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T-2 toxin inhibits gene expression and activity of key steroidogenesis enzymes in mouse Leydig cells

Jian Ying Yang [a,1], Yong Fa Zhang [a,b,*,1], Xiang Ping Meng [a], Yuan Xiao Li [c], Kai Wang Ma [a], Xue Fei Bai [a]

[a] College of Medical Technology and Engineering, Henan University of Science and Technology, Luoyang, Henan 471003, China
[b] College of Food and Bioengineering, Henan University of Science and Technology, Luo Yang, Henan 471023, China
[c] College of Animal Science & Technology, Henan University of Science and Technology, Luo Yang, Henan 471003, China

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ABSTRACT

T-2 toxin is one of the mycotoxins, a group of type A trichothecenes produced by several fungal genera including *Fusarium* species, which may lead to the decrease of the testosterone secretion in the primary Leydig cells derived from the mouse testis. The previous study demonstrated the effects of T-2 toxin through direct decrease of the testosterone biosynthesis in the primary Leydig cells derived from the mouse testis. In this study, we further examined the direct biological effects of T-2 toxin on steroidogenesis production, primarily in Leydig cells of mice. Mature mouse Leydig cells were purified by Percoll gradient centrifugation and the cell purity was determined by 3β-hydroxysteroid dehydrogenase (3β-HSD) staining. To examine T-2 toxin-induced testosterone secretion decrease, we measured the transcription levels of 3 key steroidogenic enzymes and 5 enzyme activities including 3β-HSD-1, P450scc, StAR, CYP17A1, and 17β-HSD in T-2 toxin/human chorionicgonadotropin (hCG) co-treated cells. Our previous study showed that T-2 toxin (10^{-7} M, 10^{-8} M and 10^{-9} M) significantly suppressed hCG (10 ng/ml)-induced testosterone secretion. The studies demonstrated that the suppressive effect is correlated with the decreases in the levels of transcription of 3β-HSD-1, P450scc, and StAR ($P < 0.05$) and also in enzyme activities of 3β-HSD-1, P450scc, StAR, CYP17A1, and 17β-HSD ($P < 0.05$).

1. Introduction

T-2 toxin is one of the mycotoxins, a group of type A trichothecenes produced by several fungalgenera including the *Fusarium* species (Cortinovis et al., 2013; IARC, 1993). It has been acknowledged as an unavoidable contaminant in human food, animal feed and agricultural products such as maize, wheat and oats, and has been reported in many parts of the world (WHO, 1990). Upon acute exposure to high dose of trichothecenes, animals exhibit clinical signs such as diarrhea, vomiting, leukocytosis and haemorrhage (Ueno, 1984). At extremely high dose, trichothecenes can cause shock-like syndromes that can result in death. Chronic exposure to trichothecenes can cause anorexia, weight loss, diminished nutritional efficiency, neuro-endocrine changes and immune modulation (Rotter et al., 1996).

Among the trichothecenes, T-2 toxin is the most toxic compound (Gutleb et al., 2002). Studies have demonstrated the negative effects of exposure to T-2 toxin on the immune system and digestive system, including the oral cavity, esophagus, and stomach (Canady et al., 2001). In addition, Studies have demonstrated the toxic effects of T-2 toxin on the semen quality, fertility and serum testosterone concentration in mice (Yang et al., 2010). However, these reproductive toxicities of T-2 toxin for testicular function have mostly relied on the *in vivo* approach of using animal models. Complications in pharmacokinetic distribution and secondary effects attributed to other unidentified factors may make it difficult to decipher the direct mechanistic toxicities of T-2 toxin to the cells.

It is well known that Leydig cells play a crucial role in synthesizing testosterone and regulating the process of spermatogenesis. The alteration of Leydig cell function can lead to adverse effects on testicular functions. Therefore, we researched Leydig cells in mice to determine direct biological effects of T-2 toxin to validate the *in vivo* findings, and the results showed a direct suppression of testosterone secretion (Yang et al., 2014). However, no detailed data concerning the effects of exposure to this toxin on the molecular mechanism of decreasing the testosterone secretion are available. In this study, the aim was to elucidate the effects of T-2 toxin on hCG-stimulated steroidogenesis in Leydig cells of mice. To determine the molecular mechanism, the effect of T-2

* Corresponding author at: College of Food and Bioengineering, Henan University of Science and Technology, Luo Yang, Henan 471023, China. Tel.: +86 379 64282342.
E-mail address: zyf_1001@haust.edu.cn (Y.F. Zhang).
[1] These authors contributed equally to this work.

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toxin on hCG-stimulated mRNA levels and activities of key steroidogenic enzymes were measured.

2. Materials and methods

T-2 toxin was obtained from Sigma Chemical Company (St Louis, MO). A stock solution was prepared in dimethyl sulfoxide and was stored at −20 °C. The working solution was prepared by dispensing the stock solution into fresh sterilized peanut oil.

All experimental animal use and experimental design for this study was approved by the Chinese Association for Laboratory Animal Sciences.

2.1. Cell culture

Leydig cells were isolated from the testis of 60–90 day-old Kunming mice. The cells were cultured for 2 days according to Biegel et al. (1995). The testis was decapsulated, and digested in Erlenmyer flasks within an oscillating incubator (100 r.p.m and 34 °C) for 15 min by M199 medium containing 0.05% collagenase and 1% BSA. The suspension cells were transferred to a 50 ml tube and kept on ice for 2 min to allow the tubules to settle. The supernatant containing Leydig cells was filtered through a 70 μm nylon cell strainer (BD Biosciences). The cells were centrifuged at 350 g for 20 min at 4 °C. The pellet was re-suspended in 10 ml M199 and loaded onto the top of a Percoll gradient (5%, 30%, 58% and 70%) (Sigma) and centrifuged at 800 g for 30 min at 4 °C. The cells in the third layer were collected, washed twice with M199 medium, and were re-suspended in phenol red free-DMEM/F12 (1:1) containing 10% charcoal stripped fetal calf serum (GeminiBio-Products, Woodland, CA, USA), 50 U/ml penicillin and 50 μg/ml streptomycin (GIBCO/BRL, Carlsbad, CA, USA). The cells were plated at a density of 10^5 cells/cm^2 in 24-well plates (Nunc, Nalge Nunc International, Rochester, NY, USA) at 0.5 ml/well and maintained at 37 °C with 5% CO_2.

2.2. Histochemical staining of 3β-HSD and testosterone induction assay

Following 2 days of incubation, the purity of Leydig cells was examined by histochemical staining for 3β-hydroxysteroid dehydrogenase, according to the histochemical method reported by Mendelson with some modifications (Mendelson et al., 1975). In brief, Leydig cells were incubated in a 24-well plate with 0.4 ml/well staining solution containing 0.05 M PBS, pH 7.4 supplemented with 0.2 mg/ml nitro-blue tetrazolium (Sigma Chemical Co.), 1 mg/ml NAD and 0.12 mg/ml dehydroepiandrosterone (Sigma Chemical Co) for 90 min at 34 °C. The positive cells were stained a dark blue color and the purity of the Leydig cells was observed to be over 90%. Secondly, in the testosterone induction assay, Leydig cells were exposed to 10 ng/ml human chorionic gonadotrop (hCG) (Sigma Chemical Co) for 24 h.

2.3. Cell treatment

Two-day cultured Leydig cells grown in phenol red-free DMEM/F12 medium supplemented with 10% charcoal stripped fetal calf serum and antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin) were washed three times in 0.05 M PBS pH 7.4. The conditioned media (phenol red-free DMEM/F12 medium supplemented with 50 U/mL penicillin and 50 μg/mL streptomycin and the corresponding leveled-drugs and 10 ng/mL hCG) were added to the 24-well plates at 0.3 mL/well. The Leydig cells were exposed for 24 h to one of the following four treatments: (i) 10 ng/mL hCG (Sigma) + dimethyl sulfoxide (DMSO) (Sigma) solvent control; (ii)10 ng/mL hCG + 10^{-7} M T-2 toxin (Sigma, St. Louis, Mo.); (iii) 10 ng/mL hCG + 10^{-8} M T-2 toxin; (iv) 10 ng/mL hCG + 10^{-9} M T-2 toxin. The Cell viability was determined by the trypan blue dye-exclusion test, according to Gurina in 2011 (Gurina et al., 2011). The viability of the control and treated cells was over 90%. At the end of the incubation, the treated cells were used for the measurement of mRNA in steroidogenic acute regulatory protein (StAR), cytochrome P450 side-chain cleavage enzyme (P450scc) and 3β-HSD-1.

2.4. Total RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from different treatment groups using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and was followed by deoxyribonuclease I (Life Technologies, Inc.) treatment to remove DNA contamination. One microgram of total RNA isolated from Leydig cells or the ovaries was reverse transcribed using 200 U of Superscript II RNase H-Reverse Transcriptase (Gibco BRL, Bethesda, MD) in a 50 ml reaction volume in the presence of 25 g/ml Oligo (dT), first strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$), 0.01 M dithiothreitol, and 10 mM of each dATP, dGTP, dCTP, and dTTP. The RNA and Oligo (dT) mix were heated at 65 °C for 10 min and then cooled to 4 °C. The other reagents were added and the reverse-transcription (RT) was performed at 42 °C for 1 h.

The PCR of 3β-HSD-1, P450scc, StAR and HPRT (hypoxanthine phosphoribosyl transferase, housekeeping gene) was carried out by utilizing primer-pairs as described by Akingbemi in 2003 (Akingbemi et al., 2003) and Jin in 2000 (Jin et al., 2000) (Table 1). 3β-HSD-1, P450scc and StAR cDNAs were amplified by PCR for 35 cycles (94 °C for 40 s, 55 °C for 30 s, and 72 °C for 1 min); HPRT cDNAs were amplified by PCR for 28 cycles (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 40 s). The sizes of the PCR products (3β-HSD-1, P450scc, StAR and HPRT) were determined by comparison with a gene marker (100 bp DNA, Promega) run in a parallel fashion with RT-PCR products in 1.2% agarose gels containing ethidium bromide. The relative band intensity was quantified using a computer-assisted image analysis system (Visage 2000, BioImage, Ann Arbor, MI). The integrated optical density (IOD) values for 3β-HSD-1, P450scc, StAR and HPRT in each band were normalized with the corresponding HPRT expression.

2.5. Measurement of enzyme activities

Enzyme activities were determined after 24 h of treatment. Fresh medium was added, and all cultures were washed for 1 h to remove treatment agents and to deplete endogenous substrates before the determination of enzyme activities. After the washing period, enzyme activities were determined by incubating dishes for 1 h with a saturating concentration of the appropriate ^{3}H-labeled substrate (5 μM; 0.5 μCi) dissolved in 100 mM dimethylsulfoxide in 1 ml culture medium at 32 °C. 3β-HSD-1, P450scc, StAR, CYP17A1, and 17β-HSD activities were determined according to previously described procedures (Georgiou et al., 1987; Agular et al., 1992; O'Shaughnessy, 1991; Luu-The et al., 1990).

2.6. Calculation of enzyme activity results

To facilitate the comparison of the effects of treatment at different doses of T-2 toxin, the results of enzyme activity determinations at 24 h were expressed as the ratio (%) of the respective activities of hCG-stimulated cultures at different doses of T-2 toxin