Original Article
The impact of TLR4 on insulin resistance and inflammatory factors in rats with polycystic ovary syndrome

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Abstract: Polycystic ovary syndrome (PCOS) is the most common gynecological disease that is often accompanied by insulin resistance. It has been shown that the TLR signaling pathway can participate in PCOS occurrence and development. This study was to explore the effects of regulatory TLR4 on insulin resistance and inflammatory factors in PCOS rats. Female Wistar rats were randomly divided into 3 groups: the control group; the PCOS group (rat PCOS model prepared by dehydroepiandrosterone); and the TLR4 siRNA group (recombinant TLR4-siRNA transfection) before PCOS modeling. TLR4/NF-κB signaling pathway and IRS-2 expression was detected by real-time PCR and Western blot. Testosterone (T), insulin (INS), dihydrotestosterone (DHT), and free androgen index (FAI) were tested by a chemiluminescence method. Serum tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β levels were detected by ELISA. TLR4 and NF-κB expression was increased, IRS-2 level was downregulated, and T, INS, DHT, and FAI levels were elevated. Additionally, the inflammatory factors IL-1β, TNF-α, and IL-6 were enhanced in the PCOS group compared with control (P < 0.05). TLR4 expression was downregulated, NF-κB expression was inhibited, IRS-2 level was enhanced, and T, INS, DHT, and FAI levels declined. Inflammatory cytokines IL-1β, TNF-α, and IL-6 secretion decreased in the TLR4 siRNA group compared with the PCOS group (P < 0.05). Regulation of TLR4 may thus downregulate the TLR4/NF-κB signaling pathway, inhibit the inflammatory environment, and promote IRS-2 expression to improve insulin resistance in PCOS.

Keywords: PCOS, TLR4, insulin resistance, inflammation, NF-κB

Introduction

Polycystic ovary syndrome (PCOS) is the most common gynecological disease in women of reproductive age, which is characterized as metabolic and endocrine abnormalities, as well as chronic inflammation [1, 2]. The clinical manifestations of PCOS are various, including menstrual disorders, amenorrhea, obesity, infertility, and other various complications, such as diabetes, endometrial cancer, and hypertension, which seriously affect quality of life [3, 4]. The incidence of PCOS is increasing each year as affected by genetic, environmental, and endocrine factors, which are the main reasons for female ovulatory infertility [5]. However, the pathogenesis of PCOS is complicated and the etiology is unclear [6]. PCOS is related to adrenergic hyperfunction, hypothalamic GnRH-LH pulse frequency, ovarian autocrine, and paracrine abnormalities [7, 8]. Insulin resistance is often associated with PCOS patients, leading to the decrease ability of insulin to promote glucose uptake and utilization. To maintain blood glucose stability, the body further compensate for excessive secretion of insulin, leading to hyperinsulinemia [9, 10]. Insulin resistance is closely related to the increase of inflammatory factors [11].

Toll-like receptors (TLRs) are pattern recognition receptors that recognize pathogen-associated molecular patterns in the innate immune response and can be expressed on various cell surfaces, such as T cells, B cells, and macrophages [12]. The TLR family contains at least

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTAGTCACCTGTTGCTGG	TAATACGGAGACCTGTCTGGT
TLR4	ATACTCCATGGAGATTGG	TCACCG CTCGAAGCTGGTA
NF-κB	CATGGCATGGAGAAGA	CACATGGAGATTCCG C
IRS-2	AAACGCTCCTACATGGAGA	TCACCTATGGGAGACC

13 receptors. It has been shown that TLR4 is one of the important members of the TLR family and is closely related to both immune response and inflammation [13, 14]. The TLR signaling pathway can participate in the occurrence and development of PCOS [15]. However, the role of TLR4 in insulin resistance and anti-inflammatory cytokines of PCOS has not yet been elucidated. Therefore, the aim of this study was to analyze the effect of TLR4 on insulin resistance and inflammatory factors in PCOS rats.

Materials and methods

Experimental animals

Female Wistar rats in SPF grade aged 3 weeks and weighted 100 ± 20 g were purchased from the Experimental Animal Center in Shandong University. The rats were fed by the SPF Animal Experimental Center. Feeding conditions included maintaining temperature at 21 ± 1°C, relative humidity at 50-70%, and 12 hour day/night cycle. The rats were free to eating and drinking.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Shandong University of Traditional Chinese Medicine Affiliated Hospital (Shandong China).

Main materials and instruments

Pentobarbital sodium was purchased from Shanghai Zhaohui Pharmaceutical Co., Ltd. TLR4 siRNA was purchased from Genepharma. Dehydroepiandrosterone was purchased from Sigma. Insulin and testosterone kits were purchased from LINCO. PVDF membrane was purchased from Pall Life Sciences. Western blot related chemical reagents were purchased from Beyotime. ECL reagents were purchased from Amersham Biosciences. Rabbit anti-mouse TLR4, NF-κB, and IRS-2 monoclonal

antibodies and horseradish peroxidase (HRP) labeled IgG secondary antibody were purchased from Cell Signaling. Rabbit anti-mouse IL-1β, TNF-α, and IL-6 ELISA kits were purchased from R&D. Microsurgical instruments were purchased from Suzhou Medical Instrument Factory. RNA extraction kit and reverse transcription kit were purchased from ABI. The Amp PCR System 2400 DNA Amplification System was purchased from PE Gene Corporation. Imark microplate reader was purchased from BD Company. Automated chemiluminometer was purchased from Beckman.

Methods

Experimental animals and grouping: Female Wistar rats were randomly divided into 3 groups: the control group; the PCOS group; and the TLR4 siRNA group.

Rat PCOS model establishment: Dehydroepiandrosterone dissolved in 0.2 ml of 60 mg/kg sesame oil was subcutaneous injected to the cervix of rats. The vaginal exfoliated cells keratinized for 10 consecutive days was considered as successfully PCOS establishment [16]. Insulin resistance index (HOMA-IR) = (fasting blood glucose × fasting insulin)/22.5. HOMA-IR greater than 2.8 in PCOS rats was considered as PCOS insulin resistance model [17].

TLR4 siRNA preparation: TLR4 siRNA was transfected into rat follicles by recombinant lentivirus-TLR4 SiRNA. After Lentiviral TLR4 SiRNA plasmid was constructed and amplified, high titer virus supernatant was collected and the lentivirus-TLR4 SiRNA was injected via the tail vein. The virus titer was 1×10^8 Tu/ml, 100 μl/time, and once every two weeks for a total of 2 times. Then the PCOS insulin resistance model was prepared.

Real-time PCR: Total mRNA was extracted using Trizol reagent and reverse transcribed to DNA according to the kit instructions. The primers were designed by Primer Premer 6.0 software and synthesized by Invitrogen (**Table 1**). The PCR was performed at 55°C for 1 minute, followed by 35 cycles of 92°C for 30 seconds,
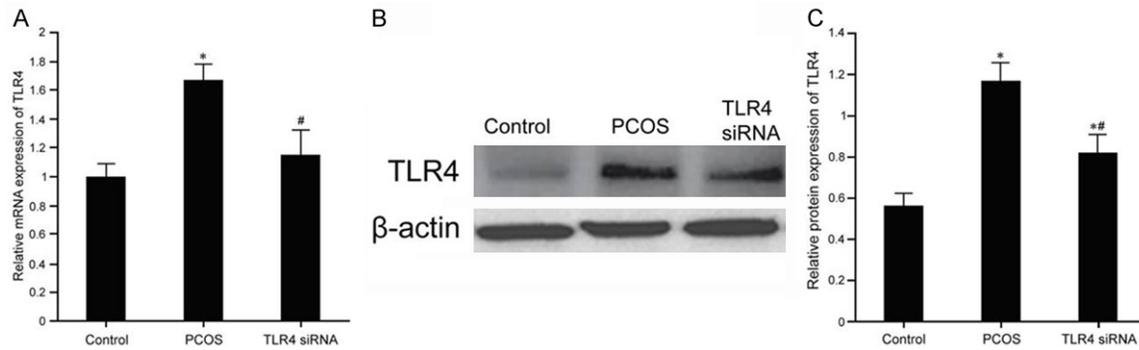
Figure 1. Impact of TLR4 regulation on TLR4 mRNA and protein expressions in PCOS rat. A. Effect of TLR4 regulation on TLR4 mRNA expression in PCOS. B. Effect of TLR4 regulation on TLR4 protein expression in PCOS. C. TLR4 protein expression analysis. *P < 0.05, compared with control, #P < 0.05, compared with PCOS.
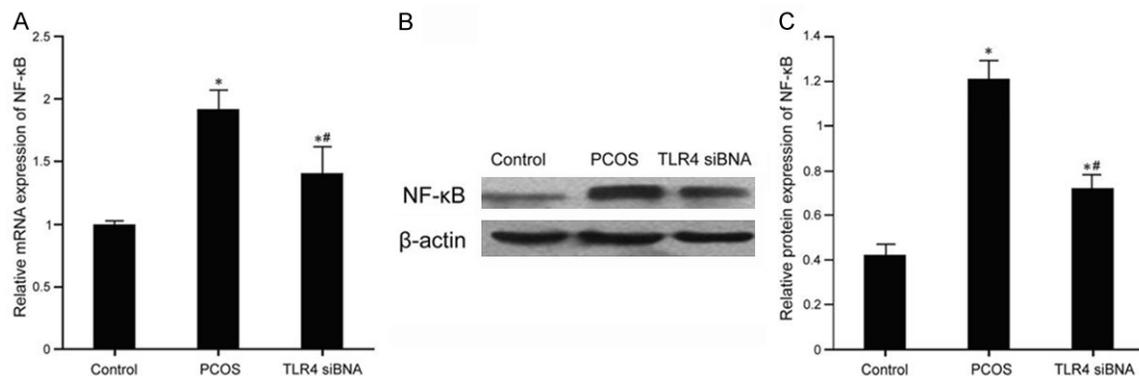

Figure 2. Influence of TLR4 regulation on NF-κB mRNA and protein expressions in PCOS rat. A. Effect of TLR4 regulation on NF-κB mRNA expression in PCOS. B. Effect of TLR4 regulation on NF-κB protein expression in PCOS. C. NF-κB protein expression analysis. *P < 0.05, compared with control, #P < 0.05, compared with PCOS.

58-60°C for 45 seconds, and 72°C for 35 seconds. For data collection, GAPDH was used as a reference. The relative expression level was calculated by $2^{-\Delta Ct}$ method.

Western blot: The tissue was added with lysate and the protein was store at -20°C. The isolated proteins were electrophoresed using 10% SDS-PAGE. The gel was transferred to PVDF membrane by semi-dry transfer method at 100 mA for 1.5 hours. After blocked for 1 hour, the membrane was incubated in TLR4, NF-κB, and IRS-2 primary antibodies (1:1500, 1:2000, 1:2000, and 1:1000) at 4°C overnight. After incubated in secondary antibody (1:2000) in the dark for 30 minutes, the membrane was imaged using chemiluminescence reagent for 1 minute and analyzed by image processing system software and Quantity one software. The experiment was repeated four times (n = 4).

Insulin resistance indicator detection: Testosterone (T), INS, dihydrotestosterone (DHT), and free androgen index (FAI) were tested by the full-scale chemiluminescence instrument. FAI = T/sex hormone-binding globulin.

ELISA: Serum levels of IL-1β, IL-6, and TNF-α were measured by ELISA. A total of 50 μl serially diluted standards and samples were added to the 96-well plate. After washing for three times, 50 μl enzyme-labeled reagent was added each well at 37°C for 30 minutes. Next, 50 μl reagent A and reagent B were added and incubated on at 37°C for 10 minutes. At last, 50 μl stop solution was added to each well and the photometric value (OD value) was measured using a microplate reader to calculate the corresponding concentration.

Statistical analysis

All data analyses were performed on SPSS 22.0 software. The measurement data are presented as mean ± standard deviation and compared by t test or one-way ANOVA. Univariate
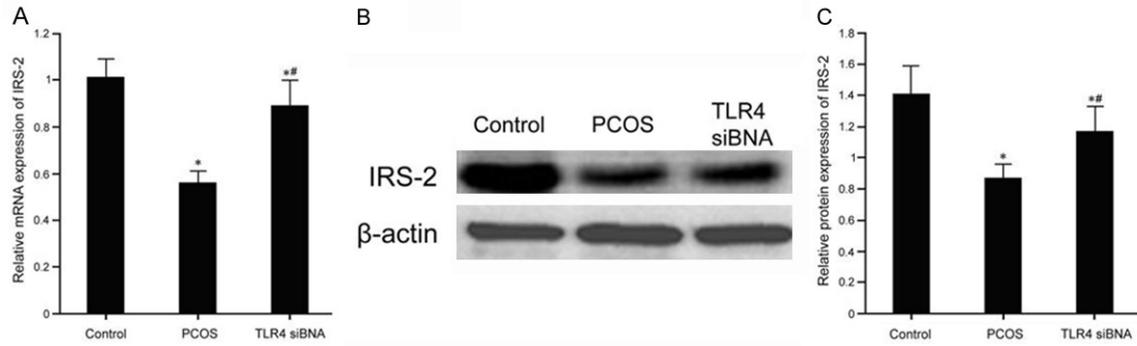
Figure 3. Effect of TLR4 regulation on IRS-2 mRNA and protein expressions in PCOS rat. A. Effect of TLR4 regulation on IRS-2 mRNA expression in PCOS. B. Effect of TLR4 regulation on IRS-2 protein expression in PCOS. C. IRS-2 protein expression analysis. $^*P < 0.05$, compared with control, $^\#P < 0.05$, compared with PCOS.

Table 2. Impact of TLR4 regulation on insulin resistance indicators in PCOS rat

Index	Control	PCOS	TLR4 siRNA
T (nmol/L)	0.96 ± 0.36	1.72 ± 0.61*	1.21 ± 0.47$^\#$
INS (mU/L)	6.13 ± 2.11	12.23 ± 2.81*	9.78 ± 1.16$^{*,\#}$
DHT (µg/dl)	209.45 ± 25.35	378.66 ± 29.88*	219.21 ± 42.16$^\#$
FAI	2.12 ± 0.35	4.27 ± 0.75*	2.67 ± 0.24$^\#$

$^*P < 0.05$, compared with control, $^\#P < 0.05$, compared with PCOS.

correlation analysis was performed by Pearson. P < 0.05 was considered as statistical difference.

Results

The impact of TLR4 regulation on TLR4 mRNA and protein expressions in PCOS rat

TLR4 mRNA and protein expression were tested by real-time PCR and Western blot. TLR4 expression was increased in the PCOS group compared with control (P < 0.05). TLR4 expression was downregulated in the TLR4 siRNA group compared with the PCOS group (P < 0.05) (**Figure 1**).

The influence of TLR4 regulation on NF-κB mRNA and protein expressions in PCOS rat

NF-κB mRNA and protein expression was detected by real-time PCR and Western blot. It was observed that NF-κB expression elevated in PCOS group compared with control (P < 0.05). NF-κB expression was reduced in the TLR4 siRNA group compared with the PCOS group (P < 0.05) (**Figure 2**).

The effect of TLR4 regulation on IRS-2 mRNA and protein expressions in PCOS rat

IRS-2 mRNA and protein expressions were detected by real-time PCR and Western blot. IRS-2 expression decreased in PCOS group compared with control (P < 0.05). IRS-2 expression was upregulated in the TLR4 siRNA group compared with the PCOS group (P < 0.05) (**Figure 3**).

The impact of TLR4 regulation on insulin resistance indicators in PCOS rat. T, INS, DHT, and FAI levels elevated in PCOS group compared with control (P < 0.05). T, INS, DHT, and FAI levels declined in TLR4 siRNA group compared with the PCOS group (P < 0.05) (**Table 2**).

The influence of TLR4 regulation on NF-κB mRNA and protein expressions in PCOS rat

Serum inflammatory factors IL-1β, IL-6, and TNF-α were tested by ELISA. IL-1β, TNF-α, and IL-6 enhanced in the PCOS group compared with control (P < 0.05). IL-1β, TNF-α, and IL-6 secretion decreased in the TLR4 siRNA group compared with the PCOS group (P < 0.05) (**Figure 4**).

Discussion

Due to less ovulation or continuous anovulation, PCOS may lead to irregular thickening of the endometrium, thereby increasing the risk of endometrial lesions, or even endometrial can-
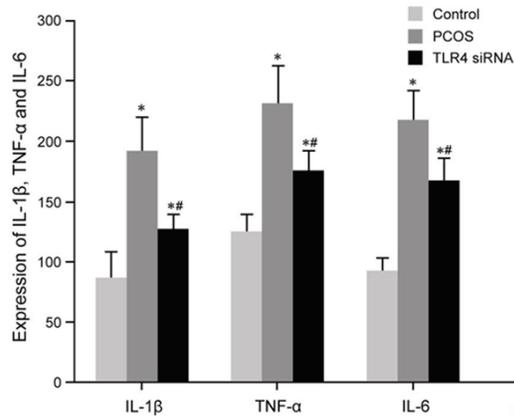
Figure 4. Influence of TLR4 regulation on NF-κB mRNA and protein expressions in PCOS rat. *P < 0.05, compared with control, #P < 0.05, compared with PCOS.

cer. Moreover, PCOS can affect the endocrine and metabolic system, increasing the risk of complications, such as hypertension and diabetes [5, 6]. Insulin resistance can lead to metabolic abnormalities, further exacerbating tissue damage [10]. TLR4 binds to its ligand and induces activation of downstream signals. Activation of its downstream signal, NF-κB, is an important part of the innate immune defense system [18]. The activation of the TLR4/NF-κB pathway is an important factor in inflammation [19]. Recent studies found that there is association between TLR4 and insulin resistance [15]. Insulin resistance can be promoted by activating TLR4. Since TLR4 is widely distributed in tissues and cells, regulation of TLR4 may improve insulin function and energy balance [19]. Therefore, in this study, a PCOS rat insulin resistance model was established and TLR4 and its related TLR4/NF-κB signaling pathway changes were analyzed. The results confirmed that the expression of TLR4 increased and TLR4/NF-κB signaling pathway activated in insulin resistant PCOS rats. IRS can be activated by the insulin receptor tyrosine kinase substrate, which has more than a dozen tyrosine residues that can be phosphorylated. Phosphorylated IRS can bind and activate downstream effectors [20]. IRS-2 plays a role in the regulation of islet cell development and insulin secretion in liver and skeletal muscle [21]. Therefore, this study also analyzed the effect of TLR4 on IRS-2 expression in insulin resistant PCOS rats. The results exhibit that expression of TLR4 was increased and IRS-2

was decreased during insulin resistance in PCOS rats. Downregulation of TLR4 expression and inhibition of TLR4/NF-κB signaling pathway could elevate expression of IRS-2 in PCOS rats with insulin resistance. Therefore, regulation of TLR4 can improve PCOS insulin resistance by regulating IRS-2.

PCOS is a low-grade chronic inflammatory disease. In patients with PCOS, especially with insulin resistance, expression of inflammatory factors such as IL-1β, TNF-α, and IL-6 are increased. Activation of TLR4/NF-κB signaling pathway and activation of NF-κB are the key fast factors that initiate and regulate inflammation. They further promote secretion of inflammatory cytokines through the expression of inflammatory mediators, leading to progression of PCOS insulin resistance [22, 23]. Therefore, this study explored the effect of regulating TLR4 on inflammatory factors in insulin resistant PCOS rats. Expression of TLR4 was found to be increased, and the secretion of inflammatory cytokines IL-1β, TNF-α, and IL-6 was enhanced in PCOS rats. Down-regulation of TLR4 expression and inhibition of the TLR4/NF-κB signaling pathway could inhibit secretion of inflammatory cytokines IL-1β, TNF-α, and IL-6 in PCOS rats with insulin resistance. Down-regulation of the TLR4/NF-κB signaling pathway, inhibiting the inflammatory environment, could further suppress expression of T, INS, DHT, and FAI, which in turn helps to alleviate insulin resistance. This study demonstrates for the first time the effect of regulating TLR4 pathway on PCOS insulin resistance and inflammatory factors, but the current sample size was small. It is proposed to increase the sample size in clinical PCOS patients in future studies.

Conclusion

Regulation of TLR4 may downregulate TLR4/NF-κB signaling pathway, inhibit the inflammatory environment, and promote IRS-2 expression to improve insulin resistance in PCOS.

Acknowledgements

Disclosure of conflict of interest

None.

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