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Chronic ethanol ingestion modifies liver microsomal phosphatidylserine inducing resistance to hydrolysis by exogenous phospholipase A_2

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Chronic ethanol ingestion leads to the acquisition of a tolerance to membrane lipid disordering, a lowered partition coefficient to hydrophobic compounds and a resistance to the hydrolysis of the phospholipids by exogenous phospholipase A_2. Anionic phospholipids have been implicated as being responsible for the resistance to lipid disordering and a number of modifications to these phospholipids are known to occur as a result of chronic ethanol-ingestion. In this study the basis of the resistance to phospholipase A_2 in hepatic microsomes was investigated. It was found that chronic ethanol-induced modifications to each of the major phospholipid classes was responsible to some extent for the resistance to phospholipase A_2, however, PS was particularly potent considering it is a compositionally minor constituent. The effect was interpreted as a reduced ability to activate the phospholipase A_2 since PS acts as an essential activator of phospholipase A_2 (along with PI). Fatty acid analysis revealed that the chronic ethanol-treatment resulted in a elevated level of docosahexaenoate with a parallel reduction in arachidonate in phosphatidylserine. Lipid packing and organization is important in the regulating the level of exogenous phospholipase A_2 activity but the activity was not found to correlate with lipid order of different phosphatidylserine species. It is concluded that subtle differences in the molecular species arrangement or disposition around the enzyme may be responsible for the altered phospholipase A_2 interaction with the membrane induced by chronic ethanol-treatment. One implication of this study is that other anionic phospholipid dependent membrane proteins, of which there are many known examples, may also be modified as a result of chronic ethanol-ingestion.

When animals are subjected to chronic ethanol exposure the cell membranes adapt by developing a resistance to the disordering of the phospholipid acyl chains by ethanol or other lipophillic compounds. This was first demonstrated in synaptosomes [1] and subsequently in a wide variety of membranes from different tissues [2–8]. Studies from our laboratory have indicated that the resistance to disordering is part of a broader alteration in biological membranes termed 'membrane tolerance'. Membrane tolerance also encompasses a decreased partitioning of ethanol and other lipophillic compounds into the membrane [9,10]. More recently it was shown that chronic ethanol treatment causes hepatic microsomes to develop a resistance to the hydrolysis of the phospholipids by exogenously added phospholipase A_2 [11]. The resistance to lipid disordering, decreased partitioning properties and reduced hydrolysis by phospholipase A_2 all appear to be due to an alteration in the phospholipids.

The resistance to lipid disordering in liver microsomal membranes from animals chronically adapted to ethanol has been shown to be due principally to an alteration in PI and to a lesser extent PS [8]. In liver mitochondria, cardiolipin appears to be modified [12]

Abbreviations: C_6-NBD-PC, 1-palmitoyl-2-*N*-(4-nitrobenz-2-oxa-1,3-diazole)aminohexanoylphosphatidylcholine; DOPS, dioleoyl-PS; DPH-PC, 1-palmitoyl-2-[[2-(4-(6-phenyl-*trans*-1,3,5-hexatrienyl) phenyl]ethyl]carbonyl]-3-*sn*-PC; NBD-HA, *N*-(4-nitrobenz-2-oxa-1,3-diazole)aminohexanoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; POPC, 1-palmitoyl-2-oleoyl-PC; POPS, 1-palmitoyl-2-oleoyl-PS; PS, phosphatidylserine; TLC, thin-layer chromatography.

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and in synaptosomes PS (Nie, Y., Ellingson, J.S., Rubin, E. and Taraschi, T.F., unpublished data). The feature common to all of these lipids is their anionic character.

Membrane proteins are dependent on the nature of the lipid environment for optimal functioning, however, the relationship between ethanol-induced adaptive lipid modifications and affects on membrane proteins has hardly been explored. There is a major class of proteins which bind peripherally to the membrane, and anionic phospholipids are intimately involved in this process since they bear a negative surface charge (for review, see Ref. 13). Membrane associated proteins that are present in the cytoplasm, but that have to be in a membrane bound form to exert functional effects include, for example, phospholipase A_2, phospholipase C and protein kinase C. Any chronic ethanol-induced modifications to the anionic phospholipids would be expected to affect this interaction. Phospholipase A_2 is of the most studied of the extrinsic membrane proteins (see reviews, Refs. 14–18) and our previous studies have shown that membranes modified as a result of chronic ethanol-treatment are resistant to hydrolysis by this enzyme [11]. In this study the hypothesis that a modification to the anionic phospholipids may be involved was investigated and it was found that in liver microsomal membranes, modifications to PS, and to a lesser extent PI, may be responsible for the resistance to phospholipase A_2 hydrolysis.

Experimental procedures

Materials. C_6-NBD-PC, POPC, POPS, DOPS and brain-PS were from Avanti Lipids (Birmingham, AL), NBD-HA was from Molecular Probes (Eugene, OR). All lipids were checked for purity by TLC.

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories) were fed for 35 days a liquid diet (Bio-serve, Frenchtown, NJ) in which ethanol comprised 36% of total calories [19]. Pair-fed littermate controls received the same diet, except that carbohydrates isocalorically replaced ethanol. Ethanol consumption averaged 14–16 g per kg of body weight per day.

Phospholipids with elevated levels of the $(n-3)22:6$ were obtained from animals fed a standard fat free rat chow diet which had been supplemented with Menhaden fish oil as previously described [20].

Membrane isolation. Rat liver microsomes were prepared and characterized as previously described [21].

Lipid Extraction, separation and analysis. Lipids were extracted [22] and the total phospholipids were obtained by adding a total lipid extract (in chloroform) to a silicic acid column and eluting the neutral lipids with chloroform. The phospholipids were then eluted by the addition of methanol.

The individual phospholipid classes were separated by HPLC, as described elsewhere [23]. After separation, the phospholipids were quantified by the determination of phospholipid phosphorus [24]. TLC separations were performed using Merck HPTLC plates [25]. Bands were identified with the aid of standards, visualized by spraying with dichlorofluorescein or iodine vapor and then scraped from the plate.

Fatty acid composition of PS was determined by saponification according to Morrison and Smith [26] and analysis by GLC on a Hewlett-Packard 5890A GLC using a diethylene glycol succinate column. The methyl esters were identified by use of authentic standards and equivalent chain length data [27].

Preparation of liposomes. Multilamellar liposomes were prepared by placing an aliquot of the lipid extract into a test tube and removing the chloroform by passing a stream of nitrogen into the tube. Tris-HCl buffer (10 mM, pH 7.4, 150 mM NaCl) was then added, and the contents of the tube agitated using a Vortex mixer.

Phospholipase assay. Phospholipase A_2 activity was determined by measuring the release of NBD-HA from C_6-NBD-PC [11]. For intact microsomes C_6-NBD-PC (5 μM) was introduced by adding 10 μl of a dispersion in buffer (with 1 mM Ca^{2+}) and incubating for 2 min at 37°C in the presence of 10 μl (0.67 μg) of phospholipase A_2 (*Crotalus durissus terrificus*, from Sigma). The value obtained was corrected for hydrolysis of the C_6-NBD-PC endogeneous phospholipases by determining the rate of hydrolysis without exogenous phospholipase A_2.

For phospholipid vesicles the C_6-NBD-PC (1 mol% of the phospholipids) was added by co-dispersing it with the phospholipids. The vesicles (100 μM phospholipids) were suspended in 0.4 ml buffer (with 1 mM Ca^{2+}), and phospholipase A_2 was added to start the reaction. The amount of phospholipase A_2 was sufficient to hydrolyze not more than 5–10% of the phospholipids in the reaction time. The reaction was stopped by the addition of chloroform/methanol (1:2, by vol), and the mixture was separated into a lower chloroform and upper aqueous phase following the lipid extraction procedure [22]. The release of NBD-HA from C_6-NBD-PC was determined by measuring the fluorescence intensity at 530 nm, determined upon excitation at 470 nm. A standard curve was obtained for NBD-HA, which was also subjected to the chloroform/methanol extraction. A similar assay has also been described elsewhere [28,29].

Results

In this study C_6-NBD-PC was used to measure phospholipase A_2 activity. This compound offers a number of advantages for the assay of phospholipase A_2 [11,28,29]. These include a rapid and complete

incorporation into intact membranes, no reincorporation of the released NBD-HA into the membrane phospholipids and a high sensitivity. NBD-HA is easily removed from the unhydrolyzed C_6-NBD-PC and can be conveniently determined by measurement of the fluorescence without recourse to TLC separations. In addition, we have previously demonstrated that the release of [^{3}H]oleate from [^{3}H]POPC is similar to that of NBD-HA from C_6-NBD-PC [11]. For comparison of membranes from ethanol-treated and control animals the method has the important advantage that the C_6-NBD-PC substrate is the same in each case, so that any differences in the rate of hydrolysis can be attributed to a difference in the physical state of the membranes or membrane micro-environments where the substrate resides. Further the C_6-NBD-PC was found to have no effect on the phospholipase A_2 activity for levels as high as 10 mol% of the native phospholipids.

It has been previously shown that in hepatic microsomes chronic ethanol ingestion leads to a resistance to hydrolysis by exogenous phospholipase A_2, relative to pair-fed controls [11]. To determine whether any single phospholipid class (PC, PE, PI or PS) was responsible for the modification, the phospholipid classes were first separated by HPLC. Aliquots of the respective phospholipid extracts in chloroform were then recombined in the same relative amounts as naturally found in liver microsomes (66.5 mol% of PC, 21.0% PE, 8.5% PI and 4.0% PS respectively [8]) except that one phospholipid class in turn was from an ethanol-treated animal while the other three were from the control. This was repeated, in turn, for each of the four phospholipid classes, and the activity of phospholipase A_2 determined against vesicles of the mixtures. The results shown in Table I reveal that the quantitatively minor PS (comprising 4% of the total) conferred the greatest degree of resistance to phospholipase A_2. PC and PE from ethanol-fed animals conferred the least resistance, with the effect of PI intermediate between these lipids and PS.

TABLE I

Relative potency of phospholipid classes in conferring resistance to phospholipase A_2 hydrolysis

Vesicles were made from a recombination of liver microsomal PC (66.5 mol%), PE (21.0%), PI (8.5%) and PS (4.0%), from control ($_{cont}$), or ethanol-fed animals ($_{eth}$), as described in Methods. The activity is expressed as% of all-control recombination (mean ± S.D. from three experiments).

		NBD-HA released (% of control)
1	$PC_{cont} + PE_{cont} + PI_{cont} + PS_{cont}$	100.0 ± 5.5
2	$PC_{eth} + PE_{eth} + PI_{eth} + PS_{eth}$	68.6 ± 7.3
3	$PC_{eth} + PE_{cont} + PI_{cont} + PS_{cont}$	82.4 ± 3.5
4	$PC_{cont} + PE_{eth} + PI_{cont} + PS_{cont}$	92.9 ± 9.9
5	$PC_{cont} + PE_{cont} + PI_{eth} + PS_{cont}$	74.9 ± 4.6
6	$PC_{cont} + PE_{cont} + PI_{cont} + PS_{eth}$	54.2 ± 2.2

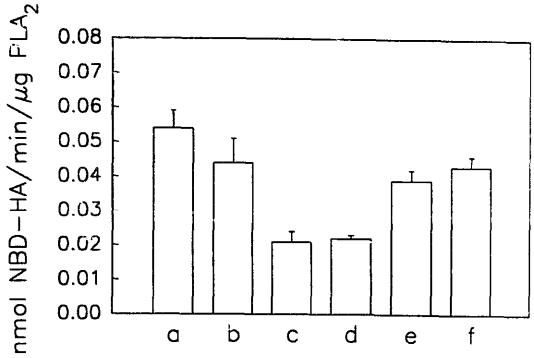

Fig. 1. The hydrolysis of C_6-NBD-PC by phospholipase A_2 in vesicles of POPC with PS (POPC/PS, 9:1). The PS was from microsomes of control (a) and ethanol-fed animals (b), POPS (c), DOPS (d), brain-PS (e) and PS from microsomes from animals fed a Menhaden oil supplemented diet (f). Details are as described in Experimental procedures.

Although the results suggest that modifications to PS and to a lesser extent PI are to a large extent responsible for the resistance to phospholipase A_2 induced by chronic ethanol-treatment, the role of the other lipid classes less clear. To investigate this, vesicles were prepared consisting of only the PS together with the synthetic POPC (PS/POPC, 9:1 molar ratio). The POPC presence prevented Ca^{2+} induced non-bilayer configurations and aggregation. The results in Fig. 1 show that a relative resistance to phospholipase A_2 conferred by PS could be shown without the presence of the other major phospholipid classes. For comparison, various other PS species were also examined, again in combination with POPC. It can be seen that the hydrolysis of C_6-NBD-PC by phospholipase A_2 differed according to the species. Vesicles made from POPC with both the monounsaturated POPS and diunsaturated DOPS were much less susceptible to hydrolysis compared to the natural PS species.

The hydrolysis of the PS itself, in vesicles comprised of PS and POPC was also determined (POPC/PS, 9:1). After phospholipase A_2 treatment the lipids were separated by TLC, the PS visualized by exposure to iodine vapor and the spot scraped off for phosphorus analysis. The rate of hydrolysis of the PS was 3.16 ± 0.48 and 1.8 ± 0.99 nmol/min per μg phospholipase A_2 for PS from microsomes prepared from livers of control and ethanol-fed animals, respectively. Thus the chronic ethanol-induced resistance to phospholipase A_2 is not solely confined to PC substrates but also the PS itself.

To determine the compositional basis of the ethanol-induced resistance to phospholipase A_2 hydrolysis by altered PS, analysis of the fatty acids were performed as shown in Table I. It can be seen that the ethanol-treatment led to a decrease in the arachidonate and an increase in the proportion of docosahex-

aenoate. To examine the importance of the level of 22:6 in the chronic ethanol-induced effects, PS was also prepared from liver microsomes from animals which had been subjected to a diet where the fat was Menhaden fish oil. This achieved a very high 22:6 level (Table II). However, while the 22:6 levels achieved by the dietary supplementation were far in excess of that achieved by the ethanol treatment, the phospholipase A_2 activity against PS/POPC vesicles was not dramatically reduced compared to PS from control liver microsomes (Fig. 1, (f)). Thus the reduced phospholipase A_2 action induced by the chronic ethanol-treatment does not appear to be simply related to the level of 22:6.

The altered fatty acid composition could modify the lipid packing properties which could be responsible for the altered level of phospholipase A_2 hydrolysis. To examine this, lipid packing differences were examined by measuring the fluorescence anisotropy of the DPH-PC, to assess lipid order. This fluorophore was chosen since DPH-PC is itself a lipid, the DPH being attached to the *sn*-2 chain. When the phospholipase A_2 interacts with the bilayer it will be surrounded mostly by the PS, and the PS directs the physico-environment around the enzyme and hence its activity. Therefore instead of probing the overall lipid order of the bulk region and that around the phospholipase A_2, vesicles were comprised solely of the PS species were examined (in a buffer containing 0.1 mM EGTA). The results indicated that there was no correlation between the fluorescence anisotropy of DPH-PC (related to lipid order) and the activity of phospholipase A_2 against PS/POPC vesicles (results not shown).

Since anionic phospholipids contribute to the surface charge, the effect of differing Na^+ concentrations and of different pH values in the buffer was investigated. In order to examine this question the hydrolysis of C_6-NBD-PC incorporated into microsomes from control and ethanol pair-fed litter mates was compared

TABLE II

Effect of chronic ethanol-treatment on the proportion of the major fatty acids of PS from liver microsomes

Mean of PS preparations from five pairs of control and ethanol-fed animals and four animal pairs for the $n-3$ supplemented experiments. Fatty acids are denoted by the number of carbon atoms:no of *cis*-double bonds.

	Control	Ethanol-fed	$n-3$ supplemented
16:0	5.07±0.76	4.38±0.90	9.87±0.44
18:0	42.74±3.43	42.10±1.58	38.12±1.14
18:1 ($n-9$)	4.63±0.31	4.77±0.32	2.68±0.99
18:2 ($n-6$)	2.75±0.60	2.49±0.29	0.62±0.22
20:4 ($n-6$)	35.45±2.56	31.72±2.76	4.53±0.67
22:6 ($n-3$)	6.85±0.66	10.11±2.43	33.38±2.47
Other	2.50±2.09	4.43±2.32	10.81±1.72

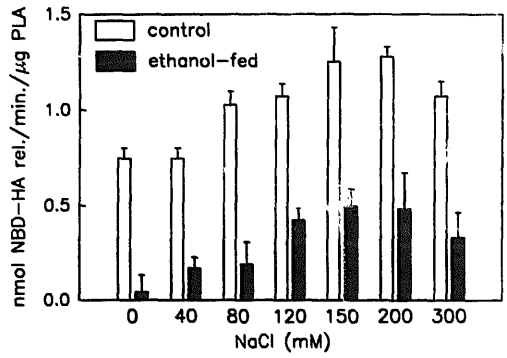

Fig. 2. Effect of different NaCl concentrations (using Tris-HCl, pH 7.4) on the hydrolysis of C_6-NBD-PC by exogenous phospholipase A_2 on intact microsomal preparations from controls and ethanol-treated animals. Details are as described in Experimental procedures.

for differing salt and pH. Microsomes were resuspended in Tris-HCl buffer (10 mM) in the presence of varying NaCl concentrations or with 150 mM NaCl and for differing pH values. Fig. 2 shows that the relative resistance of the membranes from ethanol-fed animals to hydrolysis by phospholipase A_2 diminishes at high Na + concentrations and increases at lower Na^+ concentrations. At low Na^+ concentration the main cause for the apparent increase in resistance to hydrolysis appears to be an alteration to the hydrolysis of the C_6-NBD-PC in the membranes from the ethanol-fed animals. For a pH in the range 7–9.5 the difference between the membranes from membranes from chronic and control membranes was maintained. Although at pH 6 it was no longer found the activities of the phospholipase A_2 were considerably reduced (results not shown).

Discussion

The major finding in this study is that in liver microsomes a modification to PS in particular may to a large extent be responsible for the ethanol-induced resistance to hydrolysis by phospholipase A_2. PS and PI as anionic lipids are required for phospholipase A_2 activity which is negligible in their absence so the effect can be considered as a reduced ability to activate the phospholipase A_2. Other studies from this laboratory have demonstrated that ethanol-induced alterations to liver microsomal PI [8] and mitochondrial cardiolipin [13] lead to a resistance to lipid disordering by ethanol *in vitro*. PS in synaptosomes from ethanol-fed animals also confers resistance to lipid disordering (Nie, Y., Ellingson, J.S., Rubin, E. and Taraschi, T.F., unpublished data). Thus, the results of both these studies, and the present, indicate that in general a modification to anionic phospholipids is responsible for membrane tolerance. Although other studies have shown modifications to anionic phospholipids result from chronic

ethanol-treatment this study is the first to demonstrate that there may be functional significance to these lipid changes. This is due to the fact that phospholipase A_2 is one of an increasing number of membrane proteins which have been found to require anionic lipids either for interaction with the membrane and/or for activity to ensue.

The actual substrate for the phospholipase A_2 does not appear to be a critical factor since it was previously shown that in addition to the C_6-NBD-PC substrate used here, POPC (radio-labeled) could also be used (using total microsomal phospholipids). In addition, in the present study it was shown that the natural PS itself is degraded by the phospholipase A_2 and at a reduced rate with membrane lipids derived from ethanol-fed animals. Also the source of phospholipase A_2 does not appear to be critical, since we previously demonstrated that phospholipase A_2 from *Naja naja*, pancreas and bee venom, as well as the *Crotalus durissus terrificus* used in the present study, can also be used to demonstrate the ethanol-induced resistance to phospholipase A_2 [11]. In the present study it was also shown that a modification to PS alone appears to sufficient to demonstrate the resistance to phospholipase A_2 action, since the other phospholipids can be omitted and replaced by the synthetic phospholipid POPC.

The action of phospholipase A_2 against membranes may be divided into two phases. First the enzyme interacts with the surface, where the surface charge, phospholipid head group and Ca^{2+}, all play a role. The studies on pH and different salt concentrations, while shown to affect the relative resistance to phospholipase A_2 did not appear to be critical factors. The second phase of the phospholipase A_2-membrane interaction is a Ca^{2+}-dependent conformational change in the enzyme, allowing it to penetrate partially into the lipid bilayer. Preliminary results (Stubbs, C.D., unpublished observations) indicate that the penetration of phospholipase A_2 into the bilayer, accompanying the conformational change, is probably not the focus of the inhibitory effect in membranes from animals fed ethanol.

The results of the recombination experiment allow some insight into the action of PS and PI. Without the anionic lipids the phospholipase A_2 activity was negligible. Therefore the anionic lipids can be considered as 'activating' the phospholipase A_2, so that in essence we are dealing with a *reduced ability to activate* rather than with an inhibition. To simplify the discussion it is helpful to focus on the contributions of ethanol induced PI and PS. The results show that inclusion of PS modified by ethanol-treatment renders the recombined phospholipids *less* able to activate phospholipase A_2 compared to an all control recombination (54.2% of control see Table I) i.e. the ethanol-modified-PS activates less than the control-PS. In comparison when the recombination includes ethanol-modified PI and otherwise control phospholipids, its reduced ability to activate is not so great as PS (74.9% of control). The results show that when in addition to PS modified by chronic ethanol-treatment, the PI is *also* from ethanol-fed animals, an intermediary level of activation was obtained (68.6% of control). Our present interpretation is based on the ethanol-modified lipids having a greater ability to interact with and thereby influence phospholipase A_2 activity compared to control counterparts. Thus ethanol-modified PI competes more strongly (than control-PI) with the ethanol-modified-PS and effectively reduces the latter influence (to 68.6% of control from 54%) it therefore leads to a more active phospholipase A_2.

The compositional basis of the chronic ethanol-induced resistance to exogenous phospholipase A_2 activity would appear to be the fatty acid changes in PS. In general, a number of modifications to membrane phospholipids have been described which could be involved in membrane tolerance. In brain lipids, ethanol-induced changes are mainly confined to the anionic phospholipids and in PS, docosahexaenoate is decreased and palmitate increased [30,31]. In some studies an alteration in the proportion of PS has been shown [32,33]. In liver microsomes, although some changes in the phospholipid class composition have been reported, these are modest [34,35] and the design of the present studies would in any case rule this out as being involved in the resistance to phospholipase A_2. Regarding fatty acid changes, in one study an increase in docosahexaenoate was found [36], but not in another [37]. In liver mitochondria marked fatty acid changes in cardiolipin have been noted [2] where it has been shown to be responsible for the resistance to lipid disordering [12]. Fatty acid analyses on the PS from microsomes of ethanol-fed animals in the present study showed a decrease in arachidonate and an increase in docosahexaenoate resulting from ethanol-treatment. Recent preliminary data from this laboratory (Ellingson, J.S., Nham. D and Rubin, E., unpublished observations) have shown that chronic ethanol-treatment results in a decrease in the level of the 18:0-20:4-PS species from 64.3% of the total ($\pm$1.3) to 56.2% ($\pm$1.6) and a parallel increase of the 18:0-22:6-PS species from 12.4 % ($\pm$0.2) to 18.9% ($\pm$0.3).

Since the substrate for the phospholipase A_2, C_6-NBD-PC, was the same for the membranes from both the treated and control animals one may infer that PS induces an altered physico-chemical environment in the membrane at the enzyme-PS complex. However, for various PS species lipid order did not show a correlation with the phospholipase A_2 activity. Examination of other parameters in isolation, such as the level of 22:6, proportion of saturated fatty acids etc,

also failed to reveal a correlation with activity. Thus it would appear that phospholipase A_2 interacts with the bilayer surface in a highly discriminatory manner and the different individual methods for assessing the ability of the phospholipase A_2 to interact with the lipid bilayers appear to be too crude to reveal crucial differences and the activity of the enzyme itself remains the most subtle probe of the ethanol-induced altered membrane properties.

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