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Phospholipid Content and Fatty Acid Composition in the Rat Heart after Chronic Diazepam Treatment

V. RISTIĆ, V. TEPŠIĆ, S.R. DE LUKA, S.R. VRBAŠKI

Institute for Medical Research, Belgrade, Yugoslavia

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Summary

The effects of chronic diazepam treatment (10 mg/kg/day for 180 days) on the fractional distribution and fatty acid composition of heart phospholipids were studied in male Wistar rats. It was found that diazepam treatment increased the content of phosphatidylcholine and cardiolipin in the heart and slightly increased its phosphatidylcholine fraction. There were no significant changes in fatty acid composition after diazepam treatment in heart phospholipids, with the exception of significant decrease of 20:3n-6 and 20:5n-3 fatty acids. Our findings suggest that diazepam, probably through peripheral benzodiazepine binding sites, altered the content of heart cardiolipin and caused changes in the flux of oxidative phosphorylation in the heart.

Key words
Phospholipids – Fatty acids – Heart – Rat – Diazepam

Introduction

Diazepam is widely used as an anxiolytic, anticonvulsant, muscle relaxant and psychosedative agent (Skolnick and Paul 1981). The presence of high-affinity binding sites for this drug has been demonstrated in the CNS (Möhler and Okada 1977), and these receptors have been shown to be functionally coupled to both the GABA receptor and the chloride ionophore (Olsen 1982). In addition, low-affinity binding sites for benzodiazepines appear to be present in some peripheral tissues, such as kidneys, lungs, liver, heart, skeletal muscles, smooth muscles, mast cells and even in the brain (Taniguchi *et al.* 1982).

Some observations suggested that diazepam influences lipid and carbohydrate metabolism (Madar *et al.* 1987). It was shown in our previous reports (Vrbaški *et al.* 1989, 1996, Ristić *et al.* 1988, 1989) that chronic diazepam treatment significantly changes brain, liver, erythrocyte and plasma lipids in rats.

Phospholipids represent major lipid components of cellular membranes in the brain and other tissues, but the functional role of these lipids in rats subjected to long-term diazepam treatment remains to be defined.

Therefore, the main aim of the present study was to determine possible alterations in phospholipids and their fatty acid composition in rat whole heart homogenates after 6 months of diazepam treatment.

Materials and Methods

Male Wistar rats with initial body weight of 194 g (2-month-old) were maintained on a nutritionally and energetically adequate diet (21 % protein, 62 % carbohydrate, 5 % fat, 0.25 % vitamin premix, 2.25 % mineral mixture – Veterinarski Zavod, Zemun, Yugoslavia) for 180 days. The animals were housed in separate cages in a temperature-controlled room (19±1 °C) with a 12 h light-dark cycle. All animals were matched for initial body weight and were weighed at weekly intervals. The rats were divided into two groups (diazepam – DZP and control – C) with 8 animals in each.

The test substance, diazepam (KRKA, Novo Mesto, Slovenia) was dissolved in tap water (10 mg/kg/day) and offered daily as the sole drinking fluid to 8 intact rats (DZP group) for 180 days.

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A dose of 10 mg/kg/day had been established as optimal for the oral free choice procedures in preliminary examinations, because it induced physical dependence without causing toxic or other side effects during the chronic administration (Fuch *et al.* 1984).

Eight rats (control group) received tap water as their only drinking fluid. The rats had free access to water. The daily fluid intake of the rats drinking the diazepam solution was equal to the daily water intake consumed by the control group.

After 180 days of the treatment, all animals were sacrificed during i.p. nembutal anaesthesia. The hearts were rapidly removed, weighed and processed for biochemical studies.

Heart lipids were extracted by the method of Harth *et al.* (1978). From the heart lipid total content, phospholipids were separated by thin-layer chromatography (TLC) into eight fractions, i.e. phosphatidylcholine (PC), phosphatidylethanolamine (PE), cardiolipin (CARD), sphingophospholipids (SPLs), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA) and lysophosphatidylcholine (LPC), using a two-dimensional TLC system: 1) chloroform-methanol-20 % ammonia, 65:25:5 (by volume); 2) chloroform-acetone-methanol-acetic acid-water, 75:17.5:12.5:10:4.4 (by volume).

Heart phospholipid fatty acids were isolated by TLC using hexane-diethylether-acetic acid, 87:12:1 (by volume). Total lipid phosphorus was determined by the method of Kostić *et al.* (1972). Methyl esters of phospholipid fatty acids were prepared by methods that have been reported previously (Ristić and Vrbaški 1992). The fatty acid methyl esters were then analysed by gas chromatography using a Varian GC (model 3400, Varian Associates) equipped with a flame ionisation detector and a 30 m x 0.53 mm SP-2380 fused silica capillary column (Supelco, Bellefonte). The oven temperature was held at 130 °C for 3 min, programmed to 190 °C at 3 °C/min, and then held at 190 °C for 15 min.

The chromatograph was operated with a column flow rate of 2.5 ml nitrogen/min, and an injection temperature of 220 °C. The detector was maintained at 250 °C.

Individual fatty acid methyl esters in the sample were identified from the retention times of authentic standards (Sigma Chemical Company) and/or the polyunsaturated fatty acid (PUFA)/2 standard mixture (Supelco, Bellefonte).

Peak areas were determined with a Varian 4290 integrator, and the results were expressed as percentages of total identified fatty acids.

The data were analysed using Student's t-test (Fisher 1970).

Results and Discussion

During the study, the average food intake per rat was 15 g or 244 kJ per day in both the control and experimental group.

The initial and final body weights and daily body weight gain for both groups are summarised in Table 1. Body weight gain did not differ significantly between the groups.

Table 1. Body weight and daily body weight gain in rats of the control and diazepam treated groups

	Control (n=8)	DZP (n=8)
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

Data are means±S.D.; DZP, diazepam (10 mg/kg/day)

The wet weight and total lipid content of the heart were similar in both groups. The total phospholipid content in the heart of DZP group was significantly higher than that in the control group ($p<0.05$) (Table 2).

Table 2. Heart wet weight (g), total lipid (mg/g wet weight) and phospholipid (μg/g wet weight) of the control and diazepam-treated rats

	Control	DZP
Heart wet weight	1.05±0.09	0.99±0.09
Total lipids	39.87±5.26	41.66±3.95
Phospholipids	626.80±49.90	689.2±62.1*

*Data are means±S.D., DZP, diazepam (10 mg/kg/day), * Significantly different ($p<0.05$) from the control*

A significant increase in the concentration of lipid phosphorus was found in the heart phosphatidylcholine (PC) and cardiolipin of DZP-treated rats (Table 3). There were no significant differences in the distribution of phospholipid fractions in the heart between these two groups of rats. Diazepam treatment only slightly increased the percentage distribution of PC.

Table 4 illustrates the fatty acid composition of heart phospholipids. There were no significant changes in fatty acid composition after diazepam treatment in heart phospholipids, except for the significant decrease of the 20:3 n-6 and 20:5 n-3 fatty acids.

Table 3. The concentration (μg phosphorus/g) and percentage distribution (%, means) of lipid phosphorus in the heart of rats after chronic diazepam consumption

	Control	DZP
PC (μg)	294.47±37.12	338.49±48.11*
(%)	46.9	49.0
PE (μg)	178.82±31.72	178.63±30.80
(%)	28.4	26.0
CARD (μg)	75.74±7.60	90.28±10.30**
(%)	12.2	13.3
SPLs (μg)	29.36±2.25	31.40±3.77
(%)	4.8	4.7
PI (μg)	19.37±1.89	19.25±1.19
(%)	3.1	2.8
PS (μg)	12.86±1.78	13.38±1.87
(%)	2.1	2.0
PA (μg)	8.15±2.92	8.98±2.79
(%)	1.4	1.3
LPC (μg)	8.05±1.93	8.95±1.78
(%)	1.3	1.3

*Data are means±S.D., DZP – diazepam (10 mg/kg/day), PC – phosphatidylcholine, PE – phosphatidylethanolamine, CARD – cardiolipin, SPLs – sphingophospholipids, PI – phosphatidylinositol, PS – phosphatidylserine, PA – phosphatidic acid, LPC – lysophosphatidylcholine, * Significantly different ($p<0.05$) from the control, ** Significantly different ($p<0.001$) from the control.*

The effect of chronic diazepam treatment on the content of liver, plasma and erythrocyte phospholipids (phosphatidylcholine, phosphatidylethanolamine, cardiolipin, sphingophospholipids, phosphatidylinositol, phosphatidylserine, phosphatidic acid and lysophosphatidylcholine) in rats has been described previously (Vrbaški *et al.* 1996). There was a significantly increased content of phosphatidylcholine in the liver and erythrocytes after 6 months of diazepam treatment. Such treatment did not cause statistically significant changes in the plasma of diazepam-treated rats. A recent report from our laboratory (Ristić and Vrbaški 1992) provided evidence of elevated levels of saturated and unsaturated fatty acids in plasma phospholipids due to diazepam treatment. On the other hand, diazepam produced a drastic decrease of PUFA n-3, namely 22:5 n-3 and 22:6 n-3. Diazepam also caused a significant decrease of 22:6 n-3 in liver phospholipids. The main aim of the present study was to determine possible alterations in phospholipids and their fatty acid composition in rat heart homogenates after 6 months of diazepam treatment. The main finding in the present study was the significant increase in phosphatidylcholine and particularly in cardiolipin of the heart and the decrease in the 20:3 n-6 and 20:5 n-3 phospholipid fatty acids. Cardiolipin is one of the principle phospholipids in the mammalian heart. It is localised primarily in the mitochondria and appears to be essential for the function of several enzymes of oxidative phosphorylation. Cardiolipin is also essential for energy production in the heart (Hatch 1996). The peripheral benzodiazepine binding sites (present also in the heart) are associated with the mitochondrial outer membrane (Anholt 1986). Our findings suggest that diazepam, probably through peripheral benzodiazepine binding sites, altered the content of heart cardiolipin and caused the changes in the flux of oxidative phosphorylation in the heart. The functional significance of these biochemical changes is not clear, however, number of possibilities may be considered.

Table 4. Fatty acid composition of heart phospholipids in control and diazepam-treated rats

	Control	DZP
16:0	11.07±0.22	11.04±0.50
18:0	18.72±1.02	19.59±0.83
16:1	0.74±0.11	0.67±0.10
18:1	11.71±0.54	11.34±0.45
18:2 n-6	20.90±2.39	19.14±2.39
20:3 n-6	0.54±0.05	0.37±0.63**
20:4 n-6	19.86±1.42	20.88±0.99
22:4 n-6	0.38±0.04	0.43±0.08
18:3 n-3	0.17±0.06	0.14±0.02
20:5 n-3	0.24±0.03	0.17±0.05*
22:5 n-3	2.01±0.27	1.80±0.27
22:6 n-3	12.74±1.31	13.41±1.29
Σ SFA	29.79±0.96	30.63±0.99
Σ MUFA	12.60±0.64	12.08±0.56
Σ PUFA n-6	41.68±1.22	40.56±2.24
Σ PUFA n-3	15.16±1.22	15.44±1.02
SFA/UNSFA	0.43±0.02	0.45±0.02
n-6/n-3	2.77±0.28	2.64±0.32

*Data are means±S.D., DZP – diazepam (10 mg/kg/day), SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, UNSFA – unsaturated fatty acids, * Significantly different ($p<0.05$) from the control, ** Significantly different ($p<0.01$) from the control.*

The first conclusion from our results is that a regimen of 10 mg DZP/kg/day (during the experimental period the dose was progressively increased with respect to the mean weekly body weight gain) for 6 months may lead to the development of tolerance and physical dependence. Evidence of the

tolerance and physical dependence was revealed by the changes in phospholipid constituents.

Furthermore, as phospholipids represent major lipid components of cellular membranes in the brain as in other tissues, the changes in their content provide further evidence for the lipid requirement of central and/or peripheral benzodiazepine binding sites.

Since our results were expressed as the phospholipid content in the whole tissue, some basic radioligand binding assays should be conducted to assess the influence of the observed lipid changes on the ligand recognition properties of the benzodiazepine specific binding sites. Nevertheless, these investigations are in agreement with the hypothesis that extended or chronic use of drugs, such as diazepam, may alter membrane-dependent processes.

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Reprint requests

V. Ristić, Ph.D., Institute for Medical Research, P.O. Box 721, Dr. Subotica 4a, 11000 Belgrade, Yugoslavia.