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Omega-3 polyunsaturated fatty acids modulate sphingolipid metabolism in the hippocampus of aged rats

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Neural cell membranes are rich in sphingolipids, which are powerful regulators of brain omeostasis. Ceramide is a potent signaling molecule involved in critical events in neurodegenerative brain diseases. The ω-3 polyunsaturated fatty acids (ω-3 PUFAs) are a group of essential fatty acids that serve as key components of cell membranes and energy sources, playing a vital role in maintaining normal brain function. This study aims to determine the impact of supplementing old rats with ω-3 PUFAs on hippocampal sphingolipid metabolism. To investigate the effect of ω-3 PUFAs on sphingolipid metabolism in aged rats, a comparison was made between 3-month-old and 24-month-old rats. The 24-month-old rats were divided into two groups: the experimental group received a diet supplemented with ω-3 PUFAs, and the control group received a diet supplemented with beef fat. Next, lipids were extracted from hippocampus homogenates and separated into classes (sphingomyelin, ceramide, glucosylceramide, and sphingosine) using thin-layer chromatography, followed by quantitative analysis. It has been determined that ceramide, glucosylceramide, and sphingosine synthesis increase in the hippocampus of 24-month-old rats with a non-significant decrease in the synthesis of sphingomyelin as compared to the 3-month-old animals. The nutritional factor ω-3 PUFA used in this work reduces the mass and de novo synthesis of the proapoptotic lipid ceramide in the hippocampus, which increases with age. Concurrently, ω-3 PUFAs also increase the levels of newly synthesized sphingomyelin in this region. These findings provide evidence that PUFAs act as physiological regulators of sphingolipid metabolism, reducing ceramide accumulation in the hippocampus during aging. Moreover, these results suggest that ω-3 PUFAs may help mitigate the risk of neurological diseases and alleviate age-related brain dysfunction in old age.
Key words: ω-3 polyunsaturated fatty acids; hippocampus; ceramide; glucosylceramide; sphingomyelin; sphingosine.

INTRODUCTION

Lipids constitute a major portion of the brain and are integral to both its physiological functions and pathological processes. They play crucial roles in cellular activities, including membrane formation, intercellular signaling, energy storage, and maintaining homeostasis. The hippocampus, a complex brain region involved in cognitive and behavioral functions, is particularly vulnerable to disruptions in lipid metabolism, which have been linked to the development and progression of neurodegenerative diseases and other neurological disorders [1, 2]. The ceramide pathway has recently attracted increasing attention as an important and possibly critical factor in several neuropathological processes [3]. Ceramide has been suggested to participate in

the neuronal cell death that leads to Alzheimer's disease (AD) [4]. The relevance of sphingolipids and ceramide in the progression of different neurological and neuro-inflammatory diseases, such as Alzheimer's, Parkinson's, Huntington's, and Prion diseases [5]. The hippocampus is a major component of the limbic lobe, which is further divided into the dentate gyrus and sections of Cornu Ammonis. It is an important region for learning and memory, and its subregions contribute to the formation of episodic memories. However, AD affects several brain areas, including the hippocampus. In the early stages of AD, the hippocampus exhibits fast tissue loss, which is coupled with functional separation from other areas of the brain [6]. ω-3 PUFAs are a group of essential fatty acids that act as integral membrane components and energy substrates, playing an important role in the maintenance of normal neurological function [7].

ω-3 polyunsaturated fatty acids ω-3 PUFAs, in particular docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are nutrients involved in many metabolic and physiological processes [8]. Providing evidence that vitamin E is a potent modulator of phospholipid metabolism in the hippocampus and functions at old age [9], neutral sphingomyelinase (nSMase) is a key enzyme in sphingolipid turnover; it generates ceramide through the hydrolysis of sphingomyelin. Treatment with N-acetylcysteine inhibits nSMase in the gastrocnemius muscle and blood serum of old rats [10]. Sphingolipids are bioactive molecules involved in the regulation of cell growth, differentiation, and apoptosis [11, 12]. Violation of sphingolipid metabolism leads to changes in specific functions of cells and organs. An increase in the ability of cells and tissues to form and accumulate ceramide is observed in atherosclerosis [13], diabetes [14], and neurodegenerative diseases, which most often occur in old age [15]. The level of ceramide in cells and tissues increases significantly under the conditions of normal aging of the animal and human organism [16]. It is known that the exchange of sphingolipids is closely related to the redox state of the cell. Glutathione prevents the formation and accumulation of ceramide in cells by inhibiting neutral sphingomyelinase [17]. Abnormal turnover of the brain's most abundant phospholipids: phosphatidylcholine and phosphatidylethanolamine, constitutes a major metabolic pathology in AD [18]. Activation of sphingomyelinases under conditions of oxidative stress in endothelial cells and RAW264.7 cells [19] can be prevented with the help of ω-3 fatty acids. Enrichment of the food of mice with EPA and DHA of ω-3 fatty acids leads to a decrease in the mass of ceramide in lymphocytes [20] and to an increase in the exchange of precursors of sphingolipid synthesis, L-serine and phosphatidylserine in various tissues and cells [21]. In view of this, this study aimed to examine the age-specific characteristics of sphingolipid metabolism and the synthesis of biologically active components in the diet, focusing on how these factors influence age-related changes in sphingolipid metabolism within the functional brain tissue of rats.

METHODS

Animals. The experimental group of rats was used (n = 20), male Wistar rats aged 3 and 24 months, weighing 60-90 g and 420-470 g, respectively. The rats were obtained from the vivarium at Isra University, Jordan. The animals were housed in cages of six, with each cage maintained at a 12-hour light–dark cycle, a relative humidity of 60-80%, and a controlled temperature of 24°C. The animals were kept on a "day-night" retention regimen, receiving food and water as needed. They also had unlimited access to a regular chow diet and drinking water at a temperature of 22-23°C. Every animal was kept apart based on age and kept in plastic cages with bedding as needed. Before every experiment, the animals were brought into the lab and kept in a similar quarantine for 15 days. [Guide for the Care and Use of Laboratory Animals, 2010]. The study's

Protocol (SREC/24/08/108) was approved by the Ethical Committee of Isra University. Before opening the abdominal cavity, the animals were anesthetized with diethyl ether. To study the effect of ω-3 PUFAs on the metabolism of sphingolipids, 24-month-old rats were transferred to a diet whose caloric content was increased by 25 kcal by n-3 PUFAs (experimental group) and by beef fat (control group). In general, the calorie content of the diet of the experimental and control groups was 172 kcal, while that of the standard diet was 147 kcal. [1-14C] palmitic acid (2.07 GV/mmol, Amersham, GE Healthcare UK) was used as a precursor of lipid synthesis. Extraction of lipids from hippocampus homogenates was performed according to the method [22].

Extraction and separation of lipids. Lipid extracts intended for the analysis of sphingolipids were evaporated under vacuum and incubated for 60 min at 37ºC in the medium: chloroform-methanol (1:1, v/v), which contained KOH (0.1 M) for the hydrolysis of acylglycerols. Lipids were again extracted and separated into classes (sphingomyelin, ceramide, glucosylceramide, and sphingosine) by thin-layer chromatography on commercial Sorbfil plates (JSC "Sorbpolimer") in the solvent system: chloroform - ethyl acetate - isopropyl alcohol - methanol - 0.25% KCl. Sphingomyelin, ceramide, and glucosylceramide were expressed in iodine vapor. Sphingosine (SPH) was expressed using a 3% solution of ninhydrin in butanol saturated with H_2O and identified by comparison with standards. To quantitatively determine the content of ceramides in tissues, lipid spots were transferred to test tubes and eluted with a mixture of chloroform and methanol (1:1, v/v), followed by elution with methanol. The combined eluates were evaporated in a vacuum and subjected to acid hydrolysis in 0.5 M HCl in methanol at 65°C for 15 h. The mass of ceramides was determined by measuring the release of long-chain bases during lipid hydrolysis, in accordance with the method described in [23]. The radioactivity of samples containing labeled [^{14}C] lipids was determined using a BETA radioactivity counter.

Statistical analysis. The quantitative determination of sphingolipids in chromatographic fractions was performed according to the method [24]. Numerical data were analyzed using STATISTICA 6.0 software. The effect of n-3PUFAs on the amount of newly synthesized Phospholipids in the hippocampus of old animals was estimated using the Newman – Keuls test. Experimental results are presented as the arithmetic mean ± standard error. One-way analysis of variance (ANOVA, Fisher LSD - test) and Student's t-test were used for comparison. In intergroup comparisons, $P < 0.05$ was taken as the critical significance level. OriginLab Corporation software (OriginPro 2022b) was used for graphing.

RESULTS AND DISCUSSION

It was established that an increase in the mass of ceramide in the hippocampus and cerebral cortex of 3-month-old rats compared with 24-month-old animals is correlated with the accumulation of free fatty acids in cells [16]. It is known that an increase in the level of free fatty acids serves as a prerequisite for enhancing the synthesis of ceramide and total sphingolipids in liver cells [25], adipose tissue, and skeletal muscles [14]. These studies established that by the age of 24 months, the level of newly synthesized lipids in the hippocampus increases (Fig. 1).

The mass of ceramide in the hippocampus of 24-month-old animals is higher than in the tissue of 3-month-old rats [16], which apparently occurs not due to changes in lipid synthesis de novo (Fig. 1), but due to increased degradation of complex sphingolipids, such as sphingomyelin. Changes in the metabolism of sphingolipids ceramide, glucosylceramide, and SPH, which have high biological activity, in the hippocampus in old age, as revealed in this work, may be an important cause of age-related changes in the functional activity of the investigated tissues. Thus, an increase in the

metabolism of sphingolipids and the content of ceramide and Sphingosine in the liver of old rats is one of the important reasons for the development of chronic inflammation [26]. Enhancement of ceramide synthesis de novo precedes a decrease in Bcl-2 expression, an increase in caspase 3 activity, apoptosis of kidney tubular cells, and the development of nephropathy under the synthesis of radiocontrast media [19]. Age-related disorders of kidney function (glomerulosclerosis and decrease in filtration level) are associated with significant lipid metabolism disorders, which are expressed in the accumulation of triacylglycerols [27], which, in turn, can lead to the accumulation of free fatty acids and their metabolites - ceramides. This process contributes to cell death [15], a hallmark of neurodegenerative diseases in old age. The next stage of this study involved investigating whether the metabolism of sphingolipids in the tissues of old rats could be corrected using physiological modulators of lipid metabolism, specifically dietary components such as ω-3 PUFAs. The data indicate that supplementation with additional ω-3 PUFAs in 24-month-old animals enhances sphingomyelin synthesis while reducing ceramide synthesis in the hippocampus (Fig. 2). These changes suggest that the reduction in newly synthesized ceramide content in the hippocampus is associated with an increase in sphingomyelin levels, rather than changes in glucosylceramide or sphingosine levels.

A key factor contributing to the decreased ceramide levels in the hippocampus of 24-month-old animals is its enhanced utilization for sphingomyelin synthesis under the influence of ω-3 PUF in the Hippocampus of 24-month-old animals (Fig. 2). Sphingomyelin is synthesized from ceramide through a reaction catalyzed by sphingomyelin synthase (SMS), which transfers a phosphocholine headgroup from phosphatidylcholine to ceramide, yielding sphingomyelin and diacylglycerol (DAG) as a byproduct [28]. The study of the effect of ω-3 polyunsaturated fatty acids (PUFAs) on changes in ceramide mass revealed a significant decrease by 44.3% ($P < 0.01$) in lipid levels in the hippocampal tissue of old rats. Given that SM is a substrate of sphingomyelinases, it can be assumed that the decrease in the ceramide/SM ratio is the result of the inhibition of enzyme activity by ω-3 fatty acids.

The inhibition of ceramidase can also lead to increased ceramide levels in cells. Under the given conditions of the experiment, a drop in the level of the product of the ceramidase

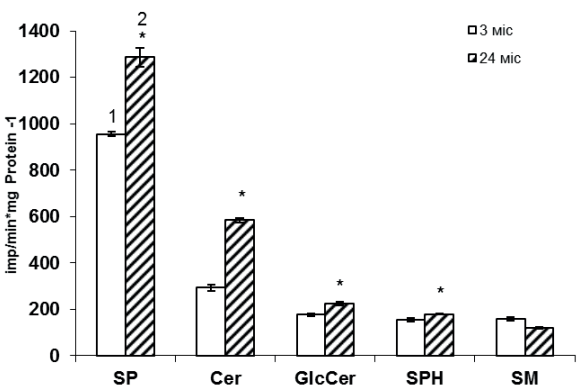

Fig. 1. The data represent normalized radioactivity levels of sphingolipid content in the hippocampus of 3-month (1) compared with 24-month-old rats (2): SP, sphingolipids; Cer, ceramide; GlcCer, glucosylceramide; SPH, sphingosine; SM, sphingomyelin (filled and open columns, respectively). Data are expressed as means ± SEM; obtained in 10 experiments performed in duplicate. *P < 0.05

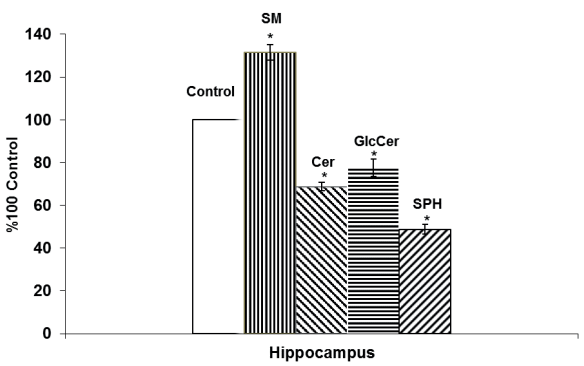

Fig. 2. The data represent the normalizing of sphingolipid levels in the hippocampus of 24-month-old rats treated intragastrically with ω-3 PUFAs daily for 14 days, compared to control 24-month-old rats fed a beef fat-supplemented diet (expressed as % of control). Results are presented as means ± SEM from 10 experiments (duplicates). *P < 0.05. Sphingolipids measured include SM, sphingomyelin; Cer, ceramide; GlcCer, glucosylceramide; SPH, sphingos

reaction - [^{14}C] sphingosine in the hippocampus was established under the synthesis of ω-3 fatty acids, which, however, occurs against the background of a decrease in the synthesis of [^{14}C] ceramide (Fig. 1). It is possible that ω-3 PUFAs can also enhance deep degradation of SPH or conversion of lipid to SPH-1-P and thus reduce its production in cells.

It is reasonable to assume that ω-3 PUFAs change the metabolism of sphingolipids in old age by suppressing the production of pro-inflammatory cytokines, which, as is known, realize part of their effects by inducing the formation of ceramides in cells [26]. In favor of this assumption, there is evidence of inhibition of sphingomyelinase activity and reduction of ceramide production under the synthesis of plant polyphenols, which have pronounced antioxidant properties and reduce the level of pro-inflammatory cytokines [29]. It was also established that the introduction of EPA and DHA of ω-3 fatty acids into the diet of humans [30] and animals [20] is accompanied by the suppression of production and secretion of cytokines. A decrease in the production of interleukin-2 under the synthesis of ω-3 PUFAs correlates with a decrease in the mass of ceramide in cells.

These studies established that long-term maintenance of animals on a diet enriched with ω-3 fatty acids is accompanied by a decrease in the content of newly synthesized ceramide in the examined tissue of 24-month-old animals compared to a control group of the same age (Fig. 2). A diet enriched with ω-3 PUFAs not only reduces the synthesis *de novo* of ceramide in the hippocampus, which is one of the reasons for the drop in levels of its various metabolites, sphingosine and glucosylceramide, and dramatically reduces the ratio between ceramide and sphingomyelin, which may indicate the contribution of sphingomyelinases to changes in the content of ceramide in investigated tissue.

Therefore, the conducted studies established significant tissue-specific changes in the metabolism of sphingolipids in old age. Inhibition of sphingolipid synthesis was noted in the hippocampus up to 24 months of age, and the increase in ceramide mass in old age occurs during the activation of sphingomyelinases and the degradation of complex sphingolipids [29]. The studies revealed that a fish-oil diet enriched with ω-3 PUFAs reduced the levels of dihydroceramides, ceramides, sphingomyelins, and glucosylceramides in the lung tissue and blood of mice. In contrast, sphingosine-1-phosphate (S1P) levels increased, suggesting that n-3 PUFAs might alter sphingolipid metabolism, potentially promoting airway hyperreactivity independent of inflammation [31]. Additionally, other research showed that ω-3 PUFAs, particularly EPA, enhance fatty acid β-oxidation and ATP production in aged organs by activating peroxisome proliferator-activated receptor alpha (PPARα). These effects help maintain lipid homeostasis and slow the aging process in organs, potentially influencing sphingolipid metabolism [32]. A comprehensive review examined the sources, functions, and health benefits of ω-3 fatty acids, highlighting their impact on neurological disorders, cardiovascular diseases, and immune system function. While not specifically focused on sphingolipids, the review offered valuable insights into the broader physiological roles of ω-3 PUFAs [33].

CONCLUSIONS

In summary, the data presented above showed that ω-3 PUFAs act as physiological regulators of the exchange of biologically active sphingolipids in various tissues of aged animals. The nutritional supplement ω-3 PUFAs used in this study decrease the amount of pro-apoptotic lipid, ceramide, which tends to increase with age in the examined tissue. *De novo* lipid synthesis processes are crucial in restoring normal ceramide levels in the hippocampus of 24-month-old animals after ω-3 PUFAs supplementation. Simultaneously, enriching the diet of experimental animals with ω-3 PUFAs causes diverse changes in lipid metabolism within hippocampal tissue, including decreased ceramide levels. The sphingolipids studied—ceramide, glucosylce-

ramide, and sphingosine—are biologically active and play crucial roles in cellular processes such as proliferation, inflammation, and apoptosis. Therefore, modulating their metabolism in aging through physiological dietary factors like ω-3 PUFAs may positively influence the functional activity of the hippocampus. This, in turn, could contribute to the correction of age-related dysfunctions and reduce the risk of neurological diseases in old age.

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ПОЛІНЕНАСИЧЕНІ ЖИРНІ КИСЛОТИ ОМЕГА-3 МОДУЛЮЮТЬ МЕТАБОЛІЗМ СФІНГОЛІПІДІВ У ГІПОКАМПІ СТАРИХ ЩУРІВ

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Мембрани нервових клітин багаті на сфінголіпіди, які є регуляторами гомеостазу мозку. Церамід – це потужна сигнальна молекула, що бере участь у критичних подіях нейродегенеративних захворювань мозку. Поліненасичені жирні кислоти ПНЖК – служать ключовими компонентами клітинних мембран та джерелами енергії, відіграючи важливу роль у підтримці нормальної функції мозку. Мета нашого дослідження – ω-3 ПНЖК на метаболізм сфінголіпідів у гіпокампі старих щурів (24 міс). Старих щурів розділили на дві групи: дослідну, яка отримувала раціон, доповнений ω-3 ПНЖК, а контрольна група – яловичим жиром. Далі ліпіди екстрагували з гомогенатів гіпокампа та розділяли на класи (сфінгомієлін, церамід, глюкозилцерамід та сфінгозин) за допомогою тонкошарової хроматографії з подальшим кількісним аналізом. Було встановлено, що синтез цераміду, глюкозилцераміду та сфінгозину збільшується в гіпокампі 24-місячних щурів з незначним зниженням синтезу сфінгомієліну порівняно з 3-місячними тваринами. Додавання ω-3 ПНЖК зменшувало масу та de novo синтез проапоптотичного ліпіду цераміду в гіпокампі, який збільшується з віком. Одночасно збільшувався вміст сфінгомієліну. Наші результати свідчать про те, що ПНЖК діють як фізіологічні регулятори метаболізму сфінголіпідів, зменшуючи накопичення цераміду в гіпокампі під час процесу старіння. А також, ці результати що свідчать про те, що ω-3 ПНЖК можуть відігравати певну роль у зниженні ризику неврологічних захворювань та полегшенні вікових дисфункцій мозку у похилому віці.

Ключові слова: ω-3 поліненасичені жирні кислоти; гіпокамп; церамід; глюкозилцерамід; сфінгомієлін; сфінгозин.

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