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Reduced testicular steroidogenesis in tumor necrosis factor-α knockout mice

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ABSTRACT

We previously demonstrated that the expression of Mullerian inhibiting substance (MIS) in Sertoli cells is downregulated by tumor necrosis factor alpha (TNF-α), which is secreted by meiotic germ cells, in mouse testes. Several studies have reported that MIS that is secreted by Sertoli cells inhibits steroidogenesis and, thus, the synthesis of testosterone in testicular Leydig cells. Here, we demonstrate that in TNF-α knockout testes, which show high levels of MIS, steroidogenesis is decreased compared to that in wild-type testes. The levels of testosterone and the mRNA levels of steroidogenesis-related genes were significantly lower after puberty in TNF-α knockout testes than in wild-type testes. Furthermore, the number of sperm was reduced in TNF-α knockout mice. Histological analysis revealed that spermatogenesis is also delayed in TNF-α knockout testes. In conclusion, TNF-α knockout mice show reduced testicular steroidogenesis, which is likely due to the high level of testicular MIS compared to that seen in wild-type mice.

1. Introduction

In testicular Leydig cells, steroidogenesis is primarily controlled by the pituitary gonadotropin luteinizing hormone (LH) [1]. LH stimulates the production of cAMP, the intracellular second messenger [1], which regulates testosterone production through both acute and chronic stimulation [2–4]. Acutely, the cAMP-dependent PKA signal pathway activates the transfer of cholesterol into the mitochondria by the steroidogenic acute regulatory protein (StAR) [2,3]. Chronically, cAMP stimulates the expression of steroidogenic enzymes and the activities of these genes. In the mitochondria, cholesterol is converted to pregnenolone by the cholesterol side-chain cleavage enzyme (P450scc). Pregnenolone is sequentially converted to progesterone by 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (3β-HSD), to 17α-hydoxyprogesterone and then to androstenedione by 17α-hydroxylase/C_{17-20} lyase (P450c17), and finally to testosterone by 17β-hydroxysteroid dehydrogenase [4].

Mullerian inhibiting substance (MIS), also known as anti-Mullerian hormone (AMH), is a hormone of the TGF-β family. MIS plays an essential role in normal sex differentiation by causing the regression of Mullerian ducts in embryos [7]. Several reports have demonstrated that MIS inhibits Leydig cell development and testicular steroidogenesis [11–15]. MIS and MIS receptor-deficient mice exhibit Leydig cell hyperplasia [8,9], and overexpression of MIS decreases the serum testosterone level in adult mice [10]. Moreover, transgenic mouse testes overexpressing MIS showed decreased expression of P450c17, while MIS knockout testes showed increased expression of P450c17 [11]. As expected, MIS inhibited the protein kinase A-induced expression of P450c17 in a mouse Leydig cell line [14]. MIS was also shown to inhibit testosterone synthesis, and to repress the expression of P450c17 in adult rats [13].

TNF-α has been reported to inhibit gonadotropin-stimulated testosterone production in goldfish [5]. Moreover, we previously showed that tumor necrosis factor alpha (TNF-α) downregulates the expression of MIS, and thus, TNF-α knockout testes show higher and more prolonged *MIS* expression than wild-type testes from around the time of puberty [6]. The downregulation of MIS expression by TNF-α was modulated through the repression of SF-1 transactivation by NF-κB, the downstream effector of TNF-α [6].

In the present study, we investigated the steroidogenesis and spermatogenesis of TNF-α knockout mice compared with those of the wild-type mice. Because TNF-α knockout testes show higher levels of MIS than wild-type testes [6], and because the MIS level

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affects testicular steroidogenesis [11–14], we proposed that TNF-α knockout testes may have altered steroidogenesis and spermatogenesis. Our results revealed that testicular steroidogenesis was inhibited and spermatogenesis was delayed in TNF-α knockout mice, producing fewer sperm than wild-type mice.

2. Materials and methods

2.1. Animals

TNF-α knockout (B6;129S6-Tnf^{tm1Gkl}, Stock No. 003008) and wild-type control (B6;129SF2, Stock No. 101045) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were kept and bred in a cage with water and chow available, and were maintained under controlled conditions (12 h light and dark photoperiod, 50% humidity, 22 °C). The ethical treatment of animals in this study was carried out according to the standards of the National Institutes of Health.

2.2. Radioimmunoassay (RIA)

The level of testicular testosterone was measured by radioimmunoassay. Dissected testes were homogenized in phosphate-buffered saline (PBS; 0.01 M, pH 7.2), and testosterones were extracted out three times with three volumes of diethyl ether. The assay procedure was performed as previously described [16], using labeled testosterone (1,2,6,7-3H-testosterone, 96 Ci/mol) obtained from NEN. The testosterone antiserum was developed in rabbit using testosterone-17-β-hemisuccinate; BSA as an immunogen. The testosterone antiserum cross-reacts 0.6% with 5α-androstene-3α,17β-diol, 1.3% with 5α-androstene-3β,17β-diol, 1.7% with androstenedione, 0.2% with dehydroepiandrostenedione, 2.8% with 11β-hydroxytestosterone, 0.1% with progesterone and estradiol-17β. Two sets of standard (5–500 pg) and samples were routinely included in each assay. The amounts of radioactivity were measured by using a liquid scintillation analyzer (Tri-carb 2300, Packard), and testosterone concentrations were calculated with the Ria smart program (Packard). Between and within assay coefficients of variation (CVs) for testosterone were 8.7% and 9.3%, respectively. The lower limits of assay sensitivity for testosterone were 2.5 pg.

2.3. Northern blot analysis

Total RNA was isolated using TRI Reagent™ (Sigma). Twenty micrograms of total RNA were fractionated by electrophoresis on a 1% agarose gel containing formaldehyde, and were then transferred to nylon membranes (Zeta-probe, Bio-Rad, Richmond, CA) by capillary blotting with 10X sodium citrate–sodium chloride (SSC). After ultraviolet (UV) cross-linking and prehybridization, membranes were hybridized overnight at 42 °C in a solution containing 50% formamide, 10% dextran sulfate, 5X SSC, 1 mM EDTA, 10 mg/ml denatured salmon sperm DNA, and a total of $(2–4) \times 10^6$ cpm of ^{32}P-labeled mouse P450c17, P450scc, and StAR cDNA probes. After hybridization, membranes were washed twice for 5 min at room temperature in 2X SSC and 0.1% SDS, followed by washing for 30 min at 65 °C in 0.5X SSC and 0.1% SDS. Membranes were then exposed using Kodak RX films (Eastman Kodak Co., Rochester, NY) for 12–24 h at −70 °C. The signals were normalized to the 18S ribosomal RNA internal control.

2.4. Testis weights and sperm counts

Mice were weighed and sacrificed. Testes were removed and weighed individually. The epididymis was enucleated to the vas deferens in mice, and was chopped in PBS. Sperm was pressed out from the epididymis and incubated at 37 °C for 15 min. Sperm was diluted with PBS to 10 times its original volume. The diluted sperm was placed in a hemocytometer and counted under a microscope.

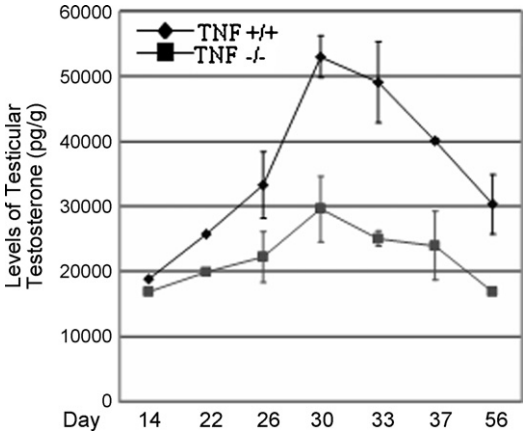

Fig. 1. Reduced testicular testosterone levels in TNF-α knockout mice. Testicular testosterone levels were measured by radioimmunoassay in 14-, 22-, 26-, 30-, 33-, 37-, and 56-day-old testes from TNF-α wild-type and knockout mice. The results indicate the mean ± S.D. of three independent experiments.

2.5. Histology

Testes were dissected from male mice at different developmental stages, and were then fixed in Bouin's fixative (Sigma) overnight at 4 °C. The tissues were embedded in paraffin blocks and cut into 5 μm sections. The sections were deparaffinized with Histoclear (Amresco) and rehydrated according to standard procedures. The tissue sections were treated with hematoxylin and periodic acid-Schiff reagent (Sigma) to stain the nucleus and acrosome, respectively. The developmental stage of seminiferous tubules was determined as described previously [17].

3. Results and discussion

3.1. Decreased testicular testosterone level in TNF-α knockout mice

MIS has been reported to regulate testosterone production *in vivo* and *in vitro* by downregulating the expression of steroidogenic enzymes, at least P450c17 [11–14]. Since previous results revealed that TNF-α knockout testes showed high and prolonged MIS expression from puberty [6,18], we investigated the testosterone level in TNF-α knockout testes to further confirm the testicular function of MIS *in vivo* (Fig. 1). In both TNF-α wild-type and knockout testes, the testosterone levels were increased by day 30, decreasing thereafter. However, the testicular testosterone level of TNF-α knockout mice from puberty to adulthood was kept significantly lower than that of wild-type mice. This result suggests that the high level of MIS resulting from the knockdown of TNF-α decreases testicular testosterone production in TNF-α knockout mice.

3.2. Reduced expression of steroidogenic enzymes in TNF-α knockout testes

To investigate the mechanism of the down-regulation of the testosterone level in TNF-α knockout mice, we first analyzed mRNA levels of steroidogenic enzymes in TNF-α knockout testes. TNF-α knockout testes expressed the mRNA encoding P450c17, P450scc, and StAR genes at a similar level to wild-type testes at postnatal day 14. However, P450c17 and P450scc mRNA levels of TNF-α knockout