

Inhibition of protein phosphatase 1 reverses alcohol-induced ciliary dysfunction

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Price ME, Pavlik JA, Sisson JH, Wyatt TA. Inhibition of protein phosphatase 1 reverses alcohol-induced ciliary dysfunction. *Am J Physiol Lung Cell Mol Physiol* 308: L577–L585, 2015. First published January 9, 2015; doi:10.1152/ajplung.00336.2014.—Airway mucociliary clearance is a first-line defense of the lung against inhaled particles and debris. Among individuals with alcohol use disorders, there is an increase in lung diseases. We previously identified that prolonged alcohol exposure impairs mucociliary clearance, known as alcohol-induced ciliary dysfunction (AICD). Cilia-localized enzymes, known as the ciliary metabolon, are key to the pathogenesis of AICD. In AICD, cyclic nucleotide-dependent ciliary kinases, which modulate phosphorylation to regulate cilia beat, are desensitized. We hypothesized that alcohol activates cilia-associated protein phosphatase 1 (PP1) activity, driving phosphorylation changes of cilia motility regulatory proteins. To test this hypothesis we identified the effects of prolonged alcohol exposure on phosphatase activity, cilia beat, and kinase responsiveness and cilia-associated phosphorylation targets when stimulated by β -agonist or cAMP. Prolonged alcohol activated PP1 and blocked cAMP-dependent cilia beat and protein kinase A (PKA) responsiveness and phosphorylation of a 29-kDa substrate of PKA. Importantly, prolonged alcohol-induced phosphatase activation was inhibited by the PP1 specific inhibitor, inhibitor-2 (I-2), restoring cAMP-stimulated cilia beat and PKA responsiveness and phosphorylation of the 29-kDa substrate. The I-2 inhibitory effect persisted in tissue, cell, and isolated cilia-organelle models, highlighting the association of ciliary metabolon-localized enzymes to AICD. Prolonged alcohol exposure drives ciliary metabolon-localized PP1 activation. PP1 activation modifies phosphorylation of a 29-kDa protein related to PKA activity. These data reinforce our previous findings that alcohol is acting at the level of the ciliary metabolon to cause ciliary dysfunction and identifies PP1 as a therapeutic target to prevent or reverse AICD.

cilia; regulation; cilia beat frequency

CILIA ARE FINGER-LIKE ORGANELLES projecting from airway epithelial cells that function to drive mucus, inhaled particles, and debris out of the lungs (18). There is a long-established association of lung disease among individuals with alcohol use disorders (AUDs) (2). One facet of alcohol-related lung disease is impaired mucociliary clearance (6, 7).

Mucociliary clearance removes inhaled pathogens from the airways as a first line of defense from the environment (6). Mechanisms to increase mucociliary clearance include but are not limited to mechanostimulation, such as a particle causing the cilium to bend, or adrenergic stimulation, which can be

elicited by clinically relevant pharmacological agonists such as albuterol or isoproterenol, which stimulate the β_2 -adrenergic receptor (13, 14). Our group has shown prolonged alcohol exposure to blunt airway cilia responsiveness at the tissue level in mouse tracheal rings, at the cellular level in mouse tracheal epithelial cells (MTEC) cultured at the air-liquid interface (ALI), and at the organelle level of isolated demembranated cilia extracted from bovine tracheas (29, 30). This cilia desensitization by prolonged alcohol exposure is termed alcohol-induced ciliary dysfunction (AICD) (29, 30).

We and others have demonstrated that cilia regulatory enzymes are intrinsic to the function of the organelle (26, 27). These key components necessary to regulate cilia beat are compartmentalized in close proximity at the organelle level of the cilium, termed the ciliary metabolon (22). Our studies with alcohol indicate key events in the pathogenesis of AICD occur in the ciliary metabolon (22, 27).

In contrast to prolonged alcohol, our laboratory has shown that brief exposure to alcohol stimulates beating of airway cilia (20, 32). This cilia stimulation occurs through nitric oxide- and cyclic nucleotide-dependent mechanisms (20). Specifically, brief alcohol activates endothelial nitric oxide synthase (eNOS) and a soluble form of adenylyl cyclase, resulting in activation of the cyclic nucleotide-dependent kinases, protein kinase G (PKG) and protein kinase A (PKA), respectively (4, 18, 20, 32). The PKG and PKA pathways can be individually stimulated to increase ciliary beat frequency (CBF) by cGMP and cAMP (32). However, maximal CBF stimulation requires that both the PKG and PKA pathways are intact and requires sequential activation of PKG followed by PKA (27, 28).

Prolonged alcohol exposure downregulates both PKG and PKA, rendering cilia unresponsive to stimulation (27). We do not fully understand, however, the mechanism of alcohol desensitization of these enzymes. In many cellular systems, phosphatase and kinase activities, with opposing actions on protein phosphorylation, regulate the function of enzymatic activity, gene transcription, and signal transduction (10, 24). We recently described a phosphorylation event in the activation of cilia following acute alcohol exposure. Brief alcohol-stimulated activity of PKA and cilia beat required upstream phosphorylation and activation of the chaperone function of heat shock protein 90 (HSP90) with eNOS (17). This observation highlighted the role of dephosphorylation of key regulators in the importance of phosphorylation regulation of cilia by alcohol.

In this context, we hypothesized that increased cilia-associated phosphatase activity plays a key role in AICD. To test this hypothesis, we studied the effects of alcohol on phosphatase

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activity, kinase activity, axoneme protein phosphorylation, and cilia beat in the presence of phosphatase inhibitors. In this study we describe that prolonged alcohol activates protein phosphatase (PP) 1, which can be inhibited by the endogenous PP1 inhibitor, inhibitor-2 (I-2), which results in the restoration of axonemal responsiveness of PKA activity and cilia beat (4).

MATERIALS AND METHODS

Materials. Reagents used include I-2 (EMD Millipore, La Jolla, CA), the phosphatase inhibitors endothall, deltamethrin, fenvalerate, and cantharidic acid from the Phosphatase Inhibition Kit (Alomone, Jerusalem, Israel), kemptide (LRRASLG) for the PKA activity assay (Bachem), alcohol (Decon Labs, King of Prussia, PA), procaterol hydrochloride (Sigma, St. Louis, MO), and 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP; Sigma).

Trachea harvesting and treatment. Tracheas were harvested as previously described (4). Briefly, tracheas were removed from dead mice and placed in serum-free M199 media (GIBCO, Grand Island, NY). Rings were cut from each trachea and placed in 35-mm culture dishes, incubated for 30 min at 37°C and 5% CO₂, equilibrated to 25°C for 10 min, and assayed for CBF. Ethanol (100 mM) was added to the experimental group and allowed to incubate for 10 days. On day 9 at hour 23, the phosphatase inhibitor I-2 (10 µM) was added (or not) to the medium to be incubated with the tissue in each condition. After a 1-h incubation with I-2, baseline CBF was recorded and then procaterol (100 nM), a β-agonist, was used to stimulate CBF for 1 h. Tracheal rings were then removed from 37°C for 10 min and allowed to equilibrate to 25°C, followed by final CBF analysis.

Cultured ciliated cells. Ciliated MTEC were cultured using an ALI (MTEC ALI) system as previously described (31). MTEC ALI were preincubated 24 h with phosphatase inhibitors (2.0 nM I-2, 90 nM endothall, 100 pM deltamethrin, 20 nM fenvalerate, and 50 nM cantharidic acid) followed by prolonged ethanol exposure (100 mM for 24 h). At hour 24 of alcohol exposure, a baseline CBF measurement was recorded and then 100 nM procaterol was added to the cells and incubated at 37°C for an additional hour. Cells were then removed from 37°C for 10 min and allowed to equilibrate to 25°C, followed by final CBF analysis.

Airway axoneme extraction and preparation. Airway axonemes were isolated from bovine ciliated epithelium by a modification of a previously described method (5, 27). Briefly, isolated axonemes were extracted from fresh bovine tracheas obtained from a local abattoir. After excess adipose and connective tissue were removed, the tracheas were washed two times with phosphate-buffered saline and incubated for 24 h in the presence (or not) of 100 mM alcohol. The proximal and distal tracheal ends were closed with large hemostats after the addition of 15 ml of extraction buffer containing 20 mM Tris-HCl, 50 mM NaCl, 10 mM calcium chloride, 1 mM EDTA, 7 mM 2-mercaptoethanol, 100 mM Triton X-100, and 1 mM dithiothreitol (DTT). Each trachea was shaken for 90 s, and the subsequent extraction buffer containing released axonemes was filtered through a 100-µm polypropylene mesh and centrifuged at 17,250 g for 7 min. After the supernatant was discarded, the pelleted axonemes were resuspended to a concentration of 1 mg/ml in resuspension buffer consisting of 20 mM Tris-HCl, 50 mM KCl, 4 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 10 mM soybean trypsin inhibitor, and 25% sucrose (wt/vol). Isolated axonemes were stimulated with 10 µM of the cell permeable analog of cAMP, 8-Br-cAMP.

Experimental treatment of axonemes. Isolated axonemes were prepared for treatment by a previously described method used by our group (27). Frozen aliquots of isolated axonemes were thawed and maintained at 4°C on ice for up to 4 h. For each experimental condition, isolated axoneme samples were diluted to a final concentration of 0.25 mg/ml in microcentrifuge tubes by adding various reagents in resuspension buffer and incubated at room temperature in the presence or absence of phosphatase inhibitors. At each condition

measured, isolated axonemes were removed from the sample microcentrifuge tube, pipetted on one well of a 48-well polystyrene tissue culture plate along with 10 µl resuspension buffer, and then placed in a Sorvall T6000D and centrifuged for 2 min at 400 g. Following centrifugation, the plate of isolated axonemes was returned to the Sisson-Ammons Video Analysis (SAVA) (21, 27) system where 10-µl aliquots of ATP and other reagents for specific experiments and a volume of resuspension buffer were added to equilibrate volumes between conditions. Following experimental conditions, isolated axonemes were flash-frozen in sample tubes for subsequent kinase and phosphatase assays.

Measurement of CBF. CBF was recorded from mouse tracheal rings and in MTEC cultured at ALI. During CBF measurement, the ambient room temperature was monitored and remained constant (24 ± 0.5°C). All recordings for analysis were taken with a Kodak Megaplug 310 analog/digital video camera (Eastman Kodak Motion Analysis Systems Division, San Diego, CA). Whole field analysis was performed, and the CBF was determined by collecting data sampled at 85 frames/s for 3 s and performing frequency spectrum analysis using the SAVA system (21).

Phosphatase activity assays. Phosphatase activity was assayed from axonemes prepared from bovine trachea in the presence or absence of 100 mM ethanol for 24 h using the Serine/Threonine Phosphatase Assay Kit (catalog no. 17-127; EMD Millipore, Billerica, MA). Briefly, protein concentrations of control and treatment groups were measured by Bradford protein assay (Bio-Rad) and normalized to equal concentrations per assay (0.2–1.8 mg/ml) by diluting in cold Tris-HCl, pH 7.4. Normalized sample (5.0 µl) was diluted in a phosphatase reaction buffer containing final reaction concentrations of 50 mM HEPES, 10 mM NaCl, 2 mM DTT, 0.01% Brij 35, pH 7.5 at 25°C, and 1 mM MnCl₂. A portion of the sample (20.0 µl) in phosphatase buffer was added in triplicate to wells of a 96-well plate on ice; 5.0 µl of a 1.0 mM solution of the phosphothreonine-peptide [K-R-pT-I-R-R (catalog 1#2-219; EMD Millipore)] was then added to each well to begin the reaction and allowed to incubate at room temperature for 5 min. The reaction was stopped with 100 µl malachite green phosphate detection solution and allowed 15 min for color development. Assay mixtures without substrate from each sample were performed to eliminate background phosphate contamination. A microplate spectrophotometer (BioTek Epoch, Winooski, VT) was used to read absorbance at 620 nm, and phosphate release was determined by a standard curve generated from a solution of 0.1 mM KH₂PO₄.

Cyclic nucleotide-dependent kinase activity assays. Kinase activity measurement was performed in a manner as previously described (27). Primary MTEC ALI were prepared by removing supernatants after experimental conditions, adding 250 µl cell lysis buffer, and then flash-freezing. Dishes were thawed and scraped into centrifuge tubes and kept on ice. The cell containing supernatant was sonicated and centrifuged at 10,000 g at 4°C for 30 min. PKA activity was then measured from the extracted cell or tissue sample. The isolated axoneme protein (1 mg/ml) was measured in the presence or absence of 10 µM 8-Br-cAMP and phosphatase inhibitors (2.0 nM I-2, 90 nM endothall, 100 pM deltamethrin, 20 nM fenvalerate, and 50 nM cantharidic acid) by a modification of the methods previously described (27) using a reaction mix consisting of 130 mM PKA heptapeptide substrate in a buffer containing 20 mM Tris-HCl (pH 7.5), 100 µM isobutyl methylxanthine, 20 mM magnesium acetate, and 200 µM [γ -³²P]ATP. Each experiment was conducted three separate times ($n = 3$), and the results were expressed as the means ± SE for each data point. Significance was determined using a one-way ANOVA and accepted at the 95% confidence interval if the P value < 0.05. Protein kinase C (PKC) activity was determined as previously described (31).

In vitro axoneme protein phosphorylation assays. Isolated axonemes (15 µl) were in vitro phosphorylated with 3 µl of 10× reaction buffer consisting of 200 mM Tris-HCl (pH 7.4), 200 mM MgCl₂, 4.5

mg/ml bovine serum albumin, and 2.0 mM ATP with the final volume (30 μ l) brought up in cilia resuspension buffer. The following final concentrations were either added or left out of the reaction mix: 8-Br-cAMP (10 μ M), PKA [0.15 μ g diluted in c-subunit dilution buffer consisting of 50 mM K_2HPO_4 (pH 6.8), 0.1 mM DTT, and 0.9 mg/ml BSA], protein kinase inhibitor (PKI, 2.5 μ g; preincubated for 5 min at 30°C), and I-2 (130 nM; preincubated for 5 min at 30°C). Each reaction mixture contained 15 μ Ci [32 P]ATP per 25- μ l sample volume, diluted in reaction buffer. The reaction assay was incubated for 10 min at 30°C, and reactions were halted by boiling in SDS-2-mercaptoethanol for 5 min at 95°C. Proteins were loaded on a precast 4–20% Tris-HCl SDS gel (Bio-Rad) and electrophoresed at 100 volts until the dye front was extruded from the bottom of the gel. Gels were stained in 0.1% Coomassie Blue R250 (Bio-Rad) in 10% acetic acid, 50% methanol, destained, dried, and exposed to autoradiographic film. Resolved bands were analyzed using a Bio-Rad Gel-Doc, and densitometry was performed using NIH-Image J.

RESULTS

Prolonged alcohol increases phosphatase activity in isolated airway cilia axonemes. The key role of phosphorylation events in AICD led us to hypothesize that phosphatase activity is increased at the cilia organelle level by alcohol (1, 8). To test this hypothesis, we directly measured axoneme preparation phosphatase activity after prolonged alcohol exposure. Prolonged alcohol exposure triggered a twofold (51.85 ± 4.83 vs. 25.93 ± 3.24 pmol phosphate/min; $n = 14$ for both groups; $P = 0.002$, respectively) increase in phosphatase activity compared with alcohol naïve axonemes (Fig. 1). In biological systems, phosphatases are often important in the dephosphorylation of kinase substrates (20). Previous data have implicated desensitization of kinases in AICD, specifically PKA, as the rate-limiting enzyme (23). The phosphatase-kinase relationship led us to determine the role of phosphatase inhibition on PKA activity.

Inhibition of PP1 phosphatase specifically blocks prolonged alcohol-associated PKA desensitization in MTECs at ALI. We have shown that the increase in CBF due to procaterol stimulation is an enzyme-mediated event dependent on the nitric oxide-mediated activation of guanylate cyclase, generation of cGMP, and subsequent activation of PKG to act on PKA as

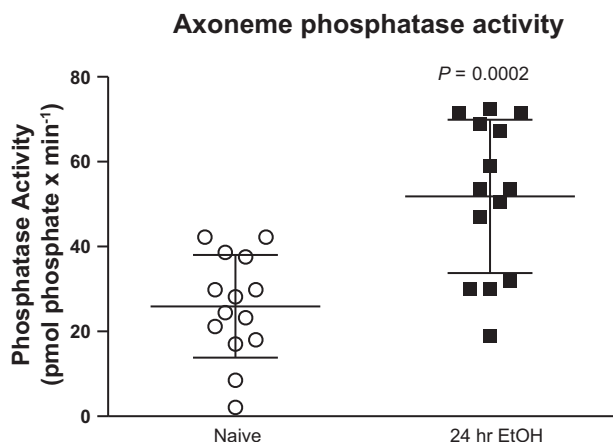


Fig. 1. Prolonged alcohol (EtOH) increases phosphatase activity in isolated airway cilia axonemes. Prolonged EtOH exposure (100 mM \times 24 h) stimulated a 2-fold increase in axoneme phosphatase activity vs. axonemes extracted from EtOH-naïve tracheas (51.85 ± 4.83 vs. 25.93 ± 3.24 pmol phosphate/min; $n = 14$ for each group; $P = 0.0002$, respectively).

well as the β -adrenergic activation of adenylate cyclase, generation of cAMP, and subsequent activation of PKA (8, 27). Alcohol utilizes these dual cGMP- and cAMP-dependent pathways to sequentially activate CBF following brief alcohol exposure (27). Therefore, we hypothesized that phosphatase activation from prolonged alcohol exposure, as seen with AICD, ultimately alters PKA activity in the CBF stimulatory pathway. To test this hypothesis, MTEC ALI exposed to prolonged alcohol (24 h with 100 mM alcohol) were incubated with an array of phosphatase inhibitors and then stimulated with 100 nM procaterol, a β -adrenergic CBF stimulus previously described (24). In the absence of phosphatase inhibitors, no increase in baseline PKA activity resulted from stimulation with 100 nM procaterol in alcohol-treated MTEC ALI (Fig. 2). Interestingly, only the inhibitor cantharidic acid, which demonstrates promiscuous PP1 and PP2A inhibition, restored procaterol-stimulated PKA activity in AICD (Fig. 2A). Incubation with the PP2B inhibitors fenvalerate and deltamethrin showed no significant change from MTEC ALI cultured in media alone or upon stimulation in the absence of phosphatase inhibitors (Fig. 2, B and C). Interestingly, endothall, a PP2A inhibitor, demonstrated a small but significant increase in PKA activity without stimulation (61.2 ± 9.1 vs. 82.3 ± 15.7 pmol \cdot min $^{-1}\cdot$ mg $^{-1}$; $P < 0.01$) but no increase from control upon procaterol stimulation. These data indicate that PP1 inhibition is closely associated with AICD reversal of PKA activity in MTEC ALI.

PP1 inhibition by I-2 restores PKA sensitivity to cAMP in MTECs at ALI. Due to the phosphatase inhibition-mediated restoration of PKA activity only by phosphatase inhibitors with activity toward PP1, we hypothesized that I-2, an endogenous and highly specific inhibitor of PP1, could restore PKA activity in AICD. To test this hypothesis, we measured PKA activity following the addition of 8-Br-cAMP, a cell permeable analog of cAMP. We found that PKA activity increased from 59.5 ± 6.9 to 185.2 ± 12.3 pmol \cdot min $^{-1}\cdot$ mg $^{-1}$ ($P < 0.05$) in media-only cultured MTEC ALI. A similar increase was seen in naïve MTEC ALI preincubated with I-2 (Fig. 3). Importantly, after prolonged alcohol exposure, PKA activity did not increase upon the addition of 8-Br-cAMP (Fig. 3). This is consistent with our previous data suggesting PKA activity as a key component of the cilia stimulation pathway impaired in AICD (24). In contrast, I-2-incubated alcohol-exposed MTEC ALI showed a significant increase in 8-Br-cAMP-stimulated PKA activity (46.3 ± 11.2 vs. 113.5 ± 15.1 pmol \cdot min $^{-1}\cdot$ mg $^{-1}$; $P < 0.05$) (Fig. 3), but this increase was significantly less ($P < 0.001$) than the 8-Br-cAMP-stimulated PKA activity from alcohol-naïve axonemes receiving no treatment or I-2 alone. These findings suggest that the cyclic nucleotide-dependent kinase PKA is downstream of PP1 action and key to the mechanism of AICD. The finding that PKA activity in AICD can be restored by PP1 inhibition led us to determine if PP1 inhibition could restore CBF in AICD.

PP1 inhibition reverses AICD. CBF measured in tracheal rings extracted from naïve mice tracheas increased from 7.2 ± 0.8 vs. 9.75 ± 0.9 Hz ($P < 0.05$) with the addition of 100 nM procaterol. In contrast, tracheal rings from 10-day alcohol-exposed mice tracheas did not demonstrate a significant change in CBF with the addition of 100 nM procaterol (Fig. 4A). These results are consistent with previous data describing stimulus desensitization of airway cilia by chronic alcohol exposure, indicating AICD (27, 30). Strikingly, after being incubated

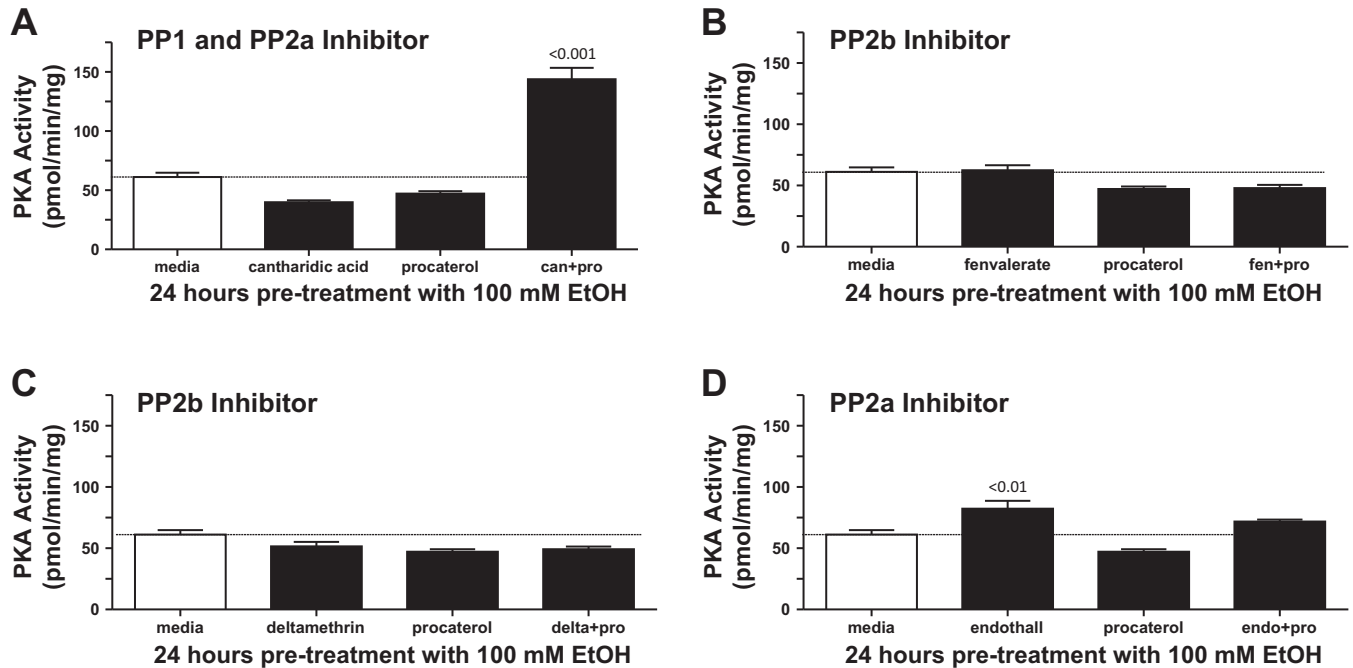


Fig. 2. Inhibition of protein phosphatase (PP) 1 specifically blocks prolonged EtOH-associated protein kinase A (PKA) desensitization in mouse tracheal epithelial cells (MTECs) at the air-liquid interface (ALI). PKA activity was determined in MTEC ALI incubated for 24 h with or without 100 mM EtOH and stimulated with 100 nM procaterol in the presence (open bar) or absence (filled bars) of the following specific phosphatase inhibitors: cantharidic acid, a PP1 and PP2A inhibitor (A); fenvalerate (B) and deltamethrin (C), inhibitors of PP2B; and endothall (D), a PP2A inhibitor.

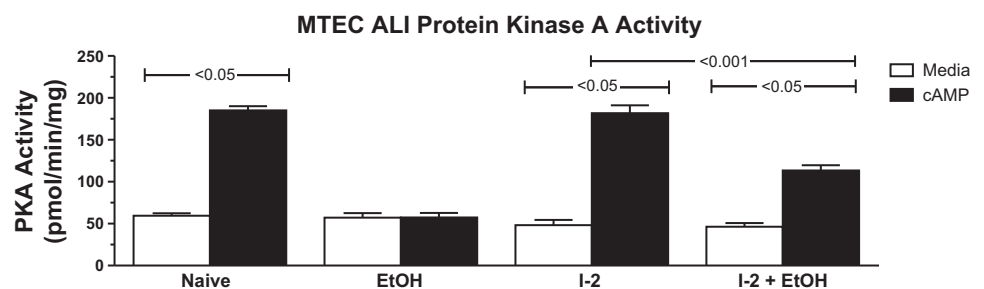
with phosphatase inhibitor I-2, alcohol-exposed tracheas maintained a 100 nM procaterol-stimulated increase in CBF similar to tracheal rings from naïve tracheas in media alone and naïve tracheas preincubated with I-2 alone (Fig. 4A). Similar results were obtained in MTEC ALI (Fig. 4B). The endogenous PP1 inhibitor I-2 reverses AICD in mouse tracheal rings as well as MTEC ALI and suggests that alcohol decreases normal stimulation of CBF through a PP1-dependent mechanism. Having established that AICD is tightly linked to PP1 activity, we next sought to determine if PKA desensitization by alcohol occurred at the cilia organelle level.

PKA responsiveness is restored by PP1 inhibition in isolated cilia extracted from tracheas exposed to prolonged alcohol. Brief alcohol exposure activates PKA, resulting in stimulated CBF, while prolonged alcohol exposure desensitizes cilia in AICD (29). Another cilia-associated kinase, PKC, is known to slow CBF (30). Because both kinases are present in isolated axoneme preparations (27), we hypothesized that alcohol differentially activates PKA vs. PKC in isolated cilia related to PP1 activation. To test this hypothesis, alcohol-exposed axonemes derived from prolonged alcohol-exposed bovine tracheas

were assayed for PKA and PKC activities stimulated by 8-Br-cAMP in the presence and absence of phosphatase inhibitors (Fig. 5). Inhibitors selecting for PP1 (I-2 and cantharidic acid) resulted in an increase in PKA activity following 8-Br-cAMP activation of prolonged alcohol-exposed isolated axonemes. Preincubation with PP2A, or PP2B phosphatase inhibitors, did not restore 8-Br-cAMP-stimulated PKA activity in alcohol-exposed axonemes. As a negative control, PKC activity did not significantly change with the presence or absence of phosphatase inhibitors. These data further suggest the presence of an axoneme dephosphorylation target of PP1 with a role to inhibit axoneme PKA activity.

PP1 inhibition enhances axoneme PKA target protein phosphorylation. The specific desensitization of PKA activity following prolonged alcohol exposure in the axoneme preparation led us to hypothesize that the target of PP1 resides in the isolated cilia axoneme preparation. To test this hypothesis, we identified phosphorylation changes by in vitro phosphorylation of naïve axoneme preparations in the presence or absence of I-2 ($0.1\text{--}1.0 \times 10^3$ nM). A protein at 41 kDa was dose dependently phosphorylated in the presence of PP1-specific

Fig. 3. PP1 inhibition by inhibitor-2 (I-2) restores PKA sensitivity to EtOH and cyclic nucleotides in MTEC at ALI. MTECs at ALI were cultured in control medium (serum free M199; GIBCO) with and without EtOH exposure (100 mM) for 24 h coincubated (or not) with I-2. Kinase activity (vertical axis, $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) was measured after the addition of 10 μM 8-bromoadenosine 3',5'-cyclic monophosphate [8-Br-cAMP (cAMP)], a PKA stimulus (filled bars), or not (open bars), for all conditions.



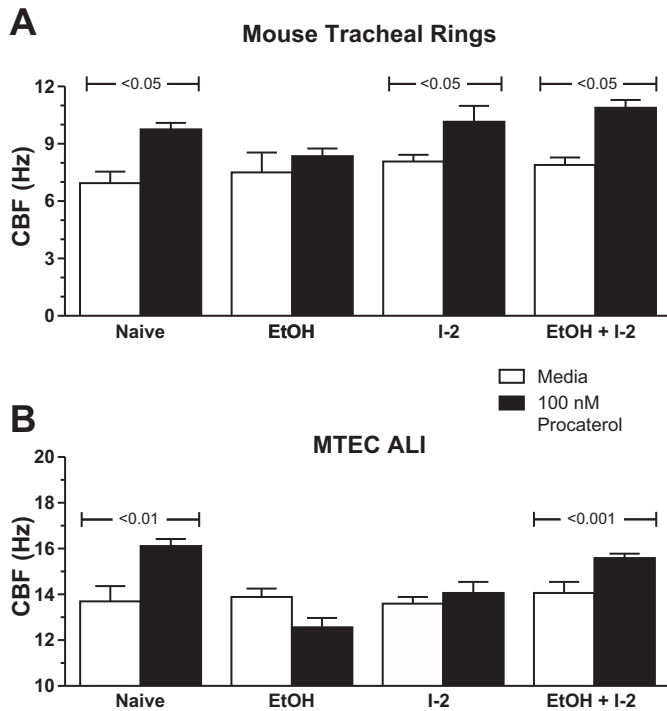


Fig. 4. PP1 inhibition reverses EtOH-induced ciliary dysfunction (AICD). *A*: tracheal rings from EtOH naïve mice tracheas were incubated in medium alone (serum-free M199; GIBCO) or medium with 100 mM EtOH for 10 days. At the end of day 9, tracheal rings were incubated as previously described with the addition of 2.0 nM I-2 or medium alone for 1 h. Ciliary beat frequency (CBF) was then measured in each condition at baseline (open bars) and after the addition of 100 nM procaterol for 1 h (filled bars). *B*: MTEC ALI were incubated 24 h in the presence or absence of 2.0 nM I-2 and then coincubated with 100 mM EtOH for an additional 24 h. CBF was then measured in each condition at baseline (open bars) and after the addition of 100 nM procaterol for 1 h (filled bars) for both *A* and *B*.

inhibition (I-2; Fig. 6A). The phosphorylation of the 41-kDa protein was not affected by other phosphatase inhibitors, suggesting PP1-specific dephosphorylation (Fig. 6B). Incubation with I-2 resulting in increases of both CBF and PKA activity led us to hypothesize that upstream activation of PP1 modulates PKA-dependent phosphorylation of specific cilia axon-

eme substrates. To test this hypothesis, PKA in naïve axonemes was stimulated by 8-Br-cAMP or inhibited by a PKI in the presence or absence of I-2 and then analyzed by in vitro protein phosphorylation (Fig. 6C). 8-Br-cAMP stimulation of isolated cilia axonemes caused phosphorylation of a 29-kDa protein. The addition of PKA holoenzyme also intensified the phosphorylation of a 29-kDa protein. In contrast, 8-Br-cAMP in the presence of a PKI was similar to control. Combined, these results suggest that the 29-kDa protein is a downstream target of PKA. Intriguingly, when preincubated with I-2, the addition of 8-Br-cAMP resulted in the appearance of the 29-kDa phosphorylated protein, which was significantly more intense than with 8-Br-cAMP alone ($23 \times 10^3 \pm 7.4 \times 10^3$ vs. $8.6 \times 10^3 \pm 1.2 \times 10^3$; $P < 0.05$) and similar to the addition of the PKA holoenzyme (Fig. 6C). These data suggest PP1 inhibits PKA-dependent axoneme target phosphorylation.

Alcohol inhibits PKA-dependent phosphorylation of a 29-kDa substrate that is restored by PP1 inhibition. The findings of a PP1-inhibitable 29-kDa PKA target in naïve axonemes led us to hypothesize that this target links PP1 activation with prolonged-alcohol-exposure (AICD) PKA activity. To test this hypothesis, we performed in vitro substrate phosphorylation gels for phosphorylation on alcohol-exposed tracheal-derived axonemes in the presence and absence of I-2 before and after 8-Br-cAMP stimulation. Interestingly, prolonged alcohol-exposed axonemes showed decreased 8Br-cAMP-dependent phosphorylation of a 29-kDa compared with naïve axonemes ($2.8 \times 10^3 \pm 1.1 \times 10^3$ vs. $17 \times 10^3 \pm 9.8 \times 10^3$; $P < 0.05$) (Fig. 7). These data are consistent with our finding that PKA activity is decreased in prolonged alcohol-exposed cilia axonemes. Axoneme preparations preincubated with I-2 and stimulated with 8-Br-cAMP resulted in the reappearance of the 29-kDa phosphorylation band in both alcohol-exposed and naïve axoneme preparations (Fig. 7). The phosphorylation protein at 29 kDa did not appear in the absence of 8-Br-cAMP stimulation, indicating PKA-dependent phosphorylation (Fig. 7). Furthermore, the 41-kDa phosphoprotein was only present in samples preincubated with I-2, in both alcohol-exposed and naïve axonemes (Fig. 7). This is consistent with I-2 inhibition of PP1 resulting in the increased phosphorylation of the 41-kDa protein. The increase in phosphorylation at 29 kDa reap-

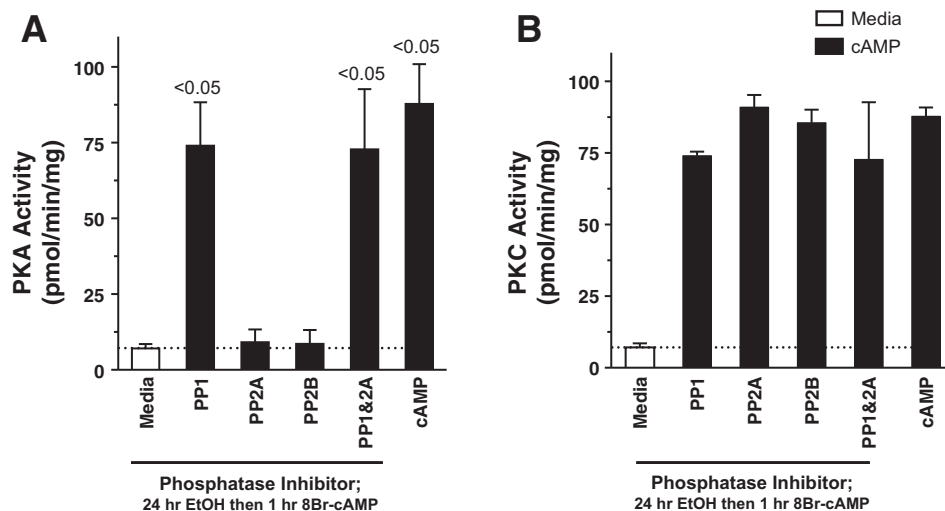


Fig. 5. PKA responsiveness is restored by PP1 inhibition in isolated cilia extracted from tracheas exposed to prolonged EtOH (100 mM for 24 h). *A*: isolated cilia, harvested from bovine tracheas exposed to 100 mM EtOH before cilia harvest, were then stimulated by 8-Br-cAMP (cAMP; 10 μ M) in the presence or absence of I-2, a PP1 inhibitor; cantharidic acid, a PP1 and PP2A inhibitor; fenvalerate and deltamethrin, inhibitors of PP2B; and endothall, a PP2A inhibitor, followed by PKA activity measurements. *B*: as a negative control, protein kinase C (PKC) activity was measured in the same EtOH-exposed cilia samples with and without the same phosphatase inhibitors as in *A*.

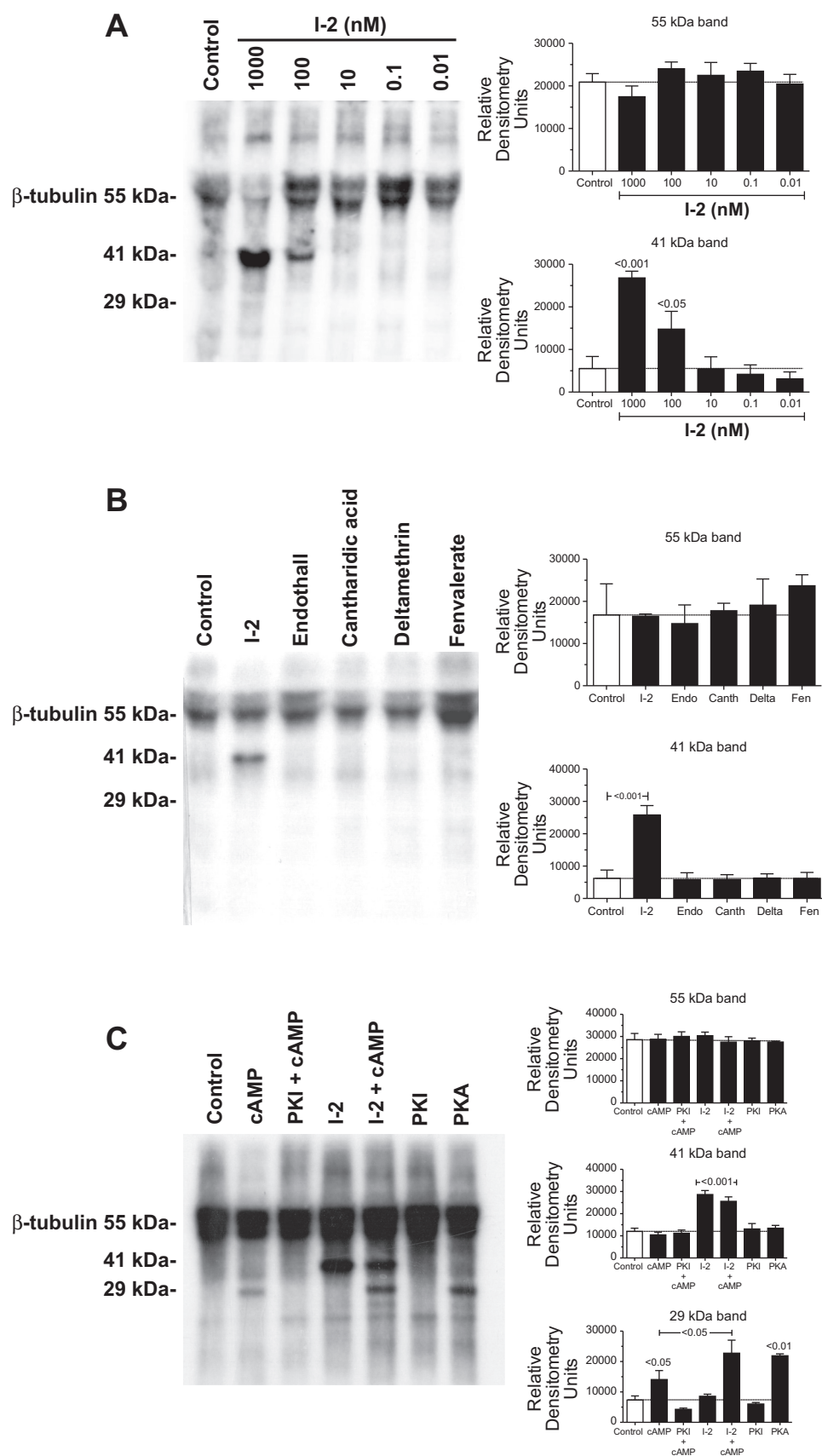


Fig. 6. A: PP1 inhibition dose responsively allows phosphorylation of a 41-kDa ciliary protein. In vitro phosphorylation analysis and densitometry of a 41-kDa phosphorylation target in axoneme preparations incubated 1 h with 0.01–1,000 nM I-2. The 55-kDa band corresponds to β -tubulin as a loading control. B: PP1 inhibition specifically allows phosphorylation of a 41-kDa axoneme substrate. In vitro phosphorylation analysis and densitometry of a 41-kDa phosphorylation target in axoneme preparations with the presence of various phosphatase inhibitors. The 55-kDa band corresponds to β -tubulin as a loading control. C: PP1 inhibition enhances axoneme PKA target protein phosphorylation. In vitro phosphorylation analysis and densitometry of 41- and 29-kDa phosphorylation targets before and after 8-Br-cAMP (cAMP) stimulation of naïve axonemes preincubated with medium only, I-2, or the PKA-specific protein kinase inhibitor PKI and then compared with addition of PKA holoenzyme. The 55-kDa band corresponds to β -tubulin as a loading control.

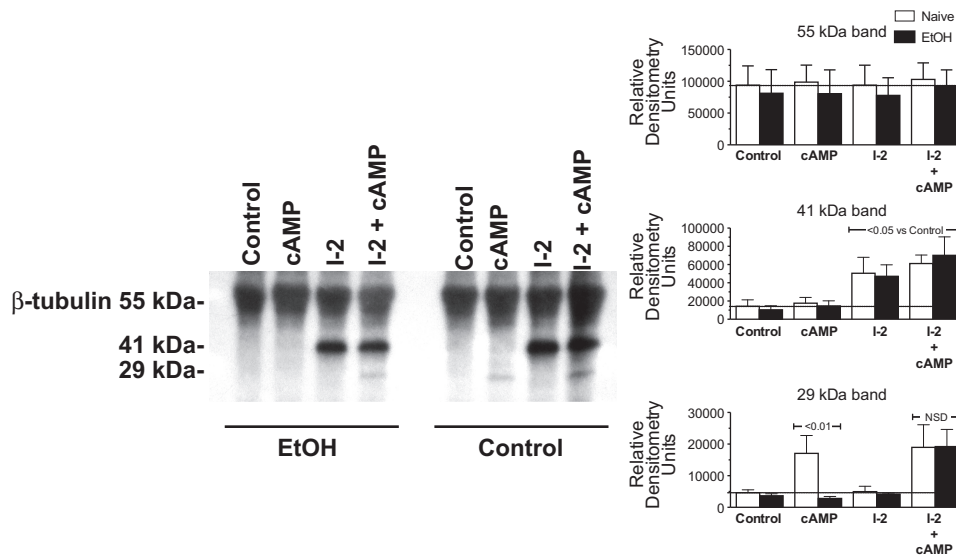


Fig. 7. EtOH inhibits PKA-dependent phosphorylation of a 29-kDa substrate that is restored by PP1 inhibition. In vitro phosphorylation gel analysis and densitometry of a 29-kDa phosphorylation target in control and prolonged EtOH-exposed axoneme preparations upon stimulation by 8-Br-cAMP (cAMP) in the presence or absence of I-2. The 55-kDa band corresponds to β -tubulin as a loading control.

peared only upon stimulation with 8-Br-cAMP and preincubation with I-2 and not when I-2 was preincubated alone in naïve or alcohol-exposed axonemes (Fig. 7). These data recapitulate alcohol-induced PP1 activity negatively regulating PKA activity.

DISCUSSION

Our data show phosphatase inhibition restores PKA sensitivity and cilia responsiveness, the loss of which are key features of AICD. Indeed, prolonged alcohol drives PP1 activation, which we confirmed in experiments using specific phosphatase inhibitors. Strikingly, the restoration of PKA-dependent cilia activity by inhibition of PP1 is conserved at the tissue, cell, and organelle level in two different species. The localization of these key regulatory components highlights the autonomy of cilium enzymatic regulation as part of the ciliary metabolon.

The ciliary metabolon represents a cluster of enzymes that regulate ciliary motility, including nitric oxide synthase, cyclases, and cyclic nucleotide-dependent kinases (27, 28). These experiments add PP1 to the ciliary metabolon (Fig. 8). To our knowledge, the finding that phosphatase activity regulates motility in mammalian airway cilia is novel. The known relationship of kinases with phosphatases (23) combined with the established role of PKA as a key regulator of cilia stimulation led us to measure kinase activity in the presence of phosphatase inhibition. The proximity of the kinases and

phosphatases in the ciliary metabolon led us to probe for PP1 phosphorylation targets within the cilium. We identified two protein phosphorylation targets, including a 41-kDa protein and a 29-kDa protein, to be regulated by PP1 and PKA, respectively.

In isolated cilia, the 41-kDa protein is only phosphorylated when PP1 is inhibited by I-2. This is consistent with the blocking of a dephosphorylating event implicating this protein as a target of PP1. Data from our laboratory suggest axoneme phosphorylation events to be related to oxidant stress. Specifically, we have shown concurrent antioxidant feeding of prolonged ethanol-fed mice prevents AICD (16). The next step in our investigation is to determine the role of phosphorylation of the 41-kDa protein in PP1-inhibited conditions. HSP90 is a protein we know to exhibit differential phosphorylation in relation to alcohol (16). In brief alcohol conditions, HSP90 is phosphorylated and activated to form a complex with eNOS, which results in increased production of nitric oxide and stimulates the guanylyl cyclase \rightarrow cGMP \rightarrow PKG activation of PKA and subsequent increase in CBF (17). To our knowledge, there are no known fractions of HSP90 that migrate at 41 kDa, suggesting that the phosphorylation of the 41-kDa protein is not related to HSP90. Furthermore, in this study, the 41-kDa protein is only phosphorylated in conditions with I-2 (Fig. 6B) while both I-2 and cantharidic acid are capable of restoring PKA activity in AICD (Figs. 2 and 3). These data suggest that the phosphorylation of the 41-kDa protein is a nonspecific

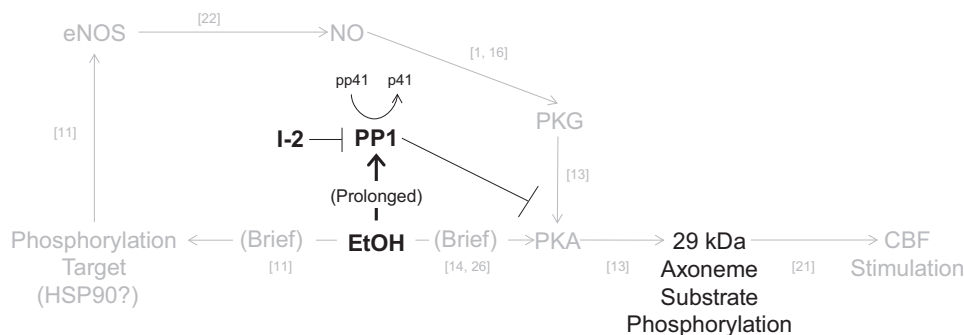


Fig. 8. Proposed pathway of prolonged EtOH activation of PP1 resulting in decreased stimulated cilia beating. Prolonged EtOH activates PP1, which dephosphorylates a 41-kDa substrate, resulting in desensitization of PKA and decreased cilia beat response. HSP90, heat shock protein 90; eNOS, endothelial nitric oxide synthase; NO, nitric oxide.

phosphorylation target related to I-2 but not functionally related to cilia beat regulation. Alternatively, the discrepancy between the appearances of the phosphorylation of the 41-kDa protein with I-2 or cantharidic acid could be related to the amplification characteristic of signal transduction. In other words, at the concentration of cantharidic acid used, the protein kinase activity could be restored as a result of phosphorylation before levels of phosphorylation of the 41-kDa protein were detectable.

The phosphorylation pattern of the 29-kDa protein is different. Three key observations point to phosphorylation of the 29-kDa protein as phosphorylated by PKA: 1) it is only phosphorylated in the presence of PKA stimulation by cAMP; 2) it is not phosphorylated in the presence of the PKA specific kinase inhibitor PKI; and 3) it is not phosphorylated in prolonged alcohol conditions following cAMP stimulation. Interestingly, in the presence of PP1 inhibition, the 29-kDa protein is phosphorylated with prolonged alcohol upon cAMP stimulation. While we have not characterized this protein in the current study, previous work in *Paramecium* and ovine cilia models has identified the cAMP-dependent phosphorylation of a protein of similar molecular weight (29 and 26 kDa, respectively) (12, 15). A 29-kDa cAMP-dependent phosphorylated protein increases swimming speed, cosediments with the 22S fraction of an axoneme sucrose gradient, and has been identified as the light chain of an outer dynein arm in *Paramecium* (15). These data suggest that the 29-kDa protein we have identified as cAMP dependently phosphorylated may be a homolog of the protein characterized in *Paramecium* that displays similar function and molecular characteristics in regulation of cilia motility.

Increased PKA activity in the presence of PP1 inhibition supports our hypothesis that PP1 activity regulates PKA. In addition, the reappearance of the AICD evanescent phosphorylation of the 29-kDa target of PKA, in the presence of I-2, further establishes PP1 activity as fundamental to AICD.

To fully understand AICD, the mechanism of PP1 activation and these phosphorylated proteins in cilia need to be identified.

Targeting phosphatases provides an attractive treatment strategy of AICD to restore mucociliary clearance and prevent pulmonary infection in individuals with AUDs. Indeed, phosphatases are an increasingly studied target of pharmacological intervention for diseases such as diabetes, cancer, and neurological immune disorders (24). The next step in contributing clinical relevance to our study is to demonstrate the effects of PP1 inhibition on mucociliary clearance in an in vivo model. This could be accomplished by genetic knockout models, siRNA knockdown, or continued application of PP1-specific phosphatase inhibitors. The use of inhibitors is limiting, since they often display promiscuity in their action and targets (25). To demonstrate PP1-specific inhibition, we used an array of inhibitors and directly measured the catalytic activity of PP1 and other phosphatases. Another limitation of this study is the use of a 100 mM in vitro concentration of alcohol. This concentration, if measured in the blood, would be approximately five times the legal limit to drive and likely only achieved among individuals with AUDs (9). Indeed there are reports of blood-alcohol concentrations from conscious human patients >100 mM, albeit uncommon (18). Despite this, in vivo studies of mice fed alcohol 6 wk ad libitum

indicate that a physiological concentration of alcohol results in AICD (15).

In summary, PP1 activation by alcohol is a key component in the pathogenesis of AICD. PP1 activation modifies phosphorylation of 41- and 29-kDa proteins related to PP1 and PKA activity, respectively. These data reinforce previous findings that the key regulatory machinery of CBF resides in the ciliary metabolon, and alcohol is acting at this level to cause ciliary dysfunction.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.E.P., J.H.S., and T.A.W. conception and design of research; M.E.P. and J.A.P. performed experiments; M.E.P., J.A.P., and J.H.S. analyzed data; M.E.P., J.A.P., J.H.S., and T.A.W. interpreted results of experiments; M.E.P. and J.A.P. prepared figures; M.E.P. and J.H.S. drafted manuscript; M.E.P., J.A.P., J.H.S., and T.A.W. edited and revised manuscript; M.E.P., J.A.P., J.H.S., and T.A.W. approved final version of manuscript.

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