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# Rats withdrawn from ethanol rapidly re-acquire membrane tolerance after resumption of ethanol feeding

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**The time course for the re-acquisition of membrane tolerance to the disordering effects of ethanol in vitro has been determined for liver microsomes obtained from chronically ethanol-fed rats that were withdrawn from ethanol for 2–4 days (during which tolerance is lost) followed by resumption of ethanol feeding. Naive rats require 28–35 days of chronic ethanol feeding to develop membrane tolerance. Microsomal membranes regain partial sensitivity to ethanol disordering after 2–3 days of withdrawal and regain the complete sensitivity observed in membranes from untreated control rats after 4 days of withdrawal. The period of ethanol re-feeding required for the reacquisition of membrane tolerance was dependent on the withdrawal period, with tolerance appearing sooner if the withdrawal period was shorter. The time course for the re-development of tolerance in previously tolerant animals was considerably faster (4–14 days) than in naive rats being administered the ethanol diet for the first time (35 days). Microsomes from rats that were withdrawn for 2 days (which retained partial tolerance) and then re-fed the ethanolic-diet required only 4 days to re-acquire membrane tolerance. Microsomes from rats withdrawn 3 days required 8 days and those withdrawn 4 days required 15 days for full tolerance to re-develop. The same timecourse for the re-acquisition of membrane tolerance was observed in either intact microsomes or in liposomes prepared from extracted mircrosomal total phospholipids. Phosphatidylinositol (PI) has previously been reported to be responsible for conferring membrane tolerance to liver microsomes in ethanol-fed rats (Taraschi, T.F., Ellingson, J.S., Wu, A., Zimmerman, R. and Rubin, E. (1986) Proc. Natl. Acad. Sci. USA 83, 9398–9402). The time course for re-acquisition of membrane tolerance by liver microsomes following ethanol withdrawal and resumption of ethanol feeding correlated with the ability of PI to confer tolerance.**

## Introduction

In our continuing studies to understand cellular adaptation to chronic ethanol ingestion we have determined the timecourse for the re-development of membrane tolerance to disordering by ethanol in vitro after a defined period of withdrawal. The membranes of a variety of cells and subcellular organelles have been shown to be disordered (fluidized) by physiologically relevant concentrations of ethanol in vitro [1–5]. By contrast, membranes from animals chronically fed ethanol develop resistance to disordering by ethanol in vitro (membrane tolerance) [1–3,6,7]. A temporal correlation between the development of membrane tolerance and of functional tolerance, e.g., the ability of an animal to balance itself on a dowel stick has been reported [8]. We have previously reported that 28–35 days of ethanol feeding are required for rat liver, microsomes and erythrocytes to develop membrane tolerance, whereas microsomes lose this property after only 4 days of ethanol withdrawal and erythrocytes after 1 day [3]. It is noteworthy that functional tolerance is also rapidly lost after ethanol withdrawal [9].

We have recently reported that modifications of specific phospholipids in rat liver microsomes (PI) and liver mitochondria (cardiolipin) are responsible for the development of membrane tolerance to ethanol disordering [1,10]. However, the factors which confer this potent property to these phospholipids and the biochemical mechanisms governing these conversions are not understood. We, therefore, investigated the time course for re-acquisition of membrane tolerance by liver microsomes after defined periods of withdrawal in an effort to provide a well-defined model to investigate these membrane adaptations at the molecular level. We report that membrane tolerance is rapidly and abruptly reacquired by liver microsomes upon resumption of ethanol feeding and that the re-acquisition of tolerance

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is dependent on the period of withdrawal and modifications in PI.

## Materials and Methods

*Animals.* Male, Sprague-Dawley rats ingested 14–16 g of ethanol/kg body weight in a nutritionally adequate diet for 35 days, while pair-fed controls consumed the same diet, except that carbohydrate isocalorically replaced ethanol [1]. Rats were then withdrawn from ethanol feeding for 2–4 days, during which time they received the control diet. Following withdrawal, the rats were again placed on the ethanol-containing diet for up to 20 days.

*Preparation of membranes.* Hepatic microsomes were prepared by differential centrifugation, as described previously [2].

*Isolation and analysis of phospholipids.* Total lipids were extracted according to Bligh and Dyer [11]. Phospholipids were separated from neutral lipids on a silicic acid column [3], and stored in $CHCl_3$ at $-20\,^{\circ}C$ under $N_2$. All procedures were performed under $N_2$ in order to prevent lipid oxidation.

*Separation of Phospholipids by preparative high pressure liquid chromatography (HPLC).* Extracted microsomal phospholipids were separated by HPLC as previously described [1]. The purity of the separated phospholipids was confirmed by chromatography on $5 \times 5$ cm HPTLC Silica gel 60 plates, using the solvent system methyl acetate/*n*-propanol/ chloroform/ methanol/ 0.25% aqueous KCl (25 : 25 : 25 : 10 : 9, by vol.). Phospholipids were identified by $I_2$ vapor and ninhydrin, Dragendorf's and phosphate-detecting spray reagents [12], and comparison of their $R_F$ values with those of standard phospholipids (Avanti Polar Lipids, Inc., Birmingham, AL). Lipid phosphate was determined according to Bartlett [13].

*Spin labeling of microsomes.* Microsomes were labeled with a spin-labeled fatty acid, $\beta$-12-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)stearic acid (12-doxylstearic acid) (Ste 12) as described previously [1–3]. The spin-labeled phospholipid, 1-palmitoyl-2-(12-doxyl-stearoyl)phosphatidylcholine (PC 12) was synthesized and incorporated into the microsomal membrane according to established procedures [2]. The final membrane/lipid probe ratio in all of the labeled membranes was 120 : 1, mole/mole.

*Preparation and spin labeling of multilamellar vesicles.* Multilamellar vesicles were prepared by (a) drying of a chloroform solution containing the desired phospholipid mixture with nitrogen, (b) high vacuum dessication of the dried lipid film for 30 min, and (c) hydration with phosphate-buffered saline (pH 7.2). The fatty acid probe, SA 12, and the phospholipid spin label, PC 12, were incorporated into the reconstituted membranes as described previously [1,3]. The ratio of membrane phospholipid/spin probe was 120 : 1, mol/mol.

*Electron spin resonance (ESR) spectroscopy.* The spin labeled sample, following the addition of the appropriate amount of ethanol, was vortexed for 2 min at room temperature, transferred to a 100 $\mu$l capillary tube and flame sealed. Capillary tubes were placed in an IBM Instruments ER 200D ESR spectrometer cavity in a standard 4 mm quartz tube, which contained silicon oil to maintain thermal stability. All samples were equilibrated for 7 min in the cavity at $37\,^{\circ}C$, prior to recording the spectrum. Temperatures were accurate to $0.5\,C^{\circ}$. Spectra were accumulated with an IMB 9000 computer interfaced to the spectrometer. The molecular order parameter, $S$, was calculated as described previously [1–3]. Typical spectrometer settings were: spectral scan, 100 G, modulation amplitude, 1.0 G, and microwave power, 5 mW. All measurements were made at $37\,^{\circ}C$.

## Results

*Time course for the re-development of membrane tolerance after withdrawal*

The addition of physiologically relevant amounts (with respect to the liver, e.g., 50–100 mM) of ethanol in vitro to liver microsomes or liposomes prepared from their extracted phospholipids causes significant membrane disordering [1–3]. The molecular order parameter, $S$, derived from the ESR spectrum, provides a measure of membrane order, since the motionally averaged spectral hyperfine splittings are influenced by the angular amplitude and rate of motion experienced by the lipid hydrocarbon chains bearing the spin label group. Molecular disordering in the presence of ethanol is characterized by an increase in the spectral splitting $2T_{\perp}$ and a decrease in $2T_{\parallel}$ leading to a decrease in $S$, an affect that reflects the greater angular displacement of the lipid fatty acyl chains from the long molecular axis of the membrane bilayer. By contrast, the order parameters determined for liver microsomes from ethanol-fed rats or their corresponding phospholipid liposomes are unchanged over a range of ethanol concentrations up to 100 mM. This condition, which we term membrane tolerance, requires a period of 28–35 days of chronic ethanol feeding for its development [3].

Membrane tolerance in liver microsomes is completely lost in only four days when rats are withdrawn from ethanol [3]. In rats withdrawn from ethanol for 2 days, liver microsomes are partially tolerant, that is they are resistant to disordering by $< 50$ mM ethanol, but are fluidized by higher concentrations (100 mM), although not to the same extent as membranes from controls [3]. The re-acquisition of membrane tolerance was studied in rats that were fed ethanol for 35 days, withdrawn for 2, 3 or 4 days and again fed the

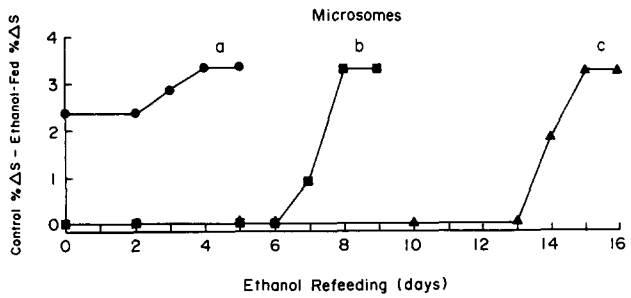


Fig. 1. Typical profile for the reacquisition of membrane tolerance by liver microsomes upon readministration of ethanol after 2 (●———●), 3 (■———■) and 4 (▲———▲) days of withdrawal. Rats were fed the ethanol diet for 35 days, withdrawn for the 2–4 days and re-fed the ethanol diet for up to 20 days. Liver microsomes were labeled with Stel2 and ESR spectra were recorded in the absence and presence of 100 mM ethanol at 37°C. Control % $\Delta S$ represents the percent difference in the order parameters, $S$, obtained for liver microsomes from control rats between 0 and 100 mM ethanol. Re-fed ethanol % $\Delta S$ represents the percent difference in the order parameters, $S$, between 0 and 100 mM ethanol for rats re-fed the ethanol diet after withdrawal. When Control % $\Delta S$ – Re-fed ethanol % $\Delta S = 0$, the microsomes from the rats re-fed ethanol are fully disordered. When Control % $\Delta S$ – Re-fed ethanol % $\Delta S = 3.4$, the microsomes from the rats re-fed ethanol are fully tolerant.

ethanol-containing diet (Fig. 1). Fig. 1a shows that liver microsomes from ethanol-fed rats withdrawn from ethanol for 2 days, which still retain partial tolerance to 100 mM ethanol, re-establish complete membrane tolerance within 4 days of ethanol refeeding. In rats withdrawn for 3 days, 8 days of ethanol consumption were required to re-establish membrane tolerance (Fig. 1b). In rats withdrawn for 4 days, after which full loss of membrane tolerance occurs [3], 15 days of ethanol feeding were needed to acquire membrane tolerance (Fig. 1c). It is clear that membrane tolerance arises from alterations in the phospholipid moiety of microsomal membranes, since the timecourse for the redevelopment of tolerance in intact microsomes was paralleled in liposomes prepared from extracted phospholipids (Fig. 2).

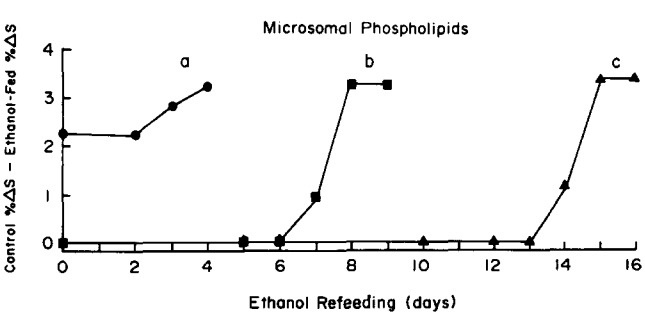


Fig. 2. Typical time-course for the re-appearance of membrane tolerance in liposomes composed of liver microsomal phospholipids from rats withdrawn for 2 (●———●), 3 (■———■) or 4 (▲———▲) days and re-fed ethanol for up to 20 days. Experimental conditions and ESR analysis were as described in Fig. 1.

Surprisingly, the re-development of membrane tolerance in these studies is abrupt rather than gradual. In animals withdrawn for 3 days, tolerance abruptly appears between the sixth and eighth days of ethanol re-feeding (Fig. 1b, 2b). The change in the order parameter caused by the in vitro addition of 100 mM ethanol to microsomes or liposomes of microsomal phospholipids labeled with either Ste 12 or PC 12 after up to 6 days of ethanol re-feeding indicated that the membranes were disordered to the same extent (Control % $\Delta S$ – Ethanol-fed % $\Delta S = 0$) as microsomes from control rats. After 7 days of resumed ethanol ingestion, the microsomes exhibited partial resistance to disordering by 100 mM ethanol (Control % $\Delta S$ – Ethanol-fed % $\Delta S = 0.9$) and were fully tolerant after 8 days of re-feeding (Control % $\Delta S$ – Ethanol-fed % $\Delta S = 3.3$). In rats withdrawn for 4 days partial tolerance became visible at day 14 and full tolerance at day 15. These results demonstrate: (1) the time required for tolerance to re-appear after ethanol withdrawal and re-feeding is dependent on the length of the withdrawal period and (2) after an initial induction period, tolerance abruptly re-develops over a two day period.

*Phospholipid(s) responsible for membrane tolerance*

We have previously reported that as little as 2.5 mol% PI extracted from liver microsomes of ethanol-fed rats confers membrane tolerance [1]. We embarked upon a study, therefore, to determine if alterations in PI are responsible for the re-acquisition of tolerance after re-sumption of ethanol feeding.

Ethanol-fed rats were withdrawn from ethanol for 3 days and re-fed the ethanol containing diet for 1–8 days. Liposomes were then prepared from extracted microsomal phospholipids. Liposomes composed of phospholipids from control animals were disordered by 100 mM ethanol as were liposomes prepared from rats re-fed ethanol for 6 days. The liposomes from the rats re-fed ethanol for 7 days were disordered by 100 mM ethanol to a lesser extent (Control % $\Delta S$ – Ethanol-fed % $\Delta S = 1.0$) compared to controls, whereas liposomes from rats re-fed 8 days were tolerant to disordering by 100 mM ethanol (Control % $\Delta S$ – Ethanol-fed % $\Delta S =$ 3.3). Liposomes were made by recombining all the individual microsomal phospholipids from the rats re-fed for 6 days, except that in each preparation one different phophoslipid class (PC, PE, PS or PI) was omitted. The missing phopholipid was replaced by an equal amount of the corresponding phospholipid class from the rats re-fed ethanol for 7 or 8 days. The only lipid that conferred membrane tolerance to the liposomes from rats re-fed ethanol was PI. PI from the rats re-fed ethanol for 7 days conferred only a partial tolerance (Control % $\Delta S$ – Ethanol-fed % $\Delta S = 1.2$) whereas after 8 days, PI provided complete protection from membrane disordering by 100 mM ethanol (Fig. 3). PC,

PE and PS from rats re-fed ethanol did not promote membrane tolerance.

In similar experiments with rats withdrawn 4 days and re-fed ethanol 13–15 days, the only lipid that conferred tolerance was PI. After 14 days, partial tolerance to 100 mM ethanol in vitro was conferred by PI and after 15 days complete tolerance was noted (Fig. 4).

## Discussion

The cells in the major body organs of animals chronically-fed ethanol manifest a variety of functional and structural alterations indicative of cellular adaptation [14,16]. Our understanding of the mechanism(s) governing these adaptations, while still poor, has become somewhat clearer in recent years. The initial acquisition of membrane tolerance by naive rats, loss of tolerance during withdrawal and re-acquisition of tolerance upon resumption of ethanol feeding all appear to be highly regulated processes. Although the initial acquisition of tolerance requires 28–35 days, full tolerance abruptly appears over a period of a few days [3]. Tolerance is rapidly and abruptly lost after 1–4 days of withdrawal depending on the membrane. Interestingly, tolerance is acquired much more quickly by previously tolerant rats than naive rats. This adaptive process also abruptly appears over a period of a few days. The time required for re-acquisition of membrane tolerance is dependent on the period of withdrawal; rat withdrawn for 3 days, which causes partial loss of tolerance, require 8 days and rats withdrawn 4 days require 15 days.

We have previously reported that membrane tolerance in liver microsomes was conferred by modifications in PI [1]. In this study, we have demonstrated using reconstituted liposomes, that the re-acquisition of membrane tolerance following withdrawal and resumption of ethanol feeding correlates with the ability of PI to cause tolerance. PI from rats withdrawn 3 days and re-fed ethanol for 7 days induced partial tolerance and PI from rats re-fed ethanol for 8 days caused complete tolerance. At day 7, the PI is modified to an extent

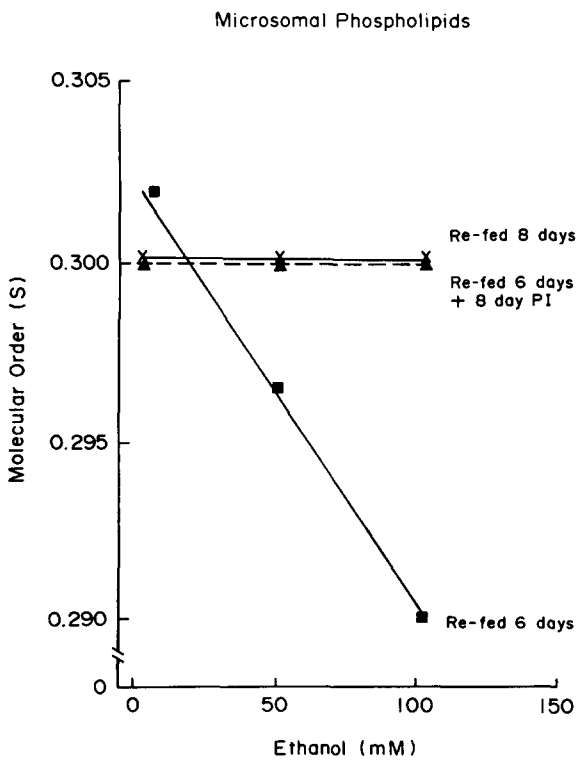


Fig. 3. Temporal correlation of the reacquisition of membrane tolerance with the ability of PI to confer tolerance to recombined phospholipid liposomes. Following 35 days of ethanol feeding, the rats were withdrawn for 3 days and re-fed ethanol up to 10 days. The order parameters in the presence of the ethanol concentrations indicated are shown for liposomes made of microsomal phospholipids from animals re-fed ethanol for 6 days (■———■) and 8 days (▲— — —▲) recombined in their naturally occurring molar ratios (66.5% PC, 21.0 % PE, 8.5% PI and 4.0% PS) and for liposomes composed of 6 day phospholipids, except that 8.5% PI from rats re-fed 8 days replaced PI from rats re-fed 6 days (×——— ×). The same results were obtained with the lipids from three other rats re-fed ethanol for 6 and 8 days.

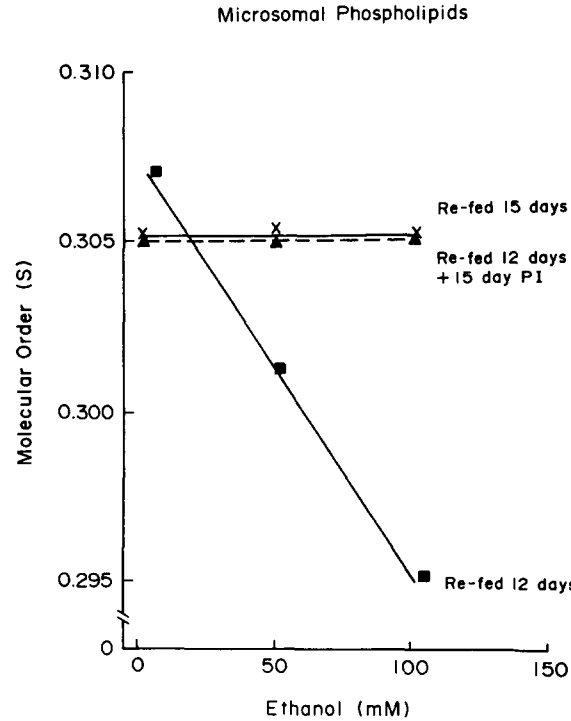


Fig. 4. Temporal correlation of the re-acquisition of membrane tolerance with the ability of PI to confer tolerance to recombined phospholipid liposomes. Rats were fed ethanol for 35 days, withdrawn for 4 days, and re-fed ethanol for up to 20 days. The order parameters in the presence of the indicated concentration of ethanol are shown for liposomes labeled with Stel2 and are composed of phospholipids from rats re-fed ethanol for 12 (■———■) and 15 days (▲— — —▲) recombined in their naturally occurring molar ratios and for liposomes containing 12 day phospholipids, except that 15 day PI replaced 12 day PI (×——— ×). The same results were obtained with the lipids from three other rats re-fed ethanol for 12 and 15 days.

where it can induce partial tolerance but is not sufficiently modified to cause complete tolerance. This final modification requires 1 further day of ethanol ingestion. In the animals withdrawn for 4 days, PI produces partial tolerance at day 14 of ethanol re-feeding and complete tolerance at day 15. Thus, in both cases, the conversion of PI from a phospholipid not able to confer tolerance to one which causes complete tolerance to disordering by 100 mM ethanol in vitro occurs over a well-defined 2-day period. A significant portion of the PI molecules could be replaced during the 2-day period since the half-life of the glycerol backbone of hepatic microsomal phospholipids is 30–40 h [15].

The mechanism by which PI is modified and the nature of the modifications remain to be identified. The re-acquisition of tolerance is a much faster process in previously tolerant rats as opposed to naive rats suggesting that the metabolic processes responsible for modification of the membrane phospholipids, particularly PI, are not completely dismantled or inactivated following 3 to 4 days of ethanol withdrawal. The processes that compensate for the chronic presence of ethanol by producing tolerance could be 'triggered' when the cell is again exposed to ethanol after a period of withdrawal. The enzymatic systems which produces tolerance must be rapidly converted to an inactive form or partially degraded during withdrawal, since increasing the withdrawal time by 1 day (i.e., from 3 to 4 days) increases the time to re-acquire tolerance twofold (i.e., from 7 to 14 days).

The timecourse for re-acquisition of tolerance after 3 or 4 days of withdrawal is characterized by an initial induction period where the membranes are fully disordered by 100 mM ethanol in vitro and the sudden appearance of tolerance over a 2-day period. It should be pointed out that this process may not be as abrupt as it seems. During the induction phase, modification of PI may proceed with each day of ethanol feeding, but not cause a large enough modification which can be detected by our ESR measurement. However, after 7 or 14 days of ethanol refeeding, depending on the period of withdrawal, enough of the PI may be modified to cause complete tolerance. We have previously reported that inclusion of at least 2.5 mol% of PI (which is approx. 30% of its naturally occurring amount in microsomes) from ethanol-fed rats into liposomes comprised of phopholipids from control rats was necessary to produce complete membrane tolerance to disordering by 100 mM ethanol. In any event, once the chemical and/or structural modifications of PI which render it a strong promoter of tolerance have been identified, the reproducible, well-defined model described in this study will provide the vehicle to identify the biochemical basis of these modifications.

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