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PHOSPHOLIPID CLASS DISTRIBUTION AND FATTY ACID COMPOSITION IN LIVER AND PLASMA FROM UNTREATED AND DIAZEPAM TREATED RATS

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The present study investigated the effect of diazepam on fatty acids bound to phospholipids and their class distribution in the plasma and liver of male Wistar rats. The rats were divided into two groups with 12 animals in each. Both groups were fed anutritionally adequate pelleted diet (18 g/d/rat). The control group (C) had free access to water. The experimental group drank diazepam in a dose of 10 mg/kg/day. Long-term consumption of diazepam (DZP) (180 days) resulted in strikingly similar alterations in the content of phospholipids and fatty acid composition in the liver. There was a significant decrease in 20:3 (n-6) and 22:5 (n-3) in liver total phospholipids in diazepam treated rats. Diazepam treatment led to a statistically significant decrease in the content of 20:3 (n-6), 22:5 (n-3), 22:6 (n-3), and total PUFA in the total and phosphatidylcholine fraction of plasma phospholipids.

Key words: diazepam, phospholipid, fatty acid, liver, plasma

INTRODUCTION

The phospholipid class and its fatty acid composition and cholesterol content in biomembranes are basic determinants of the physicalproperties of membranes and have been shown to influence a wide variety of membrane-dependent functions, such as membrane transport, enzyme activity, and receptor function (Simopoulos, 1991). After it was determined that peripheral-type benzodiazepine (BZ) recognition sites are located predominantly on mitochondria, it was hypothesized that they play a role in intermediary metabolism (Anholt,1986).It is also feasible that the role of mitochondrial BZ receptor (MBR) in intramitochondrial cholesterol transport may change the lipid distribution of mitochondrial membranes, thereby altering the physiological dynamics of mitochondria (Grueger and Papadopoulos, 1992). It has been reported for many membrane-bound enzymes, that not only the polar head groups of phospholipids, but also their acid composition, could alter enzymatic activities via modulation of the physical properties of the membrane (Sire et al., 1986). Our previous reports (Vrbaški et al., 1989; 1995; Ristić et al., 1988; 1989) showed that

chronic diazepam treatment caused significant changes in brain, plasma, erythrocyte and liver phospholipid contents.It was found that the contents of the phosphatidylethanolamine, sphingophospholipids and phosphatidylinositol+phosphatidylserine were significantly reduced in the erythrocytes of diazepam treated rats. Thus, it appears of interest to investigate phospholipid class distribution and fatty acid profile of the total phospholipid fraction and individual phospholipid classes in liver and plasma of rats after a long-term diazepam treatment.

MATERIALS AND METHODS

Male Wistar rats (8 weeks of age), were obtained from the Experimental Animal farm, Vinča, and acclimatized for 6 days in a room with controlled temperature (22-24^{0}C) and lighting on a 12-h light-dark cycle. The rats were then divided into groups (C and DZP) with 12 animals in each groups. The animals were housed individually in stainless steel suspended cages and fed a pelleted balanced nonpurified diet prepared according to the formula recommended by the American Institute of Nutrition (1977) (protein, 21%; carbohydrate, 62%; fat, 5%; vitamin premix, 0.25%, mineral mixture, 2,25%; Veterinarski Zavod, Zemun, Yugoslavia) for 180 days.

The test substance, diazepam (KRKA, Novo Mesto, Slovenia) was dissolved in tap water and consumed at 10 mg/kg daily as the sole drinking solution by 12 native rats (DZP group) for 180 days. The daily fluid intake of the rats drinking diazepam solution was monitored to be equal to to the daily water intake consumed by the control (C) group. Animals were weighed weekly and offered 18 g of diet per day.

All animals, after 180 days of treatment, were sacrificed during i. p. nembutal anaesthesia. The samples of blood were taken by heart puncture, using a heparinized syringe. Plasma was separated by centrifugation. The liver was rapidly excised and weighed. The liver and plasma were stored at -20^{0}C until further processing.

Plasma lipids were extracted by the method of Alling et al. (1982). Lipids from the liver were extracted according to the method of Harth et al. 1978. During the extraction procedure, lipids were protected against oxidation by addition of 10 mg/100 ml butylated hydroxytoluene to the solvents. Plasma phospholipids were separated by one-dimensional thin-layer chromatography (TLC) into the following fractions: phosphatidylethanolamine (PE), phosphatidylcholine (PC), and lysophosphatidylcholine (LPC) + sphingomyelin (SPH). From the liver lipid extracts, one aliquot (containing 60 mg lipid phosphorus) was spotted on Merck thin layer glass plates precoated with a 0.5 mm layer of silica H and Florisil (9:1). Phospholipids were separated by a two-dimensional TLC system: 1. Chloroform-

Abbreviations: 16:0 (n-6), palmitic acid; 16:1 (n-7), palmitoleic acid; 18:0 stearic acid; 18:1, oleic acid; 18:2 (n-6), linoleic acid; 18:3 (n-3), linolenic acid; 20:3 (n-6), eicosatrienoic acid; 20:4 (n-6), arachidonic acid; 20:5 (n-3), eicosapentaenoic acid; 22:4 (n-6), docosatetraenoic acid; 22:5 (n-3), docosapentaenoic acid; 22:6 (n-3), docosohexaenoic acid.

methanol-20% ammonia (65:25:5, v/v) and 2. Chloroform-acetone-methanol-acetic acid-water (70:17.5:12.5:10:4.4, v/v), aspirated from the plates and analyzed for their phosphorus content and for their fatty acid composition (Kostić et al., 1971).

Methylation of the fatty acids of each phospholipid class was carried out with 2M NaOH-methanol (heated at 85^{0}C for 1h) and 1M sulfuric acid-methanol (heated at 85^{0}C for 2h). Fatty acid methyl-esters were separated and quantified under isothermal conditions (190^{0}C) by gas-liquid chromatography (Varian GC, model 3400, Varian Associates) equipped with a flame ionization detector and a 30 m x 0.53 mm SP-2380 fused silica capillary column (Supelco Inc., Bellefonte). Identification of fatty acids was based on the retention times of authentic standards (Sigma Chemical Company) and/or the polyunsaturated fatty acids (PUFA)-2 standard mixture (Supelco Inc., Bellefonte). Peak areas were determined with a Varian 4290 integrator. The results were expressed as percentages of total identified fatty acids.

The data are expressed as mean ± SD. The effect of diazepam treatment was determined by Student's t-test.

RESULTS AND DISCUSSION

The initial and final body weights and daily body weight gains of rats of both groups are summarized in Table 1. The wet weight of the liver was 7.92 ± 0.39 g in control and 7.98 ± 0.71 g for DZP treated rats. The relative liver weight (g/100 g BW) was 2,20 ± 0,13 g for control and 2.24 ± 0.20 g for the DZP group. The energy intake, body weight gain, and liver weight were similar for both groups.

Table 1. Body weight and daily body weight gain in rats of the control and DZP group

	Control	DZP
Initial body weight (g)	194 ± 24.28	199 ± 21.36
Final body weight (g)	359 ± 17.16	362 ± 41.31
Daily body weight gain (g)	0.94 ± 0.14	0.93 ± 0.29

Table 2. Phospholipid class distribution of lipid phosphorus in the plasma (means± SD)

Phospholipids	Control	DZP
Total phospholipids (mmol/L)	1.60 ± 0.07	1.58 ± 0.05
	% of total phospholipids	
Phosphatidylethanolamine	13.80 ± 0.79	12.00 ± 1.11
Phosphatidylcholine	43.20 ± 1.53	43.00 ± 1.40
Sphingophospholipids	25.20 ± 0.72	28.00 ± 1.31*
Lysophosphatidylcholine	17.80 ± 1.48	17.00 ± 1.71

* Denotes significance of differences between control and DZP groups ($p<0.05$)

DZP treatment did not cause significant changes in the plasma phospholipids (Table 2). A significant increase in the distribution of plasma phospholipid fractions was found in the sphingophospholipids in the DZP treated rats.

Table 3. Phospholipid class distribution of rat liver (means± SD) in control and diazepam (DZP)-treated rats.

Phospholipids	Control	DZP
Total phospholipids (mmol/L)	36.59 ± 3.44	29.76 ± 1.80*
	% of total phospholipids	
Phosphatidylcholine	50.35 ± 3.42	51.27 ± 3.15
Phosphatidylethanolamine	28.63 ± 2.72	28.86 ± 3.72
Phosphatidylinositol	10.49 ± 2.92	10.63 ± 3.98
Sphingomyelin	3.90 ± 0.60	3.58 ± 0.69
Diphosphatidylglycerols	4.01 ± 0.85	3.54 ± 0.79
Phosphatidylserine	2.61 ± 0.47	2.04 ± 0.48

* Denotes significance of differences between control and DZP groups ($p<0.05$)

Table 3. shows the results of the chromatographic analysis of liver phospholipid class distribution in the two groups as a molar percentage of total lipid phosphorus. Whole liver phospholipid contents after diazepam treatment were significantly lower that those of the control. Our previous study demonstrated that chronic diazepam treatment provoked changes in triacylglycerol and phospholipid levels in the liver of rats (Ristić et al., 1988; 1989), but there were no differences in the distribution of phospholipid fractions in the liver between those two groups of rats (Table 3).

All phospholipid values were within the range reported by others (Wood et al., 1986; Mlekusch et al., 1993). Phosphatidylcholine (PC) accounted for 50% of the total phospholipids, phosphatidylethanolamine (PE) about 25-30%, phosphatidylinositol (PI) about 10%, diphosphatidylglycerol (DPG) about 5%, sphingomyelin (SPH) and phosphatidylserine (PS) about 7%.

Striking results were found with the fatty acids of the phospholipids, as seen in Table 4. Diazepam treated rats did not have altered levels of total saturated and total unsaturated fatty acids in liver and plasma phospholipids, compared to those levels found in the control group. In both, liver and plasma, the level of 20:3 (n-6), 22:5 (n-3) and 22:6 (n-3) decreased in the DZP treated group, that caused major shifts in the level of total n-3 PUFA. It is evident that diazepam markedly affected the metabolism of docosahexaenoic acid (DHA) (Ristić and Vrbaški, 1992; Ristić et al., 1995). In the liver, intermediary metabolism leads to secretion of DHA in the form of lipoprotein phospholipids, which are a major source of the DHA that is required by the brain. The present investigation confirms earlier observations that chronic treatment with diazepam significantly reduces brain lipids (Vrbaški et al., 1989).

Table 5 illustrates the fatty acid composition of liver PC and PE. Changes in the fatty acid profile of PC representing about 50% of total phospholipids, were

similar to those found in the total phospholipid fraction for 20:3 (n-6), 22:6 (n-3) and total PUFA (n-3). PE had a similar fatty acid pattern to that seen for the PC fraction, with the exception of 22:6 (n-3).

Table 4. Fatty acid composition of total liver and plasma phospholipids (wt%)[a/]

	LIVER		PLASMA	
Fatty acid	Control	DZP	Control	DZP
16:0	17.06 ± 1.18	16.13 ± 0.99	26.16 ± 2.06	26.36 ± 1.92
16:1 (n-7)[b/]	2.06 ± 1.04	2.09 ± 0.65	0.64 ± 0.12	0.60 ± 0.08
18:0	20.30 ± 2.35	20.33 ± 2.96	28.68 ± 2.54	28.31 ± 2.33
18:1 (n-9)	10.14 ± 1.37	11.87 ± 1.36	9.52 ± 1.48	10.14 ± 1.11
18:2 (n-6)	14.10 ± 2.29	15.12 ± 0.67	13.91 ± 1.32	14.55 ± 1.16
18:3 (n-3)	0.09 ± 0.04	0.05 ± 0.04	0.26 ± 0.07	0.41 ± 0.02
20:3 (n-6)	0.79 ± 0.26	0.47 ± 0.11*	0.77 ± 0.10	0.47 ± 0.17*
20:4 (n-6)	22.68 ± 2.85	23.10 ± 2.24	13.92 ± 2.07	13.25 ± 0.94
20:5 (n-3)	0.73 ± 0.20	0.87 ± 0.19	0.26 ± 0.03	0.24 ± 0.01
22:4 (n-6)	0.17 ± 0.05	0.16 ± 0.08	0.34 ± 0.06	0.17 ± 0.11
22:5 (n-3)	1.18 ± 0.20	0.76 ± 0.20*	0.80 ± 0.19	0.51 ± 0.09*
22:6 (n-3)	8.46 ± 1.62	6.96 ± 1.20	5.08 ± 1.26	3.14 ± 0.59**
Total saturated	37.36 ± 2.14	36.46 ± 2.14	54.84 ± 4.80	54.40 ± 3.22
Total unsaturated	60.39 ± 1.21	61.72 ± 1.69	45.49 ± 4.81	43.48 ± 3.49
Total MUFA[1/]	12.21 ± 2.36	13.97 ± 1.89	10.16 ± 1.57	10.74 ± 1.19
Total (n-6) PUFA[2/]	37.74 ± 1.76	39.15 ± 2.42	28.93 ± 3.98	28.44 ± 1.33
Total (n-3) PUFA	10.45 ± 1.91	8.57 ± 1.43	6.40 ± 1.06	4.30 ± 0.49*
n-6/n-3	3.67 ± 0.71	4.70 ± 1.00	4.52 ± 0.36	6.64 ± 0.56
PUFA/SFA[3/]	1.28 ± 0.13	1.31 ± 0.09	0.64 ± 0.11	0.60 ± 0.06

[a/] Values are means of six animals.
b/ Fatty acids are designated by the number of carbon atoms followed by the number of double bonds.
1/ MUFA, monounsaturated fatty acids.
2/ PUFA, polyunsaturated fatty acids.
3/ SFA, saturated fatty acids.
* Denotes significance of differences between control and DZP groups ($p<0.05$)

The fatty acid pattern of plasma phospholipid fractions is displayed in Table 6. The fatty acid composition of PC was characterized by low levels of 18:3 (n-3), 20:3 (n-6), 20:5 (n-3), 22:5 (n-3) and 22:6 (n-3) in the diazepam treated group. Total PUFA (n-6), total PUFA (n-3) and the n-3/n-6 ratio were greater in the control group. The fatty acid profile of LPC+SPH plasma phospholipids after diazepam treatment was similar to that observed in the control group, except for the decrease in 20:3 (n-6), which was more pronounced.

The present results show that alterations in the content of total liver phospholipids and total fatty acid composition of liver and plasma phospholipids provoke parallel changes in the amounts and composition of their major fractions.

These data suggest that diazepam treatment produced significant changes in liver and plasma fatty acid composition indicating a disturbance in fatty acid metabolism. It is evident that diazepam markedly affects the metabolism of 22:6 (n-3). Fatty acid (Meikle et al., 1989; Goodfriend et al., 1991) and phospholipid methylation (Milvae et al., 1983; Papadopoulos et al., 1987) have been implicated as playing some role in steroidogenesis and may therefore correlate with the effects observed on MBR-ligand binding.

Table 5. Fatty acid composition of liver phosphatidylcholine and phosphatidylethanolamine (wt%)

	Phosphatidylcholine		Phosphatidylethanolamine	
Fatty acid	Control	DZP	Control	DZP
16:0	21.49 ± 2.85	21.52 ± 2.49	21.35 ± 2.80	19.43 ± 1.82
16:1 (n-7)	0.91 ± 0.27	1.36 ± 0.68	0.99 ± 0.42	1.48 ± 0.77
18:0	23.64 ± 2.24	24.28 ± 2.96	31.14 ± 4.48	29.35 ± 5.24
18:1 (n-9)	8.06 ± 2.49	7.65 ± 3.03	8.62 ± 1.34	8.30 ± 1.65
18:2 (n-6)	14.15 ± 4.03	13.66 ± 1.93	9.80 ± 2.86	10.36 ± 2.46
18:3 (n-3)	0.24 ± 0.08	0.14 ± 0.11	0.25 ± 0.18	0.14 ± 0.21
20:3 (n-6)	0.74 ± 0.27	0.44 ± 0.34	0.57 ± 0.26	0.30 ± 0.11
20:4 (n-6)	22.53 ± 3.18	23.12 ± 1.56	18.95 ± 2.00	20.98 ± 1.68
20:5 (n-3)	0.79 ± 0.24	1.01 ± 0.60	0.86 ± 0.29	0.77 ± 0.23
22:4 (n-6)	0.19 ± 0.22	0.11 ± 0.10	0.24 ± 0.17	0.15 ± 0.12
22:5 (n-3)	0.78 ± 0.36	0.67 ± 0.17	0.80 ± 0.38	0.69 ± 0.40
22:6 (n-3)	6.46 ± 1.98	5.20 ± 1.43	6.36 ± 1.50	6.94 ± 1.66
Total saturated	45.13 ± 4.17	45.52 ± 4.10	52.49 ± 2.98	48.79 ± 3.76
Total unsaturated	54.84 ± 4.14	54.10 ± 3.26	47.55 ± 2.56	50.31 ± 2.92
Total MUFA[1]	8.97 ± 2.69	9.45 ± 3.09	9.61 ± 1.20	9.79 ± 2.41
Total (n-6) PUFA[2]	37.60 ± 3.30	37.42 ± 1.09	29.57 ± 3.93	31.80 ± 2.67
Total (n-3) PUFA	8.27 ± 2.37	7.11 ± 0.96	8.37 ± 1.51	8.54 ± 1.67
n-6/n-3	4.97 ± 2.03	5.34 ± 0.76	3.66 ± 0.98	3.93 ± 1.35
PUFA/SFA[3]	1.01 ± 0.14	1.00 ± 0.11	0.71 ± 0.11	0.80 ± 0.03

For details, see Table 4 legend

These findings suggest further investigations towards elucidating drug action and membrane functions. The changes in the membrane which form the basis of the phenomenon of membrane tolerance have not yet been identified at tha molecular level. Membrane tolerance is a property of the phospholipids and does not depend on the presence of membrane proteins or neutral lipid components. The observed changes can be explained as adaptive responses to antidepressantinduced fatty acid alterations.

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Table 6. Fatty acid composition of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) + sphingomyelin (SPH) plasma phospholipids (wt%)

	PC		PC ± SPH	
Faty acid	Control	DZP	Control	DZP
16:0	19.26 ± 1.01	18.55 ± 1.52	28.49 ± 3.45	28.43 ± 097
16:1 (n-7)	0.43 ± 0.02	0.48 ± 0.07	1.21 ± 0.98	1.52 ± 0.76
18:0	22.19 ± 0.81	25.60 ± 3.36	21.90 ± 6.73	24.48 ± 7.02
18:1 (n-9)	8.51 ± 0.69	9.02 ± 2.28	12.53 ± 2.26	14.34 ± 3.48
18:2 (n-6)	12.12 ± 0.01	12.56 ± 3.72	20.27 ± 3.85	17.70 ± 3.59
18:3 (n-3)	0.43 ± 0.09	0.19 ± 0.07*	0.38 ± 0.34	0.44 ± 0.18
20:3 (n-6)	0.77 ± 0.08	0.44 ± 0.18*	0.61 ± 0.18	0.31 ± 0.11*
20:4 (n-6)	22.09 ± 0.72	20.57 ± 3.23	10.21 ± 2.85	9.23 ± 2.18
20:5 (n-3)	0.90 ± 0.38	0.46 ± 0.05*	0.24 ± 0.13	0.14 ± 0.02
22:4 (n-6)	0.20 ± 0.04	0.25 ± 0.07	0.09 ± 0.00	0.06 ± 0.04
22:5 (n-3)	1.67 ± 0.19	0.87 ± 0.15**	0.38 ± 0.19	0.29 ± 0.14
22:6 (n-3)	10.35 ± 0.94	6.50 ± 1.27	1.63 ± 0.39	1.43 ± 0.55
Total saturated	41.40 ± 2.42	44.15 ± 3.79	50.39 ± 5.14	52.91 ± 6.64
Total unsaturated	57.47 ± 1.08	51.34 ± 1.69	47.55 ± 5.08	45.46 ± 6.85
Total MUFA[1]	8.90 ± 1.48	9.50 ± 1.48	13.74 ± 3.53	15.86 ± 4.14
Total (n-6) PUFA	35.18 ± 5.50	33.82 ± 4.84	31.18 ± 2.96	27.30 ± 2.84
Total (n-3) PUFA	13.35 ± 2.71	8.02 ± 1.68**	2.63 ± 0.64	2.30 ± 0.62
n-6/n-3	2.63 ± 0.52	4.41 ± 0.80*	11.86 ± 4.66	11.87 ± 3.66
PUFA/SFA	1.17 ± 0.03	0.93 ± 0.14	0.67 ± 0.03	0.56 ± 0.14

* Denotes significance of differences between control and DZP groups ($p<0.05$)
** $p < 0.01$
For details, see Table 4 legend.

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SADRŽAJ FOSFOLIPIDA I MASNIH KISELINA U JETRI I PLAZMI PACOVA NETRETIRANIH I TRETIRANIH DIAZEPAMOM

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SADRŽAJ

Ispitivan je efekat diazepama na masne kiseline fosfolipida plasme i jetre pacova, mužjaka, soja Wistar. Pacovi su bili podeljeni u dve grupe sa po 12 životinja u svakoj. Obe grupe su hranjene nutritivno odgovarajućom dijetom (18 g/d). Kontrolna grupa (C) je proizvoljno unosila vodu. Eksperimentalna grupa je pila diazepam (DZP) u dozi od 10 mg/kg/dan. Dugotrajno unošenje DZP (180 dana) dovodi do značajnih promena u sadržaju fosfolipida i sastavu masnih kiselina glavnih frakcija fosfolipida jetre. Dolazi do značajnog smanjenja 20:3 (n-6), 20:5 (n-3) ukupnih fosfolipida jetre u pacova koji su unosili diazepam. Unošenje diazepama statistički značajno smanjuje sadržaj 20:3 (n-6), 22:5 (n-3), 22:6 (n-3) i ukupnih polinezasićenih masnih kiselina fosfolipida i fosfatidilholina plazme.