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The dose-dependent effect of zearalenone on mitochondrial metabolism, plasma membrane permeabilization and cell cycle in human prostate cancer cell lines

Karolina Kowalska [a, b], Dominika Ewa Habrowska-Górczyńska [a, b], Kamila Domińska [b], Agnieszka Wanda Piastowska-Ciesielska [a, b, *]

[a] Laboratory of Cell Cultures and Genomic Analysis, Medical University of Lodz, Zeligowskiego 7/9, Lodz, 90-752, Poland
[b] Department of Comparative Endocrinology, Faculty of Biomedical Sciences and Postgraduate Training, Medical University of Lodz, Zeligowskiego 7/9, Lodz, 90-752, Poland

HIGHLIGHTS

- Zearalenone has a concentration-dependent effect on prostate cancer cells.
- The effect of zearalenone on prostate cancer cells is similar to beta-estradiol caused.
- Zearalenone is a potential modulator of prostate cancer cells proliferation.

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ABSTRACT

Zearalenone (ZEA) is a nonsteroidal mycotoxin produced by several fungi of the genus *Fusarium* spp. It is known to play various roles in the regulation of the prostate cancer cell cycle, including carcinogenesis. The present study evaluates the influence of ZEA on the mitochondrial metabolism, plasma membrane permeabilization and cell cycle of prostate cancer cells. At concentrations of 100 nM and 0.3 nM, ZEA caused a decrease in the oxidative activity of mitochondria, as well as increases in LDH release, apoptosis induction and the number of cells in the G0/G1 phase. The opposite effect was observed for lower concentrations (0.1 nM and 0.001 nM). These *in vitro* studies indicate that ZEA might have pro- and antiproliferative properties in prostate cancer cells, at concentrations 0.1 nM, 0.001 nM and 0.3 nM, 100 nM, respectively.

1. Introduction

Zearalenone (ZEA) is a nonsteroidal mycotoxin (macrocyclic β-resocyclic acid lactone), produced by several fungi of the genus *Fusarium* spp. (Wozniak and Murias, 2008), which is present in many maize, wheat and corn products (Zatecka et al., 2014). The average adult intake of ZEA in European countries ranges between 0.8 and 29 ng/kg body mass (Minervini et al., 2005). ZEA is able to mimic oestrogen and bind to oestrogen receptors which can modulate the growth of normal and cancer cells (Kumi-Diaka, 2002). ZEA has been shown to bind to both oestrogen receptors (ERs) ERα and ERβ in a similar way to β-estradiol (Zatecka et al., 2014), but with a 20-fold lower binding affinity (Takemura et al., 2007). It has also been reported to act as an endocrine disruptor in the reproductive system (Kowalska et al., 2016a). An *in vitro* study has shown that ZEA influence testosterone, progesterone and estradiol production in an H295R human adrenocortical carcinoma cell line (Frizzell et al., 2011). It is also well documented that ZEA and its metabolites are able to alter animal fertility (Kowalska et al., 2016a). This alteration has been attributed to their ability to mimic the activity of oestrogen in the reproductive tract, including such accessory glands as the prostate (Metzler et al., 2010). Moreover,

* Corresponding author. Laboratory of Cell Cultures and Genomic Analysis, Medical University of Lodz, Zeligowskiego 7/9, Lodz, 90-752, Poland.
E-mail addresses: klakus@op.pl (K. Kowalska), dominika.habrowska@umed.lodz.pl (D.E. Habrowska-Górczyńska), kamila.dominska@umed.lodz.pl (K. Domińska), agnieszka.piastowska@umed.lodz.pl (A.W. Piastowska-Ciesielska).

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ZEA has a well-documented genotoxicity and reproductive toxicity in animals (Liu et al., 2014a; Zatecka et al., 2014), and is associated with DNA adduct formation and DNA fragmentation (Liu et al., 2014b). Moreover, in addition to its oestrogenic activities, ZEA might also induce oxidative stress and ROS accumulation, influence the antioxidative enzymes, induce apoptosis via the p53 mitochondrial pathway, and modulate Ca^{2+} influx (Ayed-Boussema et al., 2008; Banjerdpongchai et al., 2010; Zatecka et al., 2014). However, the carcinogenicity of ZEA is still disputable. It has been suggested that it could influence the incidence of breast cancer (Ahamed et al., 2001) and oesophageal cancer (Pitt, 2000), but its direct role in prostate cancer has not yet been confirmed (Metzler et al., 2010; Nordby and Kristensen, 2006).

The aim of the study is to evaluate the effect of ZEA on mitochondrial metabolism, plasma membrane permeabilization and cell cycle of prostate cancer cells. As ZEA mimics oestrogens, this study was also designed to compare the influence of ZEA and β-estradiol on post-treatment cell behaviour. Therefore, the cell line chosen for the study was a human prostate adenocarcinoma PC3 line derived from a bone metastatic site of grade IV prostatic adenocarcinoma; medium metastatic (Kowalska et al., 2016b), androgen-independent cells with an average ERα/ERβ ratio (Verma et al., 2014).

It was decided to use ZEA in the range 100 nM–0.001 nM on the basis of previous studies examining the influence of ZEA on cell metabolism (Frizzell et al., 2011; Khosrokhavar et al., 2009; Minervini et al., 2005) and preliminary concentration-response experiments. Similarly, a period of 72 h was chosen for ZEA/β-estradiol exposure based on the results of previous studies which had used times ranging from 48 h (Frizzell et al., 2011), 3 or 4 days (Minervini et al., 2005) or 7 days (Khosrokhavar et al., 2009).

2. Materials and methods

2.1. Cell culture

The PC3 human prostate cancer cell line was purchased from DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The cells were cultured in a humidified incubator at 37 °C with 5% CO_2 in RPMI 1640 medium (Thermo Fisher Scientific Inc/Life technologies, Waltham, MA, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Thermo Fisher Scientific Inc/Life technologies), 1 mM Sodium Pyruvate (Thermo Fisher Scientific Inc/Life technologies), 10 mM Hepes Buffer (Thermo Fisher Scientific Inc/Life technologies), and antibiotics (Penicillin 50 U/ml; Streptomycin 50 μg/ml; Neomycin 100 μg/ml) (Thermo Fisher Scientific Inc/Life technologies). Before each experiment, PC3 cells were serum deprived and cultured in phenol red-free medium.

Stock solutions of ZEA (Sigma-Aldrich, Saint Louis, MO, USA) and β-estradiol (Sigma-Aldrich) were prepared in methanol and ethanol, respectively. The final concentrations of test ZEA and β-estradiol were achieved by the addition of phenol red-free RPMI culture medium. To exclude the potential influence of the solvent on mitochondrial activity, the cells were treated with methanol/ethanol at the highest concentrations used in the study. The methanol and ethanol treatment volume was negligible and represented an average of 0.00025% and 0.33% of the total medium volume, respectively. WST-1 test was done for those doses and no statistically significant decrease in viability was observed. Thus, for the rest of experiment non-treated cells were used as control cells. For all experiments, the cells were treated with ZEA at concentrations of 100 nM, 0.3 nM, 0.1 nM and 0.001 nM for 72 h. Additionally, the cells were exposed to β-estradiol at supraphysiological (100 nM), extremely high (100 μM), intermediate (0.3 μM) and low (1 nM) concentrations for 72 h (Fang et al., 2015; Figueira et al., 2015; Ropero et al., 2012; Yu et al., 2011).

2.2. Oxidative activity of mitochondria

The oxidative activity of mitochondria was determined by WST-1 reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. The cells were cultured on 96-well plates at an initial concentration of 1×10^4/well, and treated with ZEA/β-estradiol after 24 h. After 68 h, 10 μl of WST-1 was added per well (dilution 1:10) and the mixture was incubated for 4 h. Absorbance was measured on an ELX 808IU plate reader (BioTeck, Winooski, VT, USA) at 450 nm, with another reading taken at 600 nm as a reference (Piastowska-Ciesielska et al., 2013b). The oxidative activity of mitochondria was expressed as a percentage of oxidative activity measured in the non-treated cells. Each experiment was conducted in triplicate.

2.3. Early alteration of cell membrane permeability – lactate dehydrogenase detection (LDH)

Changes in cell membrane permeability were determined by a Cytoxicity Detection Kit^{PLUS} (LDH) (Roche) according to the manufacturer's recommendations. Cells (6×10^3/well) were cultured on 96-well plates in a similar way to previous experiments. At the end of the incubation time, 100 μl of reaction mixture was added per well and incubated in the dark for 30 min at room temperature. Following this, 50 μl of Stop Solution was added per well and shaken for 10 s. Absorbance was measured on an ELX 808IU plate reader (BioTeck) at 490 nm, with the absorbance at 690 nm used as a reference. Results were standardized to non-treated cells (Low Control) and expressed as mean optical density (OD). The analysis was performed in three independent experiments.

2.4. Analysis of changes in mitochondrial transmembrane potential

Any changes in mitochondrial membrane potential ($\Delta\Psi m$) caused by ZEA were measured by Muse™ MitoPotential Assay (Merck Millipore, Darmstadt, Germany). This assay differentiates four populations of cells: live cells with a depolarized mitochondrial membrane (MitoPotential−/7-AAD−), live cells with an intact mitochondrial membrane (MitoPotential+/7-AAD−), dead cells with a depolarized mitochondrial membrane (MitoPotential+/7-AAD+) and dead cells with an intact mitochondrial membrane (MitoPotential−/7-AAD+) (Saleh et al., 2015). The cells were cultured on six-well plates and treated as described above. MitoPotential Assay was performed according to the manufacturer's instructions. The probes were measured on a Muse™ Cell Analyzer and standardized against control probes. The analysis was performed in three independent experiments.

2.5. Apoptosis

Apoptosis was estimated by Muse™ Annexin V & Dead Cell Kit (Merck Millipore). The cells were cultured on six-well plates at a density of 62.5×10^3 cells/well for 72 h with ZEA/β-estradiol at either 100 nM or 0.3 nM. Media without ZEA/β-estradiol was used as a control. The cells were then harvested and counted on a Countess® automatic cell counter (Thermo Fisher Scientific Inc/Life technologies). Following this, 100 μl Muse™ Annexin V & Dead Cell Reagent (Merck Millipore) was added to 100 μl cell suspension (1×10^5/ml), mixed by pipetting for 3 s and incubated for 20 min in the dark. The probes were then measured on a Muse™ Cell Analyzer (Merck Millipore), standardized against a control probe. PathScan® Multi-Target Sandwich ELISA Kit #7105 (Cell Signalling,