Effects of the mycotoxin deoxynivalenol on steroidogenesis and apoptosis in granulosa cells

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Abstract

Mycotoxins can reduce fertility and development in livestock, notably in pigs and poultry, although the effect of most mycotoxins on reproductive function in cattle has not been established. One major mycotoxin, deoxynivalenol (DON), not only targets immune cells and activates the ribotoxic stress response (RSR) involving MAPK activation, but also inhibits oocyte maturation in pigs. In this study, we determined the effect of DON on bovine granulosa cell function using a serum-free culture system. Addition of DON inhibited estradiol and progesterone secretion, and reduced levels of mRNA encoding estrogenic (CYP19A1) but not progestogenic (CYP11A1 and STAR) proteins. Cell apoptosis was increased by DON, which also increased *FASLG* mRNA levels. The mechanism of action of DON was assessed by western blotting and PCR experiments. Addition of DON rapidly and transiently increased phosphorylation of MAPK3/1, and resulted in a more prolonged phosphorylation of MAPK14 (p38) and MAPK8 (JNK). Activation of these pathways by DON resulted in time- and dose-dependent increases in abundance of mRNA encoding the transcription factors FOS, FOSL1, EGR1, and EGR3. We conclude that DON is deleterious to granulosa cell function and acts through a RSR pathway.

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Introduction

Fungal contamination of animal feed is a significant problem in many parts of the world (Marin *et al.* 2013). Contamination with *Fusarium* spp is common and results in significant accumulation of the mycotoxins zearalenone (ZEN) and deoxynivalenol (DON) among others (Rodrigues & Naehrer 2012). The actions of ZEN are well known; it is estrogenic and affects the female reproductive system, particularly in pigs where symptoms include nymphomania, pseudopregnancy, and ovarian atrophy (reviewed in Cortinovis *et al.* (2013)). In cattle, ZEN intoxication is reported to result in reduced conception rates, possibly owing to toxic effects on the oocyte (Minervini *et al.* 2001).

Less is known about the effects of DON, a non-estrogenic compound, on the female reproductive system. In pigs, DON inhibited cumulus expansion and oocyte maturation *in vitro* (Alm *et al.* 2002, Malekinejad *et al.* 2007, Schoevers *et al.* 2010). The potential effects of DON on granulosa cells are unclear; DON has been reported to either increase or decrease progesterone secretion and to have a biphasic effect on estradiol (E₂) secretion from porcine granulosa cells *in vitro* (Ranzenigo *et al.* 2008, Medvedova *et al.* 2011). In cattle, there are preliminary data to suggest that DON increased levels of mRNA coding for the rate-limiting

progestagenic enzyme cytochrome P450 cholesterol side-chain cleavage (CYP11A1), but had no effect on abundance of mRNA encoding the main estrogenic enzyme cytochrome P450 aromatase (CYP19A1) in cultured granulosa cells (Pizzo *et al.* 2014); any effect of DON on abundance of mRNA encoding STAR protein (STAR), the protein involved in the transport of cholesterol across the mitochondrial membrane, was not reported. To our knowledge, no other information is available on the effects of DON on ovarian function in cattle.

The generally accepted mechanism of action of DON is through binding to ribosomes and initiation of the ribotoxic stress response (RSR). This involves activation of the p38 (MAPK14), ERK1/2 (MAPK3/1), and c-Jun N-terminal kinase (MAPK8) members of the MAP kinase (MAPK) family (Pestka 2008). As all these pathways are active in bovine granulosa cells (Evans & Martin 2000, Uzbekova *et al.* 2009, Abedini *et al.* 2015), we hypothesize that DON may activate one or more of these pathways to alter granulosa cell function. The objectives of this study were to determine the effects of DON on granulosa cell steroidogenesis and apoptosis in a non-luteinizing serum-free culture system, and to determine whether DON acts through typical RSR intracellular signaling pathways, including early response genes.

Materials and methods

Cell culture

All materials were obtained from Life Technologies, Inc. Bovine granulosa cells were cultured in serum-free conditions that maintain E_2 and progesterone secretion and responsiveness to follicle-stimulating hormone (FSH; [Gutiérrez et al. 1997](#), [Silva & Price 2000](#), [Sahmi et al. 2004](#)). Bovine ovaries were obtained from adult cows, independently of the stage of the estrous cycle, at the slaughterhouse and transported to the laboratory at 30 °C in PBS containing penicillin (100 IU) and streptomycin (100 µg/ml). Granulosa cells were harvested from follicles 2–5 mm in diameter, and the cell suspension was filtered through a 150 mesh steel sieve (Sigma–Aldrich Canada). Cell viability was assessed by Trypan blue dye exclusion.

Cells were seeded into 24-well tissue plates (Sarstedt, Inc., Newton, NC, USA) at a density of 500 000 viable cells in 500 µl DMEM/F12 containing sodium bicarbonate (10 mM), 25 mM HEPES, sodium selenite (4 ng/ml), BSA (0.1%; Sigma–Aldrich), penicillin (100 U/ml), androstenedione (10^{-6} M), and bovine FSH (10 ng/ml starting on day 2, AFP5346D; National Hormone and Peptide Program, Torrance, CA, USA). Cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air for up to 6 days.

Experimental treatments

To determine the effects of DON on granulosa cell steroidogenesis, cells were treated from day 2 with 0, 1, 10, or 100 ng/ml DON (in methanol) or vehicle (methanol alone), and cells and media were recovered on day 6; these doses were based on concentrations of 2–14 ng/ml DON reported in serum of cattle fed a contaminated concentrate ([Keese et al. 2008](#)). Apoptosis was measured using an Annexin V–FITC apoptosis detection kit (Sigma–Aldrich) after treating cells with an effective dose of DON for 4 days. To assess the effect of DON on intracellular pathway activation, cells were treated on day 5 of culture with an effective dose of DON for 0, 5, 15, 30, and 60 min, and cells were recovered in RIPA buffer to measure the phosphorylation status of key protein kinases. The dose- and time-dependent effects of DON on abundance of mRNA of early response genes were determined by treating cells on day 5 of culture with an effective dose of DON for 0, 1, 2, 4, 8, and 24 h, and by treating cells for 1 h with 0, 1, 10, or 100 ng/ml DON. Cells were recovered for RNA extraction. All experiments were carried out with three different pools of cells collected on different occasions.

Steroid assay

E_2 concentrations in a conditioned medium were measured in duplicate as described ([Jiang & Price 2012](#)) using an antibody raised in rams ([Sanford 1987](#)). Intra- and inter-assay coefficient of variation (CV) values were 6 and 9% respectively. Progesterone concentration was measured in a conditioned medium in duplicate as described ([Bélanger et al. 1990](#), [Price et al. 1995](#)) with mean intra- and interassay CV values of 7.2 and 18% respectively. Steroid concentrations in the culture medium were corrected for cell number by expressing the data

per unit mass of total cell protein. The sensitivities of these assays were 10 and 4 pg/tube for E_2 and progesterone, equivalent to 0.3 and 20 ng/µg protein respectively.

Total RNA extraction and real-time PCR

After treatments, the medium was removed and total RNA was extracted using TRIzol according to the manufacturer's instructions. Total RNA (0.5 µg) was quantified by absorbance at 260 nm and treated with 1 U DNase (Invitrogen). RNA was reverse transcribed in the presence of 1 mmol/l oligo (dT) primer and 4 U Omniscript RTase (Qiagen), and 0.25 mmol/l ddNTP mix and 19.33 U RNase inhibitor (GE Healthcare Canada, Baie D'Urfé, QC, Canada) in a volume of 20 µl at 37 °C for 1 h. The reaction was terminated by incubation at 93 °C for 5 min.

Real-time PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems) with Power SYBR Green PCR Master Mix. The bovine-specific primers have been published previously ([Jiang et al. 2013](#)). Common thermal cycling parameters (3 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C) were used to amplify each transcript. Melting curve analyses were performed to verify product identity. Samples were run in duplicate and were expressed relative to histone *H2AFZ* as a housekeeping gene. Data were normalized to a calibrator sample using the $\Delta\Delta C_t$ method with correction for amplification efficiency ([Pfaffl 2001](#)).

Western blot

After challenge with DON, cells were washed with cold PBS and lysed in 100 µl/well cold RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, and protease inhibitor cocktail). The homogenate was centrifuged at 6000 g for 5 min at 4 °C. The resulting supernatant was retained and stored at –20 °C. Protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL, USA).

Samples were resolved on 12% SDS–polyacrylamide gels (15 µg total protein/lane) and electrophoretically transferred onto nitrocellulose membrane in a Bio-Rad wet Blot Transfer Cell apparatus (transfer buffer: 39 mM glycine, 48 mM Tris–base, 1% SDS, 20% methanol, and pH 8.3). After transfer, the membranes were blocked in TTBS (10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, and pH 7.5) for 1 h. Membranes were incubated overnight with the primary antibody (rabbit anti-rat MAPK3/1, #9102, 1:2000; rabbit anti-human phospho-MAPK3/1, #9101, 1:1000; rabbit anti-human MAPK14 #9211, 1:1000; rabbit anti-human phospho-MAPK14 #9215, 1:1000; rabbit anti-human MAPK8 #9251, 1:1000; and rabbit anti-human MAPK8 #9252, 1:1000; Cell Signaling Technology, Danvers, MA, USA) diluted in 5% BSA (total MAPK8) or TTBS (all other antibodies) at 4 °C. The loading control was COX4I1 (#69359, 1:1000; Santa Cruz Biotechnology). After washing three times with TTBS, membranes were incubated for 2 h at room temperature with 1:10 000 anti-rabbit HRP-conjugated IgG (GE Healthcare Canada) diluted in TTBS. After five washes in TTBS, protein bands were revealed by ECL (Millipore, Billerica, MA, USA) using a gel imaging system

(ChemiDoc XRS system, Bio-Rad). Semiquantitative analysis was performed using the Bio-Rad ChemiDoc XRS Software.

Statistical analysis

All statistical analyses were performed using the JMP Software (SAS Institute, Cary, NC, USA). Data were transformed to logarithms if they were not normally distributed (Shapiro–Wilk test). At instances where main effects were significant, the effect of time or treatment was tested using the Tukey–Kramer honest significant difference (HSD) test. The data are expressed as least square means $\pm$ S.E.M.

Results

DON suppressed steroid secretion and steroidogenic enzyme gene expression

We first assessed the effect of DON on steroidogenesis. Cultured bovine granulosa cells were challenged with 1, 10, and 100 ng/ml DON for 4 days. At the dose of 100 ng, DON significantly inhibited E_2 and progesterone secretion (Fig. 1), and potently suppressed *CYP19A1* mRNA levels but did not alter *CYP11A1* and *STAR* mRNA levels (Fig. 2).

Figure 1 Effect of DON on estradiol and progesterone secretion from bovine granulosa cells in a serum-free medium. Cells were cultured for 4 days with the doses of DON shown, and steroid in the medium measured by RIA. Concentrations were corrected for cell number (total cell protein) and represent the amount secreted during the last 2 days of culture. Data are expressed as means ($\pm$ S.E.M.) of four independent cultures, and bars without common letters are significantly different ($P < 0.05$, Tukey–Kramer HSD). Vehicle, methanol.

Figure 2 Effect of DON on abundance of mRNA encoding steroidogenic proteins in bovine granulosa cells. Cells were cultured for 4 days with 100 ng/ml DON or methanol (vehicle), and RNA collected for real-time PCR. Data are expressed relative to a calibrator sample using the $\Delta\Delta C_t$ method with correction for amplification efficiency and are presented as means ($\pm$ S.E.M.) of three independent cultures. Asterisk denotes treatment significantly different from control ($P < 0.05$, Tukey–Kramer HSD).

DON increases granulosa cell apoptosis

The ability of DON to inhibit E_2 secretion prompted us to determine the effect of DON on granulosa cell health. Addition of an effective dose of DON (100 ng/ml) for 4 days increased the proportion of apoptotic cells by 15% (Fig. 3). In a subsequent experiment, DON was added for 24 h and increased levels of mRNA encoding the apoptosis-related genes *FASLG* and *GADD45B* (Fig. 3).

DON activates pathways related to RSR in granulosa cells

The main mechanism of action of DON is through RSR pathways; therefore, we assessed the activation of these pathways in granulosa cells. The addition of DON caused a rapid and transient increase in MAPK3/1 phosphorylation within 15 min and an increase in MAPK14 phosphorylation that was significant at 30 and 60 min (Fig. 4A and B). Furthermore, DON

Figure 3 Addition of DON increased the proportion of dead cells and abundance of *FASLG* and *GADD45B* mRNA in bovine granulosa cells. Cells were cultured for 4 days with 100 ng/ml DON and recovered for either the measurement of apoptosis by flow cytometry (Annexin-V apoptosis kit) or for RNA measurement by real-time PCR. Data are expressed as means ($\pm$ S.E.M.) of three independent cultures. Asterisk denotes treatment significantly different from control ($P < 0.05$, Student's *t*-test).

increased both MAPK8 (Fig. 4C) and phospho-MAPK8 levels (Fig. 4D), such that the ratio of phosphorylated to total MAPK8 did not change (not shown).

Our next step was to determine whether the activation of these pathways by DON affected expression of specific target genes. Addition of DON increased *EGR1*, *EGR3*, *FOS*, and *FOSL1* mRNA levels within 1–2 h, and levels declined to control values by 8 h for all genes. DON also increased *PTGS2* mRNA levels, and this was not significant until 24 h of treatment (Fig. 5). We confirmed the effect on the early response genes with a dose–response study at 1 h of treatment, and mRNA levels of all four target genes were increased at the dose of DON that inhibited E_2 secretion (Fig. 6).

Discussion

The results of this study clearly demonstrate that DON can have a significant negative impact on granulosa cell

health and function, notably on E_2 secretion and *CYP19A1* mRNA levels, and that DON acts through typical RSR pathways involving activation of MAPK3/1, MAPK8, and MAPK14 kinases.

Reports on the effects of DON on steroidogenesis are contradictory. In pigs, DON increased E_2 secretion and *CYP19A1* mRNA levels at a concentration of 10 ng/ml and inhibited both at a concentration of 100 ng/ml (Ranzenigo *et al.* 2008), whereas, in bovine granulosa cells, DON (1000 ng/ml) increased *CYP19A1* mRNA levels (Pizzo *et al.* 2014). In this study, no stimulatory effect of DON on E_2 secretion or *CYP19A1* mRNA levels was observed, although the decrease in E_2 secretion and *CYP19A1* mRNA levels with 100 ng/ml DON was consistent with the study by Ranzenigo *et al.* (2008). Similarly, for progesterone, 100 ng/ml DON increased progesterone secretion in one study carried out on pigs (Medvedova *et al.* 2011), but inhibited secretion from granulosa cells in another study (Ranzenigo *et al.* 2008) and in this study with bovine cells. One difference between the previous and present studies is the use of serum in the culture medium in all previous studies; serum is known to alter granulosa cell steroidogenesis *in vitro* (Gutiérrez *et al.* 1997).

In pig granulosa cell cultures, DON at the doses used increased cell numbers and abundance of the

Figure 4 Intracellular pathways activated by DON in granulosa cells. Bovine granulosa cells were cultured in a serum-free medium and on day 5 were challenged with DON (100 ng/ml) for the times shown. Total cell protein was recovered for western blotting with antibodies against total and phosphorylated forms of (A) MAPK3/1, (B) MAPK14, and (C and D) MAPK8. Representative blots from one replicate are shown above the graphs, and samples were loaded in the same order as in the graphs. Data are represented as the ratio of phosphorylated: total protein for MAPK3/1 and MAPK14, and of each form of MAPK8: COX411 (housekeeping protein), and are means ($\pm$ S.E.M.) of three independent cultures; bars without common letters are significantly different ($P < 0.05$, Tukey–Kramer HSD).

Figure 5 Acute effect of DON on early response genes in bovine granulosa cells. Cells were cultured in a serum-free medium and on day 5 were challenged with DON (100 ng/ml) for the times shown. Cells were recovered for RNA measurement by real-time PCR. Data are expressed relative to a calibrator sample using the $\Delta\Delta C_t$ method with correction for amplification efficiency, and are presented as means ($\pm$ S.E.M.) of three independent cultures. Bars without common letters are significantly different ($P < 0.05$, Tukey-Kramer HSD).

proliferation marker PCNA without increasing apoptosis (Ranzenigo *et al.* 2008, Medvedova *et al.* 2011), whereas, in this study, DON increased the rate of apoptosis. This difference is again likely to be owing to the absence of serum in the current culture system, as this reduces the rate of proliferation of granulosa cells (Gutiérrez *et al.* 1997). As an increase in the incidence of apoptotic granulosa cells (Irving-Rodgers *et al.* 2001) and a decrease in E_2 secretion (McNatty *et al.* 1984) are characteristics of atretic follicles *in vivo*, the ability of DON to increase apoptosis and decrease E_2 secretion suggests that it may be able to cause or promote follicle atresia.

The addition of DON increased the abundance of *FASLG* and *GADD45B* mRNAs, both of which have been linked to apoptosis. While *FASLG* is well known to induce apoptosis in a variety of cell types including granulosa cells (Porter *et al.* 2000), the role of *GADD45B* is much less clear. Granulosa cells of atretic bovine follicles contain less *GADD45B* mRNA than do those of healthy follicles (Mihm *et al.* 2008), and pro-apoptotic factors such as fibroblast growth factor 18 (FGF18) decrease *GADD45B* mRNA levels in granulosa cells *in vitro* (Portela *et al.* 2010), whereas mitogenic factors such as FGF2 increase *GADD45B* mRNA levels (Jiang *et al.* 2011). Thus, the increase in *GADD45B* mRNA levels with

increased apoptosis is not consistent with the previous data, and supports the suggestion that the regulation of *GADD45B* mRNA abundance is context (ligand?) specific (Salvador *et al.* 2013). It has been suggested that *GADD45B* may enhance or mitigate FAS-mediated apoptosis, depending on the cell type (Zazzeroni *et al.* 2003, Cho *et al.* 2010), thus the increase in *GADD45B* mRNA levels occurring with increased *FASLG* mRNA abundance may be part of either the apoptotic mechanism or a DNA repair mechanism.

The intracellular pathways activated by DON in a variety of non-reproductive cell types include MAPK3/1, MAPK8, and MAPK14. In this study, we demonstrate for the first time that DON activates these MAPKs in granulosa cells. The time-course of DON-induced phosphorylation observed herein is similar to that observed in a number of cell types, including murine macrophages (Moon & Pestka 2002, Pan *et al.* 2013), human intestine epithelial cells (Moon *et al.* 2007), and mouse skin (Mishra *et al.* 2014) among others. The activity of these pathways was demonstrated by the time- and dose-dependent increase in levels of mRNA encoding the transcription factors EGR1 and FOS; DON has previously been shown to increase *Egr1* and *Fos* mRNAs in various cell lines (Moon *et al.* 2007, Nielsen *et al.* 2009) and mouse spleen (Kinser *et al.* 2004). In this study, we also identified *FOSL1* and *EGR3* as targets of DON activity, which are novel findings. *EGR3* is a zinc finger-containing transcription factor that has been reported in breast cancer cells and in the mouse oocyte (Inoue *et al.* 2004, Shin *et al.* 2014). Although it is well known that *EGR1* mRNA abundance in granulosa cells is increased by ligands including gonadotropins and growth factors (Espey *et al.* 2000, Russell *et al.* 2003, Sayasith *et al.* 2006, Jiang *et al.* 2013), we are unaware of any reports demonstrating the regulation of *EGR3*

Figure 6 Effect of DON on early response genes is dose dependent. Bovine granulosa cells were cultured in a serum-free medium and on day 5 were challenged with DON for 1 h at the doses shown. Cells were recovered for RNA measurement by real-time PCR. Data are expressed relative to a calibrator sample using the $\Delta\Delta C_t$ method with correction for amplification efficiency and are presented as means ($\pm$ S.E.M.) of three independent cultures. Bars without common letters are significantly different ($P < 0.05$, Tukey-Kramer HSD). Vehicle, methanol.

mRNA abundance in granulosa cells. Interestingly, EGR3 but not EGR1 was shown to increase *FASLG* expression in T cells and fibroblasts (Mittelstadt & Ashwell 1998, Yoo & Lee 2004); therefore, the effect of DON on *FASLG* expression may be mediated in part through EGR3.

E₂ is a major determinant of follicle development and decreases granulosa cell apoptosis in rodents *in vivo* (Billig *et al.* 1993). Studies with bovine granulosa cells have demonstrated that E₂ can overcome the apoptotic effect of ligands such as *FASLG* and FGF18 (Quirk *et al.* 2006, Portela *et al.* 2015). Of the main RSR pathways, MAPK3/1 is known to alter *CYP19A1* mRNA levels; inhibition of MAPK3/1 phosphorylation increased *CYP19A1* mRNA levels in bovine and rodent granulosa cells (Moore *et al.* 2001, Silva *et al.* 2006) and reduced the ability of tumor necrosis factor alpha to increase apoptosis (Morales *et al.* 2006). Therefore, one mechanism for the action of DON might be via MAPK3/1 inhibition of *CYP19A1* expression and E₂ secretion, which then predisposed cells to apoptosis.

In conclusion, this study demonstrates that, *in vitro*, the mycotoxin DON has a negative impact on granulosa cell steroidogenesis and survival, and that the mechanism of action probably involves activation of the RSR. The potential impact of natural intoxication with DON on fertility in cattle warrants investigation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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