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Original Article

Regulation of lipid metabolism in rat leydig cells testosterone synthesis and proliferation

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Abstract: Aim: To induce rat model of hyperlipidemia through high fat diet feeding, exploring the effect of abnormal lipid metabolism in rat leydig cell testosterone biosynthesis and proliferation. Methods: 30 healthy male SD rats were randomly divided into normal group (NG) and the obese group (OG). Rats in NG were fed with basic diet, while rats in OG were continuously fed with high fat diet for 16 weeks to build the rat model of hyperlipidemia. After then we detected factors associated with testosterone biosynthesis and proliferation in rat Leydig cell at 0.8, 16 weeks, respectively. Results: Rats fed with high fat diet had lower blood testosterone levels compared with the normal rats, and the leydig cell testosterone biosynthesis related enzymes decreased. Moreover, the leydig cell of rats in OG decreased significantly and the expression of Ect2 (epithelial cell transforming sequence 2 oncogene) was lower than that of rats in NG. Conclusions: Rats fed with high fat diet had abnormal lipid metabolism which resulted in testosterone synthesis and proliferation capability of leydig cell decreasing. The potential mechanism was that some enzymes associated with testosterone biosynthesis, such as StAR, P450c were inhibited and the expression of Ect2 decreased, which played a role in regulating cell proliferation.

Keywords: Lipid metabolism, leydig cell, testosterone synthesis, Ect2

Introduction

With the improvement of people's living standards, there are more and more people who appear the disorder of lipid metabolism and obesity [1]. The abnormal lipid metabolism caused by high fat diet can result in many diseases threatening health such as diabetes mellitus, fatty liver and so on. Moreover, the function of the male reproductive system also has a certain negative affect by the disorder of lipid metabolism [2, 3]. It was reported that people with disorder of lipid metabolism and obesity had delayed development of reproductive system, lower level of testosterone and reproductive dysfunction compared with that of ordinary children of the same age. Correlation and the regulation mechanism between lipid metabolism and reproductive system are still unclear [4, 5]. The purpose of this study is to induce rat model of hyperlipidemia through high fat diet feeding, detecting the expression of related factors in rat leydig cell testosterone biosynthesis and proliferation, and to investigate the related pathways and mechanisms of lipid metabolism disorders affecting the reproductive function. In order to provide a new research direction and basis for the diet control and treatment of clinical reproductive endocrine disease.

Materials and methods

Animals and grouping

30 clean, inbred male SD weighing 280~310 g (purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences). After one week adaptive feeding, all rats were randomly divided into control group and lipid metabolism disorders group, and each group had 15 rats fed with basic diet and high fat diet, respectively. All rats were free fed for 12 continuous weeks. The rats weighed weekly during the experiment and serum TC, TG, HDL2C were measured before and after the experiment. The lipid metabolism disorder model had been established.

Isolation of rat leydig cells

Leydig cells were isolated as previously reported [6] with slight modifications. In brief, testes were removed, decapsulated and dispersed in 10 mL essential medium containing 0.25 mg/mL collagenase or trypsin (0.18%) at 32°C in a shaking water bath at 34°C for 10 min. Tubes were then capped and inverted several times, and the seminiferous tubules were removed by gravity sedimentation (4 minutes). The tubes containing the settled seminiferous tubules were refilled with 1% BSA, and the procedure was repeated several times to further harvest the interstitial cells. The cells were fractionated using a continuous Percoll gradient (70%, 58%, 30 and 5%), and centrifuged at 800 × g for 20 minutes at 18°C. After centrifugation, fractions at 1.056 and 1.068 g/mL were collected, washed with buffer, and counted. Isolated Leydig cells were resuspended in the culture medium containing 15 mM HEPES, nonessential amino acids, 5 μg/mL gentamicin, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were seeded on Falcon culture plates (Becton, Dickinson and Co, Lincoln Park, NJ) at a concentration of 0.5 to 0.7 × 10^6 cells/mL of medium. Viability of Leydig cells was assessed by the trypan blue dye exclusion method. Briefly, isolated Leydig cells were mixed with an equal volume of 0.4% trypan blue (Flow Laboratories, Irvine, United Kingdom), incubated for 5 minutes at 37°C, and examined under a microscope. After 24 hours of incubation, Leydig cells were almost totally viable.

The serum testosterone levels measured by radioimmunoassay

The rats were anesthetized and sacrificed by withdrawing blood from celiac artery and the hormone levels were assessed. The samples were centrifuged (1000 × g for 10 min) and the serum testosterone levels were measured by radioimmunoassay (Fangsheng Medical Bioengineering Co., Beijing, China).

Western blot analysis of StAR and P450scc of rat leydig cells

Western blot analysis was conducted. In brief, the homogenized testis samples (10 μg protein, being prepared by the same way described in the section of enzyme assay) of testes were boiled in equal volumes of sample loading buffer, a Tris-Cl buffer (pH 6.8) containing 20% glycerol, 5% SDS, 3.1% dithiothreitol and 0.001% bromophenol blue for 5 min to denature proteins. The samples were then electrophoresed on 10% polyacrylamide gels containing SDS in an electrophoresis system (Bio-Rad) at 80 V. Proteins were electrophoretically transferred onto nitrocellulose membranes, and after 30 min of exposure to 10% non-fat milk to block nonspecific binding, the membranes were incubated with a rabbit polyclonal anti-StARD7 antibody (Cell Signaling Technology, Danvers, MA, USA; dilution 1:100) and a rat anti-P450scc antibody (Cell Signaling Technology, Danvers, MA, USA; dilution 1:200) at 4°C overnight. The membranes were then washed and incubated with a 1:2500 dilution of goat anti-rabbit antiserum that was conjugated to horseradish peroxidase. The washing step was repeated, and immunoreactive bands were visualized by chemiluminescence using a kit (ECL, Amersham, Arlington Heights, IL, USA). Western blotting analysis of β-Actin (ACTB) using an ACTB antibody (Cell Signaling Technology, Danvers, MA, USA; dilution 1:1,000) was also performed, and the StAR and P450 scc levels were normalized to ACTB. The gel was placed in a Kodak image station 4000 R (Carestream Health, Inc., Rochester, NY, USA), and photos were taken. The analysis of western blot images was performed using the Kodak image station 4000 R. The pixel densities in each band were normalized to the amount of ACTB in each lane. The relative StAR levels to ACTB were calculated.

Proliferation in rat Leydig cell quantified by flow cytometry analysis

Briefly, rat Leydig cell was Closed Fc receptor and labeled Ki67 antibody (Becton Dickinson, CA, USA). Cells were collected by trypsinization and washed with PBS. Cell proliferation was measured by dual-color analysis of Annexin V binding (green fluorescence) and PI uptake (red fluorescence) using a FACSCalibur (Becton Dickinson, CA, USA) apparatus with excitation and emission settings at 488 nm plus a 515 nm band pass filter for fluorescein detection and a filter at >600 nm for PI detection.

Real-time PCR (qPCR) of Ect2 mRNA levels in rat leydig cells

Total RNAs were extracted from the pituitary and testes using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's

Table 1. The influence of weight and serum lipid levels by high fat feeding rats

Groups	Time	Weight	TC (mmol/L)	TG (mmol/L)	HDL2C (mmol/L)
Control group	0 W	305±14.6	1.66±0.24	1.48±0.09	1.36±0.04
	18 W	398±13.2	1.71±0.10	1.49±0.32	1.28±0.20
Lipid metabolism disorder group	0 W	301±19.3	1.68±0.55	1.60±0.02	1.33±0.09
	18 W	597±20.4[a,b]	4.03±0.17[a,b]	2.87±0.25[a,b]	0.64±0.07[a,b]

[a]Compared with 0 week P<0.05; [b]Compared with control group P<0.05.

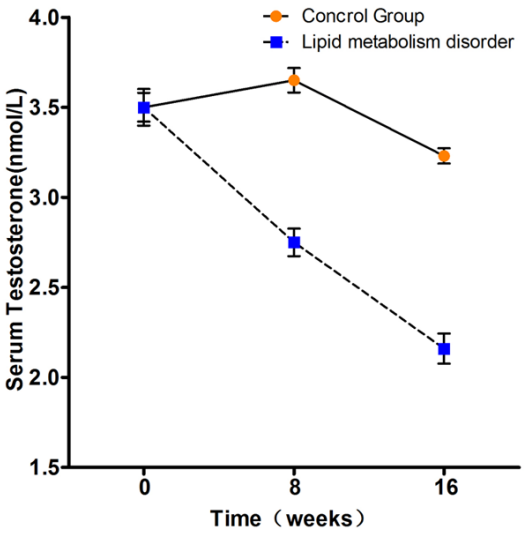

Figure 1. The serum testosterone levels measured by radioimmunoassay.

instruction. One microgram of total RNA was reverse transcribed in a final volume of 30 ml using the ImProm-II reverse transcriptase system kit (Promega Italia SRL, Milano, Italy). cDNAs were used for PCR. PCR amplification was performed using 1.5 U of Taq DNA polymerase (Promega Italia SRL) in PCR buffer containing 200 mM dNTP, 1.5 mM $MgCl_2$ and 25 pmoles of each primer in a total volume of 50 ml. The primers were listed: (1). Ect2: forward primer is 5'-CCCAAGGGACCTCATACT3-3', and the reverse primer is 5'-CAAGCCTCCAGACGACAA-3'; (2). β-actin: forward primer is 5'-CCCTGTGCTGCTCACCGA-3', and the reverse primer is 5'-ACAGTGTGGGTGACCCCGTC-3.

Statistical analysis

The data were analyzed by one-way analysis of variance followed by planned comparisons with the Sidak adjustment of the *P* value, as an estimation of the experimental error rate. All data are expressed as means ± standard error of the mean (SEM). Differences were regarded as significant at P<0.05.

Results

Lipid metabolism disorder induced by high fat feeding rats

We tested body weight and serum lipid levels of all rats at 0 week and 18 weeks, respectively. The results were showed in **Table 1**. The levels of serum total cholesterol (TC), triglyceride (TG), High density lipoprotein cholesterol (HDL2C) showed no significant difference in the two groups before the test (P>0.05). While after 18 weeks, The body weights and the levels of serum TC and TG were significantly higher in rats with abnormal lipid metabolism compared with the control group (P>0.05), and the level of HDL2C was lower in the group of abnormal lipid metabolism compared with the control group (P<0.05).

The influence of lipid metabolism disorder on serum testosterone

The level of serum testosterone was significantly lower in the group of abnormal lipid metabolism rats fed with high fat diet compared with the control group (P<0.05, **Figure 1**). Furthermore, the level of serum testosterone was lower at 16 weeks compared with that at 8 weeks, which illustrated the influence of lipid metabolism disorder on testosterone synthesis.

The influence of abnormal lipid metabolism on the key proteins expression in testosterone synthesis

Compared with the control group, the expression levels of StAR and P450 scc of rat leydig cells in the group of abnormal lipid metabolism rats fed with high fat diet were higher (P<0.05, **Figure 2**), which illustrated the influence of lipid metabolism disorder on the inhibition expression of the key enzymes in testosterone synthesis of rat leydig cells to reduce the biosynthesis of testosterones.

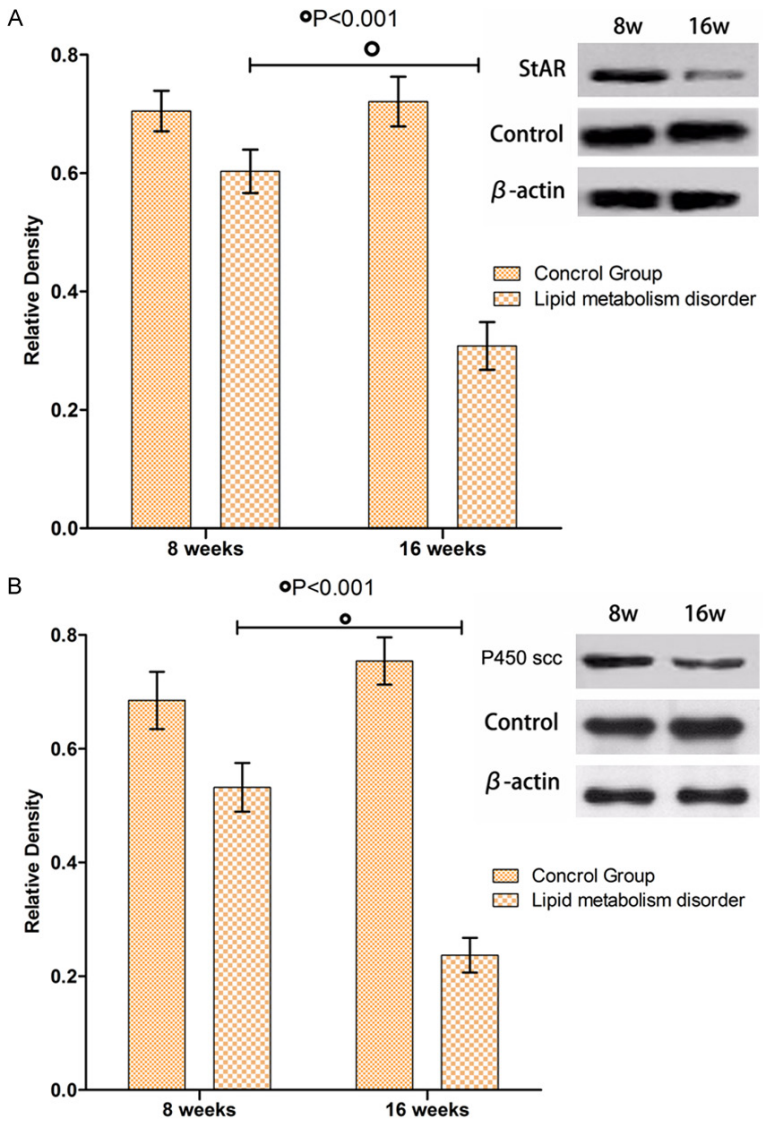

Figure 2. The expression levels of StAR and P450 scc of rat leydig cells by western blot.

The influence of abnormal lipid metabolism on the proliferation of rat leydig cells

The expression of Ki67 of rat leydig cells were detected by flow cytometry and showed a significant decreasing trend in the group of abnormal lipid metabolism rats fed with high fat diet compared with the control group ($P<0.05$, **Figure 3A**), which illustrated that lipid metabolism disorder reduced the proliferation of rat leydig cells. Moreover, the level of Ect2 mRNA of rat leydig cells was significantly lower in the group of abnormal lipid metabolism rats fed with high fat diet compared with the control group (**Figure 3B**, **3C**), which suggested that lipid metabolism disorder might inhibit rat leydig cell proliferation by inhibiting expression of Ect2.

Discussion

Some recent studies found that lipid metabolism disorders and obesity caused male reproductive dysfunction, but the particular mechanism is not very clear [7]. Leydig cells were the main cell of androgen secretion, which located on the loose connective tissue between the seminiferous tubules of the testes, synthesizing testosterone through a complex biological process [8]. The steroidogenic acute regulatory protein (StAR)[1] plays a critical role in trophic hormone-stimulated steroid biosynthesis by facilitating the transfer of cholesterol, the substrate for all steroid hormones, to the inner mitochondrial membrane where it is converted to pregnenolone by the P450 side chain cleavage enzyme (P450 scc) [9-12]. After then reacted with steroid synthetic enzyme and gradually transform into testosterone [13, 14]. With the improvement of people's living standards, irrational diet and lifestyle resulted in lipid metabolism disorder and obesity appeared in more and more people, also, the function of the male reproductive system had a certain negative affect by the abnormal lipid metabolism. Researches showed that people with obesity disorders, particularly male, had reproductive function reduced and testosterone levels decreased [15]. We found The level of serum testosterone was significantly lower in the group of abnormal lipid metabolism rats fed with high fat diet compared with the control group, which illustrated lipid metabolism inhibited the synthesis of testosterone. The western blot analysis showed that the expression levels of StAR and P450 scc of rat leydig cells in the group of abnormal

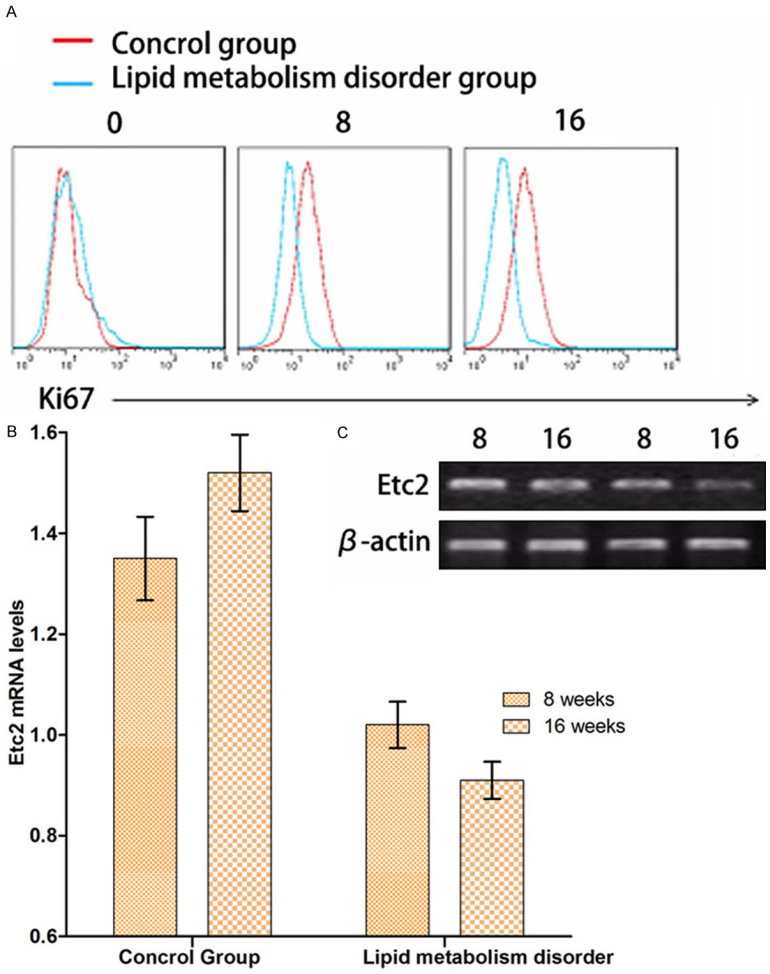

Figure 3. A: Flow cytometry showed that the expression of Ki67 was lower in the group of abnormal lipid metabolism rats fed with high fat diet compared with the control group; B, C: The level of Ect2 mRNA of rat leydig cells by RT-PCR.

lipid metabolism rats fed with high fat diet compared with the control group, which illustrated the influence of lipid metabolism disorder on the inhibition expression of the key enzymes in testosterone synthesis of rat leydig cells to reduce the biosynthesis of testosterones. The potential mechanisms might be reducing cholesterol to transport to the mitochondrial inner membrane.

We found that lipid metabolism disorder reduced the proliferation of rat leydig cells through detecting the expression of Ki67. The present study showed a significant decreasing trend of Ki67expression in the group of abnormal lipid metabolism rats fed with high fat diet compared with the control group. Epithelial cell transforming sequence 2 oncogene (Ect2) ECT2 is a guanine nucleotide exchange factor (GEF) for Rho family GTPase related to cytokinesis [16-18]. GEFs catalyze the exchange of GDP for GTP, thereby activating the Rho GTPases in signal transduction. ECT2 expression is dynamically controlled throughout the cell cycle. Upon breakdown of the nuclear envelope during mitosis [19], ECT2 is dispersed throughout the cytoplasm, then ECT2 becomes localized to the mitotic spindles during metaphase, the cleavage furrow during telophase, and the mid-body at the end of cytokinesis [20]. We suspected that Ect2 might be a critical protein regulating Leydig cell proliferation influenced by lipid metabolism disorder. The results of RT-PCR verified and showed that Ect2 mRNA of rat leydig cells was significantly lower in the group of abnormal lipid metabolism rats fed with high fat diet compared with the control group, which suggested that lipid metabolism disorder might affect rat leydig cell proliferation by regulating expression of Ect2.

This study had built the abnormal lipid metabolism rat model through high fat diet and enzymes associated with testosterone biosynthesis, such as StAR, P450c were inhibited and the expression of Ect2 decreased, which played a role in regulating cell proliferation. The different expression of some key proteins in the process of testosterone synthesis and proliferation of rat Leydig cells were analyzed. We also investigated the impact of lipid metabolism on the reproductive system and regulatory mechanisms, which provided a new therapeutic direction and ideas for the treatment of reproductive dysfunction caused by lipid metabolism disorder.

Disclosure of conflict of interest

None.

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