The food contaminant deoxynivalenol, decreases intestinal barrier permeability and reduces claudin expression

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A B S T R A C T

'The gastrointestinal tract represents the first barrier against food contaminants as well as the first target for these toxicants. Deoxynivalenol (DON) is a mycotoxin that commonly contaminates cereals and causes various toxicological effects. Through consumption of contaminated cereals and cereal products, human and pigs are exposed to this mycotoxin. Using *in vitro*, *ex vivo* and *in vivo* approaches, we investigated the effects of DON on the intestinal epithelium. We demonstrated that, in intestinal epithelial cell lines from porcine (IPEC-1) or human (Caco-2) origin, DON decreases trans-epithelial electrical resistance (TEER) and increases in a time and dose-dependent manner the paracellular permeability to 4 kDa dextran and to pathogenic *Escherichia coli* across intestinal cell monolayers. In pig explants treated with DON, we also observed an increased permeability of intestinal tissue. These alterations of barrier function were associated with a specific reduction in the expression of claudins, which was also seen *in vivo* in the jejunum of piglets exposed to DON-contaminated feed. In conclusion, DON alters claudin expression and decreases the barrier function of the intestinal epithelium. Considering that high levels of DON may be present in food or feed, consumption of DON-contaminated food/feed may induce intestinal damage and has consequences for human and animal health.

Introduction

Food safety is a major issue in Europe. In this respect, much attention needs to be paid to the possible contamination of food by fungi and the risk of toxin production. Mycotoxins are secondary metabolites produced by fungi that may contaminate all stages of the food chain. Their global occurrence is regarded as an important risk factor for human and animal health as up to 25% of the world crop production may be contaminated with mycotoxins (Oswald et al., 2005). In human, the toxicological syndromes caused by ingestion of mycotoxins range from death, induction of cancer and growth impairment (Bryden, 2007). Consumption of fungal toxins may also decrease resistance to infectious diseases (Oswald et al., 2005).

Deoxynivalenol (DON) is a mycotoxin of the trichothecenes family that is mainly produced by *Fusarium graminearum* and *F. culmorum*. DON is commonly detected in cereals and grains, particularly in wheat, barley, maize and their by-products. It is the most prevalent contaminating trichothecene in crop production in Europe and North America (CAST, 2003; SCOOP, 2003; Pestka and Smolinski, 2005). Recent surveys including 11,022 cereals samples from 12 European countries indicated that 57% of the samples were positive for DON contamination (SCOOP, 2003). Similarly, a survey including 630 samples collected in 25 states by the Federal Grain Inspection Service in United States, revealed that about 40% of the wheat samples and 57% of the barley samples contained levels greater than 2 mg/kg (Trucksess et al., 1995). Furthermore, this toxin is resistant to milling, processing and heating and, therefore, readily enters the food chain (Sugita-Konishi et al., 2006). Widespread human exposure to DON has also been demonstrated using a glucuronide metabolite as a urinary biomarker (Turner et al., 2008).

DON exhibits toxic effects in humans as well as in all animal species investigated so far (Pestka and Smolinski, 2005). Acute high dose toxicity of DON is characterized by effects such as diarrhea, vomiting, leukocytosis, hemorrhage, circulatory shock and ultimately death. Chronic low dose toxicity is characterized by anorexia, reduced weight gain, nutrients malabsorption, neuroendocrine changes and immunologic alterations (Pestka and Smolinski, 2005; Pinton et al., 2008). At the cellular level DON interacts with the peptidyltransferase at the 60S ribosomal subunit level, triggering a translational arrest (Pestka et al., 2004). As a result, protein synthesis is impaired, but also

a so-called "ribotoxic stress" is induced (Pestka et al., 2004), resulting in the activation of mitogen-activated protein kinases.

The intestinal tract represents the first barrier to ingested chemicals or food contaminants and is also the first line of defense against intestinal infection. The gut barrier is formed to a large extent by tight junctions that seal the luminal end of the intercellular space and limit transport by this paracellular route to relatively small hydrophilic molecules. These are multiple transmembrane, scaffolding and signaling proteins including Zonula Occludens-1 (ZO-1), occludin, and one or more claudin isoforms (Harhaj and Antonetti, 2004). Following ingestion of mycotoxin-contaminated food, intestinal epithelial cells could be exposed to high concentrations of toxin (Maresca et al., 2002; Bouhet and Oswald 2005; Sergent et al., 2006). However the effects of DON on the gastrointestinal tract have been poorly studied. DON was found to interfere with the TEER of human intestinal Caco-2 cells (Kasuga et al., 1998; Sergent et al., 2006) and to affect nutrients absorption (Maresca et al., 2002).

Using *in vitro*, *ex vivo* and *in vivo* approaches, the aims of the present study was to evaluate whether doses of DON commonly seen in contaminated food, could affect the barrier function of the intestine. We evaluated the effects of DON on (i) trans-epithelial electrical resistance, (ii) paracellular permeability and (iii) expression of tight junction proteins.

Materials and methods

Cell culture and reagents. The Caco-2 and IPEC-1 cell lines were derived from a human colon adenocarcinoma (ATCC HTB-37, Rockville, USA) and the small intestine of a newborn unsuckled piglet (Gonzalez-Vallina et al., 1996; Bouhet et al., 2004) respectively. Caco-2 were grown in DMEM medium (Sigma, St Quentin Fallavier, France) supplemented with penicillin (100 UI/mL), streptomycin (100 μg/mL), 15% fetal bovine serum (Eurobio, Les Ulis, France), 2 mM L-glutamine (Eurobio), MEM non-essential amino acids (Sigma). The same medium was used during the differentiation process. IPEC-1 were grown and differentiated as previously described (Bouhet et al., 2004). Both cell lines were maintained by serial passages.

Purified DON (Sigma) was dissolved in DMSO and stored at −20 °C before dilution in cell culture media. Control samples were treated with DMSO.

Animals. Animal experimentations were carried out in accordance with European Guidelines for the Care and Use of Animals for Research Purposes. Crossbred piglets, fed *ad libitum* with free access to water were killed by electrocution and exsanguination. Six animals (15–20 kg) fed with mycotoxin-free diet were used for the Ussing chamber experiments. Evaluation of claudin-4 expression was performed on 10 growing pigs fed for 5 weeks with either mycotoxin-free diet or DON-contaminated diet (2.85 mg DON/kg feed).

Measurement of trans-epithelial electrical resistance (TEER). Caco-2 or IPEC-1 cells were seeded at 10^5 cells in 0.3 cm^2 polyethylene terephtalate membrane inserts with 0.4 μm pore size (Becton Dickinson, Pont de Claix, France) in culture media and reached confluence within 2 days. Differentiation media was then used and changed every other day until complete differentiation. When differentiated, cells were treated with 0, 10, 20, 50 and 100 μM (Caco-2 cells) and 0, 10, 20, and 50 μM (IPEC-1 cells) DON and the TEER was measured during 14 days with a Millicell-ERS Voltohmmeter (Millipore, Saint-Quentin-en-Yvelines, France). TEER values were expressed as $k\Omega \times cm^2$.

Cytotoxicity assay. The cytotoxic effect of DON on Caco-2 or IPEC-1 cells was evaluated by measuring the activity of lactate dehydrogenase (LDH) released in the culture media using the CytoTox-96® Assay Kit (Promega, Charbonnières, France). Indeed, release of LDH correlates with the number of lysed cells and is widely used in cytotoxicity studies (Bouhet et al., 2004). Briefly, Caco-2 and IPEC-1 cells were seeded in 0.4 μm pore inserts and differentiated as described above before adding 0–100 μM DON for 48 h. LDH activity was then measured according to the manufacturer's instructions.

Paracellular tracer flux assay. Caco-2 or IPEC-1 were grown and differentiated in 0.4 μm pore inserts and treated with DON for 48 h as described above. 4 kDa Fluorescein isothiocyanate-dextran (FITC-dextran) (Sigma) was dissolved in cell culture medium and used at a final concentration of 2.2 mg/mL in the apical cell compartment. After 1 h of incubation the amount of fluorescence was measured in the basal compartment with a fluorimeter (Perkin Elmer LS50B, Courtaboeuf, France). The excitation and emission wavelengths were 490 and 520 nm, respectively.

Bacterial translocation. IPEC-1 cells, seeded in 3 μm pore inserts, were differentiated and treated for 48 h with DON in antibiotic-free medium. 24 h later, 2×10^6 colony forming unit of the pathogenic *Escherichia coli* strain 28C (CIP 107983 (Oswald et al., 2003)) was inoculated onto the apical surface of the cell layer. At various time points, 100 μL were collected in the basolateral compartment, diluted 1:1 in PBS and plated on Luria Broth Agar (Sigma).

Measurement of paracellular passage of FITC-dextran across porcine intestinal explants. The intestinal tract was removed and 10-cm segments from the mid-jejunum were immediately kept at 4 °C in a Krebs–Henseleit buffer (Sigma). After longitudinal incision, the underlying serosal and muscular layers were stripped off, and epithelial layer were mounted in Ussing chambers (exposed area, 0.5 cm^2).

Intestinal explants were equilibrated for 10 min in Krebs–Henseleit buffer at 38 °C continuously gassed with carbogen (95% O_2, 5% CO_2) before adding DON in the mucosal compartment (final concentration 0–50 μM). Afterwards 500 μL of FITC-dextran was added to the mucosal side. 800 μL samples were taken from the serosal side of the chambers 1 to 8 h after the addition of DON and replaced in the chambers after fluorescence measurements (as described above).

Confocal immunofluorescence microscopy. Caco-2 and IPEC-1 cells, seeded in inserts (0.3 cm^2, 0.4 μm pores), were differentiated and treated for 48 h with 30 μM DON. Cells were fixed with PBS 3.7% formaldehyde (30 min, room temperature) or for claudin-3 with methanol (5 min, −20 °C), permeabilized with PBS 0.5% Triton-X-100 for 3 min, and blocked with 10% goat serum (30 min, room temperature). Samples were incubated (1 h, room temperature) with anti-human primary antibodies diluted 1:100. These were rabbit polyclonal anti-claudin-3 (Z23.JM), occludin (Z-T22) and ZO-1 (Z-R1) or mouse monoclonal anti-claudin-4 (3E2C1) (Zymed Laboratories, South San Francisco, CA, USA). We first verified that these antibodies cross-reacted with porcine IPEC-1 cells. Cells were washed with PBS 0.1% Tween®20 (v/v) and incubated (30 min, room temperature) with FITC-conjugated goat anti-rabbit (Southern Biotech, Birmingham, AL, USA) or FITC-conjugated goat anti-mouse (Sigma) diluted 1:100. After washing, inserts were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were captured using a laser scanning confocal microscope (Olympus-IX70, Rungis, France). Optical sections (0.5 μm) were obtained using Olympus FV500 FluoView Application Software. To compare the expression of tight junction proteins in control and treated cells, images were acquired using constant acquisition parameters (laser power, confocal aperture, photomultipliers voltage and gain).

Immunohistochemistry. Jejunum fragments (1 cm) obtained from control and DON-treated animals were rinsed in PBS, fixed in 4% neutralized formalin for 24 h, embedded in paraffin-wax, cut into 5-μm-thick sections and deparaffinized. Immunohistochemical staining for

claudin-4 was performed using a standard streptavidine peroxydase procedure (apparatus Autstainer plus, Dako, France). To suppress endogenous peroxydase-like enzyme activities (undesirable background staining), the histological slides were treated by H_2O_2, after pretreatment for epitope retrieval (citrate buffer, ph 6, 95 °C, 40 min) and blocked with 10% goat serum (20 min, room temperature). After phosphate-buffered saline (PBS) wash, the samples were incubated (50 min, room temperature) with the primary antibody, a mouse anti-claudin 4, clone 3E2C1, diluted 1:100 (Zymed Laboratories). Sections were washed again with PBS 0.1% Tween®20 (v/v) and incubated (25 min, room temperature) with a biotinylated secondary antibody (kit ABC, Dako, France). After a wash, ABC complex was applied for 25 min at room temperature (streptavidine peroxidase biotinylated (Dako, France). After 2 PBS washes, 3-3′ diaminobenzidine was applied for 10 min at room temperature as a chromogen (Dako). The sections were then washed in distilled water and counterstained with Mayer hematoxylin. Intestinal sections from control and DON-treated animals were stained in parallel.

Tissue and cell protein extraction, SDS-PAGE, and immunoblotting. Caco-2 and IPEC-1 cells seeded in inserts (4.2 cm^2, 0.4 μm pore) were differentiated and treated with 30 μM DON during 48 h to analyze tight junction protein. Cells were rinsed with PBS and scraped in 80 μL of sample buffer (200 mM Tris–HCl pH 6.8, 30% glycerol, 15% β-mercaptoethanol, 6% SDS, 0.3% bromophenol blue). The samples were sonicated for 15 s, heated at 100 °C for 10 min and centrifuged (5000 g, 10 min). Supernatant were collected and proteins were quantified (Schaffner and Weissmann, 1973).

Midjejunal tissue specimens (1 cm) obtained from control or DON-treated animals were frozen in liquid nitrogen. Frozen tissue were washed in an anti-protease cocktail (4-(2-Aminoethyl; benzenesulfonyl fluoride hydrochloride; Aprotinin; Leupeptine; Antipaïne; Pepsatine A; Benzamidine, Sigma) prepared in PBS-EGTA (0.25 M). The samples were then lysed on ice in a Potter tissue grinder with lysis buffer (20 mM Tris–HCL pH8, 5 mM EDTA, 0.02% NaN3, 1% Triton X100) supplemented with antiproteases. Solution was homogenized through a 26G needle and sonicated for 3 times 20 s. Homogenate was diluted 1/2 with sample buffer (described above) and heated at 100 °C for 10 min before protein quantification.

15 μg of total proteins from cells or tissues was separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with PBS-Tween 5% skimmed milk and incubated with rabbit anti-claudin-3, mouse anti-claudin-4 (Zymed Laboratories) and rabbit anti-β-actin (Cell-Signaling, Danvers, MA) antibodies diluted 1:200, 1:500 and 1:2000 in PBS-Tween-milk respectively. After washing, they were incubated with 1:10,000 horseradish peroxidase-conjugated anti-mouse IgG (detection of claudin-4) or with 1:5000 to 1:7000 horseradish peroxidase-conjugated anti-rabbit IgG (detection of claudin-3 and β-actin). Antibody binding was detected with the SuperSignal West Pico chemiluminescent substrate (Pierce, Brebières, France) after placing the membrane against Hyperfilm™ (Amersham Biosciences, Buckinghamshire, UK). Signal intensities were estimated using Molecular-Imager® Gel Doc™ and Quantity One® software (Biorad, Marne la Coquette, France).

Statistical analysis. Data were analyzed with Statview software 5.0 (SAS Institute Inc, Cary, NC) using Student's *t* and Chi-2 tests. *P* values <0.05 were considered significant.

Results

DON causes a reduction in TEER of intestinal epithelial cell monolayers

First, we assessed, over a 14-day period, the effects of DON on TEER of polarized epithelial cells from human and porcine origin. When DON was added on human intestinal cells (Fig. 1A), a time and dose dependant reduction of the TEER was observed. After 2 days of treatment of Caco-2 cells with 5 or 20 μM DON, TEER was slightly decreased (−7%) but the decrease reached 58% after treatment with 100 μM DON. At the end of the experiment, 14 days of exposure to DON, the TEER decrease reached 19, 29, 77 and 79% for cells treated with 5, 10, 50 and 100 μM DON respectively.

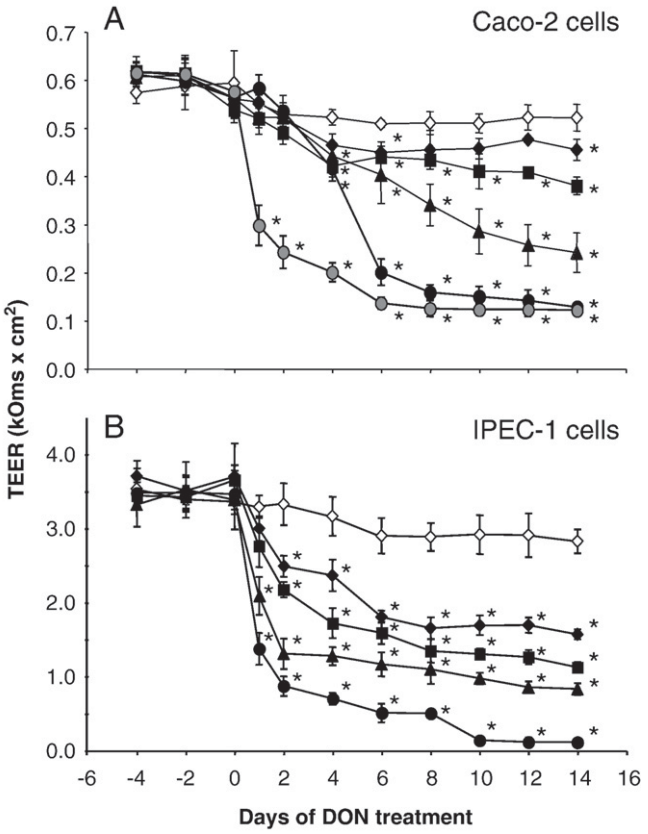

Fig. 1. Effect of DON on trans-epithelial electrical resistance (TEER) in polarized intestinal epithelial cell monolayers. Human Caco-2 cells (panel A) or porcine IPEC-1 cells (panel B) were grown and differentiated on inserts then at day 0 various concentrations of DON were added in the apical compartment: untreated inserts (◊), inserts treated with 5 (♦), 10 (■), 20 (▲), 50 (●) or 100 (⬤) μM of DON. TEER values are expressed in $k\Omega \times cm^2$ as mean ± SEM of 4 to 7 independent experiments. Student's *t*-tests were performed to determine the effect of DON treatment:*$P<0.05$.

A time-and-dose dependant reduction of TEER was also observed in porcine intestinal epithelial cells exposed to DON (Fig. 1B). One day of treatment of IPEC-1 cells with DON already induced a significant decrease of the TEER values: −25% (10 μM) to 60% (50 μM). At the end of the experiment, the TEER decrease reached 58, 69, 75 and 97% for cells treated with 5, 10, 20 and 50 μM DON respectively.

To determine whether the reduction in TEER induced by DON was due to cell death, the effect of 0–50 μM DON on the viability of the Caco-2 and IPEC-1 cell monolayers was examined using the LDH release assay. These concentrations of toxin did not significantly alter the cell viability up to 48 h after treatment (data not shown). However, exposure to higher concentrations of toxin (200 μM in IPEC-1 cells and 500 μM in Caco-2 cells) resulted in decrease cells viability.

These results indicate that non-cytotoxic doses of DON decrease the TEER of human and porcine intestinal cells in a dose and time dependant manner.

DON increases the permeability of epithelial intestinal cell monolayers to FITC-dextran and bacteria

Changes in TEER are indicative of alterations in epithelial barrier function or in the transcellular permeability of ions. To address the physiological significance of TEER decrease in polarized monolayers,

Table 1
Effect of DON on paracellular flux of FITC-dextran in polarized human or porcine intestinal epithelial cell monolayers.

Treatment	Mean fluorescence intensity measured in different cell lines	
	Caco-2	IPEC-1
Control	5.37 ± 0.25	1.36 ± 0.14
5 μM DON	5.80 ± 0.17	2.23 ± 0.31
10 μM DON	6.69 ± 0.58	2.73 ± 0.34
20 μM DON	6.73 ± 0.40	5.09 ± 1.04*
50 μM DON	8.37 ± 0.30 **	166.64 ± 42.01**
100 μM DON	21.48 ± 1.51 **	nd

Cell monolayers were grown and differentiated on inserts and treated with different concentrations of DON during 48 h. FITC-dextran was then added in the apical compartment. After 1 h, the fluorescent intensity was measured in the basolateral compartment. Results are expressed as mean fluorescence intensity in the basolateral compartment after subtraction of the background in 5 independent experiments ± SEM. nd: not done.
DON-treated and control cells were compared with Student *t*-test: *$P<0.05$; **$P<0.01$.

Table 2
Effect of DON on paracellular passage of FITC-dextran across intestinal explants.

Treatment	Mean fluorescence intensity after DON treatment	
	1 h	2 h
Control	4.3 ± 0.5	11.3 ± 1.4
5 μM DON	5.5 ± 0.6	11.7 ± 1.7
20 μM DON	8.7 ± 1.1**	19.8 ± 2.4**
50 μM DON	7.9 ± 0.9**	19.0 ± 2.9*

Explants of porcine jejunum were mounted in Ussing chambers before adding 0 to 50 μM DON and FITC-dextran in the mucosal compartment. At 1 and 2 h, the serosal compartment was collected to assess the fluorescence. Data are expressed as mean fluorescence intensity, after deduction of the background ± SEM of 6 to 8 independent experiments.
Student's *t*-tests were performed to determine the effect of DON treatment: *$P<0.05$, **$P<0.01$.

we tested whether DON also impacts on cell permeability. We first studied the effects of DON on permeability to FITC-dextran. Caco-2 and IPEC-1 monolayers were treated for 48 h with 0–50 μM DON before adding FITC-dextran in the apical compartment; one hour later, the fluorescence intensity was measured in the basolateral compartment. Untreated Caco-2 inserts were not readily permeable to FITC-dextran; however, upon 48 h treatment with 50 or 100 μM DON, the monolayer became significantly more permeable with a 1.6 and 4-fold increase in the tracer flux respectively (Table 1). In IPEC-1 monolayers, DON exposure also caused a dose-dependent increase of the tracer flux from 1.6 to 3.7-fold for treatment with 5 μM and 20 μM DON respectively (Table 1). As already observed in TEER experiments, Caco-2 cells exhibited a lower sensitivity to DON on the permeability of a paracellular tracer compared to IPEC-1 cells.

We next studied the effect of DON on bacterial translocation on porcine IPEC-1 cells as they appeared more sensitive than human cells. IPEC-1 cells were treated for 48 h with 0–50 μM DON, then *E. coli* were added on the apical side of the insert. Following 1–4 h interaction, media was collected from the basolateral compartment and plated to assess bacterial translocation. DON treatment resulted in a time- and dose-dependent increase of bacterial translocation (Fig. 2). For example, after 2 h, no bacteria were found in the basolateral medium of the 6 control inserts, whereas translocation was observed in 17, 50, 63% of inserts treated with 5, 10 and 20 μM DON respectively. At this time point, bacteria were also found in the basal compartment of all inserts treated with 50 μM.

These findings demonstrate that, in intestinal epithelial cells, DON modulates the paracellular pathway leading to an increased passage of macromolecule and bacteria.

DON increases paracellular passage of FITC-dextran across intestinal explants

Porcine intestinal explants mounted in Ussing chambers were used to investigate whether the effect of DON on paracellular passage of FITC-dextran was not limited to the cell culture insert model. These explants were treated for up to 2 h with 0–50 μM DON and the passage of FITC-dextran was measured. As shown in Table 2, a basal paracellular passage of FITC-dextran was observed in untreated explants and it increased over time. When the intestinal explants were treated with 5 μM DON, no significant difference in paracellular passage was observed compared to control explants. In contrast, the paracellular passage of FITC-dextran was increased 2 fold in explants treated with 20 μM and 50 μM DON, when compared to untreated ones.

DON selectively affects the expression of tight junction proteins

The gut barrier is formed to a large extent by tight junctions formed by multiprotein complexes that link adjacent epithelial cells near their apical border. Thus, we next examined the effect of DON on the expression of several tight junction proteins located in different cellular compartments: ZO-1 interacting in cytoplasm with actin cytoskeleton and occludin, claudin-3 or 4 interacting throughout their extracellular domain with neighbouring cells (McLaughlin et al.,

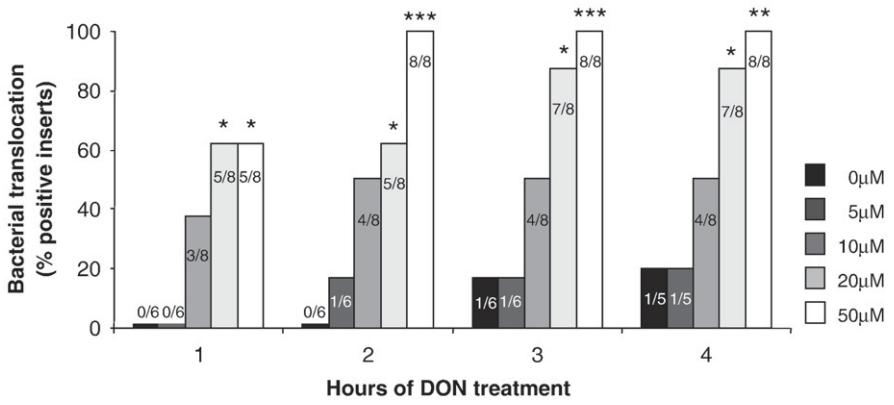

Fig. 2. Effect of DON on bacterial translocation across intestinal cell monolayers. IPEC-1 cells were grown and differentiated on 3 μm pore inserts and treated for 48 h with 0 to 50 μM DON. Pathogenic *E. coli* were added on the apical side and, at indicated time, samples from the basal compartment were plated. Data are expressed as % inserts where translocation was observed (numbers inside bars indicate number of insert positive for bacterial translocation compared to the total number of epithelial preparations observed). Chi-2 tests were performed to determine the effect of DON treatment on bacterial translocation: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

2004; Schneeberger and Lynch, 2004). Caco-2 and IPEC-1 cells, grown and differentiated on inserts, were treated with 0 or 30 μM DON for 48 h, then stained for ZO-1, occludin, claudin-3 or 4. As expected, localization of the tight junction proteins showed strong peripheral labeling in control Caco-2 and IPEC-1 cell monolayers. The overall morphology of DON-treated cells was unchanged. ZO-1 as well as occludin staining and localization were unaffected by DON treatment, irrespective of the cell line considered (Fig. 3). In contrast, DON treatment strongly reduced the intensity of the staining for claudin-3 in Caco-2 cells (Fig. 3A) and for both claudin-3 and claudin-4 in IPEC-1 cells (Fig. 3B). No increase of immunofluorescent signal was observed in other area of the cells, suggesting that, rather than being relocalized, claudins were removed from the cell tight junctional complexes.

This hypothesis was tested by western blot analysis with antibodies for claudin-3, claudin-4 and β-actin (Fig. 3). Densitometric analysis of the immunoblots revealed that in Caco-2 cells the signal for claudin-4 was reduced by 48% ($P<0.001$) in DON-treated samples

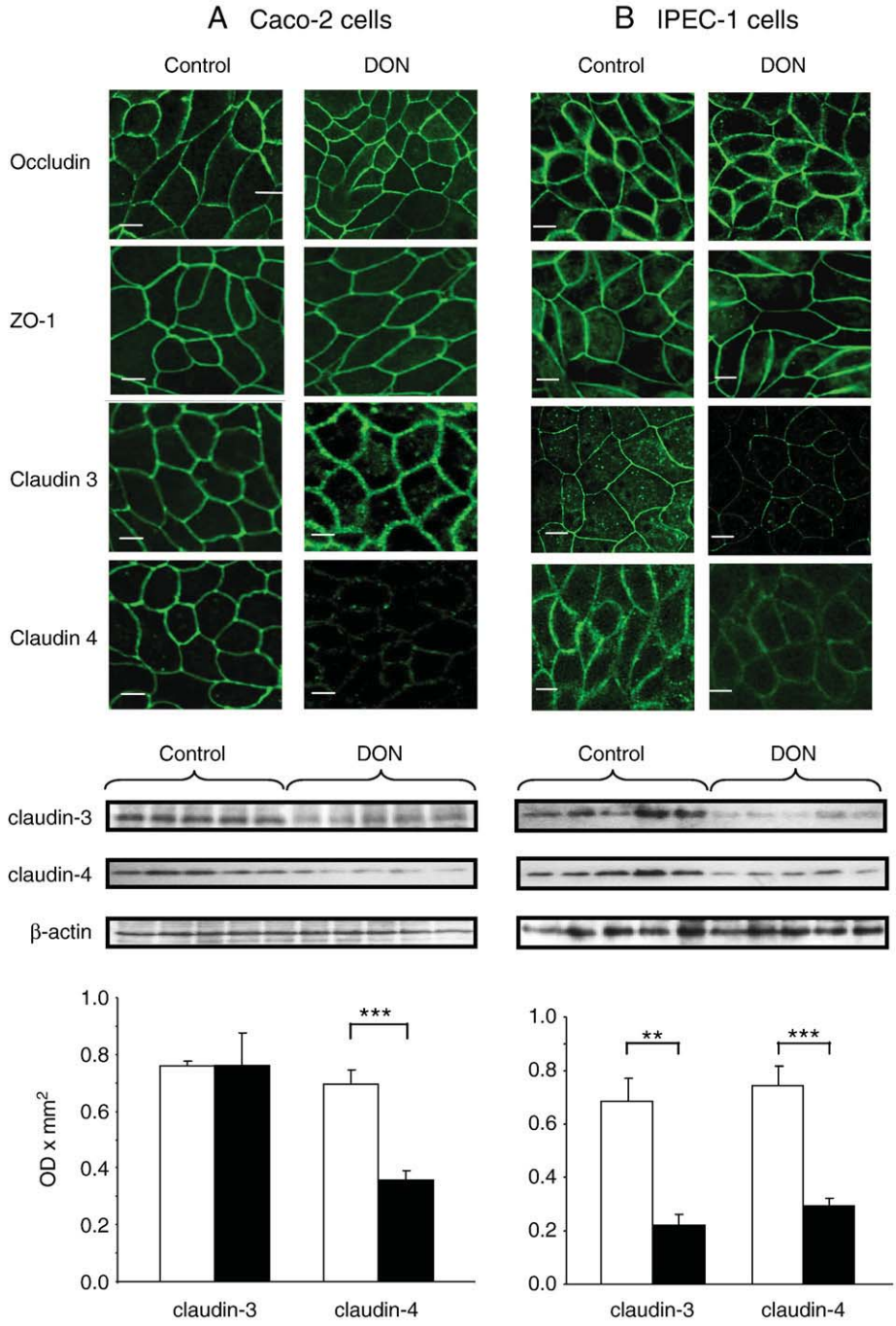

Fig. 3. Effect of DON on the expression of tight junction proteins on intestinal cell monolayers. Human Caco-2 cells (panel A) or porcine IPEC-1 cells (panel B) were grown and differentiated on inserts and treated for 48 h with 30 μM DON. Upper panel illustrates immunocytochemistry experiments: cells were stained with antibodies for occludin, ZO-1, claudin-3 and claudin-4. Confocal images ($z=0.5$ μm) were acquired using constant parameters (upper panel). The bar represents 10 μm. Middle and lower panels illustrate western blotting experiments: cells were scraped, lysed, and 15 μg of proteins was analyzed by immunoblotting with antibodies for claudin-3 and claudin-4 and β-actin as a protein loading control. The expression of the proteins was estimated by densitometry after normalization with β-actin signal and presented as mean ± SEM of 5 independent experiments. Middle panel represents the immunoblots and lower panel the densitometric analysis of control (open bars) and DON-treated (closed bars) samples. Student's *t*-tests were performed to compare control and DON-treated samples: $^{**}P<0.01$; $^{***}P<0.001$.

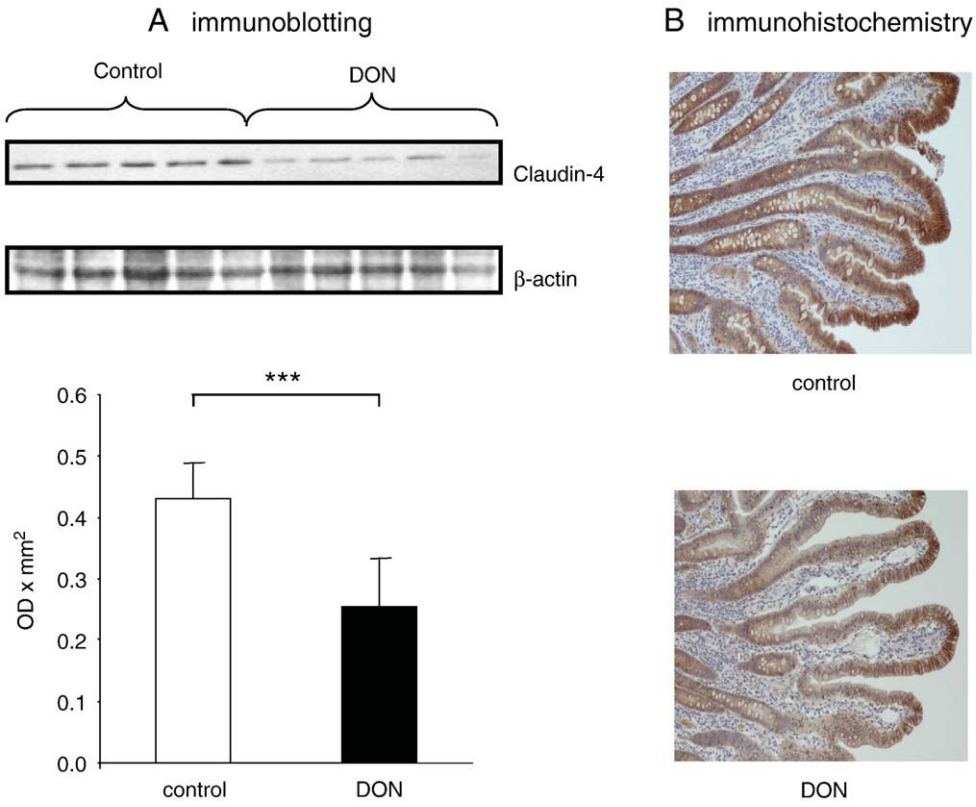

Fig. 4. Effect of DON exposure on the expression of claudin-4 in the jejunum. Midjejunal tissues were obtained from pigs fed for 5 weeks with mycotoxin-free or DON-contaminated feed. Panel A (immunoblotting): Proteins were extracted from frozen tissue and 15 μg of proteins was analyzed by immunoblotting with antibodies for claudin-4 and β-actin as a protein loading control. The expression of the proteins was estimated by densitometry after normalization with β-actin signal and presented as mean ± SEM of 5 control and 5 treated animals. Student's *t*-tests were performed to compare control and DON-treated samples: ***$P<0.001$. Panel B (immunocytochemistry): Paraffin wax-embedded jejunum fragments were obtained from control and DON-treated animals, 5-μm-thick sections were stained with antibody for claudin 4 and counterstained with Mayer hematoxylin.

when compared with untreated ones. In this cell line, the signal for claudin-3 remains unchanged in control and treated cells. In IPEC-1 cells, the signals for both claudin-3 and claudin-4 were reduced in DON-treated samples when compared to untreated samples (claudin-3: −67%, $P=0.0011$; claudin 4: −60%, $P<0.001$).

In vivo, exposure to DON-contaminated diet also significantly reduced claudin-4 expression. Pigs received for 5 weeks, either control non contaminated feed or feed contaminated with 2.85 mg/kg DON. Western blot analysis of jejunum samples revealed a 40% decrease of claudin-4 expression in samples obtained from DON exposed animals when compared with controls ones (Fig. 4A). This reduction was confirmed by immunohistochemistry, and was more pronounced in the intestinal villi than in the crypts (Fig. 4B).

We demonstrated that DON selectively decreases the expression of the claudins tight junction proteins in intestinal epithelial cells treated with the toxin as well as in the jejunum of animals exposed to DON-contaminated feed.

Discussion

An important function of gastrointestinal epithelia is to provide a barrier against the penetration of food contaminants and pathogens present in the intestinal lumen. The disruption of the intestinal barrier allows increased penetration of normally excluded luminal substances that may promote intestinal disorders (Arrieta et al., 2006; Oswald, 2006). In the present study we demonstrated that DON alters claudin expression and decreases the barrier function of the intestinal epithelium.

DON is a common contaminant of cereal grains encountered worldwide (CAST, 2003). For example, US Federal Grain Inspection Service or European Union surveys have shown significant levels of DON contamination in raw cereals: up to 50 mg/kg in wheat, 26 mg/kg in barley and 19 mg/kg in corn meal (SCOOP, 2003; Trucksess et al., 1995; Abouzied et al., 1991). In the present study, concentrations demonstrating an effect on intestinal barrier ranged between 5 and 30 μM. Assuming that DON is ingested in one meal, diluted in 1 L of gastrointestinal fluid and is totally bio accessible (Sergent et al., 2006), the *in vitro* concentrations used in this study correspond to food contamination ranging from 1.5 to 10 mg/kg. A similar approach has already been used in DON or ochratoxin A studies (Sergent et al., 2006).

In the present study, we extend to a non-transformed porcine intestinal epithelial cell line the finding that DON decreases in a dose and time dependant manner the TEER, a good indicator of the epithelium integrity. Beside DON, other mycotoxins such as Ochratoxin A, Patulin, or Fumonisin have been shown to decrease the TEER of epithelial cells (Bouhet et al., 2004; McLaughlin et al., 2004; Maresca et al., 2008). The observed reduction in TEER can be due to an alteration of the tight junction barrier properties but also to an effect on plasma membrane such as differences in transcellular ion transport (Barrett, 1993). As we observed that DON increases the permeability of Caco-2 and IPEC-1 cells to a 4 kDa paracellular tracer and decreases the expression of claudins, we would favor the hypothesis of an action on the tight junctions.

Using porcine explants, we were able to extrapolate the effect of DON on paracellular permeability from *in vitro* culture to tissue explants. Intestinal mucosal permeability from the pig correlated with those of human (Nejdfors et al., 2000). We further confirmed that DON affects the expression of tight junction protein on jejunal section obtained from piglets exposed to contaminated feed. Among animal species, pig shows great sensitivity to DON, moreover, pig can be regarded as a good model of extrapolation to humans (Rothkotter et

al., 2002; Almond, 1996). As we observed an effect of DON on human and porcine intestinal cell lines as well as on porcine explants, we can make the hypothesis that intestinal barrier function is also decreased in human or animals eating DON-contaminated food/feed.

A higher sensitivity to DON, as measured by TEER, paracellular permeability and claudin expression, was observed in IPEC-1 cells when compared to Caco-2. Several hypotheses could explain this higher sensitivity of IPEC-1 cells. First, Caco-2 cells were obtained from an adenocarcinoma whereas IPEC-1 cells were derived from normal newborn piglet (Gonzalez-Vallina et al., 1996). Second, even if Caco-2 cells express morphological and biochemical characteristics of small intestine (Pinto et al., 1983), they are derived from the colon. By contrast, IPEC-1 cells were obtained from jejunum and ileum. Third, these two cells line are from different species (pig versus human). Among animal species, pig is the most sensitive species to DON however it is difficult to assess the susceptibility of humans (Pestka and Smolinski, 2005). Our study demonstrates that IPEC-1 cells represent a very sensitive cellular model to investigate the effect of food contaminants.

The decreased barrier function (TEER and paracellular flux) was associated with an increased translocation of a pathogenic *E. coli* across the IPEC-1 cell monolayers. Using a laboratory K12 strain of *E. coli*, an increase translocation across Caco-2 cell monolayers was also demonstrated (Maresca et al., 2008). It would be important to extend this observation using a non-domesticated pathogenic strain of porcine (clinical) origin. Indeed, the *E. coli* taxon is highly versatile; the genome difference between a laboratory-adapted strain and a pathogenic clinical strain can reach as much as 60% (Welch et al., 2002) and it is recognized that a laboratory K-12 strain does not represent the archetypal, "normal" *E. coli* (Hobman et al., 2007).

As previously observed during metabolic stress, interferon-γ treatment or hypoxia (Tazuke et al., 2003; Clark et al., 2005; Nazli et al., 2006), DON treatment causes an increase in the translocation of bacteria across intestinal epithelia. This increase in the bacterial translocation following DON treatment could have major implications for human and animal health in term of susceptibility to infection as well as inflammation and sepsis. Moreover, as a synergistic toxicity between DON and LPS as been described, especially when looking at the secretion of inflammatory cytokines (Islam and Pestka, 2006; Van De Walle et al., 2008), we predict that translocated bacteria may amplify the intestinal toxicity of DON.

In mice, DON-contaminated diet accelerates *Salmonella enteritidis* infection (Hara-Kudo et al., 1996) and transiently increases the severity of reovirus infection (Li et al., 2005). In addition, inflammatory bowel diseases, such as Crohn's disease, are generally associated with an increased passage of bacteria through the gut epithelium because of a defective epithelial barrier function (Martin et al., 2004; Sasaki et al., 2007; Weber and Turner, 2007). It might be speculated that, at least in some cases, ingestion of DON-contaminated food could be involved in inducing inflammatory bowel disease.

Decreased TEER and increased FITC-dextran permeability suggested modifications of the tight junction complex (Madara, 1998). Confocal views of DON-treated cells stained for tight junction proteins showed a large reduction in the intensity of the junctional staining for claudins but not for ZO and occludin. No relocalization of the fluorescent signal to other cellular areas was observed, suggesting that claudins had disappeared from the cells. Consistent with this observation immunoblotting results demonstrate a reduction in the levels of claudins. The overall morphology of the cells was unchanged by DON treatment, suggesting specific effects on claudins rather than gross changes to the cells. At the tissue level, the decrease of the expression of claudin-4 in response to DON treatment was more important in villi than in crypts. Regarding the specific effect of toxin on claudin expression, similar results have been observed with Ochratoxin A, another mycotoxin (McLaughlin et al., 2004) and enterotoxin A from *Clostridium perfringens* (Sonoda et al., 1999). The specific removal of claudin from intercellular junctional complexes also suggests that the presence of ZO-1 and occludin alone in the tight junctions may not be sufficient to achieve a paracellular seal in intestinal epithelial cells.

As we observed that, in intestinal tissues, DON concurrently decreases barrier function and reduces the expression of claudin, we can make the hypothesis that, among other tight junction proteins, the specific decrease of claudin expression is responsible for the increase of intestinal barrier permeability. This mechanism as already been suggested in cultured cells for the increase permeability of canine intestinal epithelial cells exposed to *Campylobacter jejuni* (Lamb-Rosteski et al., 2008) and for the decrease of the barrier function of Caco-2 cells treated with Ochratoxin A (McLaughlin et al., 2004). In human biopsies, down-regulation of claudin-4 and claudin-7, and up-regulation of claudin-2 was observed in active ulcerative colitis and might lead to altered TJ structure and impaired epithelial function (Oshima et al., 2008).

Tight junction structure and function can be regulated by signaling molecules involved in MAPK pathways (Matter and Balda, 2003) and DON is known to rapidly activate MAPK (Pestka et al., 2004). Thus, we can make the hypothesis that activation of MAPK, as a consequence of DON exposure, decreases the expression of tight junction protein such as claudin-4 which in turn reduces the barrier function of the intestine evaluated by TEER, paracellular permeability and bacterial translocation.

This study, along with previous ones, strengthens the concept that intestinal epithelial cells are important targets for the toxic effects of mycotoxins (Bouhet et al., 2004, 2006; Yang et al., 2009). Considering that high levels of DON may be present in animal feeds and human food, chronic consumption of DON-contaminated food or feed can induce intestinal damage and may have consequences for animal and human health. Further epidemiological studies are needed to assess the extent to which DON may be involved in the development of infectious diseases in humans and animal.

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