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ABCC1, ABCC2 and ABCC3 are implicated in the transepithelial transport of the myco-estrogen zearalenone and its major metabolites

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ABSTRACT

The myco-estrogene zearalenone (ZEA) is a worldwide cereal contaminant, implicated in reproductive disorders in animals and humans. Intestinal cells constitute a first barrier to mycotoxins exposure, since they express membrane ABC transporters that may affect the bioavailability of food xenobiotics. In this study, we investigated the mechanisms involved in the transepithelial transfer of ZEA and its major metabolites α- and β-zearalenols (ZOLs), first using human intestinal Caco-2 cells. When exposed to ZEA, α-ZOL or β-ZOL either in the apical (AP) or basolateral (BL) compartment, cells showed asymmetry in the AP–BL and BL–AP transfer of mycotoxins. Metabolic inhibitors increased ZEA, α-ZOL and β-ZOL intracellular accumulation. Caco-2 cells apically exposed to ZEA produced metabolites (ZOLs and glucuronides) whose distribution between AP, BL and intracellular compartments was significantly modified by ABCCs inhibitor MK571. ABCB1-, ABCC1-, ABCC2 and ABCC3-transfected cells were used for studies of intracellular accumulation of ZEA, α-ZOL and β-ZOL with or without specific inhibitors, and for competitive studies using fluorescent substrates. The results showed that ZEA, α-ZOL and β-ZOL were substrates for ABCC2. ABCC1 was also involved in ZEA and α-ZOL transport, whereas ABCC3 only interacted with β-ZOL. These specific interactions suggest a role for ABCC1–3 transport proteins in zearalenone exposure and its resulting risk for human health.

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1. Introduction

Zearalenone (ZEA), 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcyclic acid lactone, is a non-steroidal estrogenic mycotoxin synthesized by a variety of *Fusarium* fungi, including *F. culmorum* and *F. graminearum*. These soil fungi are common in temperate and warm countries, and are regular contaminants of cereal crops worldwide, mainly wheat, rye, oats in European countries, and corn, wheat in Canada and the USA (as review, see Zinedine et al., 2007). ZEA and its reduced metabolites are able to activate estrogen receptors (ERs) (Kuiper et al., 1998) and to compete with natural hormones at the 3α-HSD substrate binding site (as suggested by *in vitro* studies, Malekinejad et al., 2005a, Videmann et al., 2008). Consequently, they may interfere with the balance of active steroids in receptor rich tissues. The endocrine effects of ZEA result in functional and morphological alterations (persistent estrus, ovarian atrophy, decreased fertility, stillbirth, etc.) in reproductive organs of various mammalian species (Fink-Gremmels and Malekinejad, 2007).

Via cereals and by-products, specific population groups, such as children and vegetarians, could be exposed to this toxin in quantities exceeding the tolerable weekly or daily intake levels (Leblanc et al., 2005). Following ingestion, ZEA is absorbed, widely distributed and slowly eliminated from tissues, likely resulting from enterohepatic recycling of the toxin and its metabolites (Biehl et al., 1993). Metabolism studies indicate that ZEA mainly undergoes C-6′ reduction and conjugation with glucuronic acid, resulting respectively in the formation of α-ZOL and β-ZOL (Olsen et al., 1981; Malekinejad et al., 2006) and ZEA-, α-ZOL- and β-ZOL-glucuronide (Olsen et al., 1987). This metabolism occurs both in gut and liver (Malekinejad et al., 2005b). However, the mechanisms involved in the transepithelial transport of these toxins remain unknown.

It is recognized that ATP-dependant efflux transporters (ATP-binding cassette/ABC transporters) expressed at the apical (AP) and basolateral (BL) membrane of the intestinal epithelial cells influence oral bioavailability and intestinal efflux clearance of various xenobiotics (Takano et al., 2006). In particular, ABCB1 (MDR1, P-glycoprotein) and ABCC1–3 (MRP1–3, multidrug resistance-associated proteins 1–3), expressed in tissues that are important for absorption (gut), contribute to maintain the barrier function

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of the site (Leslie et al., 2005). Indeed, the intestine is the first barrier encountered by most chemicals before distribution in the human body and reaching the target organs. As the intestine is also one of the major organs involved in ZEA biotransformation, it is of first interest to study potential interactions of ZEA and its major metabolites with ABC transporters expressed at the intestinal level. Different mammalian models indicate that ABCB1 and ABCC1–3 are directly involved in the efflux of xenobiotics and/or their metabolites, representing toxicologically relevant ABC transport proteins (Leslie et al., 2005). The current study aimed to investigate the transepithelial transport mechanisms of ZEA and metabolites and the mycotoxins interaction with these major ABC transporters, using Caco-2 and ABC transporters overexpressing cell lines.

Caco-2 cell line is a largely used model of human intestinal cells (Meunier et al., 1995; Delie and Rubas, 1997), recognized as one of the best-validated system for pharmacological and toxicological approaches. These cells are established model for ABCB1 and ABCCs-mediated transport (Artursson et al., 2001). But the expression of multiple transporters makes it difficult to design studies to elucidate the individual role of each transporter. In this context, transfected cells are useful complementary models for identifying transporters substrates. We then studied intracellular accumulation of the toxins and realized competition experiments with specific substrates in LLCPK1 and MDCKII cells overexpressing ABCB1, ABCC1, ABCC2, and ABCC3, in order to evaluate interactions of ZEA and its major metabolites with individual transport molecules.

2. Methods

2.1. Chemicals

MK571 came from Tebu-Bio (Le Perray en Yvelines, France). Valspodar (VSP) was kindly supplied by Novartis pharma (Basel, Switzerland). Zearalenone (ZEA), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), rhodamine 123 (Rho123), calcein acetoxymethyl ester (calcein-AM), dimethyl sulfoxide (DMSO), uridine diphosphate glucuronic acid (UDPGA), sodium azide (NaN_3), and all other chemicals were purchased from Sigma Aldrich (St Quentin Fallavier, France). HPLC grade water, prepared using a Milli-Q plus system (Millipore, Molsheim, France) was used for preparation of HPLC eluents.

2.2. Cell culture

The Caco-2 cell line was obtained from the American Tissue Culture Collection (ATCC No. HTB37) and was used between passages 27 and 34. LLCPK1 pig kidney epithelial cells, LLCPK1 cells transfected with human *MDR1* gene (LLCPK1-MDR1) (Schinkel et al., 1995), MDCKII (Madin-Darby canine kidney strain II) epithelial cells and MDCKII cells transfected with human *MRP1* gene (MDCKII-MRP1) (Evers et al., 1996), *MRP2* gene (MDCKII-MRP2) (Evers et al., 1998) and *MRP3* gene (MDCKII-MRP3) (Kool et al., 1999) were gifts from Prof. Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). They were used between passages 11 and 15. Caco-2, MDCKII and LLCPK1 cells were grown as described in Tep et al. (2007). Caco-2 cells were used for transport experiments at days 21–23 post-seeding, when morphological differentiation had been reached (Pinto et al., 1983). MDCKII and LLCPK1 cell lines were used at days 3–4 post-seeding (Schinkel et al., 1995; Evers et al., 1998). For Rho123 or calcein accumulation studies, cells were seeded in 24-well plates at a cell density of 1.2×10^6 cells/well and cultured in DMEM during 3 days at 37 °C until confluence. Cell culture media and reagents came from Invitrogen (Cergy Pontoise, France). Fetal bovine serum came from Perbio Science (Brebières, France). Culture flasks, and plates were purchased from Falcon (VWR international, Strasbourg, France).

2.3. Cytotoxicity studies

Caco-2 cells were exposed for 24 h to 1–200 μM ZEA, α-ZOL or β-ZOL at day 21 post-seeding. LLCPK1 and MDCKII cells were exposed for 8 h to 1–100 μM ZEA, α-ZOL or β-ZOL at day 3 post-seeding. Cell viability was assayed by the MTS test, as described in Bony et al. (2006).

2.4. Transport studies

The Caco-2 cell monolayers, grown on filters in 6-well plates, were pre-incubated for 30 min at 37 °C in Hank's balanced salt solution (HBSS), containing 10 mM glucose, and stirred gently. AP and BL chamber volumes were maintained at 1.5 and 2.3 ml HBSS (pH 7.4) respectively. 10 μM mycotoxins (ZEA, α-ZOL or β-ZOL) were added to either AP or BL compartment for the study of AP–BL and BL–AP transfer for 6 h.

The effect of inhibitors on transfer and accumulation was studied using Caco-2 cells apically exposed either to 10 μM ZEA or 10 μM ZOLs or 25 μM ZEA with 10 mM UDPGA. After a 30 min pre-incubation in the presence of metabolic inhibitor (1 mM NaN_3 with 50 mM 2-deoxy-D-glucose (2-DOG)), or ABC transport inhibitors (20 μM MK571, 10 μM VSP), mycotoxins contents in cell, AP, and BL compartments were measured after a 4 h exposure. Inhibitors concentrations were based on literature information. At the end of the pre-incubation period and at the end of the experiments, monolayer integrity was checked by measuring the transepithelial electrical resistance (TEER) using a millicell ERS (Millipore, Molsheim, France). To assess paracellular permeability, the flux of 5 mM sucrose was measured, as described in Videmann et al. (2007). Monolayers were excluded if TEER values were inferior to 300 Ω cm^2, if TEER values fell by more than 15% during the transport experiments, or if glucose concentration in the receiver compartment exceeded 100 μM.

2.5. Accumulation studies

After 4 h of incubation at 37 °C in HBSS containing the toxins, the cells were rinsed twice with cold PBS, then lysed into 400 μl Triton 1X and disrupted by 15 min sonication. After centrifugation at 10,000 × *g* for 5 min, 80 μl of the supernatant were analyzed by HPLC. The protein concentration was measured according to Bradford (1976) using bovine serum albumin as standard. Intracellular accumulation was normalized to cellular protein content.

2.6. Rho123 accumulation in LLCPK1 wild-type and LLCPK1-MDR1 cells

The transport function of ABCB1 in LLCPK1-MDR1 cells was studied by following the intracellular accumulation of Rho123, a fluorescent dye substrate of ABCB1. Cells were washed with 1 ml HBSS and exposed to 2 μM Rho123 in the absence or presence of 5 μM VSP, or ZEA, α-ZOL and β-ZOL at increasing concentration (10–100 μM) at 37 °C. After 60 min, the medium was removed, cells were washed with 0.5 ml PBS, and lysed by adding 1 ml of Triton 1X. The whole cell lysate was analyzed for fluorescence (TD-700 fluorimeter, Turner Designs, Sunnyvale, CA) with excitation at 505 nm and emission at 534 nm. Values were normalized to the cellular protein content by the method of Bradford. The measured fluorescences were corrected by subtraction of the fluorescence observed in the cells before loading.

2.7. Calcein accumulation in MDCKII wild-type, MDCKII-MRP1, MDCKII-MRP2 and MDCKII-MRP3 cells

For the functional test of ABCC transporters, we used calcein-acetoxymethyl ester (calcein-AM), a substrate that is transformed into the fluorescent calcein as soon as it enters the cells by the action of intracellular esterases (Wortelboer et al., 2005). Cells were incubated with 0.2 μM calcein-AM, in the absence or presence of 40 μM MK571, or ZEA, α-ZOL and β-ZOL at increasing concentrations (5–100 μM) at 37 °C. Inhibitors were not toxic at these concentrations according to 60 min MTT-reduction and LDH release tests (data not shown). After 20 min, the medium was removed, cells were washed with 0.5 ml PBS, then lysed in Triton 1X. The fluorescence intensity of the calcein converted from calcein-AM in cell lysates was measured, using the TD-700 fluorimeter, with excitation at 486 nm and emission at 540 nm. Intracellular accumulation was normalized to cellular protein content in each well. The measured fluorescences were corrected by subtraction of the fluorescence observed in the cells before loading.

In order to compare the effect obtained in the presence of the different toxins, VSP and MK571 were selected as reference of the transport inhibition assay since these compounds were able to respectively induce a maximal Rho123 and calcein intracellular accumulation in our experimental conditions. The results were expressed as percent of total VSP or MK571 inhibition as follows (Lespine et al., 2007):

$$\%\,\mathrm{INH} = \frac{[f_x/F_o - 1] \times 100}{F_{INH}/F_o - 1}$$

where f_x was the normalized fluorescence in cells treated with the toxin (x); F_o the mean normalized fluorescence in the control (Rho123 or calcein alone); F_{INH} the mean normalized fluorescence in cells treated with 5 μM VSP (Rho123 assay) or with 40 μM MK571 (calcein assay).

2.8. Quantification of ZEA and metabolites by the HPLC method

The analysis of ZEA and its metabolites by HPLC has been previously described in Videmann et al. (2008). The UV detection limit and the quantification limit were 0.1, 0.08, 0.08 nmol/ml and 0.2, 0.15 and 0.15 nmol/ml for β-ZOL, α-ZOL and ZEA respectively. The quantification limit was identical for each molecule and its conjugate.

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