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Testosterone production by a Leydig tumor cell line is suppressed by hyperthermia-induced endoplasmic reticulum stress in mice

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ABSTRACT

Aims: Leydig cells are characterized by their ability to produce testosterone. When the Leydig cells are unable to produce enough testosterone, spermatogenesis fails completely. Considering this, it is of great interest to investigate whether the expressions of steroidogenic enzymes are affected by testicular heat stress. This study aimed to demonstrate that heat induced ER-stress significantly influences steroidogenic enzyme expression and testosterone production in the Leydig cells.
Main methods: C57BL/6 mice were subjected to repetitive testicular heat-treatment at 42 °C for 15 min per day, and heat-treated mLTC-1 cells following hCG treatment for 1 h. The protein and RNA expressions were measured by Western blot, RT-PCR. The testosterone and progesterone levels were detected by EIA. The histological and pathological characteristics using hematoxylin and eosin (H&E) and antibody stains.
Key findings: The 3β-HSD expression was decreased by heat-stress and hCG treatment. While the GRP78/BiP and CHOP levels were increased by ER-stress inducers, those of the steroidogenic enzyme and progesterone were decreased. In contrast, an ER-stress inhibitor rescued the testosterone levels, even under heat-stress conditions. Moreover, the Leydig cells were randomly scattered, and severely damaged upon repetitive testicular heat-treatment. Additionally, immunohistochemical analyses revealed that cleaved caspase-3 was elevated in the testicular Leydig cells, and rescued by TUDCA. Thus, repetitive testicular heat-treatment in mice promotes excessive ER-stress, thereby leading to apoptosis of the Leydig cells and thus, decreased testosterone production.
Significance: Our findings help to provide an ER-stress mediate mechanistic explanation to the impairment of spermatogenesis upon elevation of the testicular temperature.

1. Introduction

The testes of most mammals are more susceptible to damage by high temperature than the other organs [29]. In concert, numerous studies across species have reported the adverse effects of hyperthermia on spermatogenesis in the normal adult testis [6,11,22]. The testosterone is produced by the Leydig cells in the testis, which plays an important role in spermatogenesis [7,37,39,42]. Unfortunately, the cellular and molecular mechanism underlying such effects of elevated testicular temperature on the testosterone production and expression of the steroidogenic enzymes in the Leydig cells are poorly described. The testosterone production in turn depends on the secretion of the luteinizing hormone (LH) by the pituitary gland [23]following the binding of LH to receptors on the Leydig cells, promoting the transfer of cholesterol to the inner mitochondrial membrane through the steroidogenic acute regulatory (StAR) protein [32,20]. Next, the conversion of cholesterol to pregnenolone is catalyzed by the P450 side chain cleavage enzyme (CYP11A1). Pregnenolone then moves out of the mitochondria to the endoplasmic reticulum (ER), where it is converted to progesterone by the 3β-hydroxysteroid dehydrogenase enzyme (3β-HSD). Finally, the progesterone is metabolized to testosterone by 17α-hydroxylase (CYP17) and 17β-hydroxysteroid dehydrogenase (17β-HSD) [25]. A testis damaged by heat-stress produces chaperone proteins such as the heat shock protein 70 (HSP70). The increase in the levels of such proteins is a cellular response to adapt and survive under elevated testicular temperatures [15]. Pertinently, the ER is an important organelle required for cell survival and maintenance of the cellular homeostasis under stress conditions. To relieve stress, the ER activates the intracellular signal transduction pathways, collectively termed the unfolded protein response (UPR) [26]. The GRP78/BiP protein is involved in sensing misfolded protein accumulation in the ER, and in conjunction with three other ER transmembrane proteins (ATF6, IRE1, and PERK), is responsible for the UPR [34]. Under normal conditions, these transmembrane proteins exist in a complex with the ER chaperone protein,

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GRP78/Bip [3]. However, under ER stress, the unfolded proteins promote the dissociation of GRP78/BiP by inducing the phosphorylation and relocalization of the transmembrane proteins. This further leads to transcription of chaperone protein, activation of ER stress-mediated degradation, and translational inhibition [28]. However, chronic or unmitigated ER stress induces apoptosis by activating the pro-apoptotic C/EBP homologous protein (CHOP) and caspase through three UPR pathways [38,33]. In the present study, therefore, we have investigated whether hyperthermia induces ER stress, and whether this stress modulates steroidogenic enzyme expression in the mLTC-1 tumor cell line. In addition, we have examined whether repetitive testicular hyperthermia in mice promotes ER stress, thereby leading to apoptosis of the Leydig cells and decreased testosterone production.

2. Materials and methods

2.1. Reagents

The hCG was commercially obtained from Intervet (Chorulon, Milton Keynes, Buckinghamshire, UK). Brefeldin A (BFA), and thapsigargin (Tg) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Tunicamycin (Tm) and tauroursodeoxycholic acid (TUDCA) were purchased from Calbiochem (La Jolla, CA, USA).

2.2. Cell culture

The mLTC-1 mouse Leydig tumor cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured at 37 °C and 5% CO_2 in a 95% air incubator. The RPMI 1640 medium was supplemented with 10% fetal bovine serum (FBS) (Hyclone, GE Healthcare Life Sciences, Logan, Utah, USA) and 1% penicillin/streptomycin (Welgene, Daegu, Korea). The cells were subcultured at a density of 2.5×10^5cells/well in 6-well plates.

2.3. Induction of heat or chemical stress on the ER of mLTC-1 cells

When the cells grew to 80% confluency in each of the 6 wells, they were pre-treated with 1% FBS in RPMI 1640 medium for 12 h, treated with 5 IU/ml of hCG for 3h, followed by incubation at 42 °C in 5% CO_2 in a 95% air incubator for 1h. For chemical stress induction, the cells grown to 80% confluency were pre-treated with 1% FBS in RPMI 1640 medium and Tm (2 μg/ml), Tg (2.5 μM), and BFA (2 μM) for 12 h. This was followed by treatment with 5 IU/ml of hCG for 3h.

2.4. Induction of testicular heat-stress

We maintained in accordance with the institutional guidelines of the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Korea) and male C57/BL6 mice (9–10 weeks of age) were purchased from Central Animal Laboratory (Korea). The mice were administered TUDCA (250 mg/kg/day) as an ER stress inhibitor by intraperitoneal injection. Control was also administered by i.p injection with PBS. One hour after the administration of TUDCA or saline, the mice were subjected to three, or five cycles of heat-treatment at 42 °C for 15 min per day. Subsequently, the animals were dried, returned to their cages, and allowed to recover from the effect of anesthesia. They were sacrificed 12 h after the heat treatment.

2.5. Western blot analysis

Lysates of the mLTC-1 cells and the total testis tissue were prepared in ice-cold PRO-PREP buffer (iNtRON Biotechnology Inc., Daejeon, Korea). The proteins were resolved on 8–12% SDS-polyacrylamide gels, and then transferred to a nitrocellulose membrane (Pall life sciences, NY, USA). The membrane was blocked with blocking buffer, and incubated with the following antibodies: anti-HSP70 (Abfrontier, Seoul, Korea) anti-GRP78/BiP, anti-CHOP (Cell Signaling, Beverly, MA, USA), and anti-3β-HSD (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following incubation, the membranes were washed and incubated with anti-goat IgG (Abfrontier, Seoul, Korea), anti-rabbit, and anti-mouse IgGs (Thermo Fisher Scientific, Waltham, MA 02454, USA) conjugated with horseradish peroxidase for 2 h at room temperature. After the removal of the excess antibodies by washing, specific binding was detected using an ECL kit (Bio-Rad, Hercules, USA). The anti-β-actin antibody (Abfrontier, Seoul, Korea) was used as a loading control. The band intensities were analyzed using the Image J software (National Institutes of Health).

2.6. RNA extraction and reverse transcript (RT)-PCR

Total RNA was isolated from both the mLTC-1 cells and the testes tissues using TRI solution (Bio Science Technology, Daejeon, Korea) according to the manufacturer's instructions. The cDNAs were synthesized using 1μg of each RNA sample and Accupower® RT-PCR premix (Bioneer, Daejeon, Korea). The PCR was carried out using the Hot Start PCR premix (Bioneer, Daejeon, Korea) containing primers specific to the ER-stress markers and steroidogenic enzymes (Table 1).

2.7. Progesterone and testosterone assays by EIA

To measure the progesterone and testosterone levels, the mLTC-1 cell culture media were collected in serum-free culture medium following the respective treatments. Meanwhile, the blood was collected from the abdominal arteries of the mice after the respective heat treatments. Both the media and the sera were separated by centrifugation at 5000 rpm for 10 min, at 4 °C and then stored at −70 °C until used for the assays. The progesterone and testosterone productions were assessed using the respective immunoassay (EIA) kits (ALPCO, Salem, NH, and Enzo Life Sciences Inc., Plymouth Meeting, PA, USA, respectively) according to the manufacturers' instructions. The progesterone and testosterone concentrations for each sample were calculated using the standard graph, and expressed in pg/mL.

2.8. Hematoxylin and eosin (H&E) staining & immunohistochemistry

The testes isolated from the mice were fixed with 10% neutral buffered formalin (Sigma-Aldrich) overnight, embedded in paraffin, and processed into 5 μm-thick sections. The sections were then stained with H&E using procedures as described previously [24].

For immunohistochemistry, deparaffinized sections were briefly heated for 4 min in a pressure cooker containing 10 mM citrate buffer (pH 6.0) for antigen retrieval. Subsequent procedures were conducted at room temperature. Sections were pretreated with 3% H2O2 in 0.1 M TBS (pH 7.4) for 30 min to quench endogenous peroxidases. The sections were treated with a protein block solution (Dako, Carpinteria, CA, USA) for 20 min and incubated with antibodies against GRP78/BiP and cleaved caspase-3 (Cell Signaling, Beverly, MA, USA) for 30 min in a humidified chamber. After washing with 0.1 M Tris base saline (TBS) containing 0.01% Tween-20 (TBST), the sections were incubated with an anti-rabbit polymer (Dako) for 30 min. The peroxidases bound to the antibody complex were visualized by treatment with a 3, 30-diaminobenzidine (DAB) chromogenic substrate solution (Dako). The DAB reaction was monitored under a microscope to determine the optimal incubation time, and stopped with several washes of 0.1 M TBS. The stained and immunolabeled sections were then dehydrated in a graded ethanol series, defatted in xylene, and mounted. The sections were observed under a BX51 microscope (Olympus, Tokyo, Japan) in a bright field, and the images were acquired with a DP 70 camera (Olympus).