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Thyroid hormone inhibits the proliferation of piglet Sertoli cell *via* PI3K signaling pathway

Yan Sun [a,b], WeiRong Yang [a], HongLin Luo [c], XianZhong Wang [a], ZhongQiong Chen [a,b], JiaoJiao Zhang [a], Yi Wang [a], XiaoMin Li [a,*]

[a] College of Animal Science and Technology, Southwest University, Beibei, Chongqing, PR China
[b] ChongQing Animal Disease Prevention and Control Center, YuBei, ChongQing, PR China
[c] Guanxi Key Laboratory for Aquatic Genetic Breeding and Healthy Aquaculture, Guangxi Institute of Fisheries, Nanning, Guangxi, PR China

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ABSTRACT

Accumulating researches show that thyroid hormone (TH) inhibits Sertoli cells (SCs) proliferation and stimulates their functional maturation in prepubertal rat testis, confirming that TH plays a key role in testicular development. However, the mechanism under the T3 regulation of piglet SC proliferation remains unclear. In the present study, in order to investigate the possible mechanism of T3 on the suppression of SC proliferation, the expression pattern of TRα1 and cell cycle–related molecules, effect of T3 on SC proliferation, and the role of phosphoinositide 3-kinase (PI3K)/Akt signaling pathway on the T3-mediated SC proliferation in piglet testis were explored. Our results demonstrated that TRα1 was expressed in all tested stages of SCs and decreased along with the ages. T3 inhibited the proliferation of SCs in a time- and dose-dependent manner, and T3 treatment downregulated the expressions of cell cycling molecules, such as cyclinA2, cyclinD1, cyclinE1, PCNA, and Skp2, but upregulated the p27 expression in SCs. Most importantly, the suppressive effects of T3 on SC proliferation seemed dependent on the inhibition of PI3K/Akt signaling pathway, and pre-stimulation of PI3K could enhance such suppressive effects. Together, our findings demonstrate that TH inhibits the proliferation of piglet SCs *via* the suppression of PI3K/Akt signaling pathway.

1. Introduction

Sertoli cells (SCs) are important in spermatogenesis [1,2]. They also establish cytoplasmic crypts around germ cells and supply nutrients, growth factors, and a stable microenvironment. Besides, SCs play an essential role on immunological responses by the establishment of the blood–testis barrier. It is known that the number of SCs affects the efficiency of spermatogenesis, because one SC can only support a limited number of germ cells. In addition, the number of SCs determines the size of testis and rate of sperm production in adult male, and hence the reproductive capacity [3–9]. It is believed that SCs proliferate at birth, decrease rapidly during the neonatal period, and stop completely at about 15 and 20 days after birth in mice and rat [5,10–13]. However, in pigs, SCs exert distinct proliferation phases. They first proliferate between birth and 1 month old and their numbers increase up to about sixfolds. Then they proliferate once again at 3 or 4 month old with double numbers to the first proliferation, and this process ceases before puberty [14]. When SCs cease proliferation and enter into differentiation, the establishment of the blood–testis barrier develops [15]. However, researches showed that proliferation and differentiation of SCs may overlap in time [16,17], suggesting that early

Yan S, WeiRong Y, and Honglin LUO have contributed equally to the work presented.

* Corresponding author. Tel.: +86 15823987891; fax: +86 2368251196.
E-mail address: lixiaomin662@sina.com (X. Li).

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perturbation of SC proliferation and/or differentiation may have long-term effects on male reproduction. Besides, prepubertal SC proliferation was shown to be closely related to FSH in several species [18], but some researches showed that the duration of SC proliferation may be strongly affected by thyroid hormones (THs) [19–21]. For example, during the neonatal period in rodents, high levels of serum 3, 3′,5-triiodothyronine (T3) result in fewer adult SCs and smaller testes [21,22]. In contrast, in adulthood, low levels of T3 lead to an increase in SC numbers, testicular size, and sperm production [12,23]. Similar effects of TH have been observed in humans. For instance, neonatal hypothyroidism leads to larger testes in prepubertal boys, whereas hyperthyroidism is associated with smaller testicular size [24,25]. Similar results to those in rodents and humans were also obtained in bull calves [26]. However, in pigs, hypothyroidism has no effect on the initiation of spermatogenesis or pubertal maturation [6]. Further researches exhibited that T3 can directly inhibit proliferation and induce neonatal rat SC differentiation [22], which upregulate cyclin-dependent kinase inhibitors [27], resulting in the elimination of mitogenic effects of FSH [19].

The effects of THs are mainly mediated by T3. T3 binds the specific intracellular thyroid hormone receptors (THRs or TRs), which function as a ligand-dependent transcription factor and control the expression of target genes [28]. Thyroid receptors are encoded by two genes, *Thra* and *Thrb*. Till date, nine peptide isoforms alternatively spliced from both genes have been isolated. Three of them have been proved to be functional: TRα1, TRβ1, and TRβ2. Researches showed that TRα1 mRNA and protein are abundant in SCs [29,30]. Although both TRα2 and TRα3 mRNA are expressed in SCs, they do not mediate T3 signaling [31,32]. Literatures also indicate that TRβ1 could be involved in SC development, but this area still keeps controversial [31,33–35]. Therefore, signaling through TRα1 may be the normal mechanism by which T3 promotes normal SC maturation [27]. A mount of T3-responsive genes have been uncovered [36,37], and evidences have been found that the biological effects of THs are mediated not only by direct transcriptional control but also by the regulation of cell signaling cascades [38]. Previous research has been shown that THs activate extracellular signal–regulated kinase-1/2 *via* the integrin αVβ3 receptor, resulting in the regulation of various cellular events, such as protein trafficking and proliferation. In addition, research has been reported that TH-mediated actin polymerization *via* phosphorylation plays an essential role in the migration of astrocytes and granular neurons [39]. None of these actions were considered to involve classical TRs. However, Cao et al. [40] reported that TR-mediated nongenomic action of T3, through which the phosphoinositide 3-kinase (PI3K)/Akt pathway was activated in primary cultured human skin fibroblasts. This action of T3 has also been found in various cell types and mouse models [41–46]. Further study showed that T3 activates PI3K/Akt through Src in neuronal cells [47]. Therefore, TRα1 may mediate T3-induced PI3K activation. However, this has not yet been investigated in piglet SCs.

Although a number of studies demonstrated that THs can regulate the SC proliferation, the mechanisms involved in this process in piglet SCs remain unclear. In the present study, we aimed to investigate the role of TH in the regulation of piglet SC proliferation and the possible role of PI3K/Akt signaling pathway during such biological process.

2. Materials and methods

2.1. Antibodies and reagents

The cell culture media DMEM/F-12 (cat no. C113305008), fetal bovine serum (cat no. 10099141), newborn bovine serum (cat no. 16010159), and trypsin (cat no. 25200056) were purchased from Gibco BRL Company. The 3, 3′, 5-triiodothyronine (synthetic T3, cat no. T2877) and porcine FSH (cat no. F2293) were acquired from Sigma–Aldrich. The anti-PCNA (cat no. bs-0754R), anti-Skp2 (cat no. bs-1096R), anti-PI3K (p85α subunit, cat no. bs-0128R), anti-phospho-PI3K (Tyr467, cat no. bs-3332R), anti-CDKN1B/p27kip1 (cat no. bs-0742R), anti-phospho-CDKN1B/p27kip1 (cat no. bs-5227R), anti-TRα1(cat no. bs-6221R), and anti-β-actin antibodies were provided by Bioss Biotech. The HRP-labeled goat anti-rabbit IgG (H + L, cat no. A0208), cell counting kit-8 (CCK-8, cat no. C0038), and Akt and phosphor-Akt (Ser473, cat no. AA326) were obtained from Beyotime Institute Biotech. The 740 Y-P (cell permeable phosphopeptide activator of PI3K, cat no. 1983) was supplied by Tocris Bioscience. RNAprep pure cell kit (cat no. DP430) was obtained from Tiangen Biotech. The cDNA synthesis kit (cat no. 170-8891) and SYBR green supermix (cat no. 1725261) were supplied by Bio-Rad Laboratories.

2.2. Animals

Three-weeks-old native healthy boar was used for SCs isolations and cell cultures. All animal studies were conducted in accordance with the guidelines for the care and use of laboratory animals issued by the Institution Committee on Animal Care and Use of the Ministry of Health of China. All the protocols had the approval of the Institutional Committee on Animal Care and Use (number: 2012-08-11).

2.3. Primary SC isolation and in vitro culture

Sertoli cells from 3-week-old native healthy boar were isolated as previously described protocol with modifications [48,49]. Briefly, each time, five boars were used to obtain the testes, and the testes were isolated from the piglet scrotum in a sterile condition after the anesthetization with 1% sodium pentobarbital for 35 to 40 mg/kg *via* intravenous injection. Then the individual testis was washed three times with precold (4 °C) PBS (with 20,000 IU/mL mycillin). The testis was cut into small pieces and homogenized after the removal of its out membrane. The tissue was then suspended in PBS and centrifuged (1000× *g* for 5 minutes) to remove the red blood cells. The sediment was then digested by incubation with 10-folds volume of collagenase IV (0.03 g/mL) in a thermostatic shaker (32 °C, 70 vibrations/minute) for 40 minutes. The suspensions were then centrifuged (1000× *g* for 5 minutes) and the sediment was incubated with 10-folds volume of trypsin (0.0025 g/mL) for the second digestion (32 °C, 70 vibrations/minute). The newborn bovine serum was added into