

Phospholipid and Free Fatty Acid Content of Rat Brain Mitochondria Following Linseed Dietary Supplementation

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In this study, we report changes in the phospholipid and free fatty acid (FFA) content in rat brain mitochondria following linseed dietary supplementation. Male Wistar rats at the age of three months were fed a standard chow diet supplemented with linseed at a dose of 3 g/day for three weeks. Afterwards, the control and experimental animals were sacrificed by decapitation, the brain mitochondrial fraction was isolated and lipids were extracted. The phospholipid content was measured by thin-layer chromatography and spectrophotometrically. FFA content was measured by gas-liquid chromatography.

In the brain mitochondria of rats fed linseed, we found 9% increase of the total phospholipids. Phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine were the predominant phospholipid classes and they together accounted for 88.8% of the total phospholipids. The content of the total FFA increased by 42.3% and stearic acid and arachidonic acid were the most pronounced components of the FFA pool.

Key words: phospholipids, free fatty acids, mitochondria, rat brain, dietary linseed.

Introduction

Brain maintains a unique lipid environment that is essential for normal brain function. It is well known that the membrane fluidity, transport of proteins and ions, and membrane enzyme activities are affected by the lipid membrane composition.

The relationship between the membrane lipid environment and its intrinsic enzymes is well documented in mitochondrial membranes [4]. The maintenance of the mitochondrial phospholipid composition is essential for the function and structure of mitochondria. It depends on the metabolism of phospholipids, their transport into and out of the mitochondrial membrane and the supply of lipids from the diet. Studies have demonstrated that dietary changes modify the fatty acid composition of the major mitochondrial phospholipids, thus influencing the membrane physical properties, respiration and other processes. Dietary interventions that contribute to the remodeling of phospholipids and mitochondrial membrane homeostasis are emerging as novel therapeutic

strategies against cancer, metabolic disorders, cardiovascular and neurodegenerative diseases and other pathologies [9].

Our experiment was conducted to examine the effect of feeding dietary linseed on the FFA and phospholipid content of rat brain mitochondria.

Materials and Methods

Experimental animals and dietary linseed intake

Twenty-five male Wistar rats at the age of three months were used in the experiment. Animals were divided into: one control group (n=5) and experimental one (n=20). The control group was fed a standard chow diet. The experimental group diet was supplemented with ground linseed at a dose of 3 g/day for three weeks. Afterwards, control and experimental rats were deprived of food for 24 hours, lightly anesthetized with diethyl ether and sacrificed by decapitation.

The animal experiments were performed in accordance with the animal protection guidelines approved by the Ethics Committee for Experimental Animal Use at IEMPAM, BAS.

Brain phospholipid and FFA analysis

A 10% brain homogenate was prepared in ice-cold 0.32M sucrose and the mitochondrial fraction was isolated as described by Venkov [20] using discontinuous two-step sucrose gradient. Lipids were extracted according to the method of Kates [21] using the following eluates: chloroform:methanol 1:2 (v/v) and chloroform:methanol:water 1:2:0.8 (v/v/v).

Total phospholipids were measured spectrophotometrically at 820 nm [2]. All major phospholipid classes were separated by thin-layer chromatography using eluate from chloroform:methanol:water 65:25:4 (v/v/v). Perkin-Elmer scanning spectrophotometer was used to estimate the concentration of migrated spots.

The FFA content was determined by gas-liquid chromatography. The fatty acids were converted to fatty acyl methylesters (FAME) by addition of methanol and 25% hydrochloric acid. The FAME were extracted by petroleum ether, then concentrated in a rotary vacuum evaporator and subjected to a gas-liquid chromatographic analysis. A gas chromatograph with a flame ionization detector and connected with Trio Vector computing integrator was used. The analysis was performed by injecting 5 μ l of the sample into a SE-35 column. The temperature was programmed from 85 °C to 205 °C (2.5 °C/min). Nitrogen was used as a carrier gas at a flow-rate of 40 ml/min.

Statistical analysis

Results are reported as mean values \pm SD and statistically analyzed by Student's *t*-test.

Results and Discussion

It is known that biochemical functions of mitochondria strongly depend on membrane lipids. The membrane phospholipids are essential for the mitochondrial respiration and the inner mitochondrial membrane integrity. Besides, free fatty acids and especially polyunsaturated fatty acids (PUFA) are an important component of the brain cellular membranes, including mitochondrial membrane. PUFA are crucial in multiple aspects of the neuronal development and function [3]. They are involved in modification of

membrane fluidity, membrane-bound enzymes activity, the number and affinity of receptors, the function of membrane ion channels [16].

The majority of membrane PUFA are synthesized from linoleic acid (LA, C18:2 n-6) and α -linolenic acid (ALA, C18:3 n-3), which act as precursors for the synthesis of longer-chain PUFA through a series of elongation and desaturation reactions [18]. α -Linolenic and linoleic acids have been identified as essential fatty acids, because they cannot be synthesized *de novo* and they must be provided in the diet. Dietary sources of LA and ALA are vegetable oils, seeds, and some vegetables. Linseed is a rich source of PUFA, including mainly LA and ALA. The percentage contribution of both these acids is around 73%. In addition, linseed oil contains 53.3% of ALA and 12.7% of LA, yielding the highest n-3/n-6 FFA ratio amongst plant sources [10]. Linseed has a high nutritional value and it is easily accessible, which makes it a beneficial rat diet supplement.

Several studies have indicated that mitochondrial lipid composition is susceptible to dietary manipulation [9, 13]. It is reported that the changes generally reflect the fatty acid pattern present in the diet [6].

In the present study, we examined the effect of linseed dietary supplementation on the FFA and phospholipid content of rat brain mitochondria. The control FFA pool was enriched in stearic acid (C_{18:0}) and arachidonic acid (AA, C_{20:4} n-6) (Fig. 1). They represented 63% and 32% of the total FFA in the mitochondria, respectively. Smaller amounts of myristic (C_{14:0}), myristoleic (C_{14:1}), palmitic (C_{16:0}) and arachidic (C_{20:0}) acids were also estimated. Among the phospholipid classes, phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were the most prominent and they together accounted for 88.6% of total phospholipids in the brain mitochondria (Fig. 2). In fact, the mitochondrial membrane is enriched in PE, in comparison to other

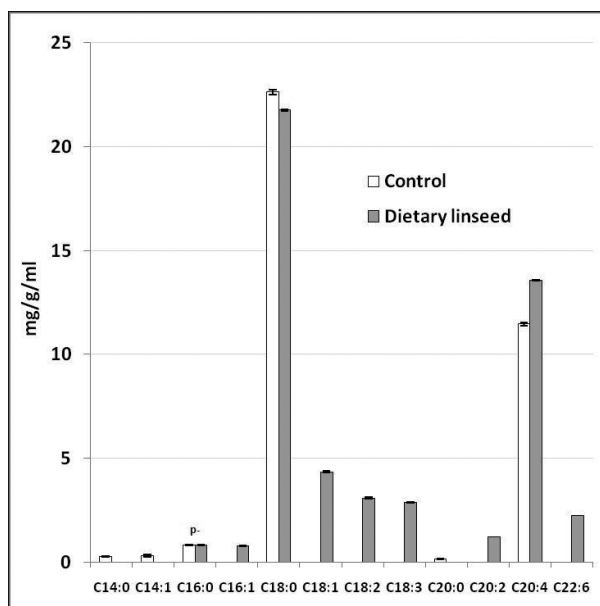


Fig. 1. Free fatty acid pool composition of the rat brain mitochondria following linseed dietary supplementation. Values are expressed in mg/g dry lipid residue/ml. P < 0.001

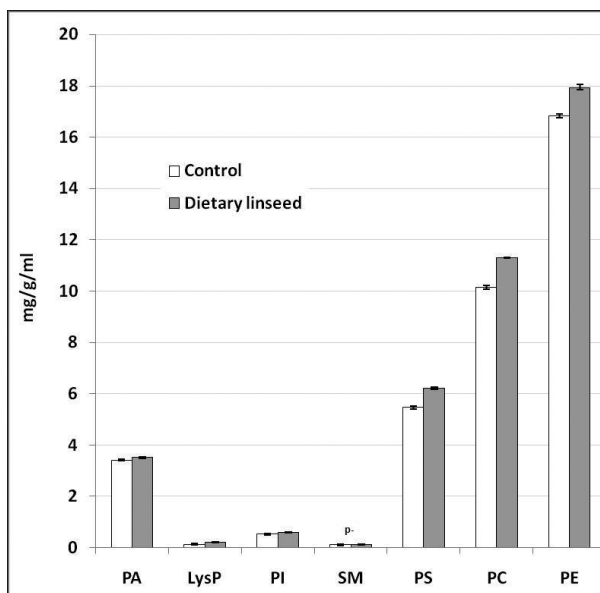


Fig. 2. Phospholipid composition of the rat brain mitochondria following linseed dietary supplementation. Values are expressed in mg/g dry lipid residue/ml. $P < 0.001$

cellular membranes. The PE is the only phospholipid that is primarily synthesized in mitochondria by the decarboxylation of PS. In contrast, the majority of phospholipids are synthesized in the endoplasmic reticulum, transported to, and imported in the mitochondria [15].

Phosphatidic acid (PA; 9.3%), lysophospholipids (LysP; 0.3%), phosphatidylinositol (PI; 1.4%) and sphingomyelin (SM; 0.3%) were also estimated in the phospholipid composition of the control animals.

Feeding linseed resulted in a significant increase in the total FFA content of brain mitochondria by 42.3% (from 35.666 ± 0.2 to 50.765 ± 0.15 mg/g dry lipid residue/ml, $p < 0.001$). The most notable effect was observed for AA, whose concentration increased by 18.5% (from 11.46 ± 0.08 to 13.578 ± 0.03 mg/g/ml, $p < 0.001$) and it comprised of 26.7% of the total FFA (**Fig. 1**). In contrast, other studies have shown that increasing dietary intake of the n-3 PUFA decreases the desaturation of LA, and thus, the production of arachidonic acid [7]. Furthermore, we found decreased content of stearic acid by 3.8% (from 22.629 ± 0.1 to 21.759 ± 0.03 mg/g/ml, $p < 0.001$), though it had the highest percentage in the FFA pool of the experimental rats. No statistically significant changes were observed in the level of palmitic acid. Besides the FFA pool size, the composition of the FFA pool was also modified by linseed supplementation. The latter was comprised of mono- and polyunsaturated FFA, some of which were absent in controls: palmitoleic acid ($C_{16:1}$ n-7) – 1.6%, oleic acid ($C_{18:1}$ n-9) – 8.6%, LA – 6.1%, ALA – 5.7%, eicosadienoic acid ($C_{20:2}$ n-6) – 2.4%, DHA – 4.5%. Other studies have also reported an increased content of ALA and long-chain PUFA following dietary linseed supplementation [1]. Our findings indicate that the FFA pattern resembles those of linseed regarding the presence of LA and ALA.

The increased PUFA content of the diet is shown to increase the metabolic rate [14]. For example, treatment of rats with n-3 PUFA is reported to decrease proton leakage from the respiratory chain and this is related to the incorporation of PUFA into mitochondrial PC, PE and cardiolipin [11]. Similarly, the presence of ALA in the diet is shown to contribute to a greater resistance to certain neurotoxic agents.

The nutritional importance of the n-3 to n-6 fatty acid ratio in the diet has aroused a great interest. It is reported that the ratio is important to avoid imbalance of membrane fluidity. Studies in animal models also demonstrate that the ratio influences various aspects of serotonergic and catecholaminergic neurotransmission, as well as prostaglandin formation [8]. Our findings in controls indicated that the n-6 series predominated among the polyenoic acids in the mitochondrial FFA pool. Feeding linseed resulted in enhanced n-3 fatty acids synthesis, though the n-6 FFA had a prevalence (n-3/n-6=0.29). In contrast, we have previously shown a higher content of the n-3 FFA in comparison to the n-6 FFA in a whole brain homogenate [12]. Moreover, the ratio of PUFA to saturated fatty acids in the mitochondrial FFA pool increased from 0.48 to 1.02 with linseed dietary supplement.

Furthermore, we also examined the changes in the phospholipid composition of brain mitochondria. Adding linseed to rat diet led to 9% higher content of the total phospholipids (from 36.598 ± 0.05 to 39.889 ± 0.16 mg/g/ml, $p < 0.001$). All the individual phospholipid classes increased as follows: PA by 3%, LysP by 65.6%, PI by 15.8%, PS by 13.6%, PC by 11.4%, PE by 6.6% (**Fig. 2**). Their concentrations rose to 3.514 ± 0.02 , 0.212 ± 0.02 , 0.602 ± 0.01 , 6.206 ± 0.05 , 11.294 ± 0.02 and 17.938 ± 0.1 mg/g/ml, respectively. As we have established in controls, PS, PC and PE were still the predominant classes in the phospholipid composition and they together accounted for 88.8% of the total phospholipids. However, no statistically significant changes were observed in the level of sphingomyelin.

As seen from the results, the most notable increase was found for LysP, though they remained poorly presented in the phospholipid composition of the brain mitochondria following linseed intake. Lysophospholipids are intermediates in phospholipid metabolism and turnover and they are now recognized as important membrane-derived bioactive lipid mediators. Although LysP are usually found in small amounts in biological cell membranes, they have been shown to play a role in a wide range of cellular processes that involve membrane-protein or membrane-membrane interactions. The “shape” of the molecules in the membrane is among the factors that affect the mechanical properties of a bilayer. Phospholipids have an approximately cylindrical molecular shape, whereas LysP are cone shaped and promote the formation of curved (or nonbilayer) structures. It has been shown that if lysophospholipids accumulate in a membrane, they will tend to lower the membrane electrical resistance, increase the permeability, inhibit the fusion and alter the channel-gating kinetics. In the bilayer phase LysP would introduce membrane tensions that may influence the conformation and activity of membrane proteins [5].

It is known that the fatty acid composition of membrane phospholipids is affected by the exogenous fatty acids from the diet [19]. The degree of fatty acid unsaturation in membrane phospholipids determines the biophysical properties of the membrane, which in turn influences many critical membrane-associated functions. For example, a phospholipid made from a saturated fat has a different structure and is less fluid than one that incorporates an essential fatty acid [17]. Moreover, studies have reported that the changes in the phospholipid fatty acid composition depend not only on the dietary fatty acid profile, but also on the classes of phospholipids affected. It has been shown that the response to dietary fatty acids varies according to the phospholipid class. PC is found to be more susceptible to variation in dietary monounsaturated fatty acid (MUFA) content than the other phospholipid classes, whereas PE is more responsive than PC to both dietary n-6 PUFA and the n-3 PUFA [8].

Although the present study does not explore the changes in the fatty acid composition of phospholipids, the results suggest that the increased PE content may cause an increase in the unsaturation index, and thus increased fluidity.

In conclusion, alterations in the phospholipid and FFA content of the rat brain mitochondria were observed in response to linseed dietary supplementation. The results indicate the tendency to synthesize high amounts of long-chain PUFA which is associated with changes in the n-3/n-6 PUFA ratio and the ratio of unsaturated to saturated FFA. These changes suggest possible dietary modifications of the membrane biophysical characteristics.

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