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Linoelaidic acid enhances adipogenic differentiation in adipose tissue-derived stromal cells through suppression of Wnt/β-catenin signaling pathway *in vitro*

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ABSTRACT

Obesity has become a major health problem which is related with high-trans fatty acids diet. Adipogenic differentiation of adipose tissue-derived stromal cells (ADSCs) plays an important role in the development of adipose tissue. In order to determine the effect of trans fatty acids on adipogenic differentiation in ADSCs, cells were treated with linoelaidic acid, as well as linoleic acid and linolenic acid. We found that linoelaidic acid significantly increased the lipid droplet formation and triglyceride content compared with linoleic acid and linolenic acid. Linoelaidic acid also down-regulated the levels of β-catenin in cells and inhibited the accumulation of β-catenin in cell nuclei. Lithium chloride, an activator of Wnt/β-catenin pathway, antagonized the enhancement of linoelaidic acid on adipogenesis and up-regulated the levels of β-catenin in ADSCs. These results indicated that linoelaidic acid could enhance the adipogenic differentiation in ADSCs *in vitro*, which is partly due to the suppression of Wnt/β-catenin pathway.

1. Introduction

Fatty acids (FAs) are not only necessary required in cells and tissues, but also important regulators of lipid absorption and metabolism [1]. FAs can be classified into saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs). UFAs occurred in nature nearly all contain double bonds in *cis* conformation including monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). According to the position of double bonds, UFAs can be divided into n-3, n-6, n-7, n-9 fatty acids and so on. Linolenic acid (9Z, 12Z, 15Z-octadecatrienoic acid, n-3) and linoleic acid (9Z, 12Z-octadecadienoic acid, n-6) have be seen as essential fatty acids, which cannot be produced by mammals' do novo synthesis and must be consumed from food [2].

There are also various trans fatty acids (TFAs) with trans conformation of double bonds, which generally have different sources. Elaidic acid (9E-octadecenoic acid) is mainly produced by the process of partial hydrogenation of vegetable oil. Linoelaidic acid (9E, 12E-octadecadienoic acid) is principally discovered in foods with fried or high-heat cooking. These TFAs contain high content in dietary such as cake, oleomargarine, biscuit, chips and so on [3]. Excess intake of TFAs is associated with a high risk of many diseases, which can cause the increase of low density lipoprotein cholesterol, the decrease of high density lipoprotein cholesterol, dyslipidemia, inflammation, endothelial dysfunction, insulin antagonist and overweight [4].

Recently, obesity has become one of major health problems around the world [5]. A high TFAs diet was confirmed as a related inducement of obesity. Kavanagh et al. [6] researched on primates for 6 years to prove the influence of TFAs on the increase of body weight. The increase of weight in TFA (8% of total calories) fed group was 4 times higher than the same percentage of *cis*-MUFA feed group in primates, and their visceral fat added more than 30%. During 9 years, tracking survey of 16587 males, the males' waist circumference increased by 2.7 cm with supplement of TFAs in diet (2% of total energy) [7]. Field et al. [8] also found that the consumption of TFAs have significant effect on gaining weight but not for saturated fatty acid and other unsaturated fatty acids after a weight measure of 41,000 women for 8 years. Although the relationship between TFAs and obesity has been well studied in previous research, the mechanism of TFAs on the development of adipose tissue is still unclear.

In the development of adipose tissue, the adipogenic differentiation of stromal stem cells and preadipocytes plays an

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important role [9]. Adipose tissue-derived stromal cells (ADSCs) are a group of multipotent adult stem cells that can be isolated from adipose tissues. ADSCs have multipotential capabilities for differentiating into a variety of cell types, including adipocytes, osteocytes, chondrocytes and so on [10]. During adipogenesis, stromal stem cells firstly proliferate and commit to preadipocyte then terminal differentiate into adipocyte [11]. And it is a complex process regulated by a series of signaling pathways.

Wnt/β-catenin signaling is one of the central pathways now known to inhibit adipogenic differentiation [12]. Wnt 10b and other Wnts initiate Wnt/β-catenin signaling by binding to transmembrane frizzled receptors and low density lipoprotein receptor-related protein (LRP) co-receptors, which inhibits glycogen synthase kinase 3β (GSK3β) leading to stabilization and hypophosphorylation of β-catenin in cytoplasm [13]. Then β-catenin transfers into nucleus where it binds lymphoid-enhancer-binding factor/T-cell-specific transcription factor (LEF/TCF) and activates downstream targets to prevent the induction of peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein-α (C/EBP-α) [14].

Several studies have explored the regulation of Wnt/β-catenin pathway by FAs. In the research of Lim et al. [15], docosahexaenoic acid and eicosapentaenoic acid inhibited hepatocellular carcinoma growth through inhibition of β-catenin. Song et al. [16] also proved that ω-3 PUFAs could suppress pancreatic cancer cell growth *in vitro* and *in vivo* via down-regulation of Wnt/β-catenin signaling.

Up to now, the effect of TFAs on the differentiation of ADSCs toward adipocyte lineage remains unknown. In the present study, we analyzed the effect of linoelaidic acid on adipogenic differentiation in ADSCs, in comparison with linolenic acid and linoleic acid. Furthermore, we evaluated the expression of β-catenin in FAs treated cells. In addition, lithium chloride (LiCl), an inhibitor of GSK-3β, also an activator of Wnt/β-catenin pathway, was employed to study the contribution of Wnt/β-catenin signaling to the enhancement of adipogenic differentiation *in vitro* by linoelaidic acid.

2. Materials and methods

2.1. Isolation and cultivation of ADSCs

Mice of C57BL/6 were obtained from Dalian Medical University Laboratory Animal Center (Dalian, China). All experiments of mice in this study were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. ADSCs were isolated from inguinal adipose tissues of C57BL/6 mice (8 weeks). Adipose tissues were finely minced and washed with phosphate-buffered saline (PBS) containing 2% P/S (100 kU/mL penicillin and 100 mg/mL streptomycin; Yuanye Biotechnology, China). Tissues were then digested with 0.1% collagenase type I (Invitrogen, USA) at 37 °C for 40 min After digestion, collagenase I was neutralized by adding an equal volume of F12/DMEM (Thermo, USA) supplemented with 10% FBS (fetal bovine serum; Haoyang Biotechnology, China). Then tissues were centrifuged for 10 min at 400 × *g* to separate ADSCs from adipocytes, cellular debris and undigested tissue. ADSCs were plated in complete F12/DMEM culture medium (containing 10% FBS, 1% P/S and 1 mmol/L GlutaMAX™I; Gibco, USA), and maintained at 37 °C under an atmosphere of 5% CO_2. The medium was replaced every 3–4 days. When ADSCs were near to confluence, cells were trypsinized with 0.25% trypsin-EDTA (Invitrogen, USA) and reseeded with the same culture medium.

2.2. Characterization of ADSCs

ADSCs were trypsinized and resuspended in complete medium. Cells were stained with FITC anti-mouse CD29 (BioLegend, USA) and CD34 (Biosynthesis Biotechnology, China) for 30 min ADSCs were then washed three times with PBS, resuspended in 200 μL PBS and analyzed on FACSCalibur flow cytometer (FCM, BD) using CELL Quest software (BD).

ADSCs were induced into adipogenic and osteogenic to evaluate their multipotency. 10^5 cells were cultured in 24-well plates for 3 days in complete medium. For adipogenesis, the medium was switched to adipogenic medium consisting of F12/DMEM with 10% FBS, 1% P/S, 1 μmol/L dexamethasone (Sigma, USA), 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX; J&K Scientific, China), 200 μmol/L indomethacin (Aladdin Industrial, China) and 10 μg/mL bovine insulin (Yuanye Biotechnology, China) for 14 days. Lipid droplets were stained by 0.5% oil red O (Sigma, USA) after fixation in 10% formalin. For osteogenesis, the medium was switched to osteogenic medium (Viraltherapy Technologies, China). Cells were cultured with osteogenic medium for 14 days and 21 days. Mineral deposits were stained by alkaline phosphatase (ALP) staining kit (Keygen Biotechnology, China) on the 14th day according to the manufacturer's recommendation, and stained by 1% Alizarin Red (Kemiou Chemical Reagent, China) on the 21st day after fixation in 10% formalin.

2.3. Fatty acid treatments

FAs, including linoleic acid, linoelaidic acid and linolenic acid (all form Cayman, USA), were dissolved in alcohol and mixed with F12/DMEM. When the cells reach between 80% and 90% confluences, the medium was switched to adipogenic medium and supplemented with three kinds of fatty acids for 3 days respectively. The final concentrations of fatty acids were 20, 40 and 60 μmol/L. To investigate the possible relationship between TFAs and Wnt/β-catenin pathway on adipogenesis, 5 mmol/L LiCl was added into adipogenic medium and co-treatment with linoleic acid and linoelaidic acid for 3 days.

2.4. Oil red O staining

Cells were fixed with 10% formalin for 10 min and stained with filtered oil red O for 10 min Next, cells were stained with haematoxylin solution for 15 min and washed twice with PBS. Images of cells were captured by an Olympus IX81 inverted microscope and analyzed by using cellSens Dimension software.

2.5. Triglyceride (TG) content assay

TG content in cells was measured using a commercially available TG assay kit (Jiancheng Biotechnology, China) according to the manufacturer's protocol. ADSCs were washed twice using PBS, and lysed with 1% TritonX-100 for 30 min Lysate was mixed with TG assay kit at 37 °C for 30 min During this process, TG was hydrolyzed into glycerol and fatty acids by lipoprotein lipase. The glycerol was then detected by enzyme coupled reduction of a dye. The absorbance values of mixture were measured at 546 nm. TG content was quantified with cell numbers.

2.6. Western blot

Total proteins of cultured cells were extracted using RIPA lysis buffer (Beyotime, China) containing 100 mmol/L phenylmethanesulfonyl fluoride (PMSF, Beyotime, China). Protein concentrations were measured using BCA protein assay kit (Keygen Biotechnology, China). Proteins were loaded into SDS-PAGE gel