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Assessing the effect of food mycotoxins on aromatase by using a cell-based system

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ABSTRACT

Zeranol, aflatoxin B1, zearalenone are mycotoxins that are commonly found as food contaminants. The chemical structures of zeranol and zearalenone resemble estrogen, and may disrupt hormone metabolism. The biosynthesis of estrogen is catalyzed by aromatase or CYP19. In the present study, effect of these mycotoxins on aromatase was evaluated by using 4 cell lines, i.e. the CYP19-overexpressing cells MCF-7*aro*, the placental cells JEG-3, the breast cells MCF-7, and the brain cells T98G. Our data indicated that zearalenone was a competitive inhibitor of aromatase with a K_i value of 1 μM. As aromatase expression is promoter-specific and regulated by alternate splicing, we employed three cell lines for investigation. Our results showed that zearalenone and zeranol could suppress aromatase expression through promoters II and I.3. For aromatase transcription dictated by promoters I.f and I.1, the expression was not affected. Taken together, zearalenone was a potential aromatase inhibitor among the three mycotoxins tested. Furthermore, this 4-cell line approach could be employed in principle to screen for compounds inhibiting or inducing estrogen synthesis.

1. Introduction

Estrogen is important in human physiology, especially for women of reproductive age. Mainly produced by the ovaries, the hormone facilitates the development of secondary sexual characteristics, regulates gonadotropin secretion for ovulation, sensitizes tissues to progesterone, and maintains bone mass (Cagnacci et al., 1992; Morishima et al., 1995; Smith et al., 1994). During the course of human pregnancy, estrogen stimulates the proliferation of the uterine and mammary tissues and increases the permeability of the blood vessels (Yallampalli et al., 1994). Reduced estrogen levels during pregnancy often correlate with miscarriage (Tuckerman et al., 2004).

Humans have the capacity in synthesizing estrogen *de novo* from cholesterol, and the rate-limiting step is catalyzed by aromatase (CYP19) (Olson et al., 2007). The major sites of CYP19 expression are the placenta, ovaries, and brain; in addition, expression in the testis, bone, and adipose tissue are also detected (Kamat et al., 2002; Mendelson and Kamat, 2007).

Aromatase is a member of the cytochrome P450 enzyme superfamily (Lephart and Simpson, 1991). Human aromatase is a 55-kDa protein encoded by a single-copy gene with 10 exons (Means et al., 1989; Toda et al., 1990). The coding region distributes in Exon II to IX of the gene, and a number of promoter regions in Exon I dictate the gene regulation. Different tissues can employ promoter(s) in Exon I for regulation, so tissue-specific regulation can be accomplished through alternate splicing (Simpson et al., 1997). The transcription starts from one segment of the promoter-associated sequence in Exon I and splices to a common site in Exon II. Therefore, the 5′-end sequence of aromatase mRNA transcript may vary in different tissues. However, the translated product from all transcripts is identical since the protein-encoding region falls in the common sequence at Exon II to IX.

Mycotoxins are toxic metabolites produced by filamentous fungi, especially those in the genus *Aspergillus*, *Penicillum* and *Fusarium*. These fungi may colonize crops in the field or during postharvest handling (Streit et al., 2012). Ingestion of mycotoxin may cause mycotoxicoses, such as immune suppression, liver and

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kidney damage, pulmonary edema, cancer, and death (Bennett and Klich, 2003; Richard, 2007; Streit et al., 2012).

Aflatoxin B1 may induce DNA damage and is labeled as a Group 1 carcinogen by the International Agency for Research on Cancer (Bedard and Massey, 2006). However, some mycotoxins classified under resorcylic acid lactones (RAL) are non-toxic (Richard, 2007). The RAL zearalenone contaminates up to 69% of all agricultural products (Rhyn and Zoller, 2003). Another RAL member, zeranol, is used as a growth promoter in livestock in the United States and Canada (Bennett and Klich, 2003). These last two compounds are xenoestrogens, and our lab has shown that some xenoestrogens interfere with estrogen biosynthesis and disrupt the endocrine systems (Huang and Leung, 2009; Li et al., 2011; Wang and Leung, 2007; Ye et al., 2009). Moreover, a previous study has shown that aflatoxin B1 may affect aromatase activities (Storvik et al., 2011). In the present study, we investigated two estrogenic mycotoxins for their interference in aromatase by using a cell-based system.

2. Materials and methods

2.1. Chemicals

Zeranol, aflatoxin B1, zearalenone were obtained from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals, if not stated, were purchased from Sigma–Aldrich.

2.2. Cell culture

Our lab has previously described using a CYP19-overexpressing cell system (MCF-7*aro*) to study aromatase inhibition (Wang et al., 2005). As the brain, ovaries, and placenta are the major organs for estrogen synthesis, we employed a combination of three model cell lines in assessing the aromatase expression. The placental and brain cell lines JEG-3 and T98G have previously been used for aromatase expression as models in the respective tissues. We used the breast cancer MCF-7 cells in place for ovarian cells because they have common promoter usage, i.e. promoter I.3 and II.

The cell lines T98G, MCF-7 were purchased from ATCC, Rockville, MD., while JEG-3 cells were a generous gift from Prof. Stephen Shiu (Department of Physiology, the University of Hong Kong, Pokfulam, Hong Kong). T98G cells were maintained in MEM medium (Invitrogen™, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) from Hyclone Laboratories, South Logan, Utah, USA. JEG-3, MCF-7, and MCF-7*aro* cells were cultured in RPMI-1640 (Invitrogen™) supplemented with 10% FBS, 2 mM L-glutamine and 1 mM sodium pyruvate. The selection antibiotic 500 μg/ml G418 (USB, Cleveland, OH, USA) was also added to the culture medium of MCF-7*aro*. All cells were incubated at 37 °C, 5% carbon dioxide and routinely sub-cultured when reaching 80% of confluency. Three days before experiments, the cells were switched to phenol-red free medium. Zeranol, aflatoxin B1, and zearalenone were diluted and administered in the solvent vehicle dimethyl sulphoxide (DMSO), and the volumes of the solubilized mycotoxins added into the culture medium were standardized to 0.1% v/v. Cells were seeded at a density of 5×10^2 cells/mm^2 in all experiments. The mycotoxin concentrations administered covered 1000-fold their respective exposure limits stipulated by different governing bodies.

2.3. "In-cell" aromatase assay

The assays were performed as previously described (Wang et al., 2005, 2006) with slight modification. In brief, cells were seeded and allowed 1 day for attachment. Assays were started by replacing the culture medium with serum-free medium containing [1β-^{3}H(N)]-androst-4-ene-3,17-dione (NET-926; specific activity 25.3 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston MA) and mycotoxin. The final concentration of androstenedione was standardized at 25 nM, and the reaction was incubated at 37 °C for 30 min for assays performed in MCF-7*aro* cells. An aliquot of the medium was then mixed with equal volume of chloroform, followed by a 13,000×*g* centrifugation at 4 °C for 10 min. The aqueous phase was removed into a new tube containing 500 μl of 5% activated charcoal suspension. After 30 min incubation, an aliquot of the supernatant fraction was taken out for scintillation counting. The protein content of the cells, on the other hand, was determined by using a BCA kit (Thermo Scientific Pierce) after dissolving the cells in 0.5 mol/l NaOH.

2.4. The design of exon-specific probes

Taqman probes for quantifying the non-discriminatory CYP19 and GAPDH (Assay-on-Demand®, Applied Biosystems) were used for the respective comparison and normalization. Probe sequences of the non-discriminatory CYP19 were selected within the protein-encoding region of the mRNA, so the promoter-specific transcription cannot be detected by using these commercial probes. Exon I.1, I.3, I.f, and II mRNA species were amplified by probes designed by Custom-designed Taqman® Gene Expression Assays (Assay-by-Demand®, Applied Biosystems, Foster City, CA, USA) as shown below. The validity of these probes has previously been described (Tan et al., 2013; Ye et al., 2009).

mRNA species	Probes designed for exon-specific mRNA species		
	Forward primer	Reverse primer	Reporter sequence
Exon I.1	CTGTGCTCGGGATCTTCCA	CATCTTGTGTTCCTTGACCTCAGA	ACGTCGCGACTCTAAAT
Exon I.3	AAATTAGTCTTGCCTAAATGTCTGATCACA	CCAAAACCATCTTGTGTTCCTTGAC	TTATAAAACAGACTCTAAATTGCC
Exon II	GCAACAGGAGCTATAGATGAACCTT	CATCTTGTGTTCCTTGACCTCAGA	CCACAGGACTCTAAATTG
Exon I.f	GAGAGCCCAGCAACTATGTAACTC	CGCTCCTGTGAACAGAGAGTAAT	ATCATGCCTCCCTTCCATG
CYP19	GGAGAATTCATGCGAGTCTGGAT	GGAACATACTTGAGGACTTGCTGAT	TCTGGAGAGGAAACACTC

2.5. Quantitative real time RT-PCR assay on aromatase expression

The protocol for real-time quantitative PCR was carried out as previously described by our lab (Wang et al., 2008). Cells were cultured and treated as depicted above. After 24 h of treatment of mycotoxin, total RNA was extracted from the cells using TRIzol reagent (Invitrogen™, Carlsbad, CA, USA). The concentration and purity of the isolated RNA were determined by the absorbance reading observed at 260 and 280 nm. 3 μg of total RNA, oligo-dT, and M-MLV