Deoxynivalenol Induces p38 Interaction with the Ribosome in Monocytes and Macrophages

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Received March 29, 2008; accepted May 16, 2008

Trichothecene mycotoxins rapidly induce p38-mediated gene expression and apoptosis in mononuclear phagocytes via a process known as the ribotoxic stress response. We hypothesized that the trichothecene deoxynivalenol (DON) induces interaction of p38 with the ribosome. Two models, U937 human monocytes and RAW 264.7 murine macrophages, were used to test this hypothesis based on their capacity to evoke rapid and robust p38 phosphorylation responses to DON. Following DON treatment of U937 cells, lysates were subjected to sucrose gradient fractionation and the resultant ribosomal fractions probed for p38 by Western blotting. p38 content in fractions containing ribosomal subunits and monosomes (RS + M) increased within 5 min of DON treatment and continued to increase up to 30 min. p38 appeared to be initially interact with the 40S subunit fraction and then subsequently with the 60S unit and monosome fractions. Although p38 phosphorylation was blocked by the inhibitor SB203580, interaction of the kinase with the ribosome was unaffected, suggesting that ribosomal binding and phosphorylation were dissociable events. In RAW 264.7 cells, radiolabeled DON uptake occurred within 15 min and this corresponded to sequential increases nonphosphorylated p38 and phosphorylated p38 in the RS + M fraction. As observed for p38, DON similarly induced both ribosomal interaction with two mitogen-activated protein kinases, c-Jun N-terminal kinase, and extracellular signal–regulated kinases (ERKs) in lymphoid tissues of experimental animals (Zhou et al., 2003a) as well as in mononuclear phagocytes and other leukocytes (Moon and Pestka, 2002; Pestka et al., 2005; Shifrin and Anderson, 1999). p38 is widely recognized to be a central mediator of many stress-activated signaling pathways (Chen-Chih et al., 2007). Notably, scaffolding proteins such as osmosensing scaffold for MEKK3 and TAK-1 binding protein 1 enable this kinase to phosphorylate substrates in a selective fashion and thus differentially modulate a variety of cellular functions (Kang et al., 2006; Raman et al., 2007). p38 activation is of particular importance relative to DON-induced gene transcription, messenger RNA (mRNA) stabilization and apoptosis (Chung et al., 2003; Moon and Pestka, 2002; Zhou et al., 2003a).

The general mechanism by which trichothecenes and other translational inhibitors such as ricin, Shiga toxin, and anisomycin induce phosphorylation of p38 and other MAPK has been termed the ribotoxic stress response (Iordanov et al., 1997; Laskin et al., 2002). This response is believed to involve disruption of the 3′-end of large 28S ribosomal RNA (rRNA) which typically functions in aminoacyl-transfer RNA binding, peptidyl transferase activity and ribosomal translocation. Although the ribosome undoubtedly plays some role in MAPK activation by ribotoxins, the nature of interactions occurring among ribosomal proteins rRNA and MAPK is not well understood.
In this study, we employed human monocyte and mouse macrophage cell lines to test the hypothesis that DON induces interaction of p38 with the ribosome. The large size of the ribosome and masking of critical epitopes makes it difficult to apply conventional immunoprecipitation method to identify potential kinase interactions with it. Therefore, we used sucrose density gradient fractionation in conjunction with ribosomal protein subunit markers to track interaction with p38 following DON stimulation. The results indicate that DON sequentially induced mobilization of p38 and other MAPks to the ribosome and their phosphorylation.

**MATERIALS AND METHODS**

**Chemicals.** DON and other chemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless otherwise noted. DON was dissolved in distilled, filter-sterilized water for addition to cell cultures. The p38 kinase inhibitor SB203580 was obtained from Calbiochem (San Diego, CA).

**Cell cultures.** All cultures were maintained at 37°C in a humidified 6% CO₂ incubator. U937 cells (ATCC, Rockville, MD) were grown in RPMI-1640 supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) ( Gibco BRL, Gaithersburg, MD), and 100 μM penicillin and 100 μg/ml streptomycin (Gibco BRL). RAW 264.7 cells (TIB 77, ATCC) were cultured in Dulbecco’s modified Eagles medium (Sigma) supplemented with 10% (vol/vol) heat-inactivated FBS, 1mM sodium pyruvate (Gibco BRL), 100 U/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma). In typical experiments, U937 cells (1 x 10⁶/ml) in 225-cm² cell culture flasks (Corning, Lowell, MA) or RAW 26.4.7 cells (5 x 10⁶/ml) in a 100-mm cell culture dishes (Corning) were cultured overnight, and then treated with DON for various time intervals prior to analysis. DON concentrations of 500 ng/ml (1.68 μM) and 250 ng/ml (0.84 μM) were used for U937 and RAW 26.4.7 cells, respectively, based on optimal effects observed in previous studies (Moon and Pestka, 2002; Zhou et al., 2003b).

**Sucrose density fractionation.** Cytoplasmic fractions were prepared and ribosome fractions isolated as previously described (Galban et al., 2003). Briefly, cells were washed with ice-cold phosphate buffered saline (PBS) twice and lysed in ice-cold polysome extraction buffer lysis buffer consisting of 0.3M sodium sulfate lysis buffer, sonicated, and then subjected to Western analysis as described below.

**RESULTS**

**DON Induces p38 Phosphorylation in U937 Monocytes**

Induction of p38 phosphorylation in U937 monocytes by DON was confirmed by Western analysis of whole cell extracts (Figs. 1A and 1B). Incubation with DON at 500 ng/ml induced marked phosphorylation of p38 after 15 min and this was further increased after 30 min.

**DON Induces p38 Association with the Ribosome in U937 Monocytes**

To determine whether p38 interacts with the ribosome during DON treatment, ribosomal subunits (40S and 60S), monosomes (80S), and polysomes were separated by sucrose gradient fractionation (Fig. 2A). The identity of the fractions...
was verified using anti-RPS6 and anti-RPL7 as markers for 40S and 60S ribosomal subunits, respectively. Fractions were pooled into a ribosomal subunit plus monosome (RS + M) fraction and a polysome (P) fraction. p38 was detectable in RS + M fraction after 5 min and increased in a time-dependent manner (Fig. 2B). p38 was also detectable in the P fraction after 15 and 30 min, but to a lesser extent than in the RS + M fraction.

**p38 Sequentially Associates with 40S and 60S Ribosomal Subunits**

Western blotting was conducted on individual fractions to identify the specific ribosomal components with which p38 interacts. Upon treatment of U937 cells with DON, p38 initially associated with the 40S ribosomal subunit fraction at 5 min (Fig. 3). However, after 15 min, p38 was predominantly found in the 60S and 80S fractions. Thus DON appeared to induce sequential p38 association with the 40S ribosomal subunit followed by the 60S subunit and 80S ribosome.

**p38 Interaction with the Ribosome does not Require prior Phosphorylation in U937 Monocytes**

To ascertain whether p38 required prior phosphorylation to bind the ribosome, cells were preincubated with the p38 kinase inhibitor SB203580 for 45 min before DON treatment. Although the inhibitor effectively suppressed phosphorylation of this kinase, p38 interaction with the ribosome was unaffected in DON-stimulated U937 cells (Fig. 4).

**Kinetics of DON Uptake Corresponds with p38 and Phosphorylation and Ribosome Interaction in RAW 264.7 Macrophages**

The kinetics of DON uptake was related to that for p38 activation in RAW 264.7 macrophages. [3H] DON uptake was rapid with 50 and 75% of maximum binding being observed after 2.5 and 5 min, respectively (Fig. 5A). Cells were saturated with [3H] DON after 15 min. Rapid DON uptake closely corresponded with p38 phosphorylation which also peaked after 15 min (Figs. 5B and 5C). Consistent with U937 cells, DON induced p38 association with the pooled RS + M fractions of RAW 264.7 cells, with marked increases in total p38 being observed in this fraction from 5 to 30 min (Fig. 5D). p38 phosphorylation was also detectable from 5 to 30 min, with peak phospho-p38 levels being observed after 15 min.

**p38 Interaction with the Ribosome does not Require prior Phosphorylation in RAW 264.7 Macrophages**

To determine cultures whether p38 requires prior phosphorylation to bind the ribosome in RAW 264.7, cells were...
preincubated with the p38 kinase inhibitor SB203580 for 45 min prior to DON treatment. As seen with U937 monocytes, the p38 kinase inhibitor blocked p38 phosphorylation but this did not affect p38 interaction with the ribosome (Fig. 6).

**FIG. 3.** DON induces sequential interaction of p38 with 40S and 60S ribosomal subunits in U937 monocytes. Cells were incubated with 500 ng/ml of DON for 5 or 15 min and p38 interactions within the ribosomal fractions compared with those for untreated cells. Data are representative of three independent experiments.

**FIG. 4.** DON-induced p38 interaction with the ribosome does not require p38 phosphorylation in U937 monocytes. Cells were preincubated with the p38 kinase inhibitor SB203580 (2µM) for 45 min and treated with 500 ng/ml of DON for 30 min. Cell lysates and pooled RS + M were analyzed by Western blotting. Data are representative of two separate experiments.

**FIG. 5.** Rapid [3H] DON uptake in RAW 264.7 macrophages corresponds with p38 phosphorylation and ribosome interaction. (A) Cells were incubated with [3H] DON (250 ng/ml) for various time intervals and uptake monitored. (B) Cells were stimulated with DON (250 ng/ml) for 15 or 30 min and p38 phosphorylation in lysates measured by in-cell Western assay. (C) Relative in-cell Western infrared intensities of phosphorylated p38 were normalized against intensities total p38. Bars without same letter differ (p < 0.05). (D) Pooled RS + M were analyzed for total and phosphorylated p38 by Western blotting. Data are representative of three separate experiments.
DON Induces JNK and ERK Ribosomal Interaction and Phosphorylation in RAW 264.7 Macrophages

The potential for other MAPKs to similarly bind to the ribosome following DON exposure was also addressed. As observed for p38, DON induced phosphorylation of JNK and ERK from 15 to 30 min in RAW 264.7 cells (Figs. 7A and 7B). Also consistent with p38, DON-induced JNK 1/2 and ERK 1/2 association with the RS + M fraction from 5 to 30 min, whereas phosphorylation of JNK 1/2 and ERK 1 was observed only from 15 to 30 min. These data suggest that DON sequentially induced ribosomal interaction with all three major MAPK families and their subsequent phosphorylation.

DISCUSSION

MAPKs function as molecular rheostats in the regulation of DON-induced immune gene expression and apoptosis in leukocytes (Pestka and Smolinski, 2005). p38 activation is particularly critical for not only DON-induced expression of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α or chemokines such as IL-8 and macrophage inflammatory protein-2 (Islam et al., 2006; Wong et al., 1998), but for DON-induced apoptosis as well (Zhou et al., 2005). Other translational inhibitors such as anisomycin, ricin and Shiga toxin can also target innate immune system and activate MAPK-driven gene expression and apoptosis (Bunyard et al., 2003; Cherla et al., 2006; Korcheva et al., 2005). Although it has been postulated that ricin and other translational inhibitors induce MAPK activation following cleavage of the 28S rRNA (Iordanov et al., 1997), the underlying mechanisms of ribotoxic stress response remain incompletely understood. The results presented here indicate that, in clonal human monocyte and murine macrophage models, DON rapidly evokes both the mobilization of p38 to the ribosome and its subsequent phosphorylation (Fig. 8).

DON and other trichothecene target actively dividing cells and are known to associate with 60S ribosomal subunits of ribosomes (Ueno et al., 1968; Wei et al., 1974). Here it was observed that, concurrent with DON uptake, p38 associated first with the 40S subunit fraction and then with fractions containing the 60S subunit and intact monosome. Using immunoprecipitation and peptide sequencing, Lin et al. (2003) demonstrated the capacity of p38 to interact with ribosomal proteins, however, stimuli capable of evoking such an interaction were not identified. As shown here, in the absence of a stimulus, p38 was minimally associated with the ribosomal components, whereas following treatment with DON, this kinase was readily detectable in ribosome fractions. This is the first report to our knowledge that links a specific stimulus to induction of p38 binding to the ribosome.

[Diagram of DON-induced phosphorylation]

**FIG. 6.** DON-induced p38 interaction with the ribosome does not require p38 phosphorylation in RAW 264.7 macrophages. Cells were preincubated with p38 kinase inhibitor (2μM) for 45 min and treated with 250 ng/ml of DON for 30 min. Cells lysates and pooled RS + M were analyzed by Western blotting. Data are representative of two separate experiments.

**FIG. 7.** DON induces JNK and ERK ribosomal interaction and phosphorylation in RAW 264.7 macrophages. (A) Cells were stimulated with DON (250 ng/ml) for various time intervals and JNK and ERK phosphorylation in lysates measured by in-cell Western assay. (B) Relative in-cell Western infrared intensities of phosphorylated JNK and ERK were normalized against intensities of total JNK and ERK. Bars without same letter differ (p < 0.05). (C) Pooled RS + M were analyzed for total and phosphorylated JNK and ERK by Western blotting. Data are representative of three separate experiments.
That observation that nonphosphorylated p38 was maximally associated with ribosomal fractions from 5 to 30 min, whereas the levels of phosphorylated form peaked at 15 min, indicated that p38 might be phosphorylated after binding to the ribosome. The p38 kinase inhibitor, SB203580, inhibits p38 phosphorylation by interacting with ATP binding site of this kinase. Our findings are consistent with those of Frantz et al. (1998) who observed that SB203580 at 0.1 and 1.0 μM suppressed p38 phosphorylation in THP-1 (human monocyte) cells that were treated for 20 min with LPS or TNF-α which are prototypical MAPK activators. These authors provided substantial evidence that pyridinylimidazoles block the p38 kinase biological activity by binding the inactive form of p38 and reducing its rate of activation. It should be noted that SB203580 sometimes yields disparate results. For example, Kong et al. (2008) observed that the inhibitor caused an increase in phosphorylation of p38 that was attributed to inhibition of a regulatory loop. However, that study differed extensively from both ours and that of Frantz et al. (1998). The former used BV2 microglial cells, 2-h SB203580 preincubation and a 20-h incubation with poly IC stimulus. A further issue is that Kong et al. (2008) did not show data for SB203580 alone making it difficult to know if this caused nonspecific stimulation of p38 phosphorylation. We have observed that prolonged (i.e., many hours) incubation with SB203580 alone can actually induce p38 phosphorylation in absence of additional stimulus.

The ability of inhibition with SB203580 to prevent DON-induced p38 phosphorylation at the Thr-Gly-Thr activation motif in our study but inability to block p38 association with the ribosome further suggest that p38 phosphorylation per se might not be required for the interaction. Accordingly, DON-induced p38 binding to the ribosome appears to be dissociable from its phosphorylation. A critical question arising from these observations relates to the identity of the binding partner(s) for p38. Further studies are needed to determine whether p38 interacts with specific ribosomal proteins, rRNA or additional intermediary proteins that bind to ribosome.

It is not yet clear how p38 is phosphorylated following interaction with the ribosome. Previous studies have shown that double-stranded RNA (dsRNA)–activated protein kinase (PKR) mediates DON-induced MAPK phosphorylation. PKR is a serine/threonine kinase known to localize with the ribosome (Wu et al., 1998; Zhu et al., 1997). PKR contains a specific dsRNA-binding motif that facilitates its activation (Williams, 2001). This kinase is known to interact with apoptosis signaling kinase 1 (ASK1) (Takizawa et al., 2002) and mitogen-activated kinase kinase 6 (MKK6) (Silva et al., 2004), both of which can drive p38 phosphorylation. Recently, PKR has been shown to form a functional complex with p38 that contributes to muscle differentiation of committed myogenic cells (Alisi et al., 2008). PKR-deficient U937 monocytes exhibit reduced induction of p38, JNK, and ERK phosphorylation by DON (Zhou et al., 2003b). It is tempting to speculate that DON alters conformation of or damages 28 rRNA in a manner that enables it to bind and activate ribosomal-bound PKR and that this subsequently drives p38 phosphorylation.

An alternative explanation for p38 phosphorylation is that, upon binding to the ribosome, the kinase undergoes a conformational change that mediates its activation (Wilson et al., 1996; Zhang et al., 1994). Unphosphorylated MAPKs contain an “activation lip,” which is a loop located near the Thr-X-Tyr motif that might block their activation (Wilson et al., 1996). It might be speculated that DON-induced p38 binding to the
ribosome disrupts this inhibitory structure thereby leading to phosphorylation of this kinase.

The kinetics of JNK and ERK association with the ribosome and activation in DON-treated murine macrophages remarkably paralleled that observed for p38. All three of the kinase families are known to coordinately mediate diverse biological functions (Raingeaud et al., 1995; Wong et al., 1998). Although p38 and JNK are activated in many stress signaling pathways, ERK is typically activated in response to growth hormone and proliferative stimuli. p38 can promote apoptosis and proinflammatory gene expression, whereas JNK and ERK are involved in regulation of both cell survival and death depending on cell types and stimulus. Competing apoptotic and survival signaling pathways are induced by p38 and ERK, respectively, in DON-stimulated RAW 264.7 cells (Zhou et al., 2005). The demonstration here that the ribosome functions as a scaffold for all three MAPKs suggests a unifying mechanism for coordinating these complex and seemingly disparate activities.

MAPKs play significant roles in protein translation. Both ERK and JNK are involved in the initiation of protein synthesis by phosphorylation of eIF-2α in human alveolar macrophages (Monick et al., 2006). p38 and ERK modulates translation initiation via eIF-4E phosphorylation, also critical to translation (Sheikh and Formace, 1999). Pharmacologic inhibition of p38 and ERK impairs AKT-dependent cyclin D1 and c-myc mRNA translation by reducing entry into the internal ribosomal entry site (Shi et al., 2005). ERK can modulate translation initiation by binding to and phosphorylating ribosomal protein 3 (Kim et al., 2005). It thus seems reasonable to suggest that DON-induced mobilization of MAPK to ribosomes would impact translation in some manner.

Taken together, the results presented herein demonstrate that following rapid uptake of DON by mononuclear phagocytes, p38, as well as JNK and ERK associate with the ribosome and are then phosphorylated. We propose that the ribosome functions first as an intracellular receptor for DON and then as scaffold to facilitate MAPK phosphorylation (Fig. 8). Ribosome-associated MAPKs might affect phosphorylation of protein substrates required for mRNA transcription, mRNA stabilization and translation. In the future it will be necessary to verify that ribosome-bound phosphorylated MAPKs are indeed activated. In addition, further study is needed to (1) identify critical ribosomal protein binding partners for p38 and other MAPK, (2) elucidate mechanisms by which these kinases are phosphorylated, (3) determine roles of ribosome-bound MAPKs in downstream events related to mRNA transcription, mRNA stability, protein translation and apoptosis, and (4) ascertain whether other translational inhibitors similarly induce MAPK binding to ribosomes.

Acknowledgments

We thank Jennifer Gray, Zahidul Islam, Yuhui Shi for technical advice and Mary Rosner for assistance with manuscript preparation.

References


Funding

Public Health Service grants (ES03553 and DK58833).

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