 0.05$ under a 1-way ANOVA and Tukey post-test analysis.

with 1 and 5% HDE exposure in comparison with cells that did not receive HDE ($p < 0.05$). EtOH, however, did not alter PKC α activity in cells exposed to HDE. Under the same conditions, cells were treated with various concentrations of HDE for 6 hours and assayed for PKC ϵ activity (Fig. 3B). HDE (1 and 5%) increased PKC ϵ activity when compared to the media-only exposed cells ($p < 0.05$). EtOH decreased the HDE-mediated PKC ϵ activity ($p < 0.05$ compared to 1% HDE; $p < 0.001$ compared to the 5% HDE). PKC ϵ activity is decreased by EtOH but PKC α activity is unaffected, which indicates that EtOH is affecting a target of HDE downstream of PKC α but upstream of PKC ϵ .

PMA Stimulation of PKC ϵ Is Not Affected by Alcohol

To determine whether alcohol has a direct inhibitory effect on PKC ϵ or an indirect effect on an upstream mediator of PKC ϵ activation, we assayed the ability of a direct PKC activator—PMA—to activate PKC ϵ in the presence of alcohol (Fig. 4). BEAS-2B were pretreated with media or 100 mM EtOH for 24 hours followed by a 6-hour treatment with either 5% HDE or 100 ng/ml PMA. Cells were then assayed for PKC ϵ activity. Consistent with the data shown in Fig. 3, HDE again stimulated PKC ϵ activity ($p < 0.01$) and EtOH significantly decreased HDE-stimulated activity. In contrast, PMA-stimulated PKC ϵ activity was not decreased by EtOH pretreatment. These data show that alcohol-mediated inhibition of PKC ϵ is specific to HDE stimulation and not due to a nonspecific effect of alcohol on the enzyme.

EtOH Inhibits TACE Activity

TACE is a sheddase that regulates sequestration of TNF- α . Cleavage of TACE results in a biologically active form of TNF- α . BEAS-2B were pretreated with or without 100 mM EtOH for 24 hours and then exposed to media or 5% HDE for 6 hours. TNF- α release was then measured

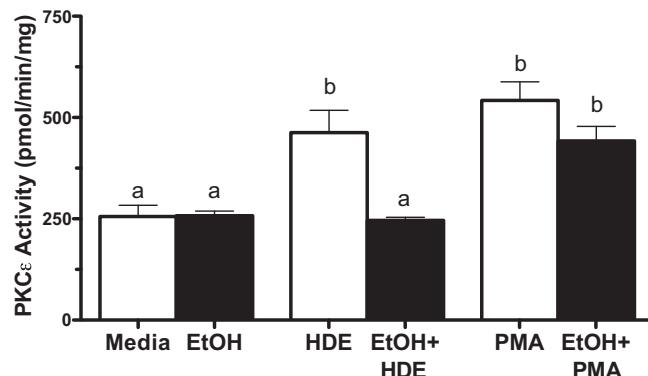


Fig. 4. Phorbol-12-myristate-13-acetate (PMA)-stimulated protein kinase C epsilon (PKC ϵ) activation is not blocked by alcohol. BEAS-2B cells were exposed to media, 5% hog barn dust extract (HDE) exposure, ethanol (EtOH; 100 mM), 5%HDE + EtOH (100 mM), PMA (100 ng/ml), and PMA + EtOH (100 mM) for 6 hours, a denotes $p < 0.05$. PKC ϵ activity increased with HDE exposure and co-exposure of HDE and EtOH decreased PKC ϵ activity significantly, b denotes $p < 0.05$. PMA-stimulated PKC ϵ activity and co-treatment of PMA and EtOH also stimulated PKC ϵ activity, b also denotes $p < 0.05$ under a 1-way ANOVA and Tukey post hoc test.

via ELISA (Fig. 5A). HDE significantly increased TNF- α release compared to control media ($p < 0.01$) while EtOH significantly decreased HDE-stimulated TNF- α release ($p < 0.05$). Media and EtOH alone controls showed low baseline levels of TNF- α release. TACE activity was then measured in BEAS-2B under the same conditions of exposure (Fig. 5B). HDE-stimulated TACE activity in comparison with the media-only exposed cells ($p < 0.05$). EtOH pretreatment blunted HDE-mediated TACE activity when compared to the HDE alone ($p < 0.05$). As a control, pre-treating BEAS-2B with TNF- α protease inhibitor 1 (Tapi-1; 20 μ M) was highly effective at blunting HDE-stimulated TACE activity.

Inhibition of PKA Results in Restoration of HDE-Mediated IL-6 Release

Because our previous studies revealed that elevations in cAMP decreased HDE-stimulated TACE activation (Wyatt et al., 2014), we targeted the PKA pathway to deduce how alcohol blocks HDE-mediated pro-inflammatory cytokine release. BEAS-2B cells were exposed to media alone, 5% HDE alone, EtOH (100 mM) and HDE alone, and EtOH and HDE with or without the PKA inhibitor H-89 (10 μ M) or the PKA antagonist Rp-cAMPS (5 μ M). Similar to IL-8 release, HDE stimulated a significant release of IL-6 ($p < 0.05$), while the combination of HDE and EtOH treatment blunted the release of IL-6 ($p < 0.05$; Fig. 6). Rp-cAMPS treatment fully restored the IL-6 response to HDE in the presence of EtOH ($p < 0.001$). H-89 treatment was also able to fully restore HDE-stimulated IL-6 release in the presence of EtOH-exposed airway cells ($p < 0.0001$). These data support a role for PKA in the alcohol-mediated inhibition of HDE-stimulated cytokine.

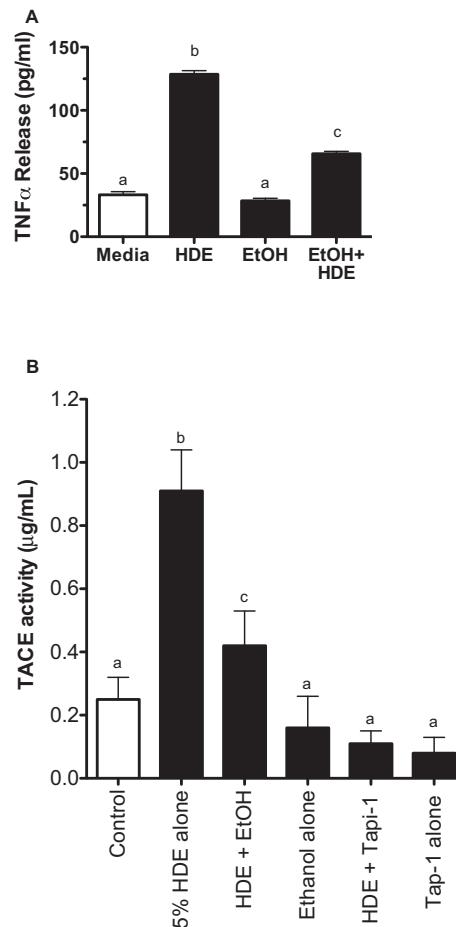


Fig. 5. Hog barn dust extract (HDE)-stimulated tumor necrosis factor alpha (TNF- α)-converting enzyme (TACE), activity and TNF release is blocked by alcohol. (A) BEAS-2B cells were exposed to media, 5% HDE, ethanol (EtOH; 100 mM), 5% HDE + EtOH (100 mM) for 6 hours a denotes $p < 0.01$. HDE stimulated an increase in TNF- α release when compared to controls, b signifies $p < 0.01$. The co-exposure of HDE and EtOH resulted in a significant decrease in TNF- α release when compared to the HDE alone, c signifies $p < 0.05$. Control and EtOH-treated cells were not significantly different. (B) BEAS-2B were subjected to the following treatments: media, 5% HDE, HDE + EtOH (100 mM), EtOH (100 mM) alone, HDE (5%) + TNF- α protease inhibitor 1 (Tapi-1) (10 μ M), and Tapi-1 alone a signifies $p < 0.05$. TACE activity was increased with HDE exposure, but ameliorated when airway cells were exposed to HDE and alcohol. b Signifies the increase of TACE activity with HDE exposure, while c denotes a decrease in TACE activity with a co-exposure of HDE and EtOH, $p < 0.05$ under a 1-way ANOVA with a Tukey post hoc analysis.

Soluble Adenylyl Cyclase Is Responsible for Partially Restoring TNF- α

Because PKA is the primary target for cAMP, we sought to determine whether inhibiting adenylyl cyclase (AC) would reverse the alcohol-mediated inhibition of HDE-stimulated TNF- α release. BEAS-2B cells were exposed to media, 5% HDE, EtOH (100 mM) and EtOH and HDE with or without the AC inhibitors SQ22,536 and KH7. In Fig. 7a, HDE-exposed cells manifested an increase in TNF- α release when compared to media-only exposed cells ($p < 0.05$) while EtOH pretreatment significantly decreased HDE-stimulated TNF- α release ($p < 0.05$). Pre-incubation with 10 μ M KH7 (soluble

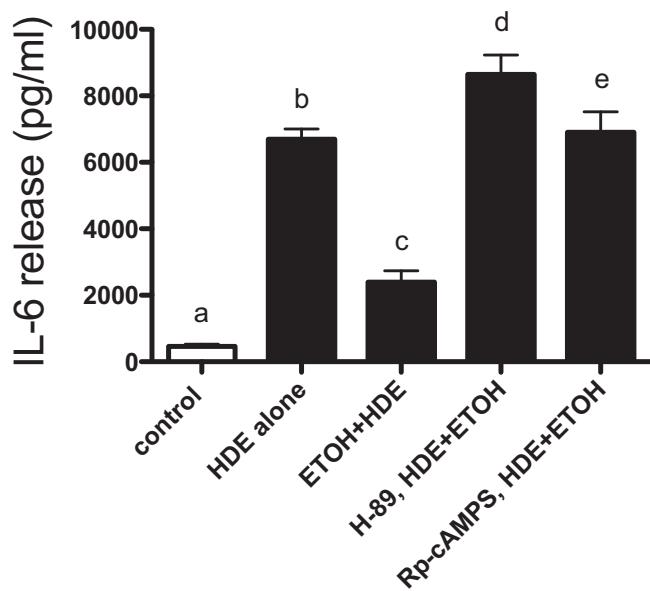


Fig. 6. Rp-cAMPS or H-89 allow hog barn dust extract (HDE)-stimulated interleukin-6 (IL-6) in presence of ethanol (EtOH). BEAS-2B cells were treated with the following treatments for 6 hours: media, HDE, HDE + EtOH and HDE + ETOH + inhibitors (Rp-cAMPS [5 μ mol] or H-89 [10 μ M]), a, b, c signify $p < 0.05$. Supernatant fractions were extracted and IL-6 was measured via ELISA. Both the antagonist analog (Rp-cAMPS) and protein kinase inhibitor (H-89) were capable of fully restoring the HDE-mediated cytokine release that was blunted by alcohol when compared to the co-exposed cultures, d denotes $p < 0.0001$ and e signifies $p < 0.001$ under a 1-way ANOVA and Tukey post hoc test.

AC [sAC] inhibitor) significantly reversed alcohol-mediated inhibition of HDE-stimulated TNF- α ($p < 0.05$). However, the inhibitor of transmembrane AC—SQ22,536—did not rescue HDE-mediated TNF- α release from alcohol inhibition. In addition to blocking the formation of cAMP through cyclase inhibition, we investigated the effects of protecting the cAMP signal through phosphodiesterase (PDE) inhibition. BEAS-2B cells were pretreated with or without PDE inhibitors IBMX (200 μ M), Rolipram (8 μ M), or Ro 20-1724 (20 μ M) followed by exposure to media, 5% HDE, EtOH (100 mM), or EtOH and HDE. At 6 hours, TNF- α release was measured. TNF- α release increased with HDE exposure while TNF- α release was decreased in HDE-exposed cells pretreated with EtOH. Importantly, both PDE4-selective inhibitors (Rolipram and Ro 20-1724) and the nonselective PDE inhibitor (IBMX) enhanced the alcohol-mediated decrease in HDE-stimulated TNF- α release (Fig. 7B). Collectively, these data suggest cAMP signaling is responsible for the alcohol-mediated decrease in pro-inflammatory cytokine release.

Inhibition of NO Fully Restores Pro-Inflammatory Cytokine Production

We have previously established that alcohol requires the generation of NO to activate the cAMP/PKA pathway (Sisson et al., 1999). We therefore examined the role of NO in

the alcohol-mediated inhibition of HDE-stimulated cytokine release. BEAS-2B cells were pretreated with or without L-NMMA (10 μ M), an NO inhibitor, for 1 hour followed by a 6-hour exposure to either media, HDE (5%), EtOH (100 mM), or HDE and EtOH (Fig. 8). Compared to controls, HDE significantly stimulated TNF- α release ($p < 0.05$) while EtOH pretreatment decreased this HDE-stimulated TNF- α ($p < 0.05$). L-NMMA reversed alcohol-mediated inhibition of HDE-stimulated TNF- α release.

NF- κ B Binding Remains Unaffected Despite EtOH Treatment

To ensure that EtOH is not inhibiting or reducing pro-inflammatory transcription factor, NF- κ B, its expression was analyzed post-EtOH and HDE treatment. In Fig. 9, we treated BEAS-2B cells with 100 mM EtOH and 5% HDE. The media-only and EtOH treatments did not significantly alter the NF- κ B binding ($p > 0.05$). However, HDE-induced NF- κ B expression ($p < 0.05$) and EtOH did not reduce this phenomenon in the combined treatment ($p < 0.05$). From this data, we conclude that EtOH does not inhibit NF- κ B expression as the mechanism for the reduction of TACE and TNF- α . These data suggest that alcohol requires the dual signaling pathways of both NO and cAMP to activate PKA for the inhibition of TACE-mediated TNF- α release in response to HDE (Fig. 10).

DISCUSSION

Alcohol has the ability to greatly modulate host innate immunity. The tracheal cilia can become desensitized when exposed to chronic alcohol, which leads to defective mucociliary clearance (Sisson, 2007). Alcohol feeding of animals (acutely) reduces TNF- α production by alveolar macrophages (Kolls et al., 1995). Alveolar macrophage phagocytosis can also be modified under acute and chronic alcohol treatments (Brown et al., 2007; Rimland and Hand, 1980; Spitzer and Zhang, 1996; Zhang et al., 1998). Mice exposed to a bacterial infection or lipopolysaccharide experienced blunted chemokine production and neutrophil recruitment (Boe et al., 2003; Zhang et al., 1998). In our current study, we investigated similar effects of alcohol, but in epithelial cells and using organic dust. Hog barn dust exposure in agricultural workers is of concern because dusts can pose respiratory problems (Poole and Romberger, 2012). Alcohol use can result in a loss of normal immunological and inflammatory responses. In the airways of heavy alcohol consumers, a condensation “rain effect” occurs, allowing alcohol to accumulate in localized high (mM) concentrations, leading to the impairment of normal airway defenses (Sisson, 2007). Using a dose of 100 mM EtOH is not cytotoxic to BEAS-2B cells (Simet et al., 2012). In fact, the highest known nonlethal blood alcohol concentration in a human at the time of hospital admission was 1,510 mg/dl or 327 mM (Johnson et al., 1982). Blood alcohol concentrations can be lower than lung alcohol concentrations due to the rapid warming and cooling

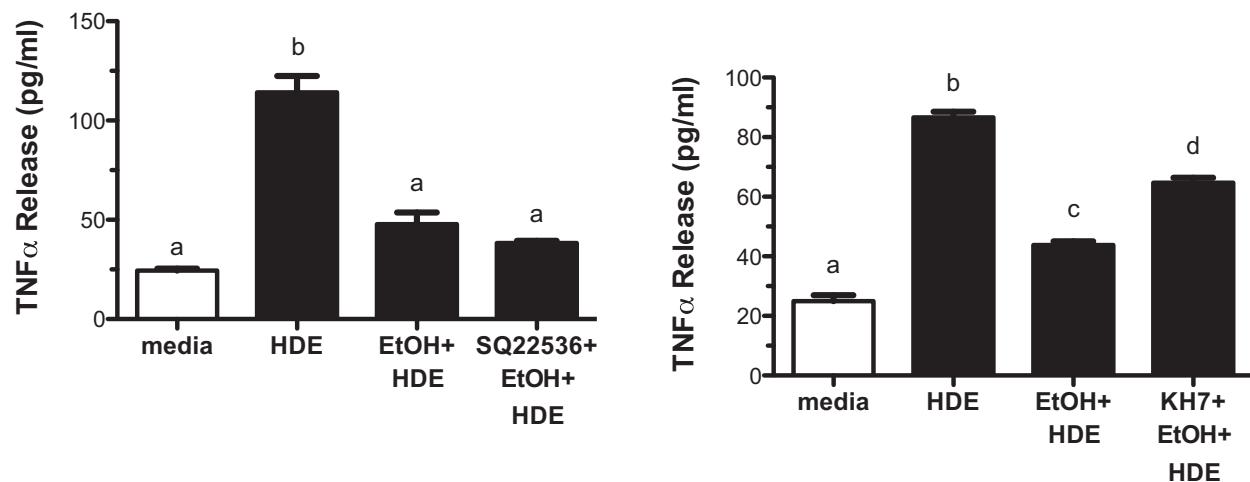
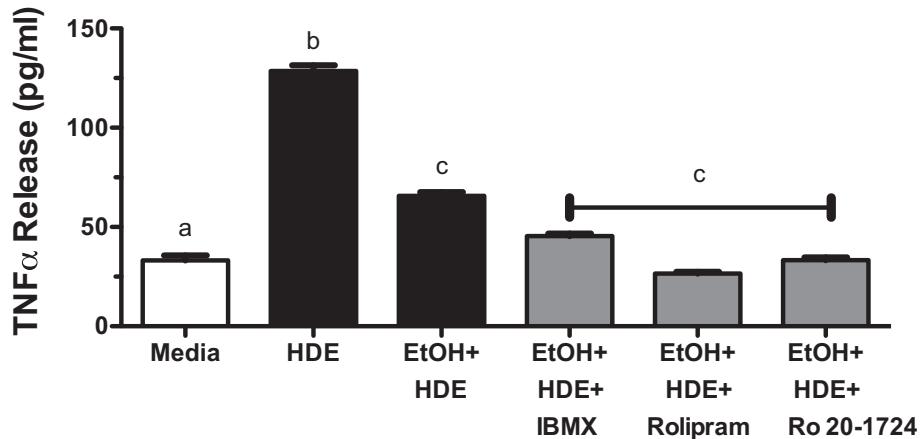
A**B**

Fig. 7. KH7 inhibitor, not SQ22,536 allowed hog barn dust extract (HDE)-stimulated tumor necrosis factor alpha/interleukin-8 (TNF- α /IL-8) in presence of ethanol (EtOH). **(A)** BEAS-2B were subjected to the following treatments for 6 hours: media, 5% HDE, HDE + EtOH (100 mM), and HDE + EtOH + inhibitor (SQ22,536, 50 μ M or KH7, 10 μ M). Supernatant fractions were extracted and TNF- α , IL-8 and IL-6 were measured via ELISA. The KH7 soluble adenylyl cyclase (AC) inhibitor was able to partially restore cytokine production (d signifies $p < 0.05$ compared to b [HDE]) whereas the SQ22,536 particulate AC inhibitor was not different from the co-exposed (HDE + EtOH) group cultures. **(B)** BEAS-2B cells were exposed to media, HDE, EtOH and HDE with or without IBMX (0.2 mmol/l), Rolipram (8 μ M), Ro 20-1724 (20 μ M). HDE increased TNF- α release while EtOH decreased HDE-induced TNF- α release b signifies $p < 0.05$ compared to media a. Treatment with phosphodiesterase inhibitors resulted in decreased TNF- α release, c denotes $p < 0.05$ compared to b. Significance was determined under a 1-way ANOVA and Tukey post hoc test.

of EtOH in the bronchial airways leading to condensation. This is known as the rain effect. Airway epithelial cells also metabolize alcohol via CYP2E1. There is evidence that EtOH can impact such pathways. In McCaskill and colleagues (2015), mice fed 20% EtOH via diet for 6 weeks experienced increased gene expression of lung CYP2E1. With this knowledge of alcohol's ability to interfere with respiratory innate defenses, we tested our hypothesis that alcohol blocks dust-mediated pro-inflammatory cytokine production through a cAMP-dependent inhibition of TACE.

Our current investigations indicate that alcohol reduces HDE-stimulated pro-inflammatory cytokine release by bronchial epithelial cells via inhibiting NO, AC, and PKA. Our

laboratory has established that alcohol activates the NO pathway, resulting in PKG activation, which is required for the downstream activation of PKA by EtOH (Sisson et al., 1999). Our data show that the NO pathway is also involved with alcohol's role in diminishing HDE-mediated inflammation. Alcohol stimulates the production of cAMP through an alcohol-sensitive AC (Tabakoff et al., 2001), and our results demonstrate that cAMP production is being activated by alcohol, leading to blunted pro-inflammatory TNF- α and downstream IL-6 and IL-8 release. By inhibiting sAC, the cytokine release that normally occurs in response to HDE was partially restored in the presence of alcohol. Interestingly, the transmembrane AC inhibitor had little effect on

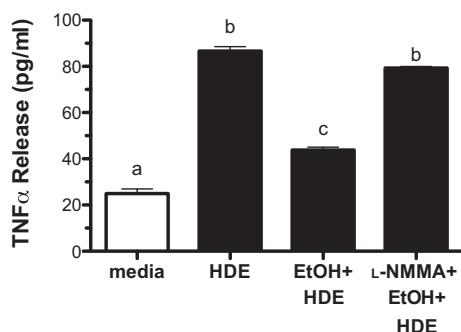


Fig. 8. L-NMMA allows hog barn dust extract (HDE)-stimulated tumor necrosis factor alpha/interleukin-8 (TNF- α /IL-8) in presence of ethanol (EtOH). BEAS-2B were subjected to the following treatments for 6 hours: media, 5% HDE, HDE + EtOH (100 mM), and HDE + EtOH + L-NMMA (100 mM), a and c denote $p < 0.05$. Supernatant fractions were extracted and pro-inflammatory cytokines TNF- α , IL-8, and IL-6 were measured via ELISA. L-NMMA restored HDE-mediated cytokine production compared to HDE-exposed cell cultures, b denotes $p > 0.05$ under a 1-way ANOVA and Tukey post hoc analysis.

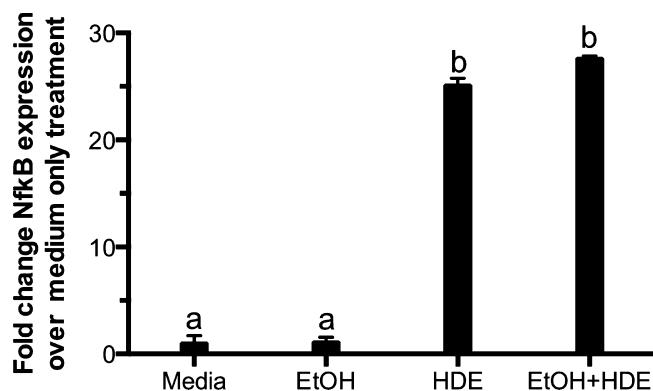


Fig. 9. Ethanol (EtOH) does not decrease tumor necrosis factor alpha (TNF- α) levels via nuclear factor-kappa beta (NF- κ B) expression. BEAS-2B cells were incubated with 100 mM EtOH 24 hours followed by a 6-hour treatment of 5% hog barn dust extract (HDE) exposure and EtOH. NF- κ B activation was assayed as described and expressed as a fold change activation over medium-only treated cells. Media and EtOH were not significantly different. a denotes $p > 0.05$, whereas HDE and HDE and EtOH significantly increased NF- κ B expression (b signifies p -value < 0.05) under a 1-way ANOVA and Tukey post hoc test.

cytokine released in response to HDE + EtOH. This observation is consistent with our previous findings that the ciliated airway epithelium contains an sAC that is stimulated by alcohol (Sisson et al., 2009). We also demonstrate the effects of inhibiting PDEs on alcohol-mediated inhibition of dust-stimulated cytokine release using both a nonselective inhibitor, IBMX, and selective PDE4 inhibitors, Rolipram and Ro 20-1724 (Fig. 7B). We have previously established that in bovine epithelial cells EtOH modulates PDE4 activity (Forget et al., 2003). Previously, we showed that by inhibiting PDE4, eventual cAMP degradation in response to alcohol-stimulated cAMP production is blocked, thus allowing for an enhanced accumulation of cAMP after alcohol exposure (Forget et al., 2003). Both selective and nonselective PDE4 inhibitors enhanced the EtOH-mediated inhibition of HDE-

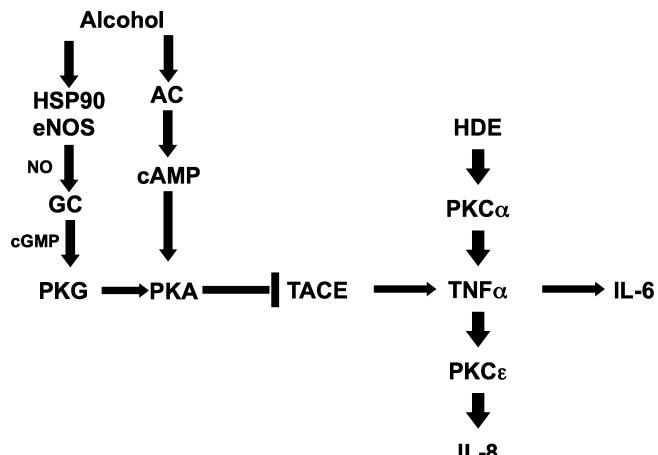


Fig. 10. Summary model diagram. Proposed model of alcohol's effect on hog barn dust extract (HDE)-stimulated pro-inflammatory cytokine release. Alcohol stimulates both nitric oxide (NO) and adenylyl cyclase, which leads to the activation cAMP and subsequent protein kinase activation, which then inhibits tumor necrosis factor (TNF)- α -converting enzyme (TACE), activity. By blunting TACE activity, the HDE-stimulated release of pro-inflammatory cytokines TNF- α , interleukin-8 (IL-8) and IL-6 are decreased. protein kinase C alpha (PKC α) is activated by dust upstream of TACE and independent of TACE action, while protein kinase C epsilon (PKC ϵ) activity is regulated by dust downstream of TACE and TNF- α .

stimulated TNF- α release. These data provide several lines of evidence that EtOH is inhibiting TNF- α release through a cAMP-dependent manner.

The observation that alcohol is working through a cAMP-dependent manner is an important key to the regulation of HDE-stimulated TACE. With the addition of cAMP, PKC ϵ activity is decreased whereas PKC α activity remains unaltered. This data led us to believe that the effects of alcohol on airway epithelial cells occurs downstream of PKC α , but upstream of PKC ϵ . Our previous study describes the sequential nature of PKC α and PKC ϵ (Wyatt et al., 2010). PKC α activation occurs around 1 hour while PKC ϵ activation occurs later, at 6 hours. PKC ϵ cannot be activated by HDE without the earlier activation of PKC α . This is the reasoning behind Fig. 3, which shows no increase of PKC α activity at 1 hour but PKC ϵ activity is increased after a 6-hour HDE exposure. In the study of Song and colleagues (2005), alcohol was shown to disrupt TACE activity. TACE is a sheddase, which cleaves TNF- α into its active signaling form (Rose-John, 2013). We have previously shown that TNF- α is released following PKC α activation and is required for PKC ϵ activation and IL-8 release (Wyatt et al., 2010, 2014). We have recently shown that HDE-mediated increases in TACE activity could be inhibited using cell-permeable cAMP analogs to directly activate PKA (Wyatt et al., 2014). In our study, alcohol-inhibited TACE activity, a concept also observed by others (Song et al., 2005). Our data confirms TNF- α release after HDE exposure corresponds temporally with TACE activity. We found that alcohol, through a cAMP-PKA dependent manner, reduces HDE-stimulated TACE activity, which corresponds with a reduction in

inflammatory cytokine release from bronchial epithelial cells stimulated with HDE. In addition, as shown as in Fig. 9, NF- κ B transcription remains unaffected after alcohol treatment, which leads us to conclude that alcohol is decreasing the HDE-mediated cytokine through the PKA pathway. The specific mechanism by which PKA inhibits TACE requires further investigation. Events upstream of TACE could be the reason for this difference. One of the proteins that could be affected is furin. Furin is a proprotein convertase that cleaves TACE into its soluble form. In the studies published by Howarth and colleagues (2012), hepatocytes that were exposed to EtOH showed endoplasmic reticulum (ER) fragmentation. This dysfunction is of importance in the case of furin because furin is manufactured in the ER and undergoes folding as well proteolytic cleavage (Creemers et al., 1995). Future studies showing furin presence and activity in relation to TACE and TNF- α in the context of alcohol and dust exposure are warranted.

HDE stimulates inflammatory cytokine release in vitro and this response is reduced by alcohol treatment. Although this reduction in inflammation associated with EtOH may be construed as a protective effect, clinical findings suggest this inhibition of inflammation in the lungs has negative consequences with regard to lung immunity. There are studies demonstrating that hog barn dust induces oxidants. In Pender and colleagues (2014), THP-1 cells that were exposed to hog barn dust (5 and 10%) show increased levels of ROS production. Wyatt and colleagues (2008) show increased NO in bovine airway cells exposed to hog barn dust. Simet and colleagues (2013) also demonstrate the efficacy of commercially available antioxidants (NAC and procysteine) to alleviate alcohol-mediated ciliary desensitization. Diallyl disulfide also reduces DNA damage and CYP2E1 levels when exposed to 80 mM alcohol in human bronchial epithelium (Sapkota et al., 2014). Chronic alcohol-exposure is known to induce alcohol-induced ciliary dysfunction in vivo and in vitro (Sisson et al., 1999; Wyatt et al., 2004). This results in the mucociliary apparatus becoming defective due to cilia desensitization. Similarly, pathogens trapped in the mucus blanket will not be cleared from the airway as efficiently, potentially leading to infection. Furthermore, alcohol use can lead to sustained infections due to impaired leukocyte functions (Happel et al., 2006). We have previously shown that alcohol exposure in BEAS-2B cells increases permeability and alters tight junction proteins (Simet et al., 2012). Concurrent HDE and EtOH treatment results in a decrease of the natural airway inflammatory response (IL-8 and TNF- α release) to dust. The normative inflammatory response incurred as a result of HDE exposure is thus altered by alcohol treatment.

These cellular observations support existing data using an in vivo animal model of dust exposure in the context of alcohol consumption. In studies conducted with a mouse model, mice that consumed ad libitum EtOH for 6 weeks and inhaled HDE for 3 weeks had fewer neutrophils and lymphocytes present in bronchoalveolar lavage fluid (BALF) compared to mice that were only exposed to HDE (McCaskill et al., 2012). In our study, we observed pro-inflammatory cytokines that are responsible for neutrophil and lymphocyte recruitment increase with a 5% HDE exposure in the BEAS-2B cell line. We also observed a blunting effect in the airway cells that were exposed to HDE and EtOH, which would be in accordance with our in vivo model's histological findings (McCaskill et al., 2012). A decrease in pro-inflammatory cytokines (TNF- α , IL-6, IL-8) as seen in Figs 1, 5, and 6 corroborates the published observations of decreased lung lavage lymphocytes and mononuclear cell aggregates in alcohol-fed mice challenged with HDE. The macrophages collected from the BALF were significantly lower in the HDE and HDE and EtOH treated groups when compared to control and EtOH exposed groups. In that study, mice fed alcohol and exposed to HDE experienced a significant reduction in body weight and 20% mortality, an observation not seen in alcohol-fed mice not exposed to dust (McCaskill et al., 2012). In the Mason and colleagues (2004) study, mice that were fed alcohol chronically and exposed to tuberculosis experienced worse infections than those who were not drinking alcohol. Thus, alcohol has the ability to dampen the immune response and therefore leave the individual immunocompromised and prone to infection. Our findings here suggest that alcohol reduces the inflammatory response of bronchial epithelial cells to HDE via a mechanism involving the inhibition of TACE by NO and PKA pathways.

In summary, alcohol exposure reduces HDE-mediated inflammatory cytokine release from bronchial epithelial cells in a cAMP-dependent manner. Our findings suggest alcohol modulates TACE activity to prevent inflammatory responses in the bronchial epithelium. Future studies are warranted to further investigate the precise mechanism of PKA-mediated TACE inhibition. We have shown that chronic alcohol use can have negative effects on the airway epithelium in response to HDE. Such observations may have important ramifications on the health of alcohol-consuming workers exposed to organic agricultural dusts.

CONFLICT OF INTEREST

There are no conflicts of interest to disclose.

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