An abnormal phospholipid in rat organs after ethanol treatment

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1. INTRODUCTION

Interaction of ethanol with essential fatty acid (EFA) metabolism has been demonstrated in man [1,2] and in experimental animals [3,4]. Several laboratories have reported on the effects of ethanol on changed proportions of polyunsaturated fatty acids in cell membranes [5,6,7]. In a series of experiments with ethanol and EFA we found increased concentrations of phospholipids in the brain of rats after 3 weeks of ethanol administration (3 g/kg body weight, daily intraperitoneal injections). This phospholipid increase was due to an increase of the acidic fraction of the phospholipids [4]. Examination of the proportions of individual neutral and acidic lipids by thin-layer chromatography (TLC) revealed the occurrence of an abnormal lipid fraction in organs from rats exposed to ethanol. This article is a report on the appearance of this abnormal lipid in various organs and the relation to the amount and duration of ethanol administration. Our currently available information on the chemical structure of the lipid is also given.

2. MATERIALS AND METHODS

2.1. *Animals*

Three different experiments were performed: two with different methods of ethanol exposure and one focusing on the time course of the appearance of the fraction. After the ethanol administration periods, rats were killed by decapitation and brain, kidney, liver, skeletal muscle and serum were taken for analysis. In expt I and II the ethanol rats were killed in an intoxicated state.

In expt I 24 Sprague–Dawley rats (female rats, 5 weeks old) received diets with either normal or low levels of EFA [8]. Ethanol (3 g/kg body weight) was given by intraperitoneal injections once a day to 12 of the rats (6 from each diet group). Each injected resulted in a maximum blood ethanol concentration of about 3 mg/ml. Isocaloric amounts of glucose were given to the 12 control animals. The ethanol treatment continued for 21 days.

In expt II, 12 Sprague–Dawley rats received ethanol in a semi-synthetic liquid diet. Six control animals received isocaloric amounts of sucrose in their diets. After 16 days half of the rats in each group were killed. The remaining rats were maintained on the liquid diet, all without ethanol, for a further 5 days, before they were killed and analysed.

Expt III was a time course test. Twelve Sprague–Dawley rats were injected intraperitoneally with ethanol (3 g/kg body weight). Two rats each were killed at 1, 2, 4, 8 and 24 h after injection.

2.2. *Lipid analyses*

Animals were analysed individually. The lipids were extracted from different organs as in [4], and the extracts were divided into one neutral, and one

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acidic fraction on an anionic ion-exchange resin [9]. The acidic lipids were separated on TLC and HPTLC-plates (Merck, Darmstadt) with the solvent system chloroform–acetone–methanol–acetic acid–water, 5.0:1.5:1.5:1.0:0.5. The chromatograms were stained with $Cu(Ac)_2$-reagent, and heated for 20 min at 130°C. The reference lipids phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, cardiolipin and phosphatidic acid were obtained from Sigma (St Louis MO). Free fatty acids were obtained after hydrolysis of corn oil. Bis(monoacylglyceryl)phosphate isolated from the muscles of chloroquine-intoxicated miniature pigs [10] and sulphatide from human brain were donated by Dr Olle Nilsson (Department of Psychiatry and Neurochemistry, University of Göteborg). For detection of carbohydrates TLC-plates were stained with orcinol, and for detection of primary amino groups with ninhydrin reagent. Fatty acid proportions were determined by gas-liquid chromatography (GLC) and quantitative determinations of lipid phosphorous were performed as in [11]. The percentage composition of phospholipids in expt III was assayed with densitometry (Zeiss KM3 densitometer) after staining of the HPTLC-plate with $Cu(Ac)_2$-reagent and heating for 20 min at 130°C. In all the other cases the determination of lipid phosphorous in individual phospholipids was made after isolation by preparative TLC [11].

3. RESULTS AND DISCUSSION

3.1. *Appearance and organ distribution in relation to ethanol exposure*

In rats given ethanol intraperitoneally for 21 days (expt I) the highest relative concentration of the abnormal lipid fraction was found in kidney,

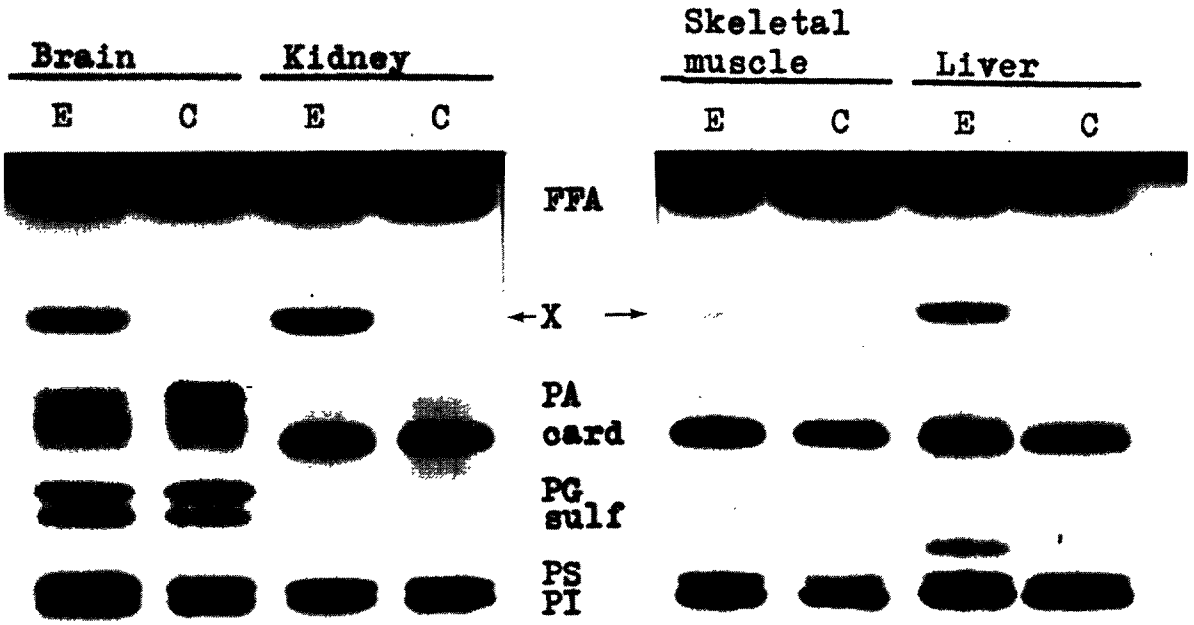

Fig.1. HPTLC of the acidic lipids from different organs in rats from expt I. The concentration of the unknown fraction (X) varies between these organs, and has the highest concentration in kidney. Reference lipids were PI, phosphatidylinositol; PS, phosphatidylserine; sulf, sulphatide; PG, phosphatidylglycerol; card, cardiolipin; PA, phosphatidic acid; FFA, free fatty acids.

followed by brain, liver and skeletal muscle (fig.1). The concentrations of the total fraction of acidic lipids in brain were higher in ethanol-treated rats fed both the low EFA and the normal EFA diets than in controls. The unknown compound constituted about 10% of these acidic fractions (table 1). The compound could not be detected in serum. The high concentration in membrane-dense organs like kidney and brain and the absence from serum indicated a localization to membrane structures. The abnormal fraction developed both in rats with low and normal EFA content in their diets.

Table 1

Lipid phosphorous in acidic fraction, in expt I

	Brain		Kidney	
	n	M ± S.D.	*n*	M ± S.D.
		μmol/g ww		
0.3 Energy-% EFA				
Controls	6	12.2 ± 1.67	6	8.6 ± 0.21
Ethanol	5	12.3 ± 1.41	4	10.0 ± 0.60
Unknown fraction	5	1.2 ± 0.15	4	1.4 ± 0.34
3.0 Energy-% EFA				
Controls	5	12.4 ± 1.21	6	8.1 ± 0.60
Ethanol	6	13.8 ± 1.00	5	10.6 ± 0.59
Unknown fraction	6	1.4 ± 0.16	5	2.1 ± 0.36

These initial findings suggested that the abnormal fraction was a degradation product in cell membranes as a result of the chronic exposure to ethanol. Therefore the next animal study (expt II) was designed in order to elucidate reversibility. In this experiment the blood–ethanol concentration varied, due to differences in the voluntary ethanol consumption. A relation was found between the concentrations of ethanol and of the abnormal fraction. Five days after interruption of ethanol exposure, the unknown compound could no longer be detected. From these results it was obvious that the abnormal fraction had a more rapid appearance and disappearance than had been initially expected.

In expt III the short-term relationship between ethanol and the abnormal fraction was studied. The pathological acidic lipid was found in rats for 1, 2, and 4 h after the ethanol injection but had already disappeared at 8 h (fig.2). The concentration of the fraction varied during the period from 1–4 h with a maximum at 2 h after ethanol injection, which indicates a slight delay in relation to the peak of the blood–ethanol profile (fig.3).

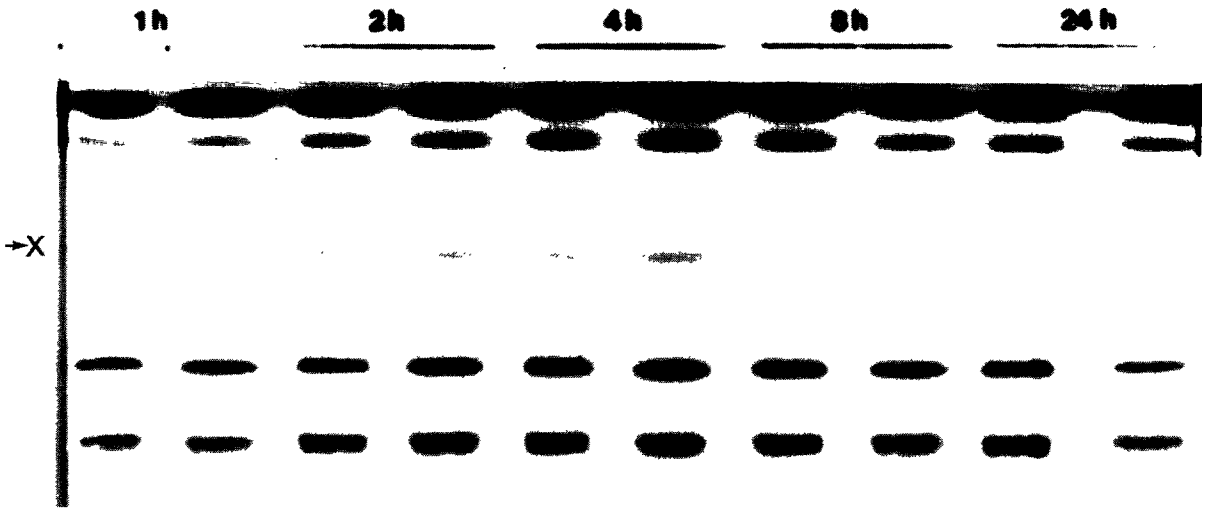

Fig.2. HPTLC of the acidic lipids from kidneys in expt III. This chromatogram shows the appearance and disappearance of the unknown compound after an injection of ethanol. The compound constituted 10.1, 11.8 and 9.0% of the major acidic lipids (phosphatidylinositol, phosphatidylserine, unknown fraction and cardiolipin) in rats 1, 2 and 4 h after injection. At 8 and 24 h the compound was undetectable.

From these experiments it is concluded that the concentration of the observed abnormal acidic lipid is strongly related to the blood–ethanol level and fully reversible. All analysed organs were affected but not blood serum. This indicates that the abnormal lipid does not appear in the tissues after formation in for example the liver and subsequent blood transport. The formation of the unknown lipid therefore takes place within the organs themselves.

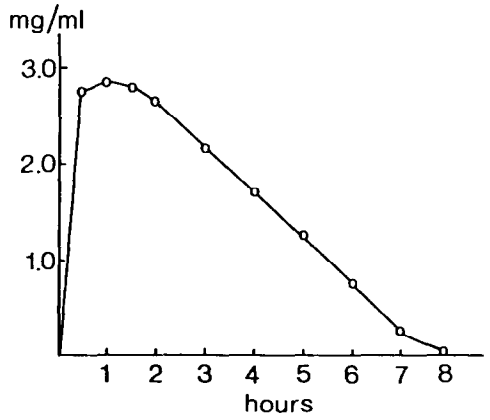

Fig.3. The blood ethanol pofile after an injection of 3 g/kg body weight in rats not previously treated with ethanol.

3.2. *Chromatographic investigations in relation to reference compounds*

A series of experiments was performed to establish the chemical nature of the abnormal lipid fraction. The fact that the compound was bound to an anionic ion-exchange resin [9] indicates acidic properties. A number of acidic lipids available in our laboratory were compared with the unknown compound for R_f-values in HPTLC (fig.4) but none of them proved to be identical. The possibility that the substance was one of the intermediates in the methylation of phosphatidylethanolamine during formation of phosphatidylcholine was also investigated [12]. This was found not to be the case judging from identity in HPTLC, in the solvent system chloroform–propionic acid–*n*-propanol–water, 2:2:3:1.

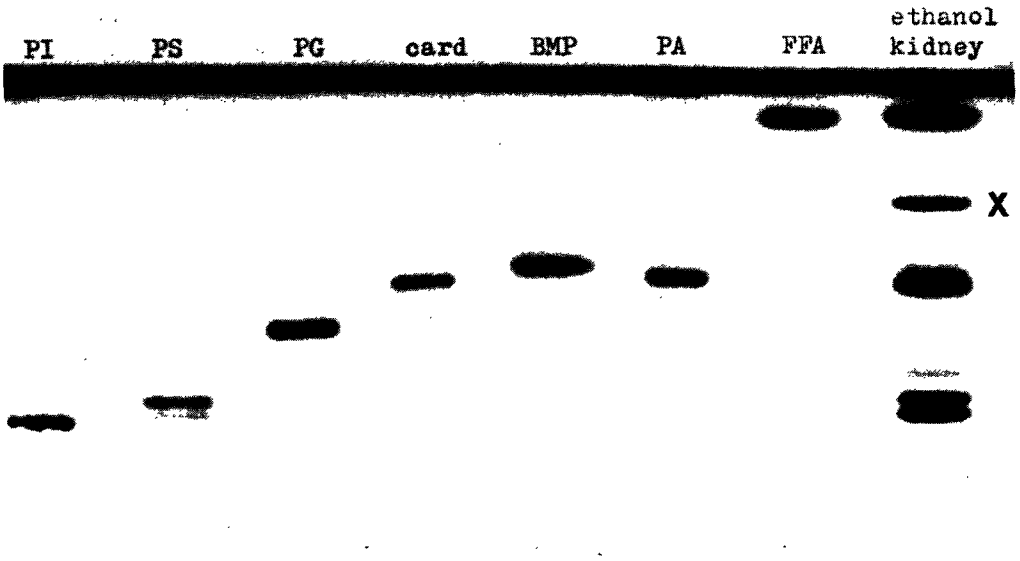

Fig.4. HPTLC for comparison of R_f-values of different references and the unknown fraction (X). The references used were: PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; card, cardiolipin; BMP, bis(monoacylglyceryl)phosphate; PA, phosphatidic acid; FFA, free fatty acids.

Table 2

Fatty acid composition of the unknown fraction in kidney from rats injected intraperitoneally with ethanol (expt I)

Fatty acids	0.3 Energy-% EFA (n = 6) M ± S.D.	3.0 Energy-% EFA (n = 6) M ± S.D.
16:0	21.7 ± 1.75	23.5 ± 1.03
18:0	11.7 ± 1.09	14.8 ± 0.51
18:1	33.4 ± 1.24	22.3 ± 0.79
18:2(*n*-6)	3.5 ± 0.20	8.0 ± 0.52
20:3(*n*-9)	7.6 ± 0.63	0.5 ± 0.41
20:4(*n*-6)	15.0 ± 1.47	22.6 ± 0.73
22:6(*n*-3)	1.0 ± 0.22	2.3 ± 0.18

3.3. *Chemical investigations of possible structure*

The chemical characterization of the unknown acidic lipid showed that the substance contained phosphorous and fatty acids in a molar ratio of about 1:2. The fatty acid composition is given in table 2. Palmitic (16:0), oleic (18:1) and arachidonic acid (20:4(*n*-6)) dominated and the fatty acid pattern was determined by the diet given to the rats. These findings indicate that the structural frame of the molecule is probably similar to that of phosphoglycerides in general and that at least part of the molecule exists prior to ethanol administration.

The abnormal fraction did not react to ninhydrin or orcinol reagents on thin-layer plates, indicating the absence of primary amino groups and carbohydrates. Addition of ethanol to the tissue homogenates prior to the lipid extraction did not give rise to the observed abnormal compound.

3.4. *Structural considerations*

There are several alternatives for the structure. Increased lipid peroxidation after ethanol administration to rats has been reported [13]. Phospholipids with peroxidized fatty acids or Schiff base-like products attributable to increased levels of malondialdehyde also have to be considered, since malondialdehyde might give rise to condensation products with amino groups of phospholipids.

Another possibility is that the ethanol might have inhibited an enzyme responsible for the metabolism of phospholipids with accumulation of an intermediate, which normally occurs only in trace quantities. This is supported by the relatively rapid appearance of the substance. An unidentified reaction between ethanol or acetaldehyde and a phospholipid or a phospholipid metabolite is also plausible. These possibilities are at present being investigated in large scale preparations which will be submitted to further structural analysis.

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