Role of Double-Stranded RNA-Activated Protein Kinase R (PKR) in Deoxynivalenol-Induced Ribotoxic Stress Response

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Trichothecene mycotoxins and other protein synthesis inhibitors activate mitogen-activated protein kinase (MAPKs) via a mechanism that has been termed the “ribotoxic stress response.” MAPKs are believed to mediate the leukocyte apoptosis that is observed following experimental exposure to these chemical agents in vitro and in vivo. The purpose of this research was to test the hypothesis that double-stranded, RNA-activated protein kinase R (PKR) is a critical upstream mediator of the ribotoxic stress response induced by the trichothecene deoxynivalenol (DON) and other translational inhibitors. DON was found to readily induce phosphorylation of JNK 1/2, ERK 1/2, and p38 in the murine macrophage RAW 264.7 cell line, within 5 min of culture addition, in a concentration-dependent fashion. Effects were maximal from 15 to 30 min and lasted up to 6 h. The translational inhibitors anisomycin and emetine also had similar effects when added to cultures at equipotent concentrations to DON. DON rapidly activated PKR within 1 to 5 min, as evidenced by autophosphorylation and by phosphorylation of eukaryotic initiation factor 2α (eIF2α). Interestingly, the latter effect was associated with rapid degradation of eIF2α. Pretreatment of RAW 264.7 cells with two inhibitors of PKR, 2-aminopurine (2-AP) or adenine (Ad), markedly impaired MAPK phosphorylation in RAW 264.7 cells according to the following rank order JNK > p38 > ERK. The capacity of DON to induce MAPK phosphorylation was also markedly suppressed in a stable transformant of the human promonocytic U-937 cell line containing an antisense PKR expression vector. This suppression followed a rank order of JNK > p38 > ERK in this PKR-deficient cell line when compared to control cells transfected with vector only. Apoptosis induction by DON and two other translational inhibitors, anisomycin and emetine, was almost completely abrogated in PKR-deficient cells. Together, the results indicate that PKR plays a critical upstream role in the ribotoxic stress response inducible by translational inhibitors.

Key Words: trichothecene; mycotoxin; mitogen-activated protein kinase; apoptosis; macrophage.

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Trichothecenes are sesquiterpenoid mycotoxins that contaminate agricultural commodities (Scott, 1990) and indoor air environments (Cooley et al., 1998). These toxins are of concern because of their documented adverse effects on human and animal health (Bhat, 1989; Etzel et al., 1998; Li et al., 1999, 2002). Trichothecenes can be immunostimulatory and immunosuppressive, depending on dose, exposure frequency, and timing of functional immune assay (Bondy and Pestka, 2000; Laskin et al., 2002). Macrophages, T cells, and B cells are all highly sensitive to trichothecenes and it is believed that induction of apoptosis by these toxins mediates immune suppression whereas induction of cytokines contributes to immune stimulation. Deoxynivalenol (DON, or vomitoxin) is the most frequently encountered trichothecene in grain-based foods worldwide, and thus its capacity to impact immune function is of particular interest (Rotter et al., 1996).

The molecular target of trichothecenes in leukocytes and other actively proliferating eukaryotic cells is the 60s ribosomal subunit (Middlebrook and Leatherman, 1989a,b; Witt and Pestka, 1990). Translational inhibitors that bind to ribosomes rapidly activate mitogen-activated protein kinases (MAPKs) and apoptosis in a process that has been termed the “ribotoxic stress response” (Iordanov et al., 1997). MAPKs impact many physiological processes, including cell growth, differentiation, and apoptosis (Cobb, 1999), and are important transducers of the immune response (Dong et al., 2002). The primary MAPK subfamilies include: (1) p44 and p42 MAPKs, also known as extracellular signal-regulated protein kinase 1 and 2 (ERK1 and 2), (2) p54 and p46 c-Jun N-terminal kinase 1 and 2 (JNK1/2), also referred to as stress-activated protein kinases (SAPK 1/2); and (3) p38 MAPK. Both our laboratory and others have demonstrated that DON and other trichothecenes activate JNK, ERK, and p38 in vitro (Moon et al., 2002; Shifrin and Anderson, 1999; Yang et al., 2000a) and in vivo (Zhou et al., 2003), suggesting that the ribotoxic stress response might be a critical transduction step during trichothecene toxicity. A major unresolved question regarding the ribotoxic stress response relates to the molecular linkage between ribosome interaction and MAPK activation (Laskin et al., 2002).
Double-stranded RNA-(dsRNA)-activated protein kinase (PKR) is a widely-expressed serine/threonine protein kinase that can be activated by dsRNA, interferon, and other agents (Williams et al., 2001). The earliest described role of PKR was translational inhibition via phosphorylation of eukaryotic initiation factor 2α (eIF2α), which is an evolutionarily conserved antiviral response. Besides eIF2α phosphorylation and auto-phosphorylation activities, PKR appears to have a wide serine-threonine kinase substrate specificity. This kinase might also act through protein–protein interactions, which do not require catalytic activity. PKR reportedly functions as a signal integrator for ligand-activated, stress-activated protein kinase pathways leading to stimulation of JNK and p38 (Goh et al., 2000; Takizawa et al., 2002; Williams, 2001). The kinase is also believed to play a key role in mediating apoptosis induced by dsRNA, LPS, and TNF-α (Der et al., 1997; Gil and Esteben, 2000; Yeung and Lau, 1998; 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). Our laboratory has previously observed that DON downregulates expression of P58(kt), which is the 58-kDa cellular inhibitor of PKR (Yang et al., 2000b). Thus, the potential exists for PKR to mediate early events leading to immunotoxicity associated with leukocyte exposure to DON and other trichothecenes and, additionally, to be an early step in the ribotoxic stress response.

The purpose of this study was to test the hypothesis that PKR is a critical upstream mediator of MAPK activation and apoptosis during the ribotoxic stress response. The results suggest that PKR can mediate activation of JNK, ERK, and p38 by DON and two other translational inhibitors, anisomycin and emetine. Furthermore, PKR contributes to the subsequent induction of apoptosis by these chemical agents.

**MATERIALS AND METHODS**

**Cell cultures.** RAW 264.7 murine macrophage cells (American Type Culture Collection, Rockville, MD) (2.5 × 10⁵ per ml) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (H-FBS, Atlanta Biologicals, Inc, Norcross, GA), 100 U/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma, St. Louis, MO) in a 5% CO₂ humidiﬁed incubator at 37°C. Macrophage cell number and viability were assessed by trypan blue (Sigma) dye exclusion using a hematocytometer. For DON-exposure studies, cells (5 × 10⁵/ml) were seeded in 10 ml of medium in 100 cm² sterile tissue culture dishes overnight to achieve 80% confluence.

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U-937 human monocyte-like cells were stably transformed with plasmids containing a parental control expression vector pRC-CMV (Invitrogen, Carlsbad, CA) or a vector constructed with anti-sense PKR and designated as U9K-C2 and U9K-A1, respectively, as described previously (Der and Lau, 1995). Cells were maintained in RPMI 1640 medium supplemented with 5% (v/v) FBS, and 400 μg/ml Geneticin ( Gibco, Rockville, MD) in 6% CO₂ at 37°C.

Cells were treated with DON, anisomycin, or emetine (Sigma) over speciﬁed time intervals in the absence or presence of various pharmacological inhibitors, and then were analyzed for MAPK phosphorylation, PKR activation, DNA fragmentation, and/or caspase activity.

**Detection of MAPK phosphorylation.** Cells were washed with ice-cold phosphate buffer, lysed in boiling lysis buffer (1% [w/v] sodium dodecysul-
EDTA. After electrophoresis, the gel was stained with ethidium bromide (0.5 g/ml), and the nucleic acids were visualized with UV transilluminator. A 100-bp DNA ladder (GIBCO) was used as a molecular size marker.

Caspase-3 assay. Cells were suspended in 200 l of CHAPS buffer (100 mM HEPES [pH 7.5] containing 10% (w/v) sucrose, 0.5% (w/v) CHAPS, 1mM EDTA, 10 mM DTT, and 100 l protease inhibitor cocktail (1:100) (Sigma), placed on ice for 30 min, sonicated briefly, and then centrifuged 10,000 g for 10 min. Following protein determination, lysates (50 g CHAPS buffer) were incubated with an equal volume (100 l) of fluorogenic substrate consisting of 25 l DEVD-AMC (Calbiochem, San Diego, CA) dissolved in CHAPS buffer at 37°C for 30 min. Substrate cleavage was detected by a CytoFluor II Microplate fluorescence reader (Biosearch, Bedford, MA) at excitation 360 nm and emission 460 nm.

RESULTS

DON and Other Translational Inhibitors Induce JNK 1/2, ERK 1/2, and p38 MAPK phosphorylation in RAW 264.7 Cells

Exposure to DON at concentrations as low as 100 ng/ml for 15 min induced phosphorylation of JNK 1/2, ERK 1/2, and p38 in RAW 264.7 cells (Fig. 1). Phosphorylation of the three MAPKs increased with increasing DON concentrations. Maximum phosphorylation was observed at 500 ng/ml DON or higher. When the kinetics of MAPK phosphorylation was assessed over a 4-h time span using 250 ng/ml of DON, JNK 1/2, ERK 1/2, and p38, phosphorylation was first observed at 5 min (Fig. 2). JNK 1/2 phosphorylation was maximal from 10 to 30 min and last detectable at 360 min. ERK 1/2 phosphorylation was maximal at 30 to 240 min and last observed at 360 min. Finally, maximal p38 phosphorylation occurred between 15 and 240 min.

The capacity of DON to inhibit leucine incorporation in RAW 264.7 cells was compared to that for the translational inhibitors anisomycin and emetine. The concentrations required to inhibit activity by 50% (IC50) were 26, 85, and 24 ng/ml for DON, emetine, and anisomycin, respectively (Fig. 3A). Since the IC50 for DON was effective at activating MAPKs, the effect of equipotent concentrations of anisomycin and emetine on MAPK phosphorylation was measured. As found for DON, markedly increased JNK, ERK, and p38 phosphorylation was observed in anisomycin-treated cultures at 15, 30, and 60 min (Fig. 3B). Emetine also induced phosphorylation of the three MAPKs, but to a much lesser extent.

DON Induces PKR Activity in RAW 264.7 Cells

The effects of DON on PKR activity were evaluated. PKR has been characterized by its capacities for autophosphorylation and to catalyze phosphorylation of eIF2α (Williams 2001). DON at 250 ng/ml induced PKR phosphorylation from 1 to 5 min (Fig. 4). DON, at the same concentration, also rapidly (1–2.5 min) induced eIF2α phosphorylation, although the end product rapidly decreased to levels below that for control (Fig. 5). The simultaneous disappearance of total eIF2α suggested that this protein was being degraded as a result of DON exposure. Inclusion of a phosphatase inhibitor prolonged the eIF2α phosphorylation response to DON as well as preventing protein degradation. Incorporation of a protease inhibitor prolonged DON-induced eIF2α phosphorylation and also pre-
vented eIF2α degradation. Overall, these results suggest that DON might rapidly induce PKR activation in RAW 264.7 cells as evidenced by its own phosphorylation, as well as eIF2α phosphorylation. DON subsequently mediated a phosphatase-dependent proteolytic degradation of this latter important translation component. Importantly, the observation that the timing of PKR activation preceded phosphorylation of MAPKs is consistent with an upstream role for PKR.

PKR Mediates DON-Induced MAPK Phosphorylation in RAW 264.7 Cells

To assess the potential role of PKR in DON-induced MAPK phosphorylation, the effect of pretreating cells with adenine (Ad) or 2-aminopurine (2-AP), two previously described PKR inhibitors (Wong and Yen, 1998), were assessed. The two compounds inhibited DON-induced JNK 1/2 phosphorylation in a concentration-dependent fashion (Fig. 6). ERK and p38 phosphorylation were slightly inhibited by 2-AP and a similar weaker trend was observed for Ad. These data suggest that PKR activation might contribute, in part, to DON-induced activation of JNK and, to a much lesser extent, the other two MAPK families.
PKR Mediates Induction of MAPK Phosphorylation by DON and Anisomycin in U-937 Cells

Fundamental limitations with inhibitor studies are the potential for the pharmacologic agents to be metabolized, to exert toxicity, or to be promiscuous with regard to their action. Therefore, we assessed the role of PKR by employing human U-937 monocyte cell lines that were stably transfected with either PKR antisense vector (U9K-A1) or control vector (U9K-C2) and that were thoroughly described previously (Der and Lau, 1995; Yeung and Lau, 1998; Yeung et al., 1996, 1999). As found in the RAW 264.7 cells, JNK 1/2 and p38 phosphorylation were effectively induced in U9K-C2 cells at DON concentrations of 100 to 1000 ng/ml, whereas ERK 2 was selectively activated (Fig. 7). However, JNK 1/2 phosphorylation was nearly completely abrogated in the PKR-deficient U9K-A1 cells with only a trace amount of phosphorylated JNK 1/2 being detectable at the 500 and 1000 ng/ml DON concentrations. To a lesser extent, PKR deficiency also partially impaired ERK 2 and p38 phosphorylation effects that were most apparent at low DON concentrations (100–250 ng/ml).

When the kinetics of DON-induced MAPK phosphorylation was assessed in U9K-C2 cells, effects were detectable from 15–240 min (Fig. 8). In U9K-A1 cells, both DON-induced JNK 1/2 and ERK 1/2 phosphorylation were detectable only from 30 to 120 min and the magnitude of this response was reduced. The time window for DON-induced p38 phosphorylation in PKR-deficient cells was not decreased; however, the magnitude of the phosphorylation was depressed. Similar MAPK phosphorylation kinetics were observed for anisomycin (Fig. 9). Overall, these results suggested that PKR was involved upstream, in part, with both DON- and anisomycin-induced JNK 1/2 phosphorylation and to a much lesser extent, with p38 and ERK 1/2 phosphorylation.

PKR Mediates Induction of Apoptosis by DON and Other Translational Inhibitors in U-937 Cells

The capacity of trichothecenes and other translational inhibitors to induce MAPKs has been previously linked to apoptosis (Shifrin and Anderson, 1999; Yang et al., 2000a). Here, DON was found to induce DNA fragmentation in
U9K-C2 cells, whereas the PKR deficient U9K-A1 cells were remarkably recalcitrant to these effects (Fig. 10). PKR-deficient cells were also resistant to apoptosis induction by anisomycin and emetine. Consistent with fragmentation data, DON, anisomycin, and emetine readily activated caspase-3 in U9K-C2 cells, whereas PKR-deficient cells did not appreciably activate this enzyme (Fig. 11). Thus, in the U937 model, PKR appeared to be required for apoptosis induction by translational inhibitors.

**DISCUSSION**

The data presented here provide evidence, for the first time, that activation of PKR by DON contributes to downstream activation of MAPK cascades and, ultimately, to apoptosis. The capacity of DON and other translational inhibitors to activate PKR suggests that this class of chemicals can be added to a growing list of agents which activate this kinase and already includes dsRNA, interferon, LPS, cytokines, growth factors, and stress signals (Williams et al., 1999). The mechanisms by which DON activates PKR are unclear but, as suggested by kinetic data, appear to precede MAPK phosphorylation. Trichothecenes are not known to bind to a membrane-specific receptor but, rather, diffuse freely into the cell and bind to eukaryotic ribosomes (Middlebrook and Leatherman, 1989a,b; Witt and Pestka, 1990). PKR is found in the endoplasmic reticulum at high concentrations and associates with ribosomes via specific recognition sites (Wu et al., 1998). Thus, one putative model would be for a DON or other translation inhibitor to first bind ribosomes and then, in turn, transduce a signal via structural modification or kinase event to a closely associated PKR molecule (Fig. 12).

An alternate explanation for PKR activation is that translational inhibition, per se, results in production of RNA polymerase III-(Pol III) directed transcripts of short, highly repetitive retro-transposed sequences (SINEs) that are analogous to human Alu sequences and can potentially drive PKR activation. In support of this possibility, Liu et al. (1995) found that cycloheximide induces SINE transcripts in mouse 3R3 cells. Furthermore, cellular exposure to different stresses, including translation inhibition, increases the abundance of human Alu RNAs that can form stable complexes with PKR (Chu et al., 1998). Williams et al. (1999) reported that, at low concentrations, Alu RNAs are efficient activators of PKR.

Recently, convincing evidence for at least one clear link between PKR and MAPK activation has been established (Takizawa et al., 2002). Specifically, a novel interaction was observed between PKR and apoptosis signal-regulating kinase (ASK1), a member of the MAPK kinase family. These conclusions were based on (1) colocalization and communoprecipitation of PKR and ASK1; (2) ASK1-mediated p38 phosphorylation in PKR deficient cells; and (3) reduced ASK-1 phosphorylation in PKR-deficient cells treated with poly (I)-poly (c), the classic activator of PKR. Further studies using these approaches could be used to better define the transduction mechanisms between PKR and MAPK during the ribotoxic stress response as well as the potential for cross-talk.
Trichothecene-induced apoptosis has been previously suggested to involve activation of JNK 1/2 and p38 (Yang et al., 2000a). Notably, DON-dependent phosphorylation of JNK might almost entirely be mediated through PKR, whereas DON-dependent phosphorylation of ERK and p38 were only partially mediated through PKR. The data presented here suggest that PKR activation may be upstream of this process. PKR-mediated apoptosis is driven by a large number of stimuli including virus infection, dsRNA, LPS, TNF-α, serum depletion, and cytokine withdrawal (Gil and Esteban, 2000). Furthermore, fibroblasts containing homozygous deletions in the PKR gene are resistant to apoptotic cell death in response to ds-RNA, TNF-α or LPS (Der et al., 1997). Although, PKR upregulates several apoptosis-related genes including Fas, Bax, TNF-α, and p53, and is known to involve the FADD-caspase 8 pathway, the precise mechanisms are not yet fully understood (Balachandran et al., 2000; Yeung et al., 1996).

PKR-mediated activation of JNK, p38, and ERK may also contribute to DON-induced upregulation of cytokines, chemokines, and cyclooxygenase-2 that have been observed previously in RAW 264.7 and U-937 cells (Ji et al., 1998; Moon and Pestka, 2002; Sugita-Konishi and Pestka, 2001; Wong et al., 1998; Zhou et al., 1997). A major consequence of MAPK phosphorylation is the activation of transcription factors (Cobb, 1999), which serve as immediate or downstream substrates of these kinases. For example, JNK 1/2 phosphorylates c-Jun, which is a component of AP-1 homodimer or heterodimer complexes (Dong et al., 2002). Also, p38 (Bhat et al., 2002) and ERK 1/2 (Hungness et al., 2002) drive activation of C/EBP. Phosphorylation/activation of CREB/ATF members is mediated by JNK (Dong et al., 2002), p38 (Bhat et al., 2002), and ERK (Belmonte et al., 2001). Finally, all three MAPK signaling pathways have been implicated in nuclear factor κB (NF-κB) activation through phosphorylation of its inhibitor IκBα (Alpert et al., 1999; Lee et al., 1998; Zhao and Lee, 1999). PKR-driven MAPK activation and subsequent activation of the aforementioned transcription factors, as observed in vitro and in vivo following DON treatment (Wong, 2002; Zhou et al., 2003), might likely play roles in these processes. Thus, it will be of interest to further define how PKR contributes to DON induction of proinflammatory genes.

Taken together, the data presented herein indicate that PKR...
is a transducer of the ribotoxic stress response that is initiated by trichothecenes and other translational inhibitors, and that evokes MAPK activation and culminates in apoptosis. Additional research is needed to discern the linkage between ribosomal binding by translational inhibitors and PKR activation as well as to understand how PKR mediates phosphorylation of the major MAPK families. In addition, there is need to examine whether these compounds can induce parallel signaling pathways that converge with PKR-directed pathways and, ultimately, generate diverse immunotoxic effects.

**FIG. 10.** PKR deficiency impairs induction of DNA fragmentation by translational inhibitors. DNA fragmentation agarose gel electrophoresis was conducted on DNA isolated from lysates control (U9K-C2) and PKR-deficient (U9K-A1) cells after 6 h of exposure to DON: 0 (lanes 1, 5), 100 (lanes 2, 6), 250 (lanes 3, 7) and 500 (lanes 4, 8) ng/ml; anisomycin 0 (lanes 1, 5), 20 (lanes 2, 6), 50 (lanes 3, 7) and 100 (lanes 4, 8) ng/ml; and emetine, 0 (lanes 1, 5), 40 (lanes 2, 6), 100 (lanes 3, 7), and 200 (lanes 4, 8) ng/ml. Gel was stained with ethidium bromide and photographed under UV light. Lane M is a 100-bp DNA ladder used for molecular size.

**FIG. 11.** PKR deficiency impairs caspase induction by translational inhibitors. U9K-C2 and U9K-A1 cells (5 × 10⁵/ml) were exposed to DON (250 ng/ml), AN (25 ng/ml), or EM (80 ng/ml) for various time intervals. Cell lysates were prepared and caspase-3 activity was measured.
FIG. 12. Depiction of PKR as transducer of ribotoxic stress response. Putative downstream effects include induction of proinflammatory and apoptotic genes. ER refers to endoplasmic reticulum.

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