

# Role of Cholecystokinin in Anorexia Induction Following Oral Exposure to the 8-Ketotrichothecenes Deoxynivalenol, 15-Acetyldeoxynivalenol, 3-Acetyldeoxynivalenol, Fusarenon X, and Nivalenol

Wenda Wu,<sup>\*,†,1</sup> Hui-Ren Zhou,<sup>†,1</sup> Kaiyu He,<sup>‡,§</sup> Xiao Pan,<sup>‡</sup> Yoshiko Sugita-Konishi,<sup>¶</sup> Maiko Watanabe,<sup>¶</sup> Haibin Zhang,<sup>\*</sup> and James J. Pestka<sup>†,‡,§,||,2</sup>

<sup>\*</sup>College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, P.R. China; <sup>†</sup>Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824; <sup>‡</sup>Center for Integrative Toxicology, Michigan State University, East Lansing, Michigan 48824; <sup>§</sup>Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824; <sup>¶</sup>Division of Microbiology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan; and <sup>||</sup>Department of Food and Life Sciences, Azabu University, 1-17-71, Fuchinobe Chuo-ku, Sagamihara, Kanagawa Pref. 252-5201, Japan

<sup>1</sup>These authors made an equal contribution to this manuscript.

<sup>2</sup>To whom correspondence should be addressed at 234 G.M. Trout Building, Michigan State University, East Lansing, MI 48824-1224. Fax: (517) 353-8963. E-mail: pestka@msu.edu.

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Cereal grain contamination by trichothecene mycotoxins is known to negatively impact human and animal health with adverse effects on food intake and growth being of particular concern. The head blight fungus *Fusarium graminearum* elaborates five closely related 8-ketotrichothecene congeners: (1) deoxynivalenol (DON), (2) 3-acetyldeoxynivalenol (3-ADON), (3) 15-acetyldeoxynivalenol (15-ADON), (4) fusarenon X (FX), and (5) nivalenol (NIV). While anorexia induction in mice exposed intraperitoneally to DON has been linked to plasma elevation of the satiety hormones cholecystokinin (CCK) and peptide YY<sub>3-36</sub> (PYY<sub>3-36</sub>), the effects of oral gavage of DON or of other 8-ketotrichothecenes on release of these gut peptides have not been established. The purpose of this study was to (1) compare the anorectic responses to the aforementioned 8-ketotrichothecenes following oral gavage at a common dose (2.5 mg/kg bw) and (2) relate these effects to changes plasma CCK and PYY<sub>3-36</sub> concentrations. Elevation of plasma CCK markedly corresponded to anorexia induction by DON and all other 8-ketotrichothecenes tested. Furthermore, the CCK1 receptor antagonist SR 27897 and the CCK2 receptor antagonist L-365,260 dose-dependently attenuated both CCK- and DON-induced anorexia, which was consistent with this gut satiety hormone being an important mediator of 8-ketotrichothecene-induced food refusal. In contrast to CCK, PYY<sub>3-36</sub> was moderately elevated by oral gavage with DON and NIV but not by 3-ADON, 15-ADON, or FX. Taken together, the results suggest that CCK plays a major role in anorexia induction following oral exposure to 8-ketotrichothecenes, whereas PYY<sub>3-36</sub> might play a lesser, congener-dependent role in this response.

**Key Words:** mycotoxin; trichothecene; anorexia; deoxynivalenol; 3-acetyldeoxynivalenol; 15-acetyldeoxynivalenol; fusarenon X; nivalenol.

Worldwide changes in climate and agricultural practices have led to increased incidence of *Fusarium* head blight in grains with a concomitant elevation in contamination by 8-ketotrichothecene mycotoxins that include five structurally related congeners: (1) deoxynivalenol (DON), (2) 3-acetyldeoxynivalenol (3-ADON), (3) 15-acetyldeoxynivalenol (15-ADON), (4) fusarenon X (FX), and (5) nivalenol (NIV) (Fig. 1) (Pestka, 2010a). DON and other 8-ketotrichothecenes have been associated with a spectrum of adverse effects that include anorexia, growth suppression, emesis, nausea, neuroendocrine changes, and immunosuppression (Pestka, 2010b). Induction of anorexia and resultant growth suppression by DON is of particular concern from the perspective of human and animal health (Forsell *et al.*, 1986; Iverson *et al.*, 1995; JECFA, 2011). Various countries have established specific regulations for DON based on the risks of this adverse outcome. Despite the importance of anorexia induction by DON and other 8-ketotrichothecenes to growth suppression (Flannery *et al.*, 2013), the pathophysiological basis for this effect remains poorly understood.

Appetite is regulated by both central and peripheral factors that influence the balance of anorexigenic and orexigenic signaling (Schwartz, 2006). Importantly, DON upregulates the expression of anorexigenic molecules including proopiomelanocortin (POMC), melanocortin 4 receptor (MC4R), and amphetamine-regulated transcript (CART) within the hypothalamic neurons of the mouse (Girardet *et al.*, 2011). A possible upstream mechanism for elevated expression of anorexigenic hormones in the hypothalamus is the increased secretion of the gut satiety peptides cholecystokinin (CCK) and peptide YY<sub>3-36</sub> (PYY<sub>3-36</sub>) by intestinal enteroendocrine cells (Steinert *et al.*, 2013). CCK is secreted by I cells within the duodenum

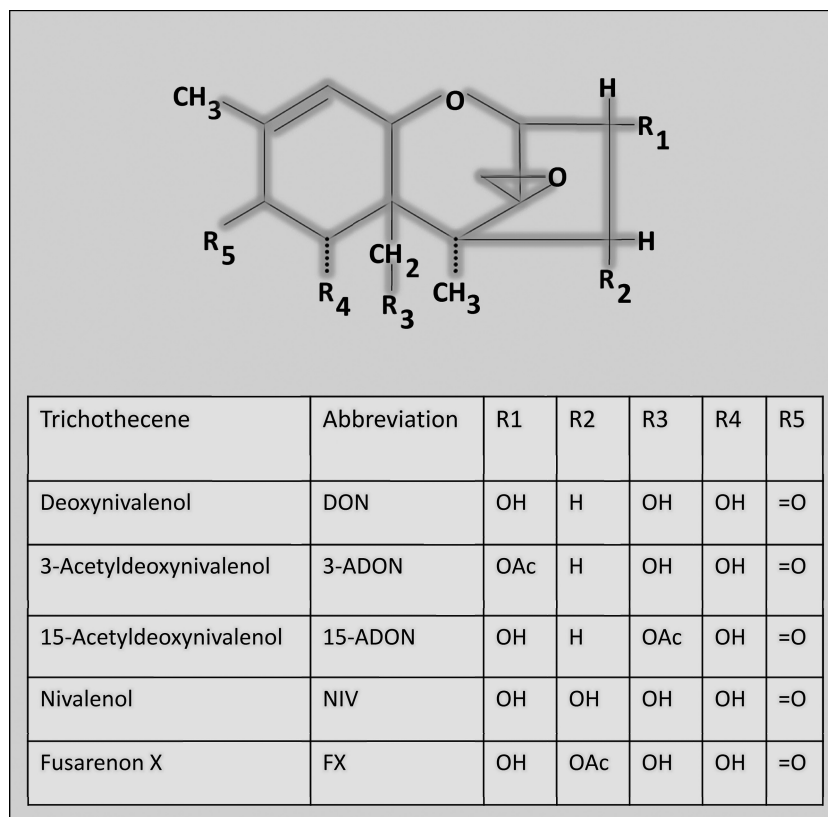


FIG. 1. Structures of 8-ketotrichothecenes included in this study.

and jejunum (Strader and Woods, 2005) and this satiety peptide upregulates POMC and MC4R expression in the hypothalamus (Kohno *et al.*, 2008). Both peripheral and central administration of CCK to animals decrease food intake (Della-Fera and Baile, 1980; Ebenezer *et al.*, 1990; Zhang *et al.*, 1986). PYY<sub>3-36</sub> is released from the L cells of the distal ileum and colon and has also been implicated in the upregulation of anorexigenic and down-regulation of orexigenic signaling molecules within the brain (Challis *et al.*, 2003). Peripheral administration of PYY<sub>3-36</sub> induces satiety in many species including humans, nonhuman primates, and rodents (Batterham *et al.*, 2002,2003a,b).

To study DON-induced food refusal, our laboratory has developed a mouse model for anorexia predicated on the placement of food and measurement of its consumption during the nocturnal feeding period (Flannery *et al.*, 2011). We have recently observed in this model that both intraperitoneal (IP) and orolingual administration of DON rapidly induce plasma CCK and PYY<sub>3-36</sub> in the B6C3F1 female mouse suggesting that these hormones are possible upstream contributors to DON-induced anorexia (Flannery *et al.*, 2012). In support of this contention, direct administration of exogenous PYY or CCK similarly caused reduced food intake in this mouse model. Food intake experiments using the NPY2 receptor antagonist BIIE0246 suggested that PYY mediated DON-induced anorexia. Although

the CCK1 receptor antagonist devazepide showed a similar trend in the reversal of DON's hypophagic (i.e., food refusal) effects, the differences were not significant.

The aforementioned mouse food refusal model has also been employed to compare the capacities of the 8-ketotrichothecenes to induce anorexia (Wu *et al.*, 2012). Anorectic responses to DON, 3-ADON, and 15-ADON following both IP exposure and oral gavage were transient with food intake recovering to control levels within 16 h. The no observed adverse effect levels (NOAEL) and lowest observed adverse effect levels (LOAEL) were 0.5 and 1 mg/kg bw following IP exposure, respectively, and 1 and 2.5 mg/kg bw after oral exposure, respectively. In contrast, food refusal persisted from 48 to 96 h following IP and oral exposure to FX and NIV. For both IP and oral FX exposure, the NOAEL was 0.025 mg/kg bw and LOAEL was 0.25 mg/kg bw, whereas the NOAELs and LOAELs for NIV were 0.01 and 0.1 mg/kg bw, respectively, after IP exposure and 0.1 and 1 mg/kg bw, respectively, following oral exposure. Accordingly, these data and the prior DON study (Flannery *et al.*, 2012) suggest that anorectic responses to 8-ketotrichothecenes were always greater or equal when administered IP as compared with oral exposure.

Because ingestion is the primary means of exposure to DON and other 8-ketotrichothecenes for humans and animals, it is im-

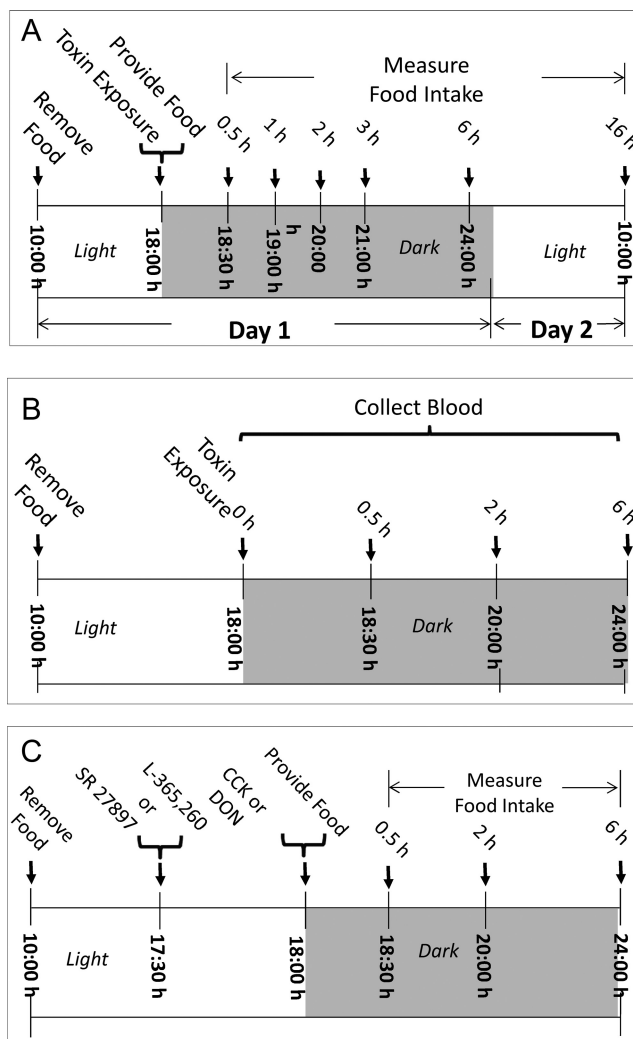
portant from a public health perspective to understand the mechanisms by which oral administration of these congeners affects plasma CCK and PYY<sub>3-36</sub>. Here, we utilized the mouse anorexia model to relate effects of oral gavage to a common anorexigenic dose of 2.5 mg/kg bw DON, 3-ADON, 15-ADON, NIV, and FX on food consumption to the release of CCK and PYY<sub>3-36</sub>. The results suggest that CCK is a major contributor to induction of anorexia after oral exposure to all 8-ketotrichothecenes whereas PYY<sub>3-36</sub> might play a lesser, congener-dependent role in this response.

## MATERIALS AND METHODS

**Toxins and drugs.** Sources of 8-ketotrichothecenes and purity (> 98%) verified by elemental analysis and LC-MS as previously described (Wu *et al.*, 2012). CCK (23–33, sulfated), CCK1 receptor antagonist SR 27897 and CCK2 receptor antagonist L-365,260, were purchased from Tocris Biosciences (Ellisville, MO). All toxins and CCK were dissolved in filter-sterilized phosphate buffered saline (PBS). SR 27897 and L-365,260 were dissolved in 5% dimethyl sulfoxide (DMSO) in filter-sterilized PBS.

**Assessment of anorexia in the mouse.** Female B6C3F1 mice (10–12 weeks old) were obtained from Charles River Breeding (Portage, MI) and housed individually in polycarbonate cages in a room maintained at 21–24°C and 40–55% relative humidity under a 12 h light (6:00–18:00 h)/dark (18:00–6:00 h) cycle. All studies were conducted in accordance with National Institutes of Health guidelines and approved by the Michigan State University Institutional Animal Care and Use Committee (MSU-IACUC). The general experimental design for the anorexia study (Fig. 2A) was based on previously described protocols (Flannery *et al.*, 2011; Wu *et al.*, 2012). Briefly, mice were acclimated a week after arriving and randomly divided into different groups according to body weight 1 day before experiment. On the day of the experiment, groups of mice ( $n = 5$ ) were fasted but water provided *ad lib* from 10:00 to 18:00 h and orally gavaged with 0 and 2.5 mg/kg bw DON, 3-ADON, 15-ADON, FX, and NIV in 100  $\mu$ l PBS, respectively, using a sterile 22 G 1.5" disposable feeding tube (Instech Solomon; Plymouth Meeting, PA). This dose was chosen based on previous data from showing significant transient anorectic effects were observed after oral exposure to all of these congeners (Wu *et al.*, 2012). Following 8-ketotrichothecene treatment, mice were then immediately provided two pre-weighted food pellets ( $\approx 7$  g) and food intake measured at 0.5, 1, 2, 3, 6, and 16 h after toxin exposure.

**Measurement of plasma CCK and PYY<sub>3-36</sub>.** Groups of mice ( $n = 6$ ) were orally gavaged with 0 or 2.5 mg/kg bw DON, 3-ADON, 15-ADON, FX, and NIV in 100  $\mu$ l PBS, respectively, and cohorts were sacrificed at 0, 0.5, 2, and 6 h after exposure



**FIG. 2.** (A) Experimental design for anorexia bioassay in mice. Mice were given oral gavage of 8-ketotrichothecenes immediately before the dark feeding cycle. Food intake was measured at 0.5, 1, 2, 3, 6, and 16 h post administration. (B) Experimental design for 8-ketotrichothecenes-induced plasma CCK and PYY<sub>3-36</sub> release in mice. Plasma was analyzed for CCK and PYY<sub>3-36</sub> at 0, 0.5, 2, and 6 h post administration. (C) Experimental design for determining the effects of CCK receptor antagonists on CCK- and DON-induced anorexia. Mice were pretreated with SR 27897 or L-365,260 and 30 min later treated with PBS, CCK, or DON immediately before the dark cycle. Food intake was then measured at 0.5, 2, and 6 h post administration.

(Fig. 2B). At experiment termination, mice were anesthetized by IP injection with 100  $\mu$ l 56 mg/ml sodium pentobarbital. Blood was collected with sterile syringes containing 20  $\mu$ l 1% (vol/vol) EDTA (pH 7.5) from the inferior vena cava and transferred to EDTA-coated tubes. Plasma was separated from blood by centrifugation at 3500  $\times$  g for 10 min at 4°C and frozen at  $-80^{\circ}$ C until analysis. Plasma hormones CCK and PYY<sub>3-36</sub> were analyzed using enzyme immunoassay kits for CCK (CCK [26–33], nonsulfated; human-, rat-, and mouse-specific) and

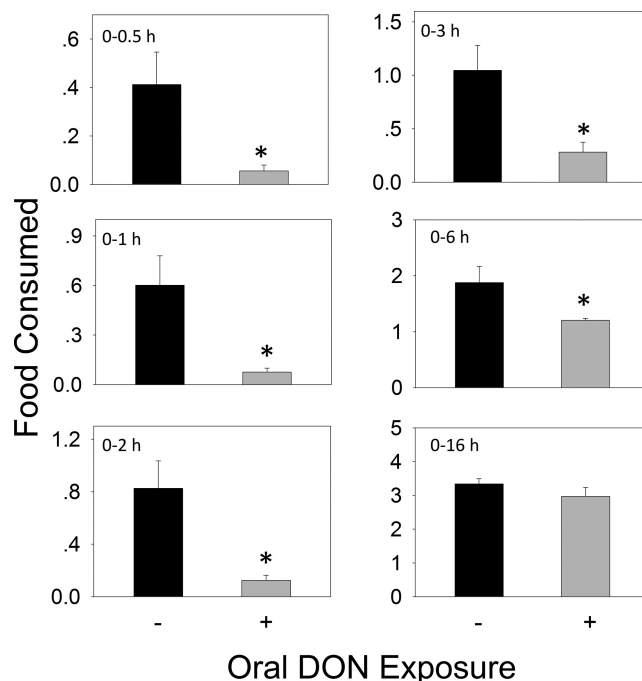
PYY (PYY [3–36]; mouse-, rat-, porcine-, and canine-specific) (Phoenix Pharmaceuticals, Burlingame, CA).

**Effects of CCK receptor antagonists on DON-induced anorexia.** The general design for receptor antagonist experiments (Fig. 2C) was identical to anorexia models except for pretreatment with antagonist 30 min before DON exposure. Doses of antagonists were chosen based on the previous demonstrated efficacies in previous *in vivo* investigations of these receptors (Blandizzi *et al.*, 1999; Cano *et al.*, 2003; Lotti *et al.*, 1986; Poncelet *et al.*, 1993). To establish that CCK1 receptor antagonist SR 27897 and CCK2 receptor antagonist L-365260 could attenuate CCK-induced anorexia, 100  $\mu$ l of each antagonist at 0, 1, 2.5, and 5 mg/kg bw doses was given by oral gavage. After 30 min, mice were given an IP injection of CCK at 0.01 mg/kg bw in 100  $\mu$ l. This dose was chosen based on previous study (Flannery *et al.* 2012). In that study, we found IP injection of CCK at 0.01 mg/kg bw significantly induced anorexia in mouse. Control groups were first gavaged with either vehicle (5% DMSO) or 5 mg/kg bw antagonist (SR 27897 or L-365260), then injected IP with PBS. Food intake was measured at 0.5, 2, and 6 h post-treatment. To ascertain the potential role of CCK in DON-induced anorexia, mice were gavaged with 100  $\mu$ l of antagonist (SR 27897 or L-365260) at 0, 1, 2.5, and 5 mg/kg bw 30 min before oral exposure to 100  $\mu$ l of DON at 2.5 mg/kg bw. Controls and food intake measurement times were the same as for the CCK study.

**Statistics.** Data were plotted and statistically analyzed using SigmaPlot 11 for Windows (Jandel Scientific; San Rafael, CA). Means were considered significantly different at  $p < 0.05$ . Food intake at specific time points was analyzed by t-test to determine significant differences between treatment and the respective control. Two-way repeated ANOVA (one-factor) using Holm-Sidak Method was used to analyze significant differences in food consumption as compared with the control over time. A one-way ANOVA using the Student-Newman-Keuls method was used to analyze significant differences between treatment groups in food consumption for CCK1 and CCK2 receptor antagonist study. A two-way ANOVA using Bonferroni *t*-test was used to assess significant differences in CCK and PYY<sub>3–36</sub> concentrations in plasma over time.

## RESULTS

When the anorectic effect of oral exposure to DON at 2.5 mg/kg bw was measured, significant reductions in cumulative food intake relative to control were observed in cumulative food consumption at 0.5 h (86%), 1 h (87%), 2 h (85%), 3 h (73%), and 6 h (36%), respectively (Figs. 3 and 4A). From 6 to 16 h, there was a trend toward increased food consumption. After 16 h, no differences being observed between control and DON group. Plasma CCK was significantly increased by DON treat-

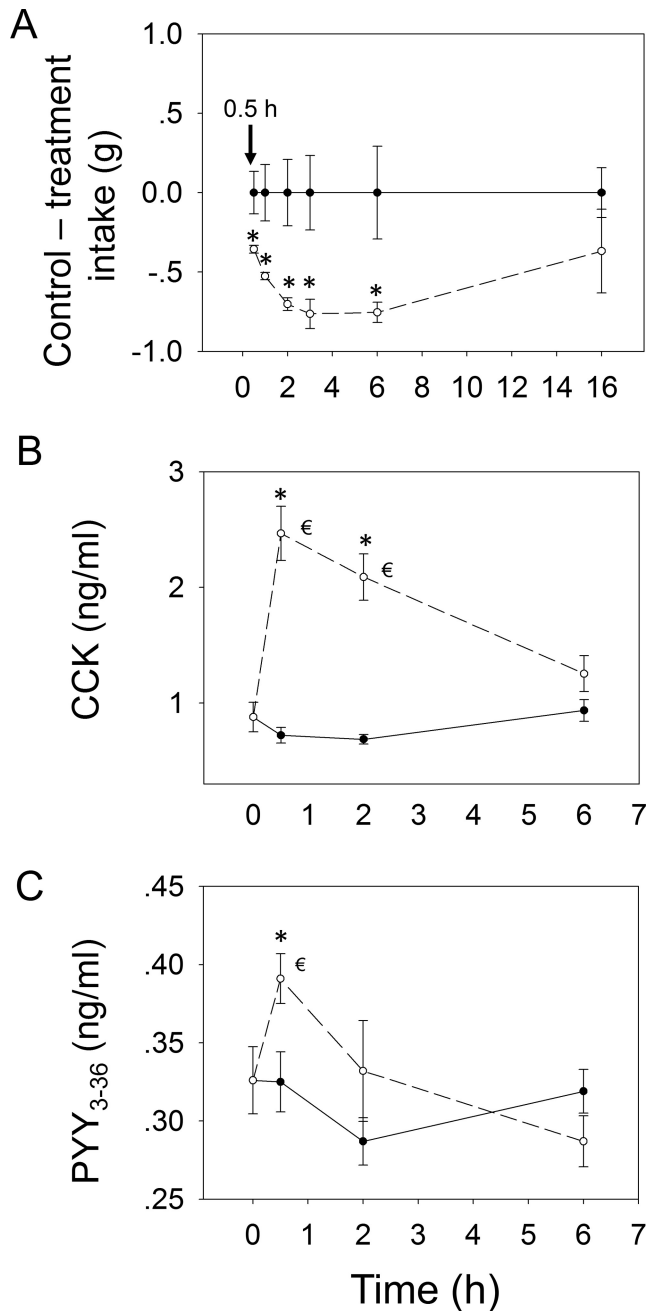


**FIG. 3.** Oral exposure to DON impairs cumulative food intake for up to 6 h. Data are mean  $\pm$  SEM ( $n = 5$ /gp) and analyzed using t-test. \* indicates significant differences between treatment and the respective control ( $p < 0.05$ ).

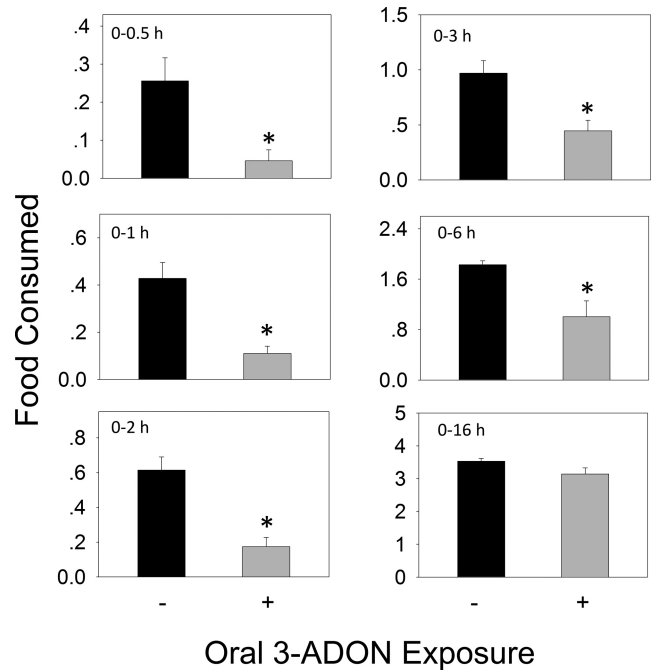
ment after 0.5 and 2 h, but recovered to control level by 6 h (Fig. 4B). PYY<sub>3–36</sub> was significantly elevated after 0.5 h but returned to control level by 2 h (Fig. 4C). Similar to DON, food intake measurements in mice orally exposed 3-ADON at 2.5 mg/kg bw indicated evoked 82, 74, 72, 54, and 45% reduction in cumulative food intake at 0.5, 1, 2, 3 and 6 h, respectively, with no differences being observed between control and 3-ADON group after 16 h (Figs. 5 and 6A). Plasma CCK was elevated at 0.5 and 2 h, but returned to basal level at 6 h after exposure to 3-ADON (Fig. 6B). Unlike DON, oral 3-ADON exposure had no effect on plasma PYY<sub>3–36</sub> (Fig. 6C).

Oral exposure to 15-ADON at 2.5 mg/kg bw also caused a similar decreased food consumption in mice during the first 6 h (Figs. 7 and 8A). Significant reduction in cumulative food intake was observed at 0.5 h (94%), 1 h (69%), 2 h (79%), 3 h (64%), 6 h (45%), and 16 h (17%). The rate of food intake by the 15-ADON group paralleled the control mice during 6–16 h, suggesting that anorexia did not occur after 6 h. Exposure to 15-ADON significantly elevated CCK concentrations at 0.5 h, which peaked at 2 h (Fig. 8B). Similar to DON and 3-ADON, plasma CCK returned to basal levels at 6 h. As observed for 3-ADON, plasma PYY<sub>3–36</sub> was not affected during the experimental period (Fig. 8C).

Following oral administration with FX at 2.5 mg/kg bw, marked reductions in food intake occurred at 0.5 h (92%), 1 h (72%), 2 h (78%), 3 h (72%), 6 h (71%), and 16 h (39%), respectively (Figs. 9 and 10A). A general trend toward reduced



**FIG. 4.** Relationship of DON-induced anorectic response to plasma CCK and PYY<sub>3-36</sub>. Mice were orally gavaged with either PBS (solid lines) or 2.5 mg/kg bw DON (broken lines). (A) Rapid and transient anorectic effect following oral exposure to DON. Data are mean  $\pm$  SEM ( $n = 5$ /gp). Two-way repeated ANOVA (one factor) using Holm-Sidak Method was used to analyze significant differences in food consumption as compared with the control. \* indicates a statistically significant difference in cumulative food consumption relative to the control at specific time point ( $p < 0.05$ ). Kinetics of DON-induced plasma (B) CCK and (C) PYY<sub>3-36</sub> elevation in mice. Data represent mean  $\pm$  SEM ( $n = 6$ /gp). Two-way ANOVA using Bonferroni  $t$ -test was used to assess significant differences in kinetics of CCK and PYY<sub>3-36</sub> concentrations in plasma as compared with the control. \* indicates difference in plasma CCK or PYY<sub>3-36</sub> concentration relative to the control at specific time point ( $p < 0.05$ ) and  $\epsilon$  indicates difference in plasma CCK or PYY<sub>3-36</sub> concentration relative to the 0 h time point ( $p < 0.05$ ).

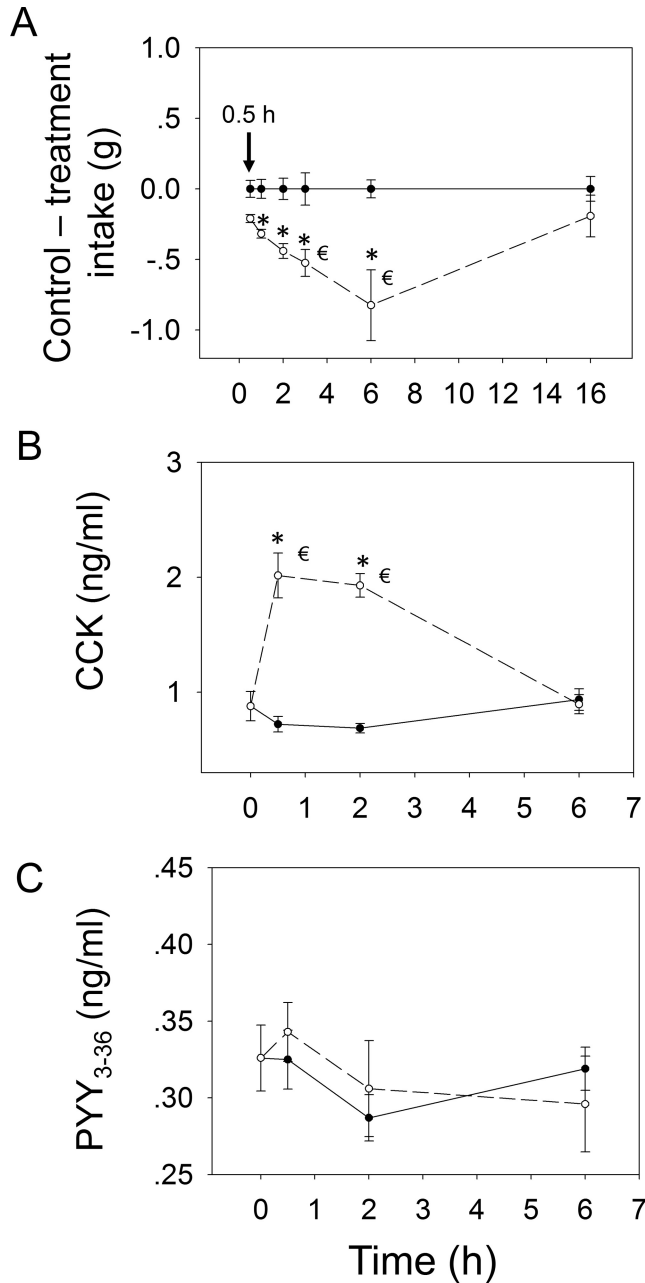


**FIG. 5.** Oral exposure to 3-ADON impairs cumulative food intake for up to 6 h. Experiment conducted and data analyzed as described in Figure 3 legend.

food consumption was observed over the entire 16 h period. Following oral exposure to FX, plasma CCK concentrations were elevated at 0.5 h, peaked at 2 h, and still markedly raised at 6 h (Fig. 10B), suggesting the prolonged elevation of CCK correlated with persistent anorexia. Again, FX had no effect on plasma PYY<sub>3-36</sub> concentrations (Fig. 10C).

Contrary to the aforementioned DON congeners, oral gavage with NIV at 2.5 mg/kg bw only modestly decreased food intake during the 16 h experimental period (Figs. 11 and 12A). Significant reduction in cumulative food consumption was observed only at 2 h (40%) and 3 h (38%) after exposure. Although differences between NIV exposure group and control were not evident thereafter, food intake was still depressed. Exposure to NIV significantly raised plasma CCK concentrations only at 2 h (Fig. 12B). Interestingly, moderate elevation of PYY<sub>3-36</sub> was observed at 2 and 6 h (Fig. 12C), suggesting that both CCK and PYY<sub>3-36</sub> might contribute to NIV-induced anorexia.

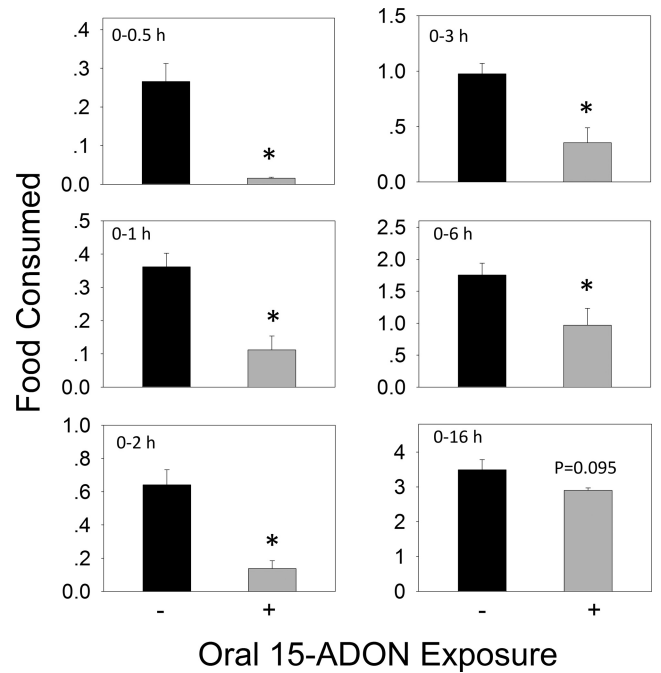
The effects of CCK1 and CCK2 receptor antagonists on both CCK- and DON-induced anorexia were evaluated (Fig. 2C). IP administration of CCK at 0.01 mg/kg bw decreased food intake by 76 and 69% relative to the control at 0.5 and 2 h, respectively (Figs. 13A and 14A). After 6 h, no differences were observed when compared with control. These effects were dose-dependently attenuated with the CCK1R antagonist SR 27897 and the CCK2R antagonist L-365,260 at 2 h post-treatment. Exposure to the highest dose (5 mg/kg bw) of either antagonist alone had no effect on food intakes therefore excluding the possibility of functional antagonism. These results suggested that,



**FIG. 6.** Relationship of 3-ADON-induced (A) anorectic response to plasma (B) CCK and (C) PYY<sub>3-36</sub>. Mice were orally gavaged with either PBS (solid lines) or 2.5 mg/kg bw DON (broken lines). Experiment conducted and data analyzed as described in Figure 4 legend except that symbol € in panel (A) indicates a significant difference in cumulative food consumption relative to the 0.5 h time point within a given dose ( $p < 0.05$ ).

in this mouse model, CCK evoked anorexia by activating both CCKR1 and CCKR2.

Consistent with the earlier experiment (Fig. 3), mice treated with DON exhibited a significant reduction in food intake relative to control (Figs. 13B and 14B). DON-exposed mice that were pretreated with SR 27985 or L-365,260 exhibited a dose-

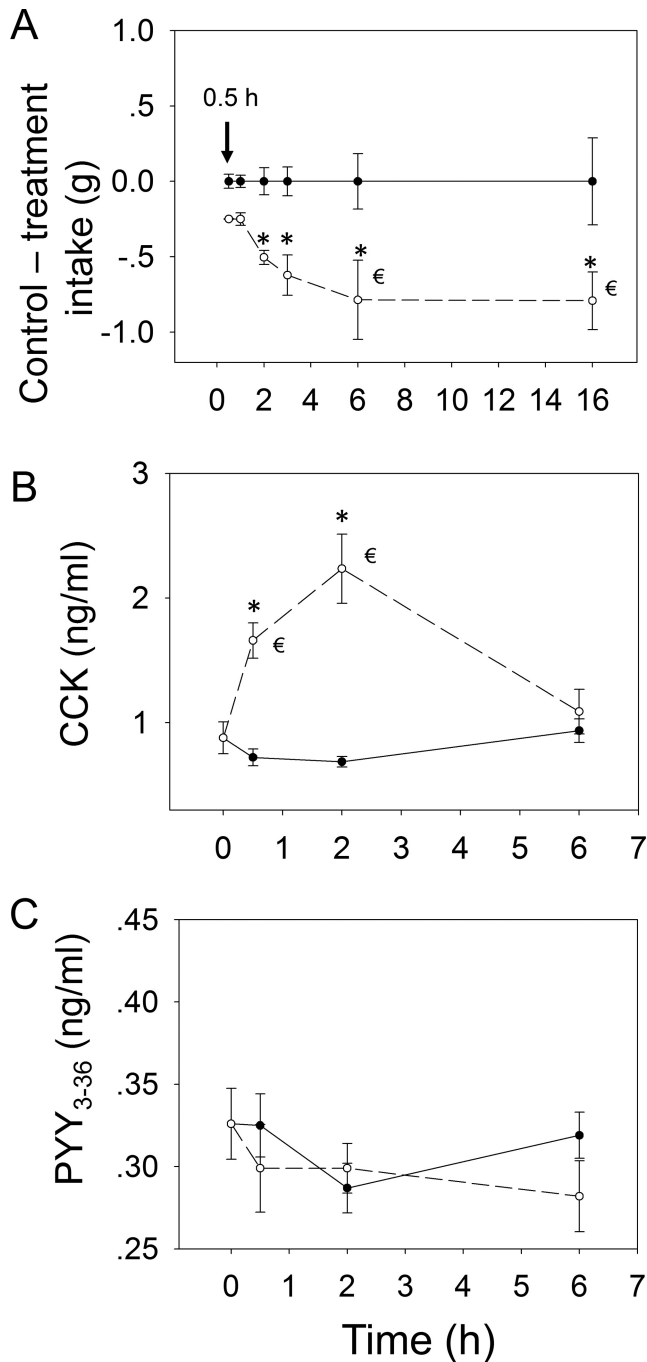


**FIG. 7.** Oral exposure to 15-ADON impairs cumulative food intake for up to 6 h. Experiment was conducted and data analyzed as described in Figure 3 legend.

dependent trend of increased food intake. Following pretreatment of SR 27897 with 1 mg/kg bw, mice consumed 19 and 15% more food at 0.5 and 2 h, respectively, than DON alone (Fig. 13B). After 6 h, no differences were observed compared with control. Mice pretreated with 2.5 and 5 mg/kg bw SR 27897 consumed 25 and 61% more food at 0.5 h than DON alone, respectively, and no differences were observed when compared with control after 2 h. Mice receiving 1, 2.5, and 5 mg/kg bw L-365,260 consumed 29, 37, and 54% more food than DON alone at 0.5 h, respectively (Fig. 14B). After 2 h, no differences in cumulative food intake were evident between L-365,260-treated and control mice. Collectively, these strong, dose-dependent antagonistic effects strongly suggest that CCK plays an essential role in the anorexia induction by DON, and operated via two distinct receptors.

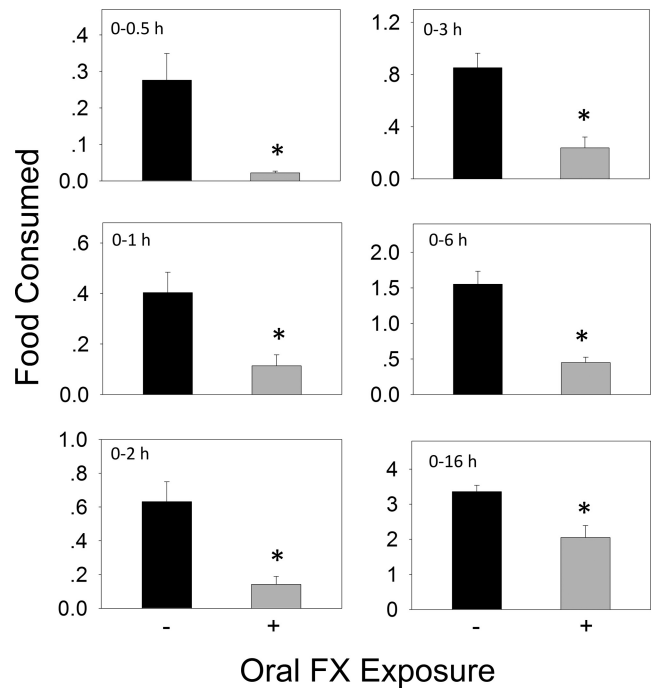
**DISCUSSION**

Although anorexia and concomitant growth suppression have long been a major concern for human and animal food exposure by 8-ketotrichothecenes, the underlying mechanisms for initiating these effects are poorly understood. Gut satiety hormones play an important upstream role in regulating appetite and appear to be important targets of this mycotoxin class. These hormones mediate complex physiological processes that sequentially involve (1) hormone secretion in gastrointestinal (GI) tract after meal consumption, (2) signaling satiation via brain-gut



**FIG. 8.** Relationship of 15-ADON-induced (A) anorectic response to plasma (B) CCK and (C) PYY<sub>3-36</sub>. Mice were orally gavaged with either PBS (solid lines) or 2.5 mg/kg bw DON (broken lines). Experiment was conducted and data analyzed as described in Figures 4 and 6 legends.

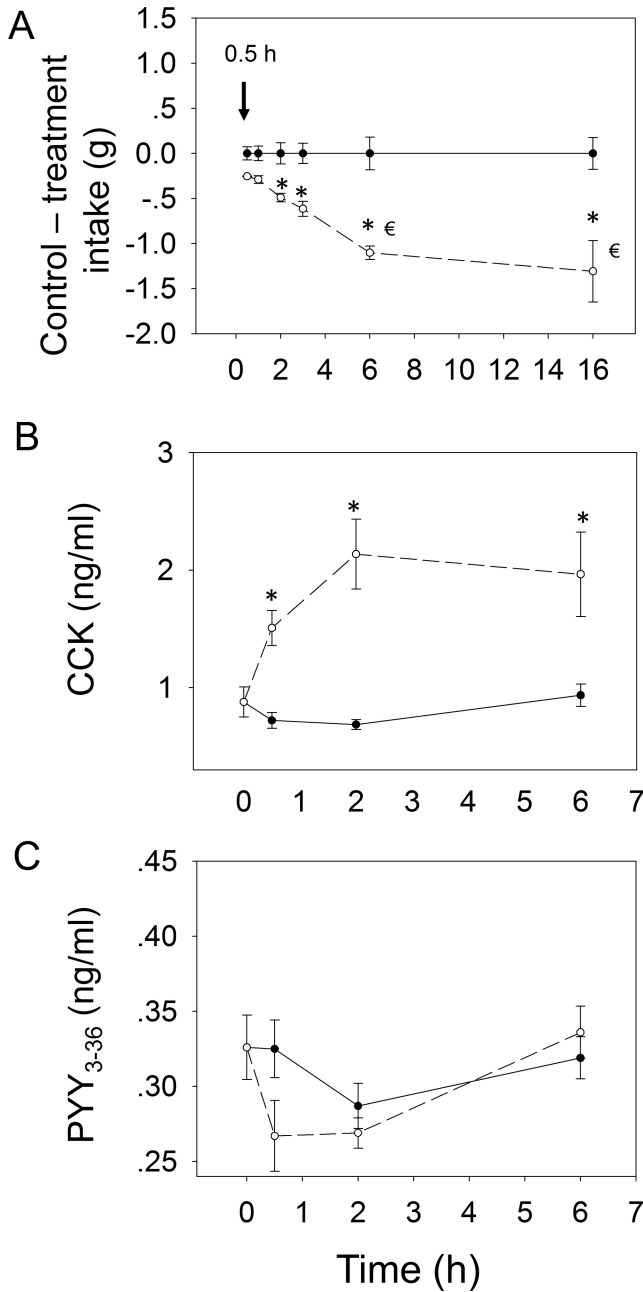
axis, (3) differential expression of anorexigenic and orexigenic signaling factors within the hypothalamus, and (4) altered regulation of appetite and GI tract motility. One of the most studied gut satiety hormones, CCK, has been demonstrated to be an effective short-term, meal-reducing signal in both human and



**FIG. 9.** Oral exposure to FX impairs cumulative food intake for up to 16 h. Experiment was conducted and data analyzed as described in Figure 3 legend.

animals (Dockray, 2009; Strader and Woods, 2005). Herein, we demonstrate for the first time that (1) oral gavage with five major 8-ketotrichothecenes caused robust increases in plasma CCK that correlated with anorexia induction and (2) both DON and CCK could mediate anorexia via CCK1R- and CCK2R-dependent mechanisms.

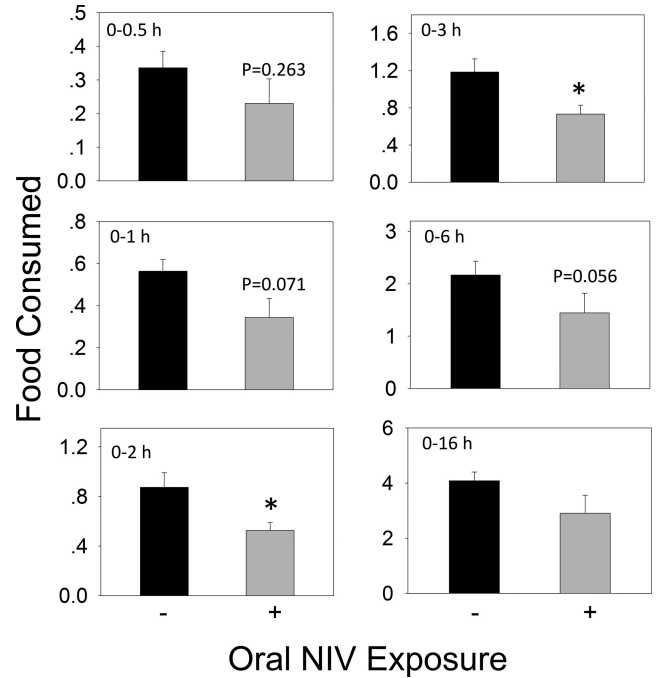
Our results are highly consistent with induction of CCK exocytosis by DON from I cells in the GI tract that normally occurs after meal consumption. When CCK is elicited in intestinal tract, it can act in a paracrine manner, and activate CCK receptors located on the abdominal vagal afferents, which relay to the hypothalamus and eventually cause anorexia (Reidelberger *et al.*, 2004). Another site of paracrine action for CCK is the spinal afferent nerves of the intestinal tract that feed into splanchnic nerve (Brown *et al.*, 2011). In addition to these paracrine effects, there is growing evidence that CCK can act in an endocrine fashion on the central nervous system (CNS), most notably at the nucleus tractus solitarius and dorsal motor nucleus of the vagus (Baptista *et al.*, 2007; Hao *et al.*, 2008; Holmes *et al.*, 2009; Reidelberger *et al.*, 2004; Rogers and Hermann, 2008). It is also important to note that, in addition to being synthesized by I cells in GI tract, CCK is also produced within CNS including various brain regions (Beinfeld *et al.*, 1981; Rehfeld *et al.*, 1985). We previously reported that DON distributes into the brain of mice within 5 min following oral exposure (Pestka *et al.*, 2008). It is possible that 8-ketotrichothecenes directly target the CNS to modulate anorectic neurocircuitry by stimulating CCK secretion in the brain. The finding that DON induces



**FIG. 10.** Relationship of FX-induced (A) anorectic response to plasma (B) CCK and (C) PYY<sub>3-36</sub>. Mice were orally gavaged with either PBS (solid lines) or 2.5 mg/kg bw DON (broken lines). Experiment was conducted and data analyzed as described in Figures 4 and 6 legends.

c-Fos in the circumventricular organs of the brain and surrounding structures are consistent with CNS being a direct target of DON (Girardet *et al.*, 2011).

CCK acts via two different receptor subtypes—CCK1R and CCK2R. CCK1R is located throughout the GI tract and CNS, and there is wide consensus that this receptor plays an essential role in the CCK-induced satiety response (Hao *et al.*,

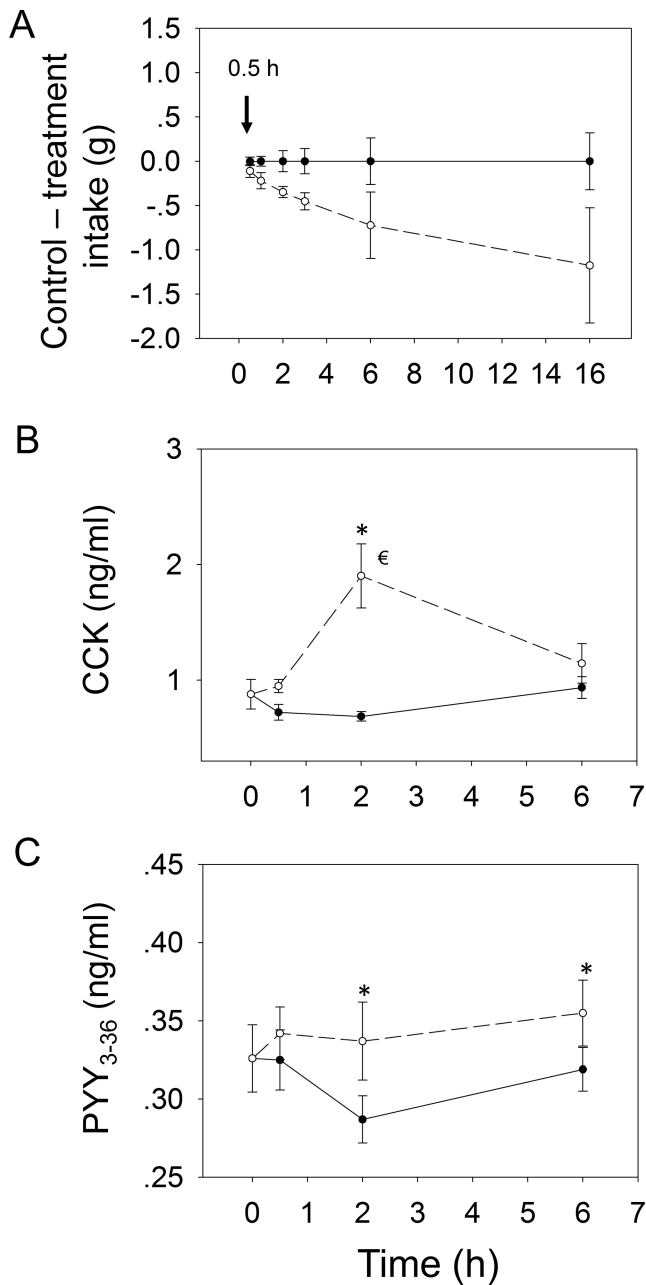


**FIG. 11.** Oral exposure to NIV impairs cumulative food intake for up to 3 h. Experiment was conducted and data analyzed as described in Figure 3 legend.

2008; Reidelberger *et al.*, 2004). It is notable that, in a prior mouse study (Flannery *et al.*, 2012), we found robust increases in plasma CCK following IP administration of DON in mice. Although a trend was observed suggesting that DON-induced anorexia was reversed of by prior IP treatment with the CCK1R antagonist devazepide, the differences were not statistically significant. There were two critical differences between the present and aforementioned studies that could explain the conflicting results. First, the CCK1R antagonist used here, SR 27897, is 7 to 10 times more effective at inhibiting CCK-induced anorexia and reduction of gut motility than devazepide in rats (Poncelet *et al.*, 1993). Second, in this study we delivered the SR 27897 via oral gavage whereas in the former study the devazepide was administered IP. Because it is likely that DON induces CCK secretion from I cells in the small intestine and that some of this elicited hormone acts in a paracrine fashion on vagal afferents, oral administration of a CCK1R antagonist might be more efficacious at attaining the inhibitory concentrations necessary for countering CCK-mediated hypophagia.

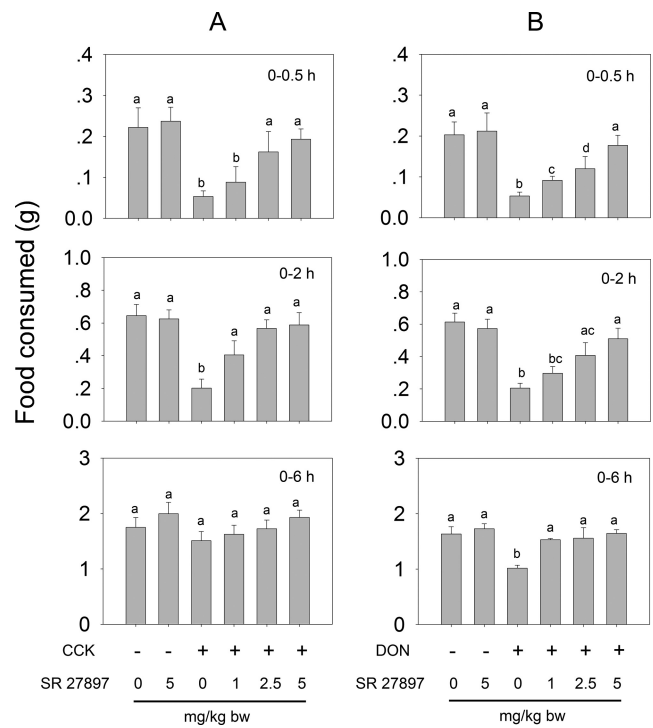
It is known that CCK2R is expressed in the brain where it integrates signaling of pain, anxiety, sexual behavior, learning, and memory (Crawley and Corwin, 1994). Besides the brain, CCK2R can be expressed in GI tissues (stomach, pancreas, and liver), vagal afferent fibers, adipocytes, adrenal gland, and mononuclear cells (Dufresne *et al.*, 2006; Guilloteau *et al.*, 2006). Studies of the role of CCK2R on appetite and feeding behavior have yielded conflicting results. Subcutaneous administration of L-365,260 increases food intake in partially satiated





**FIG. 12.** Relationship of NIV-induced (A) anorectic response to plasma (B) CCK and (C) PYY<sub>3-36</sub>. Mice were orally gavaged with either PBS (solid lines) or 2.5 mg/kg bw DON (broken lines). Experiment was conducted and data analyzed as described in Figures 4 and 6 legends.

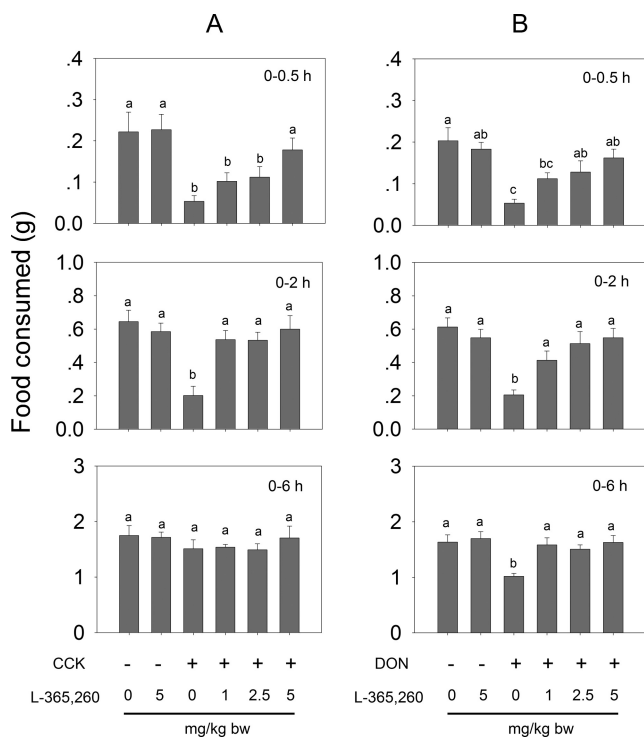
rats and postpones the onset of satiety (Dourish *et al.*, 1989). In analogous fashion, Intracerebroventricular (ICV) injection of a CCK2R antagonist into rats decreased the satiating potency of the ingested food during the meal and decreased time required for re-initiation of eating during the postprandial intermeal interval (Dorre and Smith, 1998). Genetic studies also suggest the involvement of CCK2R in the modulation of energy metabolism and food intake. CCK2R receptor-deficient mice



**FIG. 13.** CCK1 receptor antagonist SR 27897 inhibits (A) CCK- and (B) DON-induced anorectic response. Mice were pretreated with 0, 1, 2.5, and 5 mg/kg bw SR 27897 and 30 min later treated with PBS, 0.01 mg/kg bw CCK or 2.5 mg/kg bw DON. Food intake was then measured at 0.5, 2, and 6 h post administration. Data are mean ± SEM (n = 6/gp) and analyzed by one way ANOVA using Student-Newman-Keuls Method. Bars without the same letter are significantly different (p < 0.05).

exhibit significantly increased food intake compared with wild-type mice (Clerc *et al.*, 2007; Miyasaka *et al.*, 2002; Weiland *et al.*, 2004), elevated body weight (Chen *et al.*, 2006), signs of obesity (Weiland *et al.*, 2004) as well as increased plasma levels of insulin and leptin (Cano *et al.*, 2003; Clerc *et al.*, 2007). In contrast to the above-mentioned studies, ICV injection with specific CCK2R antagonists inhibits food intake in rats (Corp *et al.*, 1997; Frommelt *et al.*, 2013). Also, in another genetic investigation, CCK2R-deficient mice exhibited normal eating behavior and body weight gain (Kopin *et al.*, 1999). Clearly, results of studies of the involvement of CCK2R in eating behavior are not as clear-cut as those of CCK1R and seem to be model-dependent. Nevertheless, the demonstration here that administration of exogenous CCK evoked anorexia and that this could be inhibited by L-365,260 suggests that, in our mouse anorexia model, CCK2R indeed mediated both CCK- and DON-driven feed refusal.

The data presented herein suggest that hypophagia induction by other 8-ketotrichothecenes is also likely to involve CCK. It was notable that DON, 3-ADON, 15-ADON, and FX evoked plasma CCK elevations within 0.5 h, a time that coincided with the initiation of significant food refusal. Interestingly, elevation of CCK induced by NIV did not occur until 2 h when anorexia



**FIG. 14.** CCK2 receptor antagonist L-365,260 inhibits (A) CCK- and (B) DON-induced anorectic response. (A) Mice were pretreated with 0, 1, 2.5, and 5 mg/kg bw L-365,260 and 30 min later treated with PBS, 0.01 mg/kg bw CCK or 2.5 mg/kg bw DON. Food intake was then measured at 0.5, 2, and 6 h post administration. Data are mean  $\pm$  SEM ( $n = 6/\text{gp}$ ) and were analyzed as described in Figure 13 legend. Bars without the same letter are significantly different ( $p < 0.05$ ).

was first detected. Clearances of DON, FX, and NIV in the mouse follow two-compartment kinetics with an initial rapid phase of elimination ( $t_{1/2\alpha}$ ) and a slower terminal elimination phase ( $t_{1/2\beta}$ ) (Azcona-Olivera *et al.*, 1995; Poapolathep *et al.*, 2003). Previous studies indicate  $t_{1/2\alpha} = 0.36$  or  $0.29$  h for DON and  $0.878$  h for FX, respectively (Azcona-Olivera *et al.*, 1995; Pestka *et al.*, 2008; Poapolathep *et al.*, 2003). However, a longer initial phase of elimination was observed for NIV ( $t_{1/2\alpha} = 2.5$  h) (Poapolathep *et al.*, 2003). The possible reason for delayed CCK elevation and anorectic effect might be the slower absorption of NIV by oral exposure. Except for FX-treated group, plasma CCK returned to basal level after 6 h, and food intake recovered at 16 h after exposure. Consistent with CCK's satiety-inducing effects, FX caused persistent anorexia lasting more than 16 h, which has also been observed previously (Wu *et al.*, 2012). FX's longer elimination half-life might explain the prolonged CCK elevation and anorectic effects observed here.

Unlike CCK, our results indicated that the satiety hormone PYY<sub>3-36</sub> was not robustly elevated following oral exposure to 8-ketotrichothecenes. Although we have reported DON causes anorexia following IP exposure by inducing PYY<sub>3-36</sub> secretion (Flannery *et al.*, 2012), we found here that CCK elevation was

more consistent with anorexia induction by DON and other 8-ketotrichothecenes following oral gavage. The possible reason for differences in secretion of these satiety hormones following IP versus oral gavage might result from the different relative distributions of CCK and PYY<sub>3-36</sub> secreting cells in gut. Most CCK-producing I-cells are located in the upper GI tract including duodenal and jejunal mucosa (Polak *et al.*, 1975), whereas PYY<sub>3-36</sub> secreting L-cells are particularly numerous in the lower GI tract including the distal ileum and colon, with only a few cells presented in the upper GI tract (Hörsten *et al.*, 2004). It might be speculated that following oral gavage exposure toxins can reach the upper GI tract to induce CCK release, but are absorbed before reaching the lower GI tract and causing secretion of PYY<sub>3-36</sub>. IP exposure might evoke uniform DON concentrations in the small and large intestines from the blood, activating exocytosis of both CCK and PYY.

It is important to note that when we previously compared exposures to DON (2.5 mg/kg bw) by the IP route to the orolingual route, we observed nearly identical anorectic effects as well as similarly robust PYY and CCK responses (Flannery *et al.*, 2012). This finding could possibly relate to DON's capacity to be rapidly absorbed and distributed by either of these routes of exposure as compared with oral gavage as used in the present study. There are several inherent differences between orolingual and oral gavage exposure routes. It is likely that much the DON delivered by the orolingual route is rapidly absorbed sublingually via the reticulated vein that lies underneath the oral mucosa, and transported through the facial veins, internal jugular vein, and brachiocephalic vein and, finally, then drained into systemic circulation (Nibha and Pancholi, 2012). Another difference is the sublingual route would avoid first-pass metabolism that is encountered after oral gavage. The picture is further complicated by the recent discovery that PYY is also synthesized by taste cells in the taste buds and be detectable in saliva (Acosta *et al.*, 2011). How this would affect plasma PYY is unknown. It has also been demonstrated that orally administered PYY can induce anorexia via metabolic circuit associated with Y2R-positive cells in the oral cavity and extending through brainstem nuclei into hypothalamic satiety centers (Hurtado *et al.*, 2013). Accordingly, when an experimental animal consumes a DON-contaminated food, it will be exposed to the toxin sublingually for a short time during mastication but upon swallowing, the compound would will enter the GI tract which is best mimicked by gavage exposure. In the natural setting, DON is likely taken up both sublingually and intestinally evoking both CCK and PYY elevation. Thus, future studies should focus on the effects of consuming DON-contaminated food on satiety hormone levels in plasma and saliva as well as their functional roles in hypophagia.

To summarize, the results presented herein indicate that satiety hormone CCK was robustly induced upon oral administration of 8-ketotrichothecenes, whereas PYY<sub>3-36</sub> was not. Furthermore, CCK elevation was consistent with the induction of food refusal by these congeners. From public and veterinary

health perspectives, studies such as this will improve our understanding of how 8-ketotrichothecenes adversely affect humans and animals. Future investigations should focus on elucidating how and where 8-ketotrichothecenes activate exocytosis of CCK and other satiety hormones in the intestinal tract and CNS as well as how these hormones resulted in appetite signaling via the peripheral and CNSs.

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