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Low-Level Hyperbaric Antagonism of Ethanol's Anticonvulsant Property in C57BL/6J Mice

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This study investigated the ability of hyperbaric exposure to antagonize ethanol's anticonvulsant effect on isoniazid (INH)-induced seizures. Drug-naive, male C57BL/6 mice were injected intraperitoneally with saline, 1.5, 2.0, or 2.5 g/kg ethanol followed immediately by an intramuscular injection of 300 mg/kg of INH. The mice were then exposed to either 1 atmosphere absolute (1 ATA) air, 1 ATA helium-oxygen gas mixture (heliox), or 12 ATA heliox at temperatures that offset the hypothermic effects of helium. Ethanol increased the latency to onset of myoclonus in a dose-dependent manner. Exposure to 12 ATA heliox antagonized ethanol's anticonvulsant effect at 2.0 and 2.5 g/kg, but not at 1.5 g/kg. Ethanol also increased the latency to onset of clonus in a dose-dependent manner beginning at 2.0 g/kg. Exposure to 12 ATA heliox antagonized this anticonvulsant effect. When exposed to 12 ATA heliox, the blood ethanol concentrations at time to onset of myoclonus were significantly higher in mice treated with 2.5 g/kg of ethanol as compared with blood ethanol concentrations of mice exposed to 1 ATA air. These findings extend the acute behavioral effects of ethanol known to be antagonized by hyperbaric exposure and support the hypothesis that low-level hyperbaric exposure blocks or reverses the initial action(s) of ethanol leading to its acute behavioral effects.

Key Words: Ethanol, Isoniazid, Hyperbaric, Anticonvulsant, C57BL/6.

THE GENERAL ANESTHETIC effects of ethanol and other intoxicant-anesthetics can be reversed in a wide variety of species (tadpoles, newts, mice, and rats) by exposure to high atmospheric or hydrostatic pressures of 100–300 atmospheres absolute (ATA).[1–4] It has been postulated that this pressure-induced reversal of ethanol's acute behavioral effects may be caused by blockage of ethanol's action on critical microenvironment regions in brain cells[4–7] or by the ability of pressure to drive ethanol out of it site(s) of action.[8] These theories are supported by in vitro studies showing that high pressures can reverse the membrane-perturbing actions of these drugs.[5,9,10]

In rodents, it has been demonstrated that several of the acute and chronic behavioral effects of ethanol can be antagonized by exposure to low-level hyperbaric helium-oxygen (heliox) gas mixtures (≤12 ATA).[11–21] Pressure-induced antagonism of acute behavioral effects of ethanol by 12 ATA heliox include: reduced duration of ethanol-induced loss of righting reflex (LORR),[18,19] return of righting reflex at higher blood and brain ethanol concentrations,[11] partial or complete antagonism of the depressant effects of ethanol on spontaneous locomotor activity,[12,20] and reduction of ethanol's depression of aggressive behaviors.[13] In addition, exposure to 12 ATA heliox antagonized ethanol's excitatory effects at low doses[22] measured as stimulation of locomotor activity. Exposure to 12 ATA heliox also has been shown to antagonize the development of chronic functional tolerance to ethanol[15] and can precipitate and enhance withdrawal in ethanol-dependent animals.[14]

Mechanistic studies suggest that the antagonism by 12 ATA heliox of ethanol's effects is not caused by hyperbaric- or helium-induced changes in body temperature,[11] oxygen partial pressure,[19] or ethanol pharmacokinetics (alteration of the absorption, distribution, or elimination of ethanol).[16,19] In contrast to suggestions regarding high-pressure reversal of anesthesia,[9,10,23–27] recent results suggest that the antagonism of ethanol depression by 12 ATA heliox does not result from an increase in general CNS excitability.[17] And, it appears that exposure to 12ATA does not cause or contribute to a proconvulsant condition.[17] Moreover, the pharmacological and biophysical characteristics (dose-response, pressure-response, temperature-pressure interaction) of these low-level hyperbaric studies closely match or parallel those of high-pressure reversal of anesthesia.[3,28–31] Collectively, these results support the hypothesis that low-level hyperbaric exposure directly blocks or reverses the initial action(s) of ethanol that leads to its acute behavioral effects.

If hyperbaric exposure blocks or reverses the initial actions of ethanol leading to behavioral change, then the antagonistic effects of pressure should extend to all behavioral effects of ethanol. In earlier studies, as previously mentioned, it has been demonstrated that some of ethanol's behavioral effects are antagonized by low-level hyperbaric exposure.[11–20] However, antagonism of ethanol's behavioral effects by hyperbaric exposure has not been fully characterized. Ethanol's anticonvulsant property is one such behavioral effect that has not been demonstrated to be antagonized by low-level hyperbaric exposure.

This study begins to investigate the ability of pressure

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to antagonize the anticonvulsant effect of ethanol versus a convulsant that is known to impede GABAergic transmission.

METHODS

Experiment 1: Does Hyperbaric Exposure Antagonize the Anticonvulsant Property of Ethanol in Mice?

Experimental Design. Six-week-old male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed 4/cage in an air-conditioned, temperature-controlled (22 ± 1°C) room for at least 1 week before use in experiments. Room lighting was on a 12-hr cycle (on 0700 hr). Food (Harland Rodent Laboratory Chow) and water were freely available. Animals to be used were weighed (mean weight 24.5 ± 0.2 g) 30 min before the experiment began (0900 hr). The consequence of exposure to 12 ATA heliox on ethanol's anticonvulsant effects were investigated using a between-subjects design. Each mouse was injected with either saline or 1 of 3 doses of ethanol followed immediately by the convulsant isoniazid (INH). Within 4 min after the injection of INH, the animal was exposed to 1 of the 3 atmospheric conditions (1 ATA air, 1 ATA heliox, or 12 ATA heliox). All three atmospheric conditions were tested simultaneously using identical hyperbaric chambers. Two mice were tested concurrently within individual observation compartments inside each chamber. One of these mice was treated with ethanol + INH, and one was treated with saline + INH. Depending on the dose of ethanol administered, the experiments ran from 90–150 min after injection of INH. Mice surviving past their respective ceiling time were euthanatized by injecting an anesthetic dose of 20% ethanol followed by cervical dislocation. Mice tested at 12 ATA heliox were rapidly decompressed before euthanasia.

Drug Treatments. INH obtained from Sigma Chemical Co. (St. Louis, MO) was dissolved in normal saline before intramuscular injection at 300 mg/kg and was administered at a volume of 5 ml/kg body weight. The base form of INH was used to calculate its dose. Ethanol (20% w/v in normal saline from U.S.P. 95%) was administered by intraperitoneal injection, at doses of 1.5, 2.0, or 2.5 g/kg body weight. Mice were injected with either ethanol immediately followed by INH, or with saline followed by INH. The dose of INH was based on work from a previous study and was selected to produce reliable, clonic seizures in saline-treated mice.[17] We chose INH, rather than a more commonly used convulsant such as picrotoxin, because in a previous study INH's convulsive effect per se was the most stable when tested under pressure conditions,[17] and because its time course met the constraints of our pressure experiment. The lowest dose of ethanol used (1.5 g/kg) was the minimum dose of ethanol (based on pilot studies) that could reliably produce an anticonvulsant ED_{99} for the myoclonic effect of INH in 1 ATA air.

Determination of Drug Effect. Behavior was recorded on standard VHS cassette tapes using a videocassette recorder (Panasonic AG1270 Proline series) connected to an RCA Ultricon camera (model TC 1005/U12) or Image Sensor camera FC-06 mounted 75 cm above the clear plexiglass face-plate of each chamber. Light intensity was adjusted to 70 lux, measured at the center of each chamber's face-plate with a Lutron LX-101 Lux Meter, using appropriately positioned tensor lamps. The experimental recording time was based on the time course of anticonvulsant INH-induced activity for the dose of ethanol administered (based on pilot studies). Mice receiving 1.5 g/kg of ethanol were recorded for 90 min, mice receiving 2.0 g/kg were recorded for 120 min, and mice receiving 2.5 g/kg were recorded for 150 min after injection of INH. Convulsant drug effects were classified according to commonly used criteria composed of the following behaviors: myoclonus (whole body twitch with Straub tail activity) and clonus (clonic spasms, wild running, and bouncing or tonic hindleg extension). The latency to onset of myoclonus or clonic seizures was defined as the time interval between INH injection and the first manifestation of the seizure. Animals not demonstrating either myoclonus or clonus were assigned a latency time that was equal to the maximum postinjection recording time of their particular group (90, 120, or 150 min). Time was measured to the nearest 10th of a second from a digital chronometer positioned in the recording field outside each chamber. Mice were scored from the videotape by a observer who was blind to the experimental conditions.

Hyperbaric Treatment. Animals were exposed to 12 ATA heliox or to control atmospheric conditions of 1 ATA air or 1 ATA heliox using premixed certified compressed gases (Phoenix Distributors, Anaheim, CA) in 18-liter, cylindrical, stainless steel hyperbaric chambers as previously described.[12,14,32] Briefly, compression was conducted at a rate of 2 ATA/min, and gas flow through each chamber was adjusted to 1.2 liter/hr. Oxygen partial pressure was 0.2 ATA for all final gas conditions. For these studies, chamber temperature was set at 25°C for exposure to 1 ATA air and at 30°C for exposure to 1 or 12 ATA heliox. These temperatures were selected to offset the hypothermic effects of helium.[12] The atmospheric condition within each chamber (1 ATA air, 1 ATA heliox, and 12 ATA heliox) was alternated from day to day to remove any bias caused by differences between chambers.

Experiment 2: Does Hyperbaric Exposure Alter Blood Ethanol Concentrations (BECs) at Onset of Myoclonus in Mice?

The results of experiment 1 suggested that hyperbaric exposure antagonized ethanol's anticonvulsant property. Experiment 2 extended the investigation by examining the consequence of hyperbaric exposure on brain sensitivity to ethanol's anticonvulsant effect, measured by BEC at the onset of myoclonus. The BEC taken at the onset of myoclonus reflects the threshold ethanol concentration that no longer protects the animal against myoclonus, and thus provides a direct measure of brain sensitivity to ethanol that is independent from changes in ethanol pharmacokinetics. The experimental set-up remained the same as in experiment 1, except: (a) only one dose of ethanol was tested (2.5 g/kg body weight); (b) mice were exposed to either 1 ATA air or 12 ATA heliox (the 1 ATA heliox control was omitted on the basis of Experiment 1); (c) at the first sign of myoclonus, mice were removed from the chamber (the 12 ATA heliox mice were rapidly decompressed) and a 20-μl sample of blood was drawn from the orbital sinus.[33] The sample was processed, frozen, and analyzed later for ethanol concentration by headspace gas chromatography using procedures described previously[11] with the following modifications. After heating samples, a 50-ml headspace aliquot was injected into a Varian model 3400 gas chromatograph operated under the following conditions: column oven, 130°C; detector, 250°C; nitrogen (carrier gas), 20 ml/min; hydrogen, 30 ml/min; and air, 300 ml/min. Analysis was done on a 3′ × ¼″ o.d. × 2 mm i.d. glass column that was custom-packed by Supelco (Carbopack C, Bellefonte, PA) with 0.2% Carbowax 1500 60/80 mesh. Under these conditions, the retention times for ethanol and *n*-propanol were 0.65 and 1.20 min, respectively.

Data Analysis. Data were analyzed using the BMDP Statistical Software Package.[34] A two-way analysis of variance (ANOVA) model was chosen for initial analysis of the effects of ethanol dose, atmospheric condition (1 ATA air, 1 ATA heliox, and 12 ATA heliox), and interaction effects on latency to myoclonus and clonus. Data for BECs were analyzed by one-way ANOVA for the effect of condition on BECs at the onset of myoclonus. Because the robustness and power of ANOVA can be affected if its assumptions are not met,[35] preliminary analyses were run to determine whether data were normally distributed and possessed equality of group variances.[34] When these tests indicated failures in the assumptions, the transformation that restored variance homogeneity and induced at least near-normality was selected among a set of power transformations as suggested by Snedecor and Cochran.[36] In experiment 1, data for latency to onset of myoclonus was transformed to log(x) and time to onset of clonus was transformed to square root(x). Latency data in experiment 2 was transformed to log(x). In experiment 2, for BEC data, the hypothesis of equality of group variances was rejected as signified by Levene's test and could not be corrected by transformation. Therefore, BEC data was analyzed using Welch ANOVA that does not assume equality of group variances, but does so at the expense of a loss of degrees of freedom.[34]

When the overall F test reached significance, subsequent t tests were performed to examine the effects of helium and pressure on the time to onset of myoclonus and clonus and BECs at the onset of myoclonus. Data presented in the text are expressed as mean ± SEM. The level of statistical significance was set at $p < 0.05$ for all analyses.

RESULTS

Experiment 1: Does Hyperbaric Exposure Antagonize the Anticonvulsant Property of Ethanol in Mice?

The effects of treatment (ethanol versus saline) and condition (1 ATA air, 1 ATA heliox, and 12 ATA heliox) on time to onset of myoclonus induced by 300 mg/kg of INH are illustrated in Fig. 1. Two-way ANOVA revealed a significant effect of treatment [$F(3,111) = 184.53$, $p < 0.001$] and condition [$F(2,111) = 13.14$, $p < 0.001$] on latency to myoclonus with a significant interaction between treatment and condition [$F(6,111) = 2.20$, $p < 0.01$]. Ethanol increased the latency to onset of myoclonus in a dose-dependent manner (1.5 g/kg, $t = 4.90$; 2.0 g/kg, $t = 10.74$; 2.5 g/kg, $t = 13.11$; $p < 0.001$). Twelve ATA heliox did not significantly alter this anticonvulsant effect of 1.5 g/kg of ethanol when compared with mice exposed to 1 ATA air or 1 ATA heliox. The anticonvulsant effect of higher doses of ethanol (2.0 and 2.5 g/kg) was antagonized by 12 ATA heliox, as demonstrated by a significant reduction in latency to onset of myoclonus versus ethanol-treated mice exposed to either 1 ATA air (2.0 g/kg, $t = 3.20$; 2.5 g/kg, $t = 4.08$, $p < 0.005$) or 1 ATA heliox (2.0 g/kg, $t = 2.79$; 2.5 g/kg, $t = 3.75$, $p < 0.01$). Exposure to 12 ATA heliox did not significantly alter the time to onset of INH-induced myoclonus in saline-treated animals.

The effects of treatment and condition on time to onset of clonus induced by 300 mg/kg of INH is illustrated in Fig. 2. Two-way ANOVA revealed a significant effect of treatment [$F(3,111) = 95.21$, $p < 0.001$] and condition [$F(2,111) = 11.05$, $p < 0.001$] on latency to clonus. The interaction between treatment and condition was also significant [$F(6,111) = 2.74$, $p < 0.05$]. Ethanol significantly increased latency to clonus in a dose-dependent manner beginning at 2.0 g/kg (2.0 g/kg, $t = 5.79$; 2.5 g/kg, $t = 10.81$, $p < 0.001$). The anticonvulsant effect of 2.0 g/kg ethanol was significantly reduced in mice exposed to 12 ATA heliox compared with mice exposed to 1 ATA air ($t = 2.36$, $p < 0.05$) or 1 ATA heliox ($t = 2.58$, $p < 0.05$). Pressure also antagonized the anticonvulsant effect of 2.5 g/kg ethanol compared with ethanol-treated mice exposed to 1 ATA air ($t = 5.46$, $p < 0.001$) or 1 ATA heliox ($t = 3.91$, $p < 0.001$). Exposure to 12 ATA heliox induced a small but statistically significant reduction in the time to onset of INH-induced clonus in saline-treated mice compared with mice exposed to 1 ATA air ($t = 3.01$, $p < 0.01$) or 1 ATA heliox ($t = 3.27$, $p < 0.01$).

Experiment 2: Does Hyperbaric Exposure Alter BECs at Onset of Myoclonus in Mice?

The effects of treatment (2.5 g/kg ethanol versus saline) and condition (1 ATA air and 12 ATA heliox) on latency to onset of myoclonus induced by 300 mg/kg of INH are illustrated in Fig. 3A. In agreement with experiment 1, two-way ANOVA revealed a significant effect of treatment [$F(1,23) = 223.05$, $p < 0.0001$] and condition [$F(1,23) =$

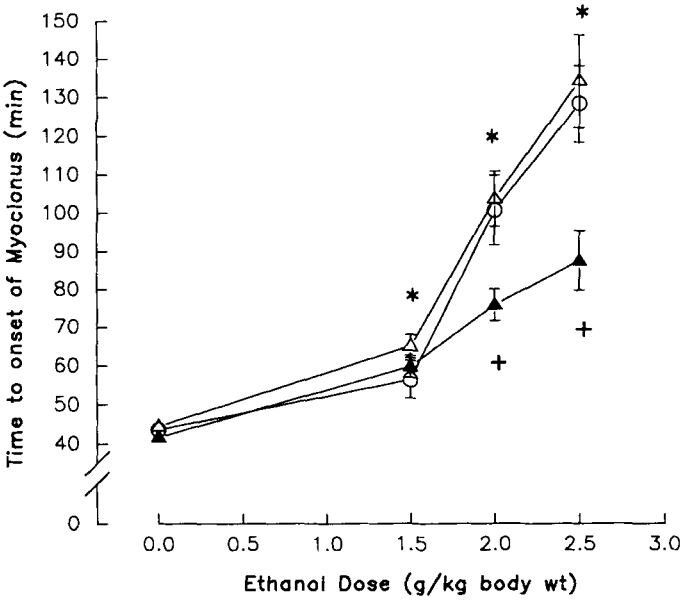

Fig. 1. Effects of ethanol and 12 ATA heliox (▲) on time to onset of myoclonus induced by 300 mg/kg INH. Ethanol significantly increased the time to onset of seizures at all doses tested. The anticonvulsant effect of higher doses of ethanol (2.0 and 2.5 g/kg) were antagonized by hyperbaric exposure. Values are mean ± SE, $n = 6$–8/group. *$p < 0.001$ vs. saline, +$p < 0.01$ vs. respective 1 ATA air (△) and 1 ATA heliox (○) groups.

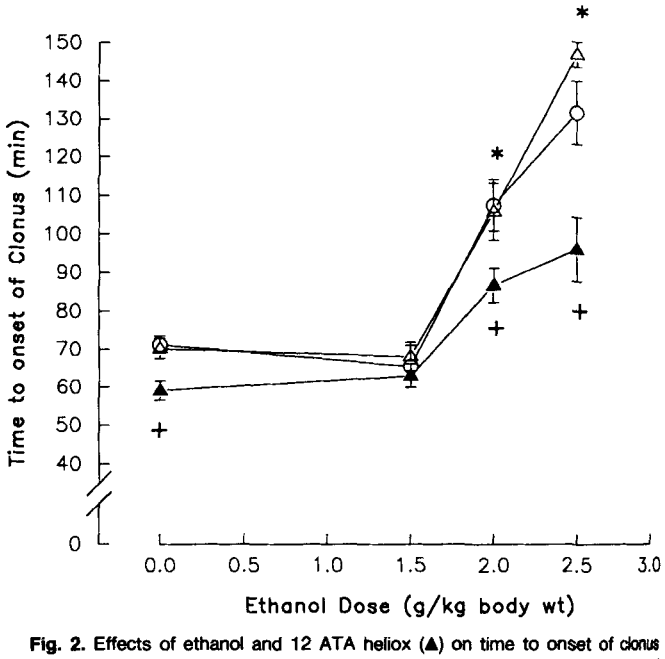

Fig. 2. Effects of ethanol and 12 ATA heliox (▲) on time to onset of clonus induced by 300 mg/kg INH. Ethanol significantly increased the time to onset of seizures at doses of 2.0 and 2.5 g/kg. Hyperbaric exposure antagonized the anticonvulsant effect of ethanol at 2.0 and 2.5 g/kg. Exposure to 12 ATA heliox caused a significant reduction in time to onset of INH-induced seizure in saline-treated mice. Values are mean ± SE, $n = 6$–8 per group. *$p < 0.001$ vs. saline, +$p < 0.01$ vs. respective 1 ATA air (△) and 1 ATA heliox (○) groups.

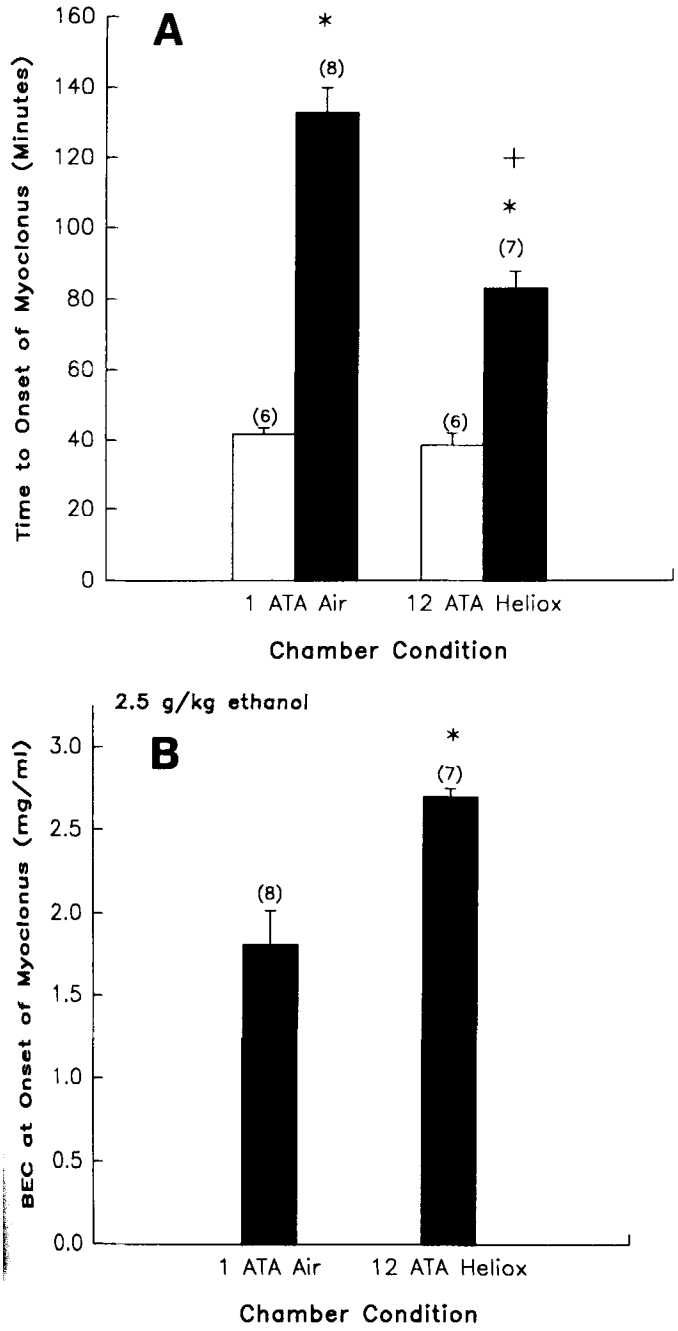

Fig. 3. (A) Effects of ethanol (2.5 g/kg; ■) and 12 ATA heliox on time to onset of myoclonus induced by 300 mg/kg INH. The anticonvulsant effect of ethanol was antagonized by exposure to 12 ATA heliox. Values are mean ± SE; the number of animals tested are given in parentheses. *$p < 0.001$ vs. respective saline (□) control, +$p < 0.01$ vs. 1 ATA air control. (B) Effects of 12 ATA heliox on BEC at the time of onset of myoclonus. Exposure to 12 ATA heliox significantly increased the BECs at time to onset of myoclonus. Values are mean ± SE for the ethanol-treated animals in (A). *$p < 0.01$ vs. 1 ATA air control.

18.96, $p < 0.0005$] on latency to myoclonus, with a significant interaction between treatment and condition [$F(1,23) = 8.44$, $p < 0.01$]. The anticonvulsant effect of 2.5 g/kg ethanol was antagonized by 12 ATA heliox, as demonstrated by a significant reduction in latency to onset of myoclonus versus ethanol-treated mice exposed to 1 ATA air ($t = 7.37$, $p < 0.0001$). Exposure to 12 ATA heliox did not significantly alter the time to onset of INH-induced myoclonus in saline-treated animals.

In addition, as determined by one-way ANOVA, there was a statistically significant effect of condition on BECs at time to onset of myoclonus, in mice treated with 2.5 g/kg ethanol, as illustrated in Fig. 3B [$F(1,8) = 17.3$, $p < 0.005$]. When exposed to 12 ATA heliox, the BECs at time of onset of myoclonus were significantly higher in mice treated with 2.5 g/kg of ethanol as compared with BECs of mice exposed to 1 ATA air ($t = 4.16$, $p < 0.005$).

DISCUSSION

Exposure to 12 ATA heliox antagonized the anticonvulsant effects of ethanol versus INH-induced seizures. This finding extends the spectrum of ethanol-induced behaviors known to be antagonized by low-level hyperbaric exposure[11-20] and supports the hypothesis that exposure to 12 ATA heliox blocks or reverses the initial action(s) of ethanol leading to its behavioral effects.

The present study provides evidence that pressure antagonism of ethanol's anticonvulsant effect is caused by a pressure-induced decrease in brain sensitivity to ethanol. First, BECs in mice exposed to 12 ATA heliox were higher at the onset of myoclonus than in mice exposed to 1 ATA air. The higher BECs at onset of myoclonus in the pressure group agrees with previous work indicating that hyperbaric exposure antagonizes ethanol by reducing brain sensitivity to ethanol, not by changing the pharmacokinetics of ethanol.[16,19] Second, in agreement with previous work indicating that 12 ATA heliox does not alter CNS excitability[17] latency to INH-induced myoclonus in saline-treated mice was not altered by exposure to 12 ATA heliox. Exposure to 12 ATA heliox did cause a small, but statistically significant decrease in latency to onset of clonus. However, this change does not appear to reflect a constant effect of 12 ATA heliox, because it was not observed in a previous study.[17] Previous work has also eliminated pressure-induced changes in oxygen partial pressure[16] and body temperature[11] as factors mediating pressure antagonism of ethanol's behavioral effects. Based on these aforementioned findings, low-level hyperbaric exposure seems to be acting as a direct antagonist of ethanol.

The neurochemical and physiological events that underlie ethanol's anticonvulsant effect against INH-induced seizures are unknown. INH is believed to induce convulsions by reducing the synthesis of GABA[37] via reduction or inhibition of pyridoxal phosphate a coenzyme essential for the conversion of glutamate to GABA by glutamic acid decarboxylase.[38] Ethanol does not affect the activity of the mammalian brain enzymes involved in the synthesis of GABA.[39] However, ethanol can enhance the affinity of the $GABA_A$ receptor for GABA.[40-45] Hence, ethanol's anticonvulsant effect on INH-induced seizures likely involves its ability to promote or enhance GABAergic transmission. Ethanol could also reduce convulsions by altering

the pharmacokinetics of INH and/or the ability of INH to inhibit GABA synthesis. Furthermore, ethanol may block INH-induced convulsions in a less direct manner by decreasing neuronal excitability via inhibition of NMDA-mediated excitatory responses[46] or through changes in other systems that lead to cellular depression. Little is known on these subjects. Therefore, although ethanol may induce its anticonvulsant effect through one or more mechanisms acting at different sites; available evidence implicates enhanced GABAergic transmission. Regardless of the underlying mechanism of ethanol's anticonvulsant effect, this effect of ethanol was antagonized by exposure to 12 ATA heliox.

In the present study, exposure to 12 ATA heliox did not completely reverse the anticonvulsant effect of ethanol, nor was there an inverse relationship between ethanol dose and the magnitude of antagonism. In contrast, previous work found that exposure to 12 ATA heliox completely antagonized the locomotor depression effect of 2.0 and 2.5 g/kg ethanol in C57 mice.[20] Furthermore, using LORR and locomotor depression, the degree of maximum ethanol antagonism by low-level hyperbaric exposure decreased as the ethanol dose increased.[18,20] This lack of correspondence between the present and previous studies probably reflects a methodological limitation imposed by the "ceiling" to latency of onset of convulsion used in the current study. The distinction might also reflect the possible complexity of mechanisms involved in mediating ethanol's anticonvulsant effect, in that some, but not all, of these mechanisms may be antagonized by pressure.

In conclusion, the present finding adds to an expanding list of ethanol-induced behaviors that have been demonstrated to be antagonized by exposure to 12 ATA heliox. These findings fit predictions based on the hypothesis that low-level hyperbaric exposure blocks or reverses the initial action(s) of ethanol leading to its acute behavioral effects. Collectively, this work suggests that low-level hyperbaric exposure may be useful as a tool for investigating the primary neurochemical site(s) of ethanol's action underlaying its behavioral effects.

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