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Deoxynivalenol affects in vitro intestinal epithelial cell barrier integrity through inhibition of protein synthesis

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ABSTRACT

Deoxynivalenol (DON), one of the most common mycotoxin contaminants of raw and processed cereal food, adversely affects the gastrointestinal tract. Since DON acts as a protein synthesis inhibitor, the constantly renewing intestinal epithelium could be particularly sensitive to DON. We analyzed the toxicological effects of DON on intestinal epithelial protein synthesis and barrier integrity. Differentiated Caco-2 cells, as a widely used model of the human intestinal barrier, were exposed to realistic intestinal concentrations of DON (50, 500 and 5000 ng/ml) during 24 h. DON caused a concentration-dependent decrease in total protein content associated with a reduction in the incorporation of [^{3}H]-leucine, demonstrating its inhibitory effect on protein synthesis. DON simultaneously increased the paracellular permeability of the monolayer as reflected through a decreased transepithelial electrical resistance associated with an increased paracellular flux of the tracer [^{3}H]-mannitol. A concentration-dependent reduction in the expression level of the tight junction constituent claudin-4 was demonstrated by Western blot, which was not due to diminished transcription, increased degradation, or NF-κB, ERK or JNK activation, and was also observed for a tight junction independent protein, i.e. intestinal alkaline phosphatase. These results demonstrate a dual toxicological effect of DON on differentiated Caco-2 cells consisting in an inhibition of protein synthesis as well as an increase in monolayer permeability, and moreover suggest a possible link between them through diminished synthesis of the tight junction constituent claudin-4.

Introduction

Intestinal epithelial cells (IECs) are in charge of two crucial but conflicting processes, which are on the one hand, the transport of nutrients and fluids and, on the other hand, the restriction of the access for luminal antigens to the internal milieu. They form a monolayer that constitutes a dynamic and selective barrier, and mediates the transport of molecules in two ways: either across the cells (i.e. the transcellular pathway) or between the cells (i.e. the paracellular pathway) (Fanning et al., 1999).

Adjacent enterocytes are connected by an intercellular junctional complex consisting of tight junctions (TJs), adherent junctions, gap junctions and desmosomes, among which the TJs represent the major determinants of the paracellular pathway (Fasano and Nataro, 2004). A complex signaling network relying on different types of proteins regulates TJ assembly and functioning. Transmembrane proteins, i.e. occludin, claudins and Junctional Adhesion Molecule (JAM), extend into the intercellular space and determine the permeability characteristics of the TJs in terms of specificity and tightness. The scaffolding proteins, among which the *zona occludens* (ZO) family, provide a structural and functional connection between the transmembrane proteins and the underlying cytoskeleton. Various signaling proteins regulate the TJ permeability either directly through the modification of the presence or function of TJ proteins, or indirectly through effects on the cytoskeleton (Lapierre, 2000; Harhaj and Antonetti, 2004).

Barrier disruption is an important etiologic factor of intestinal inflammation, causing not only defective nutrient uptake and retention, but also an increased permeability to luminal antigens with subsequent contact with and potential activation of underlying immune cells (Förster, 2008). Accordingly, the regulation of the gut barrier maintenance and functioning is an important issue to address, with the understanding of the interaction between dietary components and IECs being of particular interest, since this could lead to nutritional prevention of intestinal disorders.

Deoxynivalenol (DON or vomitoxin) is a trichothecene mycotoxin that is commonly found in raw and processed food and feed derived

Abbreviations: DON, deoxynivalenol; IBDs, inflammatory bowel diseases; IEC, intestinal epithelial cell; TJ, tight junction; TEER, transepithelial electrical resistance; LDH, lactate dehydrogenase; NF-κB, nuclear factor κB; IκB, inhibitor of κB; MAPKs, mitogen activated protein kinases.

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from cereal crops (SCF, 1999). Its toxicological effects mainly concern the immune system and the gastrointestinal tract. They have been observed mainly on experimental animals and livestock but occasionally also in humans. Chronic DON ingestion at low doses is associated with anorexia, altered nutritional efficiency and reduced growth, whereas acute higher doses provoke emesis, rectal bleeding and diarrhea (Rotter et al., 1996; SCF, 1999; Pestka and Smolinski, 2005), which are symptoms presenting strong similarities with gut disorders like Inflammatory Bowel Diseases (IBDs) (Neuman, 2007). At the cellular level, several reports indicate that DON could alter IEC differentiation (Kasuga et al., 1998), barrier functioning (Kasuga et al., 1998; Sergent et al., 2006; Pinton et al., 2009) and uptake of nutrients (Maresca et al., 2002). Trichothecenes are known to inhibit protein translation by binding to the peptidyltransferase of active ribosomes (Ueno, 1985). The intestinal epithelium, undergoing continuous cell renewal, is exposed to the highest doses of DON and could thus be particularly sensitive to the inhibitory action of DON on protein synthesis (Instanes and Hetland, 2004; Kouadio et al., 2007). We hypothesized that the IEC differentiation and permeability defects caused by DON resulted primarily from its inhibitory action on protein synthesis, and performed a comprehensive study integrating these dual toxic effects in one mechanism occurring in IEC at realistic concentrations of DON.

Materials and methods

Chemical. DON was provided by Sigma-Aldrich (St. Louis, MO). Culture reagents were purchased from Lonza (Verviers, BE) unless mentioned otherwise. Primary antibodies against claudin-4 and occludin were from Santa Cruz Biotechnology (Heidelberg, DE). The rabbit polyclonal antibody against human intestinal alkaline phosphatase was from Abcam (Cambridge, UK) and mouse monoclonal antibodies against human β-actin from Sigma-Aldrich. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit, goat anti-mouse, and rabbit anti-goat antibodies were from Dako (Glostrup, DK). [^{3}H]-Mannitol and [^{3}H]-leucine were provided by Amersham Biosciences (Buckinghamshire, UK). The protein synthesis inhibitor cycloheximide and the MAPKs inhibitors PD98059 (ERK1/2) and SP600125 (SAPK/JNK) were purchased from Sigma-Aldrich, the NF-κB inhibitor triptolide was from Tebu-Bio (Boechout, BE) and the proteasome inhibitor MG-132 from Calbiochem (Darmstadt, DE). Triton-X-100 and EGTA were from Sigma-Aldrich.

Cell culture. The human colon carcinoma Caco-2 cell line was obtained from ATCC (Rockville, MD). Caco-2 cells were grown in DMEM containing 10% (v/v) heat-inactivated FBS (Hyclone Perbio-Sciences, Erembodegem, BE), 4.5 g/l glucose, 25 mM Hepes, 2% (v/v) L-glutamine 200 mM and 1% (v/v) non-essential amino acids (NEAA) (Invitrogen, Carlsbad, CA) in a humidified atmosphere under 5% (v/v) CO_2 in air at 37 °C. For the experiments, Caco-2 cells (passage number between 30 and 50) were harvested at 80% confluency using 0.05% trypsin–0.02% EDTA (w/v) and seeded on type-I collagen (Sigma-Aldrich) precoated 6- or 12-well culture plates (Nunc, Roskile, DK) at a density of 40×10^3 cells/cm^2 unless mentioned otherwise. Cells were grown until 21 days post-confluence in standard medium supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin that was changed every 2 days. The treatments were then applied in culture medium containing 1% (v/v) FBS during 24 h.

Cytotoxicity assay. The cytotoxicity of the treatments was routinely checked using a Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, DE) as in Van De Walle et al. (2008). This assay measures the activity of the cytosolic enzyme Lactate Dehydrogenase (LDH), which is released in the extracellular medium upon cell damage or necrosis. The evaluation of LDH activity in the culture medium thus constitutes a good marker of cytotoxicity. The results were expressed in relation to the activity present in the culture medium of cells treated with 1% (v/v) Triton X-100 for maximal LDH release (positive control) or untreated cells (negative control).

Determination of total protein content. Differentiated Caco-2 cells were incubated for 24 h with DON (50, 500, 5000 ng/ml) or cycloheximide (10 µg/ml). Total proteins were extracted using 1% (w/v) deoxycholic acid (pH 11.3) and protein content was determined in µg/ml using the bicinchoninic assay (Sigma-Aldrich) and a BSA standard curve. Results were expressed in function of the negative control (untreated cells) and are presented as a mean ± standard error on the mean (SEM) of four independent experiments.

Determination of protein synthesis. Protein synthesis was assayed by measuring the incorporation of [^{3}H]-leucine into cell proteins. Differentiated Caco-2 cells were incubated for 24 h with DON (50, 500, 5000 ng/ml) or cycloheximide (10 µg/ml) in the presence of [^{3}H]-leucine (2 µCi per well, sp. act. 120–190 Ci/mmol). Cells were washed with PBS and proteins were precipitated by addition of ice-cold 10 % (v/v) trichloroacetic acid during 10 min, followed by washing and incubation with methanol for 10 min. The cellular material was then solubilized in 0.1 M NaOH and incorporation of [^{3}H]-leucine was quantified using liquid scintillation spectrometry as described below. Results are expressed as a percentage of the [^{3}H]-leucine incorporation of the negative control (untreated cells) and are presented as a mean ± SEM of four independent experiments.

Evaluation of cell barrier integrity. Caco-2 cells were seeded on transwell™ inserts with a pore diameter of 0.4 µm (Corning Costar Corp., Cambridge, MA) at a density of 120×10^3 cells/cm^2 and cultured until 21 days after confluence. DON (50, 500, 5000 ng/ml) was then applied at the apical side in 1.5 ml of culture medium containing 1 µCi of [^{3}H]-mannitol (sp. act. 26.30 Ci/mmol). The basolateral side received 2.6 ml of culture medium supplemented or not with an inhibitor of NF-κB activity (triptolide, 10 ng/ml), or of the activation of the MAPKs ERK1/2 (PD98059, 25 µM) or SAPK/JNK (SP600125, 50 µM). The efficacy of these inhibitors was previously demonstrated by Western blotting. After 24 h of treatment, a sample of 200 µl from the basolateral compartment was taken and diffusion of [^{3}H]-mannitol was measured by liquid scintillation spectrometry (Packard Tri-Carb 1600 TR, Packard, Meriden, CT) after dispersion in 2 ml of Aqualuma® (Lumac Lsc, Groningen, NL). The paracellular permeability coefficient (P_{app}) was determined in cm/min as $[dQ/dt]/[C_0{}^*A(\mathrm{cm}^2)]$, where dQ/dt is the rate of permeation, C_0 is the initial [^{3}H]-mannitol concentration in the apical compartment and A is the surface area of the culture insert. Results were expressed in function of the positive control, consisting in cells treated with 2.5 mM EGTA, as calcium chelating agent. The transepithelial electrical resistance (TEER) was measured at the beginning and the end of the experiment using an Endohm-24 chamber (World Precision Instruments, Sarasota, FL) and expressed in Ω cm^2. Results were expressed relative to the negative control (TEER of 100%) and are presented as a mean ± SEM of five independent experiments.

Western blotting analysis of claudin-4, occludin and alkaline phosphatase. Differentiated cells (21 days post-confluence) were exposed during 24 h to DON (50, 500, 5000 ng/ml) supplemented or not with 50 µM of MG-132. Proteins were extracted as in Van De Walle et al. (2008). Total protein content was determined using the bicinchoninic assay. Equal amounts (20 µg) of cell proteins were resolved by 11% (for occludin and intestinal alkaline phosphatase (ALP)) or 14% (w/v) (for claudin-4) SDS-PAGE and electrotransferred on a hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham). After blocking with 5% (w/v) nonfat-dry milk in a Tris buffer (137 mM NaCl, 20 mM Tris, pH 7.6) containing 0.05% (v/v) Tween 20 (TBST), overnight incubation with specific primary