Effects of High-Glucose and High-Fat Condition on Estrogen Receptor- and Sexual Precocity-Related Genes in GT1-7 Cells

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Background: This study was designed to investigate the effect of high-glucose and high-fat condition on estrogen receptor- and sexual precocity-related genes in GT1-7 cells.

Material/Methods: In this study, CCK8 was used to detect cell viability, and TUNEL assay was used to detect apoptosis levels of GT1-7 cells after treatment with glucosamine and palmitate. The expression level of GnRH was measured by ELISA and RT-qPCR. RT-qPCR and Western blot were used to detect the expression of ERβ, CD36, and GPR54 in GT1-7 cells, and the expression of ERβ was detected using immunohistochemistry analysis. Finally, after adding the intervening drug tamoxifen to GT1-7 cells, the expression level of GnRH was measured by ELISA and Western blot analysis was used to detect the expression of GPR54 and GnRH.

Results: GnRH secretion in the high-fat and high-glucose group increased continuously over time and peaked at 18 h, and GnRH gene expression peaked at 12 h. High-fat and high-glucose conditions also significantly increased the levels of estrogen receptors β (ERβ), fatty acid translocase protein (CD36), and G Protein-Coupled Receptors 54 (GPR54) in GT1-7 cells. After estrogen receptors β (ER) was inhibited, GnRH secretion and GPR54 expression were decreased at 12 h and 18 h.

Conclusions: Our study demonstrates that high-glucose and high-fat conditions promote the secretion of GnRH and ER and the expression of genes related to sexual precocity in GT1-7 cells.

MeSH Keywords: Abdominal Fat • Estrogen Receptor beta • Glucose

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Background

Precocious puberty (PP) is a developmental disorder that occurs before the age of 8 years in girls and 9 years in boys, accompanied by accelerated growth and elevated sex hormones [1]. In recent years, abnormal sexual development of children, especially the incidence of sexual precocity, has increased significantly, and has become one of the most common endocrine diseases in children, seriously affecting their physical and mental health [2]. According to pathogenesis, precocity can be divided into central precocious puberty, peripheral precocious puberty, and partial precocious puberty [3]. Idiopathic central precocious puberty (ICPP) is due to the initiation of the hypothalamic-pituitary-gonadal axis, which eventually leads to an increase in estrogen levels in the body [4]. However, it is clinically found that not all children with precocious puberty have increased estrogen levels, which may be related to the gene-regulation mechanism of estrogen, in addition to physiological fluctuations. Researchers found that estrogen receptors (ER) are closely related to the development of puberty in children [5]. ER is an essential member of the steroid hormone receptor gene superfamily, including 2 subtypes – ERα and ERβ – which belong to the nuclear receptor family and mediate the genotype effect of estrogen [4]. Due to differences in sex and age, the distribution and expression of estrogen receptors in tissues differ, and the expression level and biological function of estrogen receptors are also different in different tissues [6]. In recent years, a growing number of studies have shown that there is a close relationship between estrogen receptor gene polymorphism and sexual precocity [7].

Obesity is considered to be an essential factor that causes ICPP by excessive intake of lipids and sugar, which creates a series of metabolic disorders, which further affects the central nervous system [4]. Although many studies have focused on some secretion products of fat cells (such as leptin) or abnormal lipid metabolism on feeding-center related factors and transmitters [8], few studies have assessed the effects of high-glucose and high-fat condition on GnRH neurons.

Material and Methods

Cell culture and reagents

The mouse GnRH-producing hypothalamic cell line GT1-7 cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS, HyClone; GE Healthcare, Logan, UT, USA) and 1% penicillin-streptomycin at 37°C with a humidified atmosphere of 5% CO₂, Glucose with purity >98.0%, palmitic, and intervening drugs tamoxifen were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The control group cells were cultured in DMEM complete medium (glu concentration 25 mM), the high-glucose group cells were cultured in medium with glucose added to a final concentration of 45 mM, the high-fat group cells were cultured in DMEM with 1 mM palmitic acid added, and the high-fat high-glucose group cells were cultured in DMEM plus glucosamine 45 mM + palmitate 1 mM.

Cell Counting Kit-8 (CCK-8) assay

The GT1-7 cells were plated in 96-well plates at a concentration of 1×10⁴ cells/ml per well. The cells were divided into a Con (control) group, Glu (glucose) group, Pal (palmitic acid) group, and Glu+Pal group. After treatment for 1, 6, 12, 18, 24, and 30 h, 10 μl CCK-8 solution was added to each well for 2 h. The absorbance of these wells was detected using a microplate reader at 450 nm.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed to test apoptosis of alveolar epithelial cells in accordance with the manufacturer’s instructions (Millipore, Sigma, USA).

Measurement of gonadotropin-releasing hormone (GnRH) in GT-17

The level of GnRH in GT-17 was detected by enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Xitang Biotechnology Co., Shanghai, China) following the manufacturer’s instructions.

Immunofluorescence staining

We washed the cells 3 times with pre-cooled PBS after pouring out the cell culture medium, followed by immobilization with 4% paraformaldehyde on ice for 15 min. After cells were washed with TBST 3 times for 5 min each time, they were treated with 0.3% Triton 100 for 15 min and incubated with primary antibody at 4°C overnight. Then, cells were incubated with secondary antibody for 1 h in the dark, stained with 0.1% DAPI for 6 min, washed with TBST 3 times and mounted 5 min later. Images were taken in a dark room with a laser scanning confocal microscope.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA samples were extracted by using Trizol reagent according to the manufacturer’s instructions. The synthesis of cDNA was carried out with the RevertAid™ First-Strand cDNA
Synthesis Kit. QRT-PCR was performed using the SYBR Green Master Mix I (TaKaRa, Otsu, Shiga, Japan) on the ABI 7900 Fast Real-Time PCR System (ABI, Foster City, CA, USA). The reaction conditions were: 10 min at 95°C, followed by 35 cycles of 15 s at 95°C and 40 s at 55°C. The primers used were: 

- ERβ, forward primer: 5'-CCCTGCTGTG ATGAATTACAG-3' and reverse primer: 5'-TCGGTTCCCACTAACCTTCC-3';
- CD36, forward primer: 5'-CATTGGTGATGAGAAGGCAAAC-3' and reverse primer: 5'-CACCACACCAACACTGAGTAA-3';
- GnRH, forward primer: 5'-CGAGAATTGTTGGAATGAAAGCC-3' and reverse primer: 5'-CACCACACCAACACTGAGTAA-3';
- GPR54, forward primer: 5'-TACATCCAGCAGGTCTCGGTG-3' and reverse primer: 5'-ACGTACCAGCGGTCCACACT-3';
- GAPDH, forward primer: 5'-CCCACTCCTCCACCTTTGAC-3' and reverse primer: 5'-TGTTGCTGTAGCCAAATTCGTT-3'.

Figure 1. Effects of high fat and high glucose on GT-17 cells. (A) CCK-8 assay measured the OD value at 450 nm of GT-17 cells in Control, Glu, Pal, and Glu+Pal groups at 1, 6, 12, 18, 24, and 30 h. (B) TUNEL assay detected apoptosis levels of GT-17 cells in control, Glu, Pal, and Glu+Pal group. Error bars indicate±SD. All experiments were performed in triplicate.
GAPDH was used as the housekeeping gene, and relative quantification was performed using the 2^(-ΔΔCT) method. The primers were designed and synthesized by Gemma (Shanghai, China).

Western blot assay

The cells were harvested and total proteins were obtained using RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein was separated on 10% SDS-PAGE gel and then transferred to PVDF membranes (EDM Millipore). The membranes were blocked with 5% non-fat milk for 2 h, followed by incubation with antibodies overnight for 4°C. Membranes were then incubated with secondary antibody for 2 h at room temperature. Proteins were detected using enhanced chemiluminescence and imaged. β-actin was used as an internal control. The primary antibodies used were: ERβ (1: 1000, # ab3576; Abcam, USA), D36 (1: 1000, # ab133625; Abcam, USA), GnRH (1: 1000, # ab189878; Abcam, USA), and GPR54 (1: 1000, # ab100896; Abcam, USA), and the secondary antibody was Goat Anti-Rabbit IgG H&L (HRP) (1: 10000, # ab205718; Abcam, USA).

Statistical analysis

All experiments were repeated at least 3 times, and data are expressed as the mean±standard deviation (SD). SPSS 17.0 software was used to conduct all statistical analyses (SPSS, Inc, USA). A one-way ANOVA followed by a Tukey’s or Dunnett’s test was performed using GraphPad Prism 5 software (GraphPad Software, Inc.) for statistical comparisons between groups. Differences with p<0.05 were considered significant.

Results

Effects of high fat and high glucose on GT-17 cells

Glucose and/or palmitic acid were added to GT1-7 cells and cultured for 1, 6, 12, 18, 24, and 30 h. CCK-8 assay was used to detect cell proliferation of GT1-7 cells. As shown in Figure 1A, high fat and high glucose had no significant effect on the proliferation of GT1-7 cells. To assess the effect of high fat and high glucose on apoptosis of GT-17 cells, TUNEL assay was used. As exhibited in Figure 1B, high fat and high glucose had no significant effect on cell apoptosis.

Effect of high fat and high glucose on GnRH secretion and expression of GT1-7 cells

To evaluate the effect of high fat and high glucose on GnRH in GT1-7 cells, the secretion levels of GnRH were measured using kits and the expression levels of GnRH in Control, Glu, Pal, and Glu+Pal group were measured by qPCR at 1, 6, 12, 18, 24, and 30 h. We found that high fat and high glucose increased the secretion of GnRH and peaked at 18 h (Figure 2A). The expression trend of GnRH mRNA was similar to that of GnRH secretion (Figure 2B). However, the peak of GnRH gene expression in the Glu+Pal group was earlier.

Effect of high fat and high glucose on ERβ, CD36, and GPR54 expression of GT1-7 cells

To further explore the possible regulatory mechanisms underlying estrogen receptor- and sexual precocity-related genes, the expressions of ERβ, CD36, and GPR54 were measured by Western blot analysis and RT-qPCR. As shown in Figure 3A–3C, high glucose and high palmitate up regulated ERβ, CD36 and GPR54 expression in GT1-7 cells. To determine the effect of high glucose and high fat on the nucelation of ERβ, we used...
immunofluorescence assay to detect the expression and distribution of ERβ at the peak times of 12 h and 18 h of high fat and high glucose GnRH expression, respectively. As shown in Figures 4 and 5, immunofluorescence brightness increased with high glucose and high palmitate at 12 h and 18 h compared with the control group, and the results indicated that high glucose and high fat can induce upregulation of ERβ expression in GT1-7 cells.

We added the drug tamoxifen to the Glu, Pal, and Glu+Pal groups under the original culture conditions to interfere with ER in GT1-7 cells. We found that the secretion of GnRH was significantly decreased in the Glu group and was increased in the Glu+Pal group at 12 h and 18 h (Figure 6A). As shown in Figure 6B, the expression of GnRH was the same as the secretion of GnRH, and the expression of GPR54 was significantly increased in the Pal and Glu+Pal groups at 12 h and 18 h.

**Discussion**

The incidence of sexual development disorders in children, especially precocious puberty, has increased significantly in recent years and has become one of the most common pediatric endocrine diseases [9,10], and idiopathic central precocious (idiopathic central precocious puberty, ICPP) is especially important.
Figure 4. Immunofluorescence staining showing the presence and localization of ERβ in the cytoplasm under the effect of high fat and high glucose at 12 h. The image magnification is 400×.

Figure 5. Immunofluorescence staining showing the presence and localization of ERβ in the cytoplasm under the effect of high fat and high glucose at 18 h. The image magnification is 400×.
The direct cause of ICPP is early initiation of the GnRH secretion pulse in the hypothalamus and the increase of the overall level, which activates the pituitary and downstream gonadal organs such as the ovaries and uterus, and makes the HPG axis reach the puberty state prematurely [11,12]. However, the mechanism underlying the abnormal increase in GnRH secretion requires elucidation and is the subject of current research.

With economic development, dietary structure has greatly changed. The over-nutrition caused by high-sugar and high-fat diet has become common. This kind of unhealthy diet can function of multiple human organs. Excessive intake of lipids and sugar causes a series of metabolic disorders, which further affects entire body. Therefore, high-sugar and high-fat diets are an important cause of ICPP.

The hypothalamus has many different functions of organs with complex nerve nuclei; therefore, research into the related mechanism involving GnRH neurons in the human body is very difficult. Most recent studies have used primitive hypothalamic neurons of newborn mice or GT1-7 cell line as an in vitro cell model. GT1-7 cells are influenced by Kiss-1/GPR54, ER, leptin receptor, melatonin receptor, GnRH secretion function, GnRH AMPK, and MAPK signal transduction pathway, and provide an ideal cell model. However, normal hypothalamic neurons only express ERβ and not ERα, while GT1-7 cells express both. Therefore, it is necessary to consider and exclude the effect of ERα on downstream proteins and factors on the experimental results in studies using the GT1-7 cell line. In the present study, we investigated the effect of high glucose and high fat on estrogen receptor- and sexual precocity-related genes in GT1-7 cells. Estrogen receptors play a key role in the main maintenance of secondary sexual characteristics and reproductive cycles in females and affects fertility [13]. Estrogen and its receptors regulate GnRH synthesis and release it by directly or indirectly acting on GnRH neurons in the hypothalamus, and then regulate the whole reproductive system [14,15]. GT1-7 cells can express the morphology of primitive GnRH neurons, gene GnRH, KISS-1, and GPR54, and can pulse-release GnRH.

Figure 6. Effect of high fat and high glucose on GnRH secretion of GT1-7 cells. (A) Effect of high fat and high glucose on GnRH secretion of GT1-7 cell at 12 and 18 h were detected by ELISA. (B) Western blot assays were performed to measure the expression levels of GPR54 and GnRH in control, Glu, Pal, and Glu+Pal groups at 12 and 18 h. Error bars indicate±SD. * p<0.05; ** p<0.01; *** p<0.001 vs. control group. All experiments were performed in triplicate.
Our study demonstrates that high fat and high glucose have no significant effect on the proliferation and apoptosis of GT1-7 cells; therefore, we chose GT1-7 as the cell model for subsequent experiments. Through a series of in vitro experiments, we found that high fat and high glucose increased the secretion of GnRH and peaked at 18 h, the expression trend of GnRH mRNA was similar to that of GnRH secretion, and the peak of GnRH gene expression in the high fat and high glucose group was earlier.

In recent years, ERβ has been proved to exist in ovarian, kidney, brain, colon, and other tissues and to participate in a variety of physiological and pathological activities. Previous studies have focused more on the relationship between ERβ and prostate cancer, breast cancer, colon cancer, and cardiovascular and cerebrovascular diseases. Recent studies have shown that the use of the ERβ gene is promising in treatment of tendon disease, asthma, obesity, and other diseases. The physiological function of ERβ often involves multiple targets, and it is very complicated, being involved in the etiology, pathogenesis, and interrelationships among various systems in many diseases. We found that high glucose and high palmitate can induce the upregulation of ERβ, CD36, and GPR54 expression in GT1-7 cells. To determine the effect of high glucose and high fat on the nucleation of ERβ, we used immunofluorescence assay to detect the expression and distribution of ERβ at the peak times of 12 h and 18 h of high fat and high glucose GnRH expression, respectively. The results indicated that the high glucose and high fat can induce the upregulation of ERβ expression in GT1-7 cells.

GPR54 (GPR54) protein is the receptor of kisspeptin, which is encoded by the KISS-1 gene. Kisspeptin can promote the release of GnRH [16] and exert its biological activity through GPR54 [17]. Studies have found that the expression of KISS-1 and GPR54 mRNA in the hypothalamus before and after sexual maturity is significantly increased compared to in infancy [18,19]. Continuous injections of Kisspeptin-10 into primates and rats at the end of the juvenile stage can trigger the same GnRH secretion in advance as at the beginning of puberty [20–22]. KISS-1 and its receptor, GPR54, have an indispensable role in the initiation of puberty and reproduction [23]. In the present study, high glucose and high palmitate were shown to induce the upregulation of ERβ, CD36, and GPR54 expression in GT1-7 cells. After ER was inhibited, GnRH and GPR54 expression were increased under high-glucose and high-fat condition.

Conclusions

Our study demonstrates that high-glucose and high-fat condition promotes the secretion of GnRH and ER and the expression of genes related to sexual precocity in GT1-7 cells.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

None.

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