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EFFECTS OF ETHANOL ADMINISTRATION ON RAT LIVER PLASMA MEMBRANE-BOUND ENZYMES

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Abstract—Changes in the properties of rat liver plasma membranes were examined in studies designed to differentiate between direct and metabolic effects of acute and chronic ethanol ingestion. One hour after a single dose of ethanol (3 g/kg body weight) there were increases in Na^+,K^+-ATPase (32%) and 5′-nucleotidase (36%), and hepatic concentrations of ethanol and acetaldehyde were approximately 23 mM and 50 μM, respectively. Na^+,K^+-ATPase and 5′-nucleotidase activities in liver plasma membranes from control rats were not significantly changed by *in vitro* addition of 30 μM acetaldehyde or 50 mM ethanol. Increases in Na^+,K^+-ATPase (~20%) and 5′-nucleotidase (~30%) were also observed in liver plasma membranes isolated from rats 16 hr after feeding ethanol or sucrose supplements for 17 days. The intake of calories from dietary protein and lipid was decreased by about 25% in both the ethanol and sucrose-fed animals. Na^+,K^+-ATPase activities in liver plasma membranes isolated from control rats were inhibited (~20%) by 100 mM ethanol *in vitro*, whereas no inhibition was observed using membrane preparations from rats fed ethanol or sucrose supplements. Our results show that changes in liver plasma membrane enzyme activities associated with a single dose of ethanol are not a direct effect correlated with blood, hepatic or plasma membrane concentrations of ethanol or acetaldehyde. Chronic ingestion of ethanol or sucrose supplements had similar effects on liver plasma membrane enzyme characteristics and parallel changes in nutrient intake may be a more feasible explanation of these results than any analogous direct effects of the two compounds.

Ingestion of ethanol, either acutely or chronically, causes considerable metabolic derangement as well as changes in the structure and function of hepatocellular organelles [1]. Typical of such changes are those affecting the ultrastructure of the hepatic plasma membrane [2] and its associated enzyme activities, such as Na^+,K^+-ATPase [3, 4]. Together with Mg^{2+}-ATPase, Na^+,K^+-ATPase is integral to the maintenance of transmembrane ion flux and solute transport. It has been suggested that changes in Na^+,K^+-ATPase activity are related to increased cellular water and liver cell size induced by chronic ethanol ingestion [5].

Depending on the duration of exposure to ethanol and the assay conditions used, ethanol may increase [3] or decrease [6] hepatic Na^+,K^+-ATPase activity. While a single dose of ethanol increases Na^+,K^+-ATPase activity in rat hepatic plasma membranes [3], it is not known whether this is a direct result of the presence of ethanol in the liver or isolated plasma membranes. It is also unclear whether the variable changes in Na^+,K^+-ATPase activity reported following chronic ethanol consumption [3, 4] are due to ethanol directly or result from metabolic changes following repeated ethanol ingestion.

In an attempt to differentiate between the direct and indirect effects of ethanol, we have determined: firstly, whether there was any relationship between blood, liver and plasma membrane ethanol concentrations and hepatic plasma membrane Na^+,K^+-ATPase activities following the acute administration of ethanol to rats; secondly, Na^+,K^+-ATPase activity and ethanol concentrations in hepatic plasma membranes isolated from rats fed ethanol chronically; and thirdly, Na^+,K^+-ATPase activity in plasma membranes isolated from rats fed a diet supplemented with sucrose, since this can produce a fatty liver similar to that seen following chronic ethanol ingestion [7]. To ascertain whether our results were specific to Na^+,K^+-ATPase, the activities of Mg^{2+}-ATPase and 5′-nucleotidase were also measured in the various plasma membrane preparations. Ethanol (100 mM) was also added to plasma membrane preparations so that the specificity of any proposed adaptive changes to chronic ethanol consumption [8] could be examined in rats fed the different diets.

MATERIALS AND METHODS

Animals, pretreatment regimens and tissue isolation. Male Wistar albino rats (Bantin & Kingman, Hull, U.K.) were the source of plasma membrane preparations for all experiments. Animals were allowed free access to Labsure C.R.M. diet (Rank–Hovis–McDougall, Poole, U.K.).

In the single dose studies, rats weighing about 200 g were used. Food was withdrawn 2 hr prior to administration by gavage of a 50% (v/v) aqueous solution of ethanol (3 g/kg body weight). Control animals received water alone (7.5 ml/kg). Fifty minutes later all animals were anaesthetized by intraperitoneal administration of sodium pentobarbitone (40 mg/kg) and 1 hr after the initial gavage the abdomen was opened. Blood samples were withdrawn from the portal vein (1 ml) and abdominal aorta

(1 ml) into syringes containing 1 ml 0.9% saline/100 mM chloral hydrate/10 mM 4-methylpyrazole/100 units heparin. Two small (1–2 g) pieces of liver were excised into preweighed screw-capped containers, one of which was immediately transferred to a dewar flask containing liquid nitrogen in which the liver was stored until required.

In the feeding studies, 3–4 week-old rats were allocated to one of three groups which were allowed free access to CRM diet and to either tap water, 10% (v/v) aqueous ethanol or an isocaloric sucrose solution (14.4% w/v). After one week, the ethanol concentration was increased to 15% (v/v) and the sucrose to 21.6% (w/v), and those conditions were maintained for the remaining ten days of the experiment in which food and water intake and animal weight were recorded daily. Sixteen hours before sacrifice the ethanol and sucrose supplemented drinking water was replaced with tap water. Rats were killed by cervical dislocation and plasma membrane fractions were isolated and enzyme activities determined as described below.

Preparation of liver plasma membrane fractions. Plasma membrane fractions were isolated from the unfrozen piece of liver which was weighed and immersed in ice-cold $CaCl_2$ (0.5 mM) containing $NaHCO_3$ (1 mM). Liver plasma membranes were prepared by the aqueous two-phase polymer method of Brunette and Till [9] as modified by Lesko *et al.* [10] and as described previously [6]. The washed isolated liver plasma membranes were stored at −70° in 50 mM Tris buffer (pH 7.5) containing EDTA (0.1 mM). No changes in enzyme activity occurred during storage under these conditions for one month, which was twice the interval during which activities were normally determined.

Enzyme assays. All enzyme activities were determined in whole homogenates and in isolated plasma membrane preparations. The activities of Na^+,K^+-ATPase and Mg^{2+}-ATPase were assayed using slight modifications of the method of Ismail-Beigi and Edelman [11]. The incubation mixture (1 ml) consisted of 25 mM Tris buffer (pH 7.4) containing 5 mM $MgCl_2$, 120 mM NaCl, 12.5 mM KCl and 100 μl of plasma membrane suspension containing 60 μg protein. The reaction was started by the addition of 5 mM ATP ("vanadium-free" sodium salt, Sigma Chemical Company, Poole, U.K.) and terminated after 5 min incubation at 37° with 15% (w/v) ice-cold trichloroacetic acid (0.5 ml). Mg^{2+}-ATPase activity was measured in the presence of 1 mM ouabain (Sigma Chemical Company, Poole, U.K.), a specific inhibitor of Na^+,K^+-ATPase. The difference between total and Mg^{2+}-ATPase represented Na^+,K^+-ATPase activity. 5′-Nucleotidase activity was determined in tissue preparations by the method of Michell and Hawthane [12]. The incubation mixture consisted of 50 mM Tris buffer (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 10 mM KNa tartrate and 45 μg liver plasma membrane protein in a final volume of 1 ml. The reaction was started by the addition of 0.2 mM AMP and continued for 30 min before stopping with 15% trichloroacetic acid (0.5 ml).

The inorganic phosphate released during incubations determining Na^+,K^+-ATPase, Mg^{2+}-ATPase and 5′-nucleotidase was measured by the method of Fiske and Subbarow [13] as modified by Chen *et al.* [14]. The activities of the mitochondrial marker enzyme succinate dehydrogenase [15] and microsomal marker glucose-6-phosphatase [16] were also determined. The protein content of isolated fractions was measured using minor modifications of the method of Lowry *et al.* [17] with a standard of bovine serum albumin.

Determination of ethanol and acetaldehyde in blood and tissue. Portions of frozen liver were weighed and homogenized in 2 vol. isotonic saline (0.9%) containing 100 mM chloral hydrate and 10 mM 4-methylpyrazole at 4° in a Teflon–glass Potter–Elvejhem homogenizer. Aliquots (1 ml) of the whole homogenate in a screw-capped glass vial were deproteinized by addition of 0.1 ml perchloric acid (60%) to which 1 ml propan-1-ol (0.01%) was added as internal standard. The tubes were then sealed and incubated at 60° for 30 min after which 1 ml of head space was injected into a 100 m × 0.7 mm capillary column coated with Carbowax 400 and mounted in a Pye 204 gas chromatograph. Injector temperature was 125°, column temperature 55° and nitrogen carrier gas was used at a flow rate of 20 ml/min. Ethanol and acetaldehyde were quantitated with a flame ionization detector at 125° by measuring their peak heights relative to that of the propan-1-ol internal standard and using calibration curves prepared from aqueous standards.

Blood samples, collected as described earlier, were centrifuged at 1200 *g* for 10 min. An aliquot (1 ml) of the supernatant was transferred to a screw-capped vial containing 1.1 ml perchloric acid/propan-1-ol as before and retained for assay of ethanol and acetaldehyde.

Statistical analyses. Results are expressed as mean values ± S.E.M. All values were considered significantly different when the probability of their chance occurrence was less than 5% as assessed by Student's *t*-test.

RESULTS

Enzyme activities and in vivo *ethanol and acetaldehyde concentrations after acute ethanol*

The specific activity (per mg protein) of Na^+,K^+-ATPase was significantly increased (132% of control) in liver plasma membranes isolated from rats fed 3 g ethanol/kg body weight 1 hr before death (Fig. 1). An increase in 5′-nucleotidase specific activity (136% control) was also observed, but there was no significant change in Mg^{2+}-ATPase activity (104% control). No ethanol or acetaldehyde could be detected in plasma membranes isolated from these rats.

At the time of these measurements the hepatic concentration of ethanol was 22.7 ± 2.4 mM (mean ± S.E., N = 6) and that of acetaldehyde 52.9 ± 3.0 μM. Hepatic ethanol concentrations were lower than those in portal venous blood (31.6 ± 2.3 mM; $P < 0.05$) or aortic blood (30.9 ± 2.9 mM; $P < 0.05$). In contrast, hepatic acetaldehyde concentrations were approximately sixfold higher than those in portal blood (8.2 ± 1.1 μM) or in aortic blood (10.1 ± 1.2 μM).

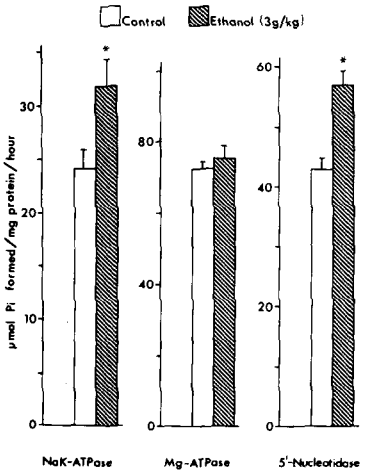

Fig. 1. Effect of a single dose of ethanol (3 g/kg) on liver plasma membrane-bound enzymes. Animals were pretreated and enzymes assayed as described in the text. Each value represents the mean ± S.E. of 3 (control) or 5 (ethanol) determinations. *Significantly different from control ($P < 0.05$, Student's *t*-test).

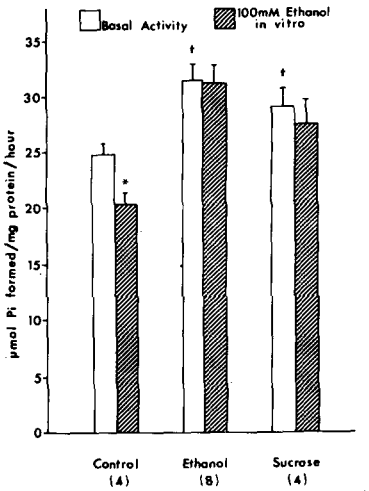

Fig. 3. Effect of ethanol *in vitro* on Na^+,K^+-ATPase from control and sucrose- or ethanol-fed rats. Animals were pretreated and Na^+,K^+-ATPase assayed as described in the text. Each value is the mean ± S.E. of the number of determinations shown in parentheses. *Effect of ethanol significant ($P < 0.05$, Student's *t*-test.) †Basal activities significantly changed by pretreatment ($P < 0.05$, Student's *t*-test).

Enzyme activities and liver histology in rats fed ethanol or sucrose supplements

Significant increases in the specific activity of Na^+,K^+-ATPase were observed in liver plasma membranes isolated from both ethanol-fed rats (123% of water-fed controls) and sucrose-fed animals (116% control) (Fig. 2). Comparable changes in 5'-nucleotidase activity were also observed in ethanol- or sucrose-fed animals (126% and 130% of control, respectively), but Mg^{2+}-ATPase was not significantly affected by ethanol or sucrose supplementation (Fig. 2). No ethanol or acetaldehyde could be detected in plasma membranes isolated from these rats. Additions of ethanol (100 mM) to liver plasma membranes isolated from control rats drinking water alone caused a significant inhibition of Na^+,K^+-ATPase activity (Fig. 3). When added to plasma membranes isolated from ethanol- or sucrose-fed rats the same concentration of ethanol had no significant effect. There was also no inhibition of Na^+,K^+-ATPase activity in plasma membrane preparations from control, ethanol- or sucrose-fed animals using 50 mM ethanol or 30–80 μM acetaldehyde.

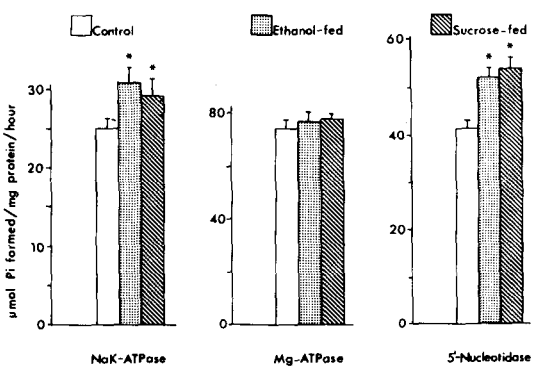

Fig. 2. Effect of dietary supplementation with ethanol or sucrose on liver plasma membrane-bound enzymes. Animals were pretreated and enzymes assayed as described in the text. Each value represents the mean ± S.E. of 4 (control, sucrose) or 8 (ethanol) determinations. *Significantly different from control ($P < 0.05$, Student's *t*-test).

Light microscopic examination of liver sections from rats fed ethanol revealed a moderate degree of fatty infiltration, particularly in the centrilobular zones. This steatosis was in the form of numerous fatty droplets which rarely coalesced into globules large enough to displace the cell nucleus. Hepatic architecture was preserved. In animals fed sucrose there was evidence of a less severe but qualitatively similar type of fatty infiltration in the hepatic parenchyma. In contrast, only occasional fat droplets were seen in the livers of rats drinking unsupplemented water.

Effects of ethanol and sucrose administration on plasma membrane properties, liver weight and nutrient intake

Acute ethanol administration had no effect on relative liver weight. Body weight increases and relative liver weights were not affected by dietary supplementation with ethanol or sucrose. Drinking water supplementation with ethanol (15%) gave a mean daily intake of 15.6 g/kg body weight. This high dosage to some extent reflects the low body weights of the young rats used.

The yield of plasma membrane proteins from rat liver homogenates and the enrichment of enzyme activities (ratio of activity in isolated plasma membranes to that in whole liver homogenate) were unchanged by any experimental pretreatment performed (Table 1). For Na^+,K^+-ATPase and 5'-nucleotidase, enrichment was >20-fold and for

Table 1. Effect of ethanol on plasma membrane protein content and enzyme enrichment

Regimen	Protein recovery (mg/g liver)	Relative enzyme activity				
		Na^+,K^+-ATPase	Mg^{2+}-ATPase	5'-Nucleotidase	Glucose 6-phosphatase	Succinate dehydrogenase
Single dose						
Control (N = 3)	1.73 ± 0.11	26.3 ± 4.0	9.1 ± 1.0	23.8 ± 1.5	0.57 ± 0.04	0.38 ± 0.08
Ethanol (N = 5)	1.88 ± 0.10	23.6 ± 2.2	9.4 ± 0.9	21.8 ± 2.1	0.56 ± 0.03	0.39 ± 0.05
Repeated doses						
Water (N = 4)	1.65 ± 0.10	23.3 ± 3.3	8.5 ± 1.6	25.2 ± 4.1	0.47 ± 0.01	0.35 ± 0.14
Ethanol (N = 8)	1.80 ± 0.13	20.3 ± 1.7	9.6 ± 0.6	24.1 ± 2.9	0.54 ± 0.10	0.35 ± 0.04
Sucrose (N = 4)	1.70 ± 0.10	24.1 ± 3.8	8.6 ± 0.8	23.4 ± 2.4	0.50 ± 0.01	0.39 ± 0.05

Values represent the mean ± S.E. of N determinations shown in parentheses. Relative enzyme activity refers to the ratio of the enzyme activity per mg plasma membrane protein to that per mg whole homogenate protein. There are no significant differences between any result obtained in measurements of the same parameter as assessed by Student's *t*-test.

Mg^{2+}-ATPase about 9-fold. In contrast, values of enrichment <0.1 were obtained for the mitochondrial and microsomal membrane marker enzymes succinate dehydrogenase and glucose 6-phosphatase, respectively. This indicates a minimal contamination of the isolated plasma membranes by other membrane fractions which was unchanged by pretreatment of the rats (Table 1).

Calorific intakes from carbohydrate, protein and lipid are shown in Table 2 for rats fed the control diet or sucrose or ethanol supplements.

DISCUSSION

Following an acute dose of ethanol, liver plasma membrane Na^+,K^+-ATPase and 5'-nucleotidase activities were increased, but Mg^{2+}-ATPase activity was unchanged. Ricci and his colleagues also found that acute ethanol administration increased hepatic plasma membrane Na^+,K^+-ATPase activity and suggested that this might be due to direct effects of ethanol on the liver surface membrane [3]. However, the increases in enzyme activities we observed were unlikely to have arisen directly from enzyme activation due to the presence of ethanol or acetaldehyde for several reasons. Firstly, no free ethanol or acetaldehyde was detected in plasma membranes isolated from rats given ethanol acutely. Secondly, the concentrations of ethanol (~25 mM) or acetaldehyde (~50 μM) present in the liver at the time of death did not affect liver plasma membrane Na^+,K^+-ATPase or 5'-nucleotidase *in vitro*. Thirdly, where ethanol did exert *in vitro* effects, these were only achieved at high (≥100 mM) concentrations, were inhibitory, were dependent on the presence of ethanol and were reversed upon its removal [18]. Finally, there was no correlation between Na^+,K^+-ATPase activity and blood or hepatic concentrations of either ethanol or acetaldehyde. Secondary metabolic consequences of ethanol ingestion have therefore to be considered. The possibilities here include ethanol-induced changes in intermediary metabolism, membrane composition and configuration and the release of endogenous compounds, all of which may affect plasma membrane function and enzymatic activities [8, 19, 20].

Liver plasma membrane Na^+,K^+-ATPase and 5'-nucleotidase activities were also increased by chronic ethanol feeding and again there was no effect on Mg^{2+}-ATPase. These results are in agreement with those of Bernstein *et al.* [4] who assayed Na^+,K^+-ATPase in whole liver homogenates, but contrast with the findings of Ricci *et al.* [3] who reported no increase in liver plasma membrane Na^+,K^+-ATPase activity. The explanation for these different findings may be in the different ethanol intakes achieved. Thus, Ricci *et al.* [3] gave 6 g ethanol/kg body weight for 5 days and the histological appearance of the livers was normal. In both the present study and that of Bernstein *et al.* [4] a higher daily dose of ethanol was ingested (~15 g/kg) and histological examination of the liver showed fatty infiltration.

Increases in Na^+,K^+-ATPase activity after chronic ethanol ingestion (like those following sucrose supplementation or acute ethanol administration) were the result of an increased specific activity (per mg

Table 2. Energy intake in rats fed solid food and drinking water supplemented with ethanol or sucrose

Water supplement	Dietary intake				
	Total (kcal./kg body wt.)	Percentage of total from:			
		Carbohydrate	Protein	Lipid	Ethanol
None (N = 4)	382	70.8	22.5	6.7	0
Sucrose (N = 4)	472	78.9	16.2	4.9	0
Ethanol (N = 8)	417	53.6	17.0	5.1	24.3

Results are mean daily values calculated for the final week of pretreatment from the dietary analysis provided by the manufacturer and calorific values of 4, 4, 9 and 7 kcal./g for carbohydrate, protein, lipid and ethanol, respectively.

membrane protein) of the enzyme since no significant changes in the yield of plasma membrane protein nor in the recovery of any plasma membrane marker enzyme activity occurred. The increase in activity which followed chronic ethanol was detected despite withdrawal of ethanol 16 hr before death, and therefore was unlikely to arise from the presence of ethanol or acetaldehyde, nor any acute metabolic effect of either compound, e.g. a change in $NAD^+/NADH$ ratios. Consequently, an alteration in the structure or composition of the plasma membrane with attendant changes in the activities of its associated enzymes seems likely. Such changes in membrane properties could explain parallel increases in both Na^+,K^+-ATPase and 5′-nucleotidase activities. These are not consistent with the lack of effect of chronic ethanol ingestion on Mg^{2+}-ATPase since this enzyme activity has been shown to be resistant to the effects of other membrane modifiers [21–23], possibly as a result of its location in the membrane.

Increases in plasma membrane Na^+,K^+-ATPase and 5′-nucleotidase activities followed dietary supplementation with sucrose as well as ethanol. Na^+,K^+-ATPase activities in plasma membranes from rats fed either diet were also resistant to inhibition by 100 mM ethanol *in vitro*. These results suggest a similar adaptive response of the membrane [8] to both dietary supplements. Because it is unlikely that chronic ethanol or sucrose ingestion has the same direct effects, analogous changes in membrane structure may be associated with consumption of both diets. It has already been demonstrated that sucrose or ethanol feeding has similar effects on membrane fluidity [19] and hepatic steatosis has been observed after feeding ethanol or sucrose supplements [7]. This morphological change has been suggested to relate to nutrient imbalance associated with both the sucrose and ethanol diets [7]. In the present study, approximately 25% decreases in the intake of calories derived from both protein and lipid followed dietary supplementation with both ethanol and sucrose. It is possible, therefore, that the similar changes in plasma membrane properties which occurred after feeding the ethanol or sucrose diets were due to the comparable decrease in calorific intake from dietary protein and lipid associated with chronic ingestion of ethanol or dietary supplementation with sucrose.

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