Rapid Publication

Decrease in Microviscosity and Cholesterol Content of Rat Liver Plasma Membranes After Chronic Ethanol Feeding

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Abstract. This investigation was performed to determine whether chronic ethanol feeding alters the lipid composition or the fluidity of liver plasma membranes. Male Sprague-Dawley rats were pair-fed nutritionally adequate liquid diets containing ethanol as 36% of energy or an isocaloric amount of carbohydrate for 4–5 wk. Contrasting with other membranes, chronic ethanol feeding resulted in an increase in hepatic plasma membrane fluidity as assessed by fluorescence anisotropy. This alteration was associated with a decrease in plasma membrane cholesterol content.

Introduction

Recent studies have demonstrated that the cell surface plays an important role in intracellular homeostasis through activities of membrane enzymes, hormone receptors, and transmembrane transport processes (1). Many of these dynamic features are determined, in part, by the fluidity of the lipid bilayer because of its effect on the mobility and/or exposure of membrane proteins (1).

There have been several reports concerning the effect of ethanol on the fluidity of membranes. A currently held view is that in vitro ethanol renders membranes more fluid and that chronic ethanol administration alters membrane lipid composition, which results in adaptation to the acute fluidizing effect. Such an adaptation has been reported to occur in brain synaptosomes (2) and erythrocytes (3), as well as in liver mitochondria and microsomes (4, 5). However, to our knowledge, there are no reports of the effect of chronic ethanol consumption on liver plasma membranes (PM).[1] Since ethanol may exert part of its hepatotoxic effect via liver PM alteration, we carried out the present study to investigate the properties of liver PM isolated from rats chronically fed an ethanol-containing diet.

Methods

Animals. Weanling male Crl:CD^R(SD)BR Sprague-Dawley rat littermates (Charles River Breeding Laboratories, Wilmington, MA) were fed chow diet until they reached a weight of 130–150 g. The rats were then housed in individual cages and pair-fed for 4–5 wk a nutritionally adequate liquid diet containing carbohydrates as 47% of total energy or an isocaloric diet with ethanol being substituted for carbohydrate as 36% of energy as previously described (6). To equalize the rate of diet consumption preceding PM isolation, one-third of the daily ration was administered at 9 a.m. and two-thirds at 5 p.m. on the day before killing. In addition, one-sixth of the daily diet was administered 4 h before the procedure.

PM isolation. The rats were killed by decapitation and their livers were immediately perfused with cold 0.9% saline. PM were prepared by the method of Hubbard et al. (7) with two modifications as follows: (*a*) The nuclear fraction was mixed with a solution that contained 76.0% (wt/vol) sucrose, 5 mM Tris-HCl (pH 7.4–7.5), and 0.5 mM $MgCl_2$ to a density of 1.20–1.21 g/cm^3. This was overlaid with 10–15 ml of sucrose-Tris buffer (d = 1.18) and then with 0.25 M sucrose-Tris buffer as described by Hubbard et al. (7). (*b*) After the crude PM fraction was collected from the interface of the discontinuous sucrose gradient, it was washed twice using the same centrifugation conditions as Hubbard et al. (7).

Biochemical determinations. 5′-nucleotidase (5′-ND) activity was measured at 30°C by the method of Arkesteijn (8) as a marker for canalicular PM. Results were expressed as units (U), each U representing 1 μmol of NADH used per minute. Gamma glutamyltranspeptidase

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1. *Abbreviations used in this paper:* DPH, 1,6-diphenyl-1,3,5-hexatriene; G6Pase, glucose-6-phosphate; GGTP, gamma glutamyltranspeptidase; 5′-ND, 5′-nucleotidase; PM, plasma membrane(s); NaK-ATPase, sodium, potassium-activated adenosine triphosphatase; U, unit(s).

(GGTP) activity was measured, as an additional marker for canalicular PM, by the method of Szasz (9) using L-glutamyl *p*-nitroanilide as substrate. The samples were preincubated with 1% deoxycholate before assay according to the method of Morland et al. (10). Results were expressed as U, 1 U representing 1 μmol of substrate used per minute at 30°C. Sodium, potassium-activated adenosine triphosphatase (Na,K-ATPase) activity was measured by the method of Ismail-Beigi and Edelman (11) with the modification of Scharschmidt et al. (12) as a marker for blood-sinusoidal PM. The final reaction mixture contained 125 mM Tris buffer (pH 7.4), 1 mM EGTA, 120 mM NaCl, 12.5 mM KCl, 5 mM NaN_3, 5 mM $MgCl_2$, and 5 mM ATP with or without 1 mM ouabain. Glucose-6-phosphatase (G6Pase) activity was measured by the method of Harper (13) as a marker for endoplasmic reticulum. Succinate dehydrogenase activity was measured by the method of King (14) as a marker for mitochondria. Protein concentrations were measured according to the method of Lowry et al. (15) using crystalline bovine serum albumin as the standard.

Fluorescence polarization studies. Fluorescence polarization was measured by the method of Cheng and Levy (16) using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe. A solution of DPH (2 mM) in tetrahydrofuran was diluted 1,000-fold by injection into a vigorously stirred solution of phosphate-buffered saline. Samples were diluted to a protein concentration of 200 μg/ml with 50 mM Tris–HCl (pH 7.5) and mixed with an equal volume of the DPH dispersion and incubated for 30 min at 37°C. Steady state fluorescence polarization measurements were made with a polarization spectrophotometer (model 4800, SLM Instruments, Champaign, IL). Temperature in the cuvettes was controlled with a thermostated circulating water pump. Excitation wavelength was 365 nm and emission was measured at 426 nm. The polarization of fluorescence was expressed as the anisotropy parameter (r): $r = 2P/(3 - P)$, where P is the degree of fluorescence polarization. $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallel and perpendicular, respectively, to the direction of polarization of the excitation beam. Measurements were made in both the presence and absence of 100 mM ethanol. The light scattering of unlabeled PM suspensions was negligible in these experiments.

Lipid analyses. Plasma membrane and homogenate lipids were extracted by the procedure of Bligh and Dyer (17). Phospholipid phosphorus was assayed according to the method of Bartlett (18). Cholesterol was determined by the method of Rudel and Morris (19) with the modification of Emerole and Thabrew (20). Phospholipid was separated by the method of Littleton and John (21) using a slurry of activated silicic acid. The phospholipids were eluted from silicic acid by repeated washing with chloroform/methanol/water (1:2:0.2; vol/vol/vol). Methyl esters of phospholipid fatty acids were prepared by the method of Beare-Rogers (22) as reported previously (23). Fatty acids were analyzed by a Hewlett Packard 5840A gas chromatograph with a 6′ × ⅛″ glass column packed with 10% SP-2330 cyanosilicone on 100/120 chromosorb. The temperature of the injector, column and flame ionization detector were 250°, 200° and 250°C, respectively; the carrier gas was nitrogen which was maintained at a flow rate of 30 ml/min. Peaks were identified by comparison with standards of methyl esters of fatty acids. The content of each fatty acid was expressed as weight percent. Each peak area was adjusted with a area response correction factor obtained by analysis of mixtures of fatty acid methyl ester standards.

Materials. All chemicals were of analytical grade. Reagents for the enzyme assays and DPH were purchased from Sigma Chemical Company, St. Louis, MO. Tetrahydrofuran was purchased from Aldrich Chemical Co., Milwaukee, WI. Standards of fatty acid methyl esters were obtained from Supelco Inc., Bellefonte, PA.

Statistics. Results were expressed as mean±SEM. Statistical comparisons were made using paired t test (24).

Results

Protein recovery, enrichment of liver PM marker enzymes, and membrane purity. Membrane protein recovery was not influenced by the ethanol diet (Table I). Also, the relative specific activities (PM/homogenate activities) of PM marker enzymes, which are measures of the enrichment of the enzymes in the membrane preparations, were similar in ethanol-fed and control groups (Table I). Microsomal (as assessed by G6Pase relative specific activity) and mitochondrial (assessed using succinate dehydrogenase relative specific activity) contamination did not differ between the two groups.

Effect of chronic ethanol feeding on the microviscosity of liver PM. Chronic ethanol feeding in rats resulted in a significant decrease in the microviscosity of liver PM as assessed with the DPH anisotropy value (Table II). In vitro addition of ethanol (100 mM) did not alter the anisotropy value.

Effect of chronic ethanol feeding on the membrane lipid composition. When compared with control values, hepatic PM from ethanol-fed animals exhibited significant decreases in cholesterol content and the cholesterol/phospholipid molar ratio (Table II). However, cholesterol content of liver homogenate was increased twofold after chronic ethanol feeding (33.3±0.66 μg/mg protein for ethanol-fed animals vs. 16.2±1.65 for controls; $n = 5$; $P < 0.001$) as previously reported (25). Chronic ethanol feeding did not affect the phospholipid content (Table II) nor the degree of saturation of phospholipid fatty acids as assessed by the percentage of saturated fatty acids or the double-bond index (Table III). However, the ratio of 18:1/16:0 was increased due to both an increase in 18:1 content and a decrease in 16:0 content (Table III).

Table I. Protein Recovery, Relative Specific Activity of PM Marker Enzymes and Contaminants in the Liver PM Fractions from Ethanol-fed Rats and Controls

	Control	Ethanol-fed
Protein recovery		
(*mg protein/g liver*)	1.94±0.24*	1.68±0.15
Relative specific activity‡		
5′-ND	12.57±0.65	10.11±0.75
GGTP	5.01±1.22	5.60±1.24
Na,K-ATPase	16.73±1.34	15.96±0.98
SDH	0.16±0.03	0.22±0.07
G6Pase	0.81±0.08	0.61±0.11

* Results are presented as mean±SEM of eight animals.
‡ Relative specific activity for each enzyme was calculated by dividing its specific activity in plasma membrane by its specific activity in homogenate.

Table II. Effect of Chronic Ethanol Feeding on the Microviscosity, Cholesterol Content and Phospholipid Content of Rat Liver PM

	Control	Ethanol-fed	Significance
DPH anisotropy*	0.1871±0.0053‡	0.1558±0.0065	$P < 0.002$
Cholesterol (*μg/mg protein*)	109.4±5.3	88.0±2.9	$P < 0.005$
Phospholipid (*μg/mg protein*)	289.0±11.3	266.5±5.0	NS§
Molar ratio of cholesterol/phospholipid	0.700±0.013	0.612±0.020	$P < 0.005$

* DPH anisotropy was calculated as described under Methods as a parameter of membrane microviscosity.
‡ Results are expressed as mean±SEM of eight observations.
§ NS, not significant.

Discussion

Although many investigations have been concerned with ethanol-induced changes in hepatic subcellular metabolism, only recently have studies concentrated on the effects of ethanol on liver plasma membrane structure and function (26–31). The present investigation has revealed that chronic ethanol feeding results in increased fluidity of liver PM associated with a decrease in the cholesterol content and in the cholesterol/phospholipid molar ratio. Since this ratio is a major determinant of membrane fluidity (32), it is likely that the decrease observed is responsible for the increased fluidity. The degree of saturation of phospholipid fatty acyl side chains (the other major determinant of fluidity [32]) was not affected by chronic ethanol feeding as assessed by percent content of fatty acids and the double-bond index. However, chronic ethanol feeding did result in an increase in the fatty acid ratio 18:1/16:0 due to both an increase in 18:1 and a decrease in 16:0. Similar fatty acid changes have been reported in liver mitochondria and microsomes of ethanol-fed animals (33) and in *Escherichia coli* incubated with ethanol (34) and have been attributed to the redox change induced by ethanol feeding (33).

Table III. Effect of Chronic Ethanol Feeding on Phospholipid Fatty Acid Composition of Rat Liver PM

	Control	Ethanol-fed	Significance
Fatty acid (*% of weight*)			
16:0	22.7±1.0*	17.4±0.7	$P < 0.005$
16:1	0.9±0.1	0.8±0.1	NS‡
18:0	29.6±1.3	32.9±1.4	NS
18:1	9.1±0.7	11.7±0.5	$P < 0.01$
18:2	6.6±0.2	6.6±0.8	NS
20:4§	30.9±1.3	30.6±1.2	NS
Saturated fatty acids (*% of total*)	52.3±1.4	50.3±1.4	NS
Double-bond index‖	1.48±0.05	1.48±0.04	NS
Ratio of 18:1/16:0	0.40±0.04	0.68±0.05	$P < 0.001$

* Results are expressed as mean±SEM of seven animals.
‡ NS, not significant.
§ Minor fatty acids or fatty acids with retention times greater than that of 20:4 have been omitted because these measurements were less reliable due to limited amounts of membrane available for analysis.
‖ Double-bond index is the sum of the fractional content of each fatty acid times the number of double-bonds in that acid.

In vitro, ethanol fluidizes membranes (2–5, 33, 35, 36). From studies of red blood cells (37) and synaptosomal membranes (37, 38), it has been theorized that membrane alterations (increased cholesterol content and/or increased fatty acid saturation) after chronic ethanol consumption reflect a "homeoviscous adaptive response" to offset this acute fluidizing effect. More recently, it has even been postulated that this may represent a universal response of membranes to chronic ethanol consumption (39). The present study revealed that this is not the case for liver plasma membranes.

As indicated by Schacter (32) in his review, there are two possible sources of error which could differentially affect hepatic PM anisotropy values in ethanol-fed and control animals: a different degree of contamination by intracellular organelles, and differences in the relative enrichment of the different PM domains (as assessed by DPH anisotropy values, the canalicular domain has been reported to be 5–25% more rigid than the others [40–42]). The former possibility is unlikely, since the degree of mitochondrial and microsomal contamination (as assessed by marker enzymes) was similar in both groups of animals. In addition, chronic ethanol feeding has been shown not to increase the fluidity of hepatic mitochondria and microsomes (4, 5). Furthermore, the results in Table I suggest that the canalicular (with 5'-ND and GGTP as markers) and sinusoidal (with Na,K-ATPase as a marker) subfractions were equally enriched. Although it is unclear why the fluidity increase is observed only in PM and not in mitochondria or microsomes (4, 5), it is noteworthy that Storch and Schacter (43) have reported a similar discrepancy between PM and microsomes after a starve-refeeding regimen.

It should be pointed out that contamination of PM by cytosol could not account for the changes in membrane cholesterol content, as chronic ethanol feeding was associated with a decrease in PM cholesterol and a simultaneous increase in homogenate cholesterol content. It is also unlikely that circulating ethanol was responsible for the decreased anisotropy values that were observed. In vitro ethanol (100 mM) did not alter the DPH anisotropy of the PM. In addition, the repeated washings of the isolation procedure most probably removed any ethanol present in the tissue.

At this time, the mechanism whereby chronic ethanol administration reduces PM cholesterol is not known, but there are a number of possible explanations. Such an alteration may reflect increased esterification of cholesterol by hepatic microsomes, since the content of cholesteryl esters in the liver is increased by ethanol feeding (25). Alternatively, this membrane

alteration may result from increased biliary excretion of cholesterol as bile acids (44, 45). Such mechanisms would be consistent with the discrepancy between the effect of ethanol feeding on liver PM (decreased cholesterol content) and its effects on other membranes such as red blood cell ghosts (increased cholesterol content) (37). Whatever its mechanism, the reduced PM cholesterol may, in part, facilitate the increased hepatic uptake of HDL after chronic ethanol feeding (46).

In summary, this study has revealed that chronic ethanol feeding in rats does not appear to result in a "homeoviscous adaptation" of liver PM as it does in other membranes. Rather, an increase in fluidity and a decrease in PM cholesterol content has been observed. Such changes may relate to the specialized role of the liver in bile excretion, lipoprotein metabolism, and ethanol oxidation. The functional significance of these changes and their role in the pathogenesis of alcohol-induced liver injury remain to be determined.

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