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Role of GRP78/BiP Degradation and ER Stress in Deoxynivalenol-Induced Interleukin-6 Upregulation in the Macrophage

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The trichothecene mycotoxin deoxynivalenol (DON) induces systemic expression of the interleukin-6 (IL-6) and other proinflammatory cytokines in the mouse. The purpose of this study was to test the hypothesis that DON triggers an endoplasmic reticulum (ER) stress response in murine macrophages capable of driving IL-6 gene expression. DON at concentrations up 5000 ng/ml. was not cytotoxic to peritoneal cells. However, DON markedly decreased protein levels but not the mRNA levels of glucose-regulated protein (GRP) 78 (BiP), a chaperone known to mediate ER stress. Inhibitor studies suggested that DON-induced GRP78 degradation was cathepsin and calpain dependent but was proteosome-independent. RNAi-mediated knockdown of GRP78 resulted in increased IL-6 gene expression indicating a potential downregulatory role for this chaperone. GRP78 is critical to the regulation of the two transcription factors, X-box binding protein 1 (XBP1) and activating transcription factor 6 (ATF6), which bind to cAMP-response element (CRE) and drive expression of CRE-dependent genes such as IL-6. DON exposure was found to increase IRE1α protein, its modified products spliced XBP1 mRNA and XBP1 protein as well as ATF6. Knockdown of ATF6 but not XBP1 partially inhibited DON-induced IL-6 expression in the macrophages. Three other trichothecenes (satratoxin G, roridin, T-2 toxin) and the ribosome inhibitory protein ricin were also found to induce GRP78 degradation suggesting that other translation inhibitors might evoke ER stress. Taken together, these data suggest that in the macrophage DON induces GRP78 degradation and evokes an ER stress response that could contribute, in part, to DON-induced IL-6 gene expression.

Key Words: **deoxynivalenol (DON); interleukin-6; ER stress, translation inhibition.**

Membrane and secretory proteins synthesized in the endoplasmic reticulum (ER) need to be folded properly with the assistance of ER chaperones and folding enzymes located in the ER (Anelli and Sitia, 2008). Cytotoxicity, nutrient limitation, and accumulation of unfolded or misfolded proteins can initiate activation of a series of self-defense mechanisms known as the ER stress response or unfolded protein response (UPR) (Schroder, 2008; Zhang and Kaufman, 2006). The ER chaperone GRP 78/BiP serves as a master controller of this stress response (Quinones *et al.*, 2008).

GRP78 interacts with two ER stress sensors known as activating transcription factor 6 (ATF6) and inositol requiring enzyme 1 (IRE1), the latter of which mediates expression of the transcription factor XBP1 (Zhang and Kaufman, 2006). Upon accumulation of unfolded proteins in the ER lumen, GRP78 is titrated away thus releasing the transcription factor ATF6 and the enzyme IRE1 α which mediates expression of the transcription factor XBP1 (Kaufman, 1999; Zhang and Kaufman, 2006; Zhang *et al.*, 2006).

ER stress is involved in inflammation, neurodegeneration, diabetes mellitus, heart diseases, and kidney diseases (Han *et al.*, 2008; Lin *et al.*, 2008; Marciniak and Ron, 2006). Both lipopolysaccharide (Endo *et al.*, 2005, 2006) and proinflammatory cytokines (Nowis *et al.*, 2007; Oliver *et al.*, 2005) induce ER stress leading to expression of acute response proteins. ER stress has also been related to autoimmune diseases including rheumatoid arthritis (Gao *et al.*, 2008; Purcell *et al.*, 2003) and autoimmune myositis (Nagaraju *et al.*, 2005). Accordingly, aberrant ER stress responses can contribute to disease.

Deoxynivalenol (DON) is a trichothecene mycotoxin produced by *Fusarium* sp. that is prevalent worldwide in cereal-based foods (Pestka and Smolinski, 2005). Toxicological effects associated with trichothecene mycotoxin poisoning in humans and animals include anorexia, gastroenteritis, emesis and hematological disorders. The innate immune system is particularly sensitive to DON with exposure to low trichothecene doses induces rapid, transient upregulation of proinflammatory cytokines causing immune stimulation and exposure to high doses causing apoptosis in lymphoid tissues resulting in immunosuppression (Pestka, 2008).

Chronic dietary exposure of mice to DON results in dramatic elevation in serum IgA, serum IgA immune complexes and IgA deposition in the mouse kidney, all of which mimic the early stages of human IgA nephropathy (Pestka, 2003; Pestka *et al.*, 1989). IgA dysregulation is mediated by DON-induced IL-6 expression

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which drives differentiation and proliferation of IgA-secreting B cells (Pestka and Zhou, 2000; Yan *et al.*, 1997, 1998). We have observed in peritoneal macrophages that the transcription factor cAMP response element binding protein (CREB) promotes IL-6 mRNA transcription by binding to cAMP response element (CRE) (Jia *et al.*, 2006; Shi and Pestka, 2006, 2009).

Using differential display, it has been demonstrated that DON exposure decreases GRP78 expression in EL-4 T cells (Yang *et al.*, 2000). Downregulation of GRP78 could potentially induce ER stress and activation of ATF6 and XBP1 (via IRE1). Because ATF6 and XBP1 belong to the CREB/ATF family, they can upregulate IL-6 expression by binding to CRE (Hai and Hartman, 2001; Kanemoto *et al.*, 2005; Schroder and Kaufman, 2005). Therefore, activation of one or both of these transcription factors might contribute to DON-induced upregulation of IL-6.

The purpose of this research was to test the hypothesis that DON triggers an ER stress response capable of driving IL-6 gene expression in the murine macrophage. We demonstrate in mouse peritoneal macrophages that DON induces GRP78 degradation but causes upregulation of IRE 1, XBP1, and ATF6 expression. Our results further indicate that these responses contribute in part to induction of IL-6 expression by DON.

MATERIALS AND METHODS

Materials. All inhibitors were purchased from Calbiochem, Inc. (San Diego, CA).

All chemicals including DON (purity ≥ 98%) and cell culture components were purchased from Sigma-Aldrich, Inc. (St Louis, MO) unless otherwise noted. DON, roridin A, satratoxin G, ricin, and T-2 toxin were dissolved in pyrogen-free water and tunicamycin (10 mg/ml) was dissolved in dimethyl sulfoxide (DMSO) as stock solutions. Inhibitors ALLN, EPO, CATI-I, and CALI-III were dissolved in DMSO at 50, 10, 50, and 25mM concentrations respectively. DON-contaminated labware and cell culture media were detoxified by sodium hypochlorite.

Animals. Female B6C3F1 mice (7 weeks old) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Housing, handling, and sample collection procedures conformed to the policies of the Michigan State University All-University Committee on Animal Use and Care in accordance with National Institutes of Health guidelines. Mice were provided free access to food and water.

Peritoneal macrophage cultures. Mice were injected ip with 1.5 ml of sterile 3% (wt/vol) thioglycollate broth. After 3 days, the animals were euthanized and macrophages collected by peritoneal lavage with ice-cold Hank's balanced salt solution (BSS) (Invitrogen Corporation, Carlsbad, CA). Cells were pelleted by centrifugation at 1100 × g for 5 min, washed with BSS once and resuspended in RPMI-1640 containing 10% (vol/vol) heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37°C under 6% CO_2 in a humidified incubator for 24 h prior to toxin treatment.

Cytotoxicity. The potential cytotoxic effects of DON in peritoneal macrophages were assessed by both the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-tetrazolium bromide (MTT) and Alamar Blue assays. For the former (Mosmann, 1983), macrophages were cultures in 96-well plates and treated with DON for 2–12 h. Filter-sterilized MTT (5 mg/ml in PBS, pH 7.4) was added at 10% (vol/vol) to each well and plates incubated for 3 h at 37°C. After removing medium, 100 μl of DMSO (Sigma) was added to each well and the plate was read at 570 nm using 690 nm as the reference wavelength on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT). In addition, the Invitrogen Alamar Blue Assay kit (Carlsbad, CA) was used according to the manufacturer's instructions. Briefly, peritoneal macrophages were cultured in 96-well with DON for 0 and 6 h, 10% (vol/vol) AlamarBlue reagent added to each well, and the cultures incubated for 6 h at 37°C. Absorbance was measured at 570 nm using 690 nm as the reference wavelength on the microplate reader.

Experimental design. In a typical experiment, macrophages were incubated with or without DON in the presence or absence of inhibitors for various time periods and analyzed for expression proteins by Western analysis or specific mRNAs by real-time PCR. For protein collection, cells (1×10^6 / ml) were incubated in 100-mm-diameter cell culture dishes (Corning Life Sciences, Lowell, MA) containing 10 ml of culture medium. Two milliliters of cell suspension (1×10^6 /ml) was incubated in each well of 6-well cell culture plates (Corning Life Sciences) for experiments requiring RNA isolation.

Western blot analysis. Macrophages were lysed in Tris buffer (10mM, pH 7.4) containing 2% (wt/vol) sodium dodecyl sulfate, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), boiled and sonicated. After centrifugation at 18,000 × g for 15 min, supernatants were subjected to Western analysis (Shi and Pestka, 2009) using specific antibodies to GRP78 or IRE1α (Cell Signaling Technology, Inc., Danvers, MA), XBP1, ATF6 (Santa Cruz Biotechnology, Inc.), and β-actin (Sigma-Aldrich). Alexa Fluor 680 goat-anti rabbit and IRDye 800 goat-anti mouse secondary antibodies were purchased from Invitrogen Corporation and Rockland Immunochemicals, Inc. (Gilbertsville, PA), respectively. Infrared fluorescence was measured using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE).

Real-time PCR. Total RNA of peritoneal macrophages was extracted using RNeasy Mini kits (Promega, Madison, WI) and mRNAs quantified by real-time PCR. TaqMan primers and probes for IL-6 mRNA were purchased from Applied Biosystems (Foster City, CA). The primer sequences for unspliced (u) and spliced XBP1 (s) were designed as follows: (forward) 5′-tgg ccg ggt ctg ctg agt ccg-3′ (u), 5′-ctg agt ccg cag cag gtg cag-3′ (s); (reverse) 5′-gtc cat ggg aag atg ttc tgg-3′ (u and s). SYBER Green PCR Master Mix (Applied Biosystems) was used for real-time PCR to detect unspliced and spliced XBP1 mRNA. β-2 Microglobulin RNA expression was not affected by DON treatment and thus was used as endogenous control to normalize target gene expression. Target gene expression levels were calculated relative to the control group.

siRNA knockdown. siRNA cocktails targeting mouse GRP78, XBP1, ATF6, or a comparable scrambled siRNAs were purchased from Dharmacon (Lafayette, CO). siRNA transfection was performed in peritioneal macrophages by electroporation using an Amaxa Nucleofector (Amaxa, Inc., Gaithersburg, MD) (Loniewski *et al.*, 2008; Shi and Pestka, 2008). Briefly, 2×10^6 cells were suspended in 100 μl of electroporation buffer (Amaxa, Inc.) and mixed with 10μM siRNA. Electroporation was performed using program D032 for macrophages according to the manufacturer's protocol. Transfection efficacy was verified by assessing reduction of GRP78, XBP1, or ATF6 proteins by Western blot after 48 h of transfection.

Statistics. All data were analyzed with SigmaStat v 3.1 (Jandel Scientific, San Rafael, CA) with the criterion for significance set at $p < 0.05$. Student's *t*-test was used for comparison of two groups of data; and one-way ANOVA was performed for comparison of multiple groups. Holm-Sidak (if normality test passed) or Dunn (ANOVA on ranks if normality test failed) tests were used for *post hoc* analysis.

RESULTS

The cytotoxic effects of DON were evaluated in cultured peritoneal macrophages. Both the MTT (Fig. 1A) and Alamar Blue (Fig. 1B) assays revealed that macrophages were remarkably recalcitrant to DON at concentrations up to 5000 ng/ml for 12 h.

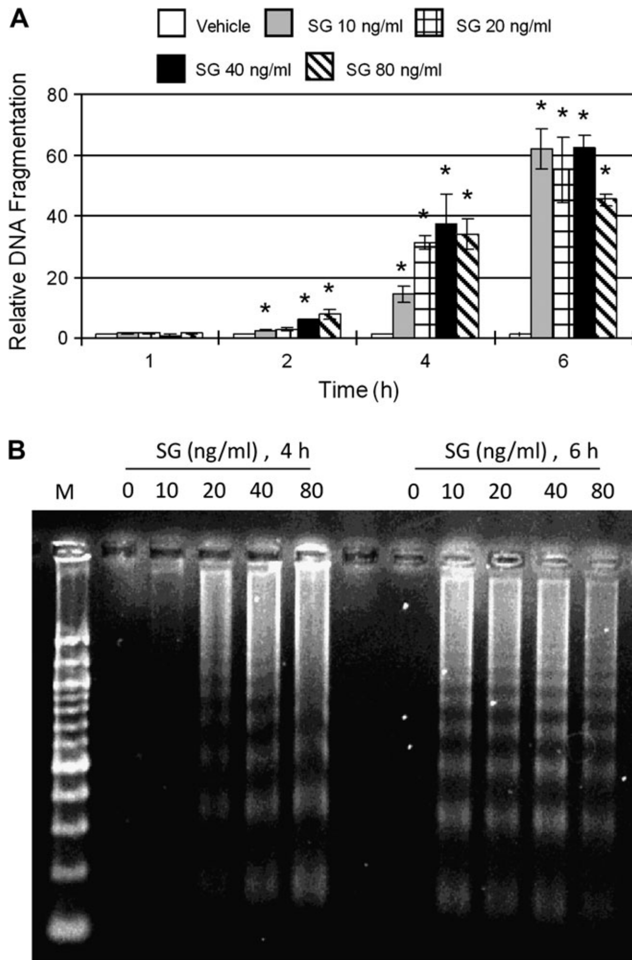

FIG. 1. DON is not cytotoxic to peritoneal macrophages. DON cytotoxicity was assessed in cultured peritoneal macrophages by the (A) MTT and (B) Alamar Blue assays.

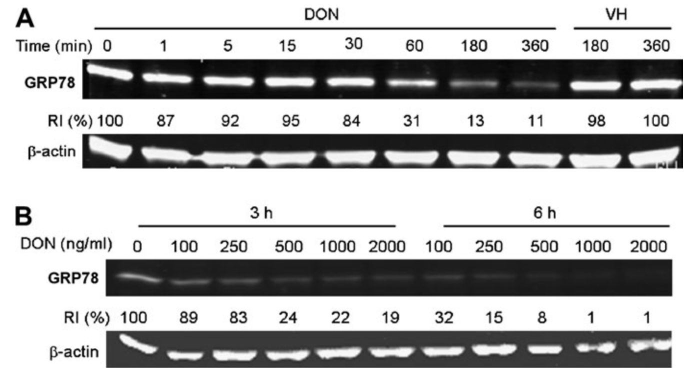

FIG. 2. Kinetics and concentration-dependence of DON-induced GRP78 degradation in peritoneal macrophages. Following exposure to DON, peritoneal macrophages were cultured with DON (500 ng/ml) or water vehicle (VH) for various intervals. Total protein was extracted and GRP78 was analyzed by Western blot. (A) Kinetics of GRP78 degradation during exposure to 500 ng/ml DON. (B) Concentration-dependent DON-induced GRP78 degradation. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.

Following incubation of peritoneal macrophages with DON (500 ng/ml), GRP78 was reduced markedly after 60 min and decreased further after 180 and 360 min as compared with vehicle-treated cells (Fig. 2A). The effects of different DON concentrations on GRP78 disappearance were assessed (Fig. 2B). DON at 500 ng/ml induced prominent decreases in GRP78 protein within 3 h, whereas as little as 100 ng/ml DON diminished GRP78 protein after 6 h. Based on these findings, a DON concentration of 500 ng/ml was chosen for subsequent studies on GRP78 reduction from the perspective of potential upstream mechanisms and downstream effects.

Although DON at 500 ng/ml induced robust time-dependent decreases in GRP78 protein (Fig. 3A), GRP78 mRNA was unaffected in peritoneal macrophages after DON treatment (Fig. 3B). Thus decreased GRP78 protein was not likely to be a direct result of decreased GRP78 gene expression.

Specific inhibitors were employed to determine if the observed reduction of GRP78 was related to proteolysis. DON-induced GRP78 degradation in peritoneal macrophages was markedly inhibited by preincubation with ALLN which inhibits both cathepsins and calpains, whereas there was no inhibition by the proteasome inhibitor epoxomicin (Fig. 4A). Preincubation with either cathepsin inhibitor I (CATI-I) and calpain inhibitor III (CALI-III) blocked DON-induced GRP78 degradation (Fig. 4B). These data suggest that GRP78 loss was possibly related to an autophagic pathway.

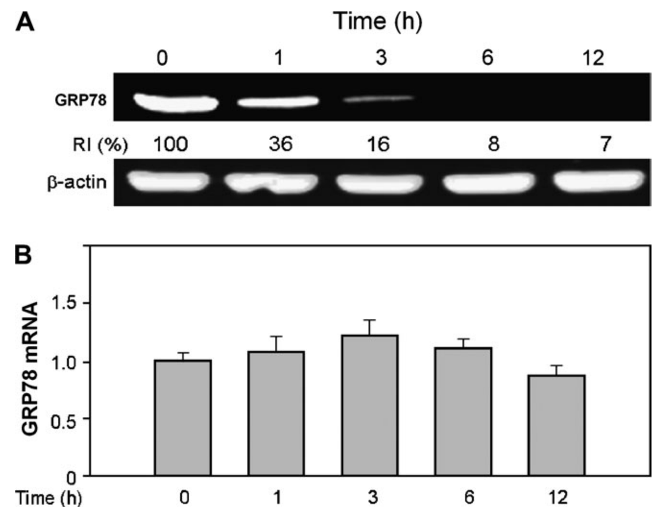

FIG. 3. DON treatment does not affect GRP78 gene expression in peritoneal macrophages. Peritoneal macrophages were treated with DON (500 ng/ml) for 0, 1, 3, 6, or 12 h. (A) GRP78 protein was detected by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane. (B) Total RNA was collected and GRP78 mRNA was analyzed by real-time PCR. Data are means ± SE.

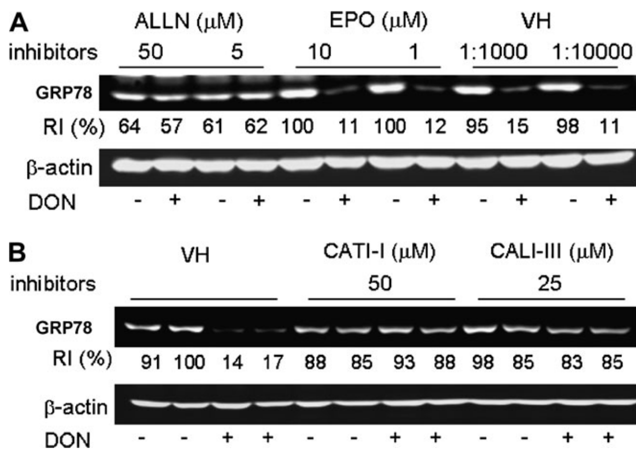

FIG. 4. DON-induced GRP78 degradation in peritoneal macrophages is cathepsin/calpain dependent. (A) Peritoneal macrophages were incubated with ALLN, epoxomicin (EPO), or equivalent concentrations of DMSO (VH) for 1 h, and then incubated with DON (500 ng/ml) for 3 h. (B) Peritoneal macrophages were incubated with inhibitors to cathepsins (CATI-I) or calpains (CALI-III) or equivalent concentrations of DMSO (VH) for 1 h, and then incubated with DON (500 ng/ml) for 3 h. Total protein was extracted and GRP78 was analyzed by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.

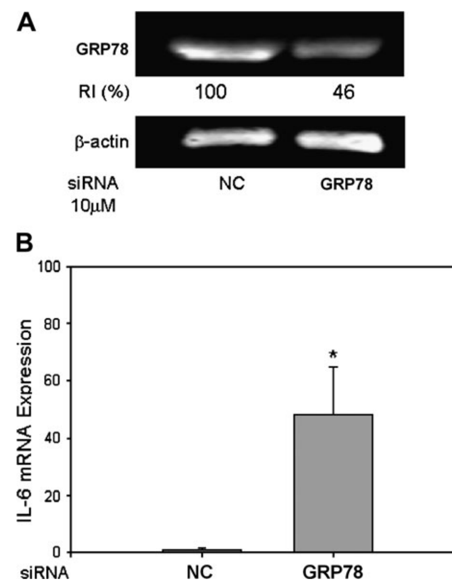

FIG. 5. GRP78 knockdown induces IL-6 gene expression in peritoneal macrophages. siRNA specific to mouse GRP78 or scrambled siRNA (negative control, NC) was transfected by electroporation into peritoneal macrophages. (A) To evaluate knockdown efficiency, total protein was collected after 48 h. GRP78 was measured by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane. (B) To detect the correlation of GRP78 knockdown and IL-6 gene expression, total RNA was extracted and IL-6 mRNA was analyzed by real-time PCR. Data are means ± SE (n = 3). Asterisk indicates statistically different from control group ($p < 0.05$).

To confirm a relationship between DON-induced GRP78 degradation and IL-6 gene expression, GRP78 was knocked down by siRNA transfection. GRP78 protein reduction was detectable within 48 h after transfection (Fig. 5A) and found to correlate with upregulated IL-6 mRNA expression (Fig. 5B).

Concentrations of IRE-1α, an ER stress sensor activated upon dissociation from GRP78, were increased in peritoneal macrophages within 1 and 3 h after DON treatment (Fig. 6A). Because XBP1 RNA is known to be spliced by activated IRE1α, levels of unspliced and spliced XBP1 mRNA were compared following DON treatment for various time intervals. Concentrations of spliced XBP-1 mRNA were upregulated by DON whereas unspliced XBP-1 mRNAs were unaffected (Fig. 6B). DON was further observed to induce modest increases in the transcription factors XBP1 and ATF6 in peritoneal macrophages (Fig. 7).

To ascertain whether DON-induced IL-6 gene expression is mediated by components of the ER stress response, ATF6 and/or XBP1 were knocked down by transfection with specific siRNAs. ATF6 and XBP1 protein concentrations decreased markedly 48 h after siRNA transfection compared with negative control (Fig. 8A). Knockdown of transcription factor ATF6 or both ATF-6 and XBP1 decreased DON-induced IL-6 expression, whereas no effect was evident following knockdown of only transcription factor XBP1 (Fig. 8B). This indicates that ATF6, but not XBP1, is involved in IL-6 expression induced by DON.

To discern if GRP78 degradation was a general effect induced by other translational inhibitors, GRP78 protein levels were measured in peritoneal macrophages that were treated with three trichothecenes roridin A, satratoxin G, and T-2 toxin, as well as the ribosomal inhibitory protein ricin. Tunicamycin, a classic ER stress inducer, was also used as a positive control. All of the toxin treatments induced GRP78 degradation at 3 and 6 h, whereas tunicamycin upregulated BiP dramatically at 6 h (Fig. 9).

DISCUSSION

The ER stress response is evoked by accumulation and aggregation of nascent, unfolded or misfolded polypeptides in the ER (Ma and Hendershot, 2001). To maintain ER homeostasis, cells initiate processes that augment the ER capacity of protein folding by evoking transient attenuation of protein translation, ER-associated degradation of misfolded proteins and the induction of molecular chaperones. If the stress is not relieved by these responses in badly damaged cells, apoptotic pathways will be activated.

GRP78, which belongs to heat shock protein 70 family, is one of the most well-characterized ER chaperones and plays a critical role in activating ER stress sensors (Zhang and Kaufman, 2006). By recognizing and binding to hydrophobic domain of unfolded proteins, GRP78 stabilizes unfolded proteins thereby enabling modification. Of particular relevance to the model described herein, GRP78 interacts directly with the ER stress sensors ATF6 and IRE1α to maintain them in inactive forms. When unfolded proteins accumulate in the ER

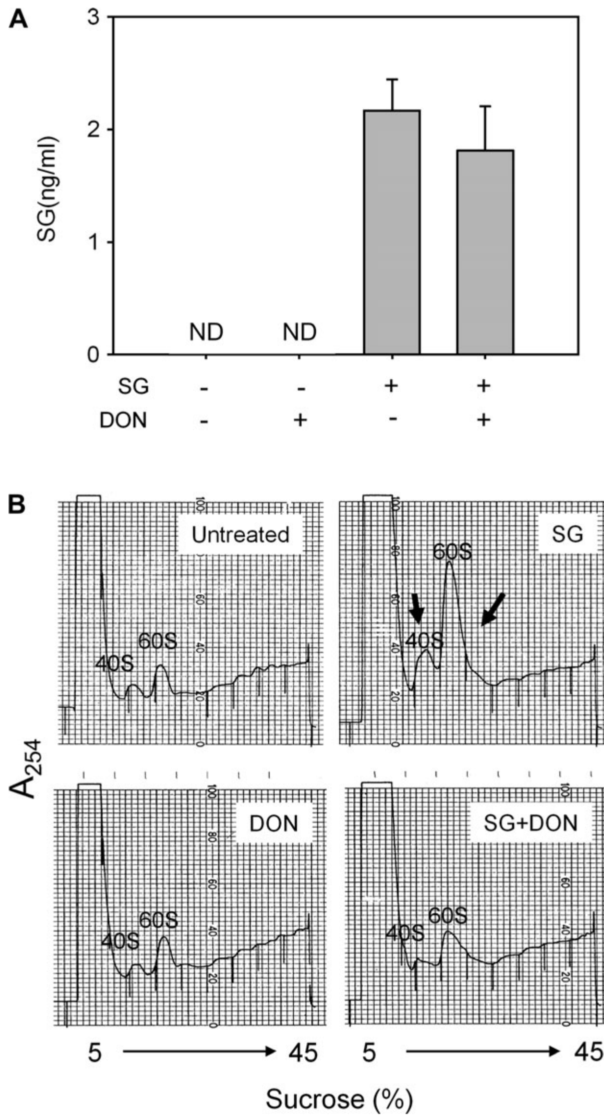

FIG. 6. DON upregulates IRE1α, and induces XBP1 mRNA splicing in peritoneal macrophages. Peritoneal macrophages were cultured with DON (500 ng/ml) for various time intervals. (A) Total protein was extracted and assessed for IRE1α . RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane. (B) Total RNA was extracted and relative concentration of unspliced and spliced XBP1 mRNA determined by real-time PCR. Data are means ± SE ($n = 3$). Bars with different letters differ ($p < 0.05$).

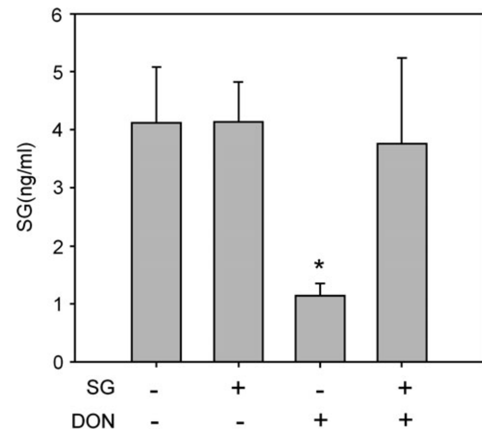

FIG. 7. DON induces XBP1 and ATF6 in peritoneal macrophages. Peritoneal macrophages were cultured with DON (500 ng/ml) for various time intervals. XBP1 and ATF6 were analyzed by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.

lumen, GRP78 is titrated away thereby releasing ATF6 and IRE1 α and enabling them to transduce signals to the cytosol and the nucleus (Kaufman, 1999; Zhang and Kaufman, 2006; Zhang *et al.*, 2006).

Activated IRE1 α will cleave XBP1 mRNA by its inherent RNase activity thereby yielding spliced XBP1 mRNA that codes for a second activated transcription factor capable of modulating gene expression (Ron and Hubbard, 2008). Activated ATF6 translocates from ER to Golgi where it is cleaved (from 90 to 50 kDa) by two proteases, S1P and S2P to become an active transcription factor (Shen *et al.*, 2002). As shown here, DON-induced degradation GRP78 in the peritoneal macrophages closely corresponded to activation and increases in IRE1, XBP1, and ATF6 proteins. It should be noted that although ER stress

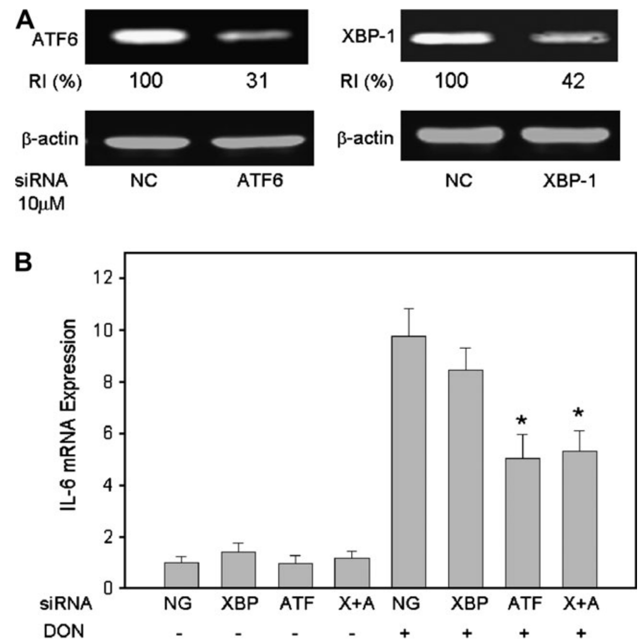

FIG. 8. DON-induced IL-6 mRNA expression correlates to ATF6 activation in peritoneal macrophages. siRNA specific to ATF6, XBP1, or scrambled siRNA (negative control, NC) was transfected into peritoneal macrophages by electroporation (A) ATF6 and XBP1 knockdown efficiency. Total protein was collected after 48 h. ATF6 and XBP1 were measured by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane. (B) Effect of ATF6 and XBP1 knockdown on IL-6 mRNA expression, cells were treated with DON after transfection with siRNA specific to XBP-1 (XBP), ATF6 (ATF), or both (X + A). Total RNA was collected 3 h later and IL-6 mRNA was analyzed by real-time PCR. Data are means ± SE ($n = 3$). Bars with different letters differ ($p < 0.05$).

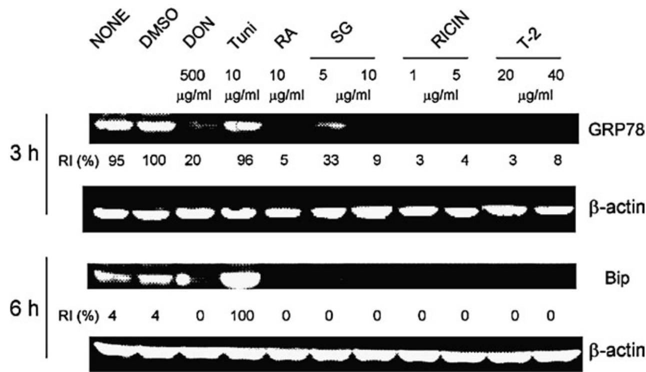

FIG. 9. GRP78 degradation is induced by different toxins in peritoneal macrophages. Peritoneal macrophages were treated with DON, tunicamycin (Tuni), roridin A (RA), satratoxin G (SG), ricin, and T-2 toxin for 3 or 6 h. GRP78 protein was measured by Western blot. RI indicates relative intensity which is the percentage of the maximal fluorescence in the same lane.

response is conventionally thought to include eventual upregulation of GRP78 as is observed for tunicamycin, DON did not have this effect. This difference might relate to selective inhibition of GRP78 translation by DON or its selective degradation.

The ER stress response has been previously associated with dysregulation of immune processes. For example, enhanced ER stress response activates transcription factor nuclear factor kappa B (NF-κB) and cyclic AMP response element binding protein H (CREBH) and induce proinflammatory gene expression (Deng *et al.*, 2004; Hu *et al.*, 2006; Hung *et al.*, 2004; Zhang *et al.*, 2006; Li *et al.*, 2005). Activation of XBP1 and ATF6 is also required for differentiation of B lymphocytes into antibody-producing plasma cells (Gass *et al.*, 2002; Iwakoshi *et al.*, 2003). Our results also showed that GRP78 knockdown directly induced IL-6 upregulation, and further indicating that the ATF6 could be upstream of IL-6 expression. Thus the ER stress response is likely to contribute to DON-induced IL-6 gene expression.

The capacity of DON and other ribotoxins to promote GRP degradation might have relevance to cancer chemotherapy. GRP78 is considered anti-apoptotic in the ER stress response and, interestingly, this chaperone is induced in a many types of cancers (Li and Lee, 2006). Both overexpression and siRNA studies have demonstrated that GRP78 contributes to tumor growth and confers drug resistance to cancer cells. There are relatively few reported examples of agents which promote degradation or downregulation of GRP78. Subtilase cytotoxin (SubAB), an AB toxin produced by Shiga-toxigenic *Escherichia coli*, causes GRP78 cleavage in Vero cells (Morinaga *et al.*, 2008). It is believed that subunit B binds to a surface receptor and that upon internalization, subunit B traffics through the Golgi where it cleaves GRP78. Sorafenib, a multikinase inhibitor that induces apoptosis in human leukemia and other malignant cells, causes reduction of GRP78 protein in U937 human monocytes. The mechanisms of these effects however, are not known (Rahmani *et al.*, 2007). Other natural compounds shown to downregulate GRP78 via as yet unidentified mechanisms include (1) deoxyverrucosidin from *Penicillium* (Choo *et al.*, 2005), (2) efrapeptin J from the marine fungus *Tolypocladium* (Hayakawa *et al.*, 2008), and (3) prunustatin A from *Streptomyces* sp. (Umeda *et al.*, 2005).

A critical question arising from this study relates to how GRP78 is degraded in the macrophage after DON treatment. This could be explained by either a decreased rate of synthesis or increased rate of degradation. Because GRP78 mRNA was unchanged following DON treatment, decreased GRP78 is not likely to be due to reduced expression. Eukaryotic cells have two major avenues for protein degradation, the ubiquitin-proteasome and autophagy-lysosomal pathways (Cecarini *et al.*, 2007; Yorimitsu and Klionsky, 2005).

Protein ubiquitination and proteasome-mediated protein degradation is a key pathway for removal of misfolded and short-lived intracellular proteins (Guerrero *et al.*, 2006; Rubinsztein, 2006). The 26S proteasome is a 2.5-MDa complex composed of two multisubunit subcomplexes: one is a 20S core particle and the other a 19S regulatory particle (Demartino and Gillette, 2007). The core particle is characterized by chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide-hydrolyzing activities (Cuervo and Dice, 1998). Epoxomicin is a potent, highly specific and irreversible inhibitor of chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide-hydrolyzing activities of the proteasome. The inability of epoxomicin to block DON-induced GRP78 degradation, suggests that proteaseome-mediated pathways are not involved.

Autophagy is another mechanism for the degradation of cytoplasmic components during ER stress and putative targets can range from single macromolecules (proteins, lipids and nucleic acids) to entire organelles (Yorimitsu and Klionsky, 2005). Once inside the lysosomal system, the substrates are degraded by cathepsins which is a mixture of more than 80 types of proteases, peptidases and other hydrolases (Cecarini *et al.*, 2007; Cuervo and Dice, 1998; Grinyer *et al.*, 2007). ALLN is an inhibitor of cathepsins, calpains and, at higher concentrations, proteasome. ALLN nearly abolished degradation of GRP78. Subsequent experiments with more specific inhibitors showed that DON-induced GRP78 degradation is cathepsin/calpain dependent. Thus, DON appeared to initiate an autophagy pathway in which GRP78 was degraded.

We have previously demonstrated the dsRNA-activated protein kinase (PKR) plays a critical role in DON-induced proinflammatory gene expression and apoptosis in mononuclear phagocytes (Pestka, 2008). The results presented herein suggest the ER stress response might be a second complementary pathway by which DON and other trichothecenes affect innate immune function. In addition to ATF6 and IRE1α, the PKR-like ER kinase (PERK) is another ER stressor sensor under regulation of GRP78 (Kincaid and Cooper, 2007). PERK, like PKR, can induce phosphorylation of eukaryotic initiation factor 2α (EIF2α) which can result in translational

inhibition. Future studies should determine whether DON and other trichothecenes affect PERK activity and whether cross-talk occurs between PERK and PKR exists.

It should be noted that DON's effects in cell cultures are likely to be very complex and will vary depending on cell type, species source and presence or absence of a costimulatory signal. For example, ATF-3, another member of CREB/ATF family that could impact IL-6, is upregulated in DON-treated epithelial cells (Yang *et al.*, 2009). In porcine pulmonary alveolar macrophages, DON induces IL-1β mRNA in unstimulated as well as LPS-stimulated macrophages, whereas tumor necrosis factor-α is overinduced in stimulated porcine macrophages and IL-6 seems to be of less importance (Döll *et al.*, 2009).

Although DON induces apoptosis in several leukocyte cell lines such as RAW 264.7, U937, and Jurkat cells (Pestka *et al.*, 2005; Yang *et al.*, 2000; Zhou *et al.*, 2005a), peritoneal macrophages were remarkably resistant to this DON at concentrations up to 5000 ng/ml for the time periods up to 12 h as indicated by two different cytotoxicity assays. It might be speculated that, in the primary cultures, anti-apoptotic signals such as ERK and AKT phosphorylation might exceed apoptotic signals, however, this requires further exploration .

The concentrations employed for the GRP78 studies (100–2000 ng/ml) here are consistent with those previously used to induce proinflammatory gene expression in mononuclear phagocytes (Gray and Pestka, 2007; Islam *et al.*, 2006; Wong *et al.*, 1998; Zhou *et al.*, 2005b). These concentrations are comparable to those attained in tissues of mice gavaged orally with DON at 5 mg/kg and sufficient to induce proinflammatory cytokine expression in spleen, liver, and lungs (Pestka and Amuzie, 2008).

To summarize, the results presented here suggest that DON induces GRP78 degradation and an ensuing ER stress response. The mechanisms for GRP78 autophagy are unclear. One possibility, however, is that it is translation suppression per se and/or that ribosome disruption evokes misfolding of proteins which are sequestered by GRP78 released from the ER. These GRP-containing complexes could enter into the autophagy pathway and be degraded. The ensuing ER stress-like response potentially contributes to DON-induced IL-6 expression in the macrophage. It is widely recognized that ER stress initially triggers evolutionarily conserved signal-transduction events designed to ameliorate the unfolded proteins accumulation in the ER, but if these events are severe or protracted they can induce apoptosis. Future research will focus on identifying critical events immediately upstream and downstream to GRP degradation as well as the relative contributions of this pathway to the upregulation of inflammatory genes and apoptosis.

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