In Vitro Effects of Deoxynivalenol on Small Intestinal D-Glucose Uptake and Absorption of Deoxynivalenol Across the Isolated Jejunal Epithelium of Laying Hens

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ABSTRACT Deoxynivalenol (DON) is a common mycotoxin contaminant in feedstuffs. It has been shown to cause diverse toxic effects in animals. The aim of the present study was to evaluate the effects of DON on the glucose transport capacity in chickens' jejunum and to investigate the permeation of DON itself by the Ussing chamber technique. Glucose uptake into chicken jejunal epithelia was measured after the addition of 200 μmol/L of ^{14}C-labeled glucose to the mucosal solution. Glucose uptake under control condition was 3.28 ± 0.53 nmol/cm^2·min. The contribution of sodium glucose-linked transporter 1 (SGLT-1) to total glucose uptake was estimated by inhibiting SGLT-1 with phlorizin (100 μmol/L). In the presence of phlorizin, glucose uptake was reduced ($P < 0.05$) to 1.21 ± 0.19 nmol/cm^2·min. Deoxynivalenol decreased ($P < 0.05$) the glucose uptake in the absence of phlorizin to 1.81 ± 0.24 nmol/cm^2·min but had no additional effect on the glucose uptake in the presence of phlorizin (0.97 ± 0.17 nmol/cm^2·min). Mucosal-to-serosal permeation of DON was proportional to the initial DON concentration over a concentration range from 1 to 10 μg/mL on the mucosal side. Apparent permeability at 10 μg/mL of DON measured 60 to 90 min after DON application was $1.7 \cdot 10^{-05}$ cm/s. It can be concluded that DON (10 mg/L) decreases glucose uptake almost as efficiently as phlorizin. The similarity between the effects of phlorizin and DON on glucose uptake evidences their common ability to inhibit Na^+-D-glucose cotransport. In addition to local effects, DON can be absorbed from the jejunum. A predominant part of DON passes across the chicken intestinal epithelium by passive diffusion, which is likely on the paracellular pathway. The results imply that the exposure to DON-contaminated feeds may negatively affect animal health and performance by local (i.e., inhibition of intestinal SGLT-1) and systemic effects.

Key words: laying hen, deoxynivalenol, Ussing chamber, glucose absorption, sodium glucose-linked transporter-1

2007 Poultry Science 86:15–20

INTRODUCTION

Mycotoxins are compounds often found in cereal grains and forages (Sweeney and Dobson, 1998). These toxins are produced by saprophytic fungi during storage or by endophytic fungi during plant growth. The presence of mycotoxins in poultry feeds is a significant factor for financial losses to animal industries. Mycotoxins like deoxynivalenol (**DON**) cause losses in performance and pose a health problem to livestock consuming contaminated cereal products (Dänicke et al., 2002). In general, poultry are less sensitive to DON compared with other species (Böhm, 2000; Razzazi-Fazeli et al., 2003). However, experimental studies with poultry show a highly variable effect of DON on performance (Dänicke et al., 2002; Sypecka et al., 2004). The latter indicates that zootechnical traits might not be a sensitive indicator of toxicity of this *Fusarium* toxin.

Toxic effects of DON in different animal species have been well documented and focus mainly on the immune system and the gastrointestinal tract. The injury to the gastrointestinal tract involves thickening of the mucosa of the stomach and changes in villi morphology in the small intestine (Rotter et al., 1994; Awad et al., 2006a,b). Consequences are alterations in feed conversion and susceptibility to diseases (Bondy and Pestka, 2000).

It seems likely that the morphological changes in the intestine and the decreased feed conversion are linked to an impaired absorption of nutrients. Intestinal absorption of sugars and amino acids occurs mainly actively through cellular pathways and small quantities passively through a paracellular or a cellular route. Given the active cotransport of D-glucose and sodium via the sodium glucose-linked transporter-1 (**SGLT-1**), the addition of D-glucose to the luminal side of different parts of small and large intestines of chickens induces a current flow across the

Received June 8, 2006.
Accepted August 23, 2006.
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epithelium (Amat et al., 1999). This current can be measured in Ussing chambers as short-circuit current (**Isc**). Awad et al. (2004) found a decreased Isc after addition of D-glucose when artificially-contaminated diets, containing 10 mg of DON/kg, were fed to broilers for 42 d. This can be taken as an indirect indication that DON interferes with SGLT-1 activity in the chicken intestine. However, direct evidence for this suggestion is lacking so far. Therefore, the aim of the first experiment was to verify an effect of DON on small intestinal glucose absorption by direct evaluation of the effect of DON on intestinal glucose uptake in chickens, using an Ussing chamber approach.

The second aim of the study was to evaluate the absorption of DON itself in our in vitro system. In vivo, DON seems to be rapidly and efficiently absorbed, most probably from the upper parts of the small intestine, and is mainly excreted in the urine, with no significant accumulation in tissues (Prelusky et al., 1988; Eriksen et al., 2003). Very little data are available on the transport mechanism of DON through the chicken digestive tract. Therefore, in the second experiment, we have investigated the kinetics of DON permeation in chicken jejunal mucosa.

MATERIALS AND METHODS

Birds and Feeding

Lohmann Brown laying hens, 18 to 22 wk of age, weighing 1 to 1.5 kg, were used in the present study. The birds were purchased from a local commercial farm (Erzeugergemeinschaft Agrarprodukte, Wildenhain, Germany). The hens were housed on deep wood shavings litter and were fed a commercial layer diet (Leikra-Qualitätsfutter, Leipzig, Germany). The diet contained 16.5% CP, 3.8% crude fat, 3.8% crude fiber, 12.9% crude ash, and 0.26% Met. The hens were provided with their diets and water ad libitum for the duration of the experiment.

Tissue Preparation

Tissue preparation, incubation in Ussing chambers, and electrophysiological recordings were performed as described earlier (Awad et al., 2004) with a few modifications specified below. Birds were slaughtered by stunning and bleeding. Immediately after exsanguination, the gastrointestinal tract was removed from the abdominal cavity. Segments were taken from the midjejunum. The intestine was rinsed with ice-cold buffer and transported to the laboratory in ice-cold incubation buffer oxygenated with carbogen (95% O_2 and 5% CO_2). The intestinal segment was opened along the mesenteric border and washed free of intestinal contents with buffer solution at 4°C. The underlying serosal layer was stripped off, and the epithelial sheets were mounted in Ussing chambers. Epithelial sheets had an exposed serosal area of 1.1 cm^2 and were incubated with 12 mL of buffer solution on their mucosal and serosal sides under short-circuit conditions.

Buffer Solutions

Buffer solutions used for washing, transport, and incubation of epithelia contained the following chemicals (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany, in mmol/L): NaCl, 115; KCl, 5; $CaCl_2$, 1.5; $MgCl_2$, 1.2; NaH_2PO_4, 0.4; Na_2HPO_4, 2.4; L-glutamine, 1; Na-DL-lactate, 5; HEPES-free acid, 10; $NaHCO_3$, 20; mannitol, 20; and NaOH, 10 (320 ± 5 mosmol/kg; pH 7.4). Isolated epithelia were incubated in D-glucose-free buffer mucosally and glucose (10 mmol/L) containing buffer serosally in Ussing chamber for at least 30 min. The incubation medium was continuously gassed with carbogen, and the temperature of the mixture was kept at 38°C by thermostated water jackets. Continuous oxygenation provided recirculation of the incubation solutions by means of a gas lift.

Glucose Uptake Technique

After a tissue stabilization period of 15 min, the basal measurements of Isc and tissue conductance (Gt) were recorded. The allotment of epithelia to different treatments was based on their tissue conductance values 30 min after mounting. Epithelial tissues used for direct comparison of different treatments were not allowed to differ in their conductance values by >30%. Forty-five minutes before uptake measurement, DON (Biopure GmbH, Tulln, Austria, 10 mg/L, final concentration) was added on the luminal side in the DON-treated group. The inhibitor of SGLT-1, phlorizin (100 μmol/L, final concentration), was added to the mucosal side 2 min before mucosal glucose addition. Deoxynivalenol and phlorizin were dissolved in ethanol. An equal volume of ethanol was added to the luminal side in the control epithelia. Thereafter, measurement of glucose uptake was started following the protocol of Aschenbach et al. (2002). ^{14}C-D-glucose (Amersham Biosciences, Little Chalfont, UK) was added to a final concentration of 200 μmol/L to the bathing solution on the mucosal side (final activity, 16.7 MBq/L). After 1 min of incubation with glucose, epithelia were washed 3 times with ice-cold buffer solution to stop protein-mediated transport processes. Ussing chambers were taken off their holders, and epithelia were transferred quickly to a precooled lysing device. The lysing device consisted of a flat plate on which the epithelia were placed (mucosal side up) and Al cylinders fixed over the epithelia. Epithelial cells were lysed by applying 4 mL of ice-cold NaOH (100 mmol/L) into the Al cylinders for 3 min (exposed area, 0.385 cm^2). The lysates were cleared by centrifugation (3,000 × g, 15 min). Glucose contents were determined in the lysate in duplicate. Glucose was measured by scintillation counting after addition of a liquid scintillation fluid (Aquasafe 300 Plus, Zinsser Analytic, Maidenhead, UK).

DON Transport Studies

Isolated epithelia were incubated in D-glucose-free buffer mucosally and D-glucose-(5 mmol/L) containing

buffer serosally in Ussing chambers. The epithelia were adapted to experimental conditions for 30 min. Thereafter, DON was introduced to the mucosal side at a final concentration of 1, 5, or 10 mg/L. Unidirectional DON fluxes from the mucosal to the serosal side were determined. At each sampling time (every 30 min), 2 mL of the buffer was withdrawn from the serosal side and replaced by fresh buffer solution. Samples were analyzed for their DON content by HPLC. Mucosal-to-serosal fluxes (J) and apparent permeability ($\mathbf{P_{app}}$) of DON were calculated from values obtained for serosal DON according to the following formulas: $J = dQ/dt \cdot A^{-1}$ and $P_{app} = J \cdot C_0^{-1}$, where dQ/dt = the amount of substance permeating per time unit; A = exposed surface area (i.e., 1.1 cm^2); and C_0 = the initial concentration in the mucosal chamber.

HPLC with Ultraviolet Detection of DON

The buffer samples from DON transport experiments were analyzed, without any extraction or cleanup, by HPLC with ultraviolet detection. The mobile phase consisted of HPLC-grade water (82%, Fisher Chemical Co., Fairlawn, NJ), methanol (9%), and acetonitrile (9%). One hundred microliters of reconstituted aliquot was injected into the HPLC system with a flow rate of 1 mL/min. The HPLC equipment consisted of an Iso Chrom LC pump (Spectra Physics Co., San Jose, CA) and autosampler AS-2000 (Merck-Hitachi, Tokyo, Japan). Separation of analytes was performed with a Synergy 4μ polar reversed-phase column (250 × 4.6 mm, 4 μm, 80A, Phenomenex, Torrance, CA) and a guard column (4 × 3 mm). The detection was achieved with an ultraviolet detector (Spectra Physics Co.) at 220 nm, and chromatograms were integrated with the help of the software Stratos V.4 (Polymer Laboratories Ltd., Shropshire, UK).

Statistics

Arithmetic means are presented together with their SEM. Data means were compared by Friedman's repeated measures ANOVA on ranks. Thereafter, Student-Newman-Keuls' test was used for the multiple comparisons of different treatments. Statements of statistical significance were based on $P \leq 0.05$, using the statistical software SigmaStat 2.0 (Systat Software GmbH, Erkrath, Germany). The same software was used to estimate linear regressions parameters.

RESULTS

Glucose Uptake Under Control Conditions

Glucose uptake was 3.28 ± 0.53 nmol/cm^2·min in the control tissues (n = 10; Figure 1). The contribution of SGLT-1 to total glucose uptake was estimated by inhibiting SGLT-1 with phlorizin. In the presence of phlorizin, glucose uptake was reduced ($P < 0.05$) to 1.21 ± 0.19 nmol/cm^2·min.

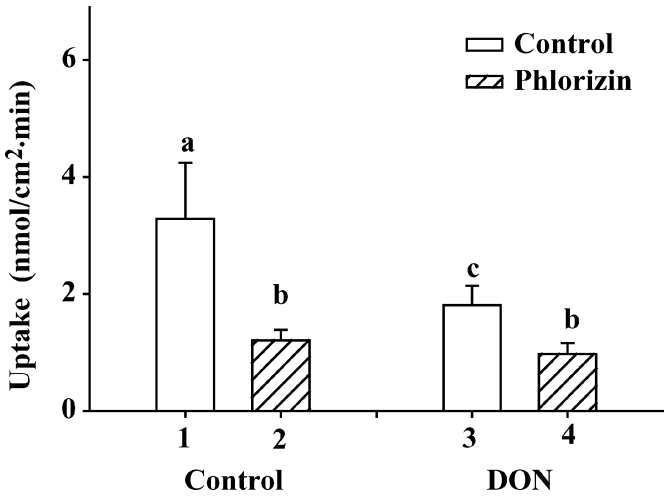

Figure 1. Influence of deoxynivalenol (DON) on apical glucose uptake by chicken jejunal epithelia in the absence or presence of phlorizin. Addition of DON (10 mg/L), phlorizin (100 μmol/L), or both to the mucosal side of intestinal epithelia was performed 45 min (DON) or 2 min (phlorizin) before a 1-min glucose uptake period. Means with different letters are significantly different ($P < 0.05$). The results are reported as means ± SEM [n = 10 (number of experiments for each treatment)].

Effect of DON on Glucose Uptake

The effect of DON was tested in the presence and absence of phlorizin. Deoxynivalenol decreased ($P < 0.05$) the glucose uptake in the absence of phlorizin to 1.81 ± 0.24 nmol/cm^2·min. However, it had no additional effect on the glucose uptake in the presence of phlorizin (0.97 ± 0.17 nmol/cm^2·min).

DON Transport Studies

In 1 set of DON transport experiments, DON was added at 10 μg/mL on the mucosal side, and its flux was determined in mucosal-to-serosal direction over consecutive 30-min flux periods (Figure 2). Flux rate of DON

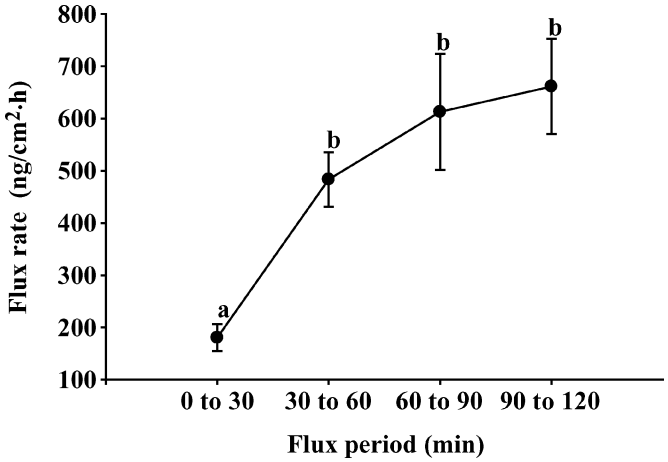

Figure 2. Time kinetics of passage of deoxynivalenol (DON; 10 μg/mL) added in the apical (mucosal) compartment to the opposite compartment (serosal). Mucosal-to-serosal flux of DON was monitored after various durations from 0.5 to 2 h. Results are means ± SEM (n = 7). Means with different letters are significantly different ($P < 0.05$).

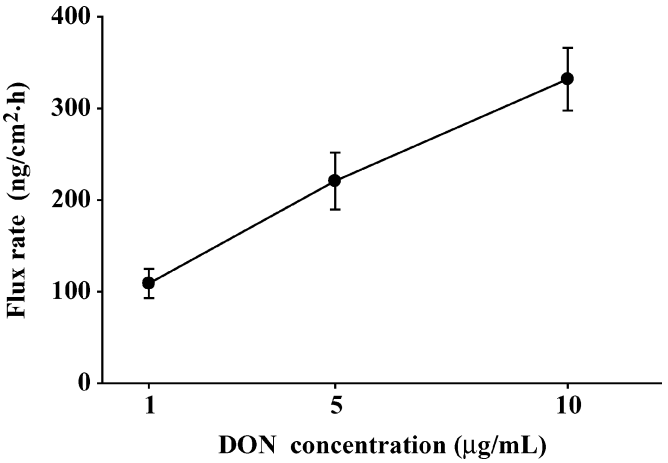

Figure 3. Concentration dependence of deoxynivalenol (DON) flux rate into the serosal compartment after addition of 1 to 10 μg/mL to the mucosal compartment of chicken jejuna mounted in Ussing chambers. The duration of incubation was 1 h [n = 3 to 7 (number of experiments)].

increased from the first (0 to 30 min) to the second flux period (30 to 60 min) and stayed relatively constant thereafter (30 to 90 min). The mean flux rates of DON in the different flux periods were 181 ± 26 (0 to 30 min), 483 ± 52 (30 to 60 min), 613 ± 111 (60 to 90 min), and 661 ± 91 ng/cm^2·h (90 to 120 min). This was equivalent to apparent permeability values of $5.01 \cdot 10^{-06}$, $1.34 \cdot 10^{-05}$, $1.70 \cdot 10^{-05}$, and $1.84 \cdot 10^{-05}$ cm/s, respectively.

The transport of DON was also investigated at different toxin concentrations, namely, 1, 5, and 10 μg/mL of DON. These concentrations represent realistic luminal DON concentrations that may be found in the gastrointestinal tract after ingestion of moderately to highly DON-contaminated food (Swamy et al., 2004). The duration of incubation was set at 1 h (Figure 3). The mean flux rate at the different concentrations of DON (1, 5, and 10 μg/mL) was 109 ± 16, 221 ± 31, and 332 ± 34 ng/cm^2·h, respectively. Thus, DON transport was proportional to the DON concentration (r = 0.998). The correlation between DON flux rate (J in ng/cm^2·h) and the initial DON concentration (C_0 in μg/mL) could be described by the following linear regression model: $J = (89.1 \pm 10.6) + (24.6 \pm 1.6) \cdot C_0$.

DISCUSSION

Influence of DON on Glucose Uptake

The gastrointestinal tract is the first barrier against ingested chemicals, food contaminants, and natural toxins. Following ingestion of mycotoxin-contaminated food or feed, intestinal epithelial cells can be exposed to high concentrations of toxins (Prelusky et al., 1996; Shephard et al., 1996). Our previous studies in chickens had suggested that the mycotoxin DON decreased both sodium-glucose and sodium-amino acid cotransport (Awad et al., 2004, 2005a,b). The activities of these transport processes were assessed indirectly by changes in Isc. It was thus of interest to analyze the effects of DON on glucose uptake in the intestine of laying hens directly and to elucidate inasmuch the active component of glucose uptake via SGLT-1 is specifically affected.

The results of the present study provided clear evidence that glucose uptake is decreased by 45-min preincubation with DON. The effect of DON was similar to the effect of SGLT-1 inhibition using phlorizin. Furthermore, a combined application of DON and phlorizin reduced D-glucose uptake to the same extent as an application of phlorizin alone. Thus, it can be concluded that the inhibitory action of DON on D-glucose uptake affects specifically the sodium-dependent component via SGLT-1.

The precise mechanism by which DON suppresses the activity of the hen intestinal SGLT-1 is unknown at this time. Previous studies have often ascribed the functional consequences of mycotoxin action to their inhibitory action on RNA and protein synthesis (Rotter et al., 1996). Accordingly, the shortening and thinning of villi in the small intestine of chickens observed in previous investigations after DON feeding (Fairchild et al., 2005; Awad et al., 2006a,b) may suggest that the decrease in glucose absorption observed in this study may just be a consequence of a general impairment of epithelial protein synthesis and function. However, Maresca et al. (2002) reported that low concentrations (<10 μmol/L) of DON applied to human intestinal epithelial cells selectively modulate the activities of specific intestinal transporters, including the D-glucose and D-galactose sodium-dependent transporter, SGLT-1. Based on this study and previous investigations, the DON-sensitive transport in chicken intestine comprise sodium-glucose cotransport by SGLT-1 (Figure 1; Awad et al., 2005a), L-proline cotransport (Awad et al., 2005b), and amiloride-sensitive sodium transport pathways (Awad et al., 2005a). It is not clear at present whether the interference with these specific intestinal transport pathways occurs mainly at the level of RNA transcription, protein synthesis, or both. The epithelia in the present study were exposed to DON for only 45 min, which might suggest that nontranscriptional inhibition of SGLT-1 could well play a role. Further studies using specific molecular characterization of Na^+-D-glucose cotransport after DON exposure may provide a better understanding.

Given the profound effects of DON on nutrient absorption in the small intestine, it is astonishing that poultry performance is often not or only moderately affected by the DON content of feedstuffs (Dänicke et al., 2002; Sypecka et al., 2004). One plausible explanation could be that the decrease in absorption of certain nutrients in the proximal small intestine (Hunder et al., 1991; Awad et al., 2004) displaces absorption to more distal intestinal sites. Chickens are able to absorb D-glucose and amino acids efficiently, even in the large intestine (Bindslev et al., 1997). Future studies should address the question of whether absorptive functions in the large intestine may be better protected against the deleterious effects of DON, either by DON absorption from the proximal digestive

tract (see below), by bacterial inactivation of DON, or both (Eriksen et al., 2002).

DON Transport Studies

Very little is known about the metabolism and kinetics of DON when it passes through the digestive tract, which is the first step governing the entry of DON into the organism.

Prelusky et al. (1988), Dänicke et al. (2004a), and Eriksen and Pettersson (2004) have detected DON appearance in the blood of pigs fed with DON-contaminated diets after <30 min following oral exposure to the toxin, with its maximum serum concentration reached 4 h after feeding. The early occurrence of DON in plasma, with its concomitant disappearance from digesta of the small intestine, indicates that the majority of the ingested DON is absorbed in the proximal part of the small intestine. Our data are in agreement with this hypothesis. The time kinetics experiments showed that DON flux rates are quickly equilibrated in the small intestinal epithelium of chickens and that steady state absorption rates are achieved within 30 min (Figure 2). The P_{app} under steady state conditions was ~$1.7 \cdot 10^{-05}$ cm/s. Although there is no comparable data from chicken jejunum in literature, this P_{app} is roughly comparable to the P_{app} of human jejunum to mannitol ($1.4 \cdot 10^{-05}$ cm/s; Mardones et al., 2004). Mannitol is a small hydrophilic molecule with predominant paracellular permeation. In a recent study in cultures of human colonic epithelial cells (Caco-2), Sergent et al. (2006) had also found very similar P_{app} for DON ($5.0 \cdot 10^{-06}$ cm/s) and the paracellular marker mannitol ($3.8 \cdot 10^{-06}$ cm/s). Because Sergent et al. (2006) also found an almost linear increase in DON permeation across Caco-2 cells with increasing DON concentrations, they concluded on a predominantly passive permeation of DON via the paracellular route. In agreement with these results from Caco-2 cultures, Doll et al. (2003) and Dänicke et al. (2004b) found a linear relationship between dietary DON and serum concentrations in pigs. In the present study, we now provide the first report on the concentration dependence of DON absorption directly at the level of the small intestinal epithelium. Our results show that DON transport from the mucosal to the serosal side (i.e., absorption) was directly proportional to its initial concentration (Figure 3). These data suggest that the major part of DON transport across the intestinal epithelium is likely due to simple diffusion. This passive diffusion is probably via the paracellular route. In support of the theory of predominantly passive permeation of DON, in vivo experiments have shown that DON does not accumulate in tissues (Prelusky et al., 1988).

In conclusion, our studies showed that DON (10 mg/L) has a specific inhibitory effect on glucose absorption mediated by SGLT-1. In the longer term, these inhibitory effects of DON on SGLT-1 would be complemented by a decrease of intestinal absorptive area. We reported previously that DON affected small intestinal morphology, especially in the duodenum and the jejunum, as evidenced by shorter and thinner villi. The decrease in villus area and specific inhibition of SGLT-1 by these mycotoxins would commonly impair digestive and absorptive functions.

Also, we found that DON is transported across the chicken intestinal epithelium by using the established Ussing chamber technique. The permeation of DON from the mucosal to the serosal side was proportional to the initial concentration. Absorption kinetics is thus compatible with predominantly passive transfer of DON. At least in part, this passive permeation could be via the paracellular route, although some transcellular permeation may not be ruled out.

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