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Physio-pathological parameters affect the activation of inflammatory pathways by deoxynivalenol in Caco-2 cells

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ABSTRACT

The intake of deoxynivalenol (DON), a mycotoxin contaminating cereal food items, causes gastro-intestinal illness in human and animal. This study investigated whether intracellular inflammatory cascades (MAPKs and NF-κB), cell maturity (proliferating *vs.* differentiated), cell state (control *vs.* inflamed) and exposure duration (chronic *vs.* acute) affect IL-8 secretion and PGE-2 synthesis in Caco-2 cells exposed to plausible intestinal concentrations (50, 500 and 5000 ng/ml) of DON. IL-8 secretion and PGE-2 synthesizing capacity were dose-dependently upregulated in differentiated Caco-2 cells exposed to DON during 24 h, reaching an increase of ~25 and 1.7-fold respectively, whereas transcript level of IL-8 and COX-2 were increased by ~40 and 17-fold. Similar results were obtained with proliferating cells. The upregulation decreased upon simultaneous incubation with inhibitors of MAPKs ERK1/2 or p38 or of transcription factor NF-κB. IL-8 secretion and PGE-2 synthesizing capacity increased respectively by ~15 and 2-fold after chronic 21 day incubation with DON (50 ng/ml). IL-8 production was exacerbated (~510-fold versus negative control) upon simultaneous exposure to inflammatory stimuli. These results suggest activation of inflammatory pathways in intestinal epithelial cells exposed chronically or acutely to DON. The sensitivity to DON, whereas not affected by cell differentiation, is exacerbated by the presence of additional stimuli.

1. Introduction

Intestinal epithelial cells (IECs) form a selective permeable barrier allowing the entry of nutrients into the internal circulation while limiting the permeation of noxious luminal dietary components. In response to various stimuli, IECs can secrete inflammatory mediators which activate the underlying immune cells, triggering inflammatory processes implicated in the intestinal mucosal defense (Jung et al., 1995; Panja et al., 1998). Defective IEC maintenance or function is considered as an important etiologic factor of various gut pathologies, affecting not only crucial activities like nutrient uptake and fluid balance, but also causing an inappropriate intestinal inflammatory response (Förster, 2008). Dietary components substantially contribute to or affect the regulation of IECs, and a better understanding of these processes is a key point for nutritional prevention and therapy of gut pathologies.

Mycotoxins are secondary fungal metabolites that commonly contaminate crops in the field and during storage. Deoxynivalenol (DON or vomitoxin) is produced by filamentous fungi of the *Fusarium* genus and is the most prevalent trichothecene mycotoxin in temperate climate regions (McMullen et al., 1997). DON survives most food processing treatments (Bullerman and Bianchini, 2007) and is thus frequently found in cereal commodities intended for animal or human consumption. According to parameters like cereal origin and nutritional behavior, the severity and frequency of exposure to DON can be important. The ingestion of DON by laboratory and farm animals was reported to trigger adverse toxicological effects mainly in the immune system and the gastro-intestinal tract. Acute intake of DON causes nausea, emesis, diarrhea, hemorrhage and leukocytosis, whereas chronic low dose ingestion of DON results in reduced food intake, impaired growth, neuroendocrine changes and immune modulation (Rotter et al., 1996; Pestka

Abbreviations: COX-2, cyclo-oxygenase 2; DON, deoxynivalenol; ERK, extracellular signal-regulated kinases; IBDs, inflammatory bowel diseases; IECs, intestinal epithelial cells; IκB, inhibitor of κB; IKK, IκB inducing kinase; MAPKs, mitogen activated protein kinases; LDH, lactate dehydrogenase; mPGES-1, microsomal PGE-synthase-1; NF-κB, nuclear factor κB; PGE-2, prostaglandin-E2; SAPK/JNK, stress-activated protein kinase/c-jun amino-terminal kinase; TNF-α, tumor necrosis factor.

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and Smolinski, 2005). Outbreaks of acute human illness with symptoms similar to gastroenteritis have been reported in China, Japan and India after consumption of mouldy maize or scabby wheat containing high levels of DON (Luo et al., 1990). Effects of chronic low dose ingestion of DON, the more common way of human exposure, remain mostly elusive.

DON binds to the ribosomal peptidyl transferase, which inhibits its protein synthesis and induces a "ribotoxic stress response" characterized by the activation of signaling pathways involving Mitogen Activated Protein Kinases (MAPKs) and their downstream targets (Shifrin and Anderson, 1999; Yang et al. 2000). Activation by DON of the MAPKs Extracellular signal-Regulated Kinases (ERK1/2 or p42/p44), Stress-Activated Protein Kinase/c-Jun Amino-terminal Kinase (SAPK/JNK1/2) and $p38^{MAPK}$ have been reported *in vitro* and *in vivo* in murine immune cells (Moon and Pestka, 2002; Zhou et al., 2003, 2005a; Pestka et al., 2005), with concurrent or subsequent activation of downstream substrates such as the transcription factors Nuclear Factor (NF)-κB, Activating Protein (AP)-1, CCAAT enhancer binding protein (C/EBP) and cyclic AMP Response Element Binding protein (CREB) (Zhou et al., 2003). NF-κB and MAPKs are major regulators of apoptosis and inflammation. The exposure of murine immune cells to DON increases the synthesis of various inflammatory mediators including interleukins IL-2, IL-4, IL-5 and IL-6 (Dong et al., 1994; Azcona-Olivera et al., 1995; Li et al., 1997; Pestka et al., 2005), Tumor Necrosis Factor (TNF)-α (Wong et al., 1998; Chung et al., 2003) and the enzyme cyclooxygenase-2 (COX)-2 (Moon and Pestka, 2002). Furthermore, the mycotoxin promotes anti-apoptotic stimuli like p90 ribosomal S6 kinase and AKT, although also the induction of the intrinsic and extrinsic apoptotic pathways that can occur through the apoptosis inductor p53 and effector caspase-3 (Zhou et al., 2005b). The effects of DON on immune cells can thus be either immunostimulatory through the increased synthesis of pro-inflammatory mediators or immunosuppressive through the induction of leukocyte apoptosis (Bondy and Pestka, 2000), and parameters like trichothecene dose and exposure duration appear crucial to determine the global response (Pestka et al., 2004). Also in human lymphocytes, DON exposure leads to increased secretion of IL-6 (Sugita-Konishi and Pestka, 2001), IL-8 (Pestka et al., 2005), IL-2, IL-4 and Interferon (IFN)-γ (Meky et al., 2001), which suggests that DON might play a role in human inflammatory disorders.

Since DON is a food contaminant, IECs are likely to constitute the first and primary target of its toxicological effects. Most perturbations of the gastro-intestinal tract observed after acute DON intake seem similar to symptoms of intestinal inflammatory diseases (IBDs) (Neuman, 2007), which provides additional evidence that DON could dysregulate inflammatory processes in the gut. The *in vitro* exposure of the human intestinal epithelium to DON negatively affects nutrient uptake (Maresca et al., 2002), IEC differentiation (Kasuga et al., 1998a; Turner et al., 2008) and barrier properties (Kasuga et al., 1998b; Sergent et al., 2006; Pinton et al., 2009). However, reports on the activation of intracellular inflammatory cascades in enterocytes are mostly limited to cultures of non differentiated IECs and/or to tests with huge DON concentrations (Instanes and Hetland, 2004; Moon et al., 2007; Van De Walle et al., 2008), which renders the extrapolation to the *in vivo* situation difficult. In order to more accurately approach the reality of DON toxicity on the intestine, Caco-2 cells were exposed to plausible intestinal concentrations of DON (50, 500 or 5000 ng/ml) to analyze how diverse physio-pathological parameters like IEC maturity (proliferating *vs.* differentiated), IEC inflammatory state (control *vs.* inflamed) and exposure duration (chronic *vs.* acute), as well as intracellular inflammatory cascades (NF-κB and MAPKs) affected the synthesis of the inflammatory mediators IL-8 and PGE-2.

2. Materials and methods

2.1. Chemicals

Culture reagents were purchased from Lonza (Verviers, BE) unless mentioned otherwise. DON, PD98059, SB203580 and SP600125, arachidonic acid and deoxycholic acid were from Sigma–Aldrich (St. Louis, MO), triptolide from Tebu-Bio (Boechout, BE), rabbit monoclonal IgG against phospho-IKKαβ (Ser176/180) and rabbit polyclonal IgG against total and phospho-MAPKs from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibodies against human β-actin were from Sigma–Aldrich and horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse antibodies from Dako (Glostrup, DK).

2.2. Cell culture

Caco-2 cells (ATCC, Rockville, MD) were cultured at 37 °C in 5% (v/v) CO_2/air 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) nonessential amino acids (NEAA) (Invitrogen, Carlsbad, CA) with weekly passage. Cells between passage 30 and 50 were seeded at a density of 80×10^3 cells/cm^2 on type-I collagen (Sigma–Aldrich) precoated 6-well plates (Nunc, Roskilde, DK) for western blotting assays and 24-well plates for ELISA. Caco-2 cells were grown for 24 h (proliferating cells) or until 21 days post-confluency (differentiated cells) in culture medium containing 100 U/ml penicillin and 100 μg/ml streptavidin. Treatment was applied in culture medium containing 1% (v/v) FBS during 24 h.

2.3. Evaluation of cell differentiation and cytotoxicity of treatments

The differentiation of cells was verified by measuring the activity of the intestinal alkaline phosphatase enzyme as described in Turner et al. (2008). Furthermore, different tests were routinely performed to check the ongoing differentiation of Caco-2 cells in this mode of culture, *i.e.* assay of the activity of (i) dipeptidyl-peptidase IV (Sergent et al., 2009b); (ii) Cyp1A1 (Sergent et al., 2009a); and (iii) Cyp3A4 (Sergent et al., 2009b). In addition, Caco-2 cells cultured in such conditions form domes, which is a hallmark of polarization and fluid transport.

At the end of the experiments, treatments were routinely checked for cytotoxicity by evaluating the activity of the enzyme Lactate Dehydrogenase (LDH) (Cytotoxicity Detection Kit, Roche Diagnostics GmbH, Mannheim, DE) in the extracellular medium as in Van De Walle et al. (2008). Results were expressed in relative terms to the activity present in the culture medium of either cells treated with 0.1% (v/v) Triton X-100 for maximal LDH release (positive control), or of untreated cells (negative control).

2.4. Evaluation of IL-8 and PGE-2 synthesizing capacity

In a first experiment, DON (50, 500 and 5000 ng/ml) was applied during 24 h on proliferating or differentiated cells. In a second experiment, DON was applied at 5000 ng/ml during 24 h in the presence or absence of the NF-κB inhibitor triptolide (10 ng/ml) or the following MAPK inhibitors: PD98059 (for ERK1/2) at 25 μM, SB203580 (for p38) at 50 μM or SP600125 (for SAPK/JNK) at 50 μM. A third experiment consisted in a 24 h incubation with DON (50, 500 and 5000 ng/ml) in the presence or absence of IL-1β at 25 ng/ml, LPS at 1 μg/ml, TNF-α at 50 ng/ml and IFN-γ at 50 ng/ml. In a forth experiment, cells were cultured in the presence or absence of DON at 50 ng/ml during 21 days, followed by a 24 h exposure to DON at 5000 ng/ml.