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Effects of deoxynivalenol on calcium homeostasis of concanavalin A—Stimulated splenic lymphocytes of chickens in vitro

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ABSTRACT

In this study, the in vitro effects of the treatment of concanavalin A (Con A)—stimulated splenic lymphocytes with DON were examined. Splenic lymphocytes isolated from chickens were stimulated with 12.5 μg/mL Con A and exposed to deoxynivalenol (DON) (0–50 μg/mL) for 48 h. The intracellular calcium concentration ($[Ca^{2+}]i$), pH, calmodulin (CaM) mRNA levels, and Na^+,K^+-ATPase and Ca^{2+}-ATPase activities were detected. With the DON exposure concentrations increased, the $[Ca^{2+}]i$ and CaM mRNA levels gradually increased in a dose-dependent manner, and all the evaluated conconcentrations affected ATPase activity to the same extent. There were significant differences ($P < 0.05$ or $P < 0.01$) between the treatment groups and the control group. These results indicate that an imbalance in calcium homeostasis and intracellular acidification are components of DON cytotoxicity in chicken lymphocytes.

1. Introduction

Mycotoxins are secondary metabolites of moulds, contaminating a wide range of crop plants and fruits before or after harvest. Among the most important mycotoxins, the most common are aflatoxins, deoxynivalenol (DON), ochratoxin A, fumonisins, zearalenone, patulin, and T-2 toxin. They are characteristically stable under changing environmental conditions and have been shown to cause a variety of toxic effects in experimental animals, livestock and humans (Chen et al., 2008). Besides, in children, ingestion of heavily DON-contaminated food results in vomiting within hours(Shiefer and Beasley, 1989; Pestka et al., 2004; Pestka and Smolinski, 2005). Thus, DON exposure is a risk factor for stunting in children.

DON is a mycotoxin that belongs to the trichothecene group of mycotoxins produced by different species of the genus *Fusarium*, particularly *F. graminearum*. DON contamination occurs worldwide in cereal crops at concentrations ranging from a few micrograms to >30 mg/kg (SCOOP, 2003). The chronic toxicity of trichotheces in animals is characterised by anorexia, reduced weight gain, diminished nutritional efficiency, neuroendocrine changes, and immune modulation (Rotter et al., 1996). DON also has a subsequent anti-proliferative effect on bone marrow cells, the gastrointestinal epithelium, immune cells (Bondy and Pestka, 2000; Nasri et al., 2006), and endometrial cells (Tiemann et al., 2003; Wollenhaupt et al., 2007). Trichothecene toxicity at the cellular level is characterised by inhibited protein synthesis, impaired membrane functions, altered intercellular communication (Pestka and Smolinski, 2005; Pestka, 2008).

Calcium homeostasis is a major factor in maintenance of cell integrity and function. Calcium had been implicated as an important second messenger and regulator of cell homeostasis (Plank et al., 2006). Calmodulin is a highly conserved calcium-binding protein that transduces calcium signals into downstream effects, influencing a range of cellular processes, including calcium homeostasis. Besides, intracellular dyshomeostasis, as occurs during oxidative stress, results in pH and ion concentration disorders, especially cellular calcium homeostasis disruptions, which may cause serious damage or death. However, very few

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studies have shown the toxicity of DON in deregulation of calcium homeostasis (Uzarski et al., 2003). The spleen is the largest peripheral lymphoid organ in the body and contains a large number of immune cells, including B lymphocytes, T lymphocytes, macrophages, dendritic-like cells, and so on. The splenic lymphocytes of chickens have been a very useful model for many studies (Okamura et al., 2004; Li et al., 2010; Villanueva et al., 2011). Therefore, we addressed the issue by evaluating the effects of DON cytotoxicity through in vitro assessment of its calcium homeostasis activity on the splenic lymphocytes of chickens.

2. Materials and methods

2.1. Reagents

Deoxynivalenol, RPMI 1640 medium, Histopaque 1077, Fluo-3/AM, and Na^+,K^+-ATPase and Ca^{2+}-ATPase analysis kits, 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein, acetoxymethyl ester (BCECF/AM) were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum was purchased from Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Trizol reagent was purchased from Invitrogen Biotechnology Co., Ltd. (Shanghai, China). SYBR PremixScript RT-PCR Kit II was purchased from TaKaRa (Shiga, Japan).

2.2. Cell Culture

All procedures in this study were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University. One hundred and eighty 1-day-old healthy male Isa Brown chickens were obtained from a comercial rearing farm (Jilin poultry farm, Jilin province) at day of hatching. One-day old chickens were placed in large pens with wood shavings and were reared with lighting regimen 23 h light and 1 h dark. The initial room temperature of 32–33 °C was reduced weekly by 1 °C to a final temperature of 28 °C. The relative humidity was within a range 50–60%. The chickens provided with water as well as feed ad libitum. And the chickens were fed only a basic commercial diet from Chai Tai feed Co., Ltd. (Sichuan, China). Nutritional requirements were adequate according to National Research Council (NRC, 1994) and the Chinese Feeding Standard of Chickens (NY/T33-2004). At 8 weeks of age, chickens in good health were anaesthetized with an intramuscular injection of 846 anesthetic mixture (haloperidol, dihydroetorphine, and 2, 4-dimethylaniline thiazole) (Duan and Zhu, 2005) using doses 0.8 mL/kg body wright. After laparotomy, spleen samples of chickens were removed from the experimental animal.

The whole spleens of chickens were ground at low temperature and teased through a 200-mesh cell strainer into a Petri dish containing phosphate-buffered saline. The cell suspension was overlaid onto Histopaque 1077 and centrifuged at $400 \times g$ for 15 min at room temperature. The lymphocytes at the interface were collected, washed twice with PBS at $250 \times g$ for 5 min at room temperature, and suspended in RPMI-1640 medium (without phenol red, a weak estrogen mimic) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 U/mL streptomycin. More than 95% of cells were viable based on trypan blue dye exclusion. The spleen cells were cultured in 6-well tissue culture plates (6×10^6 cells/mL, 2 mL/well) in triplicate and stimulated with concanavalin A (Con A; 12.5 μg/mL, to induce the proliferation of T cells) at a range of DON concentrations (0, 0.2, 0.8, 3.2, 12.5, and 50 μg/mL) at 41.5 °C in a humidified 5% CO_2 environment for 48 h. The mycotoxin concentrations were selected based on preliminary dose-response experiments (data not shown). After 48 h of incubation, cells were collected for RNA isolation, intracellular pH, intracellular calcium concentration ($[Ca^{2+}]i$), and Ca^{2+}-ATPase and Na^+,K^+-ATPase activities. All tests were performed in 3 independent experiments.

2.3. Measurement of intracellular pH with BCECF

Intracellular pH was measured by loading cells with the membrane-impermeant dye BCECF. This detailed procedure was performed according to Hirpara et al. (2001a). Briefly, harvested cells were incubated with BCECF/AM (2 μM final concentration) in 1 mL of serum-free culture medium. After 30 min of incubation in the CO_2 incubator at 37 °C, cells were pelleted, rinsed twice with PBS buffer, and resuspended at an appropriate density for fluorescence measurements. A minimum of 10,000 cells was analyzed, and the ratio of BCECF fluorescence at 525 and 610 nm was used to obtain intracellular pH from a calibration curve.

2.4. Analysis of $[Ca^{2+}]i$

Fluo-3/AM was chosen for use as an intracellular free calcium fluorescent probe for analysis of $[Ca^{2+}]i$ in DON-exposed (0, 0.2, 0.8, 3.2, 12.5, and 50 μg/mL) cells (Harrison et al., 1993; Guthrie et al., 2011). The harvested cells were loaded with Fluo-3/AM (1 μM final concentration) for 30 min in the dark at 37 °C and then washed with D-Hank's solution. Intracellular $[Ca^{2+}]i$ levels were represented by the fluorescence intensity (FL1, 530 nm) of 10,000 cells on a flow cytometer. Fluorescence intensity of cells and the blackground were measured and recorded. The relative fluorescence intensity represented the $[Ca^{2+}]i$.

2.5. Activities of Na^+,K^+-ATPase and Ca^{2+}-ATPase

A minimum of 6×10^7 cells was analyzed, and the harvested cells were homogenized in ice-cold physiological saline in an ultrasonic disintegrator. The cell homogenates were centrifuged at $1000 \times g$ for 10 min. Supernatants were obtained and their protein concentration was determined by the Folin–phenol method, using bovine serum albumin (BSA) as a standard (the protein concentration in the assay was 3–5 mg/mL) (Lowry et al., 1951). Activities of Na^+/K^+-ATPase and Ca^{2+}-ATPase from supernatants were determined as described by Bonting (1970) and Hjerten and Pan (1983), respectively. The activities were indirectly measured by estimating the phosphorous liberated after the incubation of supernatants in a reaction mixture containing the substrate ATP with the co-substrate elements at 37 °C for 15 min. The reactions were arrested by adding 1.0 mL of 10% trichloroacetic acid (TCA). The phosphorus content from the TCA supernatants was then determined as described by Sumner (1944) . ATPase activity was expressed as μmol of phosphorus liberated in 1 min per mg protein at 37 °C.

2.6. Quantification of CaM mRNA

Total RNA was isolated from cells using Trizol reagent according to the manufacturer's instructions. The RNA concentrations were determined using the GeneQuant 1300.

The reverse transcription reaction (40 μL) consisted of the following: 10 μg of total RNA, 1 μL of M-MLV reverse transcriptase, 1 μL of RNase inhibitor, 4 μL of dNTP, 2 μL of Oligo dT, 4 μL of dithiothreitol, and 8 μL of 5 × buffer. The reverse transcription was performed according to the manufacturer's instructions (Invitrogen), and the reverse transcription products (cDNA) were then stored at −20 °C for PCR.

To design primers, we used the chicken CaM mRNA GenBank sequence with the accession number NM_205005.1. Chicken β-actin (GenBank accession number L08165.1), a housekeeping gene, was used as the internal reference. The primers (Table 1) were designed using prime 5 software (Molecular Biology Insights,