Articles in PresS. Am J Physiol Lung Cell Mol Physiol (October 17, 2008). doi:10.1152/ajplung.00487.2007

Alcohol Feeding Blocks Methacholine-induced Airway Responsiveness in Mice

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Scientific Knowledge on the subject
There is very limited knowledge about the effect that alcohol has on airway responsiveness.

What this study adds to the field
These data provide an *in vivo* basis for previous clinical observations in humans and substantiate the bronchodilator properties of alcohol

Abstract:

Historical accounts of alcohol administration to patients with breathing problems suggest that alcohol may have bronchodilating properties. We hypothesized that acute alcohol exposure will alter airway responsiveness (AR) in mice. To test this hypothesis, C57Bl/6 mice were fed either 20% alcohol in drinking water or received a single intraperitoneal (ip) injection of alcohol (3 g/kg). Control groups received regular drinking water or ip saline. AR was assessed by means of ventilation or barometric plethysmography and reported as either total lung resistance (R_L) or enhanced pause (Penh) for each group of mice. To confirm alcohol exposure, elevated blood alcohol levels were documented. Alcohol feeding significantly blocked methacholine-triggered AR compared to water-fed controls. Comparable blunting of AR was also accomplished through a single ip injection of alcohol when compared to saline-injected controls. The alcohol response was slowly reversible in both routes of administration after withdrawal of alcohol: AR attenuation by alcohol persisted 12 - 20 hours (ip) or up to 2 weeks (fed) after blood alcohol cleared consistent with a sustained bronchodilator effect. These data demonstrate that brief alcohol exposure blunts AR in this murine model of alcohol exposure suggesting a role for alcohol in the modulation of bronchial motor tone.

Keywords: Alcohol, Penh, airway responsiveness, methacholine, mice

Introduction:

Alcohol is one of the most commonly used and abused substances in the United States. With the rapidly growing number of people who consume alcohol on a regular basis (66%) there is a coinciding increase in the number of people seeking medical attention for alcohol-induced problems (13). While heavy alcohol intake can clearly damage brain and liver, the lung is also a target for the toxic effects of alcohol. For example, alcohol greatly increases the risk of developing upper respiratory infections, pneumonia, and acute respiratory distress syndrome (ARDS; 20). The exposure of the airways through the volatility of alcohol likely accounts for many of the biologic effects of alcohol on lung airway functions (11).

Airflow in normal lungs is directed to zones of the lung that are well perfused by blood to preserve a normal ventilation (V) to perfusion (Q) ratio (V/Q). As regional blood flow in the lungs change, reflex bronchoconstriction and/or bronchodilation occurs to maintain normal V/Q matching. When inappropriate bronchoconstriction or bronchodilation occurs, such as that seen with asthma or pulmonary emboli, abnormal V/Q matching occurs and causes impaired gas exchange. Airway responsiveness (AR) occurs if the airways narrow too easily and too much (34, 38). AR is a cardinal feature of asthma, but the mechanisms of AR remain poorly understood. Acute narrowing of the airway lumen is caused by contraction of the airway smooth muscle (ASM; 12, 22). The myosin motor drives contraction of the ASM cell, and myosin exerts its mechanical effects within integrated cytoskeletal scaffolding. The ASM cell also plays an important role in the pathophysiology of asthma, including AR, but evidence for a causal link between AR and

an altered ASM contractility has been at best equivocal (25, 32). Although a modest number of clinical studies have focused on the role of alcohol in the treatment of asthma (2, 3), it has also been demonstrated to have potential bronchoconstrictive effects (9). With these contradicting findings reported, no one has examined the effects of alcohol on AR.

Our lab, and others, have demonstrated that brief alcohol feeding of mice stimulates airway cAMP levels in various tissues including lung through activation of an alcohol-sensitive adenylyl cyclase, AC-7 (10, 11, 36, 42). Because cAMP is a major second messenger involved in the regulation of airway motor tone, we hypothesized that brief alcohol exposure would modulate AR resulting in altered airway responsiveness that is reversible and independent of the route of administration. We sought to test this hypothesis in alcohol-exposed mice through the use of the common bronchoprovocant, methacholine.

Materials and Methods:

Mice: Male C57BL/6 mice 6 to 8 weeks old were obtained from the National Cancer Institute (Bethesda, MD). The mice were kept in community cages with 12-hour periods of light and dark cycles and were maintained on standard rodent chow with access to water *ad libitum*. All animal care and experimentation was approved and carried out in accordance with the University of Nebraska Medical Center and Columbia University College of Physicians and Surgeons institutional animal care and use committees and in

accordance with the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Chemicals: Ethyl alcohol dehydrated (200 proof) was obtained from PHARMCO-AAPER alcohol and chemical company, Louisville, KY. Acetyl-β-methylcholine chloride, N^G-methyl-L-arginine acetate salt (L-NMMA), pancuronium bromide, and sodium pentobarbital were obtained from Sigma Chemical, St. Louis, MO.

Ethanol administration: For exposure via the oral route, mice were fed alcohol *ad libitum* directly in their drinking water using a ramping method where the alcohol group drank 10% w/v alcohol for 2 days, 15% w/v alcohol for 5 days, and 20% w/v alcohol for 1 to 12 weeks while the control mice drank water only (10, 11, 33). As previously reported (11), No significant weight loss or nutritional disturbances were observed in mice fed this *ad libitum* diet. For exposure via the ip route, a single injection of ethanol (3.0 g/kg; 20% w/v in 0.9% saline) was administered, and control mice received a single ip injection of saline (16). Following the injection, an accommodation period of one hour was allowed before experiments were initialized. All doses were sufficient to achieve a BAC level of at least 45 mg/dl, which is equivalent to a human consuming 1-2 alcoholic beverages within one hour.

Non-invasive pulmonary function measurement: In order to test the pulmonary function of mice exposed to various agents, barometric plethysmography was utilized (Max II; Buxco electronics, Troy NY). This whole body plethysmography system operates without the use of anesthesia or restraint to allow real time recordings of airway

responsiveness via a dimensionless parameter known as enhanced pause (Penh) to estimate the total pulmonary resistance. This method has been demonstrated to accurately reflect airway resistance, expressed as enhanced pause (Penh) units (14). This non-invasive parameter has been shown to correlate with direct invasive measures of airway obstruction namely, airway resistance and dynamic compliance, (1, 8, 21). Airway responsiveness was assessed following a standard protocol (26). Briefly, unrestrained, non-anesthetized mice were placed in separate chambers and allowed 10 minutes to accommodate to their surroundings. After the accommodation period, a 5-minute recording was performed to obtain a baseline measure of their airway responsiveness. Following this recording, 0.9% isotonic saline was introduced to the chambers via ultrasonic nebulization for 2 minutes immediately followed by a 5-minute recording period to capture the airway responsiveness to the inhaled vehicle control. Following the saline challenge, mice were exposed to the common bronchoprovocant, methacholine (MCh), in cumulative doses (1.5 - 48.0 mg/ml) in order to establish a dose-response relationship. Readings were taken for five minutes immediately following drug aerosolization. Each consecutive dose was not administered until the mice returned to baseline Penh levels. Airway responsiveness (Penh values) was reported as an average raw value from three separate experiments.

Invasive pulmonary function measurement: The Linear first – order compartment model is the standard model of respiratory mechanics. It produces the widely used dynamic Resistance and Compliance parameters where increased resistance values signal constriction of the lungs. This parameter reflects not only central airway resistance, but is

also influenced by the lung periphery (the tissues). In these studies, Mice were injected ip with saline or ethanol (3 g/kg) one hour before being anesthetized with sodium pentobarbital (50 mg/kg), tracheostomized, and mechanically ventilated at a rate of 350 breaths per minute, tidal volume of 0.15 ml, PEEP (3-4 cmH_2O) using a computerized small animal ventilator (FlexiVent; SCIREQ Inc.; Montreal, Quebec, Canada; Finepoint; Buxco electronics, Wilmington, NC) as previously described (19, 28, 30). Dose-response curves to aerosolized methacholine (1.5 - 48.0 mg/ml) were then obtained as previously reported (24, 30), and reported as lung resisitance (R_L).

Reversibility experiments: For the alcohol-fed exposure mice received ethanol in their drinking water 6 weeks and were weaned off ethanol gradually. They drank 15% ethanol in their water for the first 5 days, 10% for the next 2 days, then regular drinking water until week 12. Following the weaning period airway responsiveness was measured. The ip-exposed mice were injected with 3 g/kg ethanol in normal saline and allowed to recover for the designated time periods of 4, 24, and 48 hours. After each time point airway responsiveness to MCh was measured. Separate groups of mice were used in each set of experiments. To avoid possible tachyphylaxis to methacholine, airway responsiveness experiments were performed on each mouse one time only.

Blood alcohol concentration (BAC): The alcohol concentration of the blood was monitored closely to verify that the mice had elevated levels of alcohol following each experiment. Upon euthanization, 0.8 - 1.0 ml of whole blood was collected into plasma separator tubes (BD Scientific; Franklin Lakes, NJ). The tubes were placed on ice for 15

minutes then centrifuged at 800 RPM for 10 minutes. Serum was removed, transferred to microcentrifuge tubes, and frozen at - 80°C until assayed. The serum was assayed using an alcohol reagent set and alcohol control (Pointe Scientific, Inc.; Canton, MI). Briefly, samples and controls were added to reconstituted reagent at 30°C, mixed, and incubated in a shaking water bath for 5 minutes. Samples and controls 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 manufacturers defined procedure.

Statistics: All experimental data are expressed as means ± SEM. Data were plotted using GraphPad Prism 4.0a (GraphPad Software Inc., San Diego, CA), and the average airway responses between different groups was analyzed by Student's t-test or analysis of variance, where applicable, followed by Bonferroni's post hoc analysis for multiple comparisons. These tests were used to determine the level of significance between all treatment groups. Probability levels less than 0.05 were considered to be statistically significant.

Results:

Ethanol feeding blocks methacholine-induced airway responsiveness. We examined baseline lung function and assessed airway responsiveness to the inhaled bronchoprovocant MCh. In mice exposed to ethanol in the drinking water, baseline Penh values were the same in control and ethanol-exposed groups of mice (0.57 ± 0.04; 0.46 ± 0.02; respectively). Six weeks of ethanol feeding completely blocked MCh-induced AR (Figure 1). Administration of MCh caused a dose-dependent increase in airway

responsiveness in the control group of mice, with a highly significant attenuation in the ethanol fed mice ($p < 0.0001$) following administration of the 12.0, 24.0, and 48.0 mg/ml doses (Figure 1). These data demonstrate that short-term ethanol consumption results in a significant attenuation of airway responsiveness to MCh.

A single ip injection of ethanol attenuates methacholine-induced AR similar to 6 weeks of oral alcohol feeding. Having established that ingested alcohol blocks AR, we next sought to determine how rapidly this effect occurs. To accomplish this we exposed groups of mice to ethanol or saline by intraperitoneal injection (ip). One hour after ip injection, MCh administration resulted in a significant increase in airway responsiveness of the saline-injected mice whereas ethanol-injected mice did not respond (5.05 ± 0.81 vs. 2.08 ± 0.23; $p < 0.0001$). We also noted that one hour following a single ip injection of ethanol (3 g/kg), baseline values of the ethanol-treated mice were significantly elevated ($p < 0.0001$) when compared to control mice (1.41 ± 0.14 vs. 0.47 ± 0.03, respectively; Figure 2). BAC analysis revealed that the ethanol treated mice, in both routes of administration, had elevated blood alcohol levels (> 45 mg/dl) whereas water/saline exposed control mice had no ethanol present (Data not shown). Figures 1 and 2 therefore demonstrate that ethanol induced attenuation of MCh responsiveness is comparable regardless of the route of administration.

Direct measures of airway resistance corroborate whole body plethysmography airway responsiveness following ethanol consumption or single dose intraperitonal injection.

Ethanol feeding model: Lung resistance values of mice exposed to MCh following 6

weeks of 20% EtOH consumption were significantly attenuated when compared to mice that drank water for 6 weeks (5.08 ± 0.58 vs. 2.88 ± 0.55; $p < 0.05$; Figure 3). Single dose intraperitoneal injection model: Administration of MCh, caused a dose-dependent increase in airway resistance in the saline-injected mice, while alcohol-injected mice demonstrated a significant attenuation of lung resistance (5.69 ± 1.7 vs. 3.35 ± .69; $p < 0.05$; Figure 4). The airway responses to alcohol using an invasive direct measurement of lung resistance method (Figures 3 and 4) complimented our non-invasive whole body plethysmography findings (Figures 1 and 2). Because alcohol rapidly affected AR, it was important to determine if this was a transient or persistent effect.

AR slowly normalizes to control levels within 48 hours of a single ip ethanol injection. Airway responsiveness was assessed at 1, 4, 24, and 48 hours after a single ethanol injection. Much to our surprise and long after alcohol was cleared from the bloodstream, blunted AR persisted for at least 24 hours after ip-ethanol injection. The divergence between the two groups decreased with time and was absent at 48 hours after injection. Interestingly, the BAC was extremely elevated (~280 mg/dl) at 1-hour post injection and by 4 hours this value drops to near control values (Figure 5). The behavior of the mice was also changed following the ip injection. At one-hour post injection the mice were extremely intoxicated and exhibited an elevated baseline and attenuated airway response when compared to saline-injected control mice. By four hours after injection however, the mice were moving around as before they were exposed to the ethanol even though the attenuated airway response persisted. Twenty-four hours after injection, the mice exhibited normal behavior and again the attenuated airway response persisted. Forty-

eight hours after injection the airway responses returned to control values. To determine the relevance of the ip reversal studies, we also examined AR in mice fed alcohol for 6 weeks and then weaned back to drinking water.

Ethanol-induced attenuation of AR normalizes when alcohol is removed from the drinking water. To determine if the attenuated airway responsiveness observed after six weeks of alcohol feeding was reversible, ethanol was slowly removed from the drinking water and airway responsiveness was assessed at 1, 2, and 3 weeks following removal. Mice weaned off of ethanol demonstrated significantly ($p < 0.05$) elevated airway responsiveness when compared to ethanol consuming mice (Figure 6). The attenuated airway responsiveness gradually normalizes and returns to the level of the water drinking control mice after 3 weeks time. We also observed that when exposure to ethanol persists for extended periods of time (12 weeks), the airway responsiveness remains attenuated as long as the mice continue to consume ethanol (data not shown). Blood alcohol levels in these weaned mice are indistinguishable from the control mice by after alcohol removal.

Discussion:

The effect of ethanol on the body is well known and includes an increasing number of effects on the lung. Some effects that ethanol exerts on the lungs include inhibition of clearance (4), impaired ciliary motility (4, 10, 40), inflammation (37), suppressing pulmonary neutrophil recruitment (7), and suppressing lung chemokine production (5). While much is known about how alcohol alters lung clearance and the susceptibility to

infection, little is known about the effects of alcohol on other airway functions, such as the regulation of bronchial motor tone and airway responsiveness (AR).

For centuries alcohol has been used as a treatment for asthma dating as far back as the Egyptian papyri (23). Several small clinical studies have shown benefits of alcohol as a treatment for asthma. In 1863, Hyde Salter reported improvement in asthma symptoms in three of his patients who self administered high amounts of oral alcohol (29). One hundred years later, Herxheimer and Stresemann saw improvements in the vital capacity (VC) of asthmatics after alcohol consumption (17). In 1947, Brown administered alcohol (intravenous) for the first time to children that were unresponsive to current asthma therapy and noted a bronchodilator effect and a rapid improvement in their symptoms (6). Because alcohol has been used as an experimental treatment for asthma, we explored the effects of alcohol on bronchial motor tone and airway responsiveness in a mouse model of alcohol exposure.

Our data demonstrate that feeding ethanol to mice attenuates AR. In mice that drank ethanol, we found a significant attenuation of AR after 1 week of consumption and a total block following 6 weeks of consumption when compared to water-drinking control mice. This blocking effect continues as long as the mice continue to consume alcohol. We also examined the effects of short-term alcohol exposure on bronchial reactivity using ip-injected mice. AR attenuation was also observed in the groups of mice that were ip injected with ethanol when compared to ip injected saline control mice. When utilizing the more invasive method of lung resistance, we observed attenuation in the total lung

resistance of mice that either consumed ethanol for 6 weeks or mice that received a single i.p. injection of ethanol. We can therefore conclude that the degree of ethanol-mediated attenuation of MChinduced hyperresponsiveness caused by ethanol is equivalent when these two independent methods are compared in this model. Taken together, we have shown that alcohol exposure, via two different routes of administration, and two separate methods of analysis of airway response yields the same outcome and supports historical evidence that the effect of alcohol as a bronchodilator is dependent on the concentration, duration, and route of exposure (31).

The attenuated AR caused by ethanol was reversed over time. To our surprise, however this alcohol-mediated effect persists for days (ip) or for weeks (fed) after removal of alcohol. When the ethanol was replaced with water in the alcohol-feeding model, the attenuated airway responsiveness slowly returned to that of the control mice. As long as ethanol remained in the water, an attenuated/blocked response was observed suggesting that the alcohol has modified a pathway or changed how the airway cells are reacting to the methacholine challenge. The same finding was also observed in mice that were injected with ethanol. These data demonstrate a sustained alcohol-induced change in the behavior of the airway. Blood alcohol levels were monitored through out the experiments and showed elevated levels of alcohol in the ethanol groups of mice. At 24 hours after alcohol injection, the attenuated AR persisted long after alcohol was absent from the blood and cleared from the tissues suggesting an unknown mechanism that requires further exploration.

Several possible mechanisms for the ethanol-induced attenuation of airway responses in these mice may include: 1) a change in membrane potential; 2) an altered sensitivity to calcium; 3) altered cAMP-PKA levels; and 4) a change in NO/PKG levels. Ethanol-induced central nervous system suppression of lung responsiveness to methacholine is not the likely mechanism as we observe similar ethanol-mediated relaxation responses in both whole lung slices and isolated cultures of airway smooth muscle cells.

One study in canine tracheal smooth muscle demonstrated that alcohol caused hyperpolarization and a suppression of membrane action potentials (27). By suppressing the membrane action potentials, a larger signal would be required to produce contraction.

Various studies also support alcohol-induced modifications in calcium sensitivity as possibly having a role (15, 35). By altering the sensitivity to calcium, the airway smooth muscle will contract or relax accordingly. In our studies, alcohol exposure may change how the cell utilizes calcium enough to allow the cell to remain in a more relaxed state.

Increased relaxation through the modification of the regulatory kinases including PKA and PKG (40, 41) may also be a productive mechanism to pursue. By altering PKA or PKG activation in the airway smooth muscle cells you can directly affect a key step in multiple known relaxation pathways.

Increased NO production could lead to increased PKG activation resulting in enhanced relaxation. Accordingly, the release of nitric oxide (NO) represents an additional feasible mechanism for this attenuated response to MCh.

NO has been shown to be a weak bronchodilator in asthmatics, but not in normal subjects (18). Since nitric oxide can act as a weak bronchodilator, and increased production of NO with alcohol stimulation in cultured bronchial epithelial cells has been reported (39), the cGMP/NO pathway seems a strong candidate for this response.

In summary, we have demonstrated, for the first time, that ethanol blocks AR regardless of the route of exposure. This effect is slowly reversible over time and surprisingly persists for at least 24 hours after alcohol is absent in the blood. Our data have intriguingly demonstrated a novel approach to utilizing alcohol to modify the responsiveness of the airways in mice. Further studies will be necessary to define the specific mechanism(s) of alcohol-induced attenuation of AR.

Acknowledgments:

We greatly acknowledge Susmit Das for his technical assistance with the mouse ventilator studies and Lisa Chudomelka for her help with the preparation of this manuscript.

Grant Support:

This work was supported by a Ruth K. Kirschstein National Research Service Award (NRSA) Grant 1F32AA017024-01 and National Institutes of Health grant 5R37AA008769-16.

Figure Legends:

1. *Ethanol feeding blocks methacholine-induced airway responsiveness.* Methacholine-induced airway responsiveness (AR) was measured in mice drinking water alone (solid line, solid squares) or 20% ethanol (dashed line, solid triangles) for 6 weeks. The vertical axis represents enhanced pause (Penh), am indirect measurement of airway responsiveness. The horizontal axis represents the dose of methacholine administered. Ethanol feeding completely blocked AR.

*** $p < 0.0001$, * $p < 0.05$. Data are represented as means ± SEM (n = 8-10 mice per group).

2. *Ethanol injection blocks methacholine-induced airway responsiveness.* One hour following a single ip dose of ethanol (3g/kg) or saline, ethanol-injected mice displayed a significant attenuation of airway responsiveness to aerosolized methacholine compared to saline injected controls. *** $p < 0.0001$. The data are represented as means ± SEM (n = 8-10 mice per group).

3. *Direct lung resistance is decreased in EtOH fed mice following MCh challenge.* Total lung resistance measurements, using a mechanically ventilated mouse system, confirm that 6 weeks of EtOH consumption attenuates methacholine-induced bronchoconstriction. * $p < 0.05$, ** $p < 0.001$. Data are expressed as means ± SEM (n = 7-9 mice per group).

4. *Direct lung resistance is decreased in ethanol-injected mice following MCh challenge.* Total lung resistance measurements, using a mechanically ventilated mouse system, confirm that ethanol injection attenuates methacholine-induced bronchoconstriction. * $p < 0.05$. Data are expressed as means ± SEM (n = 7-9 mice per group).

5. *AR blunting persists up 24 hours after alcohol injection.* The left vertical axis (bars) represents the change in enhanced pause (Penh) between saline and ethanol-injected mice at the 48 mg/ml methacholine dose for each time point. The right vertical axis (dashed line) represents the blood alcohol concentrations (BAC) for each time point. The horizontal axis represents the time (hours) after a single injection of saline or alcohol. The BAC levels in the mice peak around 1 hour and are rapidly declined to near control levels by 4 hours after injection. C represents control mice that were not exposed to any ethanol. AR is blunted in the alcohol-injected mice for at least 24 hours and long after BAC levels have dropped. Data are represented as means ± SEM (n = 8-10 mice per group).

6. *Attenuated airway responsiveness is slowly reversible.* Mice that drank 20% ethanol showed an attenuation of responsiveness to methacholine challenge. After 3 weeks time, the mice weaned off of ethanol showed a recovery in their response to methacholine challenge with significant differences observed between the EtOH only and 3 week weaned groups (* $p < 0.01$). The 1 and 2 week weaned groups demonstrated no significant difference from the EtOH only group ($p > 0.05$). Data are represented as means ± SEM (n = 8-10 mice per group).

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Figure 1

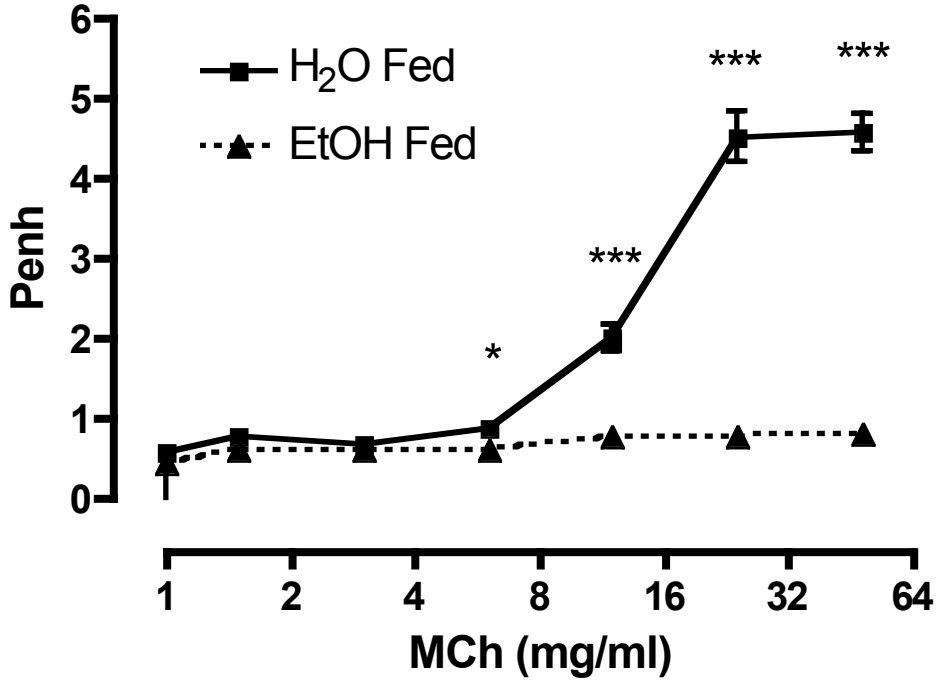


Figure 2

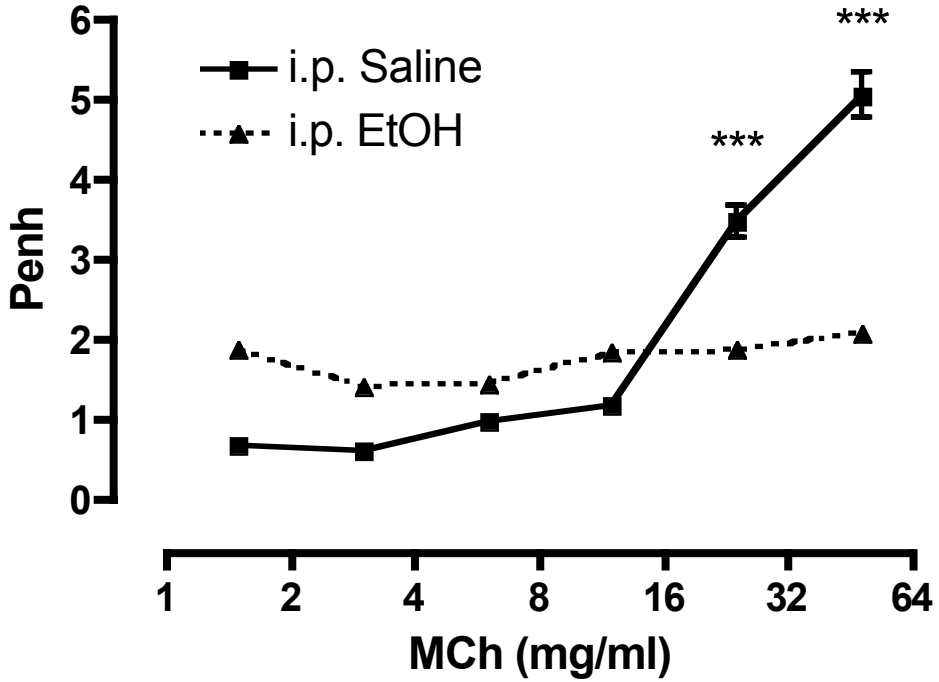


Figure 3

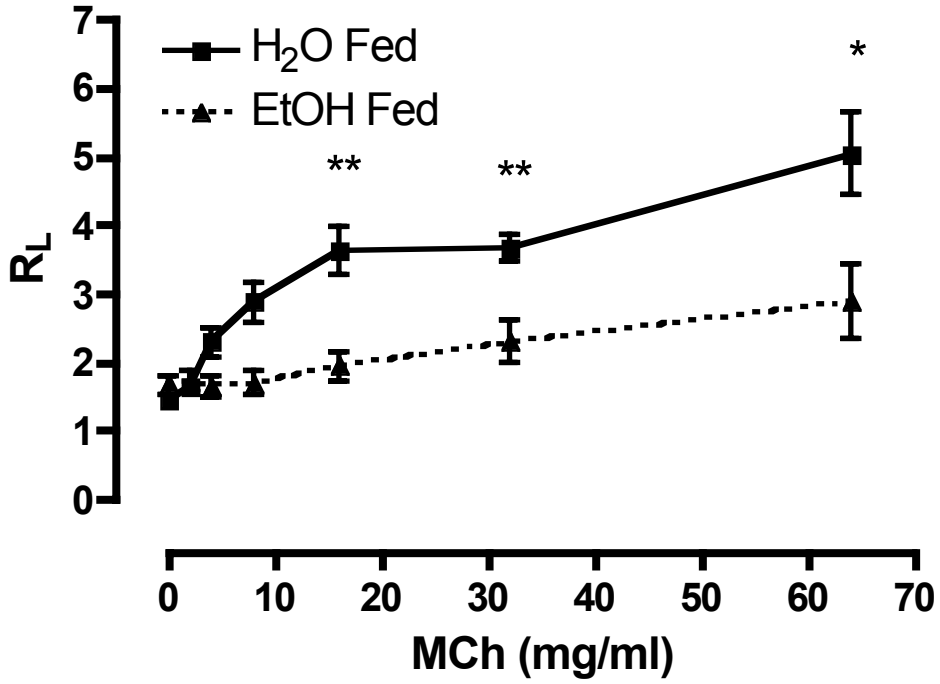
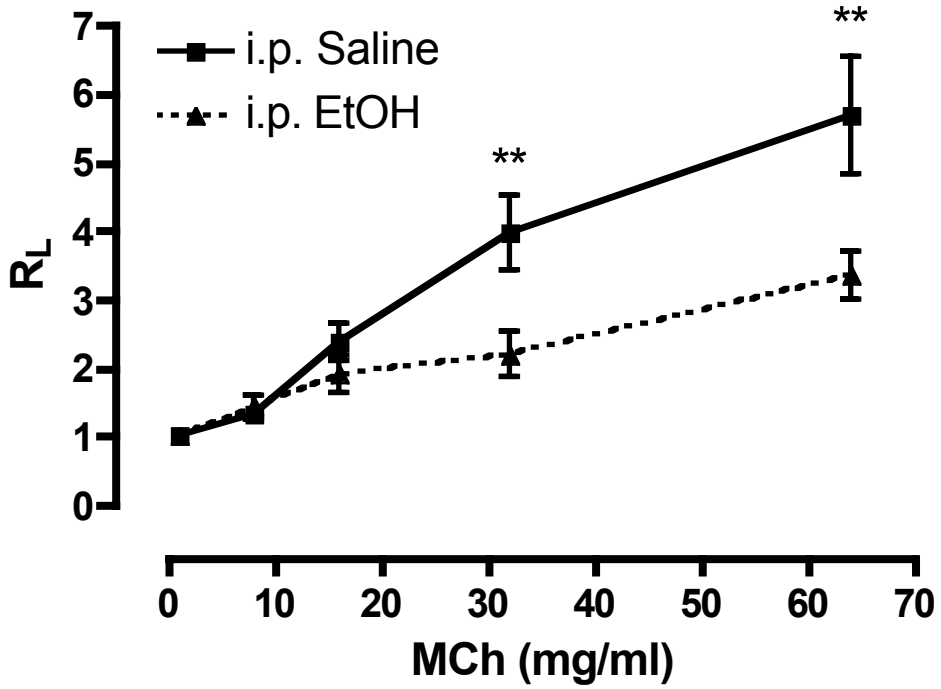


Figure 4



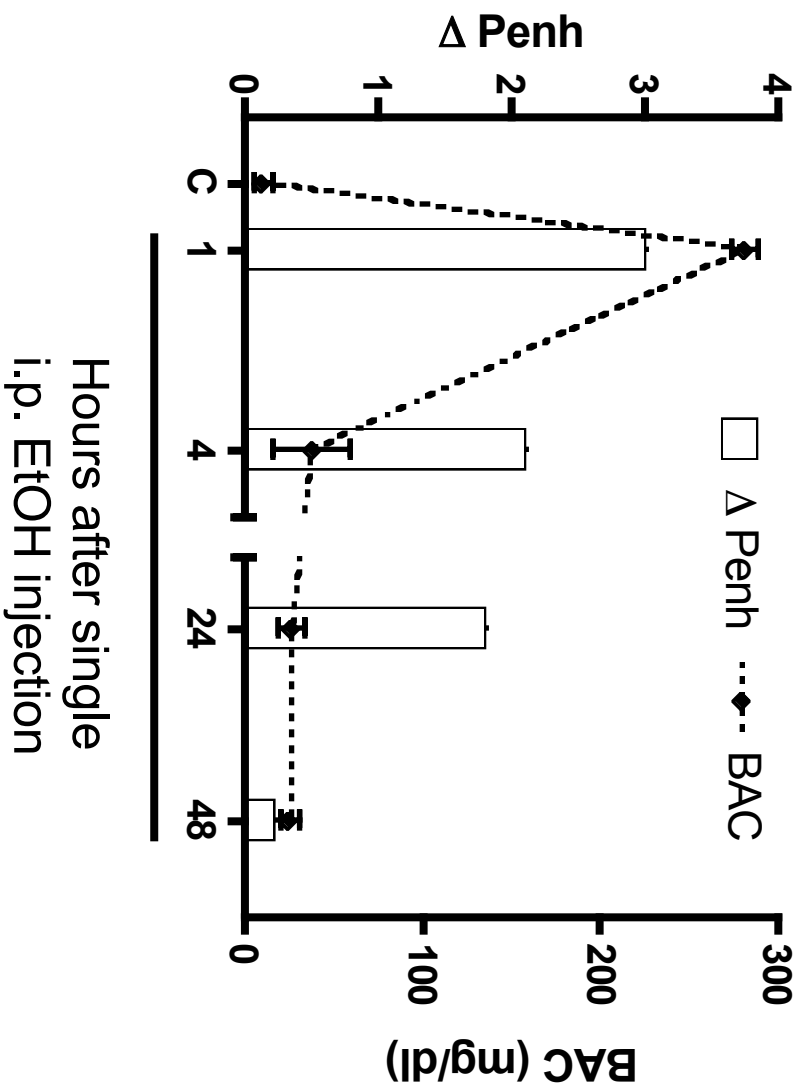

Δ Penh
0
1
2
3
4
C
1
4
24
48
0
100
200
300
BAC (mg/dl)
Δ Penh
BAC
Hours after single
i.p. EtOH injection

Figure 5

Figure 6