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Asymmetric distribution of a fluorescent sterol in synaptic plasma membranes: effects of chronic ethanol consumption

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Ethanol-induced structural changes in membranes have in some studies been attributed to an increase in total membrane cholesterol. Consistent changes in cholesterol content, however, have not been observed in membranes of ethanol consuming animals and alcoholic patients. This study examined the hypotheses that cholesterol was asymmetrically distributed in synaptic plasma membranes (SPM) and that chronic ethanol consumption alters the transbilayer distribution of cholesterol. Dehydroergosterol, a fluorescent cholesterol analogue was used to examine sterol distribution and exchange in chronic ethanol-treated and pair-fed control groups. The cytofacial leaflet was found to have significantly more dehydroergosterol as compared to the exofacial leaflet. This asymmetric distribution was significantly reduced by chronic ethanol consumption as was sterol transport. Total cholesterol content did not differ between the two groups. Chronic ethanol consumption appeared to alter transbilayer sterol distribution as determined by the incorporation and distribution of dehydroergosterol in SPM. The changes in transbilayer sterol distribution are consistent with recent reports on the asymmetric effects of ethanol in vitro ((1988) Biochim. Biophys. Acta 946, 85–94) and in vivo ((1989) J. Neurochem. 52, 1925–1930) on membrane leaflet structure. The results of this study also underscore the importance of examining membrane lipid domains in addition to the total content of different lipids.

Recent evidence indicates that alcohols including ethanol act specifically on membrane lipid domains [1–7]. Studies in model membranes have shown that alcohols have a differential interaction on certain phospholipids [1,2]. Such effects have also been observed in intact membranes using fluorescence photobleaching techniques [3]. The diffusion of PC and PE were significantly different in the presence of benzyl alcohol. In synaptic plasma membranes (SPM) it has been reported that ethanol has an ordering and disordering effect on the membrane surface and interior, respectively, as measured by nuclear magnetic resonance [4].

The studies described above examined the effects of alcohols on lateral domains in the membrane. Two other membrane domains that are differentially affected by alcohols are the membrane exofacial and cytofacial leaflets [5–7]. Benzyl alcohol had a greater fluidizing effect on the cytofacial leaflet in erythrocytes as compared to the exofacial leaflet [5]. It was reported in that study that the exofacial leaflet was significant less fluid than the cytofacial leaflet. The membrane leaflets of synaptic plasma membranes (SPM) are asymmetric in fluidity and ethanol fluidized the less rigid leaflet [6]. Significant effects of ethanol were seen at concentrations as low as 25 mM. The asymmetric effect of ethanol in vitro was shown to be markedly diminished as a result of chronic ethanol consumption in a subsequent study [7]. It also was found that difference in fluidity between the two leaflets was reduced in SPM of chronic ethanol-treated animals.

Previous studies on chronic ethanol-induced changes in membrane structure have measured changes in bulk membrane lipids (reviewed in Refs. 8 and 9). Cholesterol has been examined in a large number of studies concerned with effects of ethanol on the membrane structure [10]. The interest in cholesterol was based on the

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finding that cholesterol had a rigidifying effect on both model and intact membranes above the phase transition temperature [11]. Data on ethanol-induced increases in cholesterol content, however, have not been consistent and may be due in part to differences in ethanol dose and method of administration, species and duration of exposure [10]. An alternative explanation is that cholesterol may play a key role in ethanol-induced effects on membrane structure and function but that the changes occurring are not in the total amount of cholesterol but in the distribution of cholesterol in the exofacial and cytofacial leaflets. Cholesterol has been reported to be asymmetrically distributed in membrane leaflets, e.g., erythrocytes, LM fibroblast membranes [12,13].

The earlier reported asymmetric effects of ethanol in vitro and in vivo on SPM membrane leaflets may be associated with cholesterol distribution [6,7]. It was proposed in the present study that cholesterol would be asymmetrically distributed in SPM leaflets and that chronic ethanol consumption would alter the transbilayer distribution of cholesterol. This hypothesis was tested using a cholesterol analogue, dehydroergosterol, to estimate sterol transbilayer distribution [13,14].

Methods. C57BL male mice 3 to 4 months of age were used. Animals were maintained on an ethanol liquid diet for 8 weeks using procedures previously described [7]. The ethanol diet consisted of Sustacal, 6 percent (w/v) of 95 percent ethanol providing 35 percent of total calories. Pair-fed control mice received the liquid diet minus ethanol, with sucrose replacing the ethanol calories. Both diets were fortified with a vitamin and salt mixture (ICN Nutritional Biochemicals, Cleveland, OH). Synaptosomes were prepared and labeled with and without the fluorescent quenching agent, trinitrobenzenesulfonic acid (TNBS) after which SPM were prepared as described earlier [6,7]. Sterol distribution and transbilayer migration time were measured using the fluorescent sterol, dehydroergosterol and selective quenching of exofacial leaflet fluorescence by covalently linked trinitrophenyl groups that has been extensively described previously [13,17]. Basically, in 1-palmitoyl-2-oleoylphosphatidylcholine/ dehydroergosterol (POPC/DHE) small unilamellar vesicles (SUV), dehydroergosterol fluorescence is quenched when POPC/DHE ratios are greater than 0.05. When DHE is exchanged from SUV (with similar ratio of phospholipid/sterol as found in SPM) to SPM, fluorescence intensity quickly increases. At 4°, TNBS trinitrophenylates only the exofacial leaflet of SPM [13,17]. When the exchange occurs to SPM containing trinitrophenyl groups in the SPM exofacial leaflet, the fluorescence intensity for the exofacially attached trinitrophenyl groups quench fluorescence of dehydroergosterol in the exofacial leaflet [13,17]. Once dehydroergosterol migrates across the membrane bilayer to the cytofacial leaflet, fluorescence intensity increases. The increase in dehydroergosterol fluorescence intensity upon exchange is shifted to a longer time. The $t_{1/2}$ for transbilayer sterol migration is equal to the shift in minutes of the two intensity curves divided by two. The transbilayer distribution of dehydroergosterol was calculated from the plateau values of fluorescence intensity in SPM plus or minus TNBS. Absorbance-corrected fluorescence intensity of dehydroergosterol was determined for unlabeled and trinitrophenylated SPM using a computer-centered spectrofluorimeter [13,17]. Sterol uptake into SPM also was measured using the fluorescent sterol and [^{3}H]cholesterol. Both methods have been described in detail previously [13,14]. Total SPM phospholipid and cholesterol were measured in separate experiments using the methods of Bartlett [18] and Bowman and Wolfe [19], respectively.

TABLE I

Transbilayer distribution of dehydroergosterol in synaptic plasma membranes of control and chronic ethanol groups

Synaptosomes were treated with +/-TNBS under nonpenetrating conditions (4° C) and SPM isolated as described in the text. SPM were incubated with POPC/dehydroergosterol SUV (1 : 10 molar ratio of SUV : SPM) containing trace amounts of cholesteryl [1-^{14}C]oleate as a non-exchangeable marker. The exchange was stopped by centrifugation (10 000 × *g*, 4 min), the supernatant containing SUV was removed and the SPM pellet recovered. Fusion or sticking of the SPM to the SUV was less than 5% as measured by the presence of the non-exchangeable marker. Exchange of dehydroergosterol into SPM was measured as described in the text. Values represent the means ± S.E. of the percent sterol as determined from fluorescence intensity/mg protein ($n = 4$ membrane preparations per group). [a] signifies $P < 0.01$ as compared to the exofacial leaflet and [b] indicates $P < 0.01$ as compared to the control group using Student's *t*-test

Group	% Sterol	
	exofacial leaflet	cytofacial leaflet
Control	12 ± 2	88 ± 2 [a]
Ethanol	28 ± 1 [b]	72 ± 1 [ab]

Results. Dehydroergosterol was asymmetrically distributed in the two SPM leaflets. The cytofacial leaflet contained significantly more of the sterol ($P < 0.01$) as compared to the exofacial leaflet in both groups (Table I). The transbilayer distribution of dehydroergosterol was significantly altered in the SPM leaflets of the ethanol group. There was a greater than 2-fold increase ($P < 0.01$) in the percent sterol of the exofacial leaflet of the chronic ethanol group as compared to the exofacial leaflet of the control group (Table I). Percent dehydroergosterol in the cytofacial leaflet of the ethanol group was significantly reduced in contrast to the cytofacial leaflet of the control group ($P < 0.01$).

The distribution of the cholesterol analogue dehydroergosterol in SPM exofacial and cytofacial leaflets was markedly altered in ethanol-treated animals as compared to controls. However, chronic ethanol consump-

TABLE II

Total cholesterol and phospholipid content of synaptic plasma membranes from control and chronic ethanol groups

The total amount of cholesterol and phospholipid content were determined in SPM using methods described in the text. Values represent the means ± S.E. of four membrane preparations per group

Lipid	Control	Ethanol
Cholesterol (μmol/mg protein)	0.5336 ± 0.016	0.552 ± 0.030
Phospholipid (μmol/mg protein)	0.7310 ± 0.006	0.785 ± 0.036
Cholesterol/phospholipid (molar ratio)	0.7361 ± 0.015	0.702 ± 0.017

tion did not appear to significantly change the total amount of SPM cholesterol or phospholipid (Table II). There were no significant differences between ethanol and pair-fed groups in total cholesterol content, phospholipid content and the ratio of cholesterol to phospholipid in a separate experiment.

Chronic ethanol consumption also modified sterol exchange between SUV and SPM. The $t_{1/2}$ (min) for exchange was significantly slower in the ethanol group as compared to the control group (Table III). This difference was observed using two different methods for measuring sterol exchange, i.e., fluorescent dehydroergosterol and [^{3}H]cholesterol. The $t_{1/2}$ values for sterol exchange were very similar for both methods (Table III). The transbilayer migration time of dehydroergosterol from one leaflet to the other was not affected by chronic ethanol consumption. The $t_{1/2}$ (min) of the pair-fed control group was 6.7 ± 1.1 and 6.5 ± 0.2 for the ethanol group.

Discussion. The fluorescent sterol, dehydroergosterol was used to determine sterol distribution in SPM of ethanol-treated and control animals. Dehydroergosterol is a close structural analogue of membrane sterols and has been found to be functionally similar using different biochemical and biophysical techniques (reviewed in Ref. 15). In the present experiment for example, the $t_{1/2}$ for sterol exchange was similar using either dehydroergosterol or [^{3}H]cholesterol (Table III). Although the properties of dehydroergosterol and cholesterol are similar, it is possible that the various labeling and membrane isolation procedures could affect the distribution of the sterol and need to be considered. Transbilayer sterol distribution in erythrocytes has been shown to be similar using three different methods: a combination of [^{3}H]cholesterol and cholesterol oxidase [12], dehydroergosterol and fluorescence photobleaching of NBD-cholesterol (unpublished data). While perturbation of the membrane by the isolation and labeling procedures cannot be entirely ruled out, it would appear that dehydroergosterol approximates cholesterol distribution in biological membranes.

TABLE III

Sterol exchange in synaptic plasma membranes of control and chronic ethanol groups

SPM were prepared as described in Table I. SPM were incubated with POPC/dehydroergosterol SUV (1 : 10 molar ratio of SUV : SPM) containing [^{3}H]cholesterol and cholesteryl[1-^{14}C]oleate. Aliquots were taken at 0, 5, 15, 30, 60, and 90 min at which time the exchange was stopped by centrifugation (10 000 × *g*, 4 min) and the SPM pelleted. Fluorescence intensity of dehydroergosterol and quantitation of radiolabeled sterols by scintillation counting were determined for each sample. Values represent the means ± S.E. of five membrane preparations per group. [a] refers to $P < 0.04$ as compared to the control group by Student's *t*-test.

Sterol	$t_{1/2}$ for sterol exchange	
	control (min)	ethanol (min)
Dehydroergosterol	8.2 ± 1.8	13.2 ± 1.3 [a]
[^{3}H]Cholesterol	8.3 ± 1.5	14.1 ± 1.6 [a]

Dehydroergosterol was asymmetrically distributed in SPM exofacial and cytofacial leaflets and chronic ethanol consumption modified SPM dehydroergosterol transbilayer distribution. The cytofacial leaflet contained approximately four times as much dehydroergosterol as compared to the exofacial leaflet. Sterols have been shown to decrease fluidity of membranes above its phase transition temperature [11]. SPM dehydroergosterol enrichment in the cytofacial leaflet was consistent with the reported finding that the SPM cytofacial leaflet was less fluid than the exofacial leaflet [6,7]. This pattern of fluidity and sterol asymmetry has also been found in LM fibroblast membranes [13,14] and erythrocyte membranes (Ref. 12; unpublished data).

The asymmetric distribution of dehydroergosterol in SPM may explain the specific effects of ethanol in vitro on fluidity of the SPM exofacial leaflet [6]. The exofacial leaflet was fluidized more by ethanol when compared to the cytofacial leaflet. This asymmetric effect of ethanol parallels the asymmetry in dehydroergosterol distribution reported here. In a subsequent study, chronic ethanol consumption reduced the asymmetric effects of ethanol in vitro in SPM leaflets and also reduced the transbilayer differences in fluidity between the two leaflets [7]. The results of the present study indicate that these effects may be due in part to ethanol altering the transbilayer distribution of cholesterol in vivo. There was twice as much dehydroergosterol in the SPM exofacial leaflet of chronic ethanol animals as compared to controls.

While the present study did not examine the functional consequences of the changes in membrane structure, it has been reported that relatively small changes in the cholesterol content of the exofacial leaflet of erythrocytes can significantly affect fluidity, antigenic sites and exposure of protein sulfhydryl groups [20]. In SPM, fluidity, dopamine binding and dopamine-stimulated adenylate cyclase were altered by changing the cholesterol to phospholipid ratio in SPM [21]. It would

be expected that changes in cholesterol asymmetry would result in changes in the cholesterol to phospholipid ratio in each leaflet and in turn alter leaflet specific membrane functions.

An explanation as to how transbilayer cholesterol distribution is maintained in membranes is not well-understood (reviewed in Ref. 15). Various factors have been shown to alter cholesterol asymmetry in model membranes and in biological membranes, e.g., cholesterol/phospholipid ratio, specific lipids, cholesterol enrichment and depletion, fast, slow and nonexchangeable cholesterol pools [15]. Chronic ethanol consumption interfered with the capacity of the membrane to maintain normal leaflet asymmetry.

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