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Liver, Plasma and Erythrocyte Phospholipid Content after Chronic Diazepam Treatment in the Rat

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Summary

Male Wistar rats were maintained on a nutritionally adequate diet and diazepam was administered in a dose of 10 mg/kg/day. Control animals were pair-fed an adequate diet. Feeding was continued for 180 days, and the effects on the liver, plasma and erythrocyte phospholipid content were studied. It was found that the contents of sphingophospholipids and phosphatidylinositol + phosphatidylserine were significantly reduced in the erythrocytes of diazepam-treated rats. There was a significantly increased content of phosphatidylcholine in the liver and erythrocytes after 180 days of diazepam treatment. Such treatment did not cause statistically significant changes in the plasma of diazepam-treated rats. These investigations are in agreement with the hypothesis that extended or chronic use of drugs such as diazepam may alter membrane-dependent processes.

Key words

Diazepam – Liver – Plasma and erythrocyte phospholipids

Introduction

It was shown in a previous report (Vrbaški *et al.* 1989) that chronic treatment with diazepam significantly reduced brain lipids in the rat. It was found that the content of phospholipids, phosphatidylethanolamine and phosphatidylserine, the monogalactosyl glycolipids, hydroxy and non-hydroxy fatty acyl galactocerebroside, sulfoglycolipids, and the gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}, were significantly reduced in the brain of diazepam-treated rats. There was a significantly increased content of phosphatidylinositol after 180 days of diazepam treatment. Phospholipids represent the major lipid components of cellular membranes in the brain as in other tissues, but the functional role of these lipids in rats subjected to long-term diazepam treatment largely remains to be defined. We have, therefore, investigated the possible biochemical alterations in the liver, plasma and red cell phospholipids in rats after 180 days of diazepam treatment.

Materials and Methods

Male Wistar rats with initial weight of 194 g were maintained on a nutritionally and energetically adequate diet (protein, 21 %; carbohydrate, 62 %; fat, 5 %; vitamin premix, 0.25 %; mineral mixture, 2.25 %; 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 thereafter. The rats were divided into two groups (DZP and C) with 12 animals in each group.

The test substance, diazepam (KRKA, Novo Mesto, Slovenia), was dissolved in tap water and consumed 10 mg/kg daily as the sole drinking solution to 12 native rats (DZP group) for 180 days. The dose of 10 mg/kg/d had been established as optimal for the oral free choice procedure in preliminary examinations, as it induced physical dependence without causing toxic or other side effects during chronic administration (Fuch *et al.* 1984).

Twelve 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 diazepam solution was monitored to be equal to the daily water intake consumed by the control group.

After 180 days of treatment, the animals of the two groups were sacrificed. The blood was obtained by heart puncture. Blood samples were collected into

heparin-treated tubes and immediately centrifuged and the plasma was frozen until lipid phosphorus determination. The erythrocyte fraction (2–3 g) was washed three times with 5 ml of 0.9 % NaCl before homogenization and centrifugation (1000xg, 10 min). The washing solution was discarded and the globular fraction was frozen at −20 °C for 1 hour to produce rupture of the erythrocyte membrane. The livers from each group of rats were rapidly removed, weighed and processed for biochemical studies.

Lipid extraction and quantitative determination of major lipid classes

During the extraction procedure, lipids were protected against oxidation by addition of 10 mg/100 ml butylated hydroxytoluene to the solvents. Lipids were extracted from the liver according to the method of Harth *et al.* (1978). Plasma lipids were extracted by the method of Alling *et al.* (1982). Red cell membrane lipids were extracted by the method of Rose and Oklander (1965).

Serum and erythrocyte phospholipids were separated by one-dimensional thin-layer chromatography (TLC) into following fractions phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidylcholine (LL), sphingophospholipids (SPLs), while phosphatidylserine (PS) and phosphatidylinositol (PI) were separated only from erythrocytes.

From the liver lipid extracts that were dissolved in chloroform-methanol (1:1 by volume), one aliquot (containing 60 μg lipid phosphorus) was spotted 1 cm on Merck thin-layer glass plates precoated with a 500 μm layer of Silica H and Florisil (9:1). Phospholipids were separated into different fractions, i.e. phosphatidic acid (PA), phosphatidylserine (PS), sphingophospholipids (SPLs), phosphatidylcholine (PC), phosphatidylethanolamine (PE), diphosphatidylglycerols (DPGs), by a two-dimensional TLC system: 1) chloroform-methanol-20 % ammonia, 65 : 25 : 5 (by volume); 2) chloroform-acetone-methanol-acetic acid-water, 70 : 17.5 : 12.5 : 10 : 4.4 (by volume). Phospholipids were detected by exposure of the plate to iodine vapor. After complete sublimation of the iodine from the plate, the spots of individual phospholipids were scraped off for the determination of lipid phosphorus (Kostic *et al.* 1972).

No triacylglycerols were detected in lipid extracts from 500 μl of packed red cells which had been applied to TLC plates and developed in petroleumether-ethylether-acetic acid, 80 : 20 : 1 (by volume). Accordingly, contamination of red cell membrane lipid extracts by plasma lipids are unlikely.

The data were analyzed 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 groups.

The initial and final body weights and daily body weight gains for both groups are summarized 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 DZP groups

	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

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

Table 2
Changes in the concentration of lipid phosphorus in the liver of rats (μmol/g) after chronic DZP consumption

Lipid	Initial	Control	DZP[a]
PE	6.64±1.02	7.33±0.90	7.23±1.75
PC	12.94±1.89	15.91±1.47	17.04±1.74*
PS	0.55±0.10	0.72±0.04	0.77±0.10
PA	0.28±0.11	0.37±0.06	0.40±0.08
DPGs	0.59±0.17	0.45±0.08	0.45±0.10
SPLs	0.94±0.20	1.08±0.16	1.20±0.07
PI	3.74±0.60	4.26±0.22	4.06±0.55

*Data are means±S.D. DZP, diazepam (10 mg/kg/day); DPGs, diphosphatidylglycerols; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPLs, sphingophospholipids. *Significantly different ($p<0.01$) from the control.*

The liver PC (17.04±1.74 μmol/g tissue) after chronic diazepam treatment were significantly higher than those of the controls (Table 2).

DZP treatment did not cause significant changes of phospholipids in the plasma of diazepam treated rats (Table 3). The erythrocyte SPLs (0.38±0.01 μmol/g) and PS+PI (0.12±0.02 μmol/g) after chronic diazepam treatment were significantly lower than those of the control. The mean PC content in erythrocytes of rats after chronic DZP treatment (0.72±0.09 μmol/g) was significantly elevated above

control rat erythrocyte PC values (0.60±0.05 μmol/g) (Table 4).

Table 3
Changes in the concentration of lipid phosphorus in the plasma of rats (mmol/l) after chronic DZP consumption

Lipid	Initial	Control	DZP
PE	0.20±0.05	0.22±0.02	0.19±0.02
PC	0.67±0.15	0.69±0.02	0.69±0.03
SPLs	0.42±0.10	0.40±0.03	0.43±0.01
LL	0.26±0.09	0.28±0.04	0.28±0.03

Data are means±S.D. DZP, diazepam (10 mg/kg/day); LL, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPLs, sphingophospholipids.

Table 4
Changes in the concentration (μmol/g erythrocytes) and percent distribution (%, means) of lipid phosphorus in the erythrocytes of rats after chronic DZP consumption

Lipid	Initial	Control	DZP
PE (μmol)	0.41±0.08	0.42±0.03	0.39±0.02
(%)	25.0	24.7	24.2
PC (μmol)	0.58±0.10	0.60±0.05	0.72±0.09#
(%)	35.4	35.3	44.7
SPLs (μmol)	0.45±0.09	0.46±0.04	0.38±0.01*
(%)	27.4	27.1	23.6
PS+PI (μmol)	0.20±0.05	0.22±0.04	0.12±0.02#
(%)	12.2	12.9	7.45

*Data are means±S.D., DZP, diazepam 10 mg/kg/day); PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPLs, sphingophospholipids; PS+PI, phosphatidylinositol + phosphatidylserine. *Significantly different from the control at $p<0.05$. #Significantly different from the control at $p<0.01$*

The main interest in our investigation was to determine possible alterations in phospholipids in rat liver, plasma and red cells after 180 days of diazepam treatment. The duration of chronic treatment (6 months) was longer than generally used in such studies and is probably more relevant to the clinical situation (Vrbaški *et al.* 1989, Ristić *et al.* 1992).

Some observations suggest that diazepam influenced food consumption and weight gain in animals (Huupponen *et al.* 1986). Our investigation showed that the long-term diazepam treatment did not cause change of weight gain, whereas all animals (control and DZP) were limited to consume 15 g of diet daily. Daily body weight gain during entire 6-month period was similar (0.94 and 0.93 g).

The major finding in the present study is the significant increase in phosphatidylcholine content of both liver and red blood cells (RBC) and the decrease in PE, SPLs and PS + PI contents of RBC in drug-treated rats when compared to those in control animals. Moreover, such treatment did not cause statistically significant changes in the plasma phospholipid content.

The liver PC fraction was increased after 180 days of diazepam treatment compared to the control group. The absence of such a change in the plasma after chronic DZP treatment suggests that the level of this phospholipid (PC) during chronic administration appears to be controlled by a balance between synthesis and breakdown, with the regulation of this balance on the catabolic side.

The synthesis of triacylglycerols and PC during benzodiazepine or alcohol consumption is regulated in rat hepatocytes (Ristić *et al.* 1988, 1989, Ristić 1991). The alternative explanation that hypertriglyceridaemia could be associated with a stimulation of PC synthesis in certain cell types, including hepatocytes and mature red blood cells or their precursors, cannot be excluded.

The present results indicate that distinct changes in erythrocyte membrane phospholipids are observed in diazepam-treated rats. In these rats, membrane PC was elevated while SPLs were reduced. The PC/SPLs ratio is considered to be an important marker of membrane lipid fluidity (Bahrenholz 1984, Hucho 1986, Engelmann *et al.* 1992), a parameter thought to influence various membrane functions. Accordingly, the elevation in PC/SPLs ratio after chronic DZP treatment observed in erythrocytes (by about 41 %) probably induces a rise in membrane lipid fluidity which is likely to be restricted to the outer layer of the membrane where more than 80 % of total RBC PC and SPLs are located. In rat brain homogenates, the content of lipid phosphorus (Vrbaški *et al.* 1989) in the PS fraction was decreased after chronic diazepam treatment. However, the lipid phosphorus level in PI fraction was significantly higher in the brain of diazepam-treated rats. The significantly lower content of anionic phospholipids PI + PS, occurring in the RBC after diazepam treatment, indicates that in diazepam-treated rats different cell membranes can respond differently to diazepam due to their different original lipid composition. This possibility will require further research. At the very least, additional analysis of RBC functions (haemolysis, transport and/or determination of some RBC-specific enzymes such as cholinesterase) caused by changes in phospholipid composition after the drug treatment are necessary. Alternatively, a study of the time course of changes in phospholipid composition in brain and liver subcellular

fractions, particularly in isolated plasma membranes as well as in RBC, may provide some evidence as to how the drug affects phospholipids metabolism chronically in the rat.

Much of the attention in benzodiazepine research has been focused on benzodiazepine-induced chronic changes in the membrane lipid composition that could explain the adaptation to the presence of these drugs. Adaptation to a fluidizing effect includes a decrease in the degree of unsaturation of phospholipid fatty acids (Engelmann *et al.* 1992). This points to the high complexity of the action of diazepam upon cell membranes. Concerning the effects of diazepam on plasma and liver membrane phospholipid fatty acid composition, a disturbance in fatty acid metabolism was observed (Ristic *et al.* 1995). Chronic diazepam treatment of rats increases the proportions of saturated and monounsaturated fatty acids in plasma phospholipids. On the other hand, diazepam drastically decreased 22:6n-3 (docosahexaenoic acid) in plasma and liver phospholipids. The findings in this study extend these changes to the brain and other tissues, i.e. fatty acid composition in the brain, heart, plasma and red cell phospholipid fractions after long-term diazepam treatment.

However, the data obtained are too preliminary for any definite conclusions to be drawn and further investigations towards elucidating the drug action and membrane functions are warranted.

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Reprint Requests

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