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Variations in Alcohol Metabolism: Influence of Sex and Age[1]

ALLAN C. COLLINS, TONI N. YEAGER, M. E. LEBSACK AND S. SCOTT PANTER

School of Pharmacy and Institute for Behavioral Genetics, University of Colorado Boulder CO 80302

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COLLINS, A. C., T. N. YEAGER, M. E. LEBSACK AND S. S. PANTER. *Variations in alcohol metabolism: influence of age and sex.* PHARMAC. BIOCHEM. BEHAV. 3(6) 973–978, 1975. – Male and female C57BL/Ibg mice were divided into two age groups: 50–60 and 95–110 days of age. Alcohol-induced sleep time was measured subsequent to the intraperitoneal injection of a 3.5 g × kg^{-1} dose. The old male group had a sleep time approximately 4 times that of the young male group and approximately twice that of the old female group. Blood alcohol concentrations at time of awakening were nearly identical in all groups, indicating the difference in sleep time is not due to an altered CNS sensitivity. Measurement of in vivo alcohol disappearance rate indicates the old male group is different from the other groups because of a slower rate of alcohol metabolism. Although changes in hepatic alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) activities were seen, the changes do not explain the observed decrease in alcohol metabolism observed in the old male group. These data provide further evidence that hepatic ADH and ALDH activities are not rate limiting in alcohol metabolism.

Alcohol Alcohol dehydrogenase Aldehyde dehydrogenase Sex differences Developmental processes

A number of variables seem to be directly involved in controlling duration and intensity of the effects of alcohol. Central nervous system (CNS) sensitivity to the depressant effects of this agent, rate of elimination, and rate of absorption should be of primary importance in controlling the duration of a response such as loss of the righting reflex, i.e., alcohol-induced sleep time [13]. It is very likely that the first two of these parameters are influenced by the genotype of the test organism. For example, McClearn and Kakihana [14] have obtained by selective breeding from a heterogeneous stock of mice two lines of mice which differ substantially in duration of alcohol-induced sleep time. Recently, it has been reported that these two selected lines differ in alcohol sleep time because of a difference in CNS sensitivity to alcohol [9]. While no attempts have been made to selectively breed two lines of mice which differ in rate of alcohol metabolism, it seems likely that this would be possible. Various inbred strains of mice demonstrate vast differences in alcohol-induced sleep time following the injection of an hypnotic dose of ethanol [11]. Rogers *et al.* [16] and Sheppard *et al.* [17] have determined that these same inbred strains differ in hepatic alcohol dehydrogenase (ADH) in such a way that an excellent inverse correlation exists between alcohol-induced sleep time and ADH activity as measured in vitro. More recently, others have reported differences in alcohol metabolism in three inbred strains of mice. [3].

Strain differences in alcohol metabolism are not restricted to mice. Eriksson [4] has reported differences in the in vivo elimination rate of ethanol in two lines of rats which had been selectively bred for high and low alcohol preference [5]. Within both of these selected lines, Eriksson noted that the females had a greater ethanol elimination rate than did the males. Liver ADH activity was not assessed in these experiments. However, strain and sex differences apparently do exist in these parameters, since a sex difference in hepatic ADH activity in Sprague-Dawley but not Wistar rats has been observed [2].

Such observations seemed of probable significance when, in examining the effects of various pharmacological agents on ethanol-induced sleep time in our laboratory, it was observed that sex differences in sleep time might exist. These initial experiments had utilized sexually mature mice of both sexes, ranging in age from 55 to 95 days, from the C57BL/Ibg strain. The sex difference seemed to be greater as a function of age. Thus, experiments were designed to estimate the magnitude of this observation and to determine possible underlying mechanisms.

METHOD

Animals

The animals were divided into 2 age groups: 50–60 days of age and 95–110 days of age. Alcohol-induced sleep time was assessed following the intraperitoneal injection of 3.5 g × Kg^{-1} ethanol. The alcohol solution was prepared in isotonic saline and contained 0.35 g × ml^{-1} of ethanol. At this concentration, the appropriate dose is achieved if each

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mouse is injected with 0.01 ml × g^{-1} body weight. The animals were injected intraperitoneally and restrained for approximately 30 sec after which they were placed on their backs in a trough. Sleep time was judged terminated when the animals could right themselves successfully 2 times in 30 sec.

Procedure

Since differences in sleep time might arise as a consequence of alterations in CNS sensitivity to the depressant effects of ethanol or as a consequence of differing rates of metabolism of ethanol, experiments were performed to test these possibilities. A convenient way to estimate CNS sensitivity to ethanol in individual animals is to measure alcohol concentration in the blood at the time of regaining the righting reflex. The mice were injected with the standard 3.5 g × Kg^{-1} ethanol dose; immediately after the righting reflex was regained, a central venous blood sample was obtained from each animal by puncturing the retro-orbital sinus with a 40 microliter capillary pipet. The blood sample was added to 0.96 ml of a heparinized isopropyl alcohol solution. The final isopropyl alcohol concentration was 40 mg percent and served as an internal standard. A 5 microliter aliquout of this solution was injected onto a Porapak Q column in a Beckman GC-45 gas chromatograph, equipped with a flame ionization detector. The column and detector temperatures were 175°C and 225°C, respectively. The gas mixture consisted of air, 300 ml × min^{-1}; hydrogen, 45 ml × min^{-1}; and helium, 80 ml × min^{-1}. Each sample was run in duplicate. Blood alcohol concentration was calculated by estimating by triangulation the areas of the ethanol and isopropyl alcohol peaks and comparing the ratio obtained for each sample with a standard curve.

The rate of ethanol elimination was determined for animals from each of the 4 groups using a similar technique. Each animal was injected with the standard ethanol dose and 40 microliter blood samples were obtained at 1, 2 and 4 hr after injection. Blood alcohol concentrations were determined as described previously and the elimination rate calculated for each animal.

Hepatic alcohol dehydrogenase, ADH, and aldehyde dehydrogenase, ALDH, have long been considered the most important enzymes in ethanol metabolism. Certainly, on a kinetic basis, this appears to be the case. These considerations led us to measure hepatic ADH and ALDH activities in vitro. Animals from each of the 4 groups which had not been exposed to ethanol were sacrificed by a blow on the head, the livers quickly excised and placed in 9 volumes of cold 0.25 M sucrose. The tissue was homogenized and the 48,000 × g supernatant fraction obtained by centrifuging for 1 hr in a Sorvall RC2B refrigerated centrifuge. ADH activity was determined in this supernatant fraction by monitoring spectrophotometrically the rate of NADH formation with ethanol and NAD as cosubstrates using a modification of the method of Bonnichsen and Brink [1]. The modification consisted of the addition of 0.2 M semicarbazide to the incubations. Semicarbazide serves to trap the acetaldehyde which is formed when ethanol is oxidized; if this step is omitted, both ADH and ALDH, which convert acetaldehyde to acetic acid using NAD as a cosubstrate, will be measured.

Total hepatic ALDH activity was determined utilizing a radiochemical assay which has recently been developed in our laboratory. The assay substrate, ^{14}C benzaldehyde, was obtained from New England Nuclear as a benzene solution. The benzene was removed by evaporation under a stream of nitrogen at 0°C. The radiolabelled benzaldehyde was resolubilized in 0.5 ml of a 1:1 acetone-water solution and run across a 3.5 × 0.8 cm Dowex AG-1 column, pH 7.0. The column was washed with 2.5 ml of water and effluents combined. The benzaldehyde was diluted with unlabelled benzaldehyde to a specific activity of 0.10 microcuries × $micromole^{-1}$.

Enzyme activity was determined by incubating 50 microliters of a 10 percent homogenate in a mixture of 0.65 ml 10 mM pyrophosphate buffer, pH 9.6, 0.1 ml 10 mM NAD, 0.1 ml 10 mM pyrazole and 0.1 ml 2.5 mM benzaldehyde (0.25 microcuries). This mixture was incubated for 5 min at 37.5°C in a shaking incubator bath. The reaction was terminated by the addition of 0.1 ml of a 20 percent trichloroacetic acid (TCA) solution. Each homogenate was assayed in duplicate along with a blank which consisted of the complete reaction mixture with the TCA added prior to the enzyme. The solution was then made alkaline (pH 12) by the addition of a 20 percent NaOH solution and extracted twice with 6 ml of water saturated 1,2 dichloroethane. This extraction removes unreacted benzaldehyde and any other neutral metabolites leaving the reaction product, benzoic acid, in the aqueous layer. The radioactivity of a 0.1 ml aliquot of the aqueous layer was determined by placing it in 10 ml of a Triton X-100 toluene based scintillation cocktail and counting in a Beckman LS-133 liquid scintillation counter. Corrections for counting efficiency were made. Recovery of benzoic acid was nearly 100 percent under these conditions. Protein concentrations were determined using the biuret method.

The activities of both ADH and ALDH were calculated in terms of activity per mg of protein, per g liver, per liver and per g of body weight. Data were analyzed by analyses of variance and the means compared using Duncan's multiple range test.

RESULTS

The results of those experiments in which the potential influence of age and sex on alcohol-induced sleep time in C57BL/Ibg mice was assessed are presented in Table 1. No significant sex differential is evident in the groups tested at 50–60 days of age; however, there is a marked sex difference in the older group. The older males slept nearly twice as long as the females of the same age and approximately 4 times as long as the 50–60-day-old males. Although mean sleep time in females also increased with age, there was so much variance in this measure that a 45 percent increase was nonsignificant. The increase in ethanol-induced sleep time observed in C57BL mice is therefore dependent upon the sex of the animal. It is observed in greatest magnitude in male mice.

An increase in sleep time might arise from differing CNS sensitivities to the depressant actions of alcohol. The results of the experiment in which blood ethanol concentration was measured at time of regaining the righting reflex are presented in Table 2.

None of the groups differ significantly from the others with respect to blood alcohol concentration at time of awakening. Therefore, there probably are not any remarkable sex- or age-dependent differences in CNS sensitivity to alcohol in C57BL/Ibg mice. In addition, these data imply that the old males differed from the other groups with

TABLE 1

EFFECTS OF AGE AND SEX ON ALCOHOL-INDUCED SLEEP TIME IN C57BL/Ibg MICE

Age (days)	Sex	Sleep Time*	Comparison	p
50–60 (YF)	Female	48.4 ± 5.8 (16)	OM–YM	<0.01
50–60 (YM)	Male	33.5 ± 2.8 (16)	OM–OF	<0.01
95–110 (OF)	Female	70.4 ± 10.4 (16)	YF–YM	ns
95–110 (OM)	Male	137.9 ± 16.1 (15)	YF–OF	ns

*Sleep times were determined following the intraperitoneal injection of a 3.5 g × kg^{-1} ethanol dose. Time is measured in min. Data are expressed as mean ± standard error; number of observations is given in parentheses.

TABLE 2

BLOOD ALCOHOL CONCENTRATIONS AT TIME OF AWAKENING IN C57BL/Ibg MICE

Age (days)	Sex	Blood Alcohol (mg%)*	Comparison	p
50–60 (YF)	Female	377 ± 22 (10)	OM–YM	ns
50–60 (YM)	Male	411 ± 20 (10)	OM–OF	ns
95–110 (OF)	Female	443 ± 17 (10)	YF–YM	ns
95–110 (OM)	Male	414 ± 18 (10)	YF–OF	ns

*Blood alcohol concentration was determined using a gas chromatographic technique at the time each animal regained the righting reflex following the intraperitoneal injection of a 3.5 g × kg^{-1} ethanol dose. Alcohol concentration is expressed as mean ± standard error in mg%; number of observations is given in parentheses.

regard to rate of ethanol elimination. Only if rate of absorption or rate of elimination were altered could the old males, who slept more than twice as long as any other group, awaken with the same blood alcohol concentration.

The a priori likelihood of absorption rate being altered following an intraperitoneal injection seemed to us to be minimal. Therefore, we investigated the possibility of a significant difference in alcohol elimination rate. Rate of elimination was determined and the data were subjected to analysis of variance and Duncan's test for comparison of means (see Table 3). The old male group is significantly different from the other groups in the in vivo rate of ethanol elimination.

If the rates of ethanol elimination are expressed as mg% ethanol metabolized per 100 g body weight per hour, or as mg% per g liver weight per hour, an accentuation of the difference would be seen. This arises because mean body and liver weights of old males were greater than those of the other groups.

Differing volumes of distribution (V_D) would also result in different blood alcohol concentrations. Using our metabolism data we estimated the ethanol V_D for old males to be approximately 10 percent higher than that of the other groups. A greater V_D, if metabolism rates were identical, would result in a lower blood alcohol concentration. Blood alcohol levels of the old males were higher than those of the other groups. Had V_D's been identical, the difference in in vivo elimination between old males and the other groups would have been even greater.

Thus, we conclude that the observed difference in elimination rate was due to a difference in metabolism and not to differences in body or liver weight or in volume of distribution. Our data are in general agreement with those obtained by Eriksson [4], except that the differential in rate between the sexes in our older age group is substantially larger.

The influence of age and sex on hepatic ADH activity is presented in Table 4. Significant differences between the sexes and between age groups are seen in nearly all parameters. Specifically, the concentration of hepatic ADH (nanomoles × min^{-1} × mg^{-1} protein and micromoles × min^{-1} × g^{-1} liver) increases with age in both sexes. Females

TABLE 3

RATE OF ALCOHOL DISAPPEARANCE FROM THE BLOOD OF C57BL/Ibg MICE

Age (days)	Sex	Rate*	Comparison	p
50–60 (YF)	Female	72.0 ± 6.4 (6)	OM–YM	<0.01
50–60 (YM)	Male	82.3 ± 4.7 (6)	OM–OF	<0.01
95–110 (OF)	Female	82.9 ± 7.4 (9)	YF–YM	ns
95–110 (OM)	Male	47.6 ± 6.2 (9)	YF–OF	ns

*Blood alcohol concentration was determined using a gas chromatographic technique at 1, 2 and 4 hr after intraperitoneal injection of a 3.5 g × kg^{-1} ethanol dose. The rate of alcohol disappearance is linear and was calculated by determining the slope of the line in mg% × h^{-1}. Data are expressed as mean ± standard error; number of observations is given in parentheses.

TABLE 4

LIVER ALCOHOL DEHYDROGENASE (ADH) ACTIVITIES IN C57BL/Ibg MICE

Age (days)	Sex	n	n moles mg protein	μ moles g liver	μ moles liver	μ moles g body weight
50–60 (YF)	Female	8	19.3 ± 1.1	2.58 ± 0.06	2.78 ± 0.13	0.140 ± 0.005
50–60 (YM)	Male	8	11.3 ± 0.3	2.00 ± 0.07	1.85 ± 0.06	0.092 ± 0.003
95–110 (OF)	Female	8	24.5 ± 1.3	3.08 ± 0.12	3.16 ± 0.11	0.136 ± 0.004
95–110 (OM)	Male	8	18.9 ± 0.9	2.35 ± 0.07	3.16 ± 0.12	0.110 ± 0.005
			Statistical Comparisons (p values)			
YM–YF			<0.01	<0.01	<0.01	<0.01
OM–OF			<0.01	<0.01	ns	ns
YM–OM			<0.01	<0.01	<0.01	ns
YF–OF			<0.01	<0.01	<0.05	ns

Liver ADH activity was determined in the high speed supernatant fraction of 10 percent homogenates. Data are expressed as mean ± standard error per min.

always have a greater concentration than do the age-matched males. However, the old males have significantly heavier livers than do the old females, so there is no sex difference in total hepatic ADH activity in the older age group. Thus, the observed decrease in rate of alcohol metabolism with age in the male group cannot be easily explained by a differing hepatic ADH activity.

Table 5 presents the results of those experiments in which hepatic ALDH activities were determined. There are only modest differences between the various groups with regard to the activities of these enzymes except the old male group which has slightly elevated activity. The decrease in rate of alcohol disposition in vivo in the old male group cannot be easily explained, therefore, in terms of differences in the activities of those enzymes which are directly involved in alcohol metabolism.

TABLE 5

LIVER ALDEHYDE DEHYDROGENASE (ALDH) ACTIVITIES IN C57BL/Ibg MICE

Age (days)	Sex	n	n moles mg protein	μ moles g liver	μ moles liver	μ moles g body weight
50–60 (YF)	Female	8	19.90 ± 0.71	4.63 ± 0.26	4.26 ± 0.17	0.23 ± 0.01
50–60 (YM)	Male	8	20.33 ± 1.55	4.49 ± 0.19	5.30 ± 0.41	0.23 ± 0.01
95–110 (OF)	Female	8	20.46 ± 1.06	4.91 ± 0.25	4.36 ± 0.29	0.21 ± 0.01
95–110 (OM)	Male	8	24.53 ± 0.91	5.75 ± 0.29	7.23 ± 0.44	0.25 ± 0.02
			Statistical Comparisons (p values)			
YM–YF			ns	ns	<0.05	ns
OM–OF			<0.01	<0.05	<0.01	ns
YM–OM			<0.01	<0.01	<0.01	ns
YF–OF			ns	ns	ns	ns

Liver ALDH activities were determined in 10 percent homogenates. Data are expressed as mean ± standard error per min.

DISCUSSION

The data reported in this paper are not the first indication that sex differences in alcohol related behaviors exist. Eriksson and Pikkarainen [6] have observed higher voluntary alcohol consumption coupled with a greater hepatic ADH activity in C57BL female mice. Subsequently, these investigators [7] determined using F_2 hybrids derived from C57BL (alcohol preferring) and CBA (alcohol avoiding) mice that a poor correlation between alcohol preference and hepatic ADH activity exists in individual animals. However, the mean consumption and ADH activity was greater in female than in male F_2 hybrids. Eriksson and Pikkarainen did not measure in vivo ethanol metabolism in their studies. A partial explanation for the sex difference in alcohol preference in C57BL mice may come from the observation that male C57BL mice eliminate significantly higher amounts of acetaldehyde through the lungs than do females following ethanol administration [15]. Castration of the male mice eliminated the sex difference in acetaldehyde exhalation. Wiberg *et al.* [19] have reported developmental changes in in vivo alcohol metabolism and hepatic ADH activity in male Wistar rats which resemble those we report here for male C57BL mice. These investigators did not examine female rats for possible developmental changes. Our data provide evidence which indicates that male and female mice may be expected to differ in alcohol related behaviors because the in vivo rate of alcohol disposition decreases significantly with age in males but not in females.

We have observed an increase in hepatic ADH activity in male C57BL mice with age while in vivo alcohol metabolism decreased. This observation supplies compelling evidence to suggest that ADH activity is not the rate limiting step in alcohol disposition. Such a conclusion is consistent with the argument that some other parameter such as oxidation of NADH to NAD [10,18] is kinetically important in ethanol and acetaldehyde oxidation. Eriksson [4] detected differences in hepatic oxygen consumption between male and female rats which, if ADH activity is similar or is not rate limiting, could explain the modest sex differences in alcohol metabolism he observed. Since NADH oxidation should be coupled to oxygen consumption, it seems possible that males have a slower rate of ethanol metabolism than do females, because NADH is not as rapidly oxidized in males.

Lindros *et al.* [12] have suggested that at high concentrations, acetaldehyde can reduce ethanol metabolism. This is reasonable in that ADH is a readily reversible enzyme. Since an elevated acetaldehyde exhalation in male C57BL mice has been observed [15], it seems plausible to suspect the decreased alcohol elimination rate in our old male group may be related to an alteration in acetaldehyde metabolism. The observed increase in hepatic ALDH activity argues against this hypothesis. In addition, we have tested this hypothesis by castrating 50–60 day old male C57BL mice and then determined rate of alcohol metabolism and length of ethanol-induced sleep time at 95–110 days of age. We detected no difference between the castrated and noncastrated animals. The cause of this anomalous behavior is under investigation. In addition, we have observed that BALB mice demonstrate a substantial

sex difference in in vivo alcohol elimination at 60 days of age. Thus, the rate of development of the sex difference appears to be controlled by genotype.

The experiments described in this paper demonstrate that age and sex have an influence on the duration of alcohol-induced sleep time in C57BL/Ibg mice. An increase in sleep time appeared to be correlated with a decrease in rate of ethanol metabolism. Furthermore, data are presented which clearly support the hypothesis that hepatic ADH activity is not the rate limiting step in ethanol oxidation. These data may also have implications with regard to susceptibility to the development of dependence on alcohol. Goldstein and Pal [8] have indicated that high, constant levels of alcohol are necessary for the development of alcohol dependence. If this hypothesis is true, it seems likely that those animals which metabolize alcohol more slowly should be more susceptible to the development of dependence on alcohol. Such data lead to the interesting speculation that perhaps alcoholism in humans is more frequently encountered in males because of a slower rate of metabolism.

REFERENCES

1. Bonnichsen, R. K. and N. G. Brink. Liver alcohol dehydrogenase. In: *Methods in Enzymology*, Vol. 1, edited by S. P. Colowick and N. O. Kaplan. New York: Academic Press, 1955, pp. 495–500.
2. Büttner, H. Aldehyd- und Alkoholdehydrogenase – Aktivität un Leber und Niere der Ratte. *Biochem. Z.* **341**: 300–314, 1965.
3. Damjanovich, R. P. and J. W. MacInnes. Factors involved in ethanol narcosis: Analysis in mice of three inbred strains. *Life Sci.* **13**: 55–65, 1973.
4. Eriksson, C. J. P. Ethanol and acetaldehyde metabolism in rat strains genetically selected for their ethanol preference. *Biochem. Pharmac.* **22**: 2283–2292, 1973.
5. Eriksson, K. Rat strains specially selected for their voluntary alcohol consumption. *Annls Med. exp. Biol. Fenn.* **49**: 67–72, 1971.
6. Eriksson, K. and P. H. Pikkarainen. Differences between the sexes in voluntary alcohol consumption and liver ADH activity in inbred strains of mice. *Metabolism* **17**: 1037–1042, 1968.
7. Eriksson, K. and P. H. Pikkarainen. Strain and sex differences in voluntary alcohol consumption, liver ADH activity and aldehyde-oxidizing capacity in inbred strains of mice. *Jap. J. stud. Alcohol* **5**: 1–7, 1970.
8. Goldstein, D. B. and N. Pal. Alcohol dependence produced in mice by inhalation of ethanol: Grading the withdrawal reaction. *Science* **172**: 288–292, 1971.
9. Heston, W. D. W. and V. G. Erwin. A comparison of the effects of alcohol on mice selectively bred for differences in ethanol sleep-time. *Life Sci.* **14**: 365–370, 1974.
10. Israel, Y., J. M. Khanna and R. Lin. Effect of 2.4-Dinitrophenol on the rate of ethanol elimination in the rat *in vivo*. *Biochem. J.* **120**: 447–448, 1970.
11. Kakihana, R., D. R. Brown, G. E. McClearn and I. R. Tabershaw. Brain sensitivity to alcohol in inbred mouse strains. *Science* **154**: 1574–1575, 1966.
12. Lindros, K. O., R. Vihma and O. A. Forsander. Utilization and metabolic effects of acetaldehyde and ethanol in the perfused rat liver. *Biochem. J.* **126**: 945–952, 1972.
13. McClearn, G. E. Genetic differences in the effect of alcohol upon behavior of mice. *Proc. Int. Conf. Alcohol Rd Traff.* **3**: 153–155, 1962.
14. McClearn, G. E. and R. Kakihana. Selective breeding for ethanol sensitivity in mice. *Behav. Genet.* **3**: 409–410, 1973.
15. Redmond, G. P. and G. Cohen. Sex difference in acetaldehyde exhalation following ethanol administration in C57BL mice. *Nature* **236**: 117–119, 1972.
16. Rodgers, D. A. Alcohol preference as a function of its caloric utility in mice. *J. comp. physiol. Psychol.* **56**: 666–672, 1963.
17. Sheppard, J. R., P. Albersheim and G. E. McClearn. Enzyme activities and ethanol preference in mice. *Biochem. Genet.* **2**: 205–212, 1968.
18. Videla, L. and Y. Israel. Factors that modify the metabolism of ethanol in rat liver and adaptive changes produced by its chronic administration. *Biochem. J.* **118**: 275–281, 1970.
19. Wiberg, G. S., H. Trenholm and B. B. Coldwell. Increased ethanol toxicity in old rats: Changes in LD50, *in vivo* and *in vitro* metabolism, and liver alcohol dehydrogenase activity. *Toxic. appl. Pharmac.* **16**: 718–727, 1970.