SP1 transcriptionally upregulates the expression of APOC3 in KGN cells to promote disease progression by regulating TLR2/NF-κB signalling pathway

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Abstract

Introduction: Apolipoprotein C3 (APOC3) is known for its important functions in metabolism-related diseases. However, the function and molecular mechanism of APOC3 in polycystic ovarian syndrome (PCOS) have not been reported.

Material and methods: Quantitative polymerase chain reaction and western blot assays were used to detect the expression of APOC3 in KGN cells. Small interference APOC3 (siAPOC3) was applied to reduce APOC3 expression, and the proliferation ability of human granulosa cell line (KGN cells) was measured by cell counting kit-8 and colony formation assays. The protein levels of key genes related to apoptosis were detected by western blot assay. The transcriptional regulator of APOC3 was predicted by the UCSC and PROMO website, and verified by dual luciferase assay. siAPOC3 and pcDNA3.1-specific protein 1 (SP1) vector were co-transfected into KGN cells to detect the function of SP1 and APOC3 in KGN cells.

Results: APOC3 was overexpressed in KGN cells, and siAPOC3 transfection significantly reduced the growth ability of KGN cells and increased the apoptosis ability of KGN cells. SP1 directly bound to the promoter of APOC3 and transcriptional regulated APOC3 expression. Overexpression of SP1 increased the growth ability of KGN cells and decreased the apoptosis ability of KGN cells, which were reversed after siAPOC3 transfection. The increased levels of toll-like receptor 2 (TLR2) and p65 phosphorylation (p-P65) nuclear factor kappa B (NF-κB) caused by SP1 overexpression were inhibited by siAPOC3 transfection. APOC3, transcriptionally regulated by SP1, promoted the growth of KGN cells, and inhibited the apoptosis by regulating TLR2/NF-κB signalling pathway. (Endokrynol Pol 2023; 74 (5): 553–560)

Key words: apolipoprotein C3; apoptosis; polycystic ovarian syndrome; proliferation; transcription

Introduction

Polycystic ovarian syndrome (PCOS) is one of the most common complicated reproductive endocrine disorders, characterized by hyperandrogenaemia, chronic anovulation, and polycystic ovaries [1]. PCOS is often associated with abdominal obesity, insulin resistance, obesity, metabolic disorders, and cardiovascular risk factors [2]. The pathogenesis of PCOS is largely unclear. The follicles of patients with PCOS are blocked in the small antral follicular stage, and they cannot be recruited to develop into mature follicles, thus preventing ovulation [3]. Despite insulin-sensitizers such as metformin being used in the treatment of PCOS, these drugs have adverse effects that affect quality of life [4]. Therefore, studies have focused on exploring the molecular mechanisms underlying PCOS to excavate the therapeutic targets for the complete treatment of PCOS.

Granulosa cells (GCs) are somatic cells that originate from the sex cord, and they are a crucial cell type in the ovary [5]. Throughout oocyte development, previous studies have shown that there is an interdependence between the oocyte and its surrounding GCs, and the support of GCs is crucial for the oocyte to provide a suitable microenvironment, such as nutrients and growth regulators [6, 7]. In addition, recent studies have confirmed that dysfunction of GCs in PCOS, ranging from decreased proliferation and increased apoptosis to impaired hormone production, is closely

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related to abnormal follicle development [8]. To sum up, GCs have a higher proliferation rate in PCOS ovaries, which are implicated in ovarian dysfunction; however, the mechanism is still unknown.

Apolipoprotein C3 (APOC3) is encoded by APOA1/C3/A4/A5 gene clusters [9], and increasing evidence has demonstrated that APOC3 is a critical regulator of plasma triglyceride metabolism [10, 11]. Decreasing APOC3 protected against high-fat diet-induced metabolic derangements [12]. Moreover, increased APOC3 drove cardiovascular risk in type 1 diabetes [13]. Recently, APOC3 was identified as a potential serum biomarker of insulin resistance (IR) in patients with polycystic ovary syndrome by bioinformatics analysis [14]. However, the function of APOC3 and its molecular mechanism in PCOS have not been revealed.

In this study, we detected APOC3 expression in KGN cells, and small interference APOC3 (siAPOC3) was used to transfect KGN cells to explore the function of APOC3 in KGN cells. Then, we also investigate the upstream transcriptional regulator of APOC3 and its regulatory effect on APOC3.

Material and methods

Cell culture and transfection

Human granulosa-like tumour cell line (KGN) was purchased from American Type Culture Collection (ATCC, Manassas, VA, United States), and IOSE80 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) including 10% foetal bovine serum at 37°C for 14 days until visible clones appeared. Subsequently, cells were collected and fixed with 4% paraformaldehyde followed by staining with 0.1% crystal violet solution. The number of colonies was counted under a fluorescence microscope.

RNA interference

Knockdown of RNA in KGN cells was achieved by transfection with small interference RNA (siRNA) synthesized by Sangon (Shanghai, China). KGN cells were seeded in 6-well plates and transfected with siRNA using Lipofectamine 2000 based on the manufacture’s instruction.

Specific protein 1 (SP1) overexpression in KGN cells

The coding sequence of SP1 was subcloned into pcDNA3.1 vector, and empty vector was regarded as the negative control. After 24-h transfection, cells were gathered for the next step.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted from KGN cells by using TRIzol reagent. Then, cDNA was synthesized with the assistance of the One-Step PrimeScript RT-PCR Kit. qRT-PCR was performed on an ABI 7500 qRT-PCR instrument by using the SYBR Green PCR Kit. The relative expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the 2^(-ΔΔCT) method.

Western blot

Cells were lysed with pre-cooling radioimmunoprecipitation (RIPA) buffer, and the protein concentration was measured by the BCA method. The protein samples were denatured by heating at 95°C for 5 min, and a total of 20 μg protein samples were loaded onto per lane on a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. After transferring protein to a polyvinylidene fluoride membrane, the membrane was sealed with 5% skimmed milk for 1 h and incubated with the primary antibodies at 4°C overnight. The primary antibodies were listed as below: B-cell lymphoma 2 (Bcl2), Bcl-2-associated X protein (BAX), cleaved caspase 3 and GAPDH. After washing with TBST 3 times, the membrane was incubated with the secondary antibody at room temperature for 2 h. Then, the membranes were washed by TBST and treated by enhanced chemiluminescence. The greyscale values of band intensity were acquired by using QUANTITY ONE software. GAPDH was deemed as the internal reference control.

Cell viability assay

Cell viability was estimated by using cell counting kit 8 (CCK-8, Dojindo, Japan). First, the cells (3000) were implanted in 96-well plates and cultured at 37°C in a 5% CO2 humidified atmosphere. Then, 10 μL CCK-8 solution was put into each well at the indicated time and the cells were incubated for 2 h. Finally, the absorbance at 450 nm was measured to estimate the number of viable cells.

Colony formation assay

In brief, following cell transfection, the cells were seeded in the 60 mm culture dishes at a density of 500 cells/dish. Then, the cells were cultured in DMEM appended with 10% foetal bovine serum (FBS) for 14 days until visible clones appeared. Subsequently, cells were collected and fixed with 4% paraformaldehyde followed by staining with 0.1% crystal violet solution. The number of colonies was counted under a fluorescence microscope.

Dual luciferase assay

Fragments of the cDNAs including the promoter region of APOC3 were amplified by PCR and were inserted into the luciferase reporter gene of pGL3. Then, cells were cultured, and recombinant plasmids were co-transfected together with siPO1 or empty vector into cells. After 48-h culture, the luciferase activity of each sample was measured using the Dual-Luciferase Reporter Assay System (Promega, WI, United States).

Statistical analysis

All data were expressed as mean ± standard deviation of 3 independent experiments. GraphPad Prism 8.0 and SPSS 22.0 software were applied to perform statistical analysis. Statistical significance was determined by Student’s t-test (difference between 2 groups) or one-way analysis of variance (ANOVA) test (difference among multiple groups) followed by Bonferroni post hoc test. A p-value less than 0.05 was considered statistically significant.

Results

APOC3 was highly expressed in KGN cells, and siAPOC3 transfection significantly reduced APOC3 expression

By performing qPCR and western blot assays, we observed that APOC3 was highly expressed in KGN cells compared with the IOSE80 cells (Fig. 1AB). To further explore the function of APOC3 in KGN cells, we used siAPOC3 to transfect KGN cells to reduce APOC3 expression. As displayed in Figure 1C, we discovered that the treatment of siAPOC3-1 or siAPOC3-2 both significantly reduced APOC3 expression in KGN cells.
Knockdown of APOC3 diminished the growth ability and promoted the apoptosis of KGN cells

Subsequently, CCK-8, colony formation and western blot assays were applied to detect the function of APOC3 on KGN cell growth and apoptosis. The results in Figure 2A show that the optical density (OD) values of KGN cells reduced obviously after siAPOC3-1 or siAPOC3-2 treatment. Analogously, the number of KGN cell clones was obviously reduced after siAPOC3-1 or siAPOC3-2 treatment (Fig. 2BC). Moreover, we also discovered the effect of siAPOC3 on the expression of apoptosis-related genes. The protein levels of Bcl-2-associated X protein (BAX) and cleaved caspase 3 were significantly increased in KGN cells after siAPOC3-1 or siAPOC3-2 treatment, whereas the protein level of B-cell lymphoma 2 (Bcl-2) was reduced obviously after siAPOC3-1 or siAPOC3-2 transfection (Fig. 2C).

SP1 directly bound to the promoter of APOC3 and transcriptionally regulated APOC3 expression

To further detect the molecular mechanism of APOC3 in KGN cells, the UCSC (https://genome.ucsc.edu/) and PROMO (http://alggen.lsi.upc.es/) websites were applied to detect the upstream transcriptional regulator of APOC3. SP1 has 2 binding sites to the promoter region of APOC3 (Fig. 3A). Then, luciferase reporter vectors containing wild type (WT) or mutant (MUT) SP1 targeting sites (MUT-1 and MUT-2) were amplified and subsequently transfected into cells with SP1 overexpression plasmid or negative control. The data from Figure 3B show that SP1 overexpression increased luciferase activity, whereas MUT-1 or MUT-2 treatment resulted in decreased luciferase activity. The overexpression efficiency of SP1-OE and interference efficiency of si-SP1 are presented in Figure 3CD. Additionally, the data from Fig. 3C show that upregulation of SP1 increased APOC3 expression, whereas depletion of SP1 obviously reduced the protein expression of APOC3 in KGN cells (Fig. 3D). All the above results demonstrated that SP1 bound to the promoter region of APOC3 and regulated APOC3 expression.

The combined effects of SP1 and APOC3 on KGN cells were explored

Then, the relationship between SP1 and APOC3 in KGN cells was explored. After transfection with the control, SP1-OE, or SP1-OE+siAPOC3 into KGN cells, several relevant assays were employed. The data from CCK-8 assay showed that the OD values of KGN cells were obviously increased after SP1 overexpression, which was diminished after siAPOC3 transfection (Fig. 4A). The increase in the number of KGN cell clones caused by SP1 overexpression was suppressed by siAPOC3 treatment (Fig. 4BC). The reduced protein levels of BAX and cleaved caspase-3 in KGN cells induced by SP1 overexpression were blocked after siAPOC3 treatment, and the increased protein level of Bcl-2 in KGN cells caused by SP1 overexpression were suppressed after siAPOC3 treatment (Fig. 4D).

Function of SP1/APOC3 on KGN cells was achieved by regulating TLR2/NF-κB pathway

Toll-like receptor 2/nuclear factor kappa B (TLR2/NF-κB) signalling pathway has been reported to play an important role in the development of PCOS [15]. In our study, we used western blot assay to detect the relation between SP1/APOC3 and TLR2/NF-κB signalling pathway. The protein levels of TLR2 and p65 phosphorylation (p-P65) NF-κB were obviously increased after SP1 overexpression, while knockdown of APOC3 suppressed the protein levels of TLR2 and p-P65 NF-κB (Fig. 4E).
Discussion

The association between APOC3 and PCOS has not been well studied. Li et al. reported that APOC3 expression was positively related to the homeostasis model assessment of insulin resistance in PCOS patients [14]. Moreover, APOC3 was regarded as a member of a gene signature associated with the progression of PCOS [16]. In our study, we discovered that APOC3 was highly expressed in KGN cells, and siAPOC3 treatment reduced the growth and increased the apoptosis of KGN cells. Moreover, the transcriptional regulation of APOC3 has been explored.

Specific protein 1 (SP1) is a transcriptional activator widely implicated in life-sustaining activities, including cell cycle, differentiation, proliferation, and DNA damage [17, 18]. Moreover, the function of SP1 in the occurrence and progression of cardiovascular diseases has also been investigated [19]. In addition, SP1 was discovered to be an important regulator in the pro-
Liferation and metastasis of tumour cells [20]. The function of SP1 in ovarian cancer has been reported. For example, SP1-induced IncRNA DANCR contributed to proliferation and invasion of ovarian cancer [21], and SP1 promoted ovarian cancer cell migration by repressing miR-335 expression [22]. Based on topological and functional enrichment analyses, SP1 was identified as a potentially important regulator in the development of PCOS [23]. However, the detailed function of SP1 in PCOS has not been revealed. In our study, SP1 was identified as a transcriptional regulator of APOC3, and SP1 overexpression increased the growth ability of KGN cells and suppressed the apoptosis ability of KGN cells.

TLR2/NF-κB signalling pathway is extensively found in diverse tissues and cells and is implicated in the occurrence and modulation of multiple diseases [24]. Inactivation of TLR2/NF-κB signalling pathway could improve cardiac structure, myocardial remodeling, and cardiac function [24]. The function of NF-κB in ovarian cancer has been studied in-depth. NF-κB signalling pathway is a master and commander in ovarian cancer that promotes cancer stem cell maintenance, metastasis, chemoresistance, and immune evasion [25]. Moreover, activation of NF-κB signalling pathway could lead to inflammatory cascade in KGN cells [26]. TLR2 overexpression could promote inflammatory response and oxidative stress in the granulosa cells [27]. Several studies have reported the relationship between APOC3 and NF-κB signalling pathway. For example, knockdown of APOC3 could inhibit oxidative stress and inflammatory reaction in placenta cells from mice with preeclampsia by suppressing NF-κB signalling pathway [28], and APOC3 could aggravate diabetic nephropathy in type 1 diabetes via activating the renal TLR2/NF-κB signalling pathway [26]. In our study, SP1 overexpression increased the protein levels of TLR2 and p-P65 NF-κB, while knockdown of APOC3 suppressed the protein levels of TLR2 and p-P65 NF-κB.

Some deficiencies in this study should be pointed out. First, the experimental results obtained in this study are only based on some in vitro cell experiments and need to be verified in in vivo experiments in the future. Second, several relative indicators have not been detected in our study, which need to be analysed in future.

Figure 3. Specific protein 1 (SP1) directly bound to the promoter region of apolipoprotein C3 (APOC3) and regulated APOC3 expression. A. The targeting sequences between SP1 and APOC3 are presented; B. In the mutant APOC3 (MUT-APOC3) group, the luciferase activity was significantly reduced after SP1 overexpression; C. Knockdown of SP1 suppressed the expression of APOC3; D. SP1 upregulation increased the protein level of APOC3. SP1-OE — specific protein 1 overexpression; WT — wild type GAPDH — glyceraldehyde 3-phosphate dehydrogenase; si-con — small interference control; si-SP1 — small interference specific protein 1.
Figure 4. Function of specific protein 1/apolipoprotein C3 (SP1/APOC3) on KGN cells was achieved by regulating toll-like receptor 2/nuclear factor kappa B (TLR2/NF-κB) signalling pathway. A. The promoting effect of SP1 overexpression (SP1-OE) on KGN cell viability was suppressed by small interference APOC3 (siAPOC3) treatment; BC. Treatment with siAPOC3 suppressed the promoting effect of SP1-OE on KGN cell clones; D. SP1-OE treatment increased the protein level of B-cell lymphoma 2 (Bcl-2) and decreased the protein levels of Bcl-2-associated X protein (BAX) and cleaved caspase-3. However, siAPOC3 transfection reversed the above phenomena; Specific protein 1 treatment with siAPOC3 inhibited the promoting effect of SP1-OE on TLR2 and p65 NF-κB phosphorylation (p-P65) NF-κB expression. si-con — small interference control; GAPDH — glyceraldehyde 3-phosphate dehydrogenase
Conclusions
Our results showed that APOC3 was highly expressed in KGN cells, and knockdown of APOC3 obviously reduced the growth ability and increased the apoptosis of KGN cells. Moreover, SP1, as a transcription regulator, could bind to the promoter region of APOC3 and regulate APOC3 expression. SP1 overexpression could increase the growth ability and reduce the apoptosis of KGN cells, which were blocked after siAPOC3 treatment. The protein levels of key genes related to TLR2/NF-κB signalling pathway were altered after SP1-OE or siAPOC3 treatment.

Conflicts of interest
All authors claim no conflict of interest in the study.

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Ethics approval
Not applicable.

Consent to participate
Not applicable.

Consent to publication
All authors agreed to the article’s publication.

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

Code availability
Not applicable.

Authors’ contributions
N.Q. designed and performed the experiments and wrote the manuscript. L.W. performed the experiments and wrote the manuscript. S.L. analysed the data and wrote the manuscript. J.L. analysed the data and wrote the manuscript. C.F. conceived and supervised the experiments and wrote the manuscript.

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