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Deoxynivalenol induced mouse skin cell proliferation and inflammation *via* MAPK pathway

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ABSTRACT

Several toxicological manifestations of deoxynivalenol (DON), a mycotoxin, are well documented; however, dermal toxicity is not yet explored. The effect of topical application of DON to mice was studied using markers of skin proliferation, inflammation and tumor promotion. Single topical application of DON (84–672 nmol/mouse) significantly enhanced dermal hyperplasia and skin edema. DON (336 and 672 nmol) caused significant enhancement in [^{3}H]-thymidine uptake in DNA along with increased myeloperoxidase and ornithine decarboxylase activities, suggesting tissue inflammation and cell proliferation. Furthermore, DON (168 nmol) caused enhanced expression of RAS, and phosphorylation of PI3K/Akt, ERK, JNK and p38 MAPKs. DON exposure also showed activation of transcription factors, c-fos, c-jun and NF-κB along with phosphorylation of IkBα. Enhanced phosphorylation of NF-κB by DON caused over expression of target proteins, COX-2, cyclin D1 and iNOS in skin. Though a single topical application of DMBA followed by twice weekly application of DON (84 and 168 nmol) showed no tumorigenesis after 24 weeks, however, histopathological studies suggested hyperplasia of the epidermis and hypertrophy of hair follicles. Interestingly, intestine was also found to be affected as enlarged Peyer's patches were observed, suggesting inflammatory effects which were supported by elevation of inflammatory cytokines after 24 weeks of topical application of DON. These results suggest that DON induced cell proliferation in mouse skin is through the activation of MAPK signaling pathway involving transcription factors NFκB and AP-1, further leading to transcriptional activation of downstream target proteins c-fos, c-jun, cyclin D1, iNOS and COX-2 which might be responsible for its inflammatory potential.

Introduction

Mycotoxins are toxic secondary metabolites produced by numerous species of fungi (Bennett and Klich, 2003). Deoxynivalenol (DON) is one of the most frequently occurring *Fusarium* mycotoxins that predominantly contaminate wheat, maize and barley in temperate regions (CAST, 2003; Schothorst and Van Egmond, 2004). DON ingested at higher doses causes nausea, vomiting, and diarrhea; while at lower dose it exhibits weight loss and food refusal (Rotter et al., 1996). Due to the toxic effects of DON, its presence in food commodities is gaining global attention. DON is not only the most commonly detected trichothecene in cereal grains, but has been found at exceedingly high levels (Almeida et al., 2012; Canady et al., 2001; Edwards et al., 2011; Lee et al., 2011; Mishra et al., 2013; Van Der Fels-Klerx et al., 2012). Considering the toxicological data on DON, Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2010) has recommended a PMTDI (provisional maximum tolerable daily intake) of 1 μg/kg bw.

There are a few reports on DON showing gastrointestinal and immune-toxicity in humans (Li et al., 1999; Pestka and Smolinski, 2005). An acute outbreak of DON intoxication was reported in the Kashmir valley, India affecting about 50,000 persons following consumption of contaminated wheat flour (Bhat et al., 1989). The incidence of esophageal cancer in China and Africa has been linked to DON contamination; however, no clear cut evidence has been demonstrated (Hsia et al., 1988; Luo et al., 1990). Although DON lacks the ability to cause gene mutation, it is certain that DON not only alters chromosome aberration, DNA damage and repair but can also make mammalian cells transform into malignant ones, which suggests that DON has the potential to be a genotoxic carcinogen (Ma and Guo, 2008). However, IARC (1993) has categorized DON in group 3, "not classifiable as to its carcinogenicity to humans" because of lack of sufficient evidences particularly related to *in vivo* studies. In

Abbreviations: DON, deoxynivalenol; DMBA, 7,12-dimethylbenz[α]anthracene; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappaB; TPA, 12-O-tetradecanoyl phorbol-13-acetate; ODC, ornithine decarboxylase; MPO, myeloperoxidase.

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a 2-year study, dietary exposure to DON showed no incidence of tumors in B6C3F1 mice (Iverson et al., 1995). However, a high incidence of lung adenocarcinoma and dysplasia of glandular stomach was observed in NIH mice orally exposed to DON after 24 weeks (Huang et al., 2004).

In the recent past WHO (1998) has highlighted the need for toxicological studies on mycotoxins through dermal exposure as limited data is available following dermal exposure risk to mycotoxins. This is of importance in developing countries situated in tropical region; manual labor employed during pre- and post-harvest stages of agricultural production is vulnerable to mycotoxin exposure through dermal route. In this context our prior studies have revealed that dermal exposure to some mycotoxins *viz* aflatoxin B1 (AFB1), patulin, citrinin, and ochratoxin A (OTA) caused skin toxicity including tumor formation (Kumar et al., 2011, 2012; Rastogi et al., 2006; Saxena et al., 2009, 2011). In a recent study, the transdermal kinetics of AFB1, OTA, fumonisin B1, citrinin, zearalenone and T-2 toxin were quantitatively evaluated indicating that mycotoxins can penetrate through the skin, which may lead to toxic manifestations (Boonen et al., 2012). Due to the widespread contamination of DON, it is likely that humans involved in agricultural practices may be repeatedly exposed to DON through dermal route.

A correlation between stimulation of cell proliferation, inflammation and tumor promotion is well established as continuous induction of cell proliferation may lead to cell transformation and tumor development (Poirier, 2004). Studies have shown that skin application of tumor-promoting agent results in inflammatory responses, such as development of edema, hyperplasia, and induction of proinflammatory cytokines, induction of dermal ornithine decarboxylase (ODC) and cyclooxygenase-2 (COX-2) protein expression, as well as activation of NF-κB (Chun et al., 2002; Katiyar et al., 1995, 1996; Seo et al., 2002). Activation of mitogen-activated protein kinases (MAPKs)/NF-κB pathways has been shown to be involved in tumor growth and development (Afaq et al., 2003; Shishodia et al., 2003).

The present study was designed to gain a better understanding of the cellular events leading to DON mediated *in vivo* dermal toxicity and more particularly to investigate whether DON causes induction of cell proliferation *via* activation of MAPK pathway which may further lead to tumor formation.

Materials and methods

Chemicals. Deoxynivalenol (DON), dithiothreitol (DTT), phenylmethylsulphonyl fluoride (PMSF), and diphenylamine (DPA) 7,12-dimethylbenz [α]anthracene (DMBA), 12-O-tetradecanoyl phorbol-13-acetate (TPA), phenylmethylsulfonyl fluoride (PMSF), 2-mercaptoethanol, acrylamide, protease inhibitor cocktail set-I, ethylenediamine tetraacetic acid (EDTA) disodium salt, Tris buffer, Triton X-100, bovine serum albumin (BSA), and HRP conjugated β-actin were obtained from Sigma Chemicals Co. (St. Louis, MO). [^{14}C]-Ornithine (specific activity, 56 mCi/mol) and [^{3}H]-thymidine (specific activity, 1.0 mCi/ml) were purchased from Amersham Biosciences (Chicago, IL). Rabbit polyclonal antibodies against phospho-ERK1/2, ERK1/2, phospho-p38, phospho-JNK, anti-p-AKT, anti-c-jun, anti-c-fos, anti-p-NFκB, anti-p-IκBα, anti-COX-2 and anti-cyclin-D1 were procured from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody was obtained from Bangalore Genei (Bangalore, India). Anti-p-PI3K, anti-RAS and cytometric bead array kit was purchased from BD Biosciences (San Diego, CA). All other chemicals used were of the highest purity commercially available.

Animals and ethics statement. Six to seven week old female Swiss albino mice (20 ± 3 g), derived from the animal breeding colony of CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow, were acclimatized under standard laboratory conditions and given a commercial pellet diet (Ashirwad Industries, Chandigarh, India) and water *ad libitum*. Animals were housed in plastic cages having rice husk as bedding and maintained in a controlled atmosphere of 12 h dark/light cycle, 22 ± 2 °C temperature and 50–60% humidity as per rules laid down by the Animal Welfare Committee of CSIR-IITR. All the experiments involving animals were approved by the Institutional Animal Ethics Committee (IAEC) via approval # ITRC/IAEC/30/11-22/12, CSIR-IITR. Mice were shaved with an electric clipper (Oster, WI, USA) one week prior to the beginning of the experiment. Mice showing no signs of hair growth or in resting phase of hair cycle were used for further experiments. Animals were sacrificed by cervical dislocation as per CSIR-IITR guidelines.

Edema and dermal hyperplasia. To assess the effect of DON on skin edema, animals were distributed randomly into six groups, having five mice per group. The mice of 1st group received a single topical application of 0.2 ml acetone to serve as a control, while animals of 2nd, 3rd, 4th and the 5th groups received a single topical application of 84, 168, 336, and 672 nmol DON in 0.2 ml acetone, respectively. The 6th group received topical application of TPA (4 nmol) and served as positive control. The topical doses of DON (84 nmol and 168 nmol) were chosen from the earlier study (Lambert et al., 1995). Following 24 h and 48 h of treatment, mice were sacrificed and 1 cm diameter punches of skin from the vehicle, and DON treated and TPA-treated animals were removed, made free of fat and weighed quickly. After drying for 24 h at 50 °C, the skin punches were reweighed, and the loss of water content was determined (Saleem et al., 2004). The difference in the amount of water gain between the control (vehicle treated) and DON or TPA treated groups represented the extent of edema induced by DON. For the hyperplasia study, skin was immediately removed, washed in cold normal saline solution, fixed in 10% buffered formalin and embedded in paraffin after processing. Sections of 5.0 μm thickness were cut and stained with hematoxylin and eosin for microscopic examination.

[^{3}H]-thymidine incorporation assay. For estimation of cell proliferation, female Swiss albino mice of the same age were used as described in the earlier section. Mice were divided into 6 groups of 5 mice each: (i) acetone (0.2 ml) topical application, as vehicle control; (ii) TPA (4 nmol) was topically applied as positive control; and (iii) DON (84, 168, 336, 672 nmol/mouse). The animals of all the groups were given i.p. injection of [^{3}H]-thymidine 2 h prior to sacrifice. Animals were sacrificed by cervical dislocation and skin was excised. Dermal DNA was isolated and assessment of incorporation of [^{3}H]-thymidine into DNA was carried out according to the method of Gupta and Mehrotra (1992).

Myeloperoxidase assay. Myeloperoxidase (MPO) was determined as a marker of inflammation in the skin. The activity of MPO was assessed in skin samples obtained from the mice. Mice were divided into 5 groups of 5 animals each. Mice were topically treated with 84, 168, 336, and 672 nmol of DON following 24 h treatment. MPO was measured as a marker of inflammation in skin homogenate samples following the procedure of Bradley et al. (1982). Briefly, the skin samples were homogenized in 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide followed by sonication of the homogenate at 4 °C for 10 s bursts (three times). Further the samples were centrifuged at 13,000 ×*g* for 10 min at 4 °C and the resulting supernatants were used for MPO estimation. MPO activity in the supernatant (0.1 ml) was assayed by mixing with 50 mM phosphate buffer (2.9 ml) pH 6.0, containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance resulting from decomposition of H_2O_2 in the presence of o-dianisidine was measured using Power Wave XS2 plate reader (Biotek, Winooski, VT) at 460 nm. The activity was calculated using an extinction coefficient of 178 mM^{-1} cm^{-1}, and the data are expressed as the mean MPO U/mg protein.