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Effects of n-3PUFAs on autophagy and inflammation of hypothalamus and body weight in mice

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ABSTRACT

Objective: Fat-1 transgenic mice were used as a model to study the effect of endogenous n-3 polyunsaturated fatty acids (n-3PUFAs) on the body weight, inflammatory factors and autophagy proteins in hypothalamus to explore the mechanism of n-3PUFAs inhibiting obesity.
Method: The mice were divided into two groups after genotype identification: fat-1 transgenic mice and wild-type mice. The body weight and body length of mice were measured at 14th week, and calculated the Lee 's index. The autophagosome in arcuate nucleus neurons was observed through electron microscopy; the expression of autophagy protein P62, LC3 and ATG7 in hypothalamus were detected and analyzed quantitatively by immunofluorescence and Western blot techniques. The mRNAs of inflammatory factor TNF-α, IL-6, IL-1β, NF-kB, chemokine MCP-1, CCL5, CXCL12, CX3CL1, microglia markers TMEM119, GFAP were detected by real-time fluorescence quantitative PCR.
Result: The Lee's index of *fat-1* transgenic mice was lower than that of wild-type mice($P < 0.05$). The autophagosome of the arcuate nucleus in *fat-1* transgenic mice were more than those in wild-type mice, and the expression of autophagy-related protein P62 was significantly decreased ($P < 0.05$) in hypothalamus of *fat-1* transgenic mice, while the expression of autophagy related protein ATG7 was significantly up-regulated ($P < 0.05$), and the ratio of LC3 II/I was significantly increased ($P < 0.05$), The results of qPCR showed that the mRNAs of TNF-α, IL-6, IL-1β, NF-kB, MCP-1, CCL5, CXCL12, and GFAP was significantly down regulated ($P < 0.05$), but CX3CL1 was significantly up-regulated ($P < 0.05$) in hypothalamus of *fat-1* transgenic mice.
Conclusion: Fat-1 gene or n-3 PUFAs possesses the function of reducing body weight, which involves the enhancement of autophagy and reduction of inflammatory factor in hypothalamus.

1. Introduction

At present, the proportion of obesity in the population appears an upward trend significantly. Obesity often leads to many health problems such as hypertension, hyperlipidemia, type 2 diabetes and cardiovascular diseases [1]. The cause of obese occurrence is imbalance of energy metabolism. Obesity displays more calorie intake than expenditure and excess energy is transformed into fat stored in the body [2].

Mammal hypothalamus, an important component of the central nervous system, receives signal from peripheral hormones and nutrients and subsequently integrates the signal to prompote the body's energy balance through regulating feeding behavior, energy consumption and metabolism. Obesity is often accompanied by chronic, low-grade inflammation of the central nervous system, especially in the hypothalamus [3]. Research shows that chemokines, as an important mediator of inflammation in the state of obese, can recruit immune cells to produce inflammatory cytokines, then activate the proliferate microglia and astrocytes, which are immune cells of the nervous system, to mediating the inflammation of hypothalamus. Thereby the cause of obesity is the disturbance energy balance regulation mechanism of hypothalamic [4].

Autophagy is a highly conserved life phenomenon of eukaryotes to maintain homeostasis of the internal environment and adapt change of microenvironment, which is intracellular self-repair and self-renew of organism in response to external stimuli and internal

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changes. Therefore, autophagy plays an important role in cell differentiation, metabolism and energy balance.

Controlled by autophagy-related genes (ATG), lysosome degrades and recycles intracellular macromolecules and damaged organelles [5]. Autophagy plays a negative role in regulation of inflammation since autophagy can weaken inflammatory response by inhibiting the expression of inflammatory cytokine and promoting the clearing up of apoptotic cell in the hypothalamus. The body weight is related to the above processes [6,7].

Arachidic acid derived from the N-6 polyunsaturated fatty acids (n-6PUFAs) promotes inflammation, relating to the occurrence of many diseases [8]. However, n-3 polyunsaturated fatty acids (n-3PUFAs) can inhibit the function of n-6PUFAs, which is beneficial to human body: lowering blood fat, anti-inflammatory and lowering blood sugar. EPA and DHA are the very important 2 kinds of n-3PUFA. The intake of EPA and DHA can significantly reduce the levels of triglyceride, lipid and lipoprotein in pasma, decrease body fat content in adults and promote the utilization of fat [9,10]. Therefore, n-3PUFAs are essential fatty acids in the body. Recently it has been discovered that n-3PUFAs also have the effect of to inhibit obesity [11], but the mechanism is unknown.

At present, the main fatty acids in our diet are saturated fatty acids and n-6 PUFAs, Our diet is short of n-3 PUFAs. Mammals have no ability to synthesize PUFAs, which only come from die. [12] But *fat-1* gene of *C. elegans* can transform n-6 PUFAs into n-3 PUFAs to increase endogenous n-3 PUFAs [13]. We found that *fat-1* transgenic mice suppressed diet induced obesity. Because obesity is related to inflammation of hypothalamic, we use the *fat-1* transgenic mice as a model to investigate the effect of n-3PUFAs on autophage and inflammation in hypothalamic and explore the mechanism of n-3 PUFAs inhibiting obesity.

2. Materials and methods

2.1. Identifying and raising fat-1 transgenic mice

fat-1 transgenic SPF mice were given by Professor JingXuan Kang from Harvard Medical School in the United States, and transferred to our laboratory by Dr. JianBo Wan from the University of Macau. SPF heterozygous *fat-1* transgenic mouse(*fat-1* mouse) was hybridized with the wild-type C57BL/6(WT mice). 1 cm long tail tip was cut from 2 weeks old mice to extract DNA, then PCR was performed to identify *fat-1* gene. Mice were divided into two groups: 10 transgenic male mice and 10 wild type male mice. Two groups were fed a diet rich in ω-6 and deficient in ω-3 PUFA, free drinking, light and dark cycle of 12 h, 22−25 °C.

2.2. Determining mice body weight and length

The body weight and body length of 14 weeks old mice were measured, and the lee's index was calculated.

2.3. Analyzing inflammation factor and chemokine by qRT-PCR

Total RNA was extracted from the hypothalamus and peripheral adipose tissue by *TAKARA RNAiso plus* kit according to the protocol, reverse-transcribed into cDNA by the kit (TranScript One-Step gDNA Removal and cDNA Synthesis SuperMix). Real-time fluorescence quantitative PCR was performed through the TransStart Tip Green qPCR SuperMix kit. All reactions were performed in triplicate and the data was analyzed by $2^{-\Delta\Delta CT}$ method. The primers list in Table 1.

2.4. Detecting autophagosome through transmission electron microscopy

About 1mm3 of hypothalamic arcuate nucleus was embedded in 2.5% glutaraldehyde. The embedded tissue was washed with 0.1 M sodium cacodylate buffer once, and fixed in 0.1 M sodium cacodylate buffer containing 2% osmium tetroxide for 1 h (RT), then dehydrated through a graded series of ethanol and acetone solutions. After infiltration with resin, the sample was embedded in Epon812 (Sigma−Aldrich, USA). Ultra-thin sections were cut by ultramicrotome and collected on copper grids (Sigma−Aldrich, USA), double-stained with uranyl acetate and lead citrate. The sample sections were examined using a JEM 100CX (JEOL, Japan) TEM operating at 60 kV.

2.5. Locating P62, LC3, ATG7 in hypothalamus through immunofluorescence

The mice were anesthetized by 10% chloral hydrate, given cardiac perfusion with 4% paraformaldehyde. The brain was taken out, dehydrated, embedded with OCT, frozen and sectioned. The frozen section was 10 μm thick. The section was incubated with primary antibodies (p62 1: 100, LC3 1: 350, atg7 1: 100) at 4 °C overnight, incubated with second antibody (1: 200)at room temperature dark for 1 h, then mounted with anti-fluorescent quencher, observated under fluorescent microscope and took pictures.

2.6. Analyzing expression of P62, LC3, ATG7 by western blot

The hypothalamus tissue was homogenated in a RIPA lysis buffer containing PMSF, centrifuged at 10000 g for 10 min. Discarding precipitate and keeping supernatant. The protein in supernatant was quantified using the Micro BCA™ Protein Assay Kit (Thermo Scientific™), loaded on 12%SDS-PAGE for electrophoresis and transferred onto PVDF membrane. The membranes was blocked with 5% skim milk for at room temperature for 2hrs, incubated with primary antibody ATG7(1:100; RD, USA), LC3(1:1000, NOVUS, USA), P62(1:2000, Proteintech, China), β-actin(1:1000, zhongshan, China) at 4 °C overnight, then incubated with secondary antibody (anti-mouse IgG 1: 7000 or anti-rabbit IgG 1: 7000; Multi Sciences, China) for 1 h (room temperature). All images were captured and analyzed using Image Lab software (Bio-Rad Universal Hood II, USA). The expression levels of the above proteins were normalized to those of β-actin.

2.7. Statistical analysis

All results are expressed as mean ± standard deviation. One-way ANOVA was used to analyze the body weight, body length and Lee's index of mice. Independent sample *t*-test was used to analyze Real-time quantitative PCR result and Western result. The significant level difference was set as $P < 0.05$.

3. Result

3.1. Body weight and body length of mice

The body weight and body length of 14 weeks old mice were measured, and the Lee 's index (Lee' s $= \sqrt[3]{(\text{body weight} \times 1000)} \div \text{body length}$) was calculated because Lee 's index was an effective index to reflect the degree of obesity in mice. The Lee' s index showed that there was a significant difference between transgenic mice and wild mice. Shown in Table 2. *: $P < 0.05$ vs WT.

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