Ethanol specifically alters the synthesis, acylation and transbilayer movement of aminophospholipids in rat-liver microsomes

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Abstract

By experimenting with the aminoalcohols [3–3H]serine and [2–14C]ethanolamine we have been able to relate the effects of ethanol upon the biosynthesis of radioactive aminophospholipids (APL) in rat-liver microsomes and their distribution within the bilayer. The translocation of newly synthesized molecules of aminophospholipids labeled with different fatty acids was also investigated. The synthesis of phosphatidylserine (PS) and phosphatidylethanolamine (PE) by base-exchange reaction (BES) was inhibited in membranes exposed to ethanol in direct response to its concentration. In addition, 100 mM ethanol specifically inhibited the transport of newly synthesized PS to the inner leaflet, resulting in similar levels of PS in both leaflets of the bilayer. The inhibition of PE synthesis by ethanol caused a decrease in its distribution in both inner and outer leaflets. An in vitro study of the incorporation of radioactive palmitate and oleate into the PS and PE of microsomes incubated with ethanol showed a decrease in the radioactivity levels of PE, suggesting that ethanol was specifically inhibiting the corresponding acyltransferase. It specifically altered the transbilayer movement of newly acylated phospholipids, modifying the distribution of palmitoyl- and oleoyl-acylated PS and PE in both leaflets. These results demonstrate for the first time that ethanol interferes with both the synthesis and intramembrane transport of aminophospholipids in endoplasmic reticulum (ER) membranes. Bearing in mind that if a membrane is to function properly its structure must be in optimum condition; it is evident that the observed processes may be responsible to some degree for the pathophysiological effects of alcohol upon cells.

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Introduction

Overwhelming evidence exists to demonstrate that ethanol exerts its pharmacological effects by modulating the function of many components of intracellular signal transduction pathways (Nagy, 2004). It has also been suggested that lipid composition and the degree of phospholipid unsaturation may play a role (as a signaling system) in the modulation of membrane–protein functions (Litman and Mitchell, 1996). In previous publications we have described significant changes in the composition of lipid and phospholipid acyl groups after both chronic (Carrasco et al., 1996a) and acute (Marco et al., 1986) ethanol consumption and consequent alterations in the properties of brain and liver membranes.

The aminophospholipid (APL) phosphatidylserine (PS) accounts for 3–10% of the total phospholipid content of animal cell membranes. Apart from its presumed contribution to membrane structure, PS is involved in numerous key cell processes: it activates several enzymes and its externalization onto the cell surface is a critical signal for platelet activation during the blood-clotting cascade, for skeletal muscle development, and for macrophage recognition of cells undergoing apoptosis (reviewed by Buckland and Wilton, 2000). PS has also recently been shown to be enriched in lipid rafts (Ishii et al., 2005).

In mammalian cells it is synthesized via a base-exchange reaction (BES) that converts pre-existing phospholipid into PS. Two PS synthases have been identified, each encoded by different genes and located on separate chromosomes. Phos-
phatidylerine synthase-1 (PSS1) catalyzes the exchange of serine for the choline head-group of phosphatidylcholine (PC), whereas phosphatidylethanolamine synthase-2 (PSS2) catalyzes the exchange of serine for the ethanolamine moiety of phosphatidylethanolamine (PE) (reviewed by Vance, 2003).

The other APL, PE, is synthesized on the endoplasmic reticulum (ER) via the CDP-ethanolamine pathway and is also produced in mitochondria by PS decarboxylation. A third, quantitatively minor pathway for the biosynthesis of PE is the BES. It has recently been suggested that the synthesis of PE by this reaction may be directed towards repairing any damage to unsaturated fatty acid chains of phospholipids caused by reactive oxygen species under oxidative stress (Jasinska et al., 1996). In addition, as has already been observed in synthetic membranes, the arrangement of phospholipid molecules in the bilayer is a significant factor in determining resistance to free-radical attack during lipid peroxidation, and importantly in this respect, the primary amine group of PE and PS in the external leaflet possesses antioxidant activity, thus protecting the polyunsaturated fatty acids from lipid peroxidation (Kubo et al., 2005).

It is interesting to note that the base-exchange enzymes PSS1 and PSS2 seem to be firmly embedded in the matrix of the membrane and show a marked preference for the endogenous substrates contained within their environment (Ilincheta de Boschero et al., 2000). In this context, any modification to the activity of these enzymes is likely to be related to changes in the membrane microenvironment.

The transbilayer distribution of lipids across eukaryotic plasma membranes is asymmetric. The choline-bearing lipids PC and SM are enriched in the external leaflet of the membrane whilst the aminophospholipids PE and PS are located preferentially in the cytoplasmic leaflet (Pomorski et al., 2004). The transverse distribution of phospholipids in the ER, on the other hand, is still a matter of debate after conflicting observations (Devaux, 1993). Nevertheless, recent publications suggest that the distribution of most phospholipid classes in the ER is largely symmetrical (Buton et al., 1996; Kol et al., 2002). Thus it is important to view the biosynthesis of PS and PE in the biogenic membrane, ER, as being an asymmetric process that results in the insertion of new lipids into the cytoplasmic leaflet. Because of the thermodynamic barrier to spontaneous transbilayer movements these lipids should remain enriched on the cytoplasmic side of the membrane, thus resulting in an asymmetric phospholipid distribution. The asymmetric addition of newly synthesized phospholipids to one leaflet of the bilayer generates an unstable membrane however, inducing membrane bending and consequent shape changes (Zwaal and Schroit, 1997). It has recently been suggested that these problems might be rectified by the presence of lipid transporters (i.e. dedicated flippases), which could redistribute ER phospholipids across the membrane (Chang et al., 2004), but it must be admitted that the mechanism of flip-flop movement in biogenic membranes is not yet fully understood.

Chronic ethanol consumption alters the distribution of phospholipids in the exofacial and cytofacial leaflets of plasma membrane without changing the total quantity of any individual phospholipid (Wood et al., 1991). Moreover, ethanol (Schwichtenhovel et al., 1992) and other alcohols (Bassé et al., 1992) in vitro profoundly alter the phospholipid flip-flop in human erythrocyte membranes, accelerating the internalization rate of NBD-labeled phospholipids due to an increase in APL translocase activity. There is no experimental evidence on the other hand to describe the effect of ethanol on the transbilayer distribution of APL in biogenic membranes and for this reason we decided to investigate its effect in vitro on the microsomal synthesis and acylation of PE and PS and their translocation between the two leaflets of the membrane bilayer.

Materials and methods

Animals

Male Sprague–Dawley rats (200–250 g) were supplied by the Technical Services of the University of Granada (Spain) and fed ad libitum on a standard chow diet in a chamber with a 10-h illumination period and a constant temperature of 25 °C. The rats were deprived of food overnight before the liver microsomes were isolated.

Preparation of rat-liver microsomes

Microsomes were obtained as described elsewhere (Carrasco et al., 2001) with minor modifications. Briefly, the rats were killed by cervical dislocation and their livers were immediately removed, weighed, minced and homogenized in 220 mM manitol, 70 mM sucrose, 2 mM HEPES and 0.5 mg/mL BSA (pH 7.4) with a Potter–Elvehjem homogenizer. The homogenate was centrifuged twice for 10 min at 660 g and the supernatant of this last centrifugation was recentrifuged at 105,000 g for 60 min. The supernatant of this last centrifugation was recentrifuged at 17,000 g for 20 min. Microsomes were obtained by centrifugation of the supernatant at 105,000 g for 60 min. The microsomal pellet was homogenized in the appropriate buffer and used without storage.

Assay of ethanolamine and serine base-exchange reaction

BES reaction activity was determined in microsomes by measuring the formation of radiolabeled PS and PE from the exogenous substrates L-[3-3H]serine and [2,14C]ethanolamine, respectively. The assay mixture contained 20 mM HEPES pH 7.4, 1 mM CaCl2, and approximately 0.15 mg of microsomal protein up to a volume of 450 μL. Tubes were preincubated at 37 °C for 10 min with or without different quantities of ethanol. The enzyme reaction was started with 50 μL of 0.5 mM L-[3-3H]serine (20 dpm/pmol) or 0.5 mM [2,14C]ethanolamine (10 dpm/pmol), continued at 37 °C for 20 min and then stopped by adding 6 mL of a chloroform/methanol (2/1 by vol.) mixture. Lipids were extracted by the method of Folch et al. (1957). In all experiments blanks were routinely “stopped before starting”, i.e. the chloroform/methanol mixture was added before the addition of the radioactive aminoalcohol.
PS and PE were separated by TLC using silica gel plates and chloroform/methanol/acetic acid/water (60/50/1/4 by vol.). Spots corresponding to PS or PE were visualized by iodine exposure and identified by the Rf values (0.60 for PE and 0.28 for PS) and by comparison with phospholipid standards; the corresponding spots were scrapped and transferred to scintillation vials to be measured by liquid scintillation. Under our experimental conditions the radioactivity in PS and PE accounted for more than 95% of the total radioactivity found in the organic phase of lipid extraction.

Study of the transbilayer distribution of newly synthesized PS and PE

In some experiments the transbilayer distribution of newly produced APL was examined. PS and PE produced by BES were synthesized as described above except that the final reaction volume was doubled. In these experiments the reactions were stopped by cooling the samples to 0 °C. The microsomes were then rapidly made to react with the non-penetrating chemical probe 2,4,6-trinitrobenzenesulphonic acid (TNBS) at 0 °C, which covalently labels the PE and PS molecules of the outer leaflet of the membrane, producing the corresponding trinitrophenyl-derivatives (TNP-derivatives). TNBS-bearing samples were protected from the light as much as possible. To this end, 1 mL of 340 mM NaHCO3 (pH 8.0) was added and the covalent labeling was done as described above. TNP-derivatives were separated from the non-reacted APL by TLC as described above.

Other analyses

Protein was estimated by the method of Lowry et al. (1951) using bovine albumin as standard. Results are expressed as the mean ± SEM for three different experiments. Statistical comparisons were made by Student’s t-test using the SPSS 9.0 program. Values of P<0.05 were considered to be statistically significant.

Results and discussion

Serine and ethanolamine base-exchange enzyme activities were assayed in parallel in rat-liver microsomes. As can be seen in Table 1, the specific activities of BES for PS and PE synthesis were quantitatively similar, confirming the capacity of this enzymatic system to use both aminobases. Other authors have reported that in vitro PSS1 can use choline, ethanolamine and serine as exogenous substrates for its base-exchange activity, producing PC, PE and PS, whereas PSS2 can use serine and ethanolamine but not choline (Saito et al., 1997).

The results of incubating rat-liver microsomes in the presence of varying concentrations of ethanol revealed that both the ethanolamine and serine base-exchange reactions were inhibited dose-dependently (Table 1). Interestingly, at 100 mM ethanol, a not unusual concentration in chronic alcoholism, the synthesis of PS and PE was reduced by up to 28% and 36%, respectively, compared to the control membranes.

Ethanol is known to modify considerably the activity of numerous membrane proteins (Carrasco et al., 2001; Sánchez-Amate et al., 1992; Wójcik et al., 2000). Its effects on membrane–enzyme activity are often related to an increase in membrane fluidity, as is also the case with other alcohols and local anaesthetics. It has also been postulated that ethanol may have a direct effect on proteins. There are no reports, however, on the effects of alcohol in vitro on the synthesis of PE via BES and conflicting results have been obtained when analyzing PS synthesis after long-term treatment: for example, chronic ethanol exposure decreased PS synthesis in C6 cells (Wójcik et al., 2000) and in the brains of rat pups (Hu et al., 1992).

Table 1

<table>
<thead>
<tr>
<th>Effect of ethanol in vitro upon serine and ethanolamine base-exchange activity</th>
<th>Phosphatidylycerine</th>
<th>Phosphatidylethanolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>29.23 ± 1.06</td>
<td>30.57 ± 1.91</td>
</tr>
<tr>
<td>Ethanol 50 mM</td>
<td>24.48 ± 2.32</td>
<td>22.61 ± 3.39</td>
</tr>
<tr>
<td>Ethanol 100 mM</td>
<td>21.20 ± 0.98</td>
<td>19.78 ± 1.35</td>
</tr>
<tr>
<td>Ethanol 200 mM</td>
<td>21.72 ± 1.53</td>
<td>13.52 ± 0.97</td>
</tr>
<tr>
<td>Ethanol 400 mM</td>
<td>12.69 ± 1.27</td>
<td>14.54 ± 0.84</td>
</tr>
</tbody>
</table>

Microsomes were preincubated with or without different quantities of ethanol. The activity of serine and ethanolamine base-exchange was determined by measuring the formation of phosphatidylserine and phosphatidylethanolamine, radiolabeled with [1-3H]serine (50 μM, 20 dpm/pmol) or [2-14C]ethanolamine (50 μM, 10 dpm/pmol) as described in Materials and methods. Results are expressed as pmoI/min×mg protein and are the mean ± SEM of three measurements. *p<0.02; **p<0.001 as compared to control values.
whereas it increased its synthesis in NG 108-15 cells (Rodríguez et al., 1996) and in hepatocytes isolated from alcoholic rats (Carrasco et al., 1996a). We have also reported previously that exposure of rat hepatocytes to ethanol increased the incorporation of radiolabeled serine into PS, although this effect was counteracted when the metabolism of ethanol was curbed by 4-methylpyrazole, an inhibitor of ADH (Carrasco et al., 1996b).

Since BES is the only mechanism by which PS can be synthesized in eukaryotes it is evident that an impairment of this biosynthetic pathway will lead to a deficiency in the level of arrangement of PS in the membrane, thence perturbing several processes essential for normal cell development and homeostasis. As far as PE is concerned, although few data exist concerning its synthesis by BES, it has been suggested that when synthesized by this pathway it may constitute an element of the superfine cell machinery involved in the repair of molecular species of phospholipids altered under oxidative stress. Since one of the most often reported effects of ethanol is the production of reactive species of oxygen and lipid peroxidation (Reinke, 2002), any inhibition of the formation of PE may contribute to the deleterious effects of alcohol on membranes and cell function.

Phospholipid synthesis in eukaryotic cells occurs on the cytoplasmic side of the ER, i.e. its synthesis is asymmetric. This implies that growth of the ER membrane, which is essential for cell proliferation, requires rapid phospholipid translocation. As we have already said, ethanol specifically alters the physical properties of microsomal membranes and the BES reaction, although no data exist concerning the possible effects of ethanol upon the phospholipid transbilayer movement. Thus we went on to analyze the synthesis and distribution of newly synthesized APL on both leaflets of the microsomes and the effect of ethanol upon this process. To this end we used TNBS, which covalently binds to the amine group of APL, producing the corresponding TNP-PE and TNP-PS.

As can be seen in Table 2, in control membranes the newly synthesized PE and PS, originally present only on the cytoplasmic side of the membrane, where they are produced, are transferred across the bilayer. This observation suggests that inward APL transport is a fast and energy-independent process. Interestingly the inward transport of PS seems to be more effective than that of PE, since newly synthesized PE became symmetrically distributed between both membrane leaflets, whilst more than 60% of the radioactivity of PS was found on the inner side of the ER membrane. This result contrasts with the marked asymmetry described for PE and PS in plasma membranes (Pomorski et al., 2004).

In a similar way, a rapid flip-flop has been reported in microsomal membranes at half-times of seconds to minutes (Buton et al., 1996) in a process that has proved to be ATP-independent and protein-mediated. It has been suggested that this flipping machinery could translocate most, if not all, of the phospholipid classes across the bilayer non-vectorially (Kol et al., 2002).

It is noteworthy that exposure to ethanol in vitro clearly alters the transbilayer distribution of newly synthesized APL in ER membranes (Table 2). Thus, because of the inhibition of the BES reaction produced by ethanol, the total incorporation of L-[3-3H]serine and [2-14C]ethanolamine was reduced significantly, the reduction being quantitatively different for PS and PE in both membrane leaflets. In this way the decrease in total PS synthesized was only reflected in a reduction in the levels of radioactive PS in the inner leaflet whilst the amount of PS in the outer one was not affected. As a consequence of this ethanol-produced alteration both leaflets contained the same amount of newly synthesized PS. On the other hand, the inhibition in PE synthesis is reflected in a decrease in labeled PE in both leaflets, although the levels of labeled PE at the internal surface of the membrane were more profoundly affected.

The asymmetric distribution of phospholipids in the plasma membrane is maintained and regulated by a specific aminophospholipid carrier protein, APL translocase (Pomorski et al., 2004). Translocase activity in erythrocytes has been found to be sensitive to certain membrane perturbations such as a variation in the cholesterol/phospholipid ratio or chlorpromazine incorporation (Rosso et al., 1988). Interestingly, some fluidizing treatments such as benzyl alchohol (Bassé et al., 1992) and ethanol (Schwichtenhovel et al., 1992) increase the rate of APL translocase activity and enhance the passive transverse diffusion of any type of phospholipid.

Two populations of glycerophospholipid flippase in rat-liver ER that are clearly different from plasma membrane APL translocase have been described recently (Chang et al., 2004). To our knowledge there are no reports concerning the effects of ethanol in vitro upon transverse distribution in biogenic membranes and so our results constitute the first experimental evidence that the exposure of ER membranes to ethanol causes a decrease in the inward movement of newly synthesized APL.

It is interesting to note that the phospholipid fatty acid composition of both membrane leaflets is different and thence the fluidity of individual leaflets is dissimilar. Since it has been reported that ethanol tends to fluidize the more fluid leaflet, information on the effects of ethanol upon the acylation and translocation of newly acylated APL is essential to supplement the above data if a cohesive picture is to be

Table 2: Effect of ethanol in vitro on the synthesis and distribution of newly synthesized aminophospholipids in both microsome leaflets

<table>
<thead>
<tr>
<th></th>
<th>Total incorporation</th>
<th>Inner leaflet incorporation</th>
<th>Outer leaflet incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylserine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.605±0.007</td>
<td>0.374±0.017</td>
<td>0.232±0.013</td>
</tr>
<tr>
<td>Ethanol 100 mM</td>
<td>0.424±0.020a</td>
<td>0.208±0.007*</td>
<td>0.216±0.014</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.443±0.008</td>
<td>0.204±0.014</td>
<td>0.239±0.009</td>
</tr>
<tr>
<td>Ethanol 100 mM</td>
<td>0.332±0.007*</td>
<td>0.134±0.015*</td>
<td>0.198±0.008*</td>
</tr>
</tbody>
</table>

Microsomes were preincubated with or without 100 mM of ethanol and then incubated with L-[3-3H]serine (50 μM, 20 dpm/pmol) or [2-14C]ethanolamine (50 μM, 10 dpm/pmol). The reaction was stopped by cooling the samples to 0 °C and the microsomes were then treated with TNBS at 0 °C. TNP-derivatives and unreacted APL were isolated as described in Materials and methods. Results are expressed as nmol/mg protein and are the mean±SEM of three measurements. *p<0.05; **p<0.005; *p<0.001 as compared to control values.
developed. Accordingly, in this study we have tried to determine the influence of ethanol upon the incorporation of palmitate and olate into PS and PE and the potential movement (flipping) of these APLs to the inner leaflet. Since the catalytic site of phospholipid acyltransferase is located in the outer leaflet of the microsomal vesicles our results demonstrate that once acylated in the outer leaflet PS and PE molecules are translocated to the inner leaflet, but the rate of translocation seems to depend upon the phospholipid class and the type of fatty acid incorporated.

The incubation of microsomes with radioactive fatty acyl-CoAs showed that $^{14}$C-palmitate-bearing species of PS tended to be situated in the inner leaflet whereas those containing $^{14}$C-oleate were located mainly in the outer one (Table 3). In fact, as can be deduced from the external/internal ratios, which give an indication of the apparent rate of transfer of the molecules from the outer to the inner leaflet (Table 4), the ratio of PS labeled with palmitate was 0.4, a value that was clearly lower than that of the 1.7 obtained for $^{14}$C-oleate PS species. These results indicate that under our experimental conditions the PS molecular species labeled with the saturated fatty acid in the control microsomes were translocated to the inner leaflet in preference to those containing olate.

The results set out in Table 5 show that newly acylated PE is translocated to the inner leaflet much less efficiently than PS and so in control microsomes incubated with radioactive palmitoyl-CoA the quantity of fatty acids incorporated into PE in the outer leaflet was about 2.3 times higher than that of the inner leaflet. In addition, the asymmetric distribution of olate-bearing species of PE was much more evident because radioactive PE appeared mainly upon the external surface of the membrane. In fact, the ratio of external/internal radioactive PE was higher than 13 (Table 4). In line with our results, Williams et al. (2000) have reported that PE in lipid vesicles is randomly distributed between the inner and outer leaflets of the bilayer, whilst the transbilayer distribution of molecular species of PE is not random and is influenced significantly by several different factors.

Exposure to 100 mM ethanol decreased the level of PS acylated with palmitate upon the inner surface (Table 3). As a consequence of this alteration, the external/internal ratio of PS was increased to a value of 0.6 compared to the value of 0.4 found in the control microsomes. On the other hand, ethanol produced a higher distribution of olate-bearing species of PS within the inner leaflet accompanied by a concomitant decrease within the outer leaflet, thus decreasing markedly the value of the corresponding ratio. From these results it could be seen that ethanol altered the rate of translocation in a specific way depending upon the fatty acid incorporated. Thus, it favours the transport of oleoyl-PS to the inner side whilst impeding the inward transport of palmitoyl-acylated PS. Bearing in mind therefore the distribution of these molecular species of PS in both the inner and outer leaflets of microsomes, the final result was that ethanol caused less asymmetry in their fatty acid composition.

With regard to the effects of ethanol upon the acylation process and the transbilayer movement of newly acylated PE molecules (Table 5), the incorporation of both palmitate and olate into PE was significantly reduced in microsomes exposed to ethanol when compared to the controls, suggesting that it inhibits the specific acyltransferase of PE. As for the translocation of PE, the effect of ethanol was different according to the fatty acid assayed. Thus, whilst the decrease in the levels of palmitic-bearing PE was reflected in a reduction in radioactive PE in both leaflets, $^{14}$C-olate-acylated PE species diminished only in the outer leaflet. As a consequence of these changes, the ratio of external/internal olate-bearing species of PE decreased significantly (Table 4).
In summary, we have shown that ethanol alters the array of PE and PS molecules and molecular species of these APLs present in both leaflets of the ER membranes. To our knowledge this is the first report providing information on the effects of ethanol upon the transport of specific molecular species of APL from the external to internal surfaces in rat-liver microsomes. This is a new way of viewing how ethanol acts upon membranes, inducing changes in lipid domains linked with specific membrane functions that could have profound effects on a number of critical cell processes.

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References


