

TNF- α promotes insulin resistance in obstructive sleep apnea-hypopnea syndrome

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Abstract. Obstructive sleep apnea hypopnea syndrome (OSAHS) is the most serious among children with sleep disordered breathing. The present study aimed to investigate whether TNF- α could decrease the glucose transporter type 4 insulin-responsive (GLUT-4) expression to promote insulin resistance through the TNF- α /IKK β /IK β /NF- κ B signaling pathway in OSAHS. In total, 30 obese children with OSAHS and 30 non-OSAHS obese children were enrolled into the present study. TNF- α expression in adenoid tissues was detected by western blot analysis and immunohistochemistry. The expression of inflammatory factors (IL-1 β , IL-6 and IFN- γ) and TNF- α /IKK β /IK β /NF- κ B signaling pathway-associated proteins was also detected by western blot analysis. The expression of insulin resistance-associated factors, insulin receptor substrate 1 (IRS1) and GLUT4, was determined by western blot analysis and immunohistochemistry. TNF- α expression was increased in adenoid tissues of children with OSAHS, which was also confirmed by immunohistochemistry. The expression levels of IL-1 β , IL-6 and IFN- γ were all upregulated in adenoid tissues of children with OSAHS. The expression of IRS1 and GLUT4 was decreased in adenoid tissues of obese children with OSAHS and the result of immunohistochemistry was consistent with the result of western blot analysis. The protein level of TNF- α , and ratio of phosphorylated (p-)/total (t)-IKK β , p/t-IK β and p/t-NF- κ B was increased in adenoid tissues of children with OSAHS. TNF- α could suppress the GLUT4 expression to promote insulin resistance by TNF- α /IKK β /IK β /NF- κ B signaling pathway in OSAHS.

Introduction

Obstructive sleep apnea-hypopnea syndrome (OSAHS) is characterized by partial or total upper airway obstruction during sleep, with different degrees of blood oxygen saturation, hypercapnia and hypoxemia (1). Adenoid hypertrophy is one of the most common causes of OSAHS in children (2). According to an epidemiological investigation, the incidence of children with OSAHS is 1.2-5.7%, whilst 50% children with OSAHS exhibit moderate and severe symptoms in China (3). The incidence of OSAHS among obese children is two times greater compared with healthy weight children, and OSAHS in obese children is accompanied by abnormal lipid metabolism, with abnormal high islet glucose tolerance and hypertension, which are referred to as the insulin resistance syndrome (4). The association between OSAHS and insulin resistance has been confirmed by observational and experimental studies, which demonstrate that OSAHS is an independent risk factor for insulin resistance in children (3,5,6). Adenoid hypertrophy is an important risk factor for OSAHS in children and the severity of OSAHS is positively associated with the severity of adenoid (2). Adenoid hypertrophy can result in the downregulation of the expression of insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding protein-3, and inhibit the growth and development of children (7). IGF-1 can ameliorate insulin resistance by promoting glucose uptake and insulin secretion (8,9). Therefore, the present study hypothesized that there might be an association between adenoid hypertrophy and insulin resistance.

Several studies have demonstrated that inflammatory response markers play an important role in glucose tolerance and insulin resistance, including TNF- α and IL-6. TNF- α induces insulin resistance by mediating serine phosphorylation of the insulin receptor substrate, which becomes a tyrosine kinase inhibitor for the insulin receptor (10). Furthermore, TNF- α inhibits the insulin-stimulated glucose transport by downregulating the expression of the glucose transporter type 4 insulin-responsive (GLUT-4) (11). TNF- α can also promote the decomposition of adipose tissue and the release of free fatty acids to regulate glucose metabolism, which indirectly leads to insulin resistance (12). Clinical studies have demonstrated that patients with sleep apnea have higher plasma levels of IL-6 and TNF- α compared with healthy individuals (13,14).

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Hotamisligil *et al* (11) reported that TNF- α is an important intermediate factor that induces insulin resistance and metabolic syndrome. In addition, TNF- α has the ability to impair insulin signaling, and inhibition of TNF- α increases insulin sensitivity (15,16). Under physiological conditions, NF- κ B binds to IK β , which is present in the cytoplasm without activity (17). However, phosphorylation of IKK β caused by external stimulation results in phosphorylation of IK β , and, following dissociation of NF- κ B, IK β enters the nucleus to regulate the secretion and expression of inflammatory factors (18). Previous studies have demonstrated that obesity and a high-fat diet lead to fat deposits in the liver that activate the IKK β /NF- κ B pathway, which induces insulin resistance (19,20).

The present study aimed to investigate TNF- α expression in adenoid tissues of obese children with OSAHS to determine how TNF- α regulates insulin resistance via the TNF- α /IKK β /IK β /NF- κ B pathway in OSAHS.

Materials and methods

Human specimens. The present study enrolled 30 obese children with OSAHS (8-12 years old; mean age, 10.13 \pm 1.65 years; 15 male/15 female) and 30 non-OSAHS obese children (8-12 years; mean age, 10.38 \pm 1.24 years; 15 male/15 female) from the Nantong First People's Hospital (Jiangsu, China) between March 2018 and October 2019. The obesity criteria was referring to the 'Screening for overweight and obesity in school-age children and adolescents' (WS/T 586-2018). Patient inclusion criteria were as follows: i) One or more of the following clinical histories of sleep: Mouth breathing, snoring, frequent waking, suffocating and enuresis; ii) hypertrophy of the adenoids resulting in oropharyngeal and/or nasopharyngeal stenosis; and iii) overnight polysomnography monitoring performed within 1-2 weeks before surgery, with the results meeting the diagnostic standard of OSAHS (21). The adenoid tissue samples obtained from biopsy and plasma were stored at -80°C until subsequent experimentation. The present study was approved by the Ethics Committee of Nantong First People's Hospital (grant no. 20180209NT) and informed consent was provided by the patients or their family members.

Western blotting. Total protein was extracted from adenoid tissues with cell lysis buffer (Beyotime Institute of Biotechnology) and quantified using the BCA protein analysis kit (cat. no. ab102536; Abcam). Protein samples (50 μ g/well) were separated by 10% SDS-PAGE, transferred onto PVDF membranes and subsequently blocked with 5% skim milk powder at room temperature for 2 h. The membranes were incubated with primary antibodies against: TNF- α (cat. no. ab215188; dilution, 1:1,000; Abcam), IL-1 β (cat. no. ab216995; dilution, 1:1,000; Abcam), IL-6 (cat. no. ab233706; dilution, 1:1,000; Abcam), IFN- γ (cat. no. 8455; dilution, 1:1,000; Cell Signaling Technology, Inc.), insulin receptor substrate 1 (IRS1; cat. no. ab40777; dilution, 1:1,000; Abcam), GLUT4 (cat. no. ab216661; dilution, 1:1,000; Abcam), IKK β (cat. no. ab124957; dilution, 1:1,000; Abcam), phosphorylated (p)-IKK β (cat. no. 5441R; dilution, 1:500; Shanghai YaJi Biological Technology Co., Ltd.; https://china.guidechem.com/trade/pdetail22435375.html#f_2), IK β

(cat. no. ab109509; dilution, 1:1,000; Abcam), p-IK β (cat. no. HK6658, dilution, 1:500, Shanghai Hushi Pharmaceutical Technology Co., Ltd.), NF- κ B (cat. no. 8242; dilution, 1:1,000; Cell Signaling Technology, Inc.), p-NF- κ B (cat. no. HK5704; dilution, 1:500; Shanghai Hushi Pharmaceutical Technology Co., Ltd.) and GAPDH (cat. no. ab8245; dilution, 1:1,000; Abcam), overnight at 4°C. Following the primary antibody incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (cat. no. 7074; 1:1,000; Cell Signaling Technology, Inc.) for 1-2 h at room temperature. Protein bands were visualized using ECL reagent (EMD Millipore) and Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.) was used for densitometry analysis. Protein expression was presented as the ratio of the absorbance value of the targeted protein to the internal reference absorbance value of the internal control.

Reverse transcription-quantitative PCR (RT-qPCR). The adenoid tissues obtained from obese children with OSAHS and control obese subjects were treated with 1 ml Trizol (Thermo Fisher Scientific, Inc.) for lysis to extract the total RNA. RNA level and purity were determined using an ultra-micro ultraviolet spectrophotometer. cDNA was synthesized by reverse transcription using total RNA as template with Transcriptor First Strand cDNA Synthesis kit (Roche Molecular Systems, Inc.) at 42°C for 30 min and 85°C for 5 min. PCR amplification was performed using cDNA as template and GAPDH as internal reference. The thermocycling conditions used for the qPCR were as follows: Initial denaturation at 95°C for 30 sec; 95°C for 20 sec, 60°C for 30 sec and 72°C for 40 sec for 35 cycles. The following primer pairs were used for the qPCR: TNF- α forward, 5'-ACTTTAAGGGTTACCTGGGTTG-3' and reverse, 5'-TCACATGCGCCTTGATGTCTG-3'; IRS1 forward, 5'-TGTCACCCAGTGTAGTTGCTC-3' and reverse, 5'-CTCTCAACAGGAGGT TGGCATG-3'; GLUT4 forward, 5'-GCCCCGAAAGAGTCTAAAG-3' and reverse, 5'-AGAGCCACGGTCATCAAG-3' and GAPDH forward, 5'-GATGCTGGTGCTGAGTATGTCG-3' and reverse, 5'-TGGTGCAGGATGCATTGCTGA-3'. The expression levels of TNF- α , IRS1 and GLUT4 were determined using the SYBR-Green Realtime PCR kit (Beyotime Institute of Biotechnology) and calculated using the $2^{-\Delta\Delta C_q}$ method (22).

ELISA assay. Plasma samples obtained as aforementioned were taken from the -80°C refrigerator and thawed at room temperature. According to the manual of ELISA kits, the levels of TNF- α , IL-1 β , IL-6 and IFN- γ in plasma of obese children with OSAHS and control obese subjects were detected by human TNF- α , IL-1 β , IL-6 IFN- γ ELISA kit, respectively.

Immunohistochemistry. Adenoid tissues were fixed in 4% paraformaldehyde for 48 h at room temperature, dehydrated and embedded in paraffin. Paraffin-embedded tissue samples were cut into 3- μ m-thick sections, dewaxed in ethyl alcohol solution and washed in xylene solution. Tissue sections were incubated with 3% hydrogen peroxide for 10 min at room temperature to inhibit endogenous peroxidase activity, and antigen retrieval was subsequently performed in EDTA buffer at 75°C. Tissue sections were blocked with goat serum (Beyotime Institute

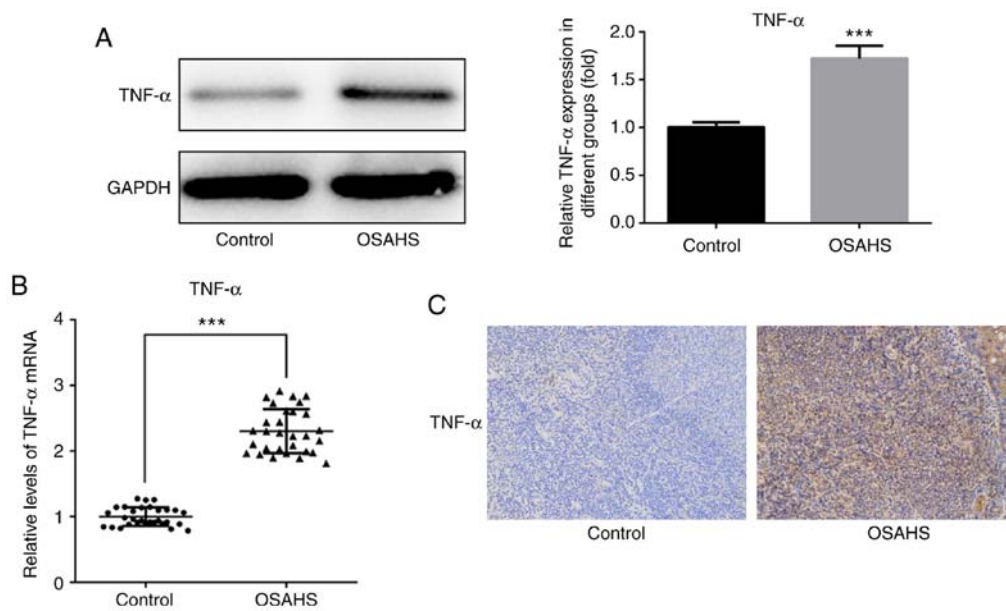


Figure 1. The expression of TNF- α in adenoid tissues. (A) Protein expression of TNF- α in adenoid tissues of obese children with OSAHS and control obese subjects was analyzed by western blot analysis. (B) mRNA expression of TNF- α in adenoid tissues of obese children with OSAHS and control obese subjects was detected by reverse transcription-quantitative PCR analysis. *** $P < 0.001$ vs. control group. (C) Expression of TNF- α in adenoid tissues of obese children with OSAHS and control obese subjects was determined by immunohistochemistry. OSAHS, obstructive sleep apnea hypopnea syndrome.

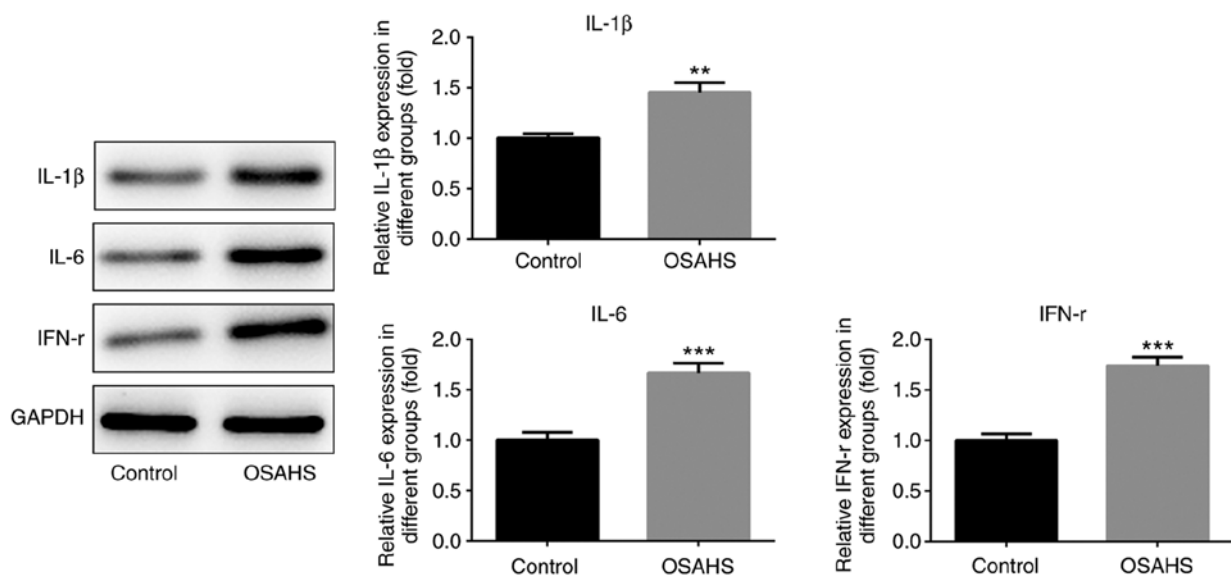


Figure 2. Expression of inflammatory factors, IL-1 β , IL-6 and IFN- γ , in adenoid tissues. The expression of IL-1 β , IL-6 and IFN- γ in adenoid tissues of obese children with OSAHS and control obese subjects was analyzed by western blot analysis. ** $P < 0.01$ and *** $P < 0.001$ vs. control group. OSAHS, obstructive sleep apnea hypopnea syndrome.

of Biotechnology) for 1 h at room temperature and incubated with primary antibodies against TNF- α (cat. no. ab215188; dilution, 1:100; Abcam), IRS1 (cat. no. ab40777; dilution, 1:500; Abcam) and GLUT4 (cat. no. ab654; dilution, 1:1,000; Abcam) at 37°C for 30 min. Following the primary antibody incubation, the sections were incubated with biotinylated goat anti-rabbit IgG antibody (cat. no. ab150077; dilution, 1:200; Abcam) at 37°C for 30 min, and subsequently treated with streptavidin-biotin-peroxidase solution (Beyotime Institute of Biotechnology). The slides were subsequently stained with 3,3'-diaminobenzidine and finally observed under a light microscope (magnification, $\times 200$).

Statistical analysis. All experiments were repeated three times. Statistical analysis was performed using SPSS 19.0 software (IBM Corp.). Data are presented as the mean \pm standard deviation. Unpaired Student's t-test was used to compare differences between two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

TNF- α expression in adenoid tissues. Western blot analysis was performed to detect TNF- α expression in adenoid tissues. The results demonstrated that TNF- α expression was notably

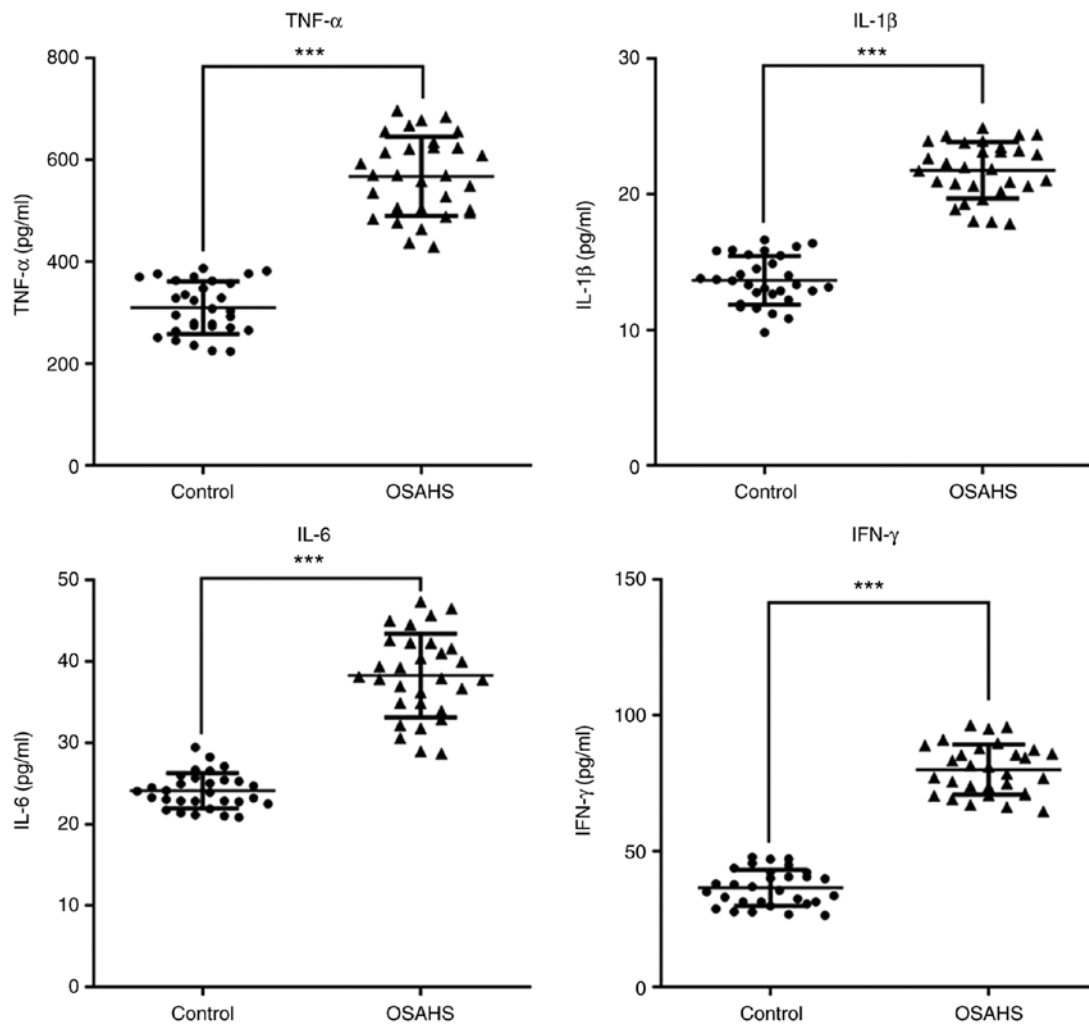


Figure 3. Expression of inflammatory factors, TNF- α , IL-1 β , IL-6 and IFN- γ , in plasma. The levels of IL-1 β , IL-6 and IFN- γ in plasma of obese children with OSAHS and control obese subjects was analyzed by ELISA assay. ***P<0.001 vs. control group. OSAHS, obstructive sleep apnea hypopnea syndrome.

higher in adenoid tissues of the OSAHS group compared with that in the control group (Fig. 1A). The results of western blot analysis were verified via RT-qPCR analysis (Fig. 1B) and immunohistochemistry analysis (Fig. 1C).

Expression levels of the inflammatory factors, TNF- α , IL-1 β , IL-6 and IFN- γ , in adenoid tissues and plasma. Western blot analysis was performed to detect the protein expression levels of IL-1 β , IL-6 and IFN- γ in adenoid tissues. As presented in Fig. 2, the protein expression levels of IL-1 β , IL-6 and IFN- γ were higher in adenoid tissues of the OSAHS group compared with those in the control group. The results presented in Fig. 3 indicated that the levels of TNF- α , IL-1 β , IL-6 and IFN- γ were also increased in plasma of the OSAHS group compared with those in the control group.

Expression levels of the insulin resistance-associated factors, IRS1 and GLUT, in adenoid tissues. Western blot and immunohistochemistry analyses were performed to detect IRS1 and GLUT4 expression in adenoid tissues. The results demonstrated that IRS1 and GLUT4 expression levels were downregulated in adenoid tissues of the OSAHS group compared with those in the control group (Fig. 4A),

which was consistent with RT-qPCR analysis (Fig. 4B) and immunohistochemistry analysis (Fig. 4C).

TNF- α may regulate insulin resistance by activating the TNF- α /IKK β /IK β /NF- κ B signaling pathway. Western blot analysis was performed to detect the protein expression levels of TNF- α , IKK β , p-IKK β , IK β , p-IK β , NF- κ B and p-NF- κ B in adenoid tissues. As presented in Fig. 5, the protein expression levels of TNF- α , p-IKK β , p-IK β and p-NF- κ B were higher in adenoid tissues of the OSAHS group compared with the control group, while no significant changes were observed in the expression levels of IKK β , IK β and NF- κ B between the two groups.

Discussion

The present study aimed to investigate whether TNF- α decreases GLUT4 expression to promote insulin resistance via the TNF- α /IKK β /IK β /NF- κ B signaling pathway in OSAHS. The results demonstrated that TNF- α expression was upregulated in adenoid tissues of obese children with OSAHS, and TNF- α promote insulin resistance via the TNF- α /IKK β /IK β /NF- κ B pathway in OSAHS.

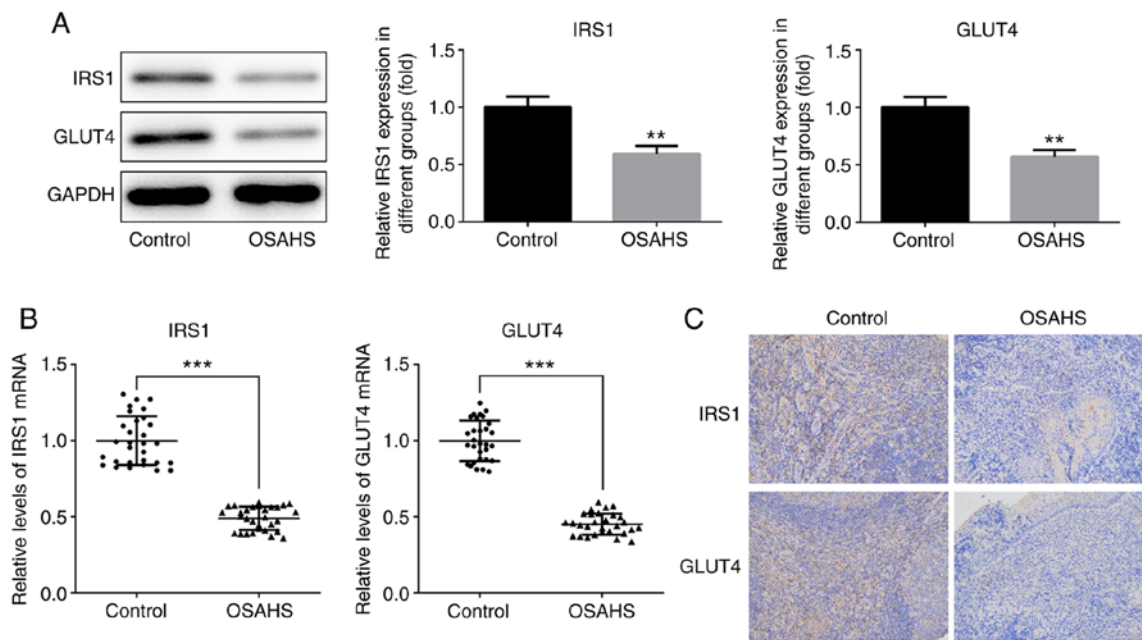


Figure 4. Expression of insulin resistance-associated factors, IRS1 and GLUT4, in adenoid tissues. (A) Protein expression of IRS1 and GLUT4 in adenoid tissues of obese children with OSAHS and control obese subjects was analyzed by western blot analysis. (B) mRNA expression of IRS1 and GLUT4 in adenoid tissues of obese children with OSAHS and control obese subjects was detected by reverse transcription-quantitative PCR analysis. ** $P < 0.01$ and *** $P < 0.001$ vs. control group. (C) Expression of IRS1 and GLUT4 in adenoid tissues of obese children with OSAHS and control obese subjects was determined by immunohistochemistry. GLUT4, glucose transporter type 4 insulin-responsive; OSAHS, obstructive sleep apnea hypopnea syndrome; IRS1, insulin receptor substrate 1.

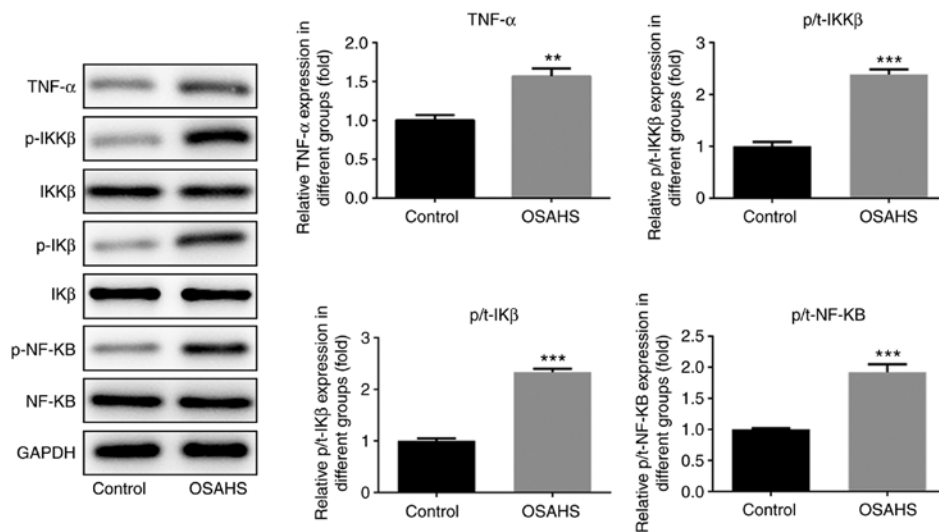


Figure 5. TNF- α may regulate the insulin resistance by activating the TNF- α /IKK β /IK β /NF- κ B signaling pathway. The protein levels of TNF- α , IFN- γ , IKK β , p-IKK β , IK β , p-IK β , NF- κ B and p-NF- κ B in adenoid tissues of obese children with OSAHS and control obese subjects was analyzed by western blot analysis. ** $P < 0.01$ and *** $P < 0.001$ vs. control group. OSAHS, obstructive sleep apnea hypopnea syndrome; p, phosphorylated; t, total.

A previous study demonstrated that children with OSAHS account for 1-5% of all childhood diseases (23). OSAHS can occur in children of all ages, most of whom are 2-8 years old, because the tonsil and adenoid hypertrophy account for important parts of the upper airway in children of this age range (24). OSAHS in children is commonly associated with impaired neurocognition, behavior, decreased quality of life, systemic hypertension, increased risk of pulmonary heart disease and increased health care spending (25-30). OSAHS also causes metabolic syndrome in children, as well as in adults (31). Previous studies have demonstrated that OSAHS

can induce insulin resistance independently of confounding factors, such as the amount of body fat and age. Furthermore, insulin resistance, as one of the core components of metabolic syndrome, may play an important role in the systemic damage caused by OSAHS (32,33).

TNF- α is a small-molecule protein mainly secreted by macrophages as a key regulator of systemic inflammation and has several inflammatory biological functions (34,35). Most patients with OSAHS are affected by pharyngeal inflammation, which can induce a series of changes in the level of inflammatory mediators, including increased

secretion of TNF- α (36). The serum TNF- α level of patients with OSAHS significantly increases during the day, which results in a disordered sleep structure (37). Ming *et al* (38) suggested that TNF- α may be involved in the occurrence and development of OSAHS, and is closely associated with OSAHS. Thus, TNF- α can be used to assess the severity of OSAHS. The results of the present study demonstrated that TNF- α expression was notably higher in adenoid tissues of obese children with OSAHS, and the levels of inflammatory factors, IL-1 β , IL-6 and IFN- γ , also increased in adenoid tissues of obese children with OSAHS.

Previous studies have reported that the main molecular mechanism of insulin resistance caused by several inflammatory factors is regulated by the association between the signal transduction of inflammatory factors and the signal transduction of pancreatic insulin receptors. The IKK β /NF- κ B pathway is the primary signaling pathway that serves as a link between inflammatory cytokines and insulin resistance (39,40). Inflammatory factors stimulate the IKK β /NF- κ B pathway, which contributes to the amplification and maintenance of the inflammatory response through NF- κ B, and leads to insulin resistance following inhibition of the insulin signaling pathway via IKK β (41-43). The results of the present study demonstrated that upregulated TNF- α expression activated the IKK β /IK β /NF- κ B pathway to effectively induce insulin resistance, which in turn decreased the expression levels of IRS1 and GLUT4.

Obesity, especially abdominal obesity, is an independent risk factor for insulin resistance, and the accumulation of solid fat has been shown to be highly correlated with the onset of insulin resistance, type 2 diabetes, metabolic syndrome and other diseases (44). Insulin signaling disorder is closely associated with the development of impaired glucose metabolism and insulin resistance in cells. In obesity, the effector pathway of insulin will be affected, resulting in insulin resistance, due to increased free fatty acids (FFA), lipid deposition, activation of inflammatory pathways, endoplasmic reticulum stress and mitochondrial dysfunction (45). In addition, FFA interferes with the insulin signal transduction pathway by promoting the expression of inflammatory factors, thereby interfering with mitochondrial function, the expression of glucose/lipid metabolism-related genes and related enzymes, increasing muscle reactive oxygen species levels and reducing GLUT-4 expression (46). Therefore, in the present study, it was hypothesized that obesity could affect the TNF- α /IKK β /IK β /NF- κ B pathway, which should be verified by future investigation. The single factor (obesity) could be studied to see how it could affect the TNF- α /IKK β /IK β /NF- κ B pathway.

In conclusion, the present study investigated the role of TNF- α in insulin resistance caused by OSAHS in obese children and demonstrated that TNF- α promoted insulin resistance in OSAHS. The results presented here provide novel insight for the effective therapy of insulin resistance in obese children with OSAHS. However, the present study has certain limitations. No experiments were included to determine how obesity affected the TNF- α /IKK β /IK β /NF- κ B pathway, how this signaling pathway affected the GLUT4 expression and insulin resistance and how treatment affected the present factors detected in this study.

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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

XW conceived and designed the study. LZ performed the experiments. LZ and YL analysed, interpreted and authenticated the data. LZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Nantong First People's Hospital (grant no. 20180209NT; Nantong, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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