Pharmacology Biochemistry and Behavior, Vol. 57, No. 4, pp. 651–657, 1997

0091-3057/97 $17.00 + .00

ELSEVIER

PII S0091-3057(96)00128-1

Phenobarbital Sensitivity in HAS and LAS Rats Before and After Chronic Administration of Ethanol

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Received 10 November 1995; Revised 15 February 1996; Accepted 26 February 1996

DRASKI, L. J., R. A. DEITRICH AND J.-F. MENEZ. *Phenobarbital sensitivity in HAS and LAS rats before and after chronic administration of ethanol.* PHARMACOL BIOCHEM BEHAV **57**(4) 651–657, 1997.—Rats selectively bred for high alcohol sensitivity (HAS) or low alcohol sensitivity (LAS) were tested for initial sensitivity to hypnotic doses of ethanol and a locomotor-altering dose of phenobarbital. Following 6 weeks of either a pair-fed control or 33% ethanol-derived calorie diet, animals were tested again for tolerance to ethanol and cross-tolerance to phenobarbital. HAS and LAS rats did not differ in baseline open field or Rotarod activity before chronic ethanol treatment. However, HAS rats were more sensitive to 50 mg/kg phenobarbital relative to LAS rats. Both control- and ethanol-diet rats appeared to be less sensitive to phenobarbital after the 6-week treatment period. Chronic ethanol-exposed HAS and LAS rats demonstrated tolerance to ethanol and cross-tolerance to phenobarbital, and in particular LAS rats were even more active in the open field following phenobarbital relative to controls. In summary, significant differences in response to phenobarbital were observed between HAS and LAS rats. These observations suggest that initial sensitivity and tolerance to ethanol are associated with differences in phenobarbital sensitivity and are influenced by similar genes. © 1997 Elsevier Science Inc.

HAS LAS Ethanol Phenobarbital Tolerance Cross tolerance Selective breeding Activity

BOTH ethanol and barbiturates have been shown to exert part of their pharmacologic effects by increasing the conductance through chloride channels associated with the γ-aminobutyric acid (GABA) receptor chloride complex (12,18,21,26). Barbiturates appear to bind directly to sites on the chloride channel complex, thereby enhancing chloride flux through allosteric alterations (10,22). Neurochemical studies show that ethanol acts indirectly at the GABA receptor to enhance chloride flux in isolated brain membranes in the absence and presence of added GABA (2,28). Studies in behavioral pharmacology also have demonstrated that the actions of ethanol are potentiated by GABA agonists and attenuated by GABA antagonists and inverse agonists (1,15,27).

A powerful approach in elucidating the mechanisms shared by ethanol and barbiturates is to examine genetically selected lines of laboratory animals that differ in response to one or more of these compounds. Rat lines exhibiting differential sensitivity to soporific effects of acute ethanol treatment have been produced by selective breeding of high alcohol sensitivity (HAS) and low alcohol sensitivity (LAS) responding animals (8). These lines also are differentially sensitive to hypnotic doses of pentobarbital in the same direction as ethanol (5,8).

More recent studies using the HAS and LAS selected rat lines show that whole brain microsacs prepared from HAS rats have greater potentiation of $^{36}Cl^-$ influx following in vitro treatment with ethanol or phenobarbital relative to LAS rats (3). Examination of GABA-activated chloride channels from long-sleep (LS) and short-sleep (SS) mice, also selected for differences in initial ethanol sensitivity, indicates that membranes from LS cerebellum are more sensitive than SS membranes to stimulation by the GABA agonist muscimol and to augmentation by ethanol as measured by chloride flux (2). These results support the hypothesis that a genetic correlation exists between ethanol sensitivity and GABA receptor function. The goal of this study was to examine behavioral effects of phenobarbital in HAS and LAS rats by measuring changes in motor activity and coordination.

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Mechanisms of action shared between alcohol and barbiturates could influence the development of tolerance and cross-tolerance between these drugs (12). It is also interesting to test whether rats that differ in sensitivity to acute effects of ethanol differ in the development and magnitude of tolerance to ethanol or cross-tolerance to phenobarbital. Thus, we examined differences in ethanol tolerance in high and low sensitive rats following 6 weeks of chronic ethanol administration, as well as the development of cross-tolerance to a locomotor-altering dose of phenobarbital.

METHODS

Animals

We used 32 HAS and 32 LAS naive males from the 12th generation of selection at the University of Colorado Health Sciences Center (Denver, CO) in this study. Equal numbers of both replicate lines (HAS-1 and HAS-2; LAS-1 and LAS-2) were used. Animals were group-housed five per cage from the time of weaning (approximately 25 days of age) until the onset of experimentation (approximately 45 days of age), at which time all animals were singly housed. Prior to experimentation, rats received food and water ad lib. Subjects were assigned randomly to receive either a pair-fed control diet or a 33% ethanol-derived calorie diet.

Diet Administration

Rats were given water and the revised AIN-76A Liquid Diet (EtOH) provided by Dyets (Bethlehem, PA) as their sole food source ad lib. Animals in the chronic ethanol groups received increasing concentrations of ethanol for the first week of the experiment: 20% of calories as ethanol for the first 3 days, 25% for 2 days, 30% for 2 days, and 33% until the end of the experiment. Control rats received a pair-fed diet adjusted to the liquid consumption of the alcohol-treated rats. Calories from ethanol were replaced by maltose-dextrose in the control diet.

Sensitivity to Ethanol and Phenobarbital Before and After Chronic Ethanol Treatment

At approximately 45 days of age and before the initiation of the experimental diet, 20 HAS and 20 LAS rats were tested for sensitivity to an acute dose of ethanol. Owing to the differential sensitivity of the lines, HAS rats were injected intraperitoneally (IP) with a dose of 3.0 g/kg ethanol and LAS rats were injected with 4.5 g/kg ethanol (15% w/v). The sleep-time duration of each animal was defined as the time between loss and recovery of the righting reflex. An animal was identified as recovered when it was able to right itself in a V-shaped trough three times within 1 min. At this time, a 40-μl blood sample was taken from the retro-orbital sinus for determination of awakening blood ethanol concentration (BEC). The blood samples were analyzed by a standard enzymatic procedure modified from Smolen and Smolen (25) using alcohol dehydrogenase.

The day after alcohol testing, half of the rats from each line and diet group received an IP injection of saline; the other half was injected with 40 mg/kg phenobarbital in saline. A minimum of 24 h from the time of ethanol testing was allowed to minimize overlapping drug effects. All rats were tested 30 min after injection in an open field, and 60 min after injection on a rotating rod. Rats were tested for open-field activity in a circular open field measuring 120 cm in diameter with a 20×20-cm grid covering the floor. Animals were placed in the center of the open field, and the number of squares entered with two front feet (crosses) and number of times an animal raised itself on its two hind legs (rears) were recorded during a 3-min test. Motor coordination was assessed on a Rotarod treadmill for rats (Model 7700) with a dowel measuring 7.5 in. in circumference rotating at a speed of 14 rev/min. Animals were held on the rod until they became orientated and replaced on the rod in the same fashion in the event of a fall. Total seconds on the rod out of 120 s maximum were measured.

After the collection of these data, it was decided that a higher dose of phenobarbital would be necessary to induce a more readily measurable level of motor debilitation, particularly considering the 6 weeks of chronic ethanol treatment some animals would be receiving. Consequently, a second, naive group of 12 HAS and 12 LAS rats was administered 50 mg/kg phenobarbital and tested as above for the prediet comparison. At 90 min after phenobarbital administration, an 80-μl retro-orbital blood sample was obtained for determination of blood phenobarbital levels, and the animals were terminated from the experiment. Phenobarbital concentrations were determined by gas chromatography with ethyl,bicyclo (3,2,1) octen-1-yl barbituric acid (10 μg) added to each sample as an internal standard. Samples were diluted, acidified with 2% perchloric acid, and extracted with methylene chloride. The organic phase was back-extracted with 0.1 N NaOH. The aqueous phase was acidified by 1 N HCl and reextracted with methylene chloride. Samples were taken to dryness and derivatized using methyl iodide as previously described (20).

Following 6 weeks of liquid diet treatment, the original animals were retested for differences in ethanol and phenobarbital sensitivity over a 3-day period. Animals were maintained on the ethanol or control diet until all behavioral testing was completed. All rats were tested for ethanol sensitivity on the first day, for open-field and Rotarod activity after an IP injection of 50 mg/kg phenobarbital on the second day, and for baseline open-field and Rotarod activity following an injection of an equivalent volume of saline on the third day. An overview of this experimental protocol is provided in Fig. 1.

Statistical Tests

No differences were observed between the replicate lines 1 and 2, and the data were collapsed over this measure. Because age has been demonstrated to alter sensitivity to ethanol significantly in the HAS and LAS rat lines (7), prediet vs. postdiet within-line statistical comparisons were not conducted. Prior to diet administration, initial differences between HAS and LAS rats in body mass, ethanol, and phenobarbital blood levels were analyzed by Student's *t*-tests. Prediet locomotor and Rotarod activity data were assessed by two-way analysis of variance (ANOVA) with line and dose of phenobarbital as the main factors. After the 6 weeks of diet administration, ethanol-induced sleep times were compared within each line by Student's *t*-tests for the effects of diet, since differential doses of ethanol were used for the HAS and LAS rats. Body mass, blood ethanol levels, and phenobarbital blood levels were analyzed by two-way ANOVAs with line and diet as the main factors. Locomotor and Rotarod activity following phenobarbital and saline was analyzed by three-way repeated-measure ANOVA with line and diet as the main factors and phenobarbital condition as the repeated measure. Where appropriate, posthoc pairwise comparisons were analyzed by

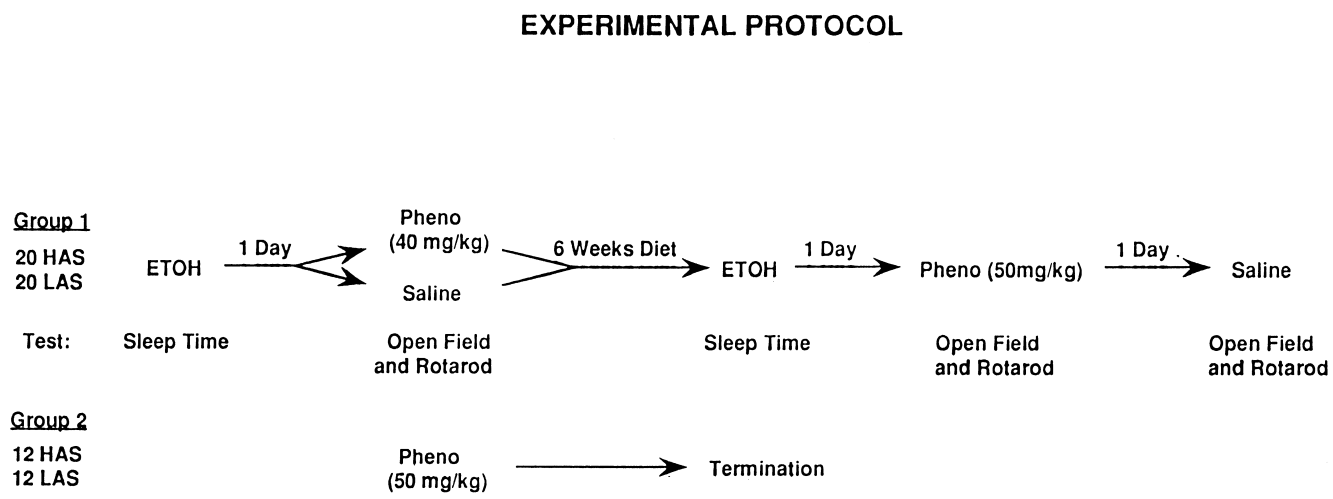

FIG. 1. Overview of the experimental protocol. Rats were tested for ethanol and phenobarbital sensitivity before and after 6 weeks of either a pair-fed control or 33% ethanol-derived calorie diet in each study.

Fisher protected least significant difference (PLSD) tests. Criterion for significance was set at $p < 0.05$ for all tests.

RESULTS

Body Mass

No significant differences in starting body mass were observed between the lines (154 ± 6 g and 157 ± 6 g, mean ± SEM, in HAS and LAS rats, respectively). However LAS rats weighed significantly more (307 ± 10 and 278 ± 65 g) than HAS rats (273 ± 4 and 247 ± 9 g) within the control and alcohol diet groups, respectively, following the 6 weeks of treatment [$F(1, 35) = 17.76$, $p < 0.001$]. In addition, rats in each alcohol diet group weighed significantly less than their corresponding control diet groups at the completion of the study [approximately 10% less in each line; $F(1, 35) = 12.74$, $p < 0.001$].

Ethanol Sensitivity

Owing to their differential sensitivities to ethanol, a higher dose of ethanol was required for LAS rats (4.5 g/kg) to induce a similar sleeping time as in HAS rats (3 g/kg) (Table 1). Because different doses of ethanol were used, no prediet sleep-time comparisons were made, and only within-line sleep-time comparisons were conducted in the postdiet analysis to examine the effects of chronic ethanol vs. control diet on this measure. No significant differences in sleep times were observed in the chronic ethanol vs. control diet groups for either HAS or LAS rats following the 6 weeks of diet administration.

BEC determined at the time of regain of the righting reflex more accurately reflects differences in CNS sensitivity and could be compared between lines as well as between diet conditions. Prior to diet administration, the mean BECs analyzed when rats regained the righting reflex were significantly higher in LAS rats than in HAS rats [$t(34) = 9.42$, $p < 0.001$;

TABLE 1

ETHANOL SLEEP TIMES (min) AND BLOOD ETHANOL CONCENTRATIONS (mg/dl)

		Prediet		Postdiet			
				Sleep Time		BEC	
Line	Dose (g/kg)	Sleep Time	BEC	Control	Etoh diet	Control	Etoh diet
HAS	3.0	58 ± 11	352 ± 9	187 ± 26	170 ± 29	264 ± 14	384 ± 23†
LAS	4.5	97 ± 10	457 ± 9*	273 ± 18	320 ± 17	409 ± 18[a]	500 ± 31†

PHENOBARBITAL CONCENTRATIONS (μg/ml)
90 MINUTES AFTER INJECTION

			Postdiet	
Line	Dose (mg/kg)	Prediet	Control	Etoh Diet
HAS	50	32.5 ± 3.3	20.5 ± 1.7	20.8 ± 1.6
LAS	50	30.7 ± 4.0	20.6 ± 1.0	20.0 ± 1.7

*Significantly different from similarly treated HAS rats at $p < 0.05$, Fisher PLSD.

†Significant within-line difference from control-diet rats.

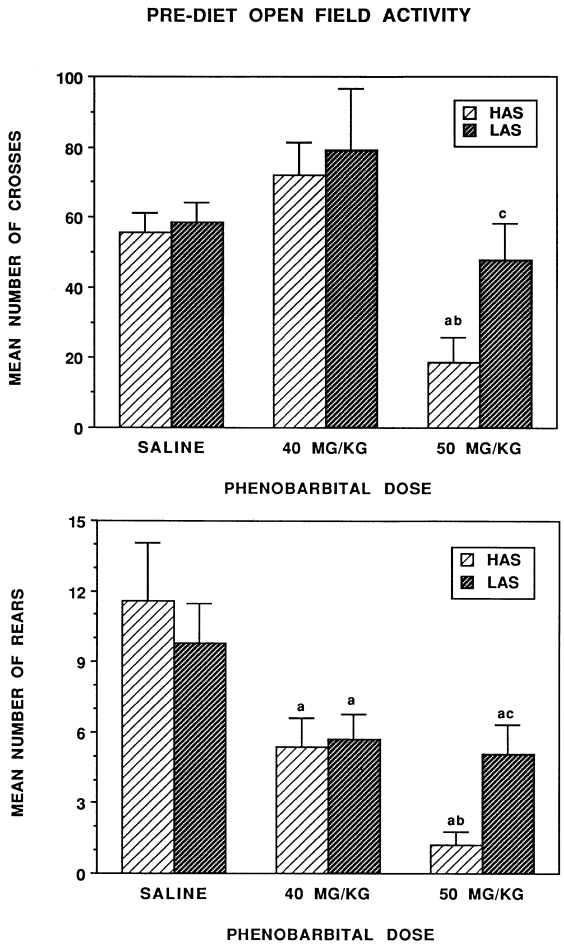

FIG. 2. Mean number of crosses (top panel) and rears (lower panel) during a 3-min test in an open field before liquid diet administration. Rats were injected with one of three doses of phenobarbital (n = 9–12/group) and tested 30 min later. [a]Significantly different from saline-injected control group by Fisher PLSD posthoc test ($p < 0.05$). [b]Significantly different from same line 40-mg/kg group. [c]Significantly different from similarly treated HAS group. Vertical lines indicate standard error of each mean.

Table 1]. Following 6 weeks of diet administration, significant effects of line [$F(1, 33) = 33.93, p < 0.001$] and diet [$F(1, 33) = 22.09, p < 0.001$] were observed. In general, HAS rats regained the righting reflex at significantly lower BECs than LAS rats, and rats of either line chronically fed alcohol had significantly higher BECs than similar rats receiving the control diet. These results suggests that both HAS and LAS rats develop tolerance to an acute dose of alcohol following chronic administration of alcohol.

Prediet Locomotor Effects of Phenobarbital

Saline-injected HAS and LAS rats did not differ in activity before diet administration. In general, HAS rats were less active in the open field and less coordinated on the Rotarod following phenobarbital administration relative to LAS rats (Figs. 2 and 3). Both 40- and 50-mg/kg phenobarbital doses led to a significant reduction in the number of rears in the open field by HAS and LAS rats relative to saline-injected

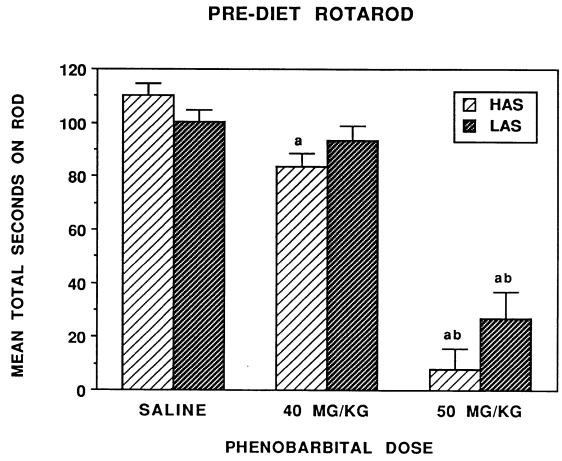

FIG. 3. Mean total seconds on the Rotarod (120 s maximum) before liquid diet administration. Rats were tested 60 min after injection of saline (0), and 40 or 50 mg/kg phenobarbital (n = 9–12/group). [a]Significantly different from saline-injected control group by Fisher PLSD posthoc test ($p < 0.05$). [b]Significantly different from same line 40-mg/kg group. Vertical lines indicate standard error of each mean.

controls [$F(2, 57) = 14.74, p < 0.001$]. However, although HAS rats were more affected by the 50-mg/kg dose than the 40-mg/kg one, 50 mg/kg phenobarbital had no greater effect on open-field rearing of LAS rats than the 40-mg/kg dose. HAS rats given 50 mg/kg phenobarbital also crossed significantly fewer squares than either control rats or similarly treated LAS rats [$F(2, 57) = 9.20, p < 0.001$]. Administration of either dose of phenobarbital failed to alter the locomotor activity of LAS rats significantly in the open field. On the Rotarod, HAS rats demonstrated a dose-dependent reduction in time spent on the rod following administration of 40 and 50 mg/kg phenobarbital; LAS rats were impaired significantly only at the 50-mg/kg dose relative to controls [$F(2, 55) = 92.81, p < 0.001$].

Postdiet Locomotor Effects of Phenobarbital

No significant effects of diet or rat line were observed in the open field after an injection of saline (Fig. 4). Relative to their saline scores, open-field crossing and rearing were unaltered following phenobarbital administration in control-diet HAS and LAS rats and significantly greater in ethanol-diet HAS and LAS rats. Of particular interest, LAS rats of both diet conditions crossed significantly more squares than HAS rats given the same diet following administration of phenobarbital [significant effects of Line ($F[1, 30] = 9.80, p < 0.01$), Dose ($F[1, 30 = 32.42], p < 0.001$), and Dose × Diet ($F[1, 30] = 14.98, p < 0.001$)]. Ethanol-diet LAS rats given phenobarbital also reared significantly more than any other group, whereas ethanol-diet HAS rats demonstrated a tendency toward an increase in rearing relative to other HAS groups following phenobarbital administration [significant effects of Line ($F[1, 30] = 12.94, p < 0.001$), Diet ($F[1, 30] = 7.89, p < 0.01$), Dose ($F[1, 30] = 15.48, p < 0.001$), Dose × Line ($F[1, 30] = 12.84, p < 0.001$), Dose × Diet ($F[1, 30] = 49.32, p < 0.001$), and Dose × Line × Diet ($F[1, 30] = 5.18, p < 0.05$)]. These results show that cross-tolerance to phenobarbital was obtained following chronic ethanol exposure.

In general, there were no significant differences among any of the LAS treatment groups in rotarod performance (Fig. 5).

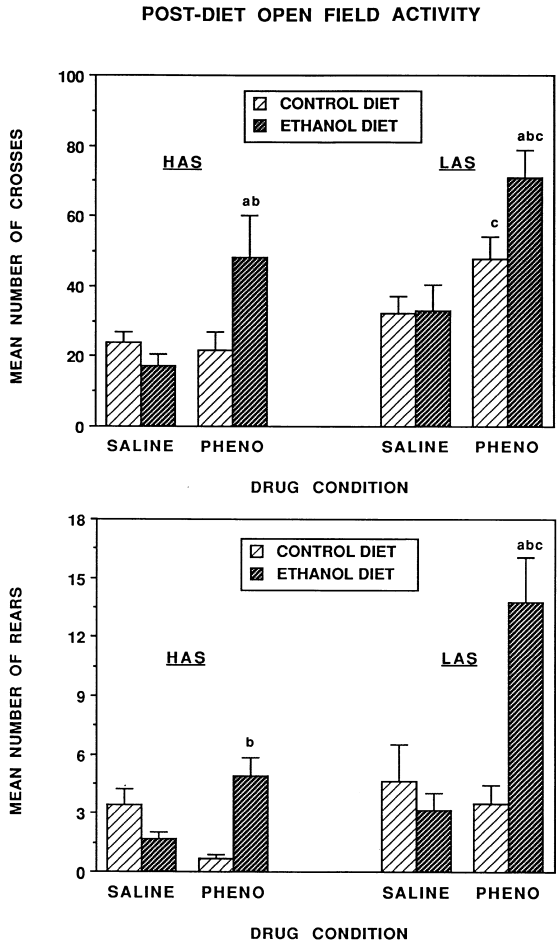

FIG. 4. Mean number of crosses (top panel) and rears (lower panel) in a 3-min open-field test after 6 weeks of either a control or ethanol liquid diet. Rats were tested 30 min after injection in both saline and 50-mg/kg phenobarbital conditions (n = 8–10/group). [a]Significant within-line difference from same diet saline-injected group by Fisher PLSD posthoc test ($p < 0.05$). [b]Significant within-line difference from similarly injected control diet rats. [c]Significantly different from similarly treated HAS rats. Vertical lines indicate standard error of each mean.

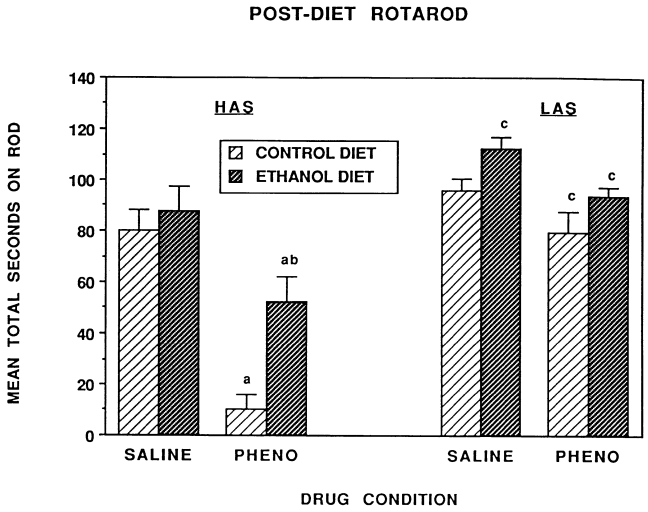

FIG. 5. Mean total seconds on the Rotarod (120 s maximum) after 6 weeks of liquid diet administration. Rats were tested 60 min after injection in both saline and 50-mg/kg phenobarbital conditions (n = 8–10/group). [a]Significant within-line difference from same diet saline-injected group by Fisher PLSD posthoc test ($p < 0.05$). [b]Significant within-line difference from similarly injected control diet rats. [c]Significantly different from similarly treated HAS rats. Vertical lines indicate standard error of each mean.

The lack of an effect of phenobarbital on the LAS rats appears to be due to a ceiling effect, with LAS rats appearing to be less sensitive to phenobarbital at this time. On the other hand, HAS rats tested following phenobarbital administration stayed on the Rotarod for significantly fewer seconds than saline-injected HAS rats, with control-diet HAS rats being significantly more debilitated than ethanol-diet HAS ones. Control- and ethanol-diet HAS rats also were less coordinated than LAS ones after phenobarbital injection [significant effects of Line ($F[1, 29] = 41.07$, $p < 0.001$), Diet ($F[1, 29] = 11.74$, $p < 0.01$), Dose ($F[1, 29] = 52.53$, $p < 0.001$), and Dose × Line ($F[1, 29] = 13.14$, $p < 0.001$)].

At 90 min after IP injection of 50 mg/kg phenobarbital, blood was collected and the concentration of this barbiturate determined. No differences between line or diet conditions were evident either before or after liquid diet administration (Table 1). These results suggest that differences in the metabolism of phenobarbital were not responsible for significant line or diet effects observed at this time. However, all groups, including the control diet animals, appeared to metabolize phenobarbital faster in the postdiet relative to the prediet condition.

DISCUSSION

Chronic exposure to ethanol resulted in tolerance to hypnotic doses of ethanol in both HAS and LAS rats. Although ethanol-induced sleep times were not altered by chronic ethanol exposure, chronically treated HAS and LAS rats had significantly higher BECs at the regain of the righting reflex relative to their respective control-diet groups, indicating the development of tolerance. BECs of control-diet HAS and LAS rats were lower than prediet groups, suggesting that either the liquid diet itself or aging may affect ethanol sensitivity.

In general, HAS rats appeared to be more sensitive in the same direction as ethanol to some, but not all, of the effects of nonhypnotic, initial doses of phenobarbital than LAS rats. Long-sleep and short-sleep (LS/SS) mouse lines, also selected for differences in initial ethanol sensitivity, have been shown to differ in acute sensitivity to a variety of nonethanol hypnotics, but mostly for those with lipid solubilities similar to ethanol (9,13,14,17). In general, the ethanol-sensitive LS mouse line exhibited greater sedation in response to nonethanol anesthetics than the ethanol-insensitive SS mouse line, with the most water-soluble drugs invoking the greatest soporific differences between the lines. However, although LS mice have been reported to be more sensitive to phenobarbital than SS mice (17,19), these measures have all dealt with soporific effects and do not address other ataxic and locomotor effects of phenobarbital.

Mice selected for differences in the activating effects of ethanol (fast/slow) were reported to show cross-sensitivity to the locomotor-stimulating effects of phenobarbital, with mice selectively bred for increased sensitivity to ethanol-induced stimulation (fast) being more sensitive than reduced sensitivity

(slow) mice (23). However, only locomotor-activating and not depressing doses of phenobarbital were used, and only one measure of locomotor activity, mean counts, was reported in this study. Diazepam-sensitive (DS) and diazepam-resistant (DR) mice, selectively bred for differential sensitivity to the ataxic effects of diazepam, also are differentially sensitive to ethanol in the same direction as diazepam (11). When these two lines were tested under the influence of phenobarbital, DS mice were more impaired on a Rotarod than DR mice (4), but no different from DR mice in phenobarbital-induced stimulation of open-field activity (24). One explanation for the discrepancy in DR and DS response to phenobarbital is that the genes controlling phenobarbital stimulation may be different from those controlling ataxia. With regard to the HAS and LAS rats, it also may be added that different genes may contribute to varying locomotor disturbances in open-field or Rotarod performance after nonhypnotic doses of phenobarbital.

In many instances, phenobarbital doses that originally were capable of producing locomotor alterations in prediet tests were found to be ineffective during the postdiet trials. For example, when control-diet rats were tested on the Rotarod, the LAS rats were no longer sensitive to the ataxic effects of phenobarbital evident before diet administration. Several factors may be operating to produce this discrepancy. Age-related changes in sensitivity to other drugs have been reported previously in these rat lines (6,7). Wanwimolruk and Levy (29) reported alterations in phenobarbital sensitivity with age in rats, although in their study rats became increasingly sensitive, rather than insensitive, with age. The stress of being maintained on a liquid diet also may alter the physiologic response of the rats to phenobarbital. The concentration of phenobarbital in blood was substantially lower in the postdiet, relative to prediet, conditions. Although differences in metabolism can not be predicted based on a single time point, it is possible that the older rats experienced lower pharmacologic levels of phenobarbital during testing than the prediet rats. However, the chronic ethanol or control diet did not significantly induce cytochrome P-450IIB in livers obtained from the animals used in these experiments relative to chow-fed controls (16). More likely, the difference before and after the liquid diet could be due to changes in the body composition of rats and the distribution of barbiturates with aging, with more barbiturates being stored in peripheral lipids of adult rats.

Cross-tolerance to phenobarbital was evident in both lines of rats. Ethanol-diet rats were considerably more active than control-diet rats in the open field, and ethanol-diet HAS rats stayed on the Rotarod longer than control-diet HAS rats following phenobarbital administration. In the open field, locomotor stimulation rather than depression was observed among ethanol-diet HAS and LAS rats after 50 mg/kg phenobarbital. In particular, ethanol-diet LAS rats given phenobarbital appeared to be more sensitive to the stimulating effects of phenobarbital and had significantly more crosses and rearings than any other group. However, phenobarbital testing was conducted only 1 day after ethanol sensitivity testing, and potential cross-reactivity between the drugs could have occurred. In addition, habituation and order effects may have contributed to the postdiet activation of animals in the open field, as saline-injected baseline levels of activity were collected on the day after phenobarbital testing of all the animals in the open field. Recent studies in our laboratory indicate that saline-injected HAS rats given one 15-min exposure to an automated activity chamber were significantly less active than same-age but apparatus-naive HAS rats when tested 6 weeks later (unpublished data). However, this reduction in activity was not observed in similarly experienced LAS rats.

In summary, HAS and LAS rats demonstrated tolerance to soporific doses of ethanol following a chronic ethanol liquid diet. HAS rats were more sensitive than LAS rats to prediet locomotor debilitating effects of 50 mg/kg phenobarbital. After 6 weeks of either a chronic ethanol or a control liquid diet, both HAS and LAS rats demonstrated cross-tolerance to the locomotor effects of phenobarbital, and postdiet phenobarbital administration led to locomotor activation in HAS and particularly LAS rats. Further studies identifying the activating segment of the biphasic response to phenobarbital and the differential response to pentobarbital in these selected lines are warranted. Finally, these results suggest that the mechanisms of action for initial sensitivity and the development of tolerance to ethanol and phenobarbital are influenced by similar genes that potentially exert their regulatory effect at the GABA receptor complex.

ACKNOWLEDGEMENTS

This work was supported by a NATO fellowship for J.F.M. and by Grants AA05868 and AA00093. The authors thank T. Fay for excellent technical assistance.

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