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The influence of eicosapentaenoic acid and docosahexaenoic acid on expression of genes connected with metabolism and secretory functions of ageing 3T3-L1 adipocytes

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ABSTRACT

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are *n*-3 long chain polyunsaturated fatty acids. The purpose of our study was to evaluate the influence of EPA and DHA on expression of genes connected with metabolism and secretory functions of ageing adipocytes. Young, mature and old differentiated 3T3-L1 adipocytes were cultured for 48 h in the presence of EPA, or DHA. Both fatty acids increased the expression of *Pparg, FATP1, FATP4* and *ATGL* genes, but only in young 3T3-L1 adipocytes. Moreover, in young, mature and old cells DHA elevated the expression of *CPT1* gene. In addition, EPA and DHA enhanced the expression of *leptin, adiponectin* and *apelin* genes only in young cells. Investigated fatty acids changed mRNA levels of *IL6* and *MCP1* in young, mature and old cells. EPA increased the expression of these two genes, whereas DHA decreased it. Furthermore, EPA and DHA treatment changed the expression of *IRS1* and *GLUT4* genes involved in insulin signalling pathway, but their effects were opposite. Expression of these genes was decreased by EPA and increased by DHA in young, mature and old cells. In summary, the investigated fatty acids are able to affect the expression of genes associated with lipid metabolism, secretory functions and insulin resistance in ageing 3T3-L1 adipocytes, but their impact is age-dependant. Young cells seem to be more sensitive to EPA and DHA than mature and old ones. Furthermore, the effect of these two fatty acids is not always identical, and therefore requires further investigation.

1. Introduction

Obesity has become a serious health problem worldwide. According to estimates done by World Health Organization in 2015 approximately 2.3 billion adults are considered to be overweight and at least 700 million are obese. The global trends show that the prevalence of obesity is rising in all age groups [1]. However, elderly people seem to be the group in which the risk of overweight and obesity is particularly high. Increased susceptibility to accumulation of body fat in the elderly is caused by adverse changes in the metabolism and decrease in physical activity. All major components of total energy expenditure, such as: resting metabolic rate, thermic effect of food and physical activity decrease with age. Hormonal changes related to ageing, including reduced secretion of growth hormone, decreased testosterone level in plasma, or increased tissue resistance to thyroid hormones also can promote excessive fat accumulation [2].

Ageing and obesity are accompanied by increased immune activation and chronic state of low-grade inflammation in adipose tissue [3,4]. This condition may lead to development of serious health complications, such as: insulin resistance, type 2 diabetes or cardiovascular disease [5,6]. It seems that the aforementioned conditions are tightly linked with adverse changes in adipokines secretion. Numerous studies revealed that in obesity secretion of pro-inflammatory cytokines (interleukin 6, TNF-alpha, monocyte chemoattractant protein-1, leptin) is increased, whereas the levels of anti-inflammatory cytokines (adiponectin, interleukin 10)

Abbrevations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Pparg, peroxisome proliferator-activated receptor gamma; FATP1, fatty acid transport protein 1; FATP4, fatty acid transport protein 4; ATGL, adipose triglyceride lipase; Cpt1, carnitine palmitoyltransferase I; IL6, interleukin 6; MCP1, monocyte chemoattractant protein-1; IRS1, insulin receptor substrate 1; GLUT4, glucose transporter type 4; TNF, tumor necrosis factor; LC PUFA *n*-3, long chain polyunsaturated fatty acids *n*-3; NBCS, newborn calf serum; FBS, foetal bovine serum; DMEM, Dulbecco's modified eagle's medium; PID, post induction day; BSA, bovine serum albumin.

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are decreased [7–9]. Furthermore, some data demonstrate that cellular ageing of adipocytes also compromises the secretion profile of adipokines. Old cells were shown to decrease the secretion of adiponectin, which is very important for maintenance of sensitivity to insulin at the appropriate level in various tissues [10,11].

A properly balanced diet and bioactive substances present in food are very important components of obesity treatment. Currently, great attention is focused on long chain polyunsaturated fatty acids *n*-3 (LC PUFA *n*-3), because of their beneficial health effects. Human and animal studies have shown that LC PUFA *n*-3, especially eicosapentaenoic acid (EPA; C20:5; *n*-3) and docosahexaenoic acid (DHA; C22:6; *n*-3) have preventive effects against development of obesity and obesity-related diseases [12]. Moreover, a growing body of evidence indicates that LC PUFA *n*-3 are able to improve metabolism and adipokines secretion profile of adipocytes in already obese subjects [13,14]. The positive effects of LC PUFA *n*-3 in obesity and obesity-related diseases are connected with their impact on metabolism and secretory functions of adipose tissue. In vitro studies revealed that EPA and DHA are able to decrease fat accumulation, and increase the expression of lipolytic genes in differentiating murine adipocytes [15,16]. Furthermore, DHA was shown to inhibit adipocyte differentiation and induce apoptosis in preadipocytes [17]. These fatty acids also act as secretion modulators of adipokines. Studies of a few research groups have shown that LC PUFA *n*-3 in most cases decrease secretion of pro-inflammatory adipokines, and increase synthesis of anti-inflammatory adipokines by cells of adipose tissue [14,18,19].

Dietary supplementation with LC PUFA *n*-3 in elderly people is mainly recommended because of their preventive properties related to cognitive decline and dementia development [20,21]. Unfortunately, there is a lack of data about the impact of LC PUFA *n*-3 on adipose tissue of old organisms, or a direct influence of these fatty acids on ageing adipocytes. As was previously mentioned, recent studies have shown that cellular ageing of adipocytes is related to adverse changes in adipokines secretion [11,22]. It was observed that only 10% of fat cells are renewed annually in adults, and the number of adipocytes in humans remain stable between 20 and 70 years of age [23]. On the other hand, several studies have demonstrated that ageing is associated with decreased expression of adipogenic transcription factors, such as: CCAAT/enhancer-binding protein, peroxisome proliferator-activated receptor and increased expression of anti-adipogenic C/EBP family members [24,25]. Furthermore, proliferation of preadipocytes and their ability to differentiate decrease with age [26].

Taking these data together and considering the fact that mature and old adipocytes may constitute a large part of adipose tissue especially in older subjects, the aim of the present study was to evaluate the influence of EPA and DHA on expression of genes connected with metabolism and secretory functions of ageing adipocytes.

2. Methods and materials

2.1. Cell culture

3T3-L1 mouse embryo fibroblasts were purchased from American Type Culture Collection (Rockville, MD USA) and cultured in humidified atmosphere of 5% CO_2, 95% air at 37 °C. The cells were maintained in a growth medium containing the following components: Dulbecco's modified Eagle's medium (DMEM, Sigma) with high glucose, 10% newborn calf serum (NBCS, obtained from Life Technologies, Invitrogen) and 1% penicillin-streptomycin (P/S, Sigma). Two days after the cells reached confluence (post-induction day 0), differentiation to adipocytes was initiated using differentiation medium supplemented with: 1 μM dexamethasone (Sigma), 0.5 mM isobutylmethylxanthine (Sigma) and 10 μg/ml insulin (Sigma). In differentiation medium 10% addition of NBCS was also replaced with 10% foetal bovine serum (FBS, Life Technologies, GIBCO). After 2 days (post-induction day 2) fresh medium containing only insulin was added for further 2 days. On post-induction day 4 medium was replaced with DMEM supplemented with 10% FBS and antibiotics. The cells were maintained in this type of medium until fatty acid treatment. Medium was changed every two days.

Table 1
Primers used for quantitative real-time PCR.

Gene symbol	Forward primer	Reverse primer
Pparg	ACTTCGGAATCAGCTCTGTG	ATTGGGTCAGCTCTTGTGAA
FATP1	AAGGTCAATGAGGACACGAT	CTGTGGGCAATCTTCTTGTT
FATP4	GCCAACAACAAGAAGATTGC	ACACCATAAACTGCCACATC
Atgl	CACCCTTTCCAACATGCTAC	TACCCGTCTGCTCTTTCATC
Cpt1b	CTCTACAGCTTCCAAACGTC	CTGCTTCGGAGGTAGACATA
Leptin	GACCATTGTCACCAGGATCA	TGAAGCCCAGGAATGAAGTC
Adiponectin	GTCTTCTTGGTCCTAAGGGT	ATGTTGCAGTAGAACTTGCC
Apelin	CTGCTCTGGCTCTCCTTGA	GCGCATGCTTCCTTCTTCTA
Il6	CTGGGAAATCGTGGAAATGAG	AGGACTCTGGCTTTGTCTTT
Mcp1	GTGTCCCAAAGAAGCTGTAG	TTGAGGTGGTTGTGGAAAAG
Irs1	CCAAACCTCCTGTTGAGAGT	AGGACCTTGGCAATGAGTAG
GLUT4	CCCACAGAAGGTGATTGAAC	CCTGATGTTAGCCCTGAGTA

2.2. Fatty acids treatments

On post-induction days (PID) 6, 10 and 14 differentiated 3T3-L1 adipocytes were cultured for 48 h in the presence of 100 μM EPA, or 50 μM DHA complexed to albumin; whereas, in control conditions only albumin was added to the medium. Experimental media consisted of serum-free DMEM, 1% FA-free bovine serum albumin (BSA), EPA/DHA/albumin and antibiotics. Every fatty acid treatment was preceded by 12 h starvation in serum-free DMEM supplemented with 1% FA-free BSA and antibiotics. On PID 8, 12 and 16 cells were collected and stored at −80 °C until further analyses.

2.3. Total RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from 3T3-L1 cells using RNeasy Lipid Tissue Mini Kit (Qiagen, cat. no. 74804) according to manufacturer's instructions, including DNase digestion step. The concentration of total RNA was determined spectrophotometrically using NanoDrop spectrophotometer (ThermoScientific). The quality of extracted material was checked using Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA samples which were used for the analysis of gene expression were characterized by the RIN>8 (RNA integrity number). cDNA was synthesized from 2 μg of RNA using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, cat. no. 4374966). Real time PCR was performed in a Stratagene Mx3000 P Thermocycler (Agilent Technologies) using SYBR Select Master Mix (Applied Biosystems, cat. no. 4472908). Each 10 μL reaction mixture contained a final concentration of 0.5 μM of each: forward and reverse primer, 1x master mix, 1 μL cDNA (100 ng). Cycling conditions started with an initial denaturation step at 95 °C for 15 min, followed by 40 PCR cycles. Each cycle consisted of denaturation at 95 °C for 15 s, primers' annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. In order to eliminate the possibility of a quantitative determination of non-specific products, after each reaction melting curve analysis of PCR products was made. The sequences of primers used for amplification are presented in Table 1. The ΔΔCT method was used to measure relative quantification. Results were normalized against a reference gene (β-actin).