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Mechanisms of Deoxynivalenol-Induced Gene Expression and Apoptosis

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Abstract

Fusarium infection of agricultural staples such as wheat, barley and corn with concurrent production of deoxynivalenol (DON) and other trichothecene mycotoxins is an increasingly common problem worldwide. In addition to its emetic effects, chronic dietary exposure to DON causes impaired weight gain, anorexia, decreased nutritional efficiency and immune dysregulation in experimental animals. Trichothecenes are both immunostimulatory or immunosuppressive depending on dose, frequency and duration of exposure as well as type of immune function assay. Monocytes, macrophages, as well as T and B lymphocytes of the immune system can be cellular targets of DON and other trichothecenes. *In vitro* exposure to low trichothecene concentrations upregulates expression both transcriptionally and post-transcriptionally of cytokines, chemokines and inflammatory genes with concurrent immune stimulation, whereas exposure to high concentrations promotes leukocyte apoptosis with concomitant immune suppression. DON and other trichothecenes, via a mechanism known as the “ribotoxic stress response”, bind to ribosomes and rapidly activate mitogen-activated protein kinases (MAPKs). The latter are important transducers of downstream signaling events related to immune response and apoptosis. Using cloned macrophages, we have identified two critical upstream transducers of DON-induced MAPK activation. One transducer is double-stranded RNA-(dsRNA)-activated protein kinase (PKR), a widely-expressed serine/threonine protein kinase that can be activated by dsRNA, interferon and other agents. The other transducer is hematopoietic cell kinase (Hck), a non-receptor associated Src oncogene family kinase. Pharmacologic inhibitors and gene suppression studies have revealed that Hck and PKR contribute to DON-induced gene expression and apoptosis. PKR, Hck and other kinases bind to the ribosome and are activated following DON interaction. Future studies will focus on the sequence of molecular events at the ribosome level that drive selective activation of these upstream kinases.

Keywords

mycotoxin; *Fusarium*; trichothecene; immunotoxicity

Introduction

The trichothecene mycotoxins are a large group of structurally related sesquiterpenoid metabolites produced by *Fusarium* and other fungi often found in food and other organic substrates (Grove 1993; Grove 1988; Grove 2000). Trichothecenes are of low molecular

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weight (~200–500D), can diffuse rapidly into cells and can interact with the eukaryotic ribosome thereby blocking translation (Carter and Cannon 1977; Ueno 1984). All trichothecenes have in common a 9, 10 double bond and a 12, 13 epoxide group, but extensive variation exists relative to ring oxygenation patterns. Trichothecenes belonging to three structural groups are important from a public health perspective because of their presence in food or the environment (Fig. 1). These include: Type A which have isovaleryl, hydrogen, or hydroxyl moieties at the C-8 position (e.g. T-2 toxin), Type B which have a carbonyl group at the C-8 position (e.g., deoxynivalenol [DON]); and the Type D (or macrocyclic) which have a cyclic diester or triester ring linking C-4 to C-15 (e.g. satratoxin G).

Trichothecene contamination of wheat, barley and corn during *Fusarium* colonization is an increasingly common problem because of expanded use of “no-till farming” and changing climate patterns (McMullen *et al.* 1997). DON, known colloquially as “vomitoxin”, is the trichothecene most commonly detected, often at the mg kg⁻¹ level (Abouzied *et al.* 1991; Lee *et al.* 1985; Rotter *et al.* 1996; Sugiura *et al.* 1990; Tanaka *et al.* 1990). Two acetylated forms of DON, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol co-occur with DON at much lower levels. These acetylated species have equivalent or lower toxicity than DON. Nivalenol, T-2 toxin and diacetoxyscirpenol have also been reported in grains, but to a lesser extent than DON.

Studies in laboratory and food animals reveal that trichothecenes elicit a complex spectrum of toxic effects. Upon acute exposure to high doses, animals exhibit a “radiomimetic” shock-like response that includes diarrhea, vomiting, leukocytosis and hemorrhage, with extremely high doses causing death (Ueno 1984). Chronic exposure to trichothecenes can cause anorexia, reduced weight gain, diminished nutritional efficiency, neuroendocrine changes and immune modulation (Pestka and Smolinski 2005).

Relative to human toxicity, trichothecenes in moldy grain are suspected to have caused a human illness known as “Alimentary Toxic Aleukia” (ATA) in the Orenburg district of the USSR from the 1930’s to the late 1940’s where mortality reached 60% in some years (Joffe 1978). ATA had as its symptoms vomiting, diarrhea, leukopenia, hemorrhage, shock and sometimes death. The disease was related to overwintered wheat, barley and millet. Since moldy grains obtained during ATA outbreaks were later found to contain trichothecene-producing fusaria, these mycotoxins are thought likely to be etiologic agents of this disease.

Human gastroenteritis with nausea, diarrhea and vomiting as primary symptoms were also frequently associated with *Fusarium*-infested foods between 1946 and 1963 in Japan and Korea (Yoshizawa *et al.* 1983)- findings which predated the discovery of the possible causative trichothecene mycotoxins. At least 32 outbreaks of food poisoning were linked to consumption of scabby wheat, barley or corn in China from 1961–1981 (Luo and X. 1994). Nearly 6000 persons were affected (63.9% attack rate). Illness occurred within 30 minutes and symptoms included nausea, vomiting, abdominal pain, diarrhea, headache, dizziness and fever. Similar, subsequent gastroenteritis outbreaks (1984–1991) in China associated with scabby/moldy cereals were found to contain DON and/or other trichothecenes. The largest outbreak affected 130,000 people with DON being found in 10 wheat samples ranging from 2 to 50 mg kg⁻¹. In an analogous outbreak, several thousand individuals developed gastroenteritis following consumption of rain-damaged moldy wheat products in the Kashmir Valley of India (Bhat *et al.* 1989). DON at 0.34 to 8.4 mg kg⁻¹ was detected in 11/24 samples taken from the affected area.

Given that food and feed are sometimes contaminated by DON and other trichothecenes, serious questions remain regarding risks from chronic ingestion to these toxins. Understanding the molecular mode of action of a mycotoxin can assist in predicting potential adverse human health effects and be used in risk assessment and risk management, as has been elegantly demonstrated for the aflatoxins (Groopman *et al.* 2005). The capacity to inhibit protein synthesis is thought to be central to trichothecenes' effects in cells, tissues and intact animals (Ueno, 1984). However, many of their toxic effects might actually be related to a rapidly ensuing dysregulation of intracellular cell signaling and consequent alterations in downstream gene expression. The purpose of this review is to summarize studies conducted by our laboratory and others on the molecular mechanisms underlying DON's aberrant immunologic and pathophysiologic.

Acute toxic effects

To characterize potential hazards, DON toxicity studies in animals typically focus on specific tissue targets, outcomes or mechanisms (Pestka and Smolinski 2005). Toxicity symptoms in sensitive species following acute DON poisoning include abdominal distress, increased salivation, malaise, diarrhea and emesis (Forsyth *et al.* 1977; Friend *et al.* 1982; Pestka *et al.* 1987b; Prelusky and Trenholm 1993; Young *et al.* 1983). Although DON is less toxic than other trichothecenes such as T-2 toxin, extremely high DON doses (i.e. unlikely to be encountered in food) can cause shock-like death. LD₅₀ values for mice range from 49–70 and 46–78 mg/kg bw for ip and oral exposure, respectively (Forsell *et al.* 1987; Yoshizawa 1983). Lethal DON doses evokes histopathologic effects ranging from hemorrhage/necrosis of the intestinal tract, necrosis in bone marrow and lymphoid tissues, to kidney and heart lesions (Pestka *et al.* 1994a; Yoshizawa and Morooka 1977).

Even though it is considered to be one of the least lethal trichothecenes, DON's emetic and anorectic potencies are equivalent to or exceed those reported for the more potent trichothecenes (Rotter *et al.* 1996). As little as 50 µg/kg bw ip or oral can cause vomiting in swine (Forsyth *et al.* 1977; Pestka *et al.* 1987a; Prelusky and Trenholm 1993). One study observed emesis in pigs occurring within minutes after ingesting feed containing 19.7 mg kg⁻¹ DON (Young *et al.* 1983) which can be equated to be approximately 150 µg/kg bw/day of the toxin. Ingestion of DON at concentrations of 8 mg kg⁻¹ and 11 mg kg⁻¹ induce vomiting in dogs and cats, respectively (Hughes *et al.* 1999).

Taken together, extremely high acute DON (46–78 mg kg⁻¹) doses are required to cause mortality or marked tissue injury in experimental mice, whereas acute exposure to relatively low doses (≥ 50 µg/kg bw) can cause vomiting in swine, a very sensitive species.

Chronic effects

Growth and food intake

Extended DON consumption in murine and porcine experimental models decreased weight gain, anorexia and altered nutritional efficiency (Rotter *et al.* 1996). Forsell *et al.* (Forsell *et al.* 1986) found that weight gain was depressed in weanling female B6C3F1 mice consuming 2 mg kg⁻¹ DON. Other studies have shown weight gain suppression in mice at DON feed concentrations of 6.25 mg kg⁻¹ (Arnold *et al.* 1986), 5 mg kg⁻¹ (Robbana-Barnat *et al.* 1987) and 10 mg kg⁻¹ (Hunder *et al.* 1991). In the most comprehensive DON feeding study reported to date, the effects of 0, 1, 5 and 10 mg kg⁻¹ DON for 2 years were assessed in male and female B6C3F1 mice (Iverson *et al.* 1995). Significant body weight reduction was observed in mice fed 5 and 10 mg kg⁻¹ DON. Food consumption was unaffected in females but decreased for male test animals at the two highest doses.

Early studies reported that DON at 1 to 2 mg kg⁻¹ also caused partial feed refusal in pigs ingesting naturally contaminated feedstuffs, whereas 12 mg kg⁻¹ caused complete refusal (Abbas *et al.* 1986; Forsyth *et al.* 1977; Rotter *et al.* 1994; Trenholm *et al.* 1984; Young *et al.* 1983). Pigs fed diets containing 2 and 4 mg kg⁻¹ of DON exhibited a dose-related decrease in weight gain within the first 8 weeks (Bergsjö *et al.* 1992). The 4 mg kg⁻¹ diet caused decreased feed intake, weight gain, and efficiency of feed utilization throughout the experiment. When clean and naturally contaminated corn were incorporated into basal diets formulated to contain DON at 0, 0.95, 1.78, and 2.85 mg kg⁻¹, feed intake was significantly suppressed in pigs at all DON levels (Rotter *et al.* 1994). Decreased feed intake and weight gain occurs in castrated male pigs fed diets spiked with 3 mg kg⁻¹ DON in naturally contaminated corn but not in pigs fed diet spiked with purified toxin (Prelusky *et al.* 1994). Differences between naturally contaminated feed ingredients versus feed spiked with purified DON might be attributed to the presence of additional toxins and fungal components in naturally contaminated grains that contribute additively or synergistically to DON toxicity.

The above and other studies suggest monogastric species such as mice and pigs are very sensitive to growth and weight gain suppression during subchronic and chronic DON exposure. These effects are accompanied by anorexia at higher DON concentrations in diet. The no observed adverse effect levels (NOAELs) for rodents were 0.1–0.15 mg/kg bw/day, whereas, for swine, NOAELs were 0.03–0.12 mg/kg bw/day (Pestka and Smolinski 2005).

Immunological effects

Leukocytes as targets

Investigations of host resistance, cell-mediated immune responses and humoral immunity indicate that trichothecenes are both immunostimulatory and immunosuppressive depending on dose frequency and duration of exposure as well as type of functional immune assay (Pestka *et al.* 2004). Leukocytes, which represent the functional cell repertoire of the immune system, are very sensitive to trichothecenes with macrophages, monocytes, B cells and T cells all being affected. Consistent with whole animal studies, dose-dependent decreases or increases in ex vivo B- and T-cell mitogen responses occur in lymphocyte cultures from animals exposed to T-2 toxin, DON or various macrocyclic trichothecenes (Friend *et al.* 1983; Hughes *et al.* 1988; Hughes *et al.* 1989; Hughes *et al.* 1990; Miller and Atkinson 1986; Tomar *et al.* 1987; Tomar *et al.* 1988). Similarly, *in vitro* trichothecene exposure impairs or enhances mitogen-induced lymphocyte proliferation (Atkinson and Miller 1984; Bondy *et al.* 1991; Cooray 1984; Hughes *et al.* 1988; Hughes *et al.* 1989; Hughes *et al.* 1990; Miller and Atkinson 1986; Miller and Atkinson 1987; Tomar *et al.* 1986; Tomar *et al.* 1987; Tomar *et al.* 1988). Based on concentrations required to inhibit proliferation of cloned macrophages or human peripheral blood mononuclear cells, trichothecene toxicity follows the rank order Type D > Type A > Type B (Forsell *et al.*, 1985; Moon *et al.*, 2003; Pestka and Forsell, 1988) (Fig. 1).

Gene upregulation

In addition to potentiation of mitogen responses, trichothecenes can upregulate or downregulate expression of immune- and inflammation-associated genes. Stimulation of mononuclear phagocytes by low doses or concentrations of trichothecenes elicit expression of inflammation-related genes *in vivo* and *in vitro* including cyclooxygenase-2 (COX-2) (Moon and Pestka 2002), proinflammatory cytokines (Wong *et al.* 1998; Zhou *et al.* 1997) and numerous chemokines (Chung *et al.* 2003a; Islam *et al.* 2006; Kinser *et al.* 2004). Induction

of these mediators might contribute to DON-induced growth and anorectic effects as has been described for endotoxin (Kanra *et al.* 2006; Schwartz 2002).

DON-induced IgA dysregulation in experimental mice is a pathologic outcome of immune gene upregulation that illustrates the complexity of trichothecene effects and has provided a portal for mechanistic exploration. Marked elevations in IgA production occur in mice fed diets containing DON or nivalenol that results in pathologic effects that closely mimic the common human glomerulonephritis, IgA nephropathy (IgAN) (Pestka 2003). These include elevations in serum IgA, circulating IgA immune complexes, kidney mesangial IgA deposits and hematuria (Dong and Pestka 1993; Dong *et al.* 1991; Greene *et al.* 1994b; Greene *et al.* 1994a; Greene *et al.* 1995; Pestka *et al.* 1989; Pestka *et al.* 1990c) as well as polyclonal activation of IgA secreting cells and polyreactive IgA autoantibody secretion (Pestka *et al.* 1990a; Pestka *et al.* 1990b; Rasooly *et al.* 1994; Rasooly and Pestka 1992; Rasooly and Pestka 1994). DON-induced polyclonal expansion of IgA-secreting cells in mice is mediated by increased cytokine production by macrophages (Yan *et al.* 1998) and T cells (Bondy and Pestka 1991; Pestka *et al.* 1990b; Warner *et al.* 1994). IL-6 induction is particularly important for DON-induced IgAN as evidenced by *ex vivo* studies (Yan *et al.* 1997) and *in vivo* feeding trials with IL-6 deficient mice (Pestka and Zhou 2000). Upregulation of COX-2 and resultant production of prostaglandin metabolites can also contribute to DON-induced IL-6 production *in vitro* and *in vivo* (Moon and Pestka 2003a).

Induction of apoptosis

In vivo administration of trichothecenes to rodents causes apoptosis in bone marrow, Peyer's patches and, thymus, (Ihara *et al.* 1997; Ihara *et al.* 1998; Islam *et al.* 1998b; Islam *et al.* 1998a; Miura *et al.* 1998; Shinozuka *et al.* 1997a; Shinozuka *et al.* 1997b; Shinozuka *et al.* 1998). The hematotoxicity of trichothecenes has been elegantly studied in the laboratory of Parent-Massin, who demonstrated that hematopoietic progenitors are a main target for apoptosis induction (Parent-Massin 2004). T-2 toxin causes apoptosis in human cord blood CD34⁺ hematopoietic progenitors with impaired clonal expansion of granulo-monocytes, erythrocytes and megakaryocytes precursors, whereas DON does not have the same effect (Le *et al.* 2005). Leukocytes are immune cells derived from hematopoiesis which defend the body against both infectious disease and foreign materials. High concentrations of trichothecene can also evoke rapid onset of leukocyte apoptosis. DON directly induces apoptosis in T-cells, B-cells and IgA⁺ cells *in vitro* (Pestka *et al.* 1994b). Exposure to high trichothecene concentrations also induces apoptosis in macrophages (Yang *et al.* 2000; Zhou and Pestka 2003) which might suppress innate immune function (Pestka *et al.* 2004). DON induces apoptosis in RAW 264.7 murine macrophages, however, murine peritoneal macrophages might be less susceptible (Zhou and Pestka 2005). Accordingly, trichothecene-induced apoptosis of hematopoietic progenitors and leukocytes is very likely to contribute to immunosuppression with sensitivity to various trichothecenes differing among cell types.

Molecular mechanisms for trichothecene-mediated immune dysregulation

Trichothecene-mediated immune dysregulation is best viewed as a spectrum whereby low doses stimulate immune gene expression but as dose increases, leukocyte apoptosis ensues, leading to immunosuppression. In some cases, these disparate effects can overlap leading to complex sequelae. Understanding how changes in cell signaling and gene expression occur throughout this spectrum has yielded insight into the underlying molecular effects of trichothecenes. Such data facilitate structure function studies and can assist safety assessments for these toxins. The ensuing discussion will initially focus on the underlying mechanisms for low dose-elicited immune stimulation with respect to gene expression and

then extend these concepts to high dose effects that include apoptosis and immune suppression.

Induction of gene transcription

Trichothecene-mediated elevations in cytokines, chemokines and other immune related proteins are preceded by upregulation of the respective mRNAs for these genes. Investigation of DON induction of COX-2 illustrates the underlying mechanisms for increased mRNA expression. The enzyme COX-2 catalyzes oxygenation of arachidonic acid to prostaglandin endoperoxides which are subsequently converted enzymatically into prostaglandins and thromboxane A₂ (Smith *et al.* 2000). Resembling an early response gene, COX-2 is strongly induced by mitogenic and proinflammatory stimuli, and superinduced by protein synthesis inhibitors. We have observed induction of COX-2 gene expression in the macrophage and the mouse as well as enhanced the production of prostaglandin E₂ (PGE₂) in vitro (Moon and Pestka 2002). COX-2 promoter-reporter constructs were used to demonstrate that DON induces transcription of this gene in the macrophage. Although DON and other Type B trichothecenes cause COX-2 gene transactivation at equitoxic levels, representative Type A (diacetoxyscirpenol and acetyl T-2 toxin) and Type D (satratoxin F and roridin A) trichothecenes do not (Moon and Pestka 2003b). Consistent with COX-2 findings, DON elicits transcription of TNF- α and IL-6 in macrophages (Chung *et al.* 2003b; Jia *et al.* 2006), IL-8 in monocytes (Islam *et al.* 2006; Gray and Pestka, 2007, in press) and IL-2 expression in T cells (Li *et al.* 1997e).

The effects of DON have also been assessed on the transcription factors NF- κ B, AP-1 and C/EBP β , which have binding sites in the promoters of numerous immune- and inflammation-related genes. Incubation of macrophages with 100 and 250 ng/ml of DON results in increased AP-1 and C/EBP binding in nuclear extracts after 2 and 8 hrs and increased NF- κ B binding after 8 hr (Wong *et al.*, 2002). DON also increases NF- κ B/Rel binding activity, in particular the transactivating forms, c-Rel and p65, in EL-4 and primary T cells (Ouyang *et al.* 1996b; Ouyang *et al.* 1996a) and U937 monocytes (Gray and Pestka, 2007, in press). DON increases AP-1 binding activity in a concentration- and time-dependent manner in EL-4 T cells (Li *et al.* 2000a; Li *et al.* 2000b) and primary macrophages (Jia *et al.* 2006). Both reporter studies and increased transcription factor binding are consistent with trichothecene-induced gene transcription.

Enhanced mRNA stability

DON is also able to stabilize COX-2 mRNA (Moon and Pestka 2002). mRNA stabilization is often explained by the presence of multiple copies of AUUUA motif in the 3'-untranslated region (3'-UTR) of a target mRNA, which targets a transcript for rapid degradation (Dixon *et al.* 2000). [Involvement of the COX-2 3'-UTR in DON-induced COX-2 expression was tested using a constitutive luciferase reporter gene with three different 3'-UTR sections (1/1558, 1/338 and 339/1558 from coding terminal sequence) of the COX-2 gene (Moon *et al.* 2003; Moon and Pestka 2003b). Reporter-expressing cells were treated with DON and increased luciferase was measured from cells containing reporter constructs with the COX-2 AU rich region. DON is thus likely to enhance COX-2 mRNA stability via AU-rich elements. Similarly, other COX-2-inducing Type B trichothecenes increased levels of reporter via the 3'-UTR; however, representative Type A and D trichothecenes had no marked effect at equitoxic concentrations. Accordingly, AU rich repeats in the 3'-UTR are likely to be involved in mRNA stabilization by COX-2-inducing Type B trichothecenes].

As found for COX-2, DON can stabilize mRNAs for TNF- α (Chung *et al.* 2003b; Chung *et al.* 2003a) and IL-6 (Jia *et al.* 2006) in macrophages as well as IL-2 in EL-4 T cells, (Li *et*

al. 1997a; Li *et al.* 1997b; Li *et al.* 1997c; Li *et al.* 1997d; Li *et al.* 1997e). Contrastingly, mRNA stabilization does not play a role in DON-induced IL-8 in monocytes (Gray and Pestka, 2007, in press).

Mitogen-activated protein kinase activation

Since the ribosome is the primary molecular target for trichothecenes, translational inhibition is an obvious mechanism of toxicity (Ueno 1984). Trichothecenes and other ribosome-binding translational inhibitors, however, can also rapidly activate mitogen-activated protein kinases (MAPKs) via a process termed the “ribotoxic stress response” (Iordanov *et al.* 1997; Laskin *et al.* 2002). These kinases modulate numerous physiological processes including cell growth, differentiation and apoptosis (Cobb 1999) and are crucial for signal transduction in the immune response (Dong *et al.* 2002). The primary MAPK subfamilies are (i) p44 and p42 MAPKs, also known as extracellular signal regulated protein kinase 1 and 2 (ERK1 and 2); (ii) p54 and p46 c-Jun N-terminal kinase 1 and 2 (JNK 1/2) and (iii) p38 MAPK (Cobb 1999; Schaeffer and Weber 1999; Widmann *et al.* 1999).

The capacity of DON and other trichothecenes to activate JNK, ERK and p38 *in vitro* (Moon and Pestka 2002; Shifrin and Anderson 1999; Yang *et al.* 2000; Zhou and Pestka 2003) and *in vivo* (Zhou *et al.* 2003a) led us to hypothesize that immune dysregulation and perhaps other toxic manifestations of these mycotoxins are regulated by the ribotoxic stress response. Indeed, ERK and p38 are involved in trichothecene-induced transactivation of TNF- α and COX-2, while p38 contributes to trichothecene-mediated mRNA stability (Chung *et al.* 2003b; Moon and Pestka 2002; Moon and Pestka 2003a; Moon *et al.* 2003). Similar findings have been made for IL-6 and IL-8 in primary human mononuclear blood cell cultures (Islam *et al.* 2006).

Integrated *in vivo* model for trichothecene-induced gene expression

Proinflammatory cytokine mRNA expression is induced within lymphoid tissue *in vivo* by DON in a rapid (1–2 hr) and transient (4–8 hr) fashion (Azcona-Olivera *et al.* 1995a; Zhou *et al.* 1997; Zhou *et al.* 1998; Zhou *et al.* 1999) which suggests that relevant MAPKs and transcription factors associated with cytokine expression are activated prior. To test this possibility, we treated mice with a single oral dose of DON and then their spleens were analyzed for MAPK phosphorylation and transcription factor activation over time (Zhou *et al.* 2003a). As little as 1 mg/kg bw of DON induced JNK 1/2, ERK 1/2, and p38 phosphorylation with maximal effects being observed at 5 to 100 mg/kg bw. JNK and p38 phosphorylation were also transiently induced over a 60 min time period with peak effects being observed at 15 and 30 min, respectively, whereas ERK remained phosphorylated between 15 to 120 min. MAPK phosphorylation kinetics were consistent with the absorption and clearance of DON in the mouse, which peaks at 30 min in most tissues (Azcona-Olivera *et al.* 1995c; Azcona-Olivera *et al.* 1995a; Azcona-Olivera *et al.* 1995b). Regarding transcription factors assessed at 25 mg/kg bw DON, AP-1 binding activity was elevated from 0.5 h to 8 h, whereas C/EBP binding was elevated only at 0.5 h. CREB binding decreased slightly at 0.5 h but gradually increases, reaching a maximum at 4 h. NF- κ B binding increased slightly at 4 h and 8 h. TNF- α , IL-1 β and IL-6 mRNA were found to peak at 3 h and were still significantly elevated at 6 h but not 9 h. These *in vivo* data suggest that DON induces MAPK activation and then, either concurrently or subsequently, activates transcription factors specific for regulatory motifs in cytokine promoters. The kinetics of these events are highly consistent with downstream proinflammatory gene expression in the spleens of mice exposed to DON (Azcona-Olivera *et al.* 1995a; Zhou *et al.* 1997; Zhou *et al.* 1998; Zhou *et al.* 1999).

Upstream transducers of MAPKs

Since DON and other trichothecenes activate MAPKs, the next logical investigation was to identify possible upstream transducers. Using chemical inhibitors of potential upstream kinases, we identified two putative upstream kinases - double-stranded RNA-(dsRNA)-activated protein kinase (PKR) and hemoitopoeitic cell kinase (Hck).

PKR is a widely-expressed serine/threonine protein kinase that is activated by dsRNA, interferon and other agents (Williams 2001). PKR's first identified roles were translational inhibition via phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) - an evolutionarily conserved antiviral response. In addition to eIF2 α phosphorylation and auto-phosphorylation activities, PKR has a wide serine-threonine kinase substrate specificity. PKR might also act through protein-protein interactions which do not necessitate catalytic action. PKR acts as a signal integrator for ligand-activated stress-activated protein kinase pathways leading to stimulation of JNK and p38. This kinase is also thought to mediate apoptosis induced by dsRNA, LPS and TNF- α (Der *et al.* 1997; Yeung *et al.* 1996). PKR potentially modulates induction of cytokines including TNF- α (Meusel *et al.* 2002), IL-6 and IL-12 (Goh *et al.* 2000).

In macrophages, PKR is activated within 1 to 5 min as evidenced by autophosphorylation and by phosphorylation of eIF2 α , which is rapidly then degraded (Zhou *et al.* 2003b). Both antisense PKR expression vector and PKR inhibitors suppress induction of JNK, p38 and ERK phosphorylation by DON, emetine and anisomycin. These data indicate that PKR plays a critical upstream role in the ribotoxic stress response inducible by translational inhibitors. PKR suppression also inhibits induction of TNF- α and MIP-2 by DON (Pestka *et al.* 2004) as well as IL-8 (Gray and Pestka, 2007, in press). Therefore, PKR might mediate early events leading to immunotoxicity associated with leukocyte exposure to DON and other trichothecenes and, furthermore, might be an early target for the ribotoxic stress response.

Hck belongs to the highly conserved Src oncogene family of cytoplasmic protein tyrosine kinases and is specifically expressed in myelomonocytic cell lineages (Tsygankov 2003). Hck transduces extracellular signals that modulate cellular processes involving proliferation, differentiation and migration (Ernst *et al.* 2002). Src-family-selective tyrosine kinase inhibitors effectively suppress DON-induced activation of MAPKs, which suggests Hck involvement (Zhou *et al.* 2005b). As expected for a signaling event upstream of MAPK activation, tyrosine phosphorylation of Hck is detectable as early as 1 min, maximized at 2.5 min then declines within 30 min after DON addition. Specific pharmacologic inhibition of Hck ablates DON-induced phosphorylation of c-jun, ATF-2 and p90^{Rsk}, which are substrates of JNK, p38 or ERK, respectively (Pestka and Zhou 2003). Hck inhibition also suppresses DON-induced increases in nuclear levels and binding activities of several transcription factors (NF- κ B, AP-1 and C/EBP), which correspond to decreased TNF- α production, caspase-3 activation and apoptosis. Knockdown of Hck expression with small interfering RNAs confirmed involvement of this Src in DON-induced TNF- α production and caspase activation. Taken together, activation of Hck and possibly other Src family tyrosine kinases is likely to be another critical signal that precedes both MAPK activation and induction of resultant downstream sequelae by DON and other ribotoxic stressors.

Ribosome-kinase interactions

Recently, we assessed the capacity of PKR, Hck and ERK to interact with ribosomes (Bae and Pestka 2006). Sequence analysis revealed that many ribosomal proteins subunits contain putative kinase docking sites. Indeed, extremely rapid association and/or activation of PKR, Hck, p38 and ERK are detectable in ribosomes of DON-treated macrophages. Earliest

activation of kinases (1–3 min) was detectable in the monosome fraction with a subsequent localization to the polysome fraction (3–5 min). Other kinases such PDK1, AKT and p70 RSK1, also bound to ribosomes. These observations suggest that, following DON interaction, the ribosome might function as signal transducer and/or scaffold, thereby mediating activation and binding of several protein kinases, some of which are upstream to the MAPKs (Fig. 2). These interactions are very likely to impact transcriptional and post-transcriptional regulation of stress- and immune-related genes.

Upstream transducers of trichothecene-induced apoptosis

Trichothecene-mediated apoptosis also correlates closely with activation of all three MAPK families in RAW 264.7 macrophage and U937 monocyte models suggesting a possible contributing role for these kinases (Yang *et al.* 2000). The capacity of individual trichothecenes to promote MAPK phosphorylation correlates with protein synthesis inhibition and precedes apoptosis. p38 inhibition inhibits DON- induced apoptosis, whereas ERK inhibition enhances apoptosis. Accordingly, p38 and ERK might upregulate and downregulate trichothecene-induced apoptosis, respectively.

When upstream signaling transduction mechanisms contributing to DON-mediated apoptosis were investigated in macrophages, PKR and Hck inhibition, additively inhibited DON-induced caspase-3 activity and apoptosis as well as p38, ERK and JNK phosphorylation (Zhou *et al.* 2003b; Zhou *et al.* 2005b). PKR and Hck inhibition also inhibited DON-induced p53 binding activity and subsequent phosphorylation of its substrate p21. The capacity of MAPKs to mediate both apoptosis and survival was further assessed by Zhou *et al.* (Zhou *et al.* 2005a) in DON-exposed macrophages. At concentrations which partially inhibit translation, DON induced phosphorylation of p38 and ERK 1/2 mitogen activated protein kinases within 15 min in macrophages and these effects lasted up to 3 h. DON-exposed cells exhibited marked caspase 3-dependent DNA fragmentation after 6 h which was suppressed and attenuated by the p38 inhibition and ERK inhibition, respectively. DON readily induced the phosphorylation and activity of p53 which was suppressed by p38 inhibition. DON exposure elicited BAX translocation to mitochondria and corresponding cytochrome C release but did not alter mitochondrial membrane potential. The p53 inhibitor PFT α reduced both DON-induced phosphorylation of p53 and p53 binding activity. Moreover, both PFT α and p53 siRNA transfection suppressed DON-induced caspase-3 activity and subsequent DNA fragmentation. Concurrent with p53 activation, DON activated two anti-apoptotic survival pathways as evidenced by both ERK-dependent activation of both p90 Rsk and AKT. Taken together, the results indicate that DON-induced ribotoxic stress response initiates competing apoptotic (p38→p53→Bax→Mitochondria→ Caspase-3) and survival (ERK→AKT/p90Rsk→Bad) pathways in the macrophages (Fig. 2).

Summary

Leukocytes are central targets of DON and other trichothecenes, which can be immunostimulatory and immunosuppressive depending on exposure regimen and functional immunologic endpoint. Low dose DON exposure transcriptionally and post-transcriptionally upregulates expression of cytokines, chemokines and inflammatory genes with concurrent immune stimulation. High dose DON exposure promotes leukocyte apoptosis with concomitant immune suppression. MAPKs, in particular p38, are important transducers of downstream signaling events related to trichothecene-induced immune stimulation and apoptosis. Inhibitor and gene silencing studies have revealed that Hck and PKR play upstream roles in DON-induced MAPK phosphorylation and subsequent induction of gene expression and apoptosis.. A summary of these interactions is provided in Fig. 3. It is

tempting to speculate that p38 and ERK act as molecular rheostats and define response. When ERK and p38 are activated, immune gene expression and antiapoptotic pathways are favored. When only p38 is activated, an apoptotic response ensues. Future studies should focus on (1) relating the magnitude and duration of trichothecene-induced activation of MAPKs to immune and apoptotic gene expression, (2) understanding the molecular linkages of PKR, Hck and other kinases to the ribosomes and (3) uncovering the exact mechanisms of interaction among upstream kinases and MAPKs as well as downstream mediators of trichothecene-induced gene expression and apoptosis. Ultimately, paradigms derived from such basic studies must be evaluated in the context of human leukocytes as a function of toxin distribution/metabolism, genetic polymorphisms, nutrition, environmental chemical exposure, microbial infection and ongoing inflammatory responses.

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<i>Trichothecene</i>	<i>R</i> ₁	<i>R</i> ₂	<i>R</i> ₃	<i>R</i> ₄	<i>R</i> ₅	<i>IC</i> ₂₀ (ng/ml)
<u>Type A</u>						
T-2	OH	OAc	OAc	H	OCOCH ₂ CH(CH ₃) ₂	1.5
HT-2	OH	OH	OAc	H	OCOCH ₂ CH(CH ₃) ₂	2.3
Diacetoxyscirpenol	OH	OAc	OAc	H	H	4.6
Acetyl T-2	OAc	OAc	OAc	H	OCOCH ₂ CH(CH ₃) ₂	20
3'-OH T-2	OH	OAc	OAc	H	OCOCH ₂ C(OH)(CH ₃) ₂	18
3'-OH HT-2	OH	OH	OAc	H	OCOCH ₂ C(OH)(CH ₃) ₂	7.5
<u>Type B</u>						
Deoxynivalenol (DON)	OH	H	OH	OH	=O	225
Fusarenon-X	OH	OAc	OH	OH	=O	208
Nivalenol (NIV)	OH	OH	OH	OH	=O	150
15-acetyl DON	OH	H	OAc	OH	=O	160
3-acetyl DON	OAc	H	OH	OH	=O	1500
4,15-diacetyl NIV	OH	OAc	OAc	OH	=O	52
<u>Type D</u>						
Verrucarin	H	-O-R'-O-	H	H		13
Satratoxin G	H	-O-R'-O-	H	H		0.6
Satratoxin H	H	-O-R'-O-	H	H		0.9
Satratoxin F	H	-O-R'-O-	H	H		3.1
Roridin A	H	-O-R'-O-	H	H		1.8
<u>OAc : -OCOCH₃</u>						
R' : macrocyclic ester or ester-ether bridge at the R ₂ and R ₃ positions						

Figure 1.

Structures of common trichothecenes and comparative toxicity in vitro. IC₂₀ was defined as the concentration at which 20% of proliferation of cloned murine macrophages was inhibited by each toxin. Modified from Moon et al. (2003).

Figure 2.

Mechanisms leading to DON-induced immune dysregulation. DON induces p38 and ERK MAPKs via PKR and HCK. MAPK activation drives increased proinflammatory gene expression or of proinflammatory cytokines might include anorexia, reduced weight gain, immune stimulation, tissue injury and apoptosis.

Figure 3.

Depiction of DON-induced ribotoxic stress response. Binding of DON causes conformational change and/or damage to ribosome thereby driving protein kinase mobilization, phosphorylation and activation. The ensuing intracellular kinase cascades direct gene expression and/or apoptosis.