Omega-3 polyunsaturated fatty acids play roles in skeletal muscle-adipose tissue crosstalk through myokines

Wenting Wei  
Southern Medical University

Huanting Zeng  
Southern Medical University

Limei Mao (✉ mlm912@163.com)  
Southern Medical University

Research Article

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Abstract

Skeletal muscle-adipose tissue crosstalk is important for maintaining metabolic homeostasis. Myokines play an indispensable role in the skeletal muscle-adipose tissue crosstalk. This study was conducted to elucidate the association between Omega-3 polyunsaturated fatty acids (n-3 PUFAs) and myokine in vitro and figure out the role of n-3 PUFAs in the skeletal muscle-adipose tissue crosstalk. As result, different forms of n-3 PUFAs increased the expression of multiple myokines in C2C12 myotubes. The expression of p-AMPK and PGC-1α in C2C12 myotubes were up-regulated by DHA/EPA mixture. The culture supernatant of myotube cells incubated with the DHA/EPA mixture regulated the lipid metabolism-related genes and stimulated browning in adipocytes. Therefore, n-3 PUFAs play roles in the skeletal muscle-adipose tissue crosstalk by regulating the expression and release of myokines associated with lipid metabolism and browning. Part of the mechanism of this process should be attributed to n-3 PUFAs activating AMPK signaling in C2C12 myotube.

Introduction

Skeletal muscle is known as the largest energy metabolism organ, participating in 60–80% glucose metabolism of organism[1], and plays an irreplaceable role in accelerating lipolysis and fat oxidation. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) has been determined to have multiple biological function, including lowering blood glucose and plasma TAG level, reducing inflammation, and finally exerting significant preventive and therapeutic effects on obesity, type 2 diabetes, cardiovascular diseases and other non-communicable diseases (NCDs)[2]. Studies have confirmed that a considerable part of these biological activities of n-3 PUFAs are achieved by regulating the glucose and lipid metabolism of skeletal muscle. A high-fat diet enriched in n-3 PUFAs maintain insulin receptor number, IRS-1 tyrosine phosphorylation, PI3'-kinase activity and total GLUT4[3]. Many studies have shown that n-3 PUFAs increase glucose disposal and reduce intramyocellular lipid accumulation in skeletal muscle cells by directly enhancing glucose and fat oxidation[4], which may be related to the stimulation of PGC-1α expression and mitochondrial biosynthesis in skeletal muscle by n-3 PUFAs [5].

In addition to being an effector organ, skeletal muscle is an important endocrine organ secreting hundreds of muscle-derived cytokines called myokines which involved in the body's biosynthesis and energy metabolism[6]. Myokines are expressed and released into the circulation in response to exercise, playing role in crosstalk between muscle and other organs. Working as hormone-like factors, myokines erect metabolic effects by regulating adipose tissue oxidation, insulin sensitivity, pancreas function, anti-inflammationand browning of fat, which decrease risk of chronic diseases and premature mortality[7].

However, there are very few studies about the relationship between endocrine function of skeletal muscle and factors other than exercise, especially n-3 PUFAs. In current study, we evaluated the expression of myokines related to lipid metabolism and browning in C2C12 myotubes treated with n-3 PUFAs and explored the role of AMPK, a key kinase in energy metabolism, in the regulation of myokines by n-3 PUFAs. The supernatant of C2C12 myotube cell culture medium treated with n-3 PUFAs was used to incubate 3T3-L1 adipocytes to observe the effect of n-3 PUFAs on the crosstalk between skeletal muscle and adipose tissue.

Methods And Materials

C2C12 cell culture
Murine C2C12 myoblasts were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin Solution (100X) (Solarbio, Beijing, China) with 5% CO₂ at 37°C until cell proliferation met 80% confluence. Then changed differentiation medium (DM) (2% horse serum instead of 10% FBS) for 4–5 days before cell treatment. 100 µM ALA, DHA, EPA and DHA/EPA (1:1.5) were added to DMEM/F12 containing 1% bovine serum albumin (BSA, NEFA ≤ 0.05%) (ExCell, Shanghai, China) in the fatty-acid treatment group, while 1% BSA as the control group.

3t3-l1 Adipocyte Culture

3T3-L1 preadipocytes were cultured in high glucose formulation Dulbecco's modified eagle medium (DMEM) containing 1% penicillin-streptomycin and 10% FBS until they were 100% confluent. Once the cells were confluent, incubated an additional 48 hours before initiating differentiation. Then removed the 3T3-L1 preadipocyte medium and replaced with 3T3-L1 differentiation medium which containing 10 µg/ml insulin, 1 µM dexamethasone, 10 µM rosiglitazone and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma, St. Louis, USA) to incubate for 72h. Then removed differentiation medium and replaced with 3T3-L1 adipocyte maintenance medium which was 10% FBS/DMEM containing 10µg/ml insulin until cells were ready for assay.

Cell Viability Assay

Plated and differentiatiated C2C12 myoblasts in 96-well plates. Incubated with 100 µM ALA, DHA, EPA and DHA/EPA for 24 h. Added 20μl MTT solution (5mg/ml, dissolved in PBS buffer) and incubated for 4h at 37°C and 5% CO₂. Added 150ul DMSO after aspirating the culture medium in the well carefully. Incubate at 37°C for 10 minutes and record absorbance at 570nm.

Real-time Pcr

Gene expression related to myogenesis, adipogenesis, lipolysis, oxidation and browning markers were measured by quantitative real-time PCR. Total RNA was extracted from cells with the TRIzol reagent (Accurate Biology, Changsha, China). The cDNA was synthesized from the RNA with reverse transcription reagent (Accurate Biology, Changsha, China). Real-time PCR was carried out by using SYBR Green (Accurate Biology, Changsha, China) on QuantStudio 6 Flex real-time PCR system (ABI, CA, USA). The primers were showed in Table 1. The housekeeping gene was β-actin. PCR conditions were as follows: 30 s at 95°C, 5s at 95°C and 30s at 60°C for 40 cycles. Data were analyzed by using the ΔΔCT. The primers and sequences are presented in Table 1.
<table>
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<th>Genes</th>
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<th>Reverse primer</th>
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<td>myogenin</td>
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<td>Leptin</td>
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<td>5'-CCCTGCAATTTGTTAGACC-3'</td>
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</table>
### Western Immunoblotting

Protein sample from cells was prepared by using RIPA containing 1:1000 protease inhibitor cocktail, 1:100 PMSF and 1:100 phosphatase inhibitor (Keygen, Nanjing, China). Electrophoresis, transfer of proteins, incubation with antibody, collection of digital images and gray analysis were carried out according to the previous method. Primary antibodies included β-actin (1:5000, Ray Antibody Biotech, Beijing China), AMPKα2 (1:1000, Bioss, Beijing, China), p-AMPKα2 (1:1000, Cell Signaling Technolog, MA, USA) and PGC-1α (1:500, Bioss, Beijing, China).

### Oil Red O Staining

Prepare oil red O working solution according to the instruction (Solarbio, Beijing, China). Removed the medium and washed cells with PBS, then fix in ice cold 4% paraformaldehyde for 2h. Washed cells with distilled water for 3 times. Added 60% isopropanol and soaked for 5min. Removed 60% isopropanol and added the newly prepared oil red O working solution to stain for 30min. Removed the stain solution and washed 2–5 times distilled with water. Added distilled water to cover the cells and observed with the microscope.

### Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM) and analyzed using SPSS 23 software (SPSS, Chicago, IL, USA). Differences between groups were analyzed by two-tailed Student's t-test or one-way ANOVA followed by testing using the LSD post-hoc test. Differences were considered significant if $P < 0.05$.

### Results

#### The differentiation and viability of C2C12 myotubes

After 4 days of culture in vitro with 2% horse serum, the characteristics of differentiated C2C12 cells with a tubular appearance were similar to mature skeletal muscle fiber in the body (Fig. 1A). In addition, the mRNA expression of myogenin, appearing in myotubes, increased obviously (Fig. 1B, $P < 0.001$). It meant that the myoblasts were induced into myotube cells from fibroblasts.

A methylthiazol tetrazolium (MTT) assay was used to investigate the effect of n-3 PUFAs, including ALA, DHA, EPA and DHA + EPA on the viability of C2C12 myotube cells. It was found that 100 µM of all n-3 PUFAs did not significantly suppress the viability of C2C12 differentiated cells (Fig. 1C, $P > 0.05$).

### N-3 Pufas Regulated The Transcription Of Myokines Related To Lipid Metabolism
To investigate the effects of n-3 PUFAs on myokines, the myotubes were treated with 100 µM of various n-3 PUFAs for 24 h. Real-time PCR was used to quantify the mRNA abundance of lipid metabolism-related myokines. In result, all n-3 PUFAs up-regulated the expression of IL-6, Irisin and FGF21. Moreover, ALA increased IL-15, but decreased MSTN and leptin level. DHA led to an increase of IL-15 but a decrease of leptin and TNF-α. EPA increased IL-15, adiponectin and TNF-α but decreased MSTN and Leptin level. Significantly, DHA/EPA up-regulated the expression of all the myokines except for IL-15 and MSTN (Fig. 2A-H, P < 0.05).

The Differentiation Of 3t3-l1 Adipocytes

Spindle-shaped 3T3-L1 preadipocytes before differentiation were observed under the microscope (Fig. 3A). The cells retracted and rounded at 1st day of induction (Fig. 3B). The volume of cells gradually increased, and small lipid droplets began to appear at 4-5th day of induction (Fig. 3C). And then the small lipid droplets increased rapidly and gradually merged. The lipid droplets fuse in large quantities at 8-10th days and Oil Red O staining was positive (Fig. 3D). It meant that 3T3-L1 preadipocytes were induced into mature adipocytes.

DHA and EPA enhanced myokines secretion to regulate lipid metabolism- and browning-related genes of 3T3-L1 mature adipocytes

All myokines detected in Fig. 2 have been reported to play roles in adipogenesis, lipolysis, fatty acid oxidation metabolism and browning of white adipose tissue. The proteins involved in the above processes were used as input list to search against the STRING database. The interaction maps were shown in Fig. 4A and 4B. All the information of the results against the STRING database were showed in Table 2. Briefly, the $P$ value of protein-protein interaction enrichment is lower than 1.0e-16. Figure 4A showed strongly correlation between myokines and proteins of adipocyte metabolism and function. Moreover, the reported beige adipocyte markers were showed interaction with myokines except for Cited1 (Fig. 4B).

<table>
<thead>
<tr>
<th>Network Stats</th>
<th>Lipid metabolism</th>
<th>Browning of white fat</th>
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</thead>
<tbody>
<tr>
<td>Number of nodes</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>Number of edges</td>
<td>75</td>
<td>106</td>
</tr>
<tr>
<td>Average node degree</td>
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<td>10.1</td>
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<tr>
<td>Avg. local clustering coefficient</td>
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<td>0.721</td>
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<tr>
<td>Expected number of edges</td>
<td>7</td>
<td>7</td>
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<tr>
<td>PPI enrichment p-value</td>
<td>&lt; 1.0e-16</td>
<td>&lt; 1.0e-16</td>
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</tbody>
</table>

In order to further confirm that whether n-3 PUFAs can stimulate the secretion of myokines or not, we measured the content of IL-6, the first myokine be found, in the supernatant of culture medium from myotube cells treated with 100 µM of DHA and EPA. As shown in Fig. 4E, myotube cells treated with DHA and EPA for 24 h secreted more IL-6 than control group. The secretion of IL-6 stimulated by DHA and EPA was gradually reduced after DHA/EPA was withdrawn. And the secretion level was as the same as control group after DHA and EPA were withdrawn for 24 h.
To avoid the direct impact of residual DHA and EPA on adipocytes and observe the effect of myokines stimulated by n-3 PUFAs on adipocytes, we treated 3T3-L1 adipocytes with the supernatant of the culture medium (SCM) from the first 12 hours after withdrawal of DHA and EPA. Real-time PCR results showed that SCM of myotube cells treated with DHA and EPA led to an increase of HSL, CPT1, adiponectin and leptin but a decrease of SREBP1, ACC1 and FAS (Fig. 4C) in 3T3-L1 adipocytes. Besides, SCM stimulated the expression levels of browning-promoting genes including Prdm16, PPARγ, Cidea, TMEM26, CD137, Cited1, Tbx1, HOXC9, TFAM, PGC-1α and UCP1 in 3T3-L1 adipocytes (Fig. 4D).

The Role Of Ampk Signaling Pathway In The Regulation Of Myokines By Dha And EPA

We entered all myokines into STRING. In result, KEGG pathways enrichments in the network showed that AMPK signaling pathway (KO04152, strength = 1.77, FDR = 0.00018) involved in energy metabolism was significantly enriched (Fig. 5A). It implied that such myokines regulated energy metabolism through the AMPK pathway.

Furthermore, we assessed the level of AMPK and p-AMPK in myotube cells treated with DHA and EPA for 24 h to explore whether n-3 PUFAs mediate the expression of myokines by regulating AMPK signaling. As showed in Fig. 5B, the protein expression of AMPK obviously, and the phosphorylation level of AMPK was up-regulated significantly by DHA/EPA and metformin (an AMPK activator) compared with control group. Moreover, Compound C treatment decreased the elevation of p-AMPK caused by DHA/EPA in C2C12 myotube cells. However, the expression of other myokines was further increased in C2C12 myotube cells treated with Compound C, except Irisin (Fig. 6A-G). We further detected the expression of PGC-1α expression in myotube cells. In result, DHA/EPA increased the mRNA and protein levels PGC-1α mRNA and protein levels, and Compound C blocked the effect of DHA/EPA on PGC-1α (Fig. 6H, 5B). Given that PGC-1α is an important transcription factor for irisin, the above experimental results indicate that DHA/EPA can regulate the expression of Irisin through the AMPK/PGC-1α pathway.

Discussion

A few chronic diseases such as type 2 diabetes, cardiovascular disease, colon cancer, breast cancer, dementia, depression and so on are defined as ‘a diseasome of physical inactivity’. Visceral fat accumulation leading to systemic inflammation caused by lack of exercise is an independent and strong risk factor for many chronic diseases. Given that exercise is recognized as an effective method to reduce visceral adipose tissue (VAT) mass, the protective effect of exercise on disease is attributed to the anti-inflammatory effect[8]. Myokines play a key role in exercise-mediated fat loss[7]. However, not everyone is suitable for exercise. The conclusions of this study have suggested that dietary n-3 PUFAs seem to be an alternative to exercise to reap the metabolic benefits of exercise.

Even though IL-6 is often regarded as a pro-inflammatory factor, as the first myokine discovered[9], the metabolic benefits of IL-6 cannot be ignored. In spite of the role of IL-6 on glucose metabolism is still controversial[10], its effect on lipid metabolism is outstanding. A randomized controlled study has provided direct evidence that the reduction of VAT mass after cycling depends on the body’s IL-6 level[11]. Besides, more and more studies have reported that IL-6 is an important endocrine factor stimulating white fat browning[12] which plays the biological effects of improving insulin resistance and reducing body weight by increasing the oxidation of glucose and fatty acids[13]. IL-6 level both in skeletal muscle and serum increase significantly after exercise, which confirmed that exercise is the main driving force for the production of muscle-derived IL-6[6]. The present study showed that n-3
PUFAs intervention promoted the expression and secretion of IL-6 in myotube cells. It is an interesting finding that n-3 PUFAs does not work as anti-inflammatory nutrients but may as positive dietary inducers on muscle-derived IL-6. However, it is still unclear whether n-3 PUFAs also mediate the production of IL-6 through the muscle contraction mechanism[6]. The research results of Watt MJ et al. showed that IL-6 infusion reduced hormone-sensitive lipase (HSL) in the adipose tissue of patients with type 2 diabetes[14]. In this study, the expression of HSL in 3T3-L1 adipocytes was increased, and the expression of browning markers in 3T3-L1 adipocytes was also up-regulated. These all provide strong evidence that muscle-derived IL-6 may be a key myokine regulating the skeletal muscle-adipose tissue axis by n-3 PUFAs.

Irisin, as a recently discovered new muscle factor, is always reported to be beneficial to health and its active role in muscle-adipose tissue crosstalk is particularly prominent. Irisin mainly increases thermogenesis by stimulating the browning of white adipose tissue, thereby increasing energy expenditure. It is considered to be a potential target for the treatment of obesity and related diseases[15]. A double-blind controlled trial has showed that n-3 PUFAs supplementation increases the plasma Irisin level in patients with coronary heart disease[16] and type 2 diabetes[17] and pregnant women[18]. However, such studies have not determined whether plasma Irisin is derived from skeletal muscle. Our study found that n-3 PUFAs increased the transcription level of Irisin in myotube cells, which related to the browning of 3T3-L1 adipocytes. Combined with the clinical trial results above-mentioned, it is clear that n-3 PUFAs may activate white adipose tissue browning by stimulating expression and secretion of Irisin in skeletal muscle.

IL-15 expresses in bone marrow, secondary lymphoid tissues, and many non-lymphoid tissues including adipose tissue and skeletal muscle, and its expression in skeletal muscle being particularly high[19]. In rodents, high level of IL-15 is associated with decreased adiposity. Overexpression of IL-15 in skeletal muscle has been found to reduce the mass of VAT[20] rich in a large number of IL-15 receptors[21]. In addition, IL-15 has been reported to stimulate adiponectin secretion by 3T3-L1 adipocytes[22]. All these seem to provide evidence that IL-5 works on skeletal muscle-adipose tissue crosstalk. Nevertheless, instead of in an endocrine fashion, IL-15 may likely act locally in skeletal muscle by increasing fatty acid oxidation, in turn, would increase fatty acid uptake into skeletal muscle and finally limit the availability of fatty acid for adipose tissue in humans[23]. Anyway, there is no doubt that high IL-15 level in skeletal muscle improve fatty acid utilization. Lots of studies have demonstrated that circulating IL-15 levels is increased after an acute exercise in humans[24]. Previous studies have also found that supplementing 2.5g of n-3 PUFAs per day (DHA:EPA = 1:2, for 8 weeks) increase the serum IL-15 level in women with depression[21]. However, there was no study on n-3 PUFAs regulating IL-15 expression in skeletal muscle before this article, even though a decrease of intestinal IL-15 expression in the fish oil group was reported[25]. Therefore, present study is the first to make it clear that n-3 PUFAs can stimulate skeletal muscle to express IL-15, but more research is thus needed to determine whether n-3 PUFAs promote the release of myogenic IL-15. Lebris S Quinn et al. have confirmed that IL-15 can stimulate 3T3-L1 adipocytes to secrete adiponectin[25]. However, the increase in adiponectin expression in 3T3-L1 adipocytes in our study did not seem to be related to skeletal muscle IL-15 expression. Consequently, it is still unclear whether IL-15 is involved in skeletal muscle-adipose tissue crosstalk regulating by n-3 PUFAs.

Myostatin (MSTN) is known as a key protein in the regulation of energy metabolism and muscle insulin resistance. Overexpression of MSTN can inhibit the transcription of GLUT4 and block insulin signaling[26]. Conversely, lacking MSTN presents with enhanced insulin sensitivity[27] and increased browning of the SAT[28] in mice. Marzia Bianchi et al. have discovered that maternal intake of n-3 PUFAs during pregnancy influence DNA methylation levels of MSTN in cord blood white cells of newborns, regrettably, have not done any research on the relationship
between n-3 PUFAs and skeletal muscle MSTN[28]. In this study, we have found that both ALA and EPA significantly reduce the expression of MSTN, which suggests that ALA and EPA may participate in maintaining the body’s glucose and lipid metabolic homeostasis by regulating muscle-derived MSTN.

Fibroblast growth factor (FGF) 21, a mediator of glucose and lipid metabolism, is produced by the liver, adipose tissues, and skeletal muscle. The use of FGF21 reduces hepatic glucose production and plasma glucose levels, while increasing insulin sensitivity and glucose uptake in adipose tissue. In addition, FGF21 reduces liver and plasma triglycerides and body weight, while activating brown adipose tissue[30]. Although exogenous administration of FGF21 exerts beneficial effects on glucose and lipid metabolism, circulating FGF21 levels are elevated in ob/ob and db/db mice, diet-induced obese mice and obese human[31]. Therefore, obesity may be an FGF21-resistant state. n-3 PUFAs have been found to increase the expression of FGF21 in C2C12 myotubes observed in our study. However, Katsunori Nonogak et al. reported that a diet with EPA over 6 days decreased plasma FGF21 levels of individually-housed KKAy mice, but not affected FGF21 mRNA level of soleus muscle[31]. Although the up-regulation of genes related to the lipid metabolism and browning in 3T3L1 adipocytes treated with supernatant of myotube cell culture medium treated with DHA/EPA was observed, the changes in FGF21 levels in the myotube cell culture supernatant was not be detected in our study. Nevertheless, Xavier Escoté et al. found recently that EPA supplementation increased circulating FGF21 in overweight/obese women following a hypocaloric diet[33]. In consequence, we propose that n-3 PUFAs, at least including EPA, play a role in regulating function and metabolism of adipocytes through muscle-derived FGF21.

Adiponectin and leptin are produced and secreted by adipocytes in most cases and are definite as adipokines. Most of the studies targeting this two proteins have been focused on adipose-tissue secretes adiponectin and leptin which can be found in the circulation. However, it has been described that adiponectin and leptin are also produced by muscle cells. Delaigle et al. reported early the presence of adiponectin mRNA and protein in mouse tibialis anterior muscles[33]. Moreover, the evidence that exercise induces adiponectin expression in skeletal muscle is also clear[35]. It is obvious to all that adiponectin plays the prominent role in anti-inflammatory, anti-atherosclerotic, anti-obesity and improving glucose and lipid metabolism disorder[36]. Earlier studies have proven that n-3 PUFAs are promoters of adiponectin in adipose tissue[37]. Although the secretion level was not analyzed, increased expression of adiponectin in C2C12 myotubes treated with EPA was present in current study. Such a result indicates that EPA may participate in the regulation of myogenic adiponectin. However, it is unclear whether myogenic adiponectin is involved in skeletal muscle-adipose tissue axis, because some researchers regard myogenic adiponectin plays an autocrine/paracrine role[38]. Although leptin derived from adipocytes is considered to be a risk factor for diseases in most cases, it cannot be ignored that leptin in skeletal muscle palys antiobesity and antidiabetic roles[39]. We found that the combined intervention of DHA and EPA stimulated myotube cells to express leptin, which was contrary to the down-regulation of n-3 PUFAs on adipose tissue and circulating leptin in high-fat diet mice. Similarly, whether the myogenic leptin induced by n-3 PUFAs exerts endocrine function need further research, because its local effect in skeletal muscle may be more important[40].

Although TNF-α considered to be a pro-inflammatory, it is also one of the myokines because of its expression induced by exercise and related to muscle damage caused by exercise[41]. Importantly, the promote lipolysis effect of TNF-α has been reported[42]. In this study, DHA and EPA seemed to have different effects on the expression of muscle-derived TNF-α. We believe that DHA inhibits skeletal muscle express TNF-α may be the result of DHA exerting anti-inflammatory effect[43], while further study required to figure out whether EPA-mediated skeletal muscle TNF-α expression is related to lipolysis[44].
AMPK is described as a central regulator of metabolism. In this study, we tried to find out the role of AMPK in the regulation of myokines expression by n-3 PUFAs. Although previous reports have pointed out that the production of myogenic IL-15 is also related to AMPK[45], we only found that the expression of Irisin depends on activation of AMPK signaling completely. Irisin is secreted into the circulation after the proteolytic cleavage of fibronectin type III domain-containing protein 5 (FNDC5)[46] of which production has been determined to be related to peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α)[47]. The regulatory effect of DHA/EPA on Irisin expression and PGC-1α was blocked by the AMPK antagonist compound C, suggesting that DHA/EPA increased Irisin via the AMPK pathway. The transcription of other myokines can be activated by Metformin, indicating that part of the production of these myokines should be attributed to AMPK signaling, but more mechanisms may be independent of AMPK.

**Conclusion**

Our study demonstrates that n-3 PUFAs stimulate the expression of a variety of myokines. Although the secretion of these myokines has not been confirmed one by one, the changes in expression of gene associated with lipid metabolism and browning in adipocytes have shown that n-3 PUFAs play roles in skeletal muscle-adipose tissue crosstalk through myokines, which should be targeted when further study prevention and therapy aimed at metabolic disorders and related diseases.

**Declarations**

**Ethical Approval**

Not applicable

**Conflicts of interest**

The corresponding author states that there is no conflict of interest.

**Authors’ contributions**

Conceptualization, and methodology, Wenting Wei and Limei Mao; performed experiments, Wenting Wei and Huanting Zeng; data curation formal analysis and writing-original draft, Wenting Wei; writing-review and editing, Wenting Wei and Limei Mao; project administration and supervision, Limei Mao. All the authors have read and agreed to the published version of the manuscript.

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**Availability of data and materials**

All data generated or analysed during this study are included in this published article [and its supplementary information files]

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References


**Figures**

**A**

*Before induction*  
*0day*  
*4day*

**B**

Relative mRNA expression of Myogenin

0Day  
4Day  
***

**C**

Cell viability (%)

CON  
ALA  
DHA  
EPA  
DHA/EPA

**Figure 1**

**The differentiation and viability of C2C12 myotubes.**

A. Morphological changes during differentiation of myotube cells stained with hematoxylin; Scale bar: 50 µm, 200×.  
B. The relative mRNA level of myogenin, the myotube specific gene.  
C. The survival rate of myotube cells treated
with n3-PUFAs for 24 h. Values are expressed as mean ± SEM (n=3). ***: P< 0.001 versus 0 day.

Figure 2

n3-PUFAs regulated the transcription of myokines related to lipid metabolism.

A-H. The relative mRNA level of IL-6, Irisin, IL-15, MSTN, FGF21, adiponectin, leptin and TNFα in C2C12 myotube cells. Values are expressed as mean ± SEM (n=3). Labeled means without a common letter are significantly different (P<0.05).
Figure 3

The morphological changes of 3T3-L1 mature adipocytes induced by cocktail method

A. 3T3-L1 preadipocytes stained with hematoxylin; B. 3T3-L1 adipocytes at 1st day of induction stained with hematoxylin; C. 3T3-L1 adipocytes at 5th day of induction stained with oil red O. D. 3T3-L1 mature adipocytes with positive oil red O staining; Scale bar: 50 µm, 200x.
Figure 4

DHA and EPA enhanced myokines secretion to regulate lipid metabolism- and browning-related genes of 3T3-L1 mature adipocytes

A-B. Protein-protein interaction network. C. The relative mRNA level of browning specific genes including Pradm16, PPARγ, Cidea, TMEM26, CD137, Cited1, Tbx1, HOXC9, TFAM, PGC-1α and UCP1 in 3T3-L1 adipocytes. D. The relative mRNA level of lipid metabolism related genes including Srebp1, ACC, FAS, ATGL, HSL, LPL, CPT1, adiponectin and leptin in 3T3-L1 adipocytes. E. IL-6 secretion of myotube cells treated with DHA and EPA for 24 h. Values are expressed as mean ± SEM (n=3). *: $P < 0.05$ versus control; **: $P < 0.01$ versus control; ***: $P < 0.001$
versus control. ##: $P < 0.01$ versus DHA/EPA in H0-24 ###: $P < 0.001$ versus DHA/EPA in H0-24 a: $P < 0.05$ versus control in H0-24 b: $P < 0.05$ versus control in H24-36.

**Figure 5**

**DHA and EPA activate AMPK signaling pathway in C2C12 myotube cells**

A. KEGG pathways enrichments in myokines. B. The protein level of AMPK and p-AMPK regulated by metformin, DHA/EPA and Compound C; The protein level of AMPK and p-AMPK regulated by metformin, DHA/EPA and Compound C. Values are expressed as mean ± SEM (n=3). *: $P < 0.05$ versus control; **: $P < 0.01$ versus control; ***: $P < 0.001$ versus control; ##: $P < 0.01$ versus DHA/EPA; ###: $P < 0.01$ versus DHA/EPA.
Figure 6

The role of AMPK signaling pathway in the regulation of myokines by DHA and EPA

A-H. The relative mRNA level of IL-6, Irisin, IL-15, MSTN, FGF21, adiponectin, leptin and PGC-1α in C2C12 myotube cells treated with metformin, DHA/EPA and Compound C. Values are expressed as mean ± SEM (n=3). *: \( P < 0.05 \) versus control; **: \( P < 0.01 \) versus control; ***: \( P < 0.001 \) versus control; #: \( P < 0.05 \) versus DHA/EPA; ##: \( P < 0.01 \) versus DHA/EPA; ###: \( P < 0.01 \) versus DHA/EPA

Supplementary Files

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