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INCREASED CHOLESTEROL CONTENT OF ERYTHROCYTE AND BRAIN MEMBRANES IN ETHANOL-TOLERANT MICE

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Summary

Mice were treated with ethanol for eight or nine days, using a liquid diet regimen known to produce physical dependence. In previous experiments, synaptosomal plasma membranes and erythrocyte ghosts from such ethanol-treated animals were found to be resistant to the fluidizing effects of ethanol in vitro, as measured by electron paramagnetic resonance. In the present experiments, corresponding membranes were analysed for phospholipid and cholesterol. The ratio of cholesterol to phospholipid was found to be significantly increased in both types of membrane after chronic ethanol treatment. The changed ratio was produced by an increase in cholesterol. There was little or no change in phospholipid content of the membranes. Increased cholesterol may explain the previously observed alteration of physical properties of the membranes.

Introduction

Drug tolerance and physical dependence have many characteristics suggesting that the process is basically one of adaptation to the drug. It was originally assumed that a specific protein (enzyme or receptor) would be the site of the adaptation. However, drugs such as ethanol appear to act without binding to a specific receptor. They may cause their behavioral effects simply by dissolving in cell membranes and disordering the structure of the bilayer. Recently, Hill and Bangham [1] suggested that the adapting mechanism for such drugs may be the lipid matrix of the cell membranes, rather than a protein. They pointed out that bacteria and some poikilotherms can adapt their lipids to changes in environmental temperature, and proposed that mammals might adjust their membrane lipids to offset the disordering effect of drugs such as the general anesthetics. Acting on this suggestion, we showed that ethanol did indeed fluidize normal mouse biomembranes [2] and that the ethanol

effect was much less pronounced in membranes taken from animals that had been chronically treated with ethanol [3]. We used electron paramagnetic resonance with a 5-doxyl stearic acid probe and calculated the polarity-corrected order parameter. This new kind of tolerance to ethanol was seen in red blood cell membranes and synaptosomal plasma membranes. The drug effects were small but were statistically significant and concentration related.

We have now begun to examine the chemical composition of the tolerant and control biomembranes. As shown in this paper, membranes taken from ethanol-treated animals and an increased content of cholesterol. Since cholesterol tends to increase order in membranes, such a change would offset the action of ethanol, and this may represent the postulated adaptation.

Methods

Chronic administration of ethanol. Individually housed male DBA/2J mice (Jackson Laboratories, Bar Harbor) were fed a liquid diet consisting of chocolate flavored Slender (Carnation Co.) with either ethanol or sucrose added to the extent of 33% of the total calories. The mice that received the sucrose diet were pair-fed with their ethanol-treated partners and were started on the diet two days after the ethanol group. The body weights and fluid intake of all mice were recorded daily. The data were analysed according to the concept of "effective alcohol intake" [4], i.e., those mice that had maintained their intake continuously above 10 g/kg per day were considered to be the most strongly dependent. Previous work has shown that interruption of ethanol intake (reduction below 10 g/kg per day) greatly lessens the severity of withdrawal reactions in mice. After 8 days, the six ethanol-treated mice with the highest effective intake and their sucrose partners were killed. The next day, a few more mice had reached effective intakes high enough to produce clear withdrawal reactions in this model, and these mice were also used, along with their controls. Two such experiments were run, each starting with 13 pairs of mice. In all, nineteen mice had adequate patterns of ethanol intake and these mice were used for lipid analysis, along with their partners from the sucrose group.

Erythrocyte membranes. On the day of killing, at 8 : 30—10 am, duplicate 10-μl samples of tail blood were taken for enzymatic assay of blood ethanol concentration [5]. A larger sample of blood for lipid determinations was taken from the retro-orbital sinus in a heparinized capillary tube. (To facilitate collection of blood, 50 μl heparin, 1000 units/ml, was injected intravenously.) Erythrocytes were washed three times with normal saline and a 5-μl sample of packed cells was taken for cell count (Coulter Model ZBI). The lipids were extracted with isopropanol and chloroform by the procedure of Rose and Oklander [6]. Cholesterol was assayed by the method of Rudel and Morris [7] and total phospholipid according to Morrison [8]. The value used for each mouse was the mean of duplicate assays of a single extract.

Synaptosomal plasma membranes. Immediately after blood sampling, the mice were killed by cervical dislocation. The brains were homogenized in 9 vols. of ice-cold sucrose (10%, w/w). Synaptosomal plasma membranes were isolated by the flotation-sedimentation technique of Jones and Matus [9]. Briefly, the crude mitochondrial pellet was lysed in hypotonic buffer and

sucrose was added to a concentration of 34%, w/w. A discontinuous sucrose gradient was formed by layering 28.5% and then 10% sucrose over the sample. After centrifuging at 60 000 X *g* for 2 h, the material at the lower interface, enriched in synaptosomal plasma membranes, was washed four times with glass distilled water. The protein content was determined [10], and the fractions were stored frozen under nitrogen. Suspensions of the membranes were later extracted with 0.5 ml methanol per mg protein, sonicated for 30 s and centrifuged at 18 000 X *g* for 20 min. The supernatant was set aside, and the solid residue was again extracted twice with 1 ml of chloroform/methanol (1 : 1, v/v) per mg protein. The three extracts were combined, brought to a final chloroform/methanol ratio of 2 : 1 (v/v) and washed with 0.2 vol. 0.88% KCl [11]. To cleave proteins from proteolipids [12], the lower phase was again washed with one sixth vol. 0.1 M potassium citrate "upper phase" (3 : 48 : 47 ratio of chloroform/methanol/citrate) [11]. The lower layer was evaporated to dryness under nitrogen and then dissolved in chloroform/methanol (2 : 1). Cholesterol and phospholipid were determined on this fraction as described above.

Data were analysed by Student's *t*-test for paired samples.

Results

Ethanol administration. The ethanol-treated animals selected for analysis took an average of 13 g/kg ethanol per day during the 8 or 9 day treatment period in each experiment and had an effective intake (see Methods) over 10 g/kg. Their mean effective intake was 28 g/kg, i.e., they drank this cumulative amount in excess of 10 g/kg per day without interruption, during the few days prior to withdrawal. The blood ethanol concentration at the time of killing ranged from 1.4 to 4.8 mg/ml, with a mean of 3.2 ± 0.20 (S.E., $n = 19$). Ethanol intake, body weight and blood ethanol concentrations were similar in the two experiments. In each experiment, both groups of mice (the alcohol-fed and the sucrose-fed groups) lost body weight to the same extent, about 28%.

Erythrocytes. In the first of the two replicate experiments, we measured the cholesterol and phospholipid content of the isolated erythrocyte membranes. The molar ratio of cholesterol to phospholipid was higher in membranes from ethanol-treated than from control animals. It was 0.778 ± 0.0287 (S.E.) in the 8 control mice and 0.936 ± 0.0252 in 8 ethanol-treated mice. In the second replication, we determined the cell counts and packed cell volumes of each blood sample so that we could estimate changes in cholesterol and phospholipid separately, in addition to calculating their ratio. Those data are shown in Table I. Again, the cholesterol/phospholipid ratio was increased significantly in the ethanol group. When the results were calculated per cell or per μl of packed cells, it was evident that the phospholipid was unchanged, but the cholesterol was increased by about 15%, a small but highly significant increment.

Synaptosomal plasma membranes. In the brain, as in the erythrocytes, there was an increased cholesterol/phospholipid ratio in the ethanol group (Table II). The increase was less than in erythrocytes but was significant in each experiment and highly significant ($P < 0.01$) for the two experiments combined. The amount of cholesterol per unit of membrane protein was increased by about

TABLE I

CHOLESTEROL AND PHOSPHOLIPID IN ERYTHROCYTE MEMBRANES OF ETHANOL-TREATED AND CONTROL MICE

Mice were given ethanol in a liquid diet for 8 and 9 days; controls were pair-fed an isocaloric sucrose diet. Red blood cell lipids were extracted and analysed as described under Methods. Data are mean and S.E. for 7 pairs of mice in Experiment 2. Values of P refer to comparisons of ethanol and sucrose groups by Student's t-tests for paired samples.

	Control	Ethanol
Cholesterol		
nmol/μl packed cells	3.67 ± 0.099	4.27 ± 0.122 **
pmol per million cells	205 ± 4.9	232 ± 3.7 *
Phospholipid		
nmol/μl packed cells	4.76 ± 0.183	4.90 ± 0.210
pmol per million cells	265 ± 8.7	266 ± 4.4
Cholesterol/phospholipid		
Molar ratio	0.775 ± 0.0296	0.876 ± 0.0247 *

* $P < 0.01$.
** $P < 0.001$.

TABLE II

CHOLESTEROL AND PHOSPHOLIPID IN SYNAPTOSOMAL PLASMA MEMBRANES OF ETHANOL-TREATED AND CONTROL MICE

Synaptosomal plasma membranes were prepared and analysed as described under Methods. Data are mean and S.E. for 11 pairs of mice in Expt. 1 and 7 pairs in Expt. 2. Values of P refer to comparisons of ethanol and sucrose groups by paired Student's t-tests.

	Control	Ethanol
Cholesterol (nmol/mg protein)		
Expt. 1	412 ± 16.4	463 ± 10.9 *
Expt. 2	497 ± 18.4	541 ± 10.4 *
Phospholipid (nmol/mg protein)		
Expt. 1	591 ± 24.7	641 ± 16.1
Expt. 2	693 ± 20.2	710 ± 14.4
Cholesterol/phospholipid molar ratio		
Expt. 1	0.699 ± 0.0052	0.723 ± 0.0084 *
Expt. 2	0.717 ± 0.0128	0.762 ± 0.0077 *

* $P < 0.05$.

10% in the ethanol group, whereas the total phospholipid per mg of protein was not significantly changed.

Discussion

The amounts and temporal patterns of ethanol intake during the ethanol treatment period were sufficient to produce a moderate degree of physical dependence [4]. These experiments reproduced the ethanol treatment earlier shown to result in "tolerant" membranes, that is, membranes that were resistant to the in vitro fluidizing effect of ethanol [3]. The increased membrane content of cholesterol reported here may explain the changed physical state of the membranes. In the earlier experiments we saw no difference in the order

parameter between ethanol-treated and control animals unless ethanol was added in vitro. Thus, the increased cholesterol was insufficient to produce a detectable change in membrane fluidity.

The effects of cholesterol on lipid bilayers are complex [13], so it is not entirely clear whether an increase in cholesterol content would be an appropriate adaptive response to a membrane-fluidizing agent such as ethanol. In most membrane systems, when they are above their phase transition temperature, cholesterol increases the degree of order. This has been shown in phospholipid model membranes [14,15] and biomembranes [15,16], as measured by electron paramagnetic resonance, fluorescent probes or Arrhenius plots. In vivo, addition of cholesterol to the diet of guinea pigs was sufficient to cause a substantial change in the electron paramagnetic resonance spectrum of the red cell membranes [17]. Since mammalian membranes exist above their main phase transition temperatures, cholesterol would be expected to have an ordering effect on the whole. This is opposite to the action of ethanol and would tend to offset the ethanol effect. Furthermore, cholesterol can decrease the partition of lipid-soluble substances into membranes [18,19], so it is possible that our electron paramagnetic resonance experiments are explainable as a decreased entry of ethanol into the cholesterol-enriched membranes. This would be consistent with the absence of any observed change in the order parameter unless ethanol was added in vitro. It does seem reasonable to suppose that the change in lipid ratio is related to the altered physical response of the membranes to ethanol. The recently reported increase in saturation of membrane fatty acids in ethanol-treated mice [20] may also contribute to this effect.

The increased cholesterol content of the erythrocytes may be a passive response to the rise in serum cholesterol that often occurs after chronic treatment with ethanol in animals [21] and humans [21,22]. In one long term experiment [23], where ethanol was administered to monkeys for 3 months, both cholesterol and phospholipid of red blood cells increased. In those experiments, unlike ours, the cholesterol/phospholipid ratio decreased. Our results differ from those of Rawat [24], who found no change in whole brain cholesterol after chronic treatment of mice with ethanol, but did not separate the stable pool of cholesterol in myelin from that in plasma membranes. In contrast to red cell membranes, the increased cholesterol that we observed in brain membranes is more likely to represent an adaptive response to chronic ethanol.

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