Effects of Ethanol on Phospholipases in the Mouse Brain, Heart and Liver

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The activities of microsomal phospholipase A₂ (PLA₂) and C (PLC) from mouse brain, heart and liver were determined using the substrate 1-palmitoyl-2-*N*-(4-nitrobenzo-2-oxa-1,3-diazole amino caproyl)-phosphatidylcholine (NBD-PC), and the effects of chronic ethanol treatment (ethanol) as well as *in vitro* addition of various *n*-alcohols including ethanol on these activities were evaluated. Microsomal membrane fluidity was estimated by diphenylhexatriene anisotropy (γ). The microsomes from the brain and heart of ethanol-treated mice showed significantly higher PLA₂ activity than those from controls. The brains of ethanol group showed significantly higher PLC activity, while, the heart showed significantly lower PLC activity than those of controls. The microsomes from the brain and heart of ethanol-treated mice showed significantly reduced γ values compared to those of controls. The addition of ethanol *in vitro* to microsomes was found to increase PLC activity in these tissues, while it decreased PLA₂ activity in a dose-dependent manner. The other *n*-alcohols showed similar effects on PLA₂ and PLC activity in the liver microsomes, while decreases were observed the γ values in a dose-dependent manner. These results suggest that the change in the membrane fluidity associated with addition of alcohols is a prerequisite for the changes in PLA₂ and PLC activities. In addition, our findings suggest that these changes may play a major role in the cellular injury associated with chronic ethanol treatment in the mouse.

Key words ethanol; phospholipase A₂, C; membrane fluidity; mouse

Chronic ethanol intoxication leads to the development of resistance to membrane disruption in the order of membrane-lipid-protein by ethanol, a phenomenon known as "membrane tolerance." Membrane tolerance was first reported in brain synaptosomes,¹⁾ and it has since been found in a variety of tissues, including, for example, liver microsomal, mitochondrial, and plasma membranes^{2–6)} and erythrocyte membranes.⁷⁾ Membrane tolerance is also accompanied by reduction in the partitioning of ethanol and other lipophilic compounds in the membranes; ethanol enters the hydrophobic regions of the cell membrane causing an increase in membrane fluidity.³⁾ This change in fluidity may result from disturbance of the dynamic equilibrium of membrane phospholipids. Many membrane-bound enzymes are affected by their fluid environment, and membrane fluidity is positively correlated with membrane transport activity.⁸⁾ This effect of ethanol on membranes can contribute to calcium mobilization alteration, as has been demonstrated in erythrocytes, synaptosomes, and liver cell membranes.^{9–11)} Although membrane tolerance is a well-established phenomenon, its mechanism and compositional basis remain obscure.

Previously, we investigated the changes in microsomal, synaptosomal and mitochondrial phospholipid turnover in mouse and chick embryo brain, heart and liver tissue induced by chronic ethanol treatment.^{12–15)} However, changes in the quantity of each of these phospholipids have not been studied, and brain, heart and liver phospholipase A₂ (PLA₂) and C (PLC) activities have not been well characterized. Enzymatic activities are important in a variety of cellular functions in these organs, and those of PLA₂ and PLC are important in cellular regulation. In addition, both activities are sensitive to membrane lipid structure and organization, particularly at the surface. Activation of PLA and PLC by ethanol has been observed in several tissue membrane types.^{16–18)} In this study, we

demonstrated that brain, heart and liver microsomes from mice subjected to chronic ethanol intoxication show increased resistance to hydrolysis by endogenous PLA₂ and PLC compared with those from untreated controls, and this was due to the change in membrane fluidity.

MATERIALS AND METHODS

Materials 1-Palmitoyl-2-*N*-(4-nitrobenzo-2-oxa-1,3-diazole)amino caproyl-phosphatidylcholine (NBD-PC) was obtained from Avanti Lipids (Birmingham, AL) and NBD-amino caproic acid was from Molecular Probes (Eugene, OR). All other reagents used were of the purest grade available from commercial sources.

Animals Male ddY mice (Shimizu Laboratory Supplies Co., Ltd., Kyoto, Japan) initially weighing 26–28 g were given drinking water with or without 15% ethanol for 3 weeks.¹⁴⁾ Mice in both the ethanol-treated and control groups had free access to food (MF, Oriental Yeast, Tokyo, Japan).

Preparation of Microsomes Mouse brain, heart and liver microsomes were prepared and characterized as described previously.^{19–21)} Briefly, the brain (excluding the cerebellum), heart, and liver were quickly transferred to 8 volumes of buffer containing 0.25 M sucrose, 10 mM 3-(*N*-morpholino)propanesulfonic acid at 4°C and pH 7.5, and the homogenate was then centrifuged at 900 *g* for 10 min. The pellet was discarded, and the supernatant was centrifuged at 9000 *g* for 20 min. The supernatant was centrifuged at 105000 *g* for 60 min and the pellet was taken as the microsomal fraction. These microsomes were resuspended in incubation medium for determination of PLA₂ and PLC activities, and for measurement of γ values.

Phospholipase Assays PLA₂ and PLC activities were determined by measuring the release of NBD-amino caproic acid and NBD-amino caproyl glycerol (NBD-

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DG), respectively, from NBC-PC as described in our previous report.²²⁾ For microsomes, the concentration in terms of phospholipid content for a typical experiment was 100 μ M in 50 mM Tris-HCl, pH 7.4, 5 mM Ca^{2+} . NBD-PC (5 M) was added to resuspended subcellular fractions by injection with 20 μ l of methanol dispersion vehicle. The amounts of PLA_2 or PLC used were sufficient to hydrolyze not more than 5% of the phospholipids in the given reaction time at 37 °C. The reaction was stopped by the addition of chloroform-methanol (2:1, v/v), and the mixture was separated into the lower chloroform and upper aqueous phases. For determination of the release of NBD-DG, the chloroform phase was removed, evaporated, and resolved with a small volume of chloroform, then applied to a Silica gel G column (1.3 $\times$ 1.0 cm, corresponding to about 0.5 g dry Silica gel G) eluted with 3 ml of chloroform. The fluorescence intensity at 530 nm of the eluate corresponding to NBD-DG excitation at 470 nm was determined in an F-3010 spectrofluorometer (Hitachi, Ltd., Tokyo, Japan). A standard curve was obtained using standard NBD-DG which had been hydrolyzed from NBD-PC using PLC (Type 1 from *Clostridium perfringens*, *C. welchii*). For determination of the release of NBD-amino caproic acid from NBD-PC, an aliquot of the upper aqueous phase of the lipid extract was removed, and the fluorescence intensity at 530 nm excitation at 470 nm was determined. A standard curve was obtained using standard NBD-amino caproic acid which had been treated as for lipid extraction to correct for possible incomplete recovery in the aqueous phase, as described by Stubbs *et al.*¹⁶⁾ NBD-DG and NBD-amino caproic acid were not incorporated into phospholipids in the incubation medium that I used; this was true even at concentrations of NBD-DG and NBD-amino caproic acid equivalent to the stoichiometric amount if the entire amount of NBD-PC had been hydrolyzed, and for prolonged periods of incubation ranging up to 1 h. We concluded that reacylation and reincorporation of NBD-amino caproic acid into NBD-PC cannot occur, either due to the spontaneous ejection from the membrane due to its polar nature or because the reacylation enzymes lack the capacity to process NBD-amino caproic acid.

Fluorescence Anisotropy Measurement Fluorescence anisotropy was measured by a modification of the method described by Ives *et al.*²³⁾ Briefly, fluorescence anisotropy of diphenylhexatriene (DPH) in microsomes was measured using an F-3010 Fluorescence Spectrophotometer (Hitachi, Ltd., Tokyo Japan) with fixed excitation and emission polarization filters. Cuvette temperature was maintained to 37 °C in a circulating water bath. Excitation wavelength was 360 nm, and emission wavelength was 430 nm with 10 nm wavelength bands. Fluorescence anisotropy (γ) was calculated from the fluorescence intensities when the emission polarizer was oriented parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the vertically polarized excitation beam.

$$\gamma = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}}$$

G is the correction factor for the different transmission intensities of parallel and perpendicular polarized light

and is equal to $I_{\parallel}/I_{\perp}$, where the primed intensities are measured with horizontally polarized excitation light. In the brain, heart or liver microsomes, scattered light intensity was <5% and was subtracted from the total fluorescence intensity in every measurement. Solutions used for γ measurements consisted of <0.3 mg protein/ml and 2 M DPH in 250 mM sucrose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-tris(hydroxymethyl)aminomethane (Tris) at pH 7.4. For *in vitro* studies of DPH anisotropy in liver microsomes, the different n -alcohols at the concentrations indicated were added directly to the cuvette and stirred for 30 s. DPH was then added from a 1 mM stock solution in tetrahydrofuran (THF) and stirred for an additional 5 min. Longer incubation times with n -alcohols did not result in further alterations in fluorescence parameters. All fluorescence measurements were obtained at 37 °C.

Protein Assay The protein content of subcellular fractions was determined according to Lowry *et al.*²⁴⁾

Statistical Analysis Statistical significance was determined using Student's t -test.

RESULTS

Effects of Chronic Ethanol Administration on Microsomal PLA_2 and PLC Activities PLA_2 activity in the microsomal subcellular fractions from the brain, heart and liver of ethanol-treated and non-treated mice are shown in Fig. 1, and PLC activities are shown in Fig. 2. PLA_2 and PLC activities other than those of brain microsomes continued to increase after 30 min. To measure maximal enzyme activity rate, therefore, we chose 30 min as the duration for determination of endogenous PLA_2 and PLC activities.

The effects of chronic ethanol treatment on the hydrolysis of NBD-PC by endogenous PLA_2 activity in mouse brain, heart, and liver microsomes are shown in Table 1, and on that by endogenous PLC activity are shown in

Fig. 1. Time Courses of Phospholipase A_2 Activity of Brain, Heart and Liver Microsomes in Ethanol Treated and Non-treated Mice

Data represent means $\pm$ S.E.M. from 3 trials; pooled samples of 3 tissues were used per trial. The phospholipase A_2 activity in brain, heart and liver microsomes of ethanol non-treated mice are represented by ●, ▲ and ■, respectively, and those of ethanol-treated mice by ○, △ and □, respectively.

Fig. 2. Time Courses of Phospholipase C Activity of Brain, Heart and Liver Microsomes in Ethanol Treated and Non-treated Mice

Data from the experiment described in Fig. 1 are presented, and symbols used are defined in the legend for Fig. 1.

Table 1. Effects of Chronic Ethanol Administration on Mouse Brain, Heart and Liver Microsomal Phospholipase A₂ Activity

Treatment	Enzyme activity (nmol/30 min per mg protein)		
	Brain	Heart	Liver
Vehicle	2.22 ± 0.12	7.47 ± 0.21	6.82 ± 0.69
Ethanol	2.82 ± 0.18*	8.71 ± 0.23*	7.24 ± 0.19

Values are means ± S.E.M. of 5 experiments. * $p < 0.05$ when compared to vehicle.

Table 2. Effects of Chronic Ethanol Administration on Mouse Brain, Heart and Liver Microsomal Phospholipase C Activity

Treatment	Enzyme activity (nmol/30 min per mg protein)		
	Brain	Heart	Liver
Vehicle	2.67 ± 0.14	15.23 ± 0.37	2.35 ± 0.21
Ethanol	3.36 ± 0.21*	10.01 ± 0.49**	2.124 ± 0.21

Values are means ± S.E.M. of 5 experiments. * and **, significantly different at $p < 0.05$ and $p < 0.01$, respectively, when compared to vehicle.

Table 2. Brain and heart microsomes showed significantly higher PLA₂ specific activities than the corresponding controls. The brain microsomes showed significantly higher, while those of the heart showed significantly lower PLC specific activity than the corresponding controls.

Effects of Addition of Ethanol *in Vitro* on Microsomal PLA₂ and PLC Activities The effects of addition of ethanol *in vitro* to microsomal fractions from ethanol-treated and non-treated mice were also examined. PLA₂ activity in microsomal fractions showed a dose-dependent decrease which corresponded to the concentration of ethanol added (Table 3). On the other hand, PLC activity of the same microsomal fractions showed a dose-dependent increase (Table 4).

Table 3. Effects of Ethanol Added *in Vitro* on Phospholipase A₂ Activity of Microsomal Fractions from Ethanol Treated and Non-treated Mice

Added ethanol (M):		0	0.1	0.5	1
Brain	Vehicle	100.00 ± 9.91	99.10 ± 1.80	84.23 ± 3.15	75.23 ± 0.45
	Ethanol	100.00 ± 6.38	103.19 ± 2.84	92.20 ± 2.84	75.53 ± 9.57
Heart	Vehicle	100.00 ± 3.08	102.16 ± 1.61	90.50 ± 2.78	57.16 ± 3.48
	Ethanol	100.00 ± 2.64	101.49 ± 3.56	79.22 ± 2.76	58.67 ± 1.72
Liver	Vehicle	100.00 ± 9.12	95.64 ± 7.04	83.58 ± 6.01	71.99 ± 1.76
	Ethanol	100.00 ± 2.62	96.55 ± 4.83	86.46 ± 3.32	66.85 ± 4.14

Microsomal fractions from both ethanol treated and non-treated mouse brain, heart and liver were incubated with 0.1 to 1.0M ethanol at 37°C for 10 min prior to assay. The reaction was initiated by addition of the fluorescent substrate NBD-PC. Data from 5 experiments are expressed as percentages of the value prior to addition of ethanol for each tissue. Values are means ± S.E.M. Dose-dependency correlation coefficients ranged from $r = 0.86$ to $r = 0.99$.

Table 4. Effects of Ethanol Added *in Vitro* on Phospholipase C Activity of Microsomal Fractions from Ethanol Non-treated and Treated Mice

Added ethanol (M):		0	0.1	0.5	1
Brain	Vehicle	100.00 ± 5.09	136.78 ± 6.59	145.77 ± 5.99	146.59 ± 5.62
	Ethanol	100.00 ± 6.30	133.00 ± 7.43	146.08 ± 5.41	140.96 ± 6.30
Heart	Vehicle	100.00 ± 2.43	158.80 ± 6.73	174.29 ± 9.38	184.67 ± 7.78
	Ethanol	100.00 ± 4.90	139.76 ± 7.54	174.53 ± 5.89	167.68 ± 6.14
Liver	Vehicle	100.00 ± 9.01	158.54 ± 9.03	197.28 ± 9.22	230.59 ± 6.12
	Ethanol	100.00 ± 9.72	141.04 ± 9.02	234.72 ± 8.11	230.38 ± 6.23

Data from the experiment described in Table 3 are presented. Dose-dependency correlation coefficients ranged from $r = 0.88$ to $r = 0.99$.

Fig. 3. Effects of Chronic Ethanol on Microsomal Membrane Fluidity

Values are means ± S.E.M. of 5 experiments. *, significantly different from control value at $p < 0.05$.

Effects of Chronic Ethanol on Microsomal Membrane Fluidity The effects of chronic ethanol on the brain, heart and liver microsomal γ are shown Fig. 3. Microsomal fractions from the brain and heart of ethanol-treated mice showed significantly decreased γ values in comparison with controls, while no effect was seen in the liver.

Effects of Addition of Various *n*-Alcohols *in Vitro* on Liver Microsomal PLA₂ and PLC Activities, and the Membrane Fluidity The effects of the addition of some other *n*-alcohols to mouse liver microsomes *in vitro* on PLA₂ and PLC activities were also examined. Results were found to increase PLC activity, similar to those for the *in vivo* treatment with ethanol, while it decreased PLA₂

Fig. 4. Effects of Addition of Various *n*-Alcohols *in Vitro* on Mouse Liver Microsomal Phospholipase A₂ Activity

Data points are means $\pm$ S.E.M. of 5 trials. Enzyme activity is expressed as a percentage of initial activity. In the different experiments shown, initial phospholipase A₂ activity ranged from 2.9 to 4.1 nmol/30 min/mg protein. Methanol, acetaldehyde, ethanol, *n*-propanol, and *n*-butanol are represented by ○, △, ●, □ and ■, respectively. Dose-dependency correlation coefficients ranged from $r=0.80$ to $r=0.99$.

Fig. 5. Effects of Addition of Alcohols *in Vitro* on Mouse Liver Microsomal Phospholipase C Activity

Data from the experiment described in Fig. 3 are presented. In the different experiments shown, initial phospholipase C activity ranged from 4.4 to 6.3 nmol/30 min/mg protein. Dose-dependency correlation ranged from $r=0.86$ to $r=0.99$.

activity in a dose-dependent manner (Figs. 4 and 5).

The effects of the addition of some other *n*-alcohols to mouse liver microsomes *in vitro* on membrane the fluidity were also examined, and *n*-alcohol reduced the γ values (Fig. 6).

DISCUSSION

There are a number of possible explanations for the different rates of hydrolysis of NBD-PC in the membranes from ethanol-treated mice as compared to that in those from controls. These include: differences in the rate or extent of NBD-PC incorporation into the membranes; differences in the location of NBD-PC in the membranes; alterations in the lipid structure in terms of lipid order; and alterations in the lipid structure not involving lipid order changes. It is possible that the resistance to NBD-PC hydrolysis by endogenous PLA₂ and PLC may be affected by membrane lipid fluidity. The effects of chronic ethanol treatment on endogenous phospholipase hydrolysis of phosphatidylcholine or phosphatidylethanolamine in cellular fractions has previously been reported by Stubbs *et al.*¹⁶⁾ and by Spach *et al.*,²⁵⁾ who suggested that the lipid environment may have been modified. An increase in PLA₂ in synaptosomes²⁶⁾ has also been found

Fig. 6. Effects of Addition of Various *n*-Alcohols *in Vitro* on Mouse Liver Microsomal Membrane Fluidity

Data points are means $\pm$ S.E.M. of 5 trials. Anisotropy (γ) is expressed as a percentage of the initial γ value. In the different experiments shown, initial γ ranged from 0.2 to 0.3. Methanol, acetaldehyde, ethanol, *n*-propanol, and *n*-butanol are represented by ○, △, ●, □ and ■, respectively. Dose-dependency correlation coefficients ranged from $r=0.90$ to $r=0.99$.

to result from chronic ethanol treatment. The hydrolytic action of PLA₂ and PLC on phospholipid vesicles is regulated by a number of factors which together comprise the "quality of the interface".²⁷⁾ These include orientation of the phospholipids, molecular density and its changes, hydrous structure, and lipid order.²⁷⁾ At present, it is not possible to distinguish with certainty among these factors.

The majority of the ethanol derived from chronic ethanol treatment which was associated with the membrane fraction would have been lost during the preparation of the subcellular fractions. Hence, determination of PLA₂ and PLC activities in the microsomes after chronic ethanol treatment might not accurately reflect the degree of enzyme enhancement when ethanol was present *in vitro*. In addition, both PLA₂ and PLC activities and the γ value were changed by methanol, ethanol, *n*-propanol, and *n*-butanol *in vitro*. These results indicate changes in the membrane fluidity. On the other hand, the effect of ethanol on cAMP level was observed at pharmacological concentrations in some cells and appears to dependent mostly on stimulation of adenylate cyclase.²⁸⁾ However, we examined both PLA₂ and PLC activities by inhibiting adenylyl cyclase activity in ethanol-treated and non-treated mice with 2',3'-dideoxyadenosine or adenosine 3'-monophosphate and no differences were observed between ethanol-treated and non-treated mice (data not shown).

The findings in this study clearly demonstrate that the presence of ethanol caused reduction of endogenous PLA₂ activity in microsomes from the mouse brain, heart, and liver. However, the PLA₂ activity increased as a result of chronic ethanol treatment. These differences between the effect of ethanol treatment *in vitro* and chronic ethanol treatment *in vivo* on PLA₂ activity, although these fluidity was increased both *in vitro* and *in vivo*, indicate the involvement of unsaturated fatty acids and their metabolites in chronic ethanol-related disorders as reported by Pawlosky *et al.*²⁹⁾ and Engler *et al.*³⁰⁾ Also, the unsaturated fatty acid accumulation into ethanol-exposed fetal guinea-pig brain phospholipids has been reported³¹⁾ to be the result of the action of inappropriate mechanisms which counteract ethanol-induced increased membrane fluidity by reducing the polyunsaturated fatty

acids in the brain phospholipids. On the other hand, PLC activity in brain microsomes was increased by both ethanol treatment *in vitro* and by chronic ethanol treatment *in vivo*. In the heart, *in vitro* ethanol treatment increased, while chronic ethanol administration *in vivo* reduced PLC activity. Many kinds of PLC have been isolated and characterized from various mammalian tissue. Therefore, PLC isoforms from brain or heart may be affected differently by chronic ethanol treatment, activation of brain PLC and inhibition of heart PLC and may be important in the development and physiology of these tissues. *n*-Alcohols in liver microsomes *in vitro* decreased PLA₂ activity and increased PLC activity with increased membrane fluidity suggesting that these changes are not the specific responses to ethanol. Also, PLA₂ activity and PLC activity may be different because PLC is coupled to cell surface receptors known to promote phospholipid hydrolysis, whereas PLA₂ present as intracellular or extracellular enzymes with multiple isozymes in many species. These findings may reflect interactions at the membrane surface, since this is the region where PLA₂ and PLC interact primarily. Our findings also suggest that the PLA₂ and PLC activities which changed in response to chronic ethanol treatment play a major role in cellular injury in the brain, heart and liver of mice. In addition, our finding suggest that changes in the membrane fluidity associated with the addition of alcohols are prerequisite for the changes in PLA₂ and PLC activities.

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