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Zearalenone inhibits testosterone biosynthesis in mouse Leydig cells via the crosstalk of estrogen receptor signaling and orphan nuclear receptor Nur77 expression

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ABSTRACT

Zearalenone (ZEA) directly inhibits testosterone biosynthesis in Leydig cells, although the mechanisms involved remains unclear. Various experiments were performed to elucidate the molecular pathway of ZEA-mediated androgen inhibition. Leydig cells were isolated from 6 week-old male ICR mice and subjected to ZEA pre-treatment. The levels of testosterone and a series of influncing factors were measured. The results showed that ZEA caused a concentration- and time-dependent inhibition of testosterone stimulated both by hCG and cAMP ($P < 0.05$). Exposure to ZEA did not affect the LHR binding activity nor the protein expression ($P > 0.05$). However, ZEA exposure significantly elevated the cellular cAMP levels ($P < 0.05$) in low concentrations (5 μg/ml) or for long time periods (24 h), significantly reduce the mitochondrial membrane potential ($P < 0.05$). The expression of P450scc, 17β-HSD, and P450c17 at the mRNA level were significantly decreased ($P < 0.05$). The steroidogenic acute regulatory (StAR) and 3β-HSD expression was significantly increased ($P < 0.05$). Furthermore, the ERα protein expression was not affected by ZEA, but Nur77 expression was significantly inhibited ($P < 0.05$). These observations imply that ZEA activity interferes with testosterone biosynthesis in mouse Leydig cells via the crosstalk of estrogen receptor signaling and Nur77 expression.

1. Introduction

The mycotoxin zearalenone (ZEA) is produced by *Fusarium* fungi that contaminate various foods and animal feeds. Several reproductive disorders in farm animals, as well as some hyperoestrogenic syndromes in humans, have been attributed to ZEA (Minervini and Dell'Aquila, 2008). Serious ZEA contamination of foodstuff and animal feed has been reported worldwide. The trading of cereals and live animals may have contributed to the worldwide dispersal of this *Fusarium* mycotoxin (Zinedine et al., 2007). After oral administration, ZEA is rapidly absorbed and metabolized in liver. The proposed major biotransformation pathway for ZEA is its conjugation with glucuronic acid, as catalyzed by the uridine diphosphate glucuronyl transferases (UDPGTs) (Olsen et al., 1981). Its metabolite form is dependent on the presence of cofactors and the reaction pH (Malekinejad et al., 2005). However, ZEA and the concentrations of its metabolites in liver and bile tend to increase with the administered dosage because of biliary exertion and enterohepatic cycling (Biehl et al., 1993; Doll et al., 2004).

The toxicity of ZEA includes genotoxicity, immunotoxicity, reproductive toxicity, and developmental toxicity, as well as tumorigenesis (Kuiper-Goodman et al., 1987). Its genotoxicity induces DNA adduct formation, DNA fragmentation, and micronuclei production in vitro (Abid-Essefi et al., 2003, 2004; Lioi et al., 2004). ZEA binds to estrogen receptors (ERs) in vitro, with similar affinity for both forms: ERα and ERβ (Kuiper et al., 1998) disrupting the endocrine balance by having effects similar to estrogen, and consequently inducing harm to the reproductive system and its development. Neonatal exposure to zearalenone produces persistent anovulatory estrus in the rat (Kumagai and Shimizu, 1982), In addition, the presence of ZEA (3.12 μM during maturity significantly reduced the percentage of cleaved oocytes that formed blastocysts (Malekinejad et al., 2007). High doses of ZEA (100 and 30 μg) instantly caused arrhythmia, atrioventricular dissociation, and even heart stoppage in chick embryos (Vesely and Vesela, 1995). Furthermore, ZEA can affect sperm parameters in stallions

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at 0.1 nM concentration after 2 h of exposure (Filannino et al., 2011).

Numerous studies revealed that the testis is sensitive to ZEA. Frizzell et al. found that ZEA and its metabolites can act as potential endocrine disruptors at the level of nuclear receptor signaling by altering the hormone production, including testosterone production (Frizzell et al., 2011). Yang and colleagues proved that ZEA can reduce testosterone secretion in vitro and in vivo. In addition, ZEA and its metabolites influence the expression of some steroidogenic enzymes at the mRNA level (Yang et al., 2007a,b). This finding may be crucial for understanding the mechanism by which ZEA inhibits androgen production, although the concrete mechanisms involved remains unresolved. Several experiments were designed and conducted in this study to identify the site(s) of ZEA-induced testosterone reduction.

2. Materials and methods

2.1. Chemicals

Zearalenone, collagenase type I, Percoll, trypan blue, bovine serum albumin (BSA), Nitrotetrazolium Blue chloride (TLC), and trans-Dehydroandrosterone (DHA) were purchased from Sigma, whereas NAD trihydrate (NAD^+) was from the Amresco Co. Dulbecco's modified Eagles medium with the Hams F-12 nutrient mixture (at a 1:1 ratio; DMEM-F12) was obtained from Gibco, hCG was from the ProSpec Co., and cyclic AMP (cAMP) from the Millipore Co.

2.2. Animals treatment and surgery

5–8 weeks Old male icr-mice were purchased from Comparative Medicine Center of Yangzhou University (the experimental animal production license number: SCXK2012-0004). They were maintained under conditions of controlled temperature (25 °C) and lighting (12 h light:12 h darkness), and every two of them were housed to a cage. The animals were fed with Xietong mouse feed (Xietong Co., Jiangsu, China) and water ad libitum until sacrifice.

Surgical operations were carried out under anesthesia of Pentobarbital Sodium (0.9%, 0.5 ml/100 g body weight; solarbio, Beijing, China). Briefly, abdominal cavity were opened with sterile ophthalmic scissors and testis were removed by cutting the spermatic cords. Immersed the sepreted testis with phosphate buffer solution for the next experiments.

2.3. Isolation and purification of primary Leydig cells

The icr-mouse testes were decapsulated under aseptic conditions. The Leydig cells were isolated by collagenase and purified in a discontinuous Percoll gradient, using the methods described by Kerr et al. (1985) and Lee et al. (1994). Briefly, testes were decapsulated with fine forceps, cut into pieces using ophthalmic scissors, and digested using collagenase in DMEM-F12 at 34 °C for 15 min in a thermostatic shaking water bath. After incubation, DMEM-F12 without collagenase was added to each tube. The resulting liquid aspirated using a Pasteur pipette, and filtered using stainless steel trap valves (200 screen mesh) into sterile centrifuge tubes.

Discontinuous Percoll gradients were utilized to obtain the purified Leydig cells from this crude preparation. For each of the 35, 30, 25, 20, and 15% gradients of Percoll, 2 μL was carefully overlaid onto the preceding layer, while taking care to avoid mixing. Finally, 2 μL of the crude Leydig cell suspension was applied on top of the entire discontinuous gradient. The tubes were subsequently centrifuged at 800×*g* at 4 °C for 30 min. After centrifugation, most of the purified Leydig cells were observed between the 25% and 30% gradients. These cells were carefully collected, resuspended with DMEM-F12, and centrifuged at 250×*g* at 4 °C for 10 min. The resulting supernatant was discarded. To remove any excess Percoll, the cell pellets were washed twice with DMEM-F12.

The viability of the purified Leydig cells was determined by trypan blue exclusion. Briefly, the 100 μL cell suspension was mixed with 5 μL 1% trypan blue (in PBS, pH 7.4). After incubating the samples for 2 min, the number of cells was counted for each sample using a hemocytometer. Leydig cells with at least 95% viability were used for the subsequent experiments.

The purity of the cell cultures was assayed by histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) using the method by Wiebe (1976) with some modifications (Wiebe, 1976). The enzyme substrate used in the test contained 1 mg BSA, 1.5 mg NAD^+, 0.5 mg TLC, and 0.25 mg DHA/mL.

2.4. Experimental treatments

The Leydig cells were cultured in DMEM-F12 with 10% fetal calf serum (FCS). The cells were grown in 6-well plates (with 10^6 cells per well). Two treatment groups were used in vitro: one group was exposed to different ZEA concentrations for a constant time interval (12 h), whereas the other group was exposed to a fixed ZEA concentration (2.5 μg/ml) for different time intervals. The experiments were designed to analyze the effect of ZEA on the synthesis and secretion of testosterone and cAMP, as well as the expression of LHR, ERα, the steroidogenic enzymes, and the orphan nuclear receptor Nur77. This study was conducted to determine whether:

(i) *ZEA inhibits testosterone biosynthesis in Leydig cells*. In our experiment, different groups had concentrations of 0, 1, 5, 10, and 20 μg/mL, which were exposed to ZEA for 1, 6, 12, and 24 h. After 24 h of cell culture, the cells were incubated in fresh medium containing ZEA and 10 IU hCG/ml or 0.2 nmol cAMP/10^6 cells. At the end of the incubation period, the T accumulation was measured.

(ii) *ZEA interferes with the expression or binding activity of LHR*. LHR is a receptor located at the plasmalemma; this protein functions as the only binding site for LH in the testis. After binding to this receptor, LH stimulates the catalysis of cAMP production by adenylate cyclase in the ATP; cAMP functions as a secondary messenger that induces the steroidogenesis in Leydig cells (Hancock et al., 2009). For the subsequent experiments, we chose a relatively high (20 μg/mL) and a relatively low (5 μg/mL) concentration for the concentration-based treatment group, and a relatively longer (24 h) and a relatively shorter (6 h) incubation time for the time-based treatment group.

(iii) *ZEA alters cAMP production*. cAMP is produced by adenylate cyclase, which, in turn, is activated by G-proteins. This secondary messenger is crucial for regulating steroidogenesis in various endocrine systems. After incubating the cells in a medium with ZEA and 10 IU hCG/ml, the intracellular cAMP levels were measured.

(iv) *ZEA reduces the mitochondrial function*. Mitochondria are the primary locations of testosterone biosynthesis. We measured the mitochondrial membrane potential by FCM after its exposure to ZEA.

(v) *ZEA inhibits the expression of genes encoding steroidogenic enzymes*. To avoid the effects of ZEA on LHR or cAMP activity, we used cAMP to determine the expression of genes for steroidogenic enzymes. After the Leydig cells were incubated in a medium containing cAMP (0.2 nmol/10^6 cells) and ZEA, they were collected to prepare the total RNA and to examine the levels of gene expression.