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Adipocyte differentiation is regulated by mitochondrial trifunctional protein α-subunit via sirtuin 1

Hong-Xia Liu[1], Yan-Mei Wang[1], Jian-Ping Hu, Li-Ying Huang, Ning-Yuan Fang*

Department of Geriatrics, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, No. 160 Pujian Road, Pudong New Area, Shanghai 200127, China

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ABSTRACT

Mitochondrial trifunctional protein α-subunit (MTPα) is involved in the fatty acid β-oxidation (FAO) pathway. Two MTPα activities, 3-hydroxyacyl-CoA dehydrogenase and long-chain hydratase, have been linked with the occurrence and development of obesity and obesity-related disorders. These activities catalyze two steps in the FAO pathway (the second and third reactions). However, the role of MTPα in the pathogenesis of obesity has not been evaluated, and the functional role of MTPα in adipocyte differentiation has not been determined. Here, we analyzed the functional role of MTPα using in vitro and in vivo models of adipogenesis. MTPα expression was upregulated during the differentiation of 3T3-L1 preadipocyte cells into adipocytes. MTPα gene silencing stimulated peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha(C/EBPα) expression, which promoted adipocyte differentiation. By contrast, MTPα overexpression blocked adipogenesis in 3T3-L1 cells. Further analysis showed that MTPα positively regulated sirtuin 1 (SIRT1). Injection of preadipocytes overexpressing MTPα into athymic mice significantly impaired *de novo* fat pad formation compared with that of the control, and furthermore MTPα knockdown enhances fat pad formation at a time point earlier than 5-week, such as week-2 and week-3, when the control fat pad is not fully developed. In summary, our data indicate that MTPα is a novel factor that negatively regulates adipocyte differentiation. We propose a pathway in which MTPα inhibits adipogenesis by promoting SIRT1 expression, which represses PPARγ and attenuates adipogenesis.

1. Introduction

Obesity is defined as an excess accumulation of white adipose tissue (AT). Obesity is widely regarded as an increased risk factor for several life-threatening diseases, including cardiovascular disease, type 2 diabetes mellitus (T2DM), osteoarthritis, non-alcoholic fatty liver disease (NAFLD), and some types of cancer [1]. Obesity prevalence is increasing globally, and the economic impact of obesity and associated diseases consumes a significant percentage of expenditures for health care [2]. The expansion of white AT mass observed in the obese state is due to adipocyte hypertrophy and hyperplasia [3], which are regulated by metabolic, genetic and nutritional factors [4]. A greater understanding of the molecular mechanisms regulating adipogenesis and specific gene modifications occurring in the adipocyte is essential for further clinical advance.

Previous studies have focused on the transcriptional control of adipocyte differentiation using well-established in vitro models [5–7]. This work identified a number of key transcriptional regulators of adipogenesis: peroxisome proliferator-activated receptor gamma (PPARγ) [8]; CCAAT/enhancer binding protein beta (Cebpβ) [9], delta (Cebpδ) [10], and alpha (Cebpα)[11]; and metabolic regulatory factors such as sirtuin (SIRT) proteins [12–14]. PPARγ and C/EBP-family transcription factors are crucial for both white and brown adipocyte differentiation. SIRT-family proteins also play important roles in adipogenesis. SIRT1 inhibits adipogenesis by repressing the activity of PPARγ and stimulating lipolysis, which results in lower levels of adipogenesis [12,14]. SIRT2 deacylates FOXO1, thereby suppressing adipogenesis. This promotes FOXO1 binding with PPARγ, which subsequently represses PPARγ transcriptional activity [13].

Mitochondrial trifunctional protein α-subunit (MTPα) and β-subunit (MTPβ) form an α4β4 protein complex, which is called mitochondrial trifunctional protein (MTP). MTP catalyzes the final three reactions of the long-chain fatty acid β-oxidation(FAO) pathway [15]. MTP deficiency leads to aberrant accumulation of long-chain 3-hydroxy fatty acids [16,17], which stimulates the production of reactive oxygen species and the expression of pro-inflammatory cyto-

* Corresponding author.
E-mail address: fangnysh@126.com (N.-Y. Fang).
[1] Equal contributors.

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kines [18,19]. MTP deficiencies can cause sudden, unexplained death in children, acute hepatic encephalopathy, cardiomyopathy, and rhabdomyolysis [20]. Several reports have identified a role for MTP in the FAO pathway; however, the pathophysiological functions of MTP in the occurrence and development of white adipose tissue and obesity remain largely unknown. MTPα heterozygous defective mice (MTPα$^{+/-}$) develop fatty liver and insulin resistance [21]. MTPα protein levels are significantly downregulated in the hippocampi of human Alzheimer disease patients and in streptozotocin-induced diabetic mice [22]. MTPα expression also is reduced in the omental fat of obese patients and is inversely correlated with body mass index (BMI) [23]. The transcription and activity of MTPα significantly increased in oocytes of mice eating a high fat diet that were subjected to exercise training [24]. These combined results suggest that MTPα may be involved in the occurrence and development of obesity and obesity-related disorders, in addition to its normal catalytic function in FAO. Here, we use in vitro and in vivo models to investigate the role of MTPα in adipocyte differentiation.

2. Materials and methods

2.1. Experimental reagents

Dexamethasone, isobutyl-3-methylxanthine (IBMX), insulin, Oil Red O, isopropanol, and Etomoxir were obtained from Sigma-Aldrich(St. Louis, MO, USA). Radio-immunoprecipitation assay (RIPA) buffer, Dulbecco's modified Eagle's medium (DMEM), TRIzol reagent, fetal bovine serum (FBS), penicillin/streptomycin, phenylmethylsulfonyl fluoride (PMSF), and Halt Protease and Phosphatase Inhibitor Cocktail were obtained from Thermo Fisher Scientific(Waltham, MA). The 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide (EX-527) reagent was obtained from Enzo Life Sciences(New York, USA). Phosphate-buffered saline(PBS) was obtained from GE Healthcare Life Sciences (Beijing, China). PrimeScript™ RT Reagent Kit (Perfect Real Time)(Cat. # RR037A) and SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Cat. # RR420A) were obtained from Takara(Shiga, Japan). BCA Protein Assay and Primary Antibody Dilution Buffer were obtained from Beyotime (Nanjing, China). Antibodies against PPARγ, C/EBPα, FABP4, and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against MTPα, MTPβ, CPT1 and LCAD were obtained from Santa Cruz (Dallas, TX, USA), and SIRT1 antibody was obtained from Abcam (Cambridge, MA, CA).

2.2. In vitro cell culture and cell differentiation

The 3T3-L1 preadipocyte cells were obtained from the Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). The 3T3-L1 cells were cultured to confluence in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cell cultures were incubated at 37 °C with 5% CO_2. Two days after cells reached confluence(designated as Day0), they were induced to differentiate by incubating in differentiation and induction medium (MDI), which consisted of DMEM supplemented with 0.5 mM3-isobutyl-1-methylxanthine (IBMX), 1 mM dexamethasone, 5 µg/ml insulin, and 10% FBS. Cells were incubated in MDI for 2 days. Then, the MDI was replaced with DMEM supplemented with 10% FBS and 5 µg/ml insulin (Day 2), and cell cultures were incubated for another 2 days. Thereafter, the conditioned medium was replaced on Day 4 with DMEM supplemented with 10% FBS, and this medium was refreshed every 2 days up to Day 8.

2.3. In vitro gene silencing and overexpression

The 3T3-L1 preadipocytes were grown to approximately 30–40% confluence, and then transfected with control shRNA (shCON) or MTPα-targeted shRNA (shMTPα) lentiviral particles. The target sequence used against mouse MTPα was as follows: 5′-TCTCCCAATCAATCAAATT-3′; and the sequence of the control shRNA was as follows: 5′-TTCTCCGAACGTGTCACGT-3′. The 3T3-L1 preadipocytes that carried the shRNA cassette for MTPα were selected with 3 µg/ml puromycin 72 h after infection. For long-term MTPα overexpression induction in vitro, 3T3-L1 preadipocytes were transduced with lentiviral vector containing an MTPα (ovMTPα) expression cassette. Control cells were transfected with the control vector (ovCON). Subsequent selection of transfected cells was achieved with 3 µg/ml puromycin. The shRNA or overexpression lentiviral vector and particles were constructed and synthesized by GeneChem (Shanghai, China). Lentiviral-transfected cells that were used for parallel experiments had identical passage levels.

2.4. SIRT1 activity modulation in vitro

SIRT1 was inhibited in MTPα-overexpressing 3T3-L1 cells (transfected with a lentiviral vector containing an MTPα expression cassette) by treating cells with the SIRT1 inhibitor EX-527. Subsequently, adipogenesis was induced by treating cells with DMI ± 10 µM EX-527. The cells were incubated for two days. Then, the conditioned medium was replaced with DMEM supplemented with 10%FBS, 5 µg/ml insulin, and 10 µM EX-527, and the cells were allowed to grow for a further two days. On Day4, the conditioned medium was replaced with DMEM supplemented with10% FBS and 10 µM EX-527; this medium was refreshed every two days up to Day 8. After differentiation, the cells were harvested for biochemical analysis or stained for cell biological analysis with Oil Red O.

2.5. In vivo model of adipogenesis

De novo fat pad formation was induced in athymic male Balb/c nude mice (Shanghai SLAC Laboratory Animal Co. Ltd, China) as follows. A total of 1×10^7 3T3-F442A preadipocyte cells were transfected with MTPα-overexpressing (ovMTPα) or control (ovCON) lentiviral particles and control shRNA (shCON) or MTPα-targeted shRNA (shMTPα) lentiviral particles, grown to essentially complete confluency, resuspended in 200 µl of 1×PBS, and then subcutaneously injected into the back of 6- to 8-week-old male athymic Balb/c nude mice. The 3T3-F442A preadipocyte cells were obtained from the European Collection of Cell Cultures (provided by JENNIO Biological Technology, Guangzhou, China). All animal studies were approved by the animal ethics committee of the Shanghai Jiaotong University affiliate of Renji Hospital. The mice were kept in an animal room, which was maintained at 23 ± 2 °C, 55 ± 5% humidity, and 12/12 h light/dark cycle. Each test group included five mice, which were fed a standard unrestricted diet. Five weeks after injection, the size of the de novo formed fat pads were measured with vernier caliper. Mice were euthanized by intraperitoneal injection of 60 mg/kg sodium pentobarbital at the scheduled time, then the fat pads that formed *de novo* at the injection site were removed, fixed in 4% paraformaldehyde, and then embedded in paraffin blocks. Thin sections (4 µm) were prepared for histology and immunohistochemistry.

2.6. Cell staining with Oil Red O

The 3T3-L1 preadipocyte cells were prepared for microscopic analysis as follows. Cell were washed twice with PBS, fixed for 30 min with 4% formalin, and then washed twice with distilled water. Fixed cells were immersed in a filtered solution of 0.6% (w/v) Oil Red O in 60% isopropanol for 30 min. Then, cells were washed twice with distilled water. Light microscopy and digital imaging (Olympus digital camera, Tokyo, Japan) were used to observe lipid droplets. Lipid droplet formation was quantified for statistical analysis as follows: Oil Red O dye retained in cells was dissolved by suspending cells in

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