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Genotoxic effects induced by zearalenone in a human embryonic kidney cell line☆

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ABSTRACT

Mycotoxins are considered to be significant contaminants of food and animal feed. Zearalenone (ZEA) is a hepatotoxic mycotoxin with estrogenic and anabolic activity found in cereal grains worldwide. ZEA affects hematological and immunological parameters in humans and rodents. The compound can induce cell death, cause lipid peroxidation, inhibit protein and DNA synthesis, and exert genotoxic effects. ZEA may cause increased phagolysosomal fragility in the kidney. Our research showed that exposure of human embryonic kidney (HEK293) cells to ZEA (10 or 20 μM) resulted in a concentration-dependent increase in DNA strand breaks measured with the comet assay. Damage was reduced in cells pretreated with NH_4Cl, pepstatin A, or desipramine for 1 h. Production of reactive oxygen species (ROS) was increased in cells exposed to ZEA, but DNA strand break induction could not be inhibited by the antioxidant hydroxytyrosol (HT). These results suggest that oxidative stress does not play a key role in DNA strand breaks induced by ZEA, that lysosomal injury precedes DNA strand breaks, and that the lysosome may be a primary target for ZEA in HEK293 cells.

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1. Introduction

Zearalenone (ZEA) [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-*b*resorcyclic acid lactone] is a mycotoxin produced by various *Fusarium* fungi, including *F. graminearum*, *F. culmorum*, *F. equiseti*, and *F. cerealis* [1–3] and found in grains and animal feeds; humans and animals consuming contaminated food products may be exposed to this chemical [4,5]. In rodents, ZEA causes estrogenic effects, such as decrease in fertility, reduction in litter size, increase in fetal resorptions, and changes in serum hormone levels [6]. This mycotoxin can induce liver carcinoma, pituitary adenoma, and hematological and renal toxicity [7,8]. ZEA may increase phagolysosomal fragility in the kidney. ZEA induces oxidative damage [8,9] and shows genotoxic effects, including induction of DNA adducts [10], micronuclei, DNA fragmentation, and chromosome aberrations [11,12].

Hydroxytyrosol (HT) is a potent antioxidant olive polyphenol [13]. HT can prevent oxidative DNA damage induced by acrylamide, Sudan I, deoxynivalenol and potassium bromate in human HepG2 cells [14–17] and can be used to test whether oxidative damage contributes to DNA strand breaks induced by ZEA. Lysosomal permeability may be another effect of ZEA. Changes in mitochondrial membrane potential, lysosomal permeability, and lysosomal enzymes can be involved in apoptosis [18,19]. Roberg et al. [20] noted that lysosomal translocation of cathepsin D in cultured fibroblasts led to decreases in $\Delta\Psi m$, release of cytochrome c, and apoptosis.

The aim of this research was to investigate the mechanisms of ZEA nephrotoxicity and DNA damage. Lysosomal membrane stability and mitochondrial membrane potential were tested by fluorescence spectrometry. DNA strand breaks induced by ZEA were assessed by the comet assay. Intracellular ROS was detected with 2,7-dichlorofluorescein diacetate (DCFH-DA).

2. Materials and methods

2.1. Reagents

ZEA (CAS No.17924-92-4) was obtained from Sigma–Aldrich (Germany: purity > 98%) and dissolved in dimethy sulfoxide (DMSO). Pepstatin A, desipramine, ethidium bromide (EB), DCFH-DA, acridine orange (AO), rhodamine 123 were provided by Sigma (St. Louis, USA). Normal-melting-point (NMP) agarose and low-melting-point (LMP) agarose were obtained from Gibco BRL, Life Technologies (Paisley, UK). The sample of HT was supplied by Eisai Food & Chemical Co., Ltd. (Japan). All tissue culture reagents, including minimum essential Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum, antibiotics (penicillin and streptomycin) and trypsin-EDTA solution were supplied by Gibco BRL-Life Technologies (Grand Island, NY).

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2.2. Cell culture and treatments

HEK293 cells (transformed human primary embryonic kidney cells, ATCC CRL 1573) were grown as monolayer cultures in DMEM supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 g/ml), incubated at 37 °C in an atmosphere of 5% CO_2–95% air mixture. Cells were seeded on 96-well plates at 5×10^5 cells per well, incubated at 37 °C for 24 h, and treated with ZEA. Exponentially growing cells were pretreated with 10 mM NH_4Cl for neutralization of lysosomal acidity, 100 μM pepstatin A for inhibition of the aspartic protease cathepsin D, 50 μM desipramine for inhibition of acidic sphingomyelinase activity, or HT (6.25–25 μM) as an antioxidant, for 1 h, and then treated with ZEA for 2 h.

2.3. Comet assay

The comet assay was performed as described by Singh and Stephens [21] with slight modification. Aliquots of HEK293 cells (1×10^6 cells) were suspended in DMEM, 1 ml, and incubated with ZEA (0, 2.5, 5, 10 or 20 μM) at 37 °C for 1 or 2 h. To avoid artifacts resulting from necrotic and apoptotic cells, the cell suspensions (50 μl) were mixed with Hoechst 33342 (8 μg/ml) and trypan blue (50 μg/ml). After 15 min treatment, the cells were observed under a fluorescence microscope (U-MWU2 filters).

DNA migration was determined on sample with cell viability > 90% and no apoptotic cells. Slides were viewed under an Olympus BX-51 fluorescence microscope (excitation filter 549 nm, barrier filter 590 nm). Images containing 50 randomly selected cells from each slide were analyzed with Comet Assay Software Project casp-1.2.2 (University of Wroclaw, Poland). Three independent experiments were performed.

2.4. Lysosomal membrane stability assay

Lysosomal membrane stability was determined with a modified method [19]. Cells were treated with ZEA (0, 2.5, 5, 10 or 20 μM) for 1 h, washed twice with PBS and incubated with acridine orange (AO), final concentration = 5 μM at 37 °C in the dark for 15 min. Cells were washed twice more to remove the fluorescent dye from the medium. Fluorescence intensity from cell suspensions was measured with a fluorescence spectrometer (HITACHI 650-60, Tokyo, Japan), excitation = 495 nm and emission = 530 nm.

2.5. Mitochondrial membrane potential assay

Mitochondrial membrane potential was measured using a modified rhodamine 123 method [23]. Cells were incubated with ZEA for 1 or 2 h, washed twice with PBS, the pellet resuspended in 2 ml fresh medium containing 1.5 μM rhodamine 123, and incubated at 37 °C, with gentle shaking, for 10 min. The cells were centrifuged, and fluorescence from the supernatant was measured, emission = 490 nm and excitation = 520 nm.

2.6. Measurement of intracellular ROS

The formation of intracellular ROS was measured with the DCFH-DA method [22]. Cells were harvested and treated with ZEA at 37 °C for 1 or 2 h, washed twice with cold PBS, suspended in PBS at 5×10^5 cells/ml, and incubated with DCFH-DA, final concentration = 5 μM, at 37 °C, in darkness, for an additional 40 min. The relative fluorescence intensity of the cell suspensions was measured, excitation = 485 nm and barrier wavelength = 530 nm.

2.7. Statistical analysis of data

All values were presented as mean ± standard deviation (S.D.). The data were analyzed by one-way analysis of variance (ANOVA) and Student's *t*-test, using SPSS 11.5 software. The level of significance was set at $P < 0.05$ and $P < 0.01$ for all statistical analyses.

3. Results

3.1. DNA strand breaks induced by ZEA

DNA strand breaks in HEK293 cells exposed to ZEA (0–20 μM) for 1 h or 2 h were evaluated by the comet assay. Compared to the untreated cells, ZEA increased DNA migration in a dose-dependent manner ($P < 0.05$ or $P < 0.01$) after exposure for 2 h (Table 1), but no obvious effect was seen at 1 h (data not shown).

Table 1
DNA strand breaks induced by ZEA in HEK293 cells.

ZEA (μM)	Tail length (μm)	Tail DNA (%)	Tail moment
0	3.00 ± 0.32	2.27 ± 1.17	0.06 ± 0.03
2.5	3.75 ± 1.21	2.56 ± 1.66	0.08 ± 0.05
5	5.30 ± 1.92	3.42 ± 1.42	0.13 ± 0.13
10	6.15 ± 3.16*	5.44 ± 4.64*	0.52 ± 0.96
20	21.00 ± 5.46**	14.64 ± 4.28**	3.46 ± 3.82**

DNA damage was measured by comet assay in HEK293 cells exposed to ZEA for 2 h. Data represent the mean values ± S.D. three independent experiments.
* $P < 0.05$ vs. control.
** $P < 0.01$ vs. control.

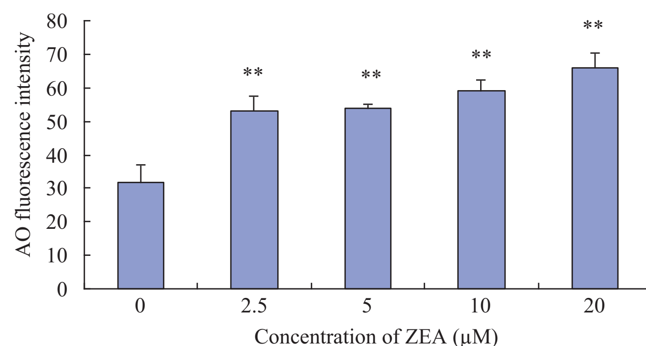

Fig. 1. Effects of ZEA on lysosomal membrane stability. HEK293 cells were exposed to ZEA (0–20 μM) for 1 h, and stained with AO. Values were expressed as mean ± S.D. of three independent experiments (**$P < 0.01$ vs. control).

3.2. Effects of ZEA on lysosomal membrane stability

AO was used to measure lysosomal membrane stability. A statistically significant increase of AO fluorescence intensity was observed in cells treated with ZEA (2.5–20 μM) for 1 h (Fig. 1).

3.3. Effects of ZEA on mitochondrial membrane potential

Mitochondrial membrane potential was examined by measuring the fluorescence of the cationic dye rhodamine 123 in control and ZEA-treated HEK293 cells. A decrease of rhodamine 123 fluorescence intensity was observed in cells treated with ZEA (20 μM) for 2 h ($P < 0.05$), but no obvious effect was seen at 1 h (Fig. 2).

3.4. Lysosomal acidic compartment contributed to ZEA-induced lysosomal membrane stability, mitochondrial membrane potential and DNA strand breaks

To evaluate the effect of the acidic lysosomal compartment on ZEA-induced lysosomal membrane stability, mitochondrial

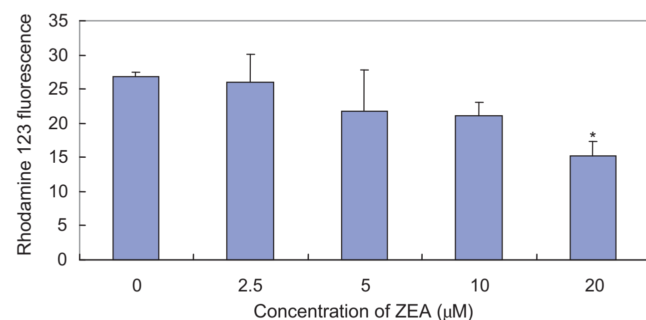

Fig. 2. Effects of ZEA on mitochondrial membrane potential. HEK293 cells were incubated with or without ZEA for 2 h, and stained with rhodamine 123. Data were the mean ± S.D. of three independent experiments (*$P < 0.05$ vs. control).

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