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Molecular Stability of Brain Plasmalogens in a Fat Free Diet

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Summary

Fat free diet (FFD) affects specifically structures of particular lipid molecules. The effect of short-term (two-week) diet on rat brain plasmalogens is reported in this paper. Two groups of eight animals (Wistar strain, average body weight 180 g) were on two different diets: the control diet (CD) was actually a standard rat chaw, while the fat free diet (FFD) was prepared in our own laboratory. Both diets were isoenergetic (CD 15.74 kJ; FFD 15.10 kJ). The average body weight gain was 4 %. For CD the mass of brain was 692.7 and for FFD 891.3 mg. Total phosphate/protein ratio (TP/P) of the brain tissue was 48.7/100 in CD and 33.2/100 in FFD, indicating the inhibited biosynthesis of phospholipids. The majority of plasmalogens in brain is associated with the phosphatidylethanolamine (PE) lipids. Plasmenyl-phosphatydilethanolamine (Pla-PE) comprised 58.3 % in CD and 59.7 % in FFD of total PE. Following effects of FFD on Pla-PE fatty acid composition were noticed: (*i*) increased content of palmitoleic acid (+3.45 %), (*ii*) increased content of stearic (+8.22 %) with decreased content of oleic acid (–6.02 %), (*iii*) decreased content of essential fatty acids (–12.55 %) and (*iv*) decreased content of polyunsaturated fatty acids followed by a simultaneous increase of eicosatrienoic acid content (+6.21 %) and a decrease of docosahexaenoic acid content (–8.47 %). The overall effect of FFD caused a moderate saturation of Pla-PE fatty acids (F_u/–7.39). The analysis of the aldehydogenic moiety of the Pla-PE resulted in: (*i*) no essential changes in the amount of total aldehydes of the PE lipids (CD 58.3 %, FFD 59.7 %); (*ii*) the alkenyl chain was composed of C 16, C 18 and C 20 homologues, while C 18 and C 20 homologues comprised almost 70 % of total aldehydes, (*iii*) no essential changes neither of the total amount of C 18 homologue (CD 59.4 %, FFD 64.1 %), nor of the ratio of *cis-trans* C 18 isomers were found, and finally (*iv*) the amount of C 20 homologue remained almost unchanged (CD 4.05 %, FFD 4.23 %). All of the represented data indicate very slight modulations of the Pla-PE molecule affected by a FFD and support the already recognized idea that plasmalogens are one of the most important stabilizers of the structure of biological membranes.

Key words: fat-free diet, plasmalogens, brain, rat

Introduction

Plasmalogens are well known constituents of a variety of membrane structures. The most important events were reviewed by Paltauf (*1*). Structural changes of plasmalogens seem to be of the essential importance for

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biomembrane properties. Only the most important roles of plasmalogens can be stressed here. The link between the phospholipase A_2 and *signal transduction* was reviewed by Farooqui *et al.* (*2,3*). Plasmalogens stabilize the structure of biological membranes, as reported by Lohner *et al.* (*4*) and Glasser and Gross (*5*). Vance (*6*) stressed the capability of plasmenyl-phosphatidylethanolamine (Pla-PE) to stimulate the synthesis of nascent lipoproteins. Plasmalogens also seem to be very effective *antioxidants* as suggested by Paltauf (*1*), Khaselev and Murphy (*7*) and Boeynaems *et al.* (*8*).

Changes of the plasmalogens structure and/or content during the aging or diseases were also extensively examined so far. Just to exemplify, Martinez and Mougan (*9*) did research on fatty acid changes of brain phospholipids in peroxisomal disorders. Structural changes of plasmalogens in Alzheimer's disease were studied by Guan *et al.* (*10*), Farooqui and Horrocks (*11*), Gingsberg *et al.* (*12*) and Farooqui *et al.* (*13*). Plasmalogens are by Roscher *et al.* (*14*) suggested to be a convenient diagnostic tool in prenatal detection of Zellweger syndrome. Fatty acid composition of plasmalogens during *normal development* was reported more recently by Martinez and Mougan (*15*), while Weisser *et al.* (*16*) and Lopez *et al.* (*l7*) described age associated changes of brain glycerophospholipids.

At the end, specific effects of nonconventional diets on structural changes of brain lipids should be pointed out. Just to exemplify, Favreliere *et al.* (*18*) reported the effect of »PUFA diets« (PUFA = polyunsaturated fatty acids) on structural changes of brain plasmalogens. Diets missing essential fatty acids (*19*) or diets composed of nontypical fatty acids (*20–22*) can remarkably change the composition of tissue lipids. In our previous studies similar effects were noticed when rats were on fat free diet (*23–25*). Since these researches did not include the effects on plasmalogens, the studies were extended in this direction and reported in this paper.

Materials and Methods

Animals and foods

Two groups of eight male albino rats (Wistar strain) of 180 g average body weight (b.w.) were on a fat free diet (FFD) and a control diet (CD) during a two-week period, respectively. The CD was a standard rat chow manufactured by Pliva, Zagreb, Croatia, while the FFD was prepared in our laboratory following the diet composed by Iritani and Narita (*26*). Both diets were almost isoenergetic (Table 1). The access to food and water was unlimited. When animals were sacrificed, the brain was removed, rinsed with cold saline, weight and frozen.

Table 1. Overall composition of control (CD) and fat free diet (FFD) expressed as fraction in %

Main ingredients	CD	FFD
Total proteins	19.37	11.94
Total lipids	3.67	0.66
Total carbohydrates	64.82	75.33
Total nitrogen	3.10	1.91
Dry weight/g	90.96	89.84
Energy (kJ/g)	15.74	15.10

Methods

The frozen tissue was homogenized and extracted with chloroform/methanol (2/1 volume fractions) following the original procedure of Folch *et al.* (*27*), slightly modified by Hoermann *et al.* (*28*). Dry total lipids (TL) were dissolved in 10 mL toluol/ethanol (3/2 volume fractions). Aliquots were subsequently used for preparative and analytical procedures. Brain proteins were determined following the method by Lowry *et al.* (*29*). Total phosphates were determined according to Parker and Peterson (*30*).

An aliquot of TL was used to separate phosphatidylethanolamine (PE) from phosphatidylcholine (PC) by a thin layer chromatography (TLC) method on silica gel G (25 nm) coated plates (E. Merck, Darmstadt, Germany). The plates were developed in chloroform/methanol/15 M ammonia (65/35/5 volume fractions) up to 12 cm from the start. Lipid lines were visualized by iodine vapors and identified according to authentic PE and PC standards. PE and PC zones were separately collected into centrifuge tubes and extracted several times with a total volume of 10 mL chloroform/methanol (1/1 volume fractions). The solvent volume was reduced to several microliters (but not to dryness) and subsequently used for separation of plasmalogens from diacylglycerophosphatides of both PE and PC lipids.

Plasmalogen aldehydes can be separated from their total PE and PC lipids by means of a 2-D HPTLC as published by Horrocks (*31*). Total phospholipids were first developed in chloroform/methanol/15 M ammonia (65/25/4 volume fractions) up to 12 cm from the start. The chromatogram was dried and exposed to vapors of concentrated hydrochloric acid in order to cleave the *sn*-1 vinyl-ether bond. Liberated aldehydes were separated from the rest of phospholipids by running the chromatogram in the rectangular direction in a developer chloroform/methanol/15 M ammonia (100/50/12 volume fractions) up to 10 cm from the start. Lipid fractions were visualized by iodine vapors, while aldehydes can be specifically identified with a 2,4-dinitrophenylhydrazine reagent.

The quantification of *sn*-1 aldehydes was carried out in a pool of eight samples by a gas-liquid chromatography (GLC) following the method by Hoermann *et al.* (*28*). Phosphatidylethanolamine (50 μg P) was mixed with the following internal standards: 100 nmol of nonadecanal and 50 nmol of acetylmyristate. The solution was applicated on the silica gel coated plate, and the solvent was blown off to dryness. The start-line was then covered with 100 μL of 2 % trichloracetic acid in 8 % aqueous hydrochloric acid. This reagent was allowed to react 10–15 min with the applicated PE lipids. The start line was dried and the HPTLC plate was subsequently developed in *n*-hexane/diethyl ether (95/5 volume fractions). Aldehydes were identified by spraying both outer vertical borders with 2,4-dinitrophenylhydrazine reagent. The unsprayed aldehyde's silica gel G line was scratched off into a centrifuge tube. Aldehydes were reextracted several times with *n*-hexan/isopropanol (3/2

volume fractions). Combined extracts were evaporated to dryness in a stream of argon. The remained substance was finally dissolved in 100 μL of *n*-hexane. An aliquote was used to quantify the aldehydes by means of GLC. The analysis was performed in a Hewlett-Packard model 5890 instrument, provided with a FID and capillary column HP-5 (25 m length; 0,2 mm i.d.). The column temperature was 160 °C iso for 25 min, followed by a linear gradient of 10 °C/min up to 230 °C. Helium was the carrier gas.

For the determination of *sn*-2 linked fatty acids of both PE and PC lipid fractions, lysophospholipids obtained by the already described 2-D HPTLC were used (Fig. 1). The samples were allowed to react 5 h with anhydrous 1 M HCl at 100 °C. The reaction mixture was cooled to room temperature and extracted with 4x10 mL of *n*-hexane. The combined extracts were washed several times in order to remove acidic traces completely, dried over anhydrous sodium sulphate and evaporated in vacuum to dryness.

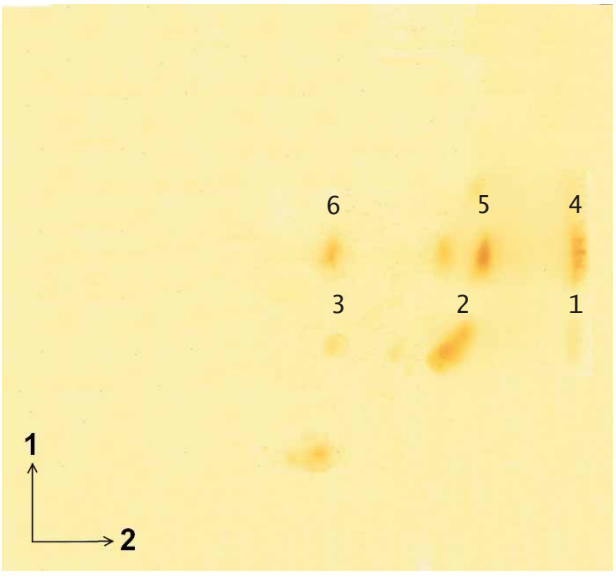

Fig. 1. 2-D HPTLC of brain phospholipids of rats on fat free diet 1. aldehyde from plasmenylphosphatidylcholine; 2. phosphatidylcholine; 3. lysophosphatidylcholine; 4. aldehyde from plasmenylphosphatidylethanolamine; 5. phosphatidylethanolamine; 6. lysophosphatidylethanolamine
Developers: 1st dimension: $CHCl_3$:CH_3OH:2 M NH_4OH = 65:25:4 (volume fractions), 2nd dimension: $CHCl_3$:CH_3OH:2 M NH_4OH = 100:50:12 (volume fractions)

The obtained fatty acid methyl esters were analyzed in a Perkin Elmer instrument, model Sigma 2, provided with FID and a capillary column SP-2340 (30 m length; 0,25 mm i.d.). The column temperature was: 8 min at 150 °C and then up to 190 °C at linear gradient 3 °C/min. The carrier gas was nitrogen. Fatty acid methyl esters were identified relative to retention times of authentic standards.

All used chemicals and organic solvents were of highest purity grade, purchased from Hospitalia, Zagreb, Croatia. Lipid standards were delivered from E. Merck, Darmstadt and Sigma, Munich, Germany.

Results

The basic composition of the both applied isoenergetic diets is shown in Table 1. The main energetic source of both diets are carbohydrates. The fatty acid composition of CD was previously published (*23*). During the diet period, following average quantities of food were ingested per animal and day: CD 20 g (314.8 kJ); FFD 28 g (452.8 kJ). The average body weight gain per animal was 4 %. The average mass of the isolated brain tissues was found as follows: CD 692.7 mg; FFD 891.3 mg, wet tissue (w.t.). Total phospholipids (TP) and proteins (P), as well as their ratios (TP/P), were determined: (*i*) CD, TP 33.6 mg/g w.t., P=6.9 mg/100 mg w. t., TP/P=48.7/100; (*ii*) FFD, TP=23.6 mg/g w.t., P=7.1 mg/100 mg w.t., TP/P = 33.2/100.

The qualitative TLC analysis of brain PC and PE lipids revealed no essential differences of the PC/PE ratio affected by FFD (data not shown).

The separation and identification of the plasmenyl--moieties is demonstrated in Fig. 1. The applied 2-D HPTLC method showed spots 1 and 4 belonging to PC and PE aldehydes, respectively. The chromatogram indicates that the majority of plasmenyl-phospholipids are a part of PE lipids. Lyso-plasmenylphospholipids of both PC and PE classes were also identified and designated as spots 3 and 6 on Fig. 1. Very mild conditions of the acidic hydrolysis (HCl vapors) do not cleave ester bonds, so intact diacyl-PC and diacyl-PE were also identified (Fig. 1, spots 2, 5).

Since the majority of plasmalogens belongs to PE lipids, only this class was submitted to further analysis of both aldehydogenic and acyl moieties. Data on Pla--PC will be referred only partially.

Plasmenyl-aldehydes were quantified by means of GLC. As it is presented in Table 2, the C 18 homologues are the predominant ones. Saturated *vs*. total unsaturated aldehydes of the C18 series were found in almost equal amounts. FFD caused no essential differences of both, *cis*- and *trans*-, C 18 aldehyde. The C 16 homologue comprised approximately the same amount as C 18:0 aldehyde, but only half as much as total C 18 aldehydes. The minor homologue was found to be the C 20 aldehyde (approximately 4 %). No quantitative changes of C 20 aldehyde fraction were caused by FFD.

Table 2. Aldehyde fraction % of brain plasmenyl phosphatidylethanolamine (Pla-PE) of rats on control (CD) and fat free diet (FFD) for two weeks

Aldehyde	CD	FFD	FFD - CD (Δ%)
C16:0	38.70	27.00	–11.70
C18:0	29.49	33.40	+3.91
C18:1c	13.10	15.01	+1.91
C18:1t	16.80	15.68	–1.12
C20:0	4.05	4.29	+0.24

The *sn*-2 fatty acids of Pla-PE were isolated and derivatized into methyl esters. The quantification was achieved by means of GLC (details in: *Methods*). Particular data are shown in Table 3. These data relative to the effects of the FFD will be discussed in the next chapter.

Table 3. Fatty acid fraction of brain plasmenyl phosphatidylethanolamine (Pla-PE) of rats on control (CD) and fat free diet (FFD) for two weeks. Results are expressed as mean value ± s. dev.

Fatty acid	CD (%)	FFD (%)	FFD – CD (Δ %)
C14:0	1.28 ± 1.34	1.60 ± 0.64	+0.32
C16:0	3.25 ± 0.25	3.84 ± 1.14	+0.59
C16:1	1.77 ± 1.23	5.22 ± 1.14	+3.45
C18:0	2.87 ± 1.32	11.09 ± 2.71	+8.22
C18:1	34.71 ± 3.86	28.69 ± 3.44	–6.02
C18:2	tr	tr	–
C18:3	7.55 ± 2.09	5.58 ± 3.02	–1.97
C20:3	5.40 ± 2.32	11.61 ± 8.41	+6.21
C20:4	15.64 ± 2.12	13.53 ± 3.98	–2.11
C22:6	26.68 ± 4.18	18.24 ± 8.04	–8.47

Table 4. Factor of unsaturation (F_u) of C16 and C18 fatty acids of brain total lipids (TL), plasmenyl phosphatidylethanolamine (Pla-PE) and plasmenyl phosphatidylcholine (Pla-PC) of rats on control (CD) and fat free diet (FFD) for two weeks

	Fu	CD	FFD	± ΔFu (FFD-CD)
TL	C 16:1/16:0	0.03	0.05	–0.04
	C 18:x/18:0	1.18	1.00	–0.18
Pla-PE	C 16:1/16:0	0.54	1.36	0.82
	C 18:x/18:0	14.75	3.09	–11.66
Pla-PC	C 16:1/16:0	0.64	0.77	0.13
	C 18:x/18:0	0.06	2.89	2.83

Most of the feeding experiments, introducing nonconventional diets, pay a special attention to the rate of desaturation of fatty acids. The ratio of unsaturated *vs.* saturated fatty acids, designated mostly as »factor of unsaturation (F_u)«, is one of the crucial parameters in determining the fluidity of biological membranes. Data relative to this issue are represented in Table 4 and Fig. 3, and will be discussed in the following chapter.

Discussion

Modulations of lipid molecules affected by nonconventional diets were extensively investigated so far. Therefore, comments on this issue will be restricted here on fat free diets only. The importance of fat free diets relative to cardiovascular diseases was repeatedly stressed by WHO, Public Health Association and other professional organizations. However, numerous problems on this issue still remained open. Starting these studies we wanted to contribute the researches for better understanding of such, yet unsolved, problems.

As already mentioned, the daily food ingestion per animal ranged from 20–27 g, while the average body weight increase was 4 %. Data relative to total phosphate/protein ratio (CD 48.7; FFD 33.2) indicate a reduced phospholipid biosynthesis influenced by a FFD. The background of this inhibited biosynthesis is not clear enough, but the lack of exogenous glycerol, originating from nutritional glycerolipids in FFD, might be

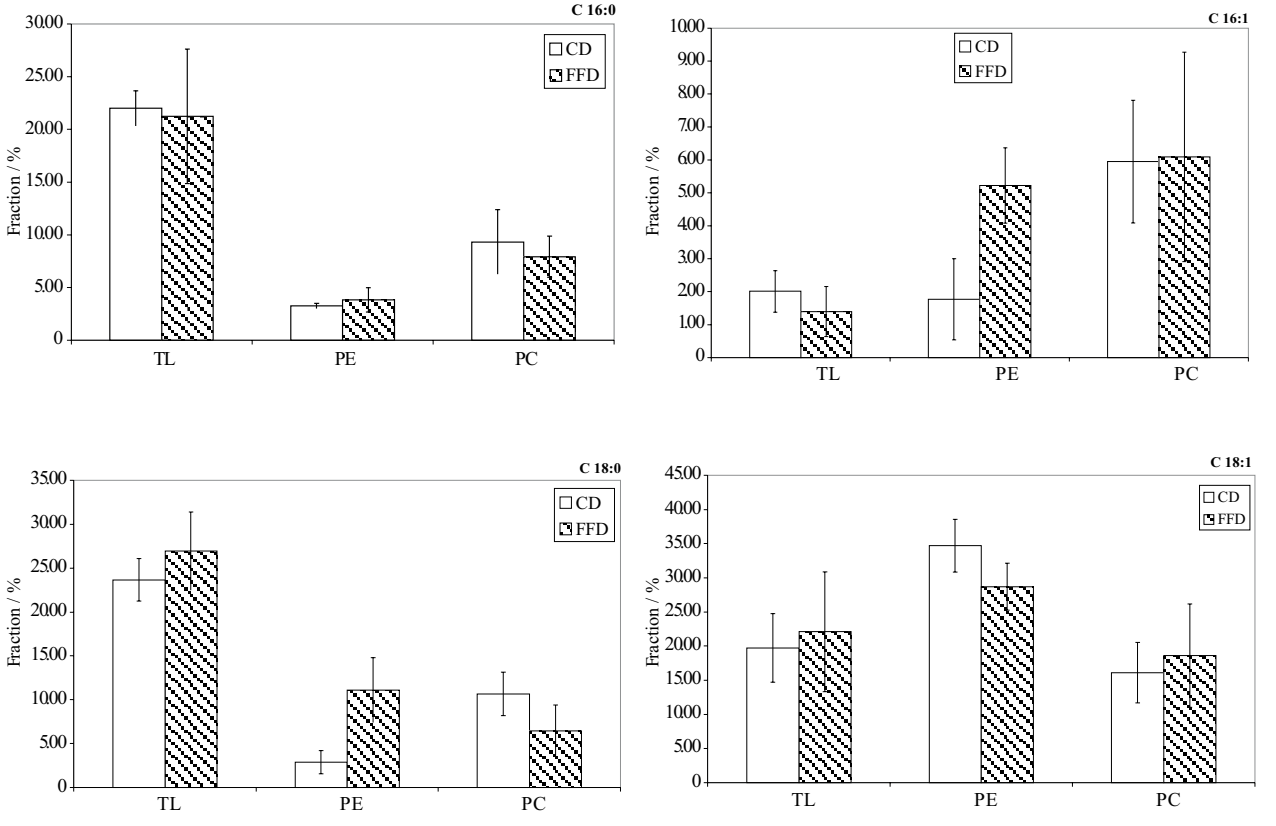

Fig. 2. Fatty acid fraction of brain total lipids (TL)*, plasmenylphosphatidylethanolamine (Pla-PE) and plasmenylphosphatidylcholine (Pla-PC) of rats on control (CD) and fat free diet (FFD), * (*25*)

one of the possible reasons of the inhibited biosynthesis of brain phospholipids.

After total brain lipids were isolated and subsequently PC and PE classes separated, both of these lipid classes were submitted to the *sn*-2-linked fatty acid analyses. Data on the composition of Pla-PE *sn*-2 fatty acids are shown in Table 3. Three main points may be stressed: (*i*) the most abundant fatty acid is oleic, C 18:1; however, no remarkable desaturation of this fatty acid was noticed at the end of FFD; (*ii*) in the case of C 16 fatty acids a doubtless effect of desaturation is obvious, while F_u for both C 16 and C 18 fatty acids can be seen in Table 4; (*iii*) the total amount of very long chain fatty acids (C 20 and C 22) remained at the end of the FFD almost unchanged (Table 3).

On the basis of the hitherto presented data of plasmalogen fatty acids it is obvious that short-termed FFD moderately affects their composition. Data represented in Table 3. suggest some main conclusions concerning the Pla-PE fatty acids: (*i*) the content of palmitoleic acid was increased, (*ii*) the content of stearic acid was increased, (*iii*) the content of essential fatty acids (EFA) was decreased (–12.55 %) and (*iv*) the content of PUFAs was decreased, too (–8.47 %).

However, the just mentioned data should not be evaluated *per se.* They must also be justified in view of factors of unsaturation (F_u). The F_u of C16 and C18 fatty acids compared with relevant data of brain total fatty acids and Pla-PC fatty acids is shown in Table 4. On the basis of these data, following conclusions can be done: (*i*) in spite of the particular increase of the C 16:0 content, the increase of unsaturation of this fatty acid was found; (*ii*) an opposite effect occurred to C 18 fatty acid, where an overall saturation was found; (*iii*) the comparison of metabolic changes of C 16 and C18 fatty acids of Pla-PE relative to Pla-PC showed (Fig. 2, Table 4) that both fatty acids were desaturated after the FFD; (*iv*) in the case of brain total fatty acids, a slight saturation of both C16 and C18 fatty acids was found. All of these data indicate that FFD will specifically affect not only different tissues of the same species, but even particular lipid classes of the same tissue. We have already pointed to similar data in some of our previous papers (*23–25*).

As represented in Table 3, the decrease of EFAs (–12.55 %) and PUFAs (–6.34 %) content was expected, because of the absence of EFAs in the FFD. The decrease of PUFAs content was minimized relative to EFAs because of the endogenous synthesis of eicosatrienoic acid (C 20:3, +6.21 %). The decreased content of C 22:6 and other PUFAs and the simultaneous endogenous synthesis of C 20:3 is actually a sophisticated biological balance which basically maintains the F_u ratio of unsaturated *vs.* saturated fatty acids and therefore ensures the fluidity of tissue membranes. Concerning the absence of EFAs in the diet it might be of a certain interest to mention that Balint *et al.* (*32*) suggested the possibility of an apparent »surplus« of Δ9-desaturase (its predominant substrates EFAs are missing), thus the palmitic acid may be increasingly desaturated by this enzyme. Finally, in order to draw a general conclusion of Pla-PE total fatty acid changes, relative to the desaturation/saturation effects caused by FFD, an overall moderate saturation with F_u –7.39, or approximately 40 %, was found (Table 4).

Plasmalogens of the brain tissue are predominantly a part of the PE lipid class, while only minor quantities belong to the PC family (Fig. 1). Williams and Mounder (*33*) reported that the metabolic echo of particular diets relative to lipid modulations would be preferentially expressed in the PE class. They proposed the following order of sensitivity to modulations: PE>PC>PI. The previous investigations (*22–24*) support this idea. Therefore the effect of FFD on the aldehydogenic moiety of Pla-PE only has been studied here. The pool of eight individual samples of Pla-PE aldehydes was analyzed by GLC. The total amount of aldehydes remained almost unchanged (CD 58.3 %, FFD 59.7 %). Data shown in Table 2. demonstrate that the aldehyde moiety was composed of C 16, C 18 and C 20 homologues. Minor changes were found in the total C 18 series: CD 59.4 %; FFD 64.1 %. This was true for the distribution of *cis-trans* isomers of C 18 aldehydes. The FFD also did not affect the distribution of C 20 aldehydes. The only homologue which was apparently affected by FFD was the C 16 one. For some, yet unknown reasons, the amount of C 16 aldehydes was reduced at the end of the experiment. Nevertheless, the majority of the aldehydogenic moiety of Pla-PE (65–70 %) remained almost intact after the short-term FFD. The aldehydogenic moiety of Pla-PE is obviously the main stabilizing factor of this molecule, which is one of the major constituents of brain biological membranes. Ford and Gross (*34*) proposed that the stability of the aldehydogenic moiety of plasmalogens might be caused by the fact that they originate from long-chain alcohols, thus their metabolic conversion into the aldehydes has a relatively small turnover. On the contrary, the incorporation of fatty acids into the *sn*-2 position of the glycerol moiety of Pla-PE is a much faster process.

Summarizing the data presented in this paper it can be concluded, that the short-term FFD did not essentially change the structure of plasmenyl-phosphatidyethanolamine (Pla-PE). The most stabile moiety of the molecule is the aldehydogenic chain. Changes of the unsaturation/saturation ratio (F_u) of *sn*-2-linked fatty acids ranged no more than ±7 % (mean value), which is considered to be a fairly moderate change. Relatively high substitution and compensation of unsaturated *vs.* saturated fatty acids allow membranes to keep their essential functions, especially the membrane fluidity. In conclusion it can be said that plasmalogens are again found to be one of the most important stabilizers of biological membranes.

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Molekularna stabilnost plazmalogena mozga u bezmasnoj dijeti

Sažetak

Istraživan je utjecaj kratkotrajne bezmasne dijete na plazmalogene mozga štakora. Dvije skupine, po osam životinja (soj Wistar, prosječna tjelesna masa 180 g), hranjene su standardnom hranom za laboratorijske životinje kao kontrolnom (CD) i bezmasnom dijetom (FFD) priređenom u našem laboratoriju. Obje su dijete bile izoenergetske (CD 15,74 kJ; FFD 15,10 kJ). Prosječni je prirast tjelesne mase pojedinih životinja bio 4 %. Masa izoliranog moždanog tkiva prosječno je iznosila kod CD 692,7 mg, a kod FFD 891,3 mg. Odnos ukupnih fosfata prema proteinima bio je za CD 48,7/100, a za FFD 33,2/100. To upućuje na inhibiranu biosintezu fosfolipida. Većina plazmalogena u mozgu pripada skupini fosfatidiletanolaminskih (PE) lipida. Nađeno je da je udjel plazmenilfosfatidiletanolamina (Pla-PE) iznosio u CD 58,3, a u FFD 59,7 %. Utvrđen je sljedeći utjecaj FFD na sastav masnih kiselina Pla-PE: (*i*) povećanje udjela palmitoleinske kiseline (+3,45); (*ii*) povećanje udjela stearinske kiseline (+8,22) uz smanjeni udjel oleinske kiseline (–6,02 %); (*iii)* smanjenje udjela esencijalnih masnih kiselina (–12,55 %) i (*iv*) smanjenje udjela polinezasićenih kiselina uz istodobno povećanje eikosatrienske kiseline (+6,21 %) i smanjenje količine dokosaheksaenske kiseline (–8,47 %). Ukupni učinak FFD na promjenu F_u (–7,39) upućuje na izvjesno zasićenje ukupnih masnih kiselina Pla-PE. Analizom aldehidnog dijela Pla-PE postignuti su ovi rezultati: (*i*) nije se bitno promijenila količina ukupnih aldehida u PE lipidima (CD 58,3 %, FFD 59,7 %); (*ii*) alkenilni lanac tvore C 16, C 18 i C 20 homolozi, a C 18 i C 20 homolozi čine oko 70 % ukupnih aldehida; (*iii*) nisu nađene bitne promjene ni u ukupnoj količini C 18 homologa (CD 59,4 %, FFD 64,1 %), ni u omjeru *cis-trans* C 18 izomera; (*iv*) količina C 20 homologa ostala je skoro nepromijenjena (CD 4,05 %, FFD 4,23 %). Svi navedeni podaci upućuju na vrlo male promjene Pla-PE molekule u uvjetima bezmasne dijete i potvrđuju pretpostavku da su plazmalogeni jedan od najvažnijih stabilizatora strukture bioloških membrana.