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Full Length Article

Apoptosis inducing factor gene depletion inhibits zearalenone-induced cell death in a goat Leydig cell line

Diqi Yang[a], Tingting Jiang[a], Pengfei Lin[a], Huatao Chen[a], Lei Wang[a], Nan Wang[a], Fan Zhao[a], Keqiong Tang[a], Dong Zhou[a], Aihua Wang[b], Yaping Jin[a,*]

[a] Key Laboratory of Animal Biotechnology of the Ministry of Agriculture, College of Veterinary Medicine, Northwest A&F University, Yangling,712100, Shaanxi, China
[b] Department of Preventive Veterinary Medicine, College of Veterinary Medicine, Northwest A&F University, Yangling, 712100, Shaanxi, China

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ABSTRACT

Zearalenone (ZEA) is a contaminant of human food and animal feedstuffs that causes health hazards. However, the signal pathways underlying ZEA toxicity remain elusive. The aims of this study were to determine which pathways are involved in ZEA-induced cell death and investigate the effect of apoptosis inducing factor (AIF) on cell death during ZEA treatment in the immortalized goat Leydig cell line hTERT-GLC. This study showed that ZEA-induced cell death in hTERT-GLCs works via endoplasmic reticulum (ER) stress, the caspase-dependent pathway, the caspase-independent pathway and autophagy. Recombinant lentiviral vectors were constructed to silence AIF expression in hTERT-GLCs. Flow cytometry results showed that knockdown of AIF diminished ZEA-induced cell apoptosis in hTERT-GLCs. Furthermore, we found AIF depletion down-regulated phosphoIRE1α, GRP78, CHOP and promoted the switch of LC3-I to LC3-II. Therefore, ZEA induces cytotoxicity in hTERT-GLCs via different pathways, while AIF-mediated signaling plays a critical role in ZEA-induced cell death in hTERT-GLCs.

1. Introduction

Zearalenone (ZEA) is a nonsteroidal estrogenic mycotoxin produced by several species of *Fusarium* that contaminates human food and animal feedstuffs around the world [1,2]. Many studies have reported that ZEA is involved in the activation of estrogen-responsive genes, which impairs the function of reproductive organs and leads to ovarian dysfunction and endometrial adenocarcinomas in females [3–5]. In male reproduction, a prior study from our group demonstrated that ZEA reduced cell proliferation and testosterone levels in mouse Leydig cells [6]. Another report showed that treatment with ZEA can activate autophagy and apoptosis in primary rat Leydig cells [7].

Apoptosis inducing factor (AIF), regarded as a key caspase-independent death effector, is a conserved mitochondrial flavoprotein [8]. AIF is cleaved into its mature form (57 kDa) and translocated to the nucleus from the mitochondria to mediate caspase-independent apoptosis, leading to chromatin condensation and DNA fragmentation [9]. Accumulating evidence has confirmed that a decrease in AIF in cells can provide protection against toxins and stress, such as neurotoxin 1-methyl-4-phenylpyridinium (MPP^+), phytosphingosin, and radiation [10–15]. Research by Yu suggested that ZEA induces caspase-independent apoptosis via AIF-mediated signaling in RAW264.7 cells [4].

The endoplasmic reticulum (ER) is an indispensable eukaryotic organelle that synthesizes secretory and transmembrane proteins and helps maintain cellular homeostasis [16]. With perturbations, the ER function experiences interferences, leading to the accumulation of unfolded or misfolded proteins in the ER, resulting in stress [17–19]. To cope with ER stress, the unfolded protein response (UPR) is triggered to promote cell survival. The UPR has three major branches: the activating transcription factor (ATF) 6 pathway, the inositol-requiring enzyme 1α (IRE1α) pathway, and the protein kinase (PKR)-like ER kinase (PERK) pathways [20,21]. Our previous studies have shown that ZEA up-regulated ER stress-related proteins in RAW 264.7 macrophages and mouse Leydig cells [1,2,6]. Other studies have reported similar conclusions that ZEA activates the ER stress response in HCT116 cells [22]. However, little is known about the role of AIF in ER stress-mediated cell death under conditions of toxicity. The present study demonstrated that ZEA induced cell death in hTERT-GLCs via ER stress, the caspase-dependent pathway, the caspase-independent pathway and autophagy. We

* Corresponding author.
E-mail address: yapingjin@163.com (Y. Jin).

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also showed that the knockdown of AIF inhibited ZEA-induced cell death and ER stress and triggered autophagy. Through this study, we aimed to investigate the mechanisms of ZEA exposure on goat Leydig cells in relation to the expression of AIF.

2. Materials and methods

2.1. Reagents

ZEA at 98% analytical standard and the anti-LC3 antibody were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Mediatech, Inc. (Cornig, Manassas, VA, USA). The anti-phosphoIRE1α antibody was purchased from Abcam (Cambridge, UK). Anti-GRP78 was purchased from Cell Signaling Technology (Boston, MA, USA). The anti-AIF, anti-caspase-3 and anti-CHOP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-PARP and anti-BCL-2 antibodies were purchased from Wanleibio Co., Ltd (Shenyang, China). The Cell Counting Kit-8 (CCK-8) proliferation assay and Z-VAD-fmk were purchased from Beyotime Biotechnology Company (Haimen, Jiangsu, China). The Annexin V-PE/7-AAD apoptosis detection kit was purchased from Nanjing Keygen Biotech Co., Ltd. (Nanjing, Jiangsu, China).

2.2. Cell culture and treatments

Primary goat Leydig cells were immortalized by transfection with human telomerase reverse transcriptase (hTERT) to establish the goat Leydig cell line (hTERT-GLCs) [23]. The hTERT-GLCs were positively stained for the Leydig cell marker 3β-hydroxysteroid dehydrogenase (3β-HSD), and hTERT-GLCs at passage 50 showed the normal goat chromosome number [23]. This cell line is well maintained in our laboratory. Therefore, hTERT-GLCs could be used as an excellent model to assess Leydig cell function. The hTERT-GLCs were previously prepared, and no animal resources were used for the present work. The hTERT-GLCs at passage 50 were seeded in 6-well plates cultured in M-199 medium containing 10% FBS and incubated at 37 °C in a humidified 5% CO_2 incubator. The culture medium was changed every 3 days. After the hTERT-GLCs reached 70–80% confluence, the cells were treated with various concentrations (0–240 μM) of ZEA. In the Z-VAD-fmk group, 20 μM Z-VAD-fmk (broad-spectrum caspase inhibitor) was added to the hTERT-GLCs for 1 h before ZEA treatment.

Table 1
Results of CCK-8 for hTERT-GLCs treated with ZEA.

Group	Cell viability (%)
0 μM	99.22 ± 0.69^{a}
15 μM ZEA	87.10 ± 1.96^{ab}
30 μM ZEA	78.20 ± 2.99^{b}
90 μM ZEA	40.55 ± 18.36^{c}
150 μM ZEA	6.00 ± 0.60^{d}
240 μM ZEA	4.54 ± 0.47^{d}

Data represent the mean ± S.E.M. from three independent experiments. Different superscripts within column indicate significant difference, $P < 0.05$.

Table 2
Results of Annexin V-PE/7-AAD apoptosis detection for hTERT-GLCs treated with ZEA.

Group	Cell viability (%)
0 μM	98.03 ± 0.76^{a}
15 μM ZEA	87.06 ± 2.13^{b}
30 μM ZEA	78.20 ± 0.75^{c}
60 μM ZEA	63.76 ± 6.17^{d}

Data represent the mean ± S.E.M. from three independent experiments. Different superscripts within column indicate significant difference, $P < 0.05$.

2.3. Cell morphological changes and viability

The hTERT-GLCs were plated into 96-well plates, and ZEA was added at 0, 15, 30, 90, 150, and 240 μM for 48 h to determine

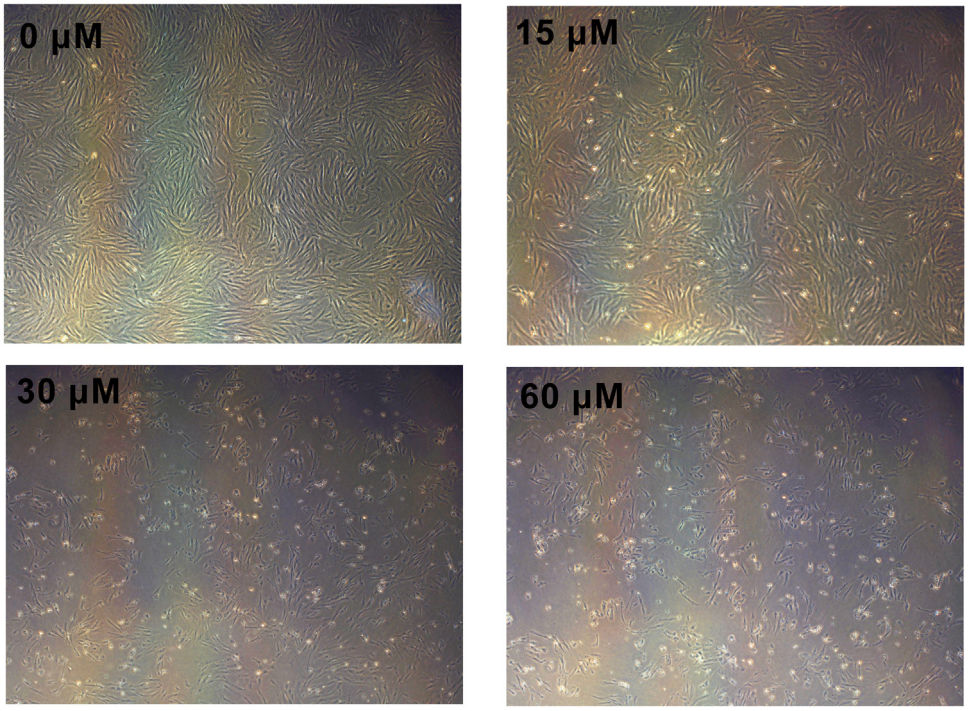

Fig. 1. ZEA induced morphological changes in hTERT-GLCs. The hTERT-GLCs were treated with different concentrations of ZEA (0–60 μM) for 48 h and were photographed to assess morphological changes under 40× magnification. The data are the means ± S.E.M of three independent experiments. Bars with different letters are significantly different ($P<0.05$).