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Nitric oxide and thyroid hormone receptor alpha 1 contribute to ovarian follicular development in immature hyper- and hypo-thyroid rats

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ABSTRACT

Thyroid dysfunction can cause ovarian cycle and ovulatory disturbances, however, the molecular link(s) between these two disorders remains largely unknown. In the current study, we examined the roles of nitric oxide synthase (NOS) and thyroid hormone receptor alpha 1 (TRα1) in these disorders using immature hyper-thyroid (hyper-T) and hypo-thyroid (hypo-T) rats. In comparison to controls, hyper-T rats had higher serum concentrations of triiodothyronine (T3) and thyroxine (T4), whereas hypo-T rats had lower serum T3 and T4. Serum estradiol (E2) level was decreased in both hyper-T and hypo-T animals and serum E2 in hyper-T rats were lower than in hypo-T rats. We found that neuronal NOS (nNOS) and TRα1 were present in oocytes, granulosa cells and theca cells of all examined rat groups. Ovarian nitric oxide (NO) content and the constitutive NOS (cNOS) activity in hyper-T rats were significantly decreased compared with control or hypo-T rats. Moreover, the number of large antral follicles was reduced in hyper-T rats, and number of primordial follicles was decreased in hypo-T rats compared with control rats. In conclusion, we observed an association between thyroid hormone and NO signaling pathways during the process of ovarian follicular development in immature rats. In hyperthyroidism, thyroid hormones induced an estrogen deficiency that inhibited the function of nNOS, resulting in the inhibition of NO synthesis and suppressed development of large antral follicles, while in hypothyroidism only development of primordial follicles was inhibited.

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1. Introduction

In addition to gonadotropins, other hormones and growth factors are also involved in the regulation of ovarian development and differentiation [1–3]. For example, adequate levels of circulating thyroid hormones appear to be important for both oocyte and granulosa cell maturation [4–7]. The intracellular action of thyroid hormones, such as triiodothyronine (T3) and thyroxine (T4), are mediated *via* thyroid hormone nuclear receptors (TRs). The receptors are encoded by two genes, α and β, with four triiodothyronine-binding receptor isoforms (α1, β1, β2 and β3). Thyroid hormone receptors were found in human ovarian surface epithelium and were reported to affect ovarian follicles [8]. Our previous studies demonstrated that thyroid hormones and their receptors play an essential role in follicular development in postnatal and immature rats [9].

Nitric oxide (NO) regulates physiological processes within the reproductive system [10–12]. NO is a reactive species synthesized from L-arginine by NO synthase (NOS), and occurs in three isoforms: brain NOS (bNOS) or neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS), and inducible NOS (iNOS) [10]. Neuronal NOS (nNOS) and eNOS together form constitutive NOS (cNOS), which requires calcium/calmodulin for its activation [10]. Our previous studies showed the presence of NOS in the ovaries of immature rats [11] and the involvement of NO in follicular development [12]. NO was also found to inhibit follicular steroidogenesis in the pig [13].

The effects of thyroid dysfunction on animal reproduction, including ovulatory failure, preterm delivery, miscarriages and cycle abnormalities, have been reported in many studies [6–9]. Hyperthyroidism increased serum LH levels during the follicular and luteal phases of the menstrual cycle [14]. In women, both hyper- and hypothyroidism were associated with menstrual disorders and reduced fertility [15]. Moreover, estrogen levels were two-to-three times higher in hyperthyroid than in hypothyroid women [16]. In addition, hypothyroidism affected the length of the menstrual cycle and amount of menstrual bleeding [17]. The aim of the present study was to evaluate the short-term influence of thyroid hormones on ovarian follicular development in prepubertal rats. To further evaluate how hyperthyroidism and hypothyroidism influence folliculogenesis, we also examined the effects of thyroid dysfunction on NOS activity and NO content in the rat ovary.

2. Materials and methods

2.1. Animals and experimental design

Thirty immature female Sprague–Dawley rats (21 days old) were obtained from the Experimental Animal Center of Nanjing Medical University, China. Rats were randomly assigned to three groups (10/group): hyperthyroid (hyper-T), hypothyroid (hypo-T), or euthyroid (control) group, and received feed and water *ad libitum*. Hypothyroidism was induced by feeding with methimazole (MMI; Sigma, St. Louis, MO, USA) at a concentration of 0.04 g/100 mL in water for 10 days [18]. Hyperthyroidism was induced by i.p. injections of T4 (30 μg/100 g BW) (Sigma, St. Louis, MO, USA) for 10 days [19]. The control group received a daily i.p. injection of 0.9% saline (2 mL/100 g BW). To evaluate the short-term effects of hyper- and hypothyroid treatment on ovarian follicular development, immature rats in all three groups were injected with equine chorionic gonadotropin (eCG; 10 IU; Ningbo Second Hormone Co. Ltd., Ningbo, China) at 30 days of age, followed by an injection of an ovulatory dose (30 IU) of human chorionic gonadotropin (hCG; Ningbo Second Hormone Co. Ltd., Ningbo, China) 48 h later (both dissolved in 0.9% saline, and administered i.p.). The animals were anesthetized with ether, and blood samples were collected from the retro-orbital sinus. The blood samples were allowed to clot, centrifuged, and the serum was collected for hormone assays. Rats were then sacrificed by cervical dislocation after euthanization with ether on PND (postnatal day) 33, and the ovaries were collected under a stereomicroscope. Ovaries were immediately washed three times with ice-cold Hank's solution. One ovary was fixed in 40 g/L paraformaldehyde at room temperature (RT) for 24 h and then kept in 70% alcohol for histology and immunohistochemistry; the contralateral ovary was frozen and kept in −80 °C for measurement of NOS activity, NO content and estradiol-17β concentration (E2). Animal protocols were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University, and were performed according to the Guide for the Care and Use of Laboratory Animals.

2.2. Radioimmunoassay of hormones

Serum concentrations of T3, T4 and E2 were determined by commercial RIA kits (Shanghai University of Traditional Chinese Medicine, Shanghai, China) by the General Hospital of Nanjing Military Area Command. The sensitivities of the T3, T4 and E2 assays were 0.2 ng/mL, 5 ng/mL and 5 pg/mL, respectively. The intra-assay coefficients of variation were <10% and inter-assay coefficients of variation were <15%.

2.3. Histology and immunohistochemistry

The ovaries kept in 70% alcohol, were dehydrated in increasing alcohol concentrations and embedded in paraffin. They were then serially sectioned at 10 μm, stained with hematoxylin and eosin (H&E), and the sections were observed under a microscope (Nikon, NY, USA). Follicular stages were determined as previously reported [20], *i.e.*, primordial follicles contained a single layer of flattened granulosa cells, primary follicles were lined by a single or two layers of cuboidal granulosa cells, secondary follicles had more than two layers of cuboidal granulosa cells and no antrum, antral follicles contained an antrum, and large antral follicles were larger than 400 μM in diameter.

Paraffin-embedded ovarian samples from all three groups were sectioned at 5 μm and mounted on slides for immunohistochemical staining with the use of anti-rabbit polyclonal antibodies for nNOS, iNOS, and eNOS (Boster Biological Technology, Wuhan, China) and TRα1 (Abcam, Cambridge, MA, USA). The sections were incubated at RT overnight with nNOS (1:100), iNOS (1:100), eNOS (1:100) and TRα1 (1:100) antibodies. The immunostaining was visualized using the Elite ABC kit (Bio Genex, San Ramon, CA, USA, containing a secondary antibody: peroxidase-conjugated goat anti-rabbit immunoglobulin G) and 0.05% 3,3′-diaminobenzidine