Biochimica et Biophysica Acta, 1062 (1991) 199–205

ADONIS 000527369100116U

BBAMEM 75140

The effect of chronic ethanol consumption on the fatty acid composition of phosphatidylinositol in rat liver microsomes as determined by gas chromatography and ^{1}H-NMR

John S. Ellingson, Nathan Janes, Theodore F. Taraschi and Emanuel Rubin

Department of Pathology and Cell Biology, Medical College of Thomas Jefferson University, Philadelphia, PA (U.S.A.)

(Received 27 September 1990)

Key words: Phosphatidylinositol; Ethanol; Membrane tolerance; Fatty acid; NMR, ^{1}H-; Microsome; Phospholipid; (Rat liver)

Cell membranes and vesicles composed of extracted phospholipids isolated from rats chronically-fed ethanol develop a resistance to disordering by ethanol in vitro (membrane tolerance) and a decreased partitioning of ethanol into the membranes. The anionic lipid phosphatidylinositol (PtdIns) is the only microsomal phospholipid from the ethanol-fed rats that confers tolerance to vesicles of microsomal phospholipids from control rats in a paradigm where phospholipid classes are sequentially swapped. To investigate the molecular basis of this adaptation, the fatty acid content of microsomal PtdIns extracted from the livers of rats chronically fed ethanol for 5 weeks and their calorically-matched controls was analyzed by gas-liquid chromatography (GLC) and ^{1}H-NMR spectroscopy. Chronic ethanol consumption caused an 8.4% decrease in arachidonic acid [20:4($n-6$)], a 20.0% increase in oleic acid [18:1($n-9$)] and a 47.1% increase in the quantitatively minor fatty acid, eicosatrienoic acid [20:3($n-6$)]. ^{1}H-NMR was used to quantitatively assay compositional changes in the Δ^5 olefinic moiety of the acyl chains in PtdIns, an approach that should be broadly applicable to other lipid systems. After chronic ethanol feeding PtdIns had decreased Δ^5 unsaturates (−7.9% NMR, −8.2% GLC) and a corresponding increase in Δ^5 saturates (+5.4% NMR, +5.3% GLC). In the other phospholipids, chronic ethanol feeding caused alterations in the fatty acid compositions specific for each phospholipid. PtdIns was the only microsomal phospholipid that exhibited a significant decrease in both the polyunsaturate pool and the ratio of the total olefinic content to the saturated fatty acid content. The major adaptive response in rat liver microsomal PtdIns to chronic ethanol administration involves a decrease in arachidonic acid [20:4($n-6$)], which is partly compensated for by increases in oleic acid [18:1($n-9$)] and eicosatrienoic acid [20:3($n-6$)], resulting in a depressed unsaturation and polyunsaturation index. The decreased unsaturation at the Δ^5 position may have special functional relevance, due to the proximity of this position to the membrane surface, where ethanol is believed to reside. Whether these acyl changes are merely coincident with, or causative of, membrane tolerance requires further elucidation.

Introduction

Membrane lipids were implicated in the actions of ethanol and anesthetics when Meyer and Overton correlated anesthetic potency with olive oil-water partitioning at the turn of the century [1]. To date, the precise molecular basis of the interaction of ethanol and anesthetics with membrane lipids has yet to be established. In the late 1960s the ability of ethanol and anesthetics to disorder (fluidize) membranes was demonstrated by Hubbell and McConnell [2] using electron paramagnetic resonance (EPR), and subsequently, by others, using fluorescence polarization [3]. Subsequently Chin and Goldstein [4] discovered that brain synaptosomes and erythrocyte membranes of mice chronically exposed to ethanol adapted by developing a resistance to the ethanol-induced disordering, a phenomeon termed 'membrane tolerance'. Development of membrane tolerance to disordering by ethanol has since been observed by EPR in rat erythrocytes, pancreatic plasma

Abbreviations: PtdIns, phosphatidylinositol; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; CL, cardiolipin; NMR nuclear magnetic resonance; EPR, electron paramagnetic resonance; GLC, gas-liquid chromatography; UI, unsaturation index; PUFA, polyunsaturated fatty acids.

Correspondence: J.S. Ellingson, Department of Pathology and Cell Biology, Medical College of Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107, U.S.A.

membranes, and hepatic mitochondrial and microsomal membranes [5–9]; and in brain synaptosomes by fluorescence polarization [10]. Evidence of altered structure is also noted by a reduced partitioning of ethanol and other anesthetics into tolerant membranes [11]. Tolerant membranes *in vitro* and vesicles prepared from their extracted phospholipids are less susceptible to hydrolysis by exogenous phospholipase A_2 [12], suggesting that the phospholipids are reorganized in an arrangement less accessible to the enzyme.

To identify the molecular basis of the biological adaptation of membranes to ethanol, we developed an *in vitro* system to directly test the ability of individual phospholipids to confer membrane tolerance to phospholipid vesicles. This experimental system has revealed that the anionic phospholipids are important agents in the development of membrane tolerance. In ethanol-fed rats, PtdIns from liver microsomes [6] and cardiolipin (CL) from hepatic mitochondria [7,8] confer tolerance to phospholipid vesicles at levels as little as 3 mol%; a property not dependent on the source of the other phospholipids in the vesicles [6,8]. By contrast, the quantitatively major non-anionic phospholipids, PtdCho and PtdEtn, do not confer tolerance at their much higher naturally occurring levels of 65 and 22 mol%, respectively.

The alterations of CL in hepatic mitochondria include a significant decrease in linoleic acid content and an increase in oleic acid [7,8,13]. The changes in PtdIns have not been well established. A 13.6% increase in the arachidonic acid content of liver microsomal PtdIns from rats fed ethanol for 31 days has been reported [13]. The amount of arachidonic acid reported was 22%, a value lower than expected for PtdIns. The opposite trend, a 10% decrease in arachidonic acid content was reported in the total PtdIns pool from livers of rats fed ethanol for 3 weeks [14].

In our investigations to identify structural changes in PtdIns, ^{1}H-NMR of the PtdIns indicated that there was an alteration in the C-5 position of the acyl chains. The unsaturated Δ^5 content decreased and the saturated Δ^5 content increased following chronic ethanol administration. This change was opposite to that reported previously for microsomal PtdIns [13]. Based on this observation, we have undertaken a more quantitative fatty acid analysis by traditional methods to elucidate the origins of the NMR change, and to validate our adaptation of the NMR method. We have used ^{1}H-NMR and gas-liquid chromatography to measure the alterations in PtdIns from rats and their pair-fed littermates which had consumed ethanol for five weeks. The combination of the two methods has allowed us to identify changes in the fatty acids present and to monitor broad positional alterations in the acyl environment, based on the differences in the electronic shielding of the acyl protons.

Methods

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC or Zivic-Miller Laboratories, Inc., Allison Park, PA) initially weighing 100–130 g were fed for 35 days a totally liquid diet (Bio-Serv, Frenchtown, NJ), in which ethanol comprises 36% of total calories [15]. Pair-fed littermate controls received the same diet except that carbohydrates isocalorically replaced ethanol. Ethanol consumption averaged 12–17 g/kg weight per day. Blood alcohol levels were determined by use of an alcohol dehydrogenase assay kit (Sigma Chemical Co., St. Louis, MO) and ranged from 25 to 50 mM.

Preparation of membranes. Rat liver microsomes were prepared and characterized as described previously [16].

Extraction, separation and analysis of lipids. Lipids were extracted by the Bligh and Dyer procedure [17]. Phospholipids were separated from neutral lipids by silicic acid chromatography [18] and stored under N_2 in $CHCl_3$ at $-20°C$. The extraction solvents, which contained 0.01% butylated hydroxytoluene to prevent oxidation of the fatty acids, were flushed with N_2. Phospholipid phosphate was determined by the Bartlett procedure [19].

To minimize oxidation of the polyunsaturated fatty acids during the lipid separation, all procedures were conducted under nitrogen. All solvents were bubbled with helium (HPLC) or nitrogen. In no instance did the lipids exhibit the characteristic 233 nm absorbance for oxidation [20], and UV/visible absorption spectra were identical for the pair-fed isolates.

Phospholipids (80–120 μmoles) were separated on a 250 × 25 mm Merck Hibar RT preparative HPLC column packed with 7-μm LIChrosorb-Si-60 silica particles (EM Science, Cherry Hill, NJ) using the gradient procedure previously described [21]. As reported earlier [6], phospholipid recovery was greater than 96%, and the distribution of phospholipid classes was as expected and did not vary after ethanol feeding.

Fatty acid analysis. Fatty acid methyl esters were prepared with 12% BF_3 in methanol [22]. They were separated by capillary gas-liquid chromatography using a Hewlett-Packard 5890A gas-liquid chromatograph and a fused silica, highly polar biscyanopropylphenyl polysiloxane stabilized phase SP-2380 column (30 m × 0.32 mm i.d., 0.2 μm film thickness) (Supelco, Bellefonte, PA). The column temperature was linearly programmed from 170°C to 195°C at 3 C°/min, with initial and final times of 3 and 20 min, respectively. The injector and detector temperatures were 250°C. Methyl esters were analyzed using a Hewlett Packard 3393A computing integrator to calculate the area percent of each fatty acid. The integrator response was checked daily using methyl ester standards (Supelco and Applied Science, State College, PA). Fatty acid methyl esters were identi-

fied by comparison of retention times of standards and/or equivalent chain lengths.

Hydrolysis of PtdIns by phospholipase A_2. Up to 2 μmol of PtdIns was incubated for 2 h at 34°C with 3.2 mg porcine pancreatic phospholipase A_2 (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in 2 ml of 0.1 M borate buffer (pH 7.1) containing 3 mg deoxycholate per μmole PtdIns and 2.5 mM $CaCl_2$. The reaction was stopped with 0.5 ml of 0.1 M EDTA, and the lipids were extracted by the addition of 5.0 ml of chloroform/methanol (2:1, v/v). The reaction was monitored by TLC and the PtdIns from control and ethanol-fed rats was completely hydrolyzed in 2 h. The organic phase was evaporated under N_2, and the fatty acids, lyso-PtdIns and deoxycholate were separated on HPTLC Si-60 plates with a preconcentration zone (E. Merck), using a three step solvent system. The first solvent system, acetone/hexane (1:3, v/v), was allowed to travel 6.5 cm; the second system, chloroform/methanol/acetic acid/water (80:13:8:0.3, by vol), 5 cm; and the third solvent system, methyl acetate/*n*-propanol/chloroform/methanol/0.25% KCl (12.5:12.5:12.5:5:4.5, by vol), 3 cm. The plates were dried for 15 min between solvent runs. The areas containing fatty acids and lyso-PtdIns were scraped from the plates and the fatty acid methyl ester were prepared. The fatty acids in position *sn*-1 were determined by analyzing the fatty acid composition of lyso-PtdIns produced, and the fatty acids from the *sn*-2 position were determined by identifying the fatty acids released.

Nuclear magnetic resonance. Spectra were obtained on a Bruker 8.5T AM spectrometer (Billerica, MA) operating at 360 MHz, at 298 K using a 5-mm probe. Typical conditions for the acquisition of the Bloch decay were as follows: 4.5 μs 45 degree pulse width, 4 KHz spectral window, 500–3000 transients, 10 s repetition rate (fully relaxed, $n = 4$) or 0.68 s (partially saturated, $n = 4$), 20 Hz sample rotation.

Since high resolution spectra could not be obtained from inverted micelles which PtdIns forms in nonpolar solvents, spectra were obtained from dilute solutions in methanol-d_4 (Cambridge Isotope, Woburn, MA). The solutions used were 0.5 mM native salt form of PtdIns (determined by atomic absorption to be about 60% Ca^{2+} and 40% Mg^{2+}; unpublished observations) and 1.5 mM for the more soluble Na^+ form, prepared as described by Kates [23].

An exponential filter (0–1 Hz) was applied to the Bloch decay before transformation. The observables, i.e., the areas of the resonances, are dependent on the baseline definition, (especially for the broad multiplets considered) as well as phasing and limits of integration. Each Bloch decay was processed pairwise in triplicate and averaged. The methyl resonance was chosen as the internal standard, assuming six methyl protons per phospholipid. Fully relaxed spectral integration of the glycerol backbone and inositol headgroup confirmed this assumption (e.g., no detectable anteiso, etc.). The results were analyzed pairwise and the differences expressed as the percent change of the preparations from ethanol-fed vs. the controls. The observed changes were independent of salt form or repetition rate. The 3-methylene resonances were tentatively identified using Gunstone's rules and confirmed using homonuclear decoupling.

Electron paramagnetic resonance. Phospholipids were assayed for the ability to confer membrane tolerance in recombined liposomes, as described previously [6]. Conditions for obtaining EPR spectra were as described previously [6,16].

Results

Changes in the fatty acid composition

The content of arachidonic acid decreased by 8.4% in the PtdIns of ethanol-fed animals (Table I). Increases in the percentages of stearic (2.2%), oleic (20.0%), and the quantitatively minor fatty acid eicosatrienoic (47.1%) acids were observed. As a result of these modifications, the total polyunsaturated fatty acid content decreased by 4.8%. The ratio of the total olefinic content (unsaturation index in Table I) to the saturated fatty acid level decreased by 7.0%. The decrease in arachidonic

TABLE I

Fatty acid composition of liver microsomal phosphatidylinositol from control and ethanol-fed rats

Fatty acid content is presented as the average percent of the total ± S.E. determined from ten sets of pair-fed littermates. For each rat, two preparations of methyl esters were made, three determinations were made for each preparation, and an average was obtained from six total determinations. Components registering less than 1% of the detector response are excluded from the Table. They include 16:1, 18:1($n-7$), 22:5 and several other unidentified species. No notable changes in the levels of these components were observed. UI, unsaturation index, the sum of the products of the percent of each fatty acid and the number of double bonds in the fatty acid. PUFA, polyunsaturated fatty acids.

Fatty acid	Control	Ethanol-fed
16:0	3.5 ± 0.2	3.3 ± 0.3
18:0	41.8 ± 0.6	42.7 ± 0.5 [b]
18:1($n-9$)	2.5 ± 0.2	3.0 ± 0.2 [a]
18:2($n-6$)	1.7 ± 0.2	2.0 ± 0.1
20:3($n-6$)	1.7 ± 0.2	2.5 ± 0.3 [b]
20:4($n-6$)	43.9 ± 0.5	40.2 ± 0.6 [a]
22:6($n-3$)	1.0 ± 0.1	1.3 ± 0.2
%PUFA	48.3 ± 0.3	46.0 ± 0.6 [a]
UI	192.6 ± 1.2	183.1 ± 2.3 [a]
UI/%saturated	4.3 ± 0.1	4.0 ± 0.1 [b]

[a] Differences between the values for control and the ethanol-fed animals are statistically significant at the $P < 0.005$ level in the two-tailed paired Student's *t*-test.

[b] Differences significant at $P < 0.05$.

TABLE II

Fatty acid composition of liver microsomal phospholipids in control and ethanol-fed rats

Fatty acid content is presented as the average percent of total ± S.E. from five sets of pair-fed littermates. For each rat, three preparations of methyl esters were prepared. One determination was made for each preparation and the average was obtained from the three determinations. C, control; EF, ethanol-fed; tr, trace levels (< 0.5%).

Fatty acid	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylserine	
	C	EF	C	EF	C	EF
16:0	20.9±0.9	14.8±0.3[b]	15.8±0.7	12.7 ±0.7[b]	5.3±0.4	4.3±0.2
18:0	24.0±0.8	26.6±0.8	30.2±1.0	29.1 ±1.0	42.5±0.6	43.5±0.5
18:1($n-9$)	9.0±0.4	11.8±0.8[b]	5.2±0.4	8.5 ±0.5[a]	5.7±0.8	5.6±0.9
18:2($n-6$)	10.8±0.5	12.1±1.4	5.3±0.2	7.8 ±0.6[b]	3.2±0.4	3.1±0.8
20:3($n-6$)	0.6±0.2	1.6±0.5	tr	tr	tr	tr
20:4($n-6$)	31.9±0.3	27.8±1.9	33.4±0.4	32.9 ±0.4	37.1±1.0	32.9±2.0[b]
22:6($n-3$)	1.9±0.7	3.3±0.6	10.1±0.2	9.00±0.9	6.2±0.3	10.7±0.9[a]
%PUFA	45.2±0.6	44.8±0.5	48.8±0.4	49.7 ±1.1	46.5±0.7	46.7±0.6
UI	171.4±3.7	171.8±5.2	210.0±1.6	209.7 ±6.3	197.7±1.8	207.6±2.0[b]
UI/% sat.	3.8±0.2	4.1±0.2	4.6±0.1	5.0 ±0.3	4.1±0.1	4.3±0.1

[a] Differences between the values for control and ethanol-fed animals are statistically significant at the $P < 0.005$ level in the two-tailed paired Student's t-test.

[b] Differences significant at the $P < 0.05$ level.

acid, an ($n-6$) fatty acid, was accompanied by increases in the percent of stearic [18:0], oleic [18:1($n-9$)] and eicosatrienoic [20:3($n-6$)] acids. No significant alterations were noted for the other quantitatively major fatty acids. Trace levels of other fatty acid species were found, but no remarkable differences were noted due to ethanol feeding.

Since PtdIns is the only microsomal phospholipid that confers membrane tolerance, we examined whether the decrease in the unsaturation content was unique to PtdIns, compared to the fatty acid content of the other microsomal phospholipids. Those compositions (shown in Table II) reveal that the % PUFA significantly decreased only in PtdIns and was essentially unchanged in the other phospholipids. The UI decreased in PtdIns, increased in PtdSer and remained essentially unchanged in PtdCho and PtdEtn. The ratio of the total olefinic content to saturated fatty acids also decreased only in PtdIns, while in the other phospholipids it increased, though not significantly.

The changes in the fatty acid composition varied with each phospholipid; however, the level of arachidonic acid was decreased to varying extents in all the phospholipids. The changes in the other anionic phospholipid, PtdSer, differed from those in PtdIns. The % PUFA remained constant, but arachidonic acid decreased by 11.3%, and docosahexaenoic acid increased by 72.6%. In the two major microsomal phospholipids, PtdCho and PtdEtn, a decrease in palmitic acid and an increase in linoleic acid occurred. In phosphatidylcholine, the composition of every fatty acid was affected. Palmitic acid decreased by 29.2% and oleic acid increased by 31.1%. The content of arachidonic acid (−12.9%), linoleic (+12.0%), and docosahexaenoic acids (+73.7%) were all altered, but these alterations did not reach statistical significance. PtdEtn exhibited a 19.6% decrease in palmitic acid, no change in the amount of stearic acid, a 63.5% increase in oleic acid and a 47.2% increase in linoleic acid.

Distribution of the fatty acid chains on the sn-1 and sn-2 positions of the glycerol moiety

If the fatty acids are rearranged between the *sn*-1 and *sn*-2 positions of the glycerolipid, the physical properties can be altered with no apparent change in fatty acid composition [24]. The subtle compositional changes observed for the major acyl species led us to examine whether such a rearrangement in the PtdIns occurred during chronic ethanol consumption. The posi-

TABLE III

Fatty acid composition of the sn-1 and sn-2 positions in microsomal phosphatidylinositol from the livers of control and ethanol-fed rats

PtdIns was hydrolyzed with phospholipase A_2, and the fatty acid composition was determined by gas chromatography of the resulting free fatty acids and lyso-PtdIns separated by TLC. The values are the mean from three sets of pair fed animals ± S.E. tr < 0.5%.

Fatty acid	Position			
	sn-1		*sn*-2	
	control	ethanol-fed	control	ethanol-fed
16:0	7.1±0.4	8.8±1.4	1.3±0.1	1.2±0.1
18:0	83.5±0.5	83.3±1.3	2.5±0.4	2.6±0.5
18:1($n-9$)	4.0±0.3	4.1±0.3	1.7±0.2	2.3±0.5
18:2($n-6$)	1.7±1.0	1.5±0.5	3.9±0.8	4.6±0.8
20:3($n-6$)	tr	tr	3.0±0.3	3.4±0.6
20:4($n-6$)	3.2±0.6	1.8±0.3	85.8±1.2	81.3±0.9
22:6($n-3$)	tr	tr	1.9±0.2	2.7±0.5

tional distribution of the fatty acids was determined by hydrolyzing the fatty acids from the *sn*-2 position with pancreatic phospholipase A_2, and separating the products on TLC. The results in Table III reveal that the *sn*-1 position contained predominantly saturated fatty acids and the *sn*-2 position contained mostly unsaturated fatty acids and that there was no major rearrangement in the acyl composition of microsomal PtdIns from the treated animals.

NMR spectroscopic measures of changes in the distribution of double bonds in the acyl chains of PtdIns

The NMR analysis is a simple nondestructive assay that allows elucidation of acyl chain composition and requires only 250 nmol of lipid. The method relies on an extension of analytical principles of substituent effects on chemical shifts, using Frost and Gunstone's rules [25]. The proton resonance position is determined by the local electronic environment. For the acyl chain methylenes, the largest perturbation is the proximity to the deshielding carboxyl or olefinic moieties. Of particular interest is the 3-methylene position, two carbons away from the carboxyl group. If this methylene is also two carbon atoms from the Δ^5 olefinic moiety, it is further deshielded, and its resonance is resolved from the resonance of a 3-methylene in a chain lacking the Δ^5 double bond (Fig. 1). The two resonances remain adjacent. Arachidonic acid [20 : 4($n-6$)] was the only fatty acid present that contained the 3-methylene group and the Δ^5 double bond. In other membrane systems, fatty acids such as eicosatrienoic acid [20 : 3($n-9$)] contribute to this resonance. Since the two resonances comprise the whole population of 3-CH_2 protons, changes in the amounts of the populations lead to increased intensity in one resonance and decreased in the other, as shown in Fig. 1. A third resonance representing fatty acids such as 22 : 5($n-6$) and 22 : 6($n-3$), in which the olefinic moiety is in the Δ^4 position, was not observed since their concentrations did not exceed the sensitivity threshold. It is crucial that perturbations cause intensity changes of opposite sign in two adjacent resonances because the method is much less dependent on extraneous factors, such as baseline definition of methyl-standard integrity, and modest changes in dilute samples are readily observed from a difference spectrum.

As shown in Figs. 1 and 2, a paired analysis of the calorically matched ethanol-fed and control animals yields a decrease in arachidonic acid upon chronic ethanol consumption ($-7.9\% \pm 1.7$ S.E.), with corresponding increase in the remainder ($+5.4\% \pm 0.9$ S.E.). This result obtained by NMR was in good agreement with the analogous pairwise results obtained by gas chromatography: $-8.2\% \pm 1.4$ S.E. and $+5.3\% \pm 1.3$ S.E., respectively (Table I).

The total olefin content determined by NMR showed

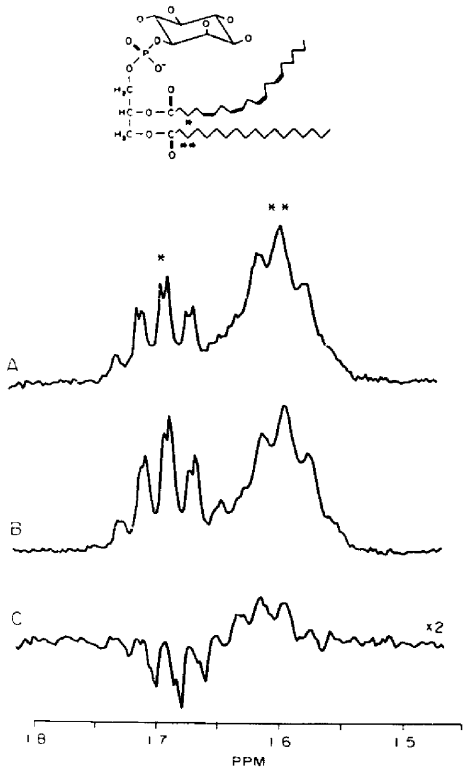

Fig. 1. ^{1}H-NMR spectra of resonances arising from 3-methylene protons in the fatty acyl chains of purified microsomal PtdIns from chronically ethanol-fed (A) and control (B) animals. The difference spectrum (A–B) magnified 2-fold is shown in C. Asterisks are used to identify the 3-methylene groups in the PtdIns structure shown and their respective resonance peaks in the spectra: *, 3-methylene protons in acyl chains containing a Δ^5 olefin; **, 3-methylene protons in acyl chains saturated from C-1 to C-5.

no overall change. Within individual pairs the variation was larger, and the precision, therefore, smaller ($-0.2\% \pm 3.0$ S.E.). The unsaturation index, calculated from the fatty acid composition data showed a $4.8\% \pm 1.1$ S.E. decrease in the total unsaturation (Table I). This decrease is in good agreement with the results obtained by NMR (i.e., the standard deviations overlap between the NMR and GC results).

We believe that the NMR method may have broad utility for the analysis of lipids with unsaturation at the Δ^5 position. While the accuracy of measuring chemical changes by ^{1}H-NMR is generally within 5%, the method is much more accurate in the measurement of the unsaturation at the Δ^5 position, in which two adjacent resonances undergo intensity changes of opposite sign. Therefore the chemical changes observed at the Δ^5 position are in better agreement with the gas chromato-

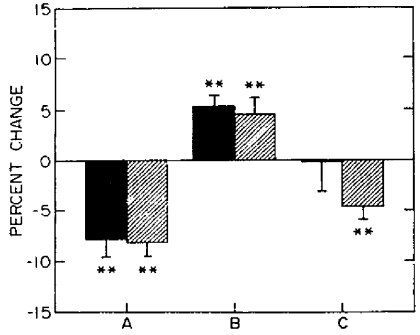

Fig. 2. Comparison of the changes of microsomal PtdIns acyl composition following chronic ethanol administration as determined by NMR (solid bars) and GLC (striped bars). (A) Percent change in the $20:4(n-6)$ which corresponds to fatty acids containing a Δ^5 olefin identified with a single asterisk in Fig. 1. (B) Percent change in the 16:0, 18:0, $18:1(n-9)$, $18:2(n-6)$, $20:3(n-6)$ sum, which corresponds to the fatty acids containing a C-5 saturated carbon identified with a double asterisk in Fig. 1. (C) Percent change in the total olefin content determined by NMR and the unsaturation index calculated from GLC data as described in Table I. Values expressed as ratio percent of ethanol-fed/pair-fed control ± standard error. $n = 6$, NMR; $n = 10$, GLC. ** Differences between the values for control and ethanol-fed animals are statistically significant at the $P < 0.005$ level in the two-tailed paired Student's t-test.

graphic measures than the good agreement observed for the total olefin content.

Discussion

Although it has been known for more than a decade that resistance to membrane fluidization by ethanol develops in several different types of membranes in ethanol-fed animals [4], the molecular modifications associated with tolerance remain to be identified. The ability to measure resistance to disordering in phospholipid vesicles has permitted a systematic approach to identifying the components responsible for membrane tolerance. After chronic ethanol feeding, PtdIns of hepatic microsomes acquires the ability to confer tolerance at levels of only one-third its naturally occurring amount, a property which is not dependent on the source or fatty acid composition of the remaining phospholipids in the vesicles [6]. We have noted that the proportions of the phospholipid classes in microsomes do not change during adaptation to ethanol [6]. This implies that tolerance results from modification of the chemical structure of the microsomal phospholipids, particularly PtdIns.

Using a combination of GLC and ^{1}H-NMR we have examined the effects of chronic ethanol consumption on the hydrophobic portion of the PtdIns molecule. In the NMR analysis each local environment gives rise to its characteristic signature, which may pinpoint broad chemical changes of potential physical relevance. NMR highlights the change in unsaturation at the acyl 5-position, and the sensitivity to chemical change is enhanced 2-fold due to opposite intensity changes in adjacent resonances. This adaptation of the NMR method should have broad utility for the analysis of lipids containing fatty acids with unsaturation at the Δ^5 position, which are predominantly lipids containing arachidonic acid, the precursor of prostaglandins and other eicosanoids. Analyses can be performed on all glycerophospholipids, since the 3-methylene resonances are not affected by the phospholipid polar groups. The NMR method requiries a relatively small amount of lipid, and in contrast to GLC analysis, is nondestructive and does not require chemical degradation. The close agreement of chemical changes observed by GLC and NMR demonstrates the utility of the NMR method as a nondestructive screening assay to elucidate lipid composition.

The most notable effects on the fatty acid composition of PtdIns from ethanol-fed rats are an 8.4% decrease in arachidonic acid $[20:4(n-6)]$, the major polyunsaturated fatty acid, a 20.0% increase in oleic acid $[18:1(n-9)]$ and a 47.1% increase in the minor eicosatrienoic acid $[20:3(n-6)]$. Our results agree with a recent study of total liver PtdIns that showed a 10% decrease in arachidonic acid (36.5 to 33%), an increased amount of oleic acid, and corresponding changes in the Δ^5 index (Δ^5 unsaturated, −10%; Δ^5 saturated, +3.2%) [14]. Our results differ from those reported from microsomal PtdIns [13], in which arachidonic acid increased from 22.1% to 25% and the Δ^5 index varied in opposite manner (Δ^5 unsaturated, +13.6%; Δ^5 saturated, −6.2% – excluding trace and unidentified fatty acids); however, only 3% microsomal PtdIns was extracted, instead of the expected 8–10% [26]. All studies agree that the acyl changes are not dramatic, leaving inferences concerning the origins of membrane tolerance tentative.

Chronic ethanol consumption disrupts the control of the fatty acid composition of microsomal phospholipids, and as a result, each phospholipid class exhibits unique fatty acid changes. These changes suggest that the action of ethanol in fatty acid modification is not limited to any one particular metabolic reaction. Chronic ethanol consumption lowers the content of the $(n-6)$ family of fatty acids in liver microsomes by reducing the levels of arachidonic acid to varying extents in the phospholipids. The decreased ratio of arachidonic acid to linoleic acid (Tables I and II) observed in all the phospholipids is characteristic of hepatic membrane lipids in ethanol-fed rats [27,28] and may be partly due to the inhibition by ethanol of microsomal fatty acyl-CoA Δ^6 and Δ^5-desaturase enzyme activities [29,30]. The increased levels of linoleic acid in PtdCho, PtdEtn, and PtdIns correlate with an inhibition of Δ^6-desaturase activity by ethanol, an effect which would decrease the conversion of linoleic to γ-linolenic acid $[18:3(n-6)]$.

The increased level of $20:3(n-6)$ and the decreased amount of arachidonic acid in PtdCho and PtdIns are consistent with an inhibition of Δ^5-desaturase activity, lowering the conversion of homo-γ-linolenic acid $[20:3(n-6)]$ to arachidonic acid.

PtdIns is the only microsomal phospholipid that is synthesized from a CDP-diacylglycerol intermediate, and chronic ethanol feeding in rats apparently affects the turnover of polyunsaturated fatty acids in PtdIns in a unique manner. As a consequence, PtdIns alone acquires an ethanol-induced decrease in both the polyunsaturated fatty acid content and the ratio of the total olefinic content to saturated fatty acid content. PtdIns serves as a precursor to polyphosphoinositides, which are hydrolyzed by a hormonally stimulated phospholipase C to produce the second messengers, phosphoinositols and diacylglycerols. It contains high levels of arachidonic aicd, which is converted to regulatory eicosanoids after release by an agonist-stimulated phospholipase A_2. The metabolic consequences of PtdIns with different physical properties in structurally altered membranes could be significant, due to its participation in signal transduction processes [31].

Neutron scattering has revealed that ethanol is largely excluded from the hydrophobic core of membranes and partitions predominantly into the polar region of membranes [32]. The thermodynamics of ethanol transfer from the aqueous to membrane environments indicate that hydrogen bonding is maintained in both phases, consistent with residence of the drug in the polar headgroup region [33]. Structural studies have indicated that the polar region extends to carbon-4 of the predominantly unsaturated *sn*-2 chain, abutting the Δ^5 olefinic moiety [34]. As a consequence of the decrease in arachidonic acid and its Δ^5 double bond in PtdIns, a depletion of the Δ^5 unsaturates occurs near the membrane surface where packing is tighter and order is higher [35] and where an olefinic alteration may magnify a modification of the interaction of the membrane with ethanol or other anesthetics. The observed alterations of PtdIns would influence both the surface and core membrane properties, the putative sites for ethanol and nonpolar anesthetics, respectively. Whether these acyl chain modifications are responsible for, or merely coincident with, membrane tolerance remains to be established.

Acknowledgements

We thank Dong Nham, Marita Barber, Eben Rosenthal, and Jack Hsu for expert technical assistance. This work was supported by Public Health Service Grants AA07215, AA07463, AA07186, and AA00088. T.F.T. is the recipient of a Research Scientist Development Award from the National Institute on Alcohol Abuse and Alcoholism.

References

1 Meyer, H. (1899) Arch. Exp. Path. U. (Naunyn-Schmiedeberg) 42, 109–118.
2 Hubbell, W.L. and McConnell, H.M. (1968) Proc. Natl. Acad. Sci. USA 61, 12–16.
3 Johnson, P.A., Lee, N.M., Cooke, R. and Loh, H.H. (1979) Mol. Pharmacol. 15, 739–746.
4 Chin, J.J. and Goldstein, D.B. (1977) Science 196, 684–685.
5 Taraschi, T.F., Ellingson, J.S., Wu, A., Zimmerman, R. and Rubin, E. (1986) Proc. Natl. Acad. Sci. USA 83, 3669–3673.
6 Taraschi, T.F., Ellingson, J.S., Wu, A., Zimmerman, R. and Rubin, E. (1986) Proc. Natl. Acad. Sci. USA 83, 9398–9402.
7 Waring, A.J., Rottenberg, H., Ohnishi, T. and Rubin, E. (1981) Proc. Natl. Acad. Sci. USA 78, 2582–2586.
8 Ellingson, J.S., Taraschi, T.F., Wu, A., Zimmerman, R. and Rubin, E. (1988) Proc. Natl. Acad. Sci. USA 85, 3353–3357.
9 Ponappa, B.C., Waring, A.J., Hoek, J.B., Rottenberg, H. and Rubin, E. (1982) J. Biol. Chem. 257, 10141–10146.
10 Harris, R.A., Baxter, D.M., Mitchell, M.A. and Hitzeman, R.J. (1984) Mol. Pharmacol. 25, 401–409.
11 Rottenberg, H., Waring, A.J. and Rubin, E. (1981) Science 213, 583–585.
12 Stubbs, C.D., Williams, B.W., Pryor, C.L. and Rubin, E. (1988) Arch. Biochem. Biophys. 262, 560–573.
13 Cunningham, C.C., Filus, S., Bottenus, R.E. and Spach, P.I. (1982) Biochim. Biophys. Acta 712, 225–233.
14 Gustavsson, L. and Alling, C. (1989) Alcohol 6, 139–146.
15 DeCarli, L.M. and Lieber, C.S. (1967) J. Nutr. 91, 331–336.
16 Taraschi, T.F., Wu, A. and Rubin, E. (1985) Biochemistry 24, 7096–7101.
17 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
18 Ellignson, J.S. (1974) Biochim. Biophys. Acta 337, 60–67.
19 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
20 Klein, R.A. (1970) Biochim. Biophys. Acta 210, 486–489.
21 Ellingson, J.S. and Zimmerman, R. (1987) J. Lipid Res. 1016–1018.
22 Morrison, W.R. and Smith, L.M. (1964) J. Lipid Res. 5, 600–608.
23 Kates, M. (1986) Techniques of Lipidology, 2nd Edn., Elsevier, New York, pp. 110–111.
24 Lynch, D.V. and Thompson, Jr., G.A. (1984) Trends Biochem. Sci. 9, 442–445.
25 Frost, P.J. and Gunstone, F.D. (1975) Chem. Phys. Lipids 15, 53–85.
26 White, D. (1973) in Form and Function of Phospholipids (Ansell, C., Hawthorne, J. and Sauson, R., eds.), pp. 441–482, Elsevier, Amsterdam.
27 Cunningham, C.C. and Spach, P.I. (1987) Ann. N.Y. Acad. Sci. 492, 181–192.
28 Reitz, R.C., Wang, L., Schilling, R.J., Starich, G.H., Bergstrom, J.D. and Thompson, J.A. (1981) Prog. Lipid Res. 20, 209–213.
29 Nervi, A.M., Peluffo, R.O., Brenner, R.R. and Leiken, A.I. (1980) Lipids 15, 263–268.
30 Wang, D.L. and Reitz, R.C. (1982) Alc. Clin. Exp. Res. 7, 220–226.
31 Augert, G., Bockino, S.B., Blackmore, P.F. and Exton, J.H. (1989) J. Biol. Chem. 264, 21689–21698.
32 Herbette, L.C., Napolitano, C.A., Messineo, F.C. and Katz, A.M. (1985) Adv. Myocardiol. 5, 333–346.
33 Miller, K.W., Hammond, L. and Porter, E.G. (1977) Chem. Phys. Lipids 20, 229–241.
34 Hauser, H., Pascher, I., Pearson, R.H. and Sundell, S. (1981) Biochim. Biophys. Acta 650, 21–51.
35 McConnell, H.M. and McFarland, B.G. (1972) Ann. N.Y. Acad. Sci. 195, 207–217.