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Dietary antioxidants prevent alcohol-induced ciliary dysfunction

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Abstract

Previously we have shown that chronic alcohol intake causes alcohol-induced ciliary dysfunction (AICD), leading to non-responsive airway cilia. AICD likely occurs through the downregulation of nitric oxide (NO) and cyclic nucleotide-dependent kinases, protein kinase G (PKG) and protein kinase A (PKA). Studies by others have shown that dietary supplementation with the antioxidants N-acetylcysteine (NAC) and procysteine prevent other alcohol-induced lung complications. This led us to hypothesize that dietary supplementation with NAC or procysteine prevents AICD. To test this hypothesis, C57BL/6 mice drank an alcohol/water solution (20% w/v) *ad libitum* for 6 weeks and were concurrently fed dietary supplements of either NAC or procysteine. Ciliary beat frequency (CBF) was measured in mice tracheas, and PKG/PKA responsiveness to β-agonists and NO_x levels were measured from bronchoalveolar lavage (BAL) fluid. Long-term alcohol drinking reduced CBF, PKG and PKA responsiveness to β-agonists, and lung NO_x levels in BAL fluid. In contrast, alcohol-drinking mice fed NAC or procysteine sustained ciliary function and PKG and PKA responsiveness to β-agonists. However, BAL NO levels remained low despite antioxidant supplementation. We also determined that removal of alcohol from the drinking water for as little as 1 week restored ciliary function, but not PKG and PKA responsiveness to β-agonists. We conclude that dietary supplementation with NAC or procysteine protects against AICD. In addition, alcohol removal for 1 week restores cilia function independent of PKG and PKA activity. Our findings provide a rationale for the use of antioxidants to prevent damage to airway mucociliary functions in chronic alcohol-drinking individuals.

Keywords

alcohol; ciliary dysfunction; nitric oxide; PKA; PKG; N-acetylcysteine; procysteine; ciliary beat frequency

Introduction

Cilia are specialized, motor-driven organelles that propel particles, mucus, and debris out of the lung via the airways. Individuals who consume heavy amounts of alcohol on a long-term basis are likely to have alveolar epithelial pathology, increased oxidant stress, an increased susceptibility to various pulmonary infections, and impaired mucociliary clearance(Boé,

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Vandivier, Burnham, & Moss, 2009; Jerrells et al., 2007; Joshi & Guidot, 2007; Laurenzi & Guarneri, 1966; Sisson, 2007).

Mucociliary clearance is the first line of innate immunity protecting the lung from infections. Our previous studies demonstrated that brief alcohol exposure stimulates airway cilia beating through generation of nitric oxide and the downstream activation of the cyclic nucleotide-dependent kinases PKG and PKA (Sisson, 1995; Wyatt, Forgèt, & Sisson, 2003). In contrast, heavy and prolonged alcohol drinking impairs mucociliary clearance through cilia desensitization (Wyatt, Gentry-Nielsen, Pavlik, & Sisson, 2004; Wyatt & Sisson, 2001). This sustained alcohol-induced ciliary dysfunction (AICD) likely occurs through the downregulation of nitric oxide and the cyclic nucleotide-dependent kinase pathway, processes which alcohol is known to stimulate in airway cells (Wyatt, Gentry-Nielsen, Pavlik, & Sisson, 2004).

Considerable evidence shows that sustained alcohol exposure increases oxidative stress within the lung, which can impair alveolar macrophage function (Brown, Ping, Harris, & Gauthier, 2007), impair epithelial barrier function (Guidot et al., 2000), increase type II cell apoptosis, and impair many other cellular functions (Brown, Harris, Bechara, & Guidot, 2001; Brown, Harris, & Guidot, 2001). Alcohol abuse has been also been shown to increase the risk of acute respiratory distress syndrome (ARDS). Prolonged alcohol abuse not only increases the risk for serious illnesses that lead to ARDS, it independently increases the risk for ARDS and complications, which can lead to organ failure and ventilator-associated trauma (Joshi & Guidot, 2007).

It is notable that the majority of alcohol-induced oxidative stress studies have examined lung alveolar tissue with little focus on the conducting airways. Studies in both rats and humans have shown that prolonged alcohol consumption promotes oxidative stress within the lung, likely by increasing reactive oxygen species generation and by depleting critical antioxidants such as glutathione (GSH) in the epithelial lining fluid (Brown, Harris, Bechera, & Guidot, 2001; Brown, Harris, & Guidot, 2001; Holguin, Moss, Brown, & Guidot, 1998; Liang, Yeligar, & Brown, 2012; Moss et al., 2000). GSH is an antioxidant that has been implicated in immune responses. Within the lung, GSH has been implicated as a critical component in protecting the lung epithelium; GSH prevents the injury and inflammation caused by reactive oxygen species (Kehrer & Lund, 1994; Morris & Bernard, 1994) which localize to specific cytoplasmic and mitochondrial pools. Prolonged alcohol exposure has been shown to decrease GSH in alveolar macrophages, which are dependent on GSH in the epithelial lining fluid for normal cellular function. Prolonged alcohol exposure selectively depletes GSH mitochondrial pools in alveolar type II cells (Das & Vasudevan, 2007; Liang, Yeligar, & Brown, 2012), rendering the mitochondria susceptible to damaging ROS. However, the generation of oxidative stress in the conducting airways may also be triggered by nitric oxide (NO).

During stress conditions, such as prolonged and heavy alcohol intake, the NO-producing enzyme nitric oxide synthase (NOS) may become “uncoupled”. This NOS uncoupling diverts NO from the canonical guanylyl cyclase, PKG activation pathway, toward the production of superoxide and peroxynitrite (Verhaar, Westerweel, van Zonneveld, & Rabelink, 2004). Peroxynitrite itself is a potent oxidant, which may also participate in alcohol-induced oxidative stress (Radi, Cassina, Hodara, Quijano, & Castro, 2002). While the mechanisms of alcohol-induced oxidative stress are still relatively unknown, it is likely that NOS uncoupling and oxidative stress generation are events involved in AICD.

Dietary supplementation with N-acetylcysteine, a GSH precursor, has been shown to restore GSH cytosolic pools, while procysteine, another GSH precursor, is able to restore both

cytosolic and mitochondrial GSH pools (Guidot & Brown, 2000). In addition, these studies demonstrate that dietary supplementation of the antioxidant N-acetylcysteine (NAC) normalizes bacterial clearance in chronic alcohol-exposed rats (Tang, Gabelaia, Gauthier, & Brown, 2009), and supplementation with procysteine (PRO) prevents alcohol-induced alveolar epithelial cell function disorder (Brown, Harris, Bechera, & Guidot, 2001; Brown, Harris, & Guidot, 2001; Guidot et al., 2000; Guidot & Brown, 2000).

Considering the recent reports that NAC and procysteine were able to restore GSH levels and normalize alveolar epithelial cell function, we hypothesize that dietary supplementation with NAC or procysteine prevents alcohol-induced ciliary dysfunction (AICD).

Materials and methods

C57BL/6 female mice

Female C57BL/6 mice were purchased from Charles River Laboratories (Willmington, MA) at 6–8 weeks of age and maintained at the UNMC Comparative Medicine Facility (Omaha, NE) under standard housing conditions. Mice were group-caged and acclimated to the facility for 1 week before the start of alcohol exposure and received standard rodent chow *ad libitum* for the entire course of the study. Mice were monitored daily and weighed weekly. All experimental protocols were reviewed in advance and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. All protocols conformed to the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health.

Alcohol feeding

Mice were given increasing concentrations of ethanol in water over a 1-week period until the target concentration of 20% was reached (Song et al., 2002). Mice in the alcohol group were given 5% alcohol (w/v) to drink *ad libitum* (95% ethanol diluted with Milli-Q water) for 2 days, 10% ethanol (w/v) for 2 days, 15% ethanol (w/v) for 3 days, and 20% ethanol (w/v) for 6, 7, 8, 9, or 12 weeks. Saccharin was added to the water in all groups. Mice in the matched control group were given water from the same source without ethanol. Mice in the alcohol removal group were given decreasing concentrations of ethanol that was removed from the water over a 1-week period: 15% ethanol (w/v) for 3 days, 10% ethanol (w/v) for 2 days, 5% alcohol (w/v) for 2 days, with the 8th day returning to water only. All durations of alcohol exposure indicated in the following text refer to the time spent on the final 20% alcohol concentration. For the alcohol removal study, mice were fed ethanol at 20% in their water for 6, 7, 8, 9, or 12 weeks as described above, or alcohol was removed from 6–12 weeks. Mice were sacrificed beginning at 6 weeks, and additional mice were sacrificed every week up until 12 weeks.

Antioxidant feeding

Animals were given water, procysteine, n-acetylcysteine (NAC), or ethanol in their water. Both the control and alcohol-fed groups were given an antioxidant drug (or not) in their drinking water for 1 week prior to beginning the alcohol feeding. Alcohol (or not) was administered with NAC (0.163 mg/mL of drinking water; Sigma) or procysteine (0.35% v/v in drinking water; Sigma) based on previous studies (Guidot & Brown, 2000; Lois, Brown, Moss, Roman, & Guidot, 1999). Saccharin was added to the water in all groups to counteract the smell of the antioxidants. Mice receiving ethanol were ramped up to a treatment concentration of 20% ethanol in water over a 1-week period. No significant difference in water consumption was observed between the antioxidant + alcohol-fed groups, antioxidant-fed groups, or alcohol-fed groups.

Blood alcohol content (BAC)

BACs were monitored following each experiment to verify that the mice had elevated levels of alcohol. Upon euthanasia, 0.8–1.0 mL of whole blood was collected into serum separator tubes (BD Scientific, Franklin Lakes, NJ). The tubes were placed on ice for 30 min and then centrifuged at 8,000 revolutions/min for 10 min. Serum was transferred to microcentrifuge tubes containing a rubber gasket and frozen at −80 °C until assayed. The serum was assayed using an alcohol reagent set and alcohol control (Pointe Scientific, Canton, MI). Briefly, samples and controls were added to reconstituted reagent at 30 °C, mixed, and incubated in a water bath with shaking for 5 min. Samples were then transferred to a 96-well flat-bottom plate, and the absorbance was read at 340 nm. The ethanol concentration in mg/dL was calculated according to the manufacturer's defined procedure. BAC analysis revealed that alcohol-drinking mice had elevated BACs (> 45 mg/dL), whereas water-drinking control mice had no ethanol present. In addition, concurrent antioxidant feeding had no effect on BAC levels in either control or alcohol-drinking mice (data not shown).

Bronchoalveolar lavage (BAL) fluid

BAL fluid was collected 3 × 1 mL in separate tubes. BAL fluid was centrifuged, and supernatant from the first-collected 1 mL of BAL fluid was stored for NOA analysis at −80 °C, while the 2nd and 3rd collections were discarded. Cell pellets were resuspended in sterile PBS and counts and differentials were collected.

Trachea harvesting and treatment

Tracheas were harvested as previously described (Elliott, Sisson, & Wyatt, 2007). Briefly, tracheas were removed from euthanized mice and placed in serum-free M199 media (Gibco). Rings were cut from each trachea and placed in 35-mm culture dishes and allowed to equilibrate for 30 min at 37 °C and 5% CO_2, then allowed to equilibrate to 25° C for 10 min prior to CBF readings. Isoproterenol (Sigma) is a β-agonist that was used to treat the rings for 1 h, followed by the final CBF analysis. The remaining tracheas were flash-frozen in liquid nitrogen for kinase assays.

Ciliary beat frequency

The motion of beating cilia on each tracheal ring was quantified using phase contrast microscopy and computerized frequency spectrum analysis. During CBF measurement, tracheal rings were maintained at a constant temperature (24 °C ± 0.5 °C) by a thermostatically controlled heated stage (Sanderson, Lansley, & Dirksen, 1992). All recordings for analysis were taken with a Kodak Megaplus 310 analog/digital video camera (Eastman Kodak Motion Analysis Systems Division, San Diego, CA). Whole-field analysis was performed and the CBF determined by collecting data sampled at 85 frames per second for 3 seconds and performing frequency spectrum analysis using the Sisson-Ammons Video Analysis (SAVA) system (Sisson, Stoner, Ammons, & Wyatt, 2003).

Procaterol stimulation

Cilia responsiveness was measured by the addition of procaterol after baseline readings were obtained. ΔCBF was then calculated.

PKA and PKG activity assays

Tracheas were homogenized in 250-μL cell lysis buffer, sonicated, and centrifuged at 10,000 g for 30 min at 4 °C. The ass ay used by our lab (Wyatt, Forgèt, Adams, & Sisson, 2005) utilizes 130 μM PKA substrate heptapeptide (LRRASLG) or PKG substrate (glasstide [RKRSRAE]), 10 μM cAMP, 0.2 mM isobutylmethyxanthine, 20 mM magnesium acetate, and 0.2 mM [γ-32P] adenosine triphosphate (ATP) in a 40-mM Tris-HCl buffer (pH 7.5).

Samples (20 μL) were added to 50 μL of the above reaction mixture and incubated for 10 min at 30 °C. Incubations were halted by spotting 50 μL of each sample onto P-81 phosphocellulose papers (Whatman, Hillsboro, OR). Papers were then washed 5 times for 5 min each in phosphoric acid (75 mM), washed once in ethanol, dried, and counted in nonaqueous scintillant as previously described. Negative controls consisted of similar assay samples with or without the substrate peptide. Kinase activity was expressed in relation to total cellular protein assayed and calculated in picomoles of phosphate incorporated per minute per milligram of protein (pmol/min/mg). Protein was determined in tracheal samples by Biorad Assay (Biorad).

Nitric oxide analysis

NO_x was measured indirectly in the liquid phase of BAL fluid from experimental animals, using a Sievers NOA 280 chemiluminescence analyzer (Boulder, CO), using the purge vessel to convert oxidation products back to nitric oxide (Wyatt, Forgèt, Adams, & Sisson, 2005). Calculations were extrapolated from a standard curve made by injections of 1 nM–100 μM sodium nitrite.

N-acetylcysteine (NAC) analysis

To determine if animals were ingesting and metabolizing NAC, NAC bronchoalveolar lavage fluid levels were measured using the BIOXYTECH GSH-400 colorimetric assay following the manufacturer's protocol (OxisResearch, Portland, OR). NAC BAL fluid concentrations were examined in all treatment groups. A biologically relevant concentration of NAC was detected in all groups. Alcohol-only animals had decreased NAC levels present in BAL fluid. This observed decrease was prevented by NAC and procysteine dietary supplementation (Supplemental Figure).

Statistical analysis

Results are expressed as the mean ± SEM of the indicated number of animals in each group. Statistical differences between the various group means were determined using the one-way ANOVA *post hoc* Bonferroni's test (Graphpad Prism, San Diego, CA). An unpaired *t* test was used to determine significance between the ΔPKA-alcohol and the ΔPKA-procysteine-alcohol groups. A *p* value of less than 0.05 was accepted as significant.

Results

Alcohol-induced ciliary dysfunction (AICD) is prevented by antioxidants

Dietary supplementation with either procysteine or N-acetylcysteine (NAC) prevented AICD in alcohol-drinking animals (Fig. 1). Our results show that procaterol-stimulated CBF is blunted in alcohol-drinking mice, resulting in significantly decreased CBF stimulation (ΔCBF $p < 0.0001$) compared to naïve animals. Conversely, alcohol-drinking mice fed NAC or procysteine had a CBF response equivalent to naïve animals.

We previously linked AICD to blunted activation of the cGMP- and cAMP-dependent kinases, PKG and PKA, respectively, which are thought to be required for cilia stimulation by β-agonists. Because dietary supplementation with procysteine or NAC prevented alcohol-induced cilia non-responsiveness, we hypothesized that dietary supplementation with procysteine or NAC preserves PKG and PKA responsiveness to β-agonists.

Alcohol-impaired activity of PKG is prevented by N-acetylcysteine

Dietary antioxidant supplementation with NAC prevented alcohol-impaired PKG responsiveness (Fig. 2A & B). Alcohol-drinking mice had significantly lower PKG

responsiveness ($p < 0.01$) compared to naïve mice. Both alcohol-drinking and non-drinking mice fed dietary procysteine had higher baseline levels of PKG activity compared to naïve mice. Although the data from mice receiving both alcohol and procysteine suggested a small response to procaterol (a β-agonist), PKG responsiveness did not reach statistical significance ($p = 0.4$). Moreover, alcohol-drinking mice fed NAC had significantly ($p < 0.01$) higher PKG responsiveness compared to alcohol-drinking mice, suggesting that NAC prevents alcohol-reduced PKG responsiveness. The summarized responsiveness data, expressed as ΔPKG responsiveness to β-agonist, are presented in Fig. 2B.

We have previously shown that alcohol stimulation of CBF activates PKG, followed by the activation of PKA. Like PKG, PKA is blunted in AICD. Because NAC blocked alcohol depression of PKG activity, we hypothesized that dietary supplementation with NAC would also preserve PKA responsiveness in alcohol-drinking mice.

Alcohol-impaired PKA responsiveness is prevented by N-acetylcysteine and procysteine

Dietary supplementation with NAC (0.163 mg/mL in drinking water) or procysteine prevents impairment of PKA responsiveness by alcohol. Procaterol-stimulated PKA responsiveness was significantly lower ($p < 0.05$) in alcohol-drinking mice than naïve mice. Although PKA responsiveness in alcohol-drinking mice fed procysteine was analogous to naïve mice, it was not significantly different from untreated alcohol-drinking mice ($p > 0.05$) because baseline PKA activity was higher in the control animals that received procysteine (Fig. 3A). Summarizing the data and presenting ΔPKA responsiveness demonstrated a significant difference in PKA responsiveness in alcohol-drinking animals fed dietary procysteine ($p = 0.02$) (Fig. 3B). In addition, alcohol-drinking mice fed NAC had significantly higher PKA responsiveness ($p < 0.01$) compared to alcohol-drinking mice (Fig. 3B).

We have previously shown stimulation of CBF by alcohol originates through the nitric oxide (NO) pathway, activating PKG and PKA. Like PKG and PKA, NO is blunted in AICD (Fig. 3). Because NAC was able to prevent impairment by alcohol of PKG and PKA responsiveness and procysteine was able to prevent impairment by alcohol of PKA responsiveness, we hypothesized that dietary supplementation with NAC would restore nitric oxide levels in lung lavage fluid.

Alcohol-impaired BAL nitric oxide levels are not prevented by antioxidants

Alcohol-drinking mice had significantly decreased levels of bronchoalveolar lavage (BAL) NO_x. However, dietary supplementation with NAC or procysteine did not prevent alcohol-triggered reduction of No_x in BAL fluid (Fig. 4). Indeed, NAC treatment alone appears to have decreased NO_x levels. We have demonstrated that dietary supplementation with antioxidants is able to prevent some aspects of AICD. However, we were also interested in whether AICD is reversible upon the removal of alcohol and if so, how long does it take for AICD to resolve after alcohol intake is discontinued?

CBF decreased responsiveness is reversible upon alcohol removal

We have demonstrated that long-term alcohol drinking leads to alcohol-induced ciliary dysfunction (AICD) in mice and that most aspects of AICD can be prevented with NAC and procysteine. Still, it is unknown how long AICD persists after the removal of alcohol and without the aid of dietary antioxidants. We hypothesized that AICD is reversible by removing alcohol from the drinking water. To test this hypothesis we induced AICD by adding alcohol to the drinking water for 6 weeks. For 6 additional weeks we either continued adding alcohol or removed alcohol from the drinking water in a subset of animals. After the initial 6 weeks of alcohol drinking, CBF responsiveness to procaterol (Δ CBF;

before and after adding a β-agonist, procaterol) was completely absent in alcohol-drinking mice consistent with AICD and our previous findings (Fig. 5). However, after 1 week of alcohol-free drinking water, cilia responsiveness normalized to that of naïve mice and persisted throughout the remainder of the experiment. These results demonstrate that alcohol-impaired CBF responsiveness is reversible after 1 week of abstinence from alcohol.

We have shown that AICD blunts PKG and PKA responsiveness. Because the absence of alcohol from the drinking water for 1 week restored cilia responsiveness, we hypothesized that removal of alcohol would restore PKG and PKA responsiveness to β-agonists.

Removal of alcohol for up to 6 weeks does not restore PKG or PKA responsiveness

We measured the β-agonist responsiveness of cilia-associated PKG and PKA activities in naïve, alcohol-continued, and alcohol-removed mice. After 6 weeks of alcohol drinking, mice had significantly reduced PKG and PKA responsiveness to procaterol compared to naïve mice (Fig. 6). Although CBF responsiveness was restored after 1 week of alcohol-free drinking water, PKG and PKA responsiveness to the β-agonist remained depressed for the duration of the experiment.

Discussion

Heavy and prolonged alcohol intake increases oxidative stress in the lung with a time course similar to alcohol-induced ciliary dysfunction (AICD). We have shown that AICD decreases ciliary beat frequency, PKG and PKA responsiveness to β-agonists, and NO-lavage levels. We hypothesized that AICD is linked to alcohol-induced oxidative stress. Under stressful conditions, such as prolonged alcohol intake, it is possible for eNOS to become uncoupled (uncoupling of NADPH oxidation and NO synthesis, with incorporation of oxygen instead of L-arginine), which leads to the shunting of NO from the canonical guanylyl cyclase pathway toward the production of peroxynitrite (Verhaar, Westerweel, van Zonneveld, & Rabelink, 2004). Peroxynitrite, a strong oxidant, is known to increase oxidative stress within cells, which can lead to dysfunction of NO-dependent signaling pathways (Pacher, Beckman, & Liaudet, 2007). Our results demonstrate that long-term alcohol-drinking mice have AICD, which can be prevented by feeding mice the dietary antioxidant, N-acetylcysteine (NAC) or procysteine. Alcohol-drinking mice fed NAC or procysteine retained ciliary beat frequency responsiveness to β-agonists. We are uncertain as to why both PKG and PKA responsiveness was protected from AICD in NAC-fed mice but only relative and not statistically significant protection was observed in PKG responsiveness in procysteine-fed animals. Although procysteine did not significantly increase PKG responsiveness, it did appear to moderate AICD and procysteine did significantly prevent alcohol-impaired ΔPKA responsiveness. Interestingly, alcohol + procysteine-fed mice maintained their higher baseline PKG activity compared to control mice. In addition to higher baseline kinase activity, these mice also maintained a lesser degree of procaterol-stimulated PKG responsiveness; nevertheless, these mice were still responsive to procaterol stimulation. Despite the fact that NAC and procysteine prevented AICD, BAL nitric oxide concentrations were significantly decreased in all but control and procysteine-only fed mice. These observed low levels of BAL NO_x suggest that PKG is likely being activated through an NO-independent pathway in which NAC antioxidant treatment shunts β-agonist responsiveness from an NO-dependent pathway to an NO-independent pathway with NAC itself down-regulating NO_x. While few studies have looked directly at the relationship between NAC and NO_x levels, one study has shown that NAC treatment prior to lipopolysaccharide (LPS)-induced shock, which increases serum NO-Hb concentrations, is able to decrease blood NO-Hb levels in LPS-injected animals (Bergamini et al., 2001). In addition, data from Xia et al. also show reduced levels of plasma NO_x in control NAC-fed animals (Xia, Nagareddy, Guo, Zhang, & McNeill, 2006). While these studies do not

explain why or how NAC downregulates NO_x production, these studies support the hypothesis that NAC downregulates NO_x production by inhibiting protein expression of NOS proteins, thereby shunting cilia responsiveness from an NO-cGMP-dependent pathway to an NO-cGMP-independent pathway.

Interestingly, procysteine has been shown to have superior effects in some published studies on alcohol and lung models. The lung contains pools of GSH stores within the cytoplasm and mitochondria of type II pneumocytes and circulating macrophages, which localize to the lung parenchyma. NAC had been shown to restore GSH pools within the cytoplasm only, while procysteine has been shown to restore both cytoplasmic and mitochondrial GSH pools. The airways do not contain type II pneumocytes and have very few, if any, circulating macrophages in the airway epithelium. The differences observed between how the lung parenchyma and airway epithelium respond to these antioxidants is likely due to the difference and localization of the cell types that make up these 2 tissues.

Because we were able to prevent AICD with NAC, we next sought to determine if AICD was reversible upon alcohol removal. After just 1 week following alcohol removal, CBF responsiveness was completely restored. Conversely, PKG and PKA responsiveness was not restored even after 6 weeks of alcohol removal. However, at 6 weeks PKG and PKA (slightly) activity appears to start trending upward. While these levels are nowhere near control PKG and PKA activities, it does suggest that PKG and PKA activity may start to recover after 6 weeks of alcohol removal. The lack of PKG and PKA activity in these mice suggest, similar to the antioxidant study, that procaterol is able to stimulate CBF through an alternative NO-independent pathway.

In summary, these data show that CBF responsiveness is restored upon alcohol removal independent of PKG and PKA responsiveness. This suggests that because β-agonists are able to stimulate cilia responsiveness, PKG, and PKA in an independent manner, AICD is not directly linked to desensitization of PKG or PKA.

Prolonged and heavy alcohol intake produces alcohol-induced ciliary dysfunction, preventing the normal clearance of inhaled pathogens, particles, and debris. AICD likely contributes to the increase of pulmonary infections, which are seen in individuals who consume alcohol in this manner. Although the removal of alcohol restores cilia responsiveness, key signaling molecules involved in regulating CBF remain unresponsive. The use of antioxidants could potentially prevent AICD from occurring in these individuals. Clearly, the linkage of decreased CBF, PKG, and PKA oversimplifies the mechanisms of oxidant-linked ciliary dysfunction in the prolonged alcohol-exposed mouse. This altered signaling cascade suggests redundant ciliary signaling pathways, which are essential to ciliary function upon the disruption of a primary signaling pathway. Future studies investigating the potential role of antioxidants in “repairing” AICD after alcohol removal are needed to address these questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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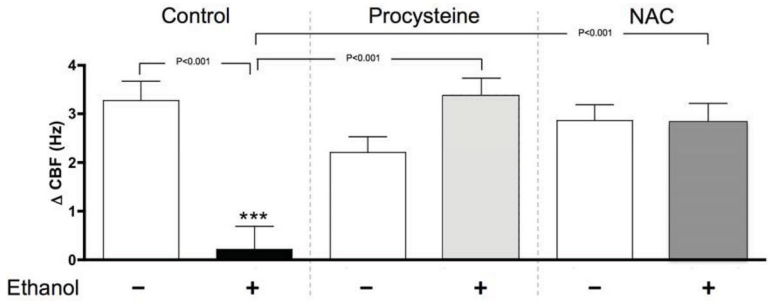

Fig. 1. Change of CBF in tracheal rings from control and alcohol-drinking mice ± supplemental dietary antioxidants (NAC or procysteine)

Change in ciliary beat frequency following procaterol (from baseline) is represented on the vertical axis in cycles/sec (Hz). A pre-activation baseline CBF measurement made for each group was subtracted from the procaterol-stimulated CBF responsiveness to calculate ΔCBF. Alcohol-only mice had significantly reduced CBF responsiveness compared to naïve mice. Alcohol-drinking mice fed dietary antioxidants of procysteine or NAC had significantly increased procaterol responsiveness compared to alcohol-only mice and were not significantly different from naïve mice.

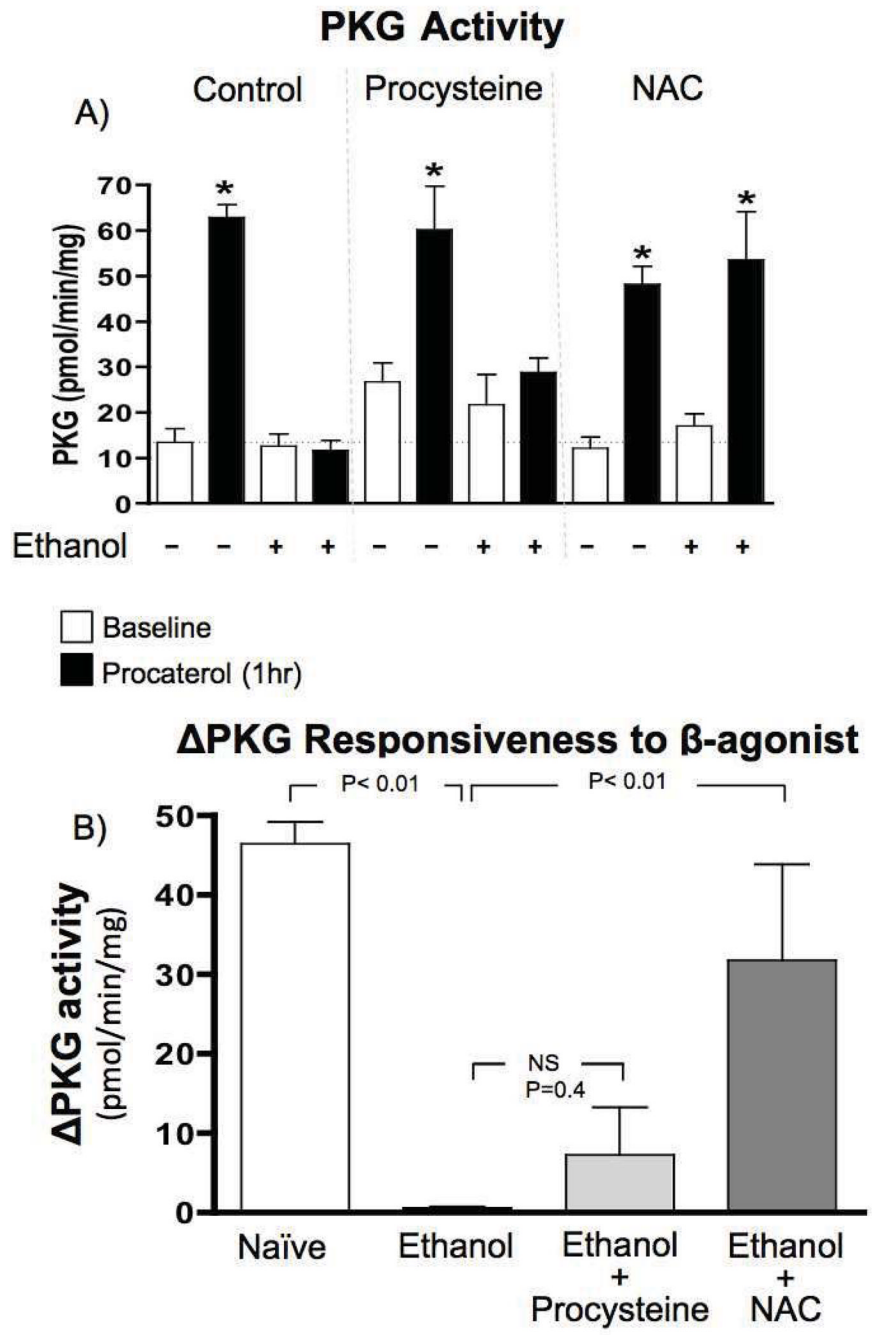

Fig. 2.

Fig. 2A. *PKG activity in 6-week control and alcohol-fed mice ± dietary supplementation with procysteine or N-acetylcysteine*

PKG activity was measured from mouse tracheas. PKG activity in naïve mice significantly increased upon procaterol stimulation ($p < 0.01$) compared to alcohol-drinking mice (Ethanol). PKG activity in alcohol-drinking mice was not stimulated with the addition of procaterol. Alcohol-drinking mice fed dietary PRO or NAC had increased PKG activity levels compared to alcohol-drinking mice. Furthermore, alcohol-drinking mice fed NAC had significantly increased PKG responsiveness to procaterol ($p < 0.01$) compared to alcohol-drinking mice (Ethanol;*$p < 0.01$ compared to baseline.

Fig. 2B. Δ*PKG activity in 6-week control and alcohol-fed mice ± dietary supplementation with N-acetylcysteine or procysteine*

PKG activity was measured from mouse tracheas. Baseline PKG measurements were taken and compared to PKG levels in procaterol-stimulated PKG activity to calculate the change of PKG activity from baseline PKG. Naïve mice and alcohol-drinking mice fed NAC had significant PKG responsiveness compared to alcohol-only drinking mice. Although alcohol + procysteine-fed mice suggest a small response to procaterol, PKG responsiveness did not reach significance ($p = 0.40$).

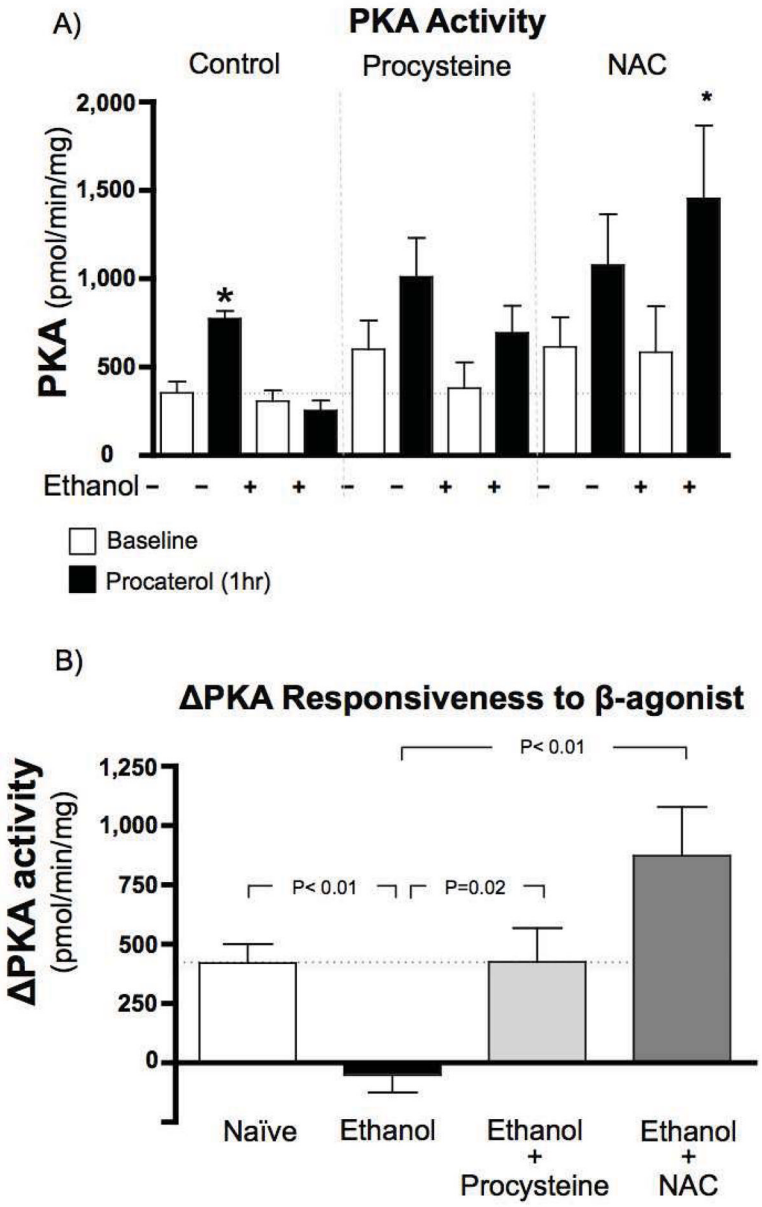

Fig. 3.

Fig. 3A. *PKA responsiveness in 6-week control and alcohol-fed mice ± dietary supplementation with procysteine or N-acetylcysteine*

PKA activity was measured from mouse tracheas. PKA activity significantly increased upon procaterol stimulation in naïve mice compared to alcohol-drinking mice (Ethanol). PKA activity in alcohol-drinking mice was unresponsive upon the addition of procaterol. Both alcohol-drinking mice fed dietary procysteine or NAC had increased PKA baseline levels compared to alcohol-drinking and naïve mice. While procysteine + alcohol mice had similar PKA activity compared to naïve mice, NAC + alcohol mice had significantly higher levels of PKA responsiveness compared to alcohol-drinking mice (Ethanol). **n* = 7–13 mice per group

Fig. 3B. Δ*PKA activity in 6-week control and alcohol-fed mice ± dietary supplementation with procysteine or N-acetylcysteine*

PKA activity was measured from mouse tracheas. Baseline PKA measurements were taken and compared to PKA levels in procaterol-stimulated PKA activity to calculate the change of PKA activity from baseline. Naïve and alcohol-drinking mice fed procysteine or NAC had significant PKA responsiveness compared to alcohol-only drinking mice.

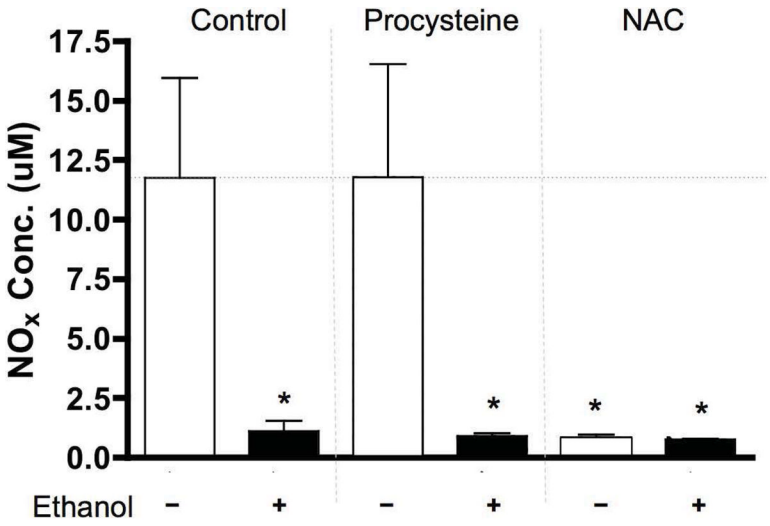

Fig. 4. Nitric oxide measurement in lung lavage fluid of 6-week control and alcohol-fed mice ± dietary procysteine or NAC

Ethanol, procysteine + ethanol, NAC, and NAC + ethanol fed mice had significantly lower NOx levels compared to control and procysteine-only mice ($p < 0.05$).

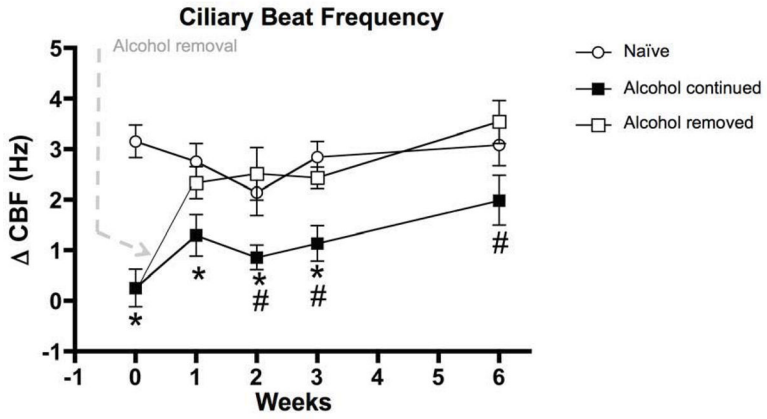

Fig. 5. Change of CBF in tracheal rings in control, alcohol-continued, and alcohol-removed mice exposed to procaterol

Mice continually fed alcohol had a significantly lower procaterol-stimulated CBF (*$p < 0.05$) compared to control mice. Mice with alcohol removed for 1 week had a procaterol-stimulated CBF comparable to control mice. By 2 weeks of alcohol withdrawal, these mice had a significantly higher procaterol-stimulated CBF than mice maintained on alcohol (#$p < 0.05$).

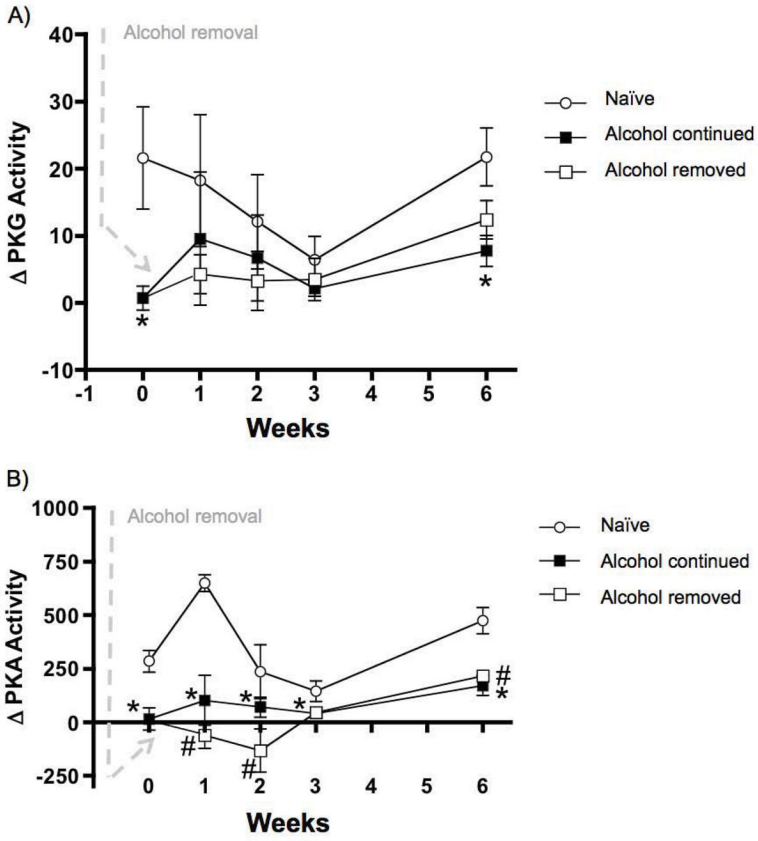

Fig. 6. Change of PKG and PKA activity levels in control, alcohol-continued, and alcohol-removed mice tracheas stimulated with procaterol

A) Procaterol-induced changes in PKG levels in control mice were significantly higher than alcohol-fed mice at 6 and 12 weeks (*$p < 0.05$). Although alcohol-removed mice had a lower PKG responsiveness level, it was not significantly lower than control animals.

B) Mice continually fed alcohol had a significantly lower procaterol-stimulated PKA responsiveness (*$p < 0.05$) compared to control mice. Alcohol-removed mice also had significantly lower PKA responsiveness (#$p < 0.05$) compared to control mice.