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ALCOHOL-INDUCED REDOX CHANGES IN THE LIVER OF THE SPONTANEOUSLY HYPERTENSIVE RAT

EFFECT OF CHRONIC ETHANOL TREATMENT

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Abstract—Acute ethanol administration (1.0 g/kg, i.p.) to naive, chow-fed male spontaneously hypertensive (SH) rats (4–13 weeks of age) had no effect on the hepatic lactate/pyruvate (L/P) ratio, whereas this ratio was elevated significantly in male Wistar rats. The β-hydroxybutyrate/acetoacetate (B/A) ratio in the liver was increased in both male SH and male Wistar rats. As in the male Wistar rats, the acute administration of ethanol led to significant elevation of the hepatic L/P and B/A ratios of females of both the SH and Wistar strains. Acute ethanol administration to chronically ethanol-fed male SH rats, in which the ethanol metabolic rate was 100 per cent higher than in controls, resulted in significantly elevated hepatic L/P and B/A ratios. Chronic ethanol administration to male SH and male Wistar rats led to marked development of metabolic tolerance in the former and to moderate tolerance in the latter. In addition, alcohol dehydrogenase (ADH) activity was elevated significantly in the ethanol-fed male SH rats, but not in the ethanol-fed male Wistar rats. The present work extends our recent observations that ADH primarily determines ethanol metabolism in the male SH rat.

Recent studies by our group have suggested that the amount of alcohol dehydrogenase (ADH) (maximum activity determined *in vitro* with saturating concentrations of NAD) in the liver appears to constitute the most important factor in regulating the rate of ethanol metabolism in the male spontaneously hypertensive (SH) rat. This enzyme, and the rate of alcohol metabolism, were found to decrease markedly with age in males of this strain. Furthermore, in male SH rats, chronic ethanol administration led to an induction of alcohol dehydrogenase, which was associated with the development of metabolic tolerance [1].

It has been proposed that the actual ADH activity measured *in vitro* be compared to the rates of alcohol metabolism *in vivo* to determine whether ADH is a primary rate-limiting step [2]. This approach, although providing an indication of the approximate ratio of enzyme activity to ethanol metabolic rate, may not be fully adequate to allow accurate extrapolation to *in vivo* conditions. ADH may be denatured during preparation, and it is assayed *in vitro* in dilute solutions that differ from its microenvironment, in the absence of activity modifers that may exist in the intact liver cell [3].

To assess the extent of the regulatory role of ADH in the SH rat strain, we have measured the changes in the NADH/NAD redox state, in both cytosol and mitochondria, induced by ethanol in naive and in chronically ethanol-treated animals. In the cytosol, changes in the NADH/NAD ratio are well represented by changes in the lactate/pyruvate (L/P) ratio, and those in the mitochondria are expressed by the β-hydroxybutyrate/acetoacetate (B/A) ratio [4–6]. A change toward a higher NADH/NAD ratio produced by ethanol in the cytosol can be taken as an indication that the production of NADH can occur at a rate higher than that at which it can be removed by reoxidation or by transfer into the mitochondria. An increase in NADH/NAD ratio after ethanol is not seen in protein malnutrition which leads to a reduction in ADH activity [7], or following the administration of pyrazole or 4-methyl pyrazole which inhibit ADH [8, 9].

The reoxidation of NADH produced during the metabolism of alcohol, although partially mediated by cytosolic mechanisms [5, 10], depends primarily on the activity of the mitochondrial electron transport [11–14]. The latter is dependent on the basal metabolic rate or ATP utilization of the organism. Videla *et al.* [15] have observed that the rates of ethanol metabolism in different species are proportional to their basal metabolic rates.

Due to the importance of oxygen consumption in determining the rate of ethanol metabolism in most species, we have also investigated, in addition to changes in the NADH/NAD ratios, the effect of age on hepatic oxygen utilization in male SH rats.

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METHODS

Animals. Male and female SH rats (purchased from the Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) and male and female Wistar rats (Biobreeding Lab, Ottawa, Ontario, Canada) were housed in wire-mesh cages and given Purina rat chow and water *ad lib.* up to the time of the experiment. In some studies, ethanol or isocaloric sucrose was administered chronically in liquid diets for 1–2 months as described previously [16]. Before each experiment, the animals receiving the alcohol diet were withdrawn for 16 hr and fed the sucrose-containing diet. Blood ethanol disappearance was determined in both naive chow-fed and in chronically ethanol-fed animals as described earlier [17].

Determination of lactate, pyruvate, β-hydroxybutyrate, and acetoacetate in rat liver. Unless stated otherwise, animals were given 1.0 g ethanol/kg in saline i.p., as a 10% solution, while controls were given an equal volume of saline. Sixty minutes later the rats were killed by quick cervical dislocation, and a portion of the liver was excised and clamped, within 7–8 sec, between aluminium tongs precooled in liquid nitrogen. The frozen liver was ground to a fine powder and further homogenized and deproteinized in 0.6 N $HClO_4$. The homogenate was centrifuged at 12,000 *g* and the supernatant fraction was neutralized with 2 M K_2CO_3 to pH 6.0 to 6.5 and further treated with florisil as described by Williamson *et al.* [5].

Lactate and pyruvate were determined in the neutralized supernatant fraction by the methods of Hohorst [18] and Bucher *et al.* [19] respectively. β-Hydroxybutyrate was determined as described by Williamson and Mellanby [20] and acetoacetate by the method of Mellanby and Williamson [21]. The recovery of these metabolites added to samples in concentrations similar to endogenous levels were as follows: lactate, 98 per cent; pyruvate, 98 per cent; β-hydroxybutyrate, 99 per cent; and acetoacetate, 95 per cent.

Oxygen consumption by liver slices. The rate of oxygen consumption by rat liver slices was measured polarographically [22] with a Clark electrode and an oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). Animals were anesthetized with sodium pentobarbital, and following a quick laparotomy, a portion of the liver was removed and slices were prepared as described by Ismail-Beiji and Edelman [23]. Liver slices were incubated in Krebs–Ringer bicarbonate solution (pH 7.4, temperature 37°) containing 10 mM glucose and oxygenated with 95% O_2: 5% CO_2.

Alcohol dehydrogenase activity. A sample of the liver was homogenized in nine times its volume of 0.05 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) buffer with 0.33 mM dithiothreitol at pH 8.4 [2], also containing 1% Triton X-100 [24]. The homogenates were further centrifuged, and alcohol dehydrogenase activity in the clear supernatant fractions was determined as described earlier [2].

Lactate dehydrogenase activity. Liver homogenates were prepared as described above for alcohol dehydrogenase. However, they were diluted 100 times further. Lactate dehydrogenase was measured by the method of Lowry [25].

RESULTS

Figure 1 shows the effect of acute ethanol administration on the lactate/pyruvate ratios (L/P) in livers of male SH rats of various ages; a group of male Wistar rats was used as a positive control. In Wistar rats, a 150 per cent increase in the L/P ratio was observed following acute ethanol administration. On the other hand, in male SH rats, the L/P ratio did not increase significantly using identical experimental conditions. This was observed at all ages examined in the male SH rats, although a trend toward an increase in the L/P ratio, albeit small, was observed in very young animals. The pattern for the β-hydroxybutyrate/acetoacetate ratio (Fig. 2), how-

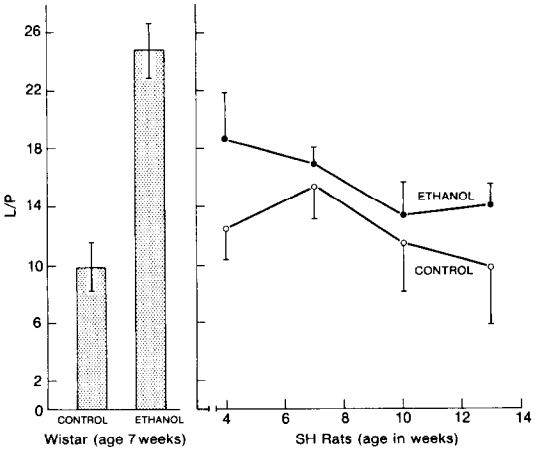

Fig. 1. Effects of acute ethanol administration on the hepatic lactate pyruvate (L/P) ratios in naive, chow-fed male SH and Wistar rats. Each point with bar represents the mean ± S.E.M. of four to six animals. Ethanol was given at a dose of 1 g/kg, i.p., in saline; controls received saline alone.

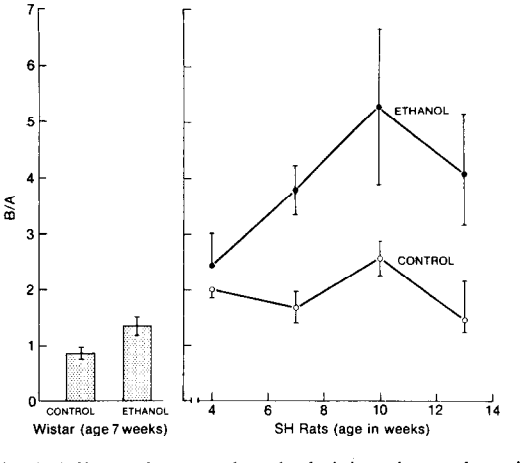

Fig. 2. Effects of acute ethanol administration on hepatic β-hydroxybutyrate/acetoacetate (B/A) ratios in naive, chow-fed male SH and Wistar rats. Each point with bar represents the mean ± S.E.M. of four to six animals. Ethanol was given at a dose of 1 g/kg, i.p., in saline; controls received saline alone.

Table 1. Effect of acute ethanol administration on the lactate/pyruvate (L/P) and β-hydroxybutyrate/acetoacetate (B/A) ratios in the livers of naive chow-fed *female* SH and Wistar rats*

Group	L/P	B/A
Wistar saline	3.88 ± 0.48 (6)	0.82 ± 0.04 (4)
	$P < 0.004$	$P < 0.004$
Wistar ethanol	12.28 ± 1.69 (5)	1.50 ± 0.14 (6)
SH saline	5.37 ± 0.67 (6)	1.22 ± 0.25 (6)
	$P < 0.03$	$P < 0.07$
SH ethanol	18.21 ± 5.61 (5)	1.94 ± 0.25 (5)

* Values are means ± S.E.M.; the number of animals is given in parentheses. Statistical significance was assessed by Student's *t*-test for unpaired data. All animals were 11 weeks old.

ever, was different. This ratio was increased by ethanol administration to older male SH rats but not to the youngest group. In the older animals, the changes were comparable to, or larger than, those in Wistar rats. A 56 per cent ($P < 0.05$) increase in the B/A ratio was obtained in 7-week-old male Wistars, and a 125 per cent ($P < 0.002$) increase was seen in male SH rats of the same age. Similar changes were observed in 10- and 13-week-old male SH rats.

In contrast to the observation that, after acute ethanol administration, L/P ratios did not increase significantly in mature (10- to 13-week-old) male SH rats, mature female SH rats showed marked increases in L/P ratios (239 per cent, $P < 0.02$; see Table 1). These changes were comparable to those found in female Wistar rats (217 per cent, $P < 0.004$; see Table 1). Similarly, B/A ratios increased both in female SH and female Wistar rats.

Because a change in the steady state of an NADH/NAD-related redox couple depends on both the rate of production and the rate of reoxidation of NADH, the latter being mostly mitochondrial [11–14], we studied the rates of oxygen consumption by livers of male SH rats of various ages. Liver slices

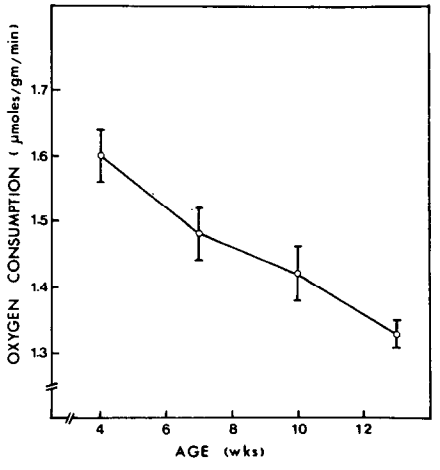

Fig. 3. Relationship between the rate of hepatic oxygen consumption and the age of naive chow-fed male SH rats. Oxygen consumption was measured *in vitro* using liver slices. Each point with bar represents the mean ± S.E.M. of eight animals. Statistical significance was assessed by a one-way analysis of variance ($P < 0.01$).

of young naive SH rats consumed oxygen at rates higher than those of older animals (Fig. 3). Although the trend toward a reduction in Q_{O_2} with age was statistically significant, the rate of liver oxygen consumption of 13-week-old animals was only 17 per cent lower than that of 4-week-old rats. The reduction in the rate of ethanol metabolism in the same period is 50–55 per cent [1].

Data in Table 2 show that the rate of ethanol metabolism was markedly increased (100 per cent) in male SH rats following chronic alcohol administration. In chronically alcohol-fed male SH rats, L/P ratios were elevated significantly following acute ethanol administration. Further, consistent with the findings in naive chow-fed male SH rats, pair-fed male SH rats fed the sucrose control diet showed no elevation in the L/P ratio following acute ethanol administration. The B/A ratio was found to be increased by acute ethanol treatment in both sucrose-control and chronically ethanol-fed male SH rats (Table 2).

The assumption normally made when extrapolating from lactate/pyruvate ratios to free NADH/NAD ratios is the existence of sufficient lactate dehydrogenase (LDH) to support an equilibrium between the NADH/NAD redox pair and the lactate/pyruvate pair. Thus, it was necessary to compare LDH activity in male SH and Wistar rats. Further, it was necessary to demonstrate that lactate dehydrogenase was not modified by the chronic ethanol treatment. Table 3 shows that male SH rats, maintained on sucrose liquid diets, actually possessed higher LDH activity than did age-matched male Wistar rats under identical conditions ($P < 0.01$). Male SH rats chronically fed ethanol did not show significantly different liver LDH activity, compared to pair-fed sucrose controls. There was a decrease in LDH following chronic ethanol feeding in male Wistar rats ($P < 0.01$). Ethanol metabolism and alcohol dehydrogenase were markedly increased in male SH rats following chronic alcohol feeding (Table 3). In contrast, the Wistar rats showed only a moderate rise in the rate of ethanol metabolism (26 per cent) and no change in alcohol dehydrogenase activity.

DISCUSSION

The marked increase in the cytosolic NADH/NAD ratio, measured by the lactate/pyruvate ratio, fol-

Table 2. Effects of acute ethanol administration on lactate, pyruvate, β-hydroxybutyrate, and acetoacetate (μmoles/g liver) and on the L/P and B/A ratios in livers of male SH rats chronically fed with ethanol and their pair-fed controls*

Chronic treatment	Ethanol Metabolism	Acute ethanol administration (1.5 g/kg)	Lactate (L)	Pyruvate (P)	L + P	L/P	β-Hydroxybutyrate (B)	Acetoacetate (A)	B + A	B/A
Sucrose		−	522 ± 73 (8)	38 ± 2 (8)	560 ± 73 (8)	13.8 ± 1.7 (8)	1025 ± 116 (8)	328 ± 45 (8)	1353 ± 156 (8)	3.26 ± 0.22 (8)
			NS†	P < 0.02	NS	NS	P < 0.03	NS	NS	P < 0.001
	203 ± 6 (10)	+	371 ± 39 (10)	31 ± 2 (10)	402 ± 40 (10)	11.9 ± 1.1 (10)	1407 ± 111 (10)	257 ± 32 (10)	1664 ± 130 (10)	5.53 ± 0.48 (10)
	P < 0.001									
Ethanol		−	603 ± 368 (6)	42 ± 5 (6)	645 ± 372 (6)	12.3 ± 5.9 (6)	806 ± 137 (6)	246 ± 50 (6)	1052 ± 184 (6)	3.40 ± 0.27 (6)
			NS	NS	NS	P < 0.05	NS	NS	NS	P < 0.01
	406 ± 14 (9)	+	848 ± 176 (10)	36 ± 3 (10)	884 ± 177 (10)	23.4 ± 4.3 (10)	852 ± 73 (10)	143 ± 12 (10)	995 ± 75 (10)	6.35 ± 0.78 (10)

* Animals were fed liquid diets from 7 to 13 weeks of age, as described in Methods. At 13 weeks of age, animals were withdrawn from ethanol for 16 hr, and the above variables were measured. Values are means ± S.E.M. The numbers of animals in the groups are given in parentheses. Statistical significance was assessed by Student's *t*-test for unpaired data. The values of the ratios were logarithmically transformed.

† Not significant.

Table 3. Effect of chronic ethanol treatment on ethanol metabolic rate and on the activities of alcohol dehydrogenase and lactate dehydrogenase in livers of male rats of the Wistar and SH strains*

Strain	Chronic treatment	Ethanol metabolism ($mg \cdot kg^{-1} \cdot hr^{-1}$)	Alcohol dehydrogenase [$\mu moles \cdot (g\ liver)^{-1} \cdot min^{-1}$]	Lactate dehydrogenase [$\mu moles \cdot (g\ liver)^{-1} \cdot min^{-1}$]
Wistar	Sucrose (10)	290.01 ± 12.76	2.42 ± 0.26	205.5 ± 12.74
		P < 0.001	NS†	P < 0.01
	Ethanol (10)	365.01 ± 9.53	1.79 ± 0.23	151.6 ± 9.70
SH	Sucrose (8)	210.88 ± 6.82	1.03 ± 0.08	272.3 ± 10.72
		P < 0.001	P < 0.0001	NS
	Ethanol (8)	366.35 ± 8.84	1.58 ± 0.07	238.5 ± 15.38

* Animals were fed liquid diets from approximately 4 to 9 weeks of age, as described in Methods. At 9 weeks of age, animals were withdrawn from ethanol for 16 hr, and the variables were tested. Values are means ± S.E.M.; statistical significance was assessed by Student's *t*-test. The number of animals is given in parentheses.

† Not significant.

lowing ethanol administration to Wistar rats is in agreement with data obtained by other investigators [26–28]. Under the same conditions, naive male SH rats show no such increase. A *change* in the cytosolic compartment from a steady state with a lower NADH/NAD ratio to one with a higher NADH/NAD ratio in Wistar rats indicates that NADH can be generated at a rate faster than that at which it can be reoxidized to NAD. This suggests that the amount of alcohol dehydrogenase present in the cell can support a rate of ethanol metabolism higher than that at which the enzyme proceeds at the steady state and, thus, that ADH is not likely to constitute a major rate-determining step in ethanol metabolism in these animals. The fact that mitochondrial uncouplers and pyruvate increase the rate of ethanol metabolism both *in vitro* and *in vivo* further supports this idea [11–14, 29–31]. The lack of change in the L/P ratio in male SH rats suggests that ADH activity cannot exceed the capacity of the cell to reoxidize NADH, and, therefore, that in these animals ADH constitutes a primary rate-determining step. This agrees with data [1] presented previously by us showing that ADH activity measured *in vitro* correlates well with the rate of ethanol metabolism at various ages in male SH rats. In very young male SH rats in which ADH activity is high, there is a trend toward accumulation of cytosolic reducing equivalents following acute ethanol, but this disappears in older animals in which ADH activity is reduced by 50–60 per cent.

The rate of removal of hydrogen equivalents from the cytoplasm is largely mediated by the activity of substrate shuttles that transfer these equivalents from the cytoplasm to mitochondria where they are subsequently oxidized [13, 32–34]. During the oxidation of ethanol by the liver, however, a significant increase of hydrogen equivalents probably is produced in the mitochondria because acetaldehyde appears to be preferentially oxidized to acetate in these organelles [31, 35–37]. Thus, the "load" of reducing equivalents in the mitochondria is higher than that in the cytosol. It is not surprising, therefore, that the B/A ratio could increase more than the L/P ratio in the male SH rats. A similar observation can be made during protein malnutrition, when an increased B/A ratio can be seen without a change in L/P ratio [7].

In the older animals, the effect of acute ethanol administration on the mitochondrial redox state was more pronounced than in the younger animals despite the fact that ADH activity and ethanol metabolism are lower in the former [1]. This may be due in part, to the fact that oxygen uptake (and thus NADH reoxidation) by the liver in the older animals is lower than in younger ones. It is clear that the rate of translocation of reducing equivalents into the mitochondria plus the rate at which they are generated by acetaldehyde oxidation intramitochondrially should be balanced against the activity of the electron transport chain. The final redox state in the mitochondria represents a combination of these factors.

In line with our previous findings [1], chronic administration of ethanol to male SH rats led to an enhanced rate of ethanol metabolism concomitant with an increase in hepatic ADH activity. This is consistent with the hypothesis that in the naive male SH rats ADH constitutes the primary rate-determining step. This view is also supported by the finding that the L/P ratios did not increase following acute ethanol in naive or sucrose-control male SH rats. Moreover, in chronically ethanol-fed male SH rats in which ADH activity was enhanced, acute ethanol administration did induce a marked shift in the L/P ratio.

We were also unable to demonstrate a change in ADH activity in male Wistar rats chronically fed ethanol, despite a 26 per cent increase in ethanol metabolism. In fact, a reduction in ADH activity per g liver was observed, which could be attributed to ethanol-induced hepatomegaly of the order of 25 per cent; thus, total hepatic ADH activity remained constant. This finding agrees with our earlier work [38] and that of other investigators [39–41], and suggests that ethanol metabolism in this strain can increase without a concomitant increase in ADH. This observation, together with a rise in the hepatic L/P and B/A ratios after acute ethanol treatment in Wistar rats, is in accord with the postulate that, in Wistar rats, activity of ADH is not a major rate-determining step but rather that another factor, probably faster NADH reoxidation, is likely to play an important role [11, 12, 14, 29, 42]. This is supported by observations in strains other than SH rats of a lower redox state in chronically alcohol-fed animals given a challenge dose of alcohol [42–44].

We have previously reported marked sex differences with respect to the rate of ethanol metabolism and hepatic ADH activity in the SH rat strain [1]. In the females, unlike in male rats, both the rate of ethanol metabolism and the ADH activity remained elevated and essentially constant with age. We have now shown that in female SH rats acute ethanol administration induced a marked shift in the hepatic cytosolic redox state as indicated by an increase in the L/P ratio. Thus, the major factor determining the rate of ethanol metabolism in female SH rats appears to be the rate of NADH reoxidation and to be different from that in the males in which ADH activity constitutes the primary rate-determining step. Data presented in this paper indicate that redox changes in cell compartments can be a valuable tool with which to assess the reactions that determine the rate of ethanol metabolism under various experimental conditions, including chronic ethanol administration. Our studies also show that care should be exercised when extrapolating from one rat strain, or sex, to another, with regard to rate-determining steps in the metabolism of alcohol. It is conceivable that individual differences, of the type reported here, may also exist in humans, who are known to vary widely in their susceptibilities to alcohol-induced liver abnormalities. Furthermore, male and female SH rats constitute interesting models for investigating the role of redox changes in the actions of alcohol, in conditions in which the administration of inhibitors of alcohol dehydrogenase is not desirable.

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