LKB1 alleviates high glucose- and high fat-induced inflammation and the expression of GnRH and sexual precocity-related genes, in mouse hypothalamic cells by activating the AMPK/FOXO1 signaling pathway

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Abstract. Precocious puberty (PP) is a developmental disorder. Hypothalamic cells can produce gonadotropin-releasing hormone (GnRH), the final output of neuroendocrine regulation that occurs during puberty. The aim of the present study was to investigate the role of lKB1 (LKB1), also known as serine/threonine kinase, in the progression of PP and identify the underlying mechanisms. First, the levels of LKB1 in peripheral blood and peripheral blood mononuclear cells of children with PP were detected by reverse transcription-quantitative (rT-q) PCR or western blotting. After the GT1-7 mouse hypothalamus cell line was treated with high glucose (HG) and high fat (HF), the expression of LKB1 and GnRH was tested. LKB1 was overexpressed by transfection with a pcDNA3.1 plasmid and the levels of inflammatory factors, GnRH, PP-related factors and proteins in the AMP-activated protein kinase (AMPK)/forkhead box protein O1 (FOXO1) pathway were determined using RT-qPCR or western blot analysis. Subsequently, Compound C, an inhibitor of AMPK/FOXO1 signaling, was used to clarify whether the effects of LKB1 on PP were mediated by the regulation of this pathway. Results indicated that children with PP exhibited a lower LKB1 expression. In addition, HG and HF culture resulted in an enhanced GnRH expression and a reduced LKB1 expression in GT1-7 cells. LKB1 overexpression inhibited the contents of TNF-α, IL-6 and GnRH in in GT1-7 cells exposed to HG and HF and reduced the expression of PP-related proteins, including estrogen receptor-β, cluster of differentiation 36 and G-protein-coupled receptor. In addition, the expression of phosphorylated (p)-AMPK and p-FOXO1 was markedly downregulated following LKB1 overexpression. Furthermore, compound C intervention partially diminished the inhibitory effects of LKB1-mediated upregulation on the levels of inflammation and PP-related factors. In conclusion, these results demonstrated that LKB1 alleviated HG- and HF-induced inflammation, as well as the expression of GnRH and sexual precocity-related genes, in GT1-7 cells by activating the AMPK/FOXO1 signaling pathway.

Introduction

Precocious puberty (PP) is one of the most common pediatric endocrine diseases defined as the development of pubertal changes before the age of 8 years in girls and 9 years in boys, coupled with accelerated growth and elevated levels of sex hormones (1). Reproduction and puberty onset are complex biological processes that involve numerous factors controlled by the hypothalamus-pituitary-gonadal (HPG) axis (2,3). Hypothalamic cells can produce gonadotropin-releasing hormone (GnRH), the final output of neuroendocrine regulation that occurs during puberty, which is released to stimulate the secretion of gonadotropins from the pituitary to then act on the gonads (4). Due to the early activation of the HPG axis in children, sex hormones reach puberty levels early, which consumes the proliferative capacity of the epiphyseal cartilage plate in advance, resulting in a reduced final height and premature presentation of the secondary sexual characteristics, which may cause psychological and mental problems (5).
Therefore, PP has received considerable attention within the medical field and from the public.

Obesity is considered to be a crucial factor that triggers idiopathic central PP (ICPP) and produces the excessive intake of lipids and sugar, which leads to multiple metabolic disorders and further affects the central nervous system (6). Compelling evidence indicates that high levels of sugar and fat can regulate the expression of estrogen receptor (ER) and PP-related genes in hypothalamic cells (7). Live kinase B1 (LKB1), also known as serine/threonine kinase 11 (STK11), is a serine-threonine kinase that participates in several cellular functions, including growth, cell energy metabolism, polarity and tumor formation (8). In recent years, LKB1 has been reported to be associated with obesity and LKB1 knockout in the hypothalamus has been shown to intensify susceptibility to obesity in mice administered with a high-fat (HF) diet, accompanied by a deterioration of hypothalamic inflammation and downregulation of neuronal expression (9). In the endometrial glands, LKB1 can promote the phosphorylation of AMP-activated protein kinase (AMPK), thereby increasing the activity of forkhead box protein O1 (FOXO1) (10). A clearly reduced release of GnRH is observed following the elevation of FOXO1 activity in the hypothalamus (11). Therefore, the effects of LKB1 on PP and whether LKB1 can regulate the AMPK/FOXO1 pathway, prompted the current study.

In the present study, LKB1 level was detected in the peripheral blood of children with PP. Then, GT1-7 mouse hypothalamic cell line was exposed to high glucose (HG) and HF conditions to stimulate a PP in vitro model, in order to explore the roles of LKB1 in the progression of PP and its regulatory effects on the AMPK/FOXO1 signaling pathway.

Materials and methods

Sample collection. The peripheral blood samples (5 ml) of healthy children (n=25) and PP children with ICPP (n=25) were collected from the Fujian Maternity and Child Health Hospital (Fuzhou, China). All patients were female (age, 5-8 years) recruited between April 2020 and August 2020. The diagnostic criteria used were consistent with the previous study (12). The inclusion criteria were as follows: Patients diagnosed with ICPP who had been treated with GnRHa with a follow-up ≥3 months and complete clinical data. Patients with precocious puberty due to tumor, organic or endocrine disease, simple breast precocity, rare syndromes, contraceptical pill abuse or other exogenous hormones were excluded. Patients with poor quality ultrasound images or incomplete clinical information were also excluded. Informed written consent was obtained from parents or guardians. This study was approved by the Ethics Committee of Fujian Maternity and Child Health Hospital (Fuzhou, China).

Cell culture. The GT1-7 mouse hypothalamic cell line was purchased from BLUEFIO Life Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; MilliporeSigma) containing 10% fetal bovine serum (FBS; Cytiva) under humidified conditions at 37°C in a 5% CO₂-containing atmosphere. Cells in the control group were maintained in complete DMEM (glucose concentration, 25 mM). The HG and HF group cells were cultured in DMEM plus 45 mM glucose + 1 mM palmitate according to the previous study (7). Following incubation for 12 h, cells were collected for subsequent experiments.

Cell transfection. pcDNA 3.1 plasmid containing LKB1 [overexpression (Oe)-LKB1; 4 µg] or empty vectors [Oe-negative control (NC); 4 µg] was synthesized by Shanghai GenePharma Co., Ltd. GT1-7 cells were inoculated at a density of 2x10⁵ cells/well in 6-well plates and cultured at 37°C until they reached 80% confluence. Transfection was then performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 48 h in a 5% CO₂-containing atmosphere, according to the manufacturer's instructions. The effect of LKB1 overexpression was validated by reverse transcription-quantitative (RT-q) PCR and western blot analysis. The transfected cells were used for subsequent experiments at 48 h after transfection.

RT-qPCR. Total RNA was extracted from 5x10⁶ GT1-7 cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse-transcribed into complementary DNA (cDNA) using the PrimeScript Strand cDNA synthesis kit (Takara Biotechnology Co., Ltd.) at 42°C for 30 min according to the manufacturer's protocol. Subsequently, using cDNA as the template, the gene expression levels were analyzed using RT-qPCR, which was conducted with an iTaq Universal One-Step iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) on an ABI 7500 instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The experiments were independently replicated ≥3 times. The following thermocycling conditions were used: Initial denaturation at 95°C for 7 min; and 40 cycles of 95°C for 15 sec and 60°C for 30 sec; and a final extension at 72°C for 30 sec. The primers used in this study were designed and synthesized by Sangon Biotech Co., Ltd. The sequences were as follows: LKB1 (human) forward, 5'-GAAGTTGGCTCTCCAGGT-3' and reverse, 5'-CGGCAAGTATGAAACAGCGC-3'; LKB1 (mouse) forward, 5'-GGGGCGACAGGACAAAGTGG-3' and reverse, 5'-CTTGACGTGGCTGCTCCAT-3'; IL-6 forward, 5'-TCCGGAGAGGAGACTTACCA-3' and reverse, 5'-TAACGCACTAGGTTGGCCA-3'; TNF-α forward, 5'-CAGCAGCATTGGTTTATCCTAT-3' and reverse, 5'-GGGGCTTCGAGGAGTAGACA-3'; GnRH forward, 5'-AGCAGCAGTCTCCTATGGTTG-3' and reverse, 5'-GGGTTTATGCGTCCCATTGGATCCA-3'; GAPDH was used as a reference gene. Gene expression levels were quantified according to the 2⁻ΔΔcq method (13).

Isolation of peripheral blood mononuclear cells (PBMCs) from normal and prematurity groups. PBMCs were collected from the peripheral blood samples of healthy children and PP children with ICPP. The blood was treated with ethylene-diaminetetraacetic acid anti-coagulant, diluted with twice the volume of phosphate-buffered saline (PBS) and mixed well (14). The cell suspension was added with caution to the lymphocyte separation liquid (dakewe Biotech Co., Ltd.) equal in volume to the blood and centrifuged horizontally at 500 x g at room temperature for 20 min. The PBMCs at the
juncation of the plasma layer and the lymphocyte separation liquid were aspirated, added with the equal amount of PBS, mixed well and centrifuged at 500 g for 10 min at 20°C. After discarding the supernatant, the cells were washed twice to remove the residual lymphocyte separation liquid.

**Western blot analysis.** For immunoblotting, cells were collected and lysed with RIPA buffer (Wuhan Boster Biological Technology, Ltd.). Protein concentration was detected using a bicinchoninic acid (BCA) kit (Beyotime Institute of Biotechnology). Normalized volumes of samples (40 μg protein per lane) were isolated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were subsequently blocked with 5% non-fat milk at room temperature for 1.5 h, prior to incubation with primary antibodies for the target proteins at 4°C overnight. The horseradish peroxidase (HRP)-labeled secondary antibody (cat. no. 7074P2; 1:5,000; Cell Signaling Technology, Inc.) was added for 1 h at room temperature. Protein bands were scanned and visualized using an enhanced chemiluminescence detection system (MilliporeSigma). The intensities of the protein bands were quantified using ImageJ software v1.8.0 (National Institutes of Health) and the gray value of the target protein was normalized to that of GAPDH. Anti-LKB1 (cat. no. 13031T; 1:1,000), anti-IL-6 (cat. no. 12912T; 1:1,000), anti-TNF-α (cat. no. 11948T; 1:1,000), anti-CD36 (cat. no. 74002S; 1:1,000), anti-phosphorylated (p)-AMPK (cat. no. 2535T; 1:1,000), anti-AMPK (cat. no. 5831T; 1:1,000), anti-estrogen receptor-β (ERβ; ab196787; 1:1,000) and anti-G-protein-coupled receptor (GPR54; cat. no. ab100896: 1:1,000) antibodies were provided by Abcam.

**Statistical analysis.** All experiments were repeated independently in triplicate. All data are expressed as the mean ± standard deviation (SD) for each group. Statistical analysis was performed with GraphPad Prism (version 8.0; GraphPad Software, Inc.). Contrastive analysis of the measurement data in multiple groups was performed applying one-way analysis of variance (ANOVA) followed by Turkey’s post hoc test, while the data in two groups was compared by unpaired Student's t test. *P*<0.05 was considered to indicate a statistically significant difference.

**Results**

**LKB1 expression is markedly downregulated in the peripheral blood of children with PP and HG- and HF-induced GT1-7 cells.** LKB1 expression in the peripheral blood of children with PP was measured using RT-qPCR. As shown in Fig. 1A, LKB1 expression was significantly reduced in the PP group compared with the normal group. Additionally, LKB1 protein expression in PBMCs was tested by western blot analysis and marked downregulation in LKB1 expression was observed in the PP group compared with the normal group (Fig. 1B). Next, GT1-7 cells were exposed to HG and HF to simulate the PP model *in vitro* and the expression of GnRH and LKB1 was examined. It was found that HF and HG induction led to a marked increase in GnRH expression in the PP group compared with the control group (Fig. 1C). Furthermore, the mRNA and protein expression levels of LKB1 were markedly decreased in the model group compared with the untreated control group (Fig. 1D and E). In conclusion, an abnormal LKB1 expression may be associated with PP.

**LKB1 overexpression alleviates inflammatory response in HG- and HF-induced GT1-7 cells.** To explore the effects of LKB1 on the inflammatory response in HG combined with HF-induced GT1-7 cells, LKB1 was overexpressed through LKB1-overexpressing vector transfection. It was observed that LKB1 mRNA and protein levels were clearly elevated in the Model + Oe-LKB1 group, when compared with the Control group (Fig. 2A and B). Subsequently, the RT-qPCR and western blot analysis (Fig. 2C and D) indicated that the IL-6 and TNF-α levels were markedly increased in GT1-7 cells compared with cells in the control group, but were decreased following further LKB1 overexpression. These findings suggested that LKB1 overexpression alleviates HG- and HF-induced inflammation in GT1-7 cells.

**LKB1 overexpression attenuates GnRH and PP-related protein expression in HG- and HF-induced GT1-7 cells.** In order to assess the function of LKB1 overexpression on GnRH, RT-qPCR was used to determine GnRH expression in GT1-7 cells following HG and HF exposure. As shown in Fig. 3A, LKB1 upregulation reduced GnRH levels compared with the Model + Oe-NC group. In addition, ERβ, CD36 and GPR54 expression was clearly decreased following LKB1 overexpression in GT1-7 cells under HG and HF exposure, compared with cells in the negative control group (Fig. 3B and C). These results suggested that LKB1 overexpression inhibited GnRH and PP-related protein expression in HG combined with HF-induced GT1-7 cells.

**LKB1 overexpression suppresses the AMPK/FOXO1 signaling pathway in HG- and HF-induced GT1-7 cells.** To investigate the potential mechanism of LKB1 in the regulation of HG- and HF-induced GT1-7 cells, the expression of AMPK/FOXO1 signaling proteins was examined using western blot analysis. A significant decrease in p-AMPK and p-FOXO1 expression was observed in the model group compared with the control group, while further LKB1 overexpression elevated both the p-AMPK and p-FOXO1 expression compared with the Model + Oe-NC group (Fig. 4). Collectively, these data provided evidence that LKB1 overexpression restrained the AMPK/FOXO1 signaling pathway in HG- and HF-induced GT1-7 cells.

**AMPK/FOXO1 signaling inactivation reverses the impact of LKB1 overexpression on inflammation and GnRH expression in HG- and HF-induced GT1-7 cells.** AMPK/FOXO1 signaling was inhibited by treatment with AMPK inhibitor compound C to examine the regulatory effect of LKB1 on this pathway in HG- and HF-induced GT1-7 cells. As shown in Fig. 5A and B, Compound C addition elevated the mRNA and protein expression level of IL-6 and TNF-α in HG- and HF-induced GT1-7 cells compared with the Model + Oe-LKB1
group. In addition, a partial increase in GnRH expression was observed in the Model + oe-LKB1 + compound C group compared with the corresponding control group (Fig. 5C). In addition, ERβ, CD36 and GPR54 expression was notably upregulated in HG- and HF-induced GT1-7 cells with LKB1 overexpression and compound C treatment, compared with the LKB1 overexpression-only group (Fig. 5D). These findings revealed that LKB1 overexpression suppresses HG- and HF-induced inflammation and GnRH expression in GT1-7 cells by activating AMPK/FOXO1 signaling.

Discussion

PP is one of the most common endocrine diseases in children; it is characterized by an early onset of puberty and has a far-reaching influence on children’s growth, development and mental health. ICPP is caused by the premature activation of the HPG axis (15). Obesity caused by excessive intake of lipids and sugars is considered to play an important role in the occurrence of ICPP (6). ICPP can lead to metabolic abnormalities and affect the central nervous system. It has been shown that a HG and HF diet can affect the expression of ER- and PP-related genes in hypothalamic cells (7). In the present study, a HG- and HF-induced GT1-7 mouse hypothalamic cell line was used as a PP in vitro model to simulate the physiological environment of PP in the body, as previously described (7). The role of LKB1 in the progression of PP in this in vitro model was also explored. It was demonstrated that LKB1 could alleviate HG- and HF-induced inflammation and GnRH expression in mouse hypothalamic cells through the activation of the AMPK/FOXO1 signaling pathway.

LKB1, also known as STK11, is a serine/threonine kinase that is widely expressed in mammalian tissues (16,17). A recent study demonstrated that LKB1 is closely associated with obesity and LKB1 deletion in the hypothalamus intensifies susceptibility to obesity in mice administered with a HF diet, accompanied by a deterioration of hypothalamic inflammation and decreased neuronal expression (9). Wu et al (18) also report LKB1 as a novel potential therapeutic target, due to its significant suppressing effects on hypothalamic inflammation and alleviating effects on diet-induced obesity in mice.
as a key regulator of energy metabolism, an intraventricular injection of LKB1 in rats induced by diet is found to suppress the occurrence of obesity through the activation of the AMPK-proopiomelanocortin neuronal axis (19). The present study found that LKB1 expression was significantly decreased in the peripheral blood and PBMCs of children with PP, as well as HG- and HF-induced mouse hypothalamic cells, suggesting that the abnormal LKB1 expression may be associated with PP, an obesity-related disease.

A number of studies have confirmed that LKB1 serves a crucial anti-inflammatory role in multiple diseases. For instance, activating LKB1 relieves thioacetamide-induced hepatic fibrosis and inflammation in mice (20). Chen et al (20) demonstrate that LKB1 contributes to a decreased inflammatory state in skeletal muscle by suppressing the expression of inflammation-related genes, including IL-6 and TNF-α. Another previous study suggests that LKB1 upregulation reduces the production of inflammatory cytokines in macrophages infected with mycobacterium tuberculosis (21). Notably, LKB1 elevation ameliorates hypothalamic inflammation and relieves diet-induced obesity in mice (18). In the present study, LKB1 overexpression markedly decreased the HG- and HF-induced elevation of IL-6 and TNF-α expression. Furthermore, ERs exert strong effects on the maintenance of female secondary sexual characteristics and reproductive cycles and affect fertility (22). Estrogen and its receptors adjust the synthesis and release of GnRH by acting on GnRH neurons in the hypothalamus, thereby regulating the entire reproductive system (23). ERβ, CD36 and GPR54 are important ER- and sexual precocity-related genes, which have been shown to be expressed in GT1-7 cells (7). The present study showed that the expression of ERβ, CD36 and GPR54 was

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clearly decreased following LKB1 overexpression in GT1-7 cells under HG and HF conditions, compared with the control group, which was in line with a previous study performed by Wang et al (7).

The cellular functions of LKB1 are considered to be achieved through the phosphorylation of a MPK (19). As the upstream kinase capable of AMPK, LKB1 can activate AMPK through the phosphorylation of p-AMPKα of its catalytic α subunit (24). In the endometrial glands, LKB1 can promote the phosphorylation of AMPK, thereby increasing the activity of FOXO1 (10). A clear reduction in GnRH release is observed following the elevation in the FOXO1 activity in the
hypothesize (11). The results of the present study suggested that LKB1 gain-of-function increased the expression of p-AMPK and p-FOXO1 in HG- and HF-induced GT1-7 cells. In order to explore whether LKB1 could regulate inflammation, as well as the expression of GnRH and sexual precocity-related genes by activating AMPK/FOXO1 signaling, compound C, an inhibitor of AMPK/FOXO1 signaling, was used to treat GT1-7 cells. It was found that the use of compound C suppressed the effect of LKB1 overexpression on inflammation, GnRH and sexual precocity-related gene expression.

In conclusion, LKB1 suppressed the inflammation, GnRH and sexual precocity-related gene expression by inactivating AMPK/FOXO1 signaling in HG- and HF-induced GT1-7 cells. LKB1 may serve as an effective biomarker for PP and therefore a novel target for PP treatment. The lack of the upstream mechanism of LKB1 and the animal study are limitations of the present study, therefore, comprehensive analysis is required in the future.

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

Authors' contributions
HL, LG and QZ designed the study and performed the experiments. HL and QZ drafted and revised the manuscript. LG,
HH and LX analyzed the data. HH and LX performed the literature search. All authors have read and approved the final manuscript. HL and LX confirm the authenticity of all the raw data.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Fujian Maternity and Child Health Hospital (approval number is 2020KY043).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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