INDUCTION OF RIBOTOXIC STRESS RESPONSE BY MYCOTOXIN DEOXYNIVALENOL: A PROTEOMIC VIEW

By

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ABSTRACT

INDUCTION OF RIBOTOXIC STRESS RESPONSE BY MYCOTOXIN DEOXYNIVALENOL: A PROTEOMIC VIEW

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The trichothecene mycotoxin deoxynivalenol (DON) is a common food contaminant that is of public health significance (Pestka, 2010) because it is a translational inhibitor that targets the innate immune system. DON-induced proinflammatory gene expression and apoptosis in the lymphoid tissue have been associated with a ribotoxic stress response (RSR) that involves rapid phosphorylation of mitogen-activated protein kinases (MAPKs). While it is recognized that DON-induced RSR involves protein phosphorylation and that DON targets the ribosome, a comprehensive assessment of how these events contribute to signaling, modulation of ribosome function and regulation of key biological processes is lacking.

To encapture global signaling events mediating DON-induced RSR and immunotoxicity, we employed quantitative proteomics to evaluate the dynamics of protein phosphorylation during early (≤30 min) DON-induced RSR in RAW 264.7 murine macrophage treated with a toxicologically relevant concentration of DON (250 ng/mL) and in the spleens of mice orally exposed to 5 mg/kg body weight DON. Large-scale phosphoproteomic analysis employing stable isotope labeling of amino acids in cell culture (SILAC) for RAW 264.7 or stable isotope dimethyl labeling for mouse spleen, in conjunction with titanium dioxide chromatography revealed that DON-induced RSR involves extensive phosphorylation alterations. In RAW 264.7, transcriptional regulation
was the main target during early DON-induced RSR involving transcription factors/cofactors and epigenetic modulators. In vivo DON exposure in the mouse affected biological processes such as cytoskeleton organization, regulation of apoptosis, and lymphocyte activation and development in the spleen, which likely contribute to immune dysregulation previously reported for the toxin. Some of these processes could be mediated by signaling networks involving MAPK-, NFκB-, AKT- and AMPK-linked pathways.

To understand the role of the ribosome in the spatiotemporal regulation of translational inhibition and the RSR, we evaluated dynamic changes in ribosome-associated proteome and phosphoproteome in RAW 264.7 cells similarly labeled and treated with DON. There was an overall decrease in translation-related proteins interacting with the ribosome, concurrently with a compensatory increase in proteins that mediate protein folding, biosynthetic pathways, and cellular organization. Alterations in the ribosome-associated phosphoproteome reflected proteins known to modulate translational and transcriptional regulation, as well as others that converged with known signaling pathways, suggesting the role of the ribosome as a platform.

Taken together, the proteomic analyses presented in this dissertation revealed extensive phosphorylation events mediate signaling and regulation key biological processes including transcriptional regulation leading to DON-induced RSR and immunotoxicity. Serving as a platform for stress-related proteins and phosphoproteins, the ribosome facilitates spatiotemporal regulation of translation inhibition and RSR at the subcellular level.
DEDICATION

For my parents
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<td>COX-2</td>
<td>Cyclooxygenase-2</td>
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<tr>
<td>cPLA2</td>
<td>Cytosolic phospholipase A2</td>
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<td>CRE</td>
<td>cAMP-response element</td>
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<tr>
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<td>β-catenin</td>
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<td>Cytochrome 26</td>
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<td>Dalton</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAVID</td>
<td>Database for Annotation, Visualization and Integrated Discovery</td>
</tr>
<tr>
<td>DED</td>
<td>Death effector domain</td>
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</tbody>
</table>
DFF: DNA fragmentation factor
DHB: 2,5-dihydroxy benzoic acid
DMEM: Dulbecco's Modified Eagle Medium
DNMT1: DNA methyltransferase 1
DOCK2: Dedicator of cytokinesis 2
DON: Deoxynivalenol
DTT: Dithiothreitol
eEF: Eukaryotic elongation factor
eIF: Eukaryotic initiation factor
ER: Endoplasmic reticulum
ERK: Extracellular signal-regulated kinases
ESI: Electrospray ionization
FASP: Filter-aided sample preparation
FCM: Fuzzy c-means
FDR: False discovery rate
FLNA: Filamin A
FOXP2: Forkhead box protein P2
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GO: Gene Ontology
GRP78: Glucose regulated protein 78
GSK3: Glycogen synthase kinase 3
HCK: Hematopoietic cell kinase
HDAC: Histone deacetylase
HDGF  Hepatoma-derived growth factor
HIPK3  Homeodomain interacting protein kinase 3
HIST1H1B  Histone H1.5
HP1  Heterochromatin protein 1
HPLC  High performance liquid chromatography
HSP  Heat shock protein
IAP  Inhibitor of apoptosis protein
IEF  Isoelectric focusing
IgA  Immunoglobulin A
IKK  I-kappaB kinase
IL  Interleukin
IMAC  Immobilized metal affinity chromatography
IP3  Inositol triphosphate
IRE1α  Inositol requiring kinase 1
IRES  Internal ribosome entry site
JNK  c-Jun N-terminal kinase
LCP1  Lymphocyte cytosolic protein 1
LKB1  Liver kinase B1
LMNA  Lamin A/C
LPS  Lipopolysaccharide
LTQ-FT  Linear trap quadrupole-fourier transform
LYRIC  Metadherin
Lys  Lysine
MALDI Matrix-assisted laser desorption ionization
MAPK Mitogen-activated protein kinase
MCP-1 Monocyte chemoattractant protein-1
MIP-2 Macrophage inflammatory protein 2
miRNA microRNA
MLTK MLK-like mitogen-activated protein triple kinase
MNK MAP kinase-interacting serine/threonine-protein kinase
MS Mass spectrometry
MTA3 Metastasis-associated protein 3
mTOR Mammalian target of rapamycin
MYBBP1A MYB binding protein (P160) 1a
MYH9 Myosin, heavy chain 9, non-muscle
NFKB Nuclear factor kB
NIPBL Nipped-B-like protein
n-MYC V-myc myelocytomatosis viral related oncogene, neuroblastoma derived
NPM1 Nucleophosmin
NuRD Nucleosome remodeling and deacetylase
p70S6K p70 S6 kinase
p90RSK 90 kDa ribosomal S6 kinase
PDCD11 Programmed cell death protein 11
PDK1 3-phosphoinositide-dependent protein kinase-1
PEA15 Astrocytic phosphoprotein PEA15
PERK Protein kinase RNA-like endoplasmic reticulum kinase
PGC-1α  PPARγ co-activator 1α
PGE2  prostaglandin E2
PI3K  Protein kinase RNA-like endoplasmic reticulum kinase
PI4K  Phosphatidylinositol 4-kinase
PIK3CG  Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma
PIP3  Phosphatidylinositol 3,4,5-trisphosphate
PKA  Protein kinase A
PKD2  Protein kinase D2
PKR  RNA-dependent protein kinase
PLC  Phospholipase C
PML  Sodium-hydrogen exchanger regulatory factor 1
PRKRA  Interferon inducible double stranded RNA dependent activator
PTC  Petidyltransferase center
RAC  Ribosome-associated complex
RBM  RNA binding motif protein
RIP  Ribosome-inactivating protein
RP  Ribosomal protein
rRNA  Ribosomal RNA
RSR  Ribotoxic stress response
SCX  Strong cation exchange
SEM  Standard error of the mean
Ser  Serine
SHIP  SH2-containing inositol-5'-phosphatase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SILAC</td>
<td>Stable isotope labeling of amino acids in cell culture</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SLTM</td>
<td>SAFB-like, transcription modulator</td>
</tr>
<tr>
<td>SNIP1</td>
<td>Smad nuclear interacting protein</td>
</tr>
<tr>
<td>SNW1</td>
<td>SNW domain containing 1</td>
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<td>SRRM2</td>
<td>Serine/arginine repetitive matrix 2</td>
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<td>STMN1</td>
<td>Stathmin</td>
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<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor-beta</td>
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<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TiO2</td>
<td>Titanium dioxide</td>
</tr>
<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TRIM28</td>
<td>Tripartite motif protein 28</td>
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<tr>
<td>TUB</td>
<td>Tubulin</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>U2AF2</td>
<td>U2 small nuclear RNA auxiliary factor 2</td>
</tr>
<tr>
<td>UHRF1</td>
<td>Ubiquitin-like, containing PHD and RING finger domains 1</td>
</tr>
<tr>
<td>UNC13D</td>
<td>Unc-13 homolog D</td>
</tr>
<tr>
<td>uORF</td>
<td>Upstream open reading frame</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra Performance Liquid Chromatography</td>
</tr>
<tr>
<td>USP39</td>
<td>Ubiquitin specific peptidase 39</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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</table>
w/v  Mass/volume
XBP1  X-box binding protein 1
XIC  Extracted ion chromatogram
ZO-1  Tight junction protein 1
INTRODUCTION

The trichothecene mycotoxin deoxynivalenol (DON) is of public health significance because it commonly contaminates human and animal foods and targets the innate immune system (Pestka, 2010b). DON’s immunomodulatory effects have been demonstrated in vivo in mouse systemic and mucosal immune organs (Zhou et al., 1999) as well as in primary and cloned murine monocyte/macrophage cultures derived from mice and humans (Pestka, 2010a). Innate immune system activation is central to both shock-like and autoimmune effects associated with acute and chronic DON exposure, respectively (Pestka, 2010a). DON-induced immunotoxicity is paradoxical. Low dose DON exposures are immunostimulatory as evidenced by robust upregulation of mRNAs and proteins for a diverse array of cytokine, chemokine, and other inflammation-related genes (Kinser et al., 2004; He et al., 2012b). High dose DON exposures evoke apoptosis in actively dividing immune tissues including spleen, thymus and bone marrow, as well as in myeloid cell models, which could contribute to immunosuppression (Yang et al., 2000; Zhou et al., 2000).

DON-induced proinflammatory gene activation and apoptosis are mediated by the activation of mitogen-activated protein kinases (MAPKs) via a process known as the ribotoxic stress response (RSR) (Pestka, 2010a). Besides MAPKs and their substrates, DON-induced RSR involves the rapid and transient activation of at least two upstream ribosome-associated kinases in vitro, double-stranded RNA-dependent protein kinase (PKR) and hematopoietic cell kinase (HCK), suggesting the pivotal role of protein phosphorylation in DON-induced RSR (Zhou et al., 2003; Zhou et al., 2005b; Bae et al., 2010). Phosphoproteomic changes in cloned immune cell lines, including human T and
B cell lines (Nogueira da Costa et al., 2011a) have been performed to model molecular mechanisms of DON-induced RSR in the responsive cell populations, as well as to identify biomarkers of effect for DON. However, DON’s effects of protein phosphorylation during RSR have not yet been comprehensively characterized in the macrophage, this toxin’s primary target cell type of the innate immune system, or in the immune system of an intact animal.

Interestingly, in addition to the toxin itself (Bae and Pestka, 2008), p38 and known upstream mediators of DON-induced RSR have been reported to bind to the ribosome. DON not only activates the ribosome-associated MAPK p38, but also causes rapid phosphorylate the early sentinel kinases PKR and HCK (≤ 5 min), which constitutively associate with the ribosome (Bae and Pestka, 2008; Bae et al., 2010). In addition, DON causes the ribosome mobilization and phosphorylation of other upstream components in the MAPK cascade, ASK1 (a MAPKKK) and MKK6 (Bae et al., 2010), suggesting the potential role of the ribosome as a platform for stress-related kinases.

DON-induced translation inhibition has been well documented in vivo and in vitro (Azcona-Olivera et al., 1995; Zhou et al., 2003b). It has been shown that DON induces translational inhibition in at least four ways. First, DON and other trichothecenes are canonically known to interfere with peptidyl transferase function on the ribosome, and consequently impair of initiation and elongation (Shifrin and Anderson, 1999). Second, DON can induce the activation of ribosome-associated kinase PKR, which when activated, phosphorylates eIF2α, thereby inhibiting translation (Zhou et al., 2003b). Third, DON can promote the degradation of 18S and 28S rRNA (He et al., 2012c) which could impede ribosome function and translation. Finally, DON can upregulate a large
number of microRNAs (miRNAs) that can target mRNA for translational inhibition, most notably for ribosomal proteins (He et al., 2010).

Although the ribosome is well known to be an immensely complex molecular machine dedicated to protein translation, it has been proposed that the composition and post-translational modifications of ribosomal proteins may add greater regulatory specificity in different cell types or developmental stages (Xue and Barna, 2012). For example, ribosomal protein S3 (RPS3) is a p65-binding component of NFκB complex and is essential for the function of the complex as a transcription factor (Wan et al., 2007). In addition, there is growing recognition that the ribosome can orchestrate other fundamental aspects of cell function by interacting with myriad non-ribosomal proteins (Xue and Barna, 2012). Some members of the Akt/mTOR pathway, including 3-phosphoinositide-dependent protein kinase-1 (PDK1), AKT, mammalian target of rapamycin (mTOR) and p70 S6 kinase (p70S6K) also bind to the ribosomes (Ruggero and Sonenberg, 2005; Lee et al., 2010; Zinzalla et al., 2011). Non-ribosomal proteins can potentially interact with 40S and 60S subunits, monosomes or actively translating polysome. Knowing what the ribosome-binding proteins are and where they interact on the functional ribosome landscape can provide significant insight about their cellular roles. Many questions remain regarding DON-induced RSR in terms of the role of the ribosome, the identity of the other proteins that are recruited to the ribosome and/or activated, and how they might adversely impact cell function.

Resolving the complexity of DON-induced RSR requires a sensitive, integrative approach for dissecting the molecular events occurring at the cellular and subcellular level. Proteomics facilitates large-scale identification and quantification of proteins,
providing information on protein expression and post-translational modification (Farley and Link, 2009; Mallick and Kuster, 2010). Stable isotope labeling of amino acids in cell culture (SILAC) has been successfully used to characterize the signaling and subcellular compartmentalization for global delineation of macrophage behavior during phagocytosis and upon toll-like receptor stimulation (Rogers and Foster, 2007; Dhungana et al., 2009), suggesting the applicability of this strategy to the study of DON-induced RSR in RAW 264.7, a well-established murine macrophage model (Raschke et al., 1978; Hambleton et al., 1996). The SILAC approach, however, is not readily amenable to in vivo animal studies of phosphorylation events that precede DON-induced immunotoxicity. An alternative chemical labeling strategy, stable isotope dimethyl labeling, entails rapid and complete reductive amination of peptides, which makes it a relatively simple and economic strategy for large-scale quantitative analyses of animal tissue (Kovanich et al., 2012) and could be employed to study the effect of DON on animal tissues.

Overall Hypothesis

The **objective** of this dissertation is to expand our understanding of the molecular mechanisms for DON toxicity by generating a comprehensive view of the protein phosphorylation and interactions events during its induction of RSR. Our **long term goal** of this research is to characterize how ribotoxic agents contribute to acute and chronic diseases, and to provide mechanism-based strategies of treating and preventing these diseases.

The **guiding hypothesis** of the research is that DON-induced RSR involves alterations in the proteome and phosphoproteome of the ribosome as well as the entire
cell. Using the quantitative proteomics approach, the hypothesis was addressed in three aims:

**Specific Aim 1**: Identify the kinetic phosphorylation events and map out the signaling network upon DON exposure

**Specific Aim 2**: Determine the kinetic changes of the ribosome-associated proteome and characterize the role of the ribosome in DON-induced RSR.

**Specific Aim 3**: Characterize the kinetic proteome changes upon DON exposure, and relate to changes in phosphoproteome and ribosome-associated proteome.

**Chapter Summaries**

Chapter 1 is literature review summarizing known mechanisms of DON-induced RSR and immunotoxicity, and the use of the quantitative proteomics in investigating protein phosphorylation and organelle composition and interacting proteins.

Chapter 2 evaluates the global phosphoproteome changes in SILAC-labeled RAW 264.7 to reveal key signaling mediators and biological processes in early RSR induced by a toxicologically relevant concentration of DON.

Chapter 3 focuses on the ribosome, the subcellular target of DON, and assesses the dynamic changes in ribosome-associated proteome and phosphoproteome to clarify the role of the ribosome in the RSR induced by DON.
Chapter 4 extends the discoveries \textit{in vitro} in Chapter 2 to intact experimental animals and investigates the dynamic phosphoproteomic alterations leading to the previously characterized robust proinflammatory response in the spleen of mice \textit{in vivo}.

In Chapter 5, discoveries of the dissertation are summarized and future directions of this research are discussed.

Appendix A evaluates the effect of DON on total proteome in RAW 264.7 cells to determine the contribution of total proteome on the protein phosphorylation (Chapter 2) and ribosome interaction (Chapter 3).
CHAPTER 1: Literature Review

Deoxynivalenol (DON)

Introduction

Deoxynivalenol (DON), also known as vomitoxin, is a type B trichothecene primarily produced by *Fusarium spp.* growing on wheat, barley and corn (Hope *et al.*, 2005). DON is chemically stable and resistant to normal food processing such as cleaning, milling and baking (Trigo-Stockli, 2002), and contaminates cereal-based foods worldwide (Rodrigues and Naehrer, 2012). DON also adversely affects human health. *Fusarium*-contaminated food has been linked to the outbreaks of human gastroenteritis with typical syndrome of vomiting in Japan, Korea and China in the 20th century (Pestka, 2010b). Studies in United Kingdom showed that urinary DON was detected in over 98% of the adults consuming cereal foods, revealing a positive correlation between cereal intake and urinary DON and indicating urinary DON and DON glucuronide as biomarkers of exposure in humans (Turner *et al.*, 2010).

Experimental animals have been used to assess the acute and chronic toxic effects of DON. It is a known translational inhibitor, and therefore mainly targets the highly proliferative cells in the gastrointestinal and immune system (Pestka, 2010b). High dose, acute exposure of pigs and mink to DON elicits abdominal distress, increased salivation, malaise, diarrhea, and emesis (Pestka, 2010b; Wu *et al.*, 2012). Extended low-dose of DON causes growth retardation, as evidenced by impaired weight gain, anorexia in pigs and mice fed chronically with low dose of DON (Rotter *et al.*, 1996). With regards to the immune system, DON causes proinflammatory response with acute
DON exposures (Pestka, 2010b), and upregulates serum immunoglobulin A (IgA) chronically (Pestka, 2003), which mimics the human IgA nephropathy.

Given the prevalence and safety issues associated with DON, the U.S. Food and Drug Administration has established an advisory limit of 1 part per million (ppm) of DON for human, 10 ppm for cattle and poultry and 5 ppm for other animals (Pestka, 2003).

**Paradoxical effect of DON on the immune system**

The primary target cell types of DON in immune system are leukocytes, including macrophages, monocytes, T cells and B cells with the former two as most sensitive cell populations (Pestka et al., 2004). Depending on the dose and exposure frequency, DON induces either immunostimulatory or immunosuppressive effects by upregulating proinflammatory gene expression or apoptosis, respectively.

Low doses of DON stimulate the immune system primarily by upregulating the transcription and expression of inflammatory response genes in vitro and in vivo. In cell cultures, DON induces TNF-α, IL-6, MIP-2 and COX-2 in macrophages (Moon and Pestka, 2002; Chung et al., 2003a; Chung et al., 2003b; Jia et al., 2004), IL-8 in monocytes (Gray and Pestka, 2007; Gray et al., 2008)and IL-2 in T cells (Li et al., 1997). In *in vivo* mouse models, DON exposure induces robust upregulation of the expression of various cytokines (IL-1α, IL-1β, IL-6, IL-11) and chemokines (MIP-2, CINC-1, MCP-1, MCP-3) (Kinser et al., 2004; Kinser et al., 2005).

DON upregulates the mRNA expression of proinflammatory genes at both the transcriptional and post-transcriptional levels by elevating transcription rate and improving mRNA stability. DON upregulates the expression of transcription factors,
including c-Fos, c-Jun, Fra-2 and JunB, were found upregulated in response to DON treatment (Kinser et al., 2004; Kinser et al., 2005), and increases the activation of transcription factors, such as NF-κB and AP-1 (Ouyang et al., 1996; Li et al., 2000; Wong et al., 2002; Gray and Pestka, 2007). Alternatively, DON could enhance mRNA stability to further promote the expression of proinflammatory genes. DON is reported to enhance the mRNA stability of COX-2 (Moon et al., 2003), TNF-α (Chung et al., 2003b), IL-6 (Jia et al., 2006) and IL-2 (Li et al., 1997) via AU rich elements in the 3’-UTR of mRNA, which promotes rapid degradation of mRNA. Additionally, HuR/Elav-like RNA binding protein 1 (ELAVL1) translocates from nucleus to cytosol and binding to the 3’-UTR of IL-8 transcript, thereby contributing to DON-induced stabilization of IL-8 mRNA (Choi et al., 2009).

In contrast to the aforementioned stimulating effects, rapid apoptosis of immune cells is induced by high concentrations of DON, including macrophages, monocytes, T and B cells in vitro (Pestka et al., 1994; Uzarski et al., 2003). Mice fed DON at high doses exhibit reduced thymus weight, antibody production and stimulation of B and T cells by mitogens (Robbana-Barnat et al., 1988). Apoptosis in bone marrow, thymus, spleen and Peyer’s patches is also evident in mice exposed acutely to DON (25 mg/kg body weight) (Zhou et al., 2000).

DON induces apoptosis by both intrinsic and extrinsic pathways (Zhou et al., 2005a; He et al., 2012c). At high concentrations, DON causes pronounced caspase 3-dependent DNA fragmentation after 6 h, and activation of MAPKs, including p38 and ERK1/2, within 15 min and up till 3 h. Chemical inhibition of p38 phosphorylation alleviates DON-induced apoptosis, whereas ERK inhibition enhances apoptosis,
suggesting that p38 and ERK might upregulate and downregulate signaling events leading to DON-induced apoptosis, respectively (Yang et al., 2000). DON induces the phosphorylation and activity of p53 which was suppressed by p38 inhibition. Furthermore, inhibition of upstream kinases that initiate DON-induced RSR, i.e. PKR and HCK, inhibits DON-induced p53 binding activity, p53 phosphorylation of its substrate p21, as well as caspase-3 activity and apoptosis (Zhou et al., 2003b; Zhou et al., 2005a; Zhou et al., 2005b). DON exposure could also trigger the mitochondria translocation of BAX, a Bcl-2 family protein, and corresponding cytochrome C release. At the same time, DON is able to activate two survival pathways via phosphorylation of 1) p90RSK, which phosphorylates BAD, and reduces its interaction with anti-apoptotic Bcl2 family members thereby inhibiting mitochondrial release of cytochrome C; and 2) AKT, which exerts anti-apoptotic effects by phosphorylating BAD, NF-B-inducing kinase (NIK), forkhead transcription factor FKRH and mouse double minute 2 homolog MDM2 (Franke et al., 2003; Zhou et al., 2005a).

Ribotoxic stress response

Introduction

The immunotoxicic effects of DON are proposed to be mediated by the activation of mitogen-activated protein kinases (MAPKs) via a process termed ribotoxic stress response (RSR) (Iordanov et al., 1997; Laskin et al., 2002). RSR is induced by translational inhibitors or translation-interfering toxicants, termed ribotoxins, including chemicals, proteins and ultraviolet light radiation.

Some ribotoxins are of low-molecular-weight, such as the trichothecenes T-2 toxin, nivalenol and DON and antibiotics anisomycin, which directly bind to ribosome
and inhibit protein synthesis. They bind to the 28S rRNA petidytransferase center (PTC) and trigger RSR (Iordanov et al., 1997; Shifrin and Anderson, 1999). Ribosome-inactivating proteins (RIPs) contain an RNA N-glycosidase domain that specifically cleaves a conserved adenine off the eukaryotic 28S rRNA. RIPs have been isolated from various organisms, including plants (e.g. ricin) (Woo et al., 1998), fungi (e.g. α-sarcin) (Endo et al., 1983) and bacteria (e.g., Shiga toxin) (Gyles, 2007). Finally, instead of directly associating with the ribosome, ultraviolet C (200–290 nm) and B (290–320 nm) rapidly activate JNK and p38 kinase by nucleotide and site-specific damage to the 3'-end of 28S rRNA, which impairs PTC activity and inhibits protein synthesis (Iordanov et al., 2002).

In addition to translational inhibition, ribotoxins could induce inflammasomes in the innate immune system. For example, trichothecenes (e.g. DON, Satratoxin G, roridin A, verrucarin A, and T-2 toxin), antibiotics (e.g. anisomycin) (Kankkunen et al., 2009; Yu et al., 2013), and RIPs (e.g. ricin, Shiga toxin) (Jandhyala et al., 2012) have been shown to activates inflammasome-associated caspase-1, and further enables the secretion of IL-1β and IL-18 from the LPS-primed cells. Such induction of the inflammasome is also mediated by MAPK activation (Jandhyala et al., 2012).

Although the mechanisms for the RSR are still unclear, two hypotheses have been proposed: direct activation of ribosome-associated kinases and indirect activation via endoplasmic reticulum (ER) stress response (Pestka, 2010b) (Figure 1.1).
Known mechanisms of DON-induced ribotoxic stress response - direct activation of ribosome-associated kinases

During RSR, MAPK activation is the hallmark signaling that connect rRNA perturbation and downstream regulation of gene expression. Studies to identify the upstream mediators of DON-induced MAPK activation identified two kinases, the double-stranded RNA-activated protein kinase (PKR) and hematopoietic cell kinase (HCK) (Zhou et al., 2003b; Zhou et al., 2005b).

PKR is a widely-distributed, constitutively-expressed serine/threonine protein kinase that can be activated by dsRNA, interferon, proinflammatory stimuli, cytokines and oxidative stress (Garcia et al., 2006) and has been implicated in control of cell growth, tumor suppression, apoptosis, and antiviral infection (Garcia et al., 2006). In RAW 264.7 macrophages, DON rapidly activates PKR within 5 minutes, and the phosphorylation of MAPKs are suppressed by pretreating with PKR inhibitor (Zhou et al., 2003b), indicating that PKR mediates the activation of MAPKs. Human U-937 monocyte cell line transfected with stable PKR antisense RNA vector also showed significantly reduced MAPK activation upon DON exposure (Gray and Pestka, 2007). Upon binding to dsRNA, PKR is activated by dimerization and autophosphorylation and phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α), which leads to the higher affinity to eIF2β and results in global translation initiation inhibition (Sudhakar et al., 2000).
Figure 1.1 Known mechanism of DON-induced ribotoxic stress response mediated (A) directly by ribosome-associated kinases, or (B) indirectly by endoplasmic stress response. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.
HCK is a member of the Src family of tyrosine kinases expressed specifically in myelomonocytic cell lineages and transduces extracellular signals that regulate proliferation, differentiation and migration (Tsygankov, 2003). DON exposure induces rapid phosphorylation of HCK at 1 min and MAPK at 5 min in RAW 264.7 macrophage. HCK inhibitor dose-dependently impairs the DON-induced MAPK activation, suggesting the role of HCK as an upstream mediator of MAPKs. Pretreatment of specific HCK inhibitor also suppresses the phosphorylation of MAPK substrates c-Jun, ATF-2 and p90RSK as well as the DON-induced activation of NFkB, AP-1 and C/EBP (Zhou et al., 2005b). Similarly, HCK siRNA also suppresses DON-induced TNF-α production and caspase activation. Consistent with these data, an HCK inhibitor suppresses p38 activation and p38-driven interleukin 8 (IL-8) expression (Bae et al., 2010) in the U937 human monocyte. HCK, as well as PKR, are important transducers of ribotoxic stress-induced apoptosis in addition of DON-induced proinflammatory gene activation, the inhibition of which suppresses DON-induced p53 binding activity thus blocking the p38/p53/Bax/mitochondria/caspase-3 apoptosis pathway (Pestka, 2008).

Following binding to the ribosome, DON activates the ribosome-associated MAPK p38 (Bae and Pestka, 2008). Two kinases upstream of MAPK activation are double-stranded RNA-dependent protein kinase (PKR) and hemopoietic cell kinase (HCK) (Zhou et al., 2003b; Zhou et al., 2005b) which constitutively associate with the ribosome, and are rapidly phosphorylated as early as 5 min, serving as early sentinels for DON exposure (Bae and Pestka, 2008; Bae et al., 2010). In addition, DON causes the ribosome mobilization and phosphorylation of other upstream components in the MAPK cascade, ASK1 (a MAPKKK) and M KK6 (Bae et al., 2010). The ribosome might
function as a platform for various signaling molecules, which mobilize and/or get differentially phosphorylated to the ribosome upon ribotoxin exposure. This key step might facilitate regulation of downstream gene transcription and post-transcriptional expression in DON-induced RSR by providing spatiotemporal organization of signaling molecules.

**Known mechanisms of DON-induced ribotoxic stress response - indirect activation via endoplasmic reticulum stress response**

Endoplasmic reticulum (ER) stress refers to perturbations in the ER environment such as alterations in redox state, calcium levels, or failure to post-translationally modify secretory proteins can change ER homeostasis and subsequently lead to accumulation of unfolded or misfolded proteins in the ER lumen (Lai et al., 2007). To cope with such stress, DON-treated macrophages markedly decrease expression of cytoplasmic glucose regulated protein (GRP) 78, a chaperone known to mediate ER stress in peritoneal macrophages (Shi et al., 2009). Such decrease of GRP78 is mediated by autophagy-lysosomal pathway of protein degradation (Yorimitsu and Klionsky, 2007). GRP78 regulates 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. DON treatment increases levels of ATF6 as well as inositol requiring kinase 1 (IRE1α) protein and its modified products spliced XBP1 mRNA and XBP1 protein (Shi et al., 2009).

It is also possible that DON-induced ER stress contributes to MAPK activation. Specifically, it is known that (1) IRE 1 activation mediates ASK1 phosphorylation and (2)
DON induces ASK1 phosphorylation (Bae et al., 2010). Accordingly, the ER stress response could thus be a second pathway leading to DON-induced RSR.

**Ribosome and translational regulation**

*Translation and the ribosome*

Translation is the process of decoding mRNA into a specific amino acid chain by cellular protein synthesis via the ribosome. In response to environmental stress, organisms rapidly change their cellular protein synthesis by precise regulation of translation to adaptively cope with these stimuli. Translation can be divided into four stages: initiation, elongation, termination, and recycling (Kapp and Lorsch, 2004).

The ribosome is an immensely complex molecular machine dedicated to protein translation. There are approximately 15,000 ribosomes in a single cell, and they make up about 25% of the dry weight of cells (Luisi and Stano, 2011). In eukaryotic cells, the ribosome consists of two ribonucleoprotein subunits, 40S and 60S, each containing characteristic proteins and rRNAs. The small subunit (40S) of eukaryotic ribosomes is composed of the 18S rRNA and approximately 30 proteins; the large subunit (60S) contains the 28S, 5.8S, and 5S rRNAs and about 45 proteins. Both subunits contain three binding sites for tRNA molecules that are in three different functional states. The A site binds the aminoacyl-tRNAs that are about to be incorporated into the growing polypeptide chain, the P site positions the peptidyl-tRNA and the E site is occupied by all deacylated tRNAs before they dissociate from the ribosome (Luisi and Stano, 2011).

Translation is a well-orchestrated process. Discrepancy between the transcriptome and proteome underlines the importance of translational control in
addition to the better understood transcriptional regulation (Sonenberg and Hinnebusch, 2009).

**Translational regulation**

Eukaryotic protein translation is mainly controlled at the level of initiation, a process of elongation-competent 80S ribosome assembly from 40S and 60S subunits. p90 ribosomal S6 kinases (p90RSKs), activated by ERK1/2, phosphorylates eIF4B and eEF2K to facilitate translation (Ma and Blenis, 2009). Alternatively, mammalian target of rapamycin complex 1 (mTORC1) can be activated by PI3K or ERK1/2 to enhance translation. It activates p70 S6 kinase (p70S6K) that phosphorylates the small ribosomal subunit protein S6 (RPS6) to promote translation (Ma and Blenis, 2009).

The best characterized translation control via translation-related factors is the phosphorylation of translation initiation factors, eIF2α and eIF4E, which regulate the efficacy of translation (Clemens, 2004). In addition, the general translation rate can be regulated by controlling the availability of the cap-binding protein eIF4E. The PI3K-AKT pathway phosphorylates eIF4E-binding protein 1 to release eIF4E, which binds to the 7-methyl GTP cap of mRNAs and increase the rate of initiation (Gebauer and Hentze, 2004; Ma and Blenis, 2009). In addition, a number of kinases including PKR and PERK are activated under different stresses to suppress global translation by coordinating the phosphorylation of eIF2α at Ser51 (Gebauer and Hentze, 2004).

Some featured regulatory sequences within the 5′-UTR of mRNA are also involved in global translation (Calvo et al., 2009; Komar and Hatzoglou, 2011). The internal ribosome entry site (IRES), a unique nucleotide sequence, allows recruitment of
the translation initiation complex downstream of the 5’ cap and translates IRES-containing mRNAs in a cap-independent manner (Komar and Hatzoglou, 2011). This type of initiation is commonly used during stress conditions under which the cap-dependent translation is damaged. Another example of a regulatory sequence as a common mechanism is the upstream open reading frame (uORF), which are mRNA elements defined by a start codon in the 5’-UTR that is out-of-frame with the main ORF and coding sequence (Calvo et al., 2009). uORFs typically interfere with expression of the downstream primary ORF by increasing translation initiation (Medenbach et al., 2011).

**Known mechanisms of DON-induced translational regulation**

DON-induced translation inhibition has been well documented in vivo and in vitro (Azcona-Olivera et al., 1995; Zhou et al., 2003b). It has been shown that DON-induced RSR involves three concurrent mechanisms giving rise to inhibition of translation (Figure 1.2). First, DON is known to cause the degradation of 18S and 28S rRNA (He et al., 2012c), and interfere with peptidyl transferase function on the ribosome, with consequent impairment of initiation and elongation (Shifrin and Anderson, 1999). Second, DON can induce the activation of ribosome-associated kinase PKR, which when activated, phosphorylates eIF2α, thereby inhibiting translation (Zhou et al., 2003b). Finally, DON can upregulate a large number of microRNAs (miRNAs) which can target mRNA for translational inhibition (He et al., 2010).

The weakened translation in early DON-induced RSR can liberate the translational machinery from pre-existing translation-competent transcripts so that newly made mRNAs may be able to more effectively compete for the translational machinery.
This shift in translation, known as “translational reprogramming” (Ron and Walter, 2007), may allow for a bias in the synthesis of proteins to combat the ribotoxic stress in the cell, and has been reported for several proinflammatory genes induced by DON in RAW 264.7 cells (He et al., 2013).

**Mass spectrometry in biology**

Around 20 years after its introduction to biology research, mass spectrometry (MS) has become widely used (Cravatt et al., 2007). It has revolutionized the way in which biological information, especially related to proteins, can be obtained. With time, MS will become a routine technique to tackle a wide variety of biological questions.

**General workflow of proteomics**

Mass spectrometry measures the mass to charge ratio (m/z) of molecules. In MS-based proteomics, the m/z values of peptides or small proteins are measured, which reflects their amino acid composition and possible post-translational modifications.

Proteins are enzymatically cleaved at specific sites to yield short peptides which typically consist of 6-20 amino acid residues. Mass spectrometers firstly examine the m/z value of these peptides (survey scan or MS1 scan) and secondly measure their fragmentation. Taking a protein database as a reference, mass spectra can be correlated to amino acid sequences with the aid of computer algorithms. The found peptide sequences are then assigned to proteins, which ultimate leads to protein identification (Aebersold and Mann, 2003).

Proteomics often deals with complex protein or peptide mixtures. As dynamic range and sequencing speed are the limiting factors in the current MS technology (de Godoy et al.,
2006), sample complexity has to be reduced. On-line separation with reverse phase chromatography (HPLC) connecting on-line to the mass spectrometer has proven to be a useful separation method (Figure 1.3). The C18 reverse phase HPLC column elutes peptide mixtures with linearly increasing organic solvent, e.g. acetonitrile (ACN). The gradual elution (typically at a few hundred nanoliter per minute) with shallow gradients increases available sequencing time in the MS. Prior to such hydrophobicity-based peptide separation, complex samples can be first be separated by one-dimensional gel (Aebersold and Mann, 2003), isoelectric focusing (Graumann et al., 2008), ion-exchange (Liu et al., 2002), molecular size (Lu et al., 2009), and affinity binding such as immunoprecipitation (Blagoev et al., 2003), IMAC (Gruhler et al., 2005) and TiO2 enrichment (de Godoy et al., 2006).

For less complex samples, static electrospray, namely nanoelectrospray (ESI), can be used which often yields better identification results. In particular static spray is beneficial for some modification studies (Liuni and Wilson, 2011).

Data analysis and information mining takes place at the end of the work flow. For example, much biological information can be discovered by using different clustering algorithms. Frequently used public resources are the Gene Ontology (GO; http://www.geneontology.org/) and KEGG pathway databases (http://www.genome.jp/kegg/). Integration of MS-based proteomics with other “omics” datasets can provide deeper insights. Examples of these valuable datasets are microarray based transcriptome studies (Kim et al., 2008) and protein-protein interactome and protein complex studies (Uetz et al., 2000; Gavin et al., 2002).
Modeling of molecular networking is emerging. It is expected that integration of these large-scale studies will deliver comprehensive understanding of a system which cannot be otherwise obtained by each of the separate approaches.

**Quantitation in proteomics**

Knowledge of protein abundance and modification, and their changes under different conditions are essential to study the toxicological impact of xenobiotics. Various MS-based quantitation methods have been developed. MS-based quantitation that entirely depends on the signal intensity of unlabeled peptides, i.e. label-free quantitation, is just emerging. This method demands a very precise and accurate performance of the whole proteomics workflow. In contrast, a large group of other methods employ stable isotopes for labeling peptides. They usually better tolerate the signal fluctuations in HPLC and mass spectrometers.

Stable isotope minimizes the impact on the physiochemical properties of the peptides. Protein/peptide samples that are labeled with stable isotopes have shifted m/z values when compared to their natural, non-isotope-labeled counterparts but are otherwise identical in all respects. Thus, stable isotopes such as $^{13}$C, $^{15}$N, and $^{18}$O do not induce shifts in HPLC retention times (Zhang and Regnier, 2002). Therefore labeled and non-labeled peptides show up as pairs in mass spectra, and their relative intensities can be directly visualized (Ong *et al.*, 2002). Generally, there are two ways to label proteins or peptides with stable isotopes (Fig 1.4) (Bantscheff *et al.*, 2007). Metabolic labeling supplies stable isotopes during the growth and development of cells (Ong *et al.*, 2002) and organisms (Wu *et al.*, 2004). Chemical labeling modifies certain amino acid
Figure 1.2 Known mechanisms of DON-induced translation mediated by (A) rRNA damage, (B) activation of PKR and eIF2α and (C) miRNA.
Figure 1.3 General workflow for proteomic analysis. Proteins from biological samples such as animal tissues and cell cultures can be extracted and digested into peptides. HPLC can be coupled to MS to generate information of peptide primary structure. Data search using computer algorithms to match mass spectra to amino acid sequences could then be performed, and bioinformatic analysis reveals biological significance of proteomic alterations.
side chains with natural or isotope-labeled reagents (Gygi et al., 1999).

**Relative quantitation - metabolic labeling**

It is advantageous to mix samples at an early stage of the experiment to avoid accumulating systematic errors, which can translate into inaccurate quantitation results in the sensitive mass spectrometry measurement. From this perspective, metabolic labeling is superior to other quantitation methods. Stable isotope labeling with amino acids in cell culture (SILAC) is an established method with all the strengths of the metabolic labeling strategy (Ong et al., 2002) (Fig 1.4). SILAC labeling utilizes arginine and lysine with heavy elements of $^{13}\text{C}$, $^{15}\text{N}$, and $^2\text{H}$. The most commonly used forms are $^{13}\text{C}_6$-Arg, $^{13}\text{C}_6^{15}\text{N}_4$-Arg, $^2\text{H}_4$-Lys and $^{13}\text{C}_6^{15}\text{N}_2$-Lys. Up to three different biological conditions can be compared in a single SILAC experiment.

**Relative quantitation - chemical labeling**

iTRAQ (an isobaric tag for relative and absolute quantitation) is a chemical labeling method which can quantify up to eight different conditions in single experiment (Pierce et al., 2008). A multiplexed set of isobaric reagents are used to modify each sample. Differently modified peptides display no difference in MS scans due to the isobaric property of the mass tags. However, after peptide fragmentation the unique, low-mass reporter ion of each tag (113-121 Da) is displayed in MS/MS spectra. Quantitation is therefore carried out in MS/MS spectra rather than MS spectra.
Dimethyl labeling involves chemically labeling primary amine groups (lysine and amino termini) of peptides with different isotopically labeled formaldehyde through reductive amination (Kovanich et al., 2012). The only exception is in the rare occurrence of an N-terminal proline, for which a monomethylamine is formed. By using combinations of several isotopomers of formaldehyde and cyanoborohydride, peptide triplets can be obtained that differ in mass by a minimum of 4 Da between the different samples. Stable isotope dimethyl labeling is a relatively simple and economic strategy for large-scale quantitative analyses.

**Absolute quantitation**

Absolute quantitation is another challenge in quantitative proteomics. Using a reference sample with known amount, the aforementioned quantitation methods can be used to deduce the absolute amount of proteins in the sample. Successful examples include the precise quantitation of Grb2 copy numbers in HeLa cells by means of absolute-SILAC (Hanke et al., 2008).

**Phosphoproteomics**

One major conduit of signal transduction is mediated via reversible phosphorylation of proteins. In eukaryotes, phosphorylation occurs chiefly on serine, threonine, and tyrosine residues, with the relative abundance of phosphoserine:phosphothreonine: phosphotyrosine is 1800:200:1 in vertebrates (Mann et al., 2002). Early estimates suggest that there are over 100,000 phosphorylation sites in human proteome. While ubiquitously distributed, phosphoproteins are typically of low abundance (Reinders and Sickmann, 2005). The overall level of phosphorylation is
regulated by an interplay between the activities of protein kinases, as well as protein phosphatases within cells.

To enrich the low abundant phosphorylated proteins or peptides, various methods have been developed. Chromatographic methods include affinity binding to metal or metal oxide chromatography (Posewitz and Tempst, 1999; Larsen et al., 2005), and ion exchange (Ballif et al., 2004), and phospho-antibodies (Blagoev et al., 2004; Schmelzle et al., 2006). Among these, the chromatography methods are most widely adopted due to their high enrichment efficiency and experimental simplicity (Palumbo et al., 2011).

**TiO$_2$ chromatography**

Titanium dioxide (TiO$_2$) particles are stable with regards to mechanical, chemical and thermal stress. Heck and coworkers demonstrated that TiO$_2$ chromatography can achieve very high enrichment efficiency (90%) for phosphopeptides in simple samples (Pinkse et al., 2004). For complex samples, however, non-specific binding of acidic amino acid residues such as glutamic acid and aspartic acid becomes significant. Larsen et al. proposed the use of 2,5-dihydroxy benzoic acid (DHB) to compete with the acidic peptides from binding (Larsen et al., 2005). Because the binding strengths decrease from phosphopeptide to DHB to acidic peptides, this approach has proven to be very successful in large scale phosphoproteomic studies (Olsen et al., 2006).
Figure 1.4 Common quantitative mass spectrometry workflows. Boxes in blue and yellow represent two experimental conditions. Horizontal lines indicate when samples are combined. Dashed lines indicate points at which experimental variation and thus quantification errors can occur. From (Bantscheff et al., 2007).
**Immobilized metal affinity chromatography (IMAC)**

Phosphopeptide enrichment by IMAC is based on the high-affinity coordination of phosphates to certain trivalent metal ions. Metal ions are immobilized by loading onto porous column packing material, and phosphopeptides are subsequently captured by a metal complex formed on the distant side of the immobilized metal ion. Tempst and coworkers assessed the capacity and selectivity of IMAC for phosphopeptide binding for a variety of metals, including Fe$^{3+}$, Ga$^{3+}$, Al$^{3+}$ and Zr$^{3+}$ (Posewitz and Tempst, 1999). They observed best selectivity with iminodiacetate columns complexed with Ga$^{3+}$.

Similar to TiO$_2$, one challenge in IMAC is that strongly acidic peptides rich in glutamic and aspartic acid residues also coordinate well with metal complexes. Therefore, additional chemistry (e.g., methylation) or separation methods are often needed to prevent displacement of phosphopeptides by abundant acidic peptides (Beltran and Cutillas, 2012).

**Strong cation exchange (SCX)**

SCX is a low resolution but robust enrichment method (Ballif et al., 2004). The principle of using SCX in phosphopeptide analysis is based on reduced positive charges on the phosphorylated peptides. Most tryptic peptides carry one positive charge at each peptide terminus at pH 2.7, as specified in the SCX buffer (NH$_4^+$ from the N-terminal amino group and the positively charged side chain of trypsin or lysine). The negatively charged phosphate group can counter the positive charges, effectively reducing the charge state, and therefore decrease the binding to the SCX column. Generally multiply phosphorylated peptides bind to the column with minimum affinity, while non-
phosphorylated peptides bind strongly. However, acidic amino acids (glutamic acid and aspartic acid) can interfere with this strategy. Gygi and coworkers demonstrated large scale identification of 2,001 phosphopeptides using SCX fractionation (Beausoleil et al., 2004).

**Antibody-based enrichment**

Tyrosine phosphorylation comprises less than 2% of the cellular phosphorylation events (Mann et al., 2002). Fortunately good quality antibodies of phospho-tyrosine (pTyr) can be employed to enrich pTyr containing proteins or peptides (Blagoev et al., 2004; Schmelzle et al., 2006). Antibodies generated against kinase substrate motifs, such as AKT motif, are also used to enrich substrates of specific kinases (Barati et al., 2006).

**Organelle proteomics**

Compartmentalization of eukaryotic cells provides distinct and suitable environments for biochemical processes such as protein synthesis and degradation, provision of energy-rich metabolites, protein glycosylation, DNA replication. Protein localization is linked to cellular function, and introduces an analytical challenge for proteomics that requires the proteome analysis with subcellular resolution. The analysis of dynamic proteome changes at a subcellular level promises to yield significant insight into biological mechanisms as well as to the molecular basis of cellular dysfunction during environmental perturbation. The strategies used for protein identification in the proteome analysis at the level of subcellular structures are not principally different from that in other proteomics strategies. However, enrichment for particular subcellular
structures by subcellular fractionation and reduction of sample complexity facilitates the analysis (Dreger, 2003).

Subcellular fractionation strategies represent the centerpiece of subcellular proteome analysis. The efficiency of fractionation is critical for the information content of the whole study, such as the accuracy with which proteomics data allow one to assign potential newly discovered proteins to subcellular structures (Duclos and Desjardins, 2011). However, the accuracy of protein identification not tested by independent methods is compromised by the presence of contaminants derived from other subcellular structures, as well as by the resolution of the study (Dreger, 2003). Many separation/identification systems perform better when the sample complexity is reduced, so that the spectrum of applicable techniques is wider in subcellular proteomics than in strategies designed for more complex samples. Some commonly used methods in sample complexity reduction include 1D gel electrophoresis, isoelectric focusing (IEF) and matrix-assisted laser desorption ionization (MALDI) (Dreger, 2003).

Ribosome composition and post-translational modifications have been studied using proteomics. Basic techniques to isolate ribosomes were developed in the 1960s and 1970s, and are still widely used with relatively minor modifications (Mehta et al., 2012). Most of these techniques require differential or density gradient ultracentrifugation of cell lysates to yield ribosomes or ribosomal subunits. Affinity tagging followed by immunoprecipitation has also been applied to purify the ribosome. (Inada et al., 2002; Zanetti et al., 2005). Proteomics has been applied to ribosomal fractions for identification of ribosome composition and usually involves further fractionating of the ribosomal fractions using 1D or 2D gel electrophoresis (Chang et al., 2005; Carroll et al.,
2008). In addition, proteomics has been applied to identify covalent post-translational modification of ribosomal proteins, such as phosphorylation (Chang et al., 2005; Carroll et al., 2008).

Combining relative quantitation and organelle fractionation allows one to compare the effect of xenobiotics on a particular subcellular compartment over time or upon different treatments. Such studies enable modes of action understanding on the subcellular level.
CHAPTER 2: Global Protein Phosphorylation Dynamics during Deoxynivalenol Induced Ribotoxic Stress Response in the Macrophage


Abstract

Deoxynivalenol (DON), a trichothecene mycotoxin produced by Fusarium that commonly contaminates food, is capable of activating mononuclear phagocytes of the innate immune system via a process termed ribotoxic stress response (RSR). To encapture global signaling events mediating RSR, we quantified the early temporal (<30 min) phosphoproteome changes that occurred in RAW 264.7 murine macrophage during exposure to a toxicologically relevant concentration of DON (250 ng/mL). Large-scale phosphoproteomic analysis employing stable isotope labeling of amino acids in cell culture (SILAC) in conjunction with titanium dioxide chromatography revealed that DON significantly upregulated or downregulated phosphorylation of 188 proteins at both known and yet-to-be functionally characterized phosphosites. DON-induced RSR is extremely complex and goes far beyond its prior known capacity to inhibit translation and activate MAPKs. Transcriptional regulation was the main target during early DON-induced RSR, covering over 20 percent of the altered phosphoproteins as indicated by Gene Ontology annotation and including transcription factors/cofactors and epigenetic modulators. Other biological processes impacted included cell cycle, RNA processing,
translation, ribosome biogenesis, monocyte differentiation and cytoskeleton organization. Some of these processes could be mediated by signaling networks involving MAPK-, NFκB-, AKT- and AMPK-linked pathways. Fuzzy c-means clustering revealed that DON-regulated phosphosites could be discretely classified with regard to the kinetics of phosphorylation/dephosphorylation. The cellular response networks identified provide a template for further exploration of the mechanisms of trichothecene mycotoxins and other ribotoxins, and ultimately, could contribute to improved mechanism-based human health risk assessment.

**Introduction**

The trichothecene mycotoxin deoxynivalenol (DON) is a common food contaminant that targets the innate immune system and is therefore of public health significance (Pestka, 2010a). DON’s immunomodulatory effects have been demonstrated *in vivo* in mouse systemic and mucosal immune organs (Zhou *et al.*, 1999) as well as in primary and cloned murine monocyte/macrophage cultures derived from mice and humans (Islam *et al.*, 2006). Most notably, DON stimulates proinflammatory gene expression at low or modest concentrations via a process known as “ribotoxic stress response” (RSR) (Iordanov *et al.*, 1997; Laskin *et al.*, 2002; Pestka *et al.*, 2004). Innate immune system activation is central to both shock-like and autoimmune effects associated with acute and chronic DON exposure, respectively (Pestka, 2010a).

DON-induced RSR involves the transient activation of at least two upstream ribosome-associated kinases, double-stranded RNA-dependent protein kinase (PKR) and hematopoietic cell kinase (HCK), which are phosphorylated within minutes of DON exposure (Zhou *et al.*, 2003b; Zhou *et al.*, 2005b; Bae *et al.*, 2010). Although
phosphorylation of mitogen-activated protein kinases (MAPKs) and their substrates clearly play pivotal roles in modulating downstream events, the DON-induced RSR signaling network is not yet comprehensively understood from the perspectives of 1) identity and extent of critical proteins involved, 2) kinetics of early signaling changes and 3) early downstream events that contribute to toxic sequelae.

Resolving the complexity of DON-induced RSR requires a sensitive, integrative approach for dissecting the molecular events occurring at the cellular and subcellular level. Proteomics facilitates large-scale identification and quantification of proteins, providing information on protein expression and post-translational modification (Farley and Link, 2009; Mallick and Kuster, 2010). Proteome and phosphoproteome changes occurring after prolonged (6 h or 24 h) DON treatment have been previously measured in human B and T cell lines (Osman et al., 2010b; Nogueira da Costa et al., 2011a; Nogueira da Costa et al., 2011b). While these studies are important for identification of biomarkers of effect, they are not informative from perspective of early events and signaling within the macrophage, a primary target of DON which mediates innate immune activation (Zhou et al., 2003b; Pestka, 2008). Stable isotope labeling of amino acids in cell culture (SILAC) has been successfully used to characterize the signaling and subcellular compartmentalization for global delineation of macrophage behavior during phagocytosis and upon toll-like receptor stimulation (Rogers and Foster, 2007; Dhungana et al., 2009), suggesting the applicability of this strategy to the study of DON-induced RSR.

The goal of this study was to test the hypothesis that DON induces ordered phosphorylation changes in proteins in the macrophage that are associated with
intracellular signaling and key biological processes that enable it to adapt and respond to RSR. Specifically, we identified and quantitated early phosphoproteomic changes induced by DON in the RAW 264.7 cells, a well-established murine macrophage model (Raschke et al., 1978; Hambleton et al., 1996) that has been used to extensively to investigate the effects of this mycotoxin on the innate immune response (Pestka, 2010a). Critical features of this investigation were the use of a moderate concentration (250 ng/mL) and short time period (0 to 30 min) to mimic acute in vivo DON exposure based on the pharmacokinetic distribution and local concentration of DON in immune organs (Azcona-Olivera et al., 1995; Pestka and Amuzie, 2008). We further employed SILAC in conjunction with titanium dioxide (TiO2) chromatography and LC-MS/MS to ensure accurate quantitative phosphoproteomics (Ong et al., 2002). This large-scale phosphoproteomic analysis revealed that DON-induced RSR is extremely complex and goes far beyond its prior known capacity to activate MAPKs. These findings further provide a foundation for future exploration and elucidation of cellular response networks associated with toxicity evoked by DON and potentially other ribotoxins.

**Materials and Methods**

**Experimental design**

Protein phosphorylation changes during DON-induced RSR were measured in RAW 264.7 cells (American Type Tissue Collection, Rockville, MD) by a multitiered approach exploiting SILAC for quantification, TiO2 chromatography for phosphopeptide enrichment and high-accuracy mass spectrometric characterization (Olsen et al., 2006) (Figure 1). Briefly, RAW 264.7 cells were labeled with L-arginine and L-lysine (R0K0), L-
arginine-$^{13}\text{C}_6^{14}\text{N}_4$ and L-lysine-$^2\text{H}_4$ (R6K4), or L-arginine-$^{13}\text{C}_6^{15}\text{N}_4$ and L-lysine-$^{13}\text{C}_6^{15}\text{N}_2$ (Cambridge Isotope Laboratories, Andover, MA) (6 plates of 80% confluent cells per condition) in Dulbecco's Modified Eagle Medium (DMEM, from SILAC™ Phosphoprotein ID and Quantitation Kit, Invitrogen, Grand Island, NY). Since SILAC requires sufficient proliferation for the full incorporation of labeled amino acids into the cellular proteome, metabolic labeling was performed for six cell doubling times ($\approx 108$ h). The defined mass increments introduced by SILAC among the three RAW 264.7 populations resulted in characteristic peptide triplets that enabled relative quantification of peptide abundance.

Labeled RAW 264.7 cells were treated with 250 ng/mL of DON for 0 min, 5 min, and 30 min. A second, identically labeled set of cells were treated with DON for 1 min, 15 min, and 30 min. Nuclear and cytoplasmic fractions were extracted using a Nuclear Extract Kit (Active Motif, Carlsbad, CA). Each fraction of three treatment types were pooled equally based on protein content as determined by BCA Protein Assay (Pierce, Rockford, IL). These resultant mixtures were then subjected to phosphoproteomic analysis. Each time course set was repeated in three independent experiments (n=3) to account for biological and technical variability.

**Phosphopeptide enrichment**

Proteins were digested with trypsin according to the filter-aided sample preparation (FASP) protocol (Wisniewski et al., 2009) using spin ultrafiltration units of nominal molecular weight cut of 30,000 (Millipore, Billerica, MA). The peptides were desalted with 100 mg reverse-phase tC18 SepPak solid-phase extraction cartridges
(Waters, Milford, MA), and enriched for phosphopeptide with Titansphere PHOS-TiO Kit (GL Sciences, Torrance, CA) following manufacturer’s instructions. Enriched peptides were desalted again prior to LC-MS/MS.

**Mass spectrometry**

Peptides were resuspended in 2% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) to final concentration of 20 µg/µL. From this, 10 µL (200 µg) were automatically injected using a Waters nanoAcquity Sample Manager (www.waters.com) and loaded for 5 min onto a Waters Symmetry C18 peptide trap (5 µm, 180 µm x 20 mm) and washed at 4 µL/min with 2% ACN/0.1% Formic Acid. Bound peptides were eluted using a Waters nanoAcquity UPLC (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 99.9% ACN/0.1% Formic Acid) onto a Michrom MAGIC C18AQ column (3 u, 200 A, 100 U x 150 mm, www.michrom.com) and eluted over 360 min with a gradient of 2% B to 30% B in 347 min at a flow rate of 1 µl/min. Eluted peptides were sprayed into a ThermoFisher LTQ-FT Ultra mass spectrometer (www.thermo.com) using a Michrom ADVANCE nanospray source. Survey scans were taken in the FT (25000 resolution determined at m/z 400) and the top ten ions in each survey scan were then subjected to automatic low energy collision induced dissociation (CID) in the LTQ.

**Data analysis**

Peak list generation, protein quantitation based upon SILAC, extracted ion chromatograms (XIC) and estimation of false discovery rate (FDR) were all performed using MaxQuant (Cox and Mann, 2008), v1.2.2.5. MS/MS spectra were searched against the IPI mouse database, v3.72, appended with common environmental contaminants using the Andromeda searching algorithm (Cox et al., 2011). Further
statistical analysis of the SILAC labeled protein and peptide ratio significance was performed with Perseus (www.maxquant.org). MaxQuant parameters were protein, peptide and modification site maximum FDR = 0.01; minimum number of peptides = 1; minimum peptide length = 6; minimum ratio count = 1; and protein quantitation was done using all modified and unmodified razor and unique peptides. Andromeda parameters were triplex SILAC labeling: light condition (no modification), medium condition (Arg6, Lys4), heavy condition (Arg10, Lys8); fixed modification of Carbamidomethylation (C), variable modifications of Oxidation (M), Phosphorylation (STY) and Acetyl (Protein N-term); Maximum number of modifications per peptide = 5; enzyme trypsin max missed cleavage = 2; parent Ion tolerance = 6ppm; fragment Ion tolerance = 0.6 Da and reverse database search was included. For each identified SILAC triplet, MaxQuant calculated the three extracted ion chromatogram (XIC) values, and XICs for the light and medium member of the triplet were normalized with respect to the heavy common 30 min time point of DON treatment, which was scaled to one. A significance value, Significance B, was calculated for each SILAC ratio and corrected by the method of Benjamini and Hochberg using the statistical program Perseus (Cox and Mann, 2008). Significance B values of a phosphopeptide in the two sets of time courses are calculated independently. Significant protein ratio cutoff was set as at least one set of time course has a Significance B value ≤0.05 as calculated by MaxQuant (Cox et al., 2009). Phosphosites were mapped with PhosphoSitePlus (Hornbeck et al., 2012).

**Verification by Western analysis**

Immunoblotting analysis was performed on selected proteins as described previously (Bae and Pestka, 2008) to verify phosphoproteome quantitation. Antibodies
against the following proteins were used: p38, phospho-p38 MAPK (Thr180/Tyr182), p42/p44 MAP kinase, phospho-p42/p44 MAP kinase (Thr202/Tyr204), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), GAPDH, stathmin (Cell Signaling, Danvers, MA), phospho-stathmin (Ser25, Santa Cruz, CA). Blots were scanned on the Odyssey IR imager (LICOR, Lincoln, NE) and quantification was performed using LICOR software v.3.0.

**DAVID analysis**

Analyses for gene ontology (GO) annotation terms were performed with the functional annotation tool DAVID (http://david.abcc.ncifcrf.gov/). Uniprot accession identifiers of significantly regulated phosphoproteins were submitted for analysis of GO biological processes and KEGG pathways, with a maximal DAVID EASE score of 0.05 for categories represented by at least two proteins.

**Fuzzy c-means clustering**

Patterns in the time profiles of regulated phosphopeptides, all phosphopeptides identified were sought by fuzzy c-means (FCM) clustering (Olsen et al., 2006) in MATLAB (MathWorks). To robustly represent the ratio of a peptide being quantified multiple times, the median values of the three biological replicates were used for clustering. SILAC ratios of peptides quantified over all five time points were included, natural log transformed and normalized. From the 40 stable clusters, we selected 6 representing early, intermediate and late responders with phosphorylation levels up- or down-regulated based on the average probability of each cluster.
Results and Discussion

**DON-induced changes in phosphoproteomic profile**

DON induced the activation p38, ERK and JNK, the marker of DON-induced RSR, in SILAC-labeled RAW 264.7 (Figure 2.1) comparable to that described previously in unlabeled RAW 264.7 (Zhou et al., 2003b), demonstrating the validity of this model to study RSR. Using SILAC in conjunction with TiO₂ chromatography and LC-MS/MS (Figure 2.2), more than 96% of identified peptides were found to harbor at least one phosphorylation residue, indicating that phosphopeptide enrichment by TiO₂ was highly efficient. We reproducibly identified 1112 and 680 unique phosphopeptides corresponding to 374 and 316 proteins in the nuclear and cytoplasmic fractions, respectively, at an accepted FDR of 1% (Figure 2.3). Approximately 17% of the total phosphopeptides identified were considered significant (FDR≤0.05). The distribution of phosphorylated serine, threonine, and tyrosine sites was 84%, 14% and 2%, respectively (Table 2.1). Based on PhosphoSitePlus annotation, the extensive phosphoproteome changes occurred in the macrophage early during DON-induced RSR encompass known and yet-to-be functionally characterized phosphosites.

**Confirmation of proteomic results**

To verify the quantification of phosphorylation in SILAC-based proteomics, the phosphorylation kinetics of three representative proteins, p38, ERK1 and STMN1, were measured by Western blotting using phosphosite-specific antibodies. SILAC- and immunoblot-based relative quantifications were in agreement (Figure 2.4), indicating the SILAC method to be a viable approach to study the dynamic effects of DON-induced RSR.
**Biological analysis of altered phosphoproteins**

To discern the potential impact of DON-induced phosphorylation, changes prevalence of differentially phosphorylated proteins were related to specific biological processes using GO annotation in DAVID (Figure 2.5). The process most affected during DON-induced RSR was regulation of transcription, which constituted more than 20 percent of all significantly regulated phosphoproteins and included transcription factors (Figure 2.6A), cofactors (Figure 2.6B), and epigenetic modulators (Figure 2.6C). Functional annotation further indicated that phosphoproteins affected during DON-induced RSR might impact other biological processes including cell cycle, RNA processing, translation, ribosome biogenesis, monocyte differentiation and cytoskeleton organization. For example, cyclin-dependent kinase 1/2 (CDK1/2) dephosphorylation (Thr14), which promotes G2/M transition (Mayya et al., 2006), was observed at 15 min. Also, modulation of translation (eIF4G1, eEF1B and eEF1D) might influence protein expression during DON-induced RSR. Finally, ribosomal RNA-processing protein 8 and 9 (RRP8 and RRP9), nucleophosmin (NPM1) and other proteins that function in the ribosome biogenesis were also differentially phosphorylated, suggesting that fine-tuning of translation likely occurs in early DON-induced RSR before onset of large-scale translation inhibition.
Figure 2.1. Western blot of p38, ERK and JNK in SILAC-labeled RAW 264.7 with total and phospho-specific antibodies, with GAPDH as internal control. The activation of MAPKs was comparable to that in unlabeled RAW 264.7 ensuring the SILAC-labeled behave similarly to their unlabeled counterparts. Results shown are representative of 3 replicate experiments.
Figure 2.2. Experimental design for SILAC-based quantitative phosphoproteomic analysis of DON-induced RSR. RAW 264.7 cultured in media supplemented with normal lysine and arginine (Lys0, Arg0), medium-labeled lysine and arginine (Lys4, Arg6), and heavy-labeled lysine and arginine (Lys8, Arg10) were subjected different DON treatments in two sets of experiments. Nuclear and cytoplasmic proteins were extracted, mixed equally among different groups based on protein amount and trypsin digested. Phosphopeptides were enriched by titanium dioxide chromatography and analyzed by high-resolution mass spectrometry. Each set was repeated in three independent experiments (n=3).
Figure 2.3. DON-induced RSR involves extensive protein phosphorylation changes. The numbers of significantly regulated (grey) phosphorylated proteins (A) and peptides (B) as determined by Significance B corrected for FDR≤0.05 among all unique proteins and peptides identified (grey + black)
Table 2.1. Distribution of phospho-serine, threonine and tyrosine identified in RAW 264.7 cells

| Site | Nuclear | | | Cytoplasmic | | |
|------|---------|---------------|---------------|---------------|---------------|---------------|---------------|
|      | Number all | Percent all | Number significant | Percent significant | Number all | Percent all | Number significant | Percent significant | |
| pSer | 921 | 83.8% | 160 | 86.5% | 505 | 83.2% | 114 | 87.0% |
| pThr | 158 | 14.4% | 25 | 13.5% | 88 | 14.5% | 12 | 9.2% |
| pTyr | 20 | 1.8% | 0 | 0.0% | 14 | 2.3% | 5 | 3.8% |
Figure 2.4. Confirmation of phosphoproteome quantification. Phosphorylation levels were measured using Western blot by normalizing the signaling intensity of the bands from phosphosite-specific antibody with those from total antibody. Normalized intensities (blue, shown as mean ± SEM) were then correlated with the SILAC ratios (red, shown as median).
Figure 2.5. Gene Ontology associated with significantly regulated phosphoproteins as determined by DAVID Biological Process terms (DAVID EASE score < 0.05). Data are presented as a histogram of the relevant biological processes identified and shown as a percentage of the total identified proteins that fall within each category.
Figure 2.6. Phosphorylation changes of transcription factors (A), cofactors (B), and epigenetic regulators (C) during DON-induced RSR. The heat map depicts the log 2 transformed relative abundance (red to green) of the protein phosphorylation.
Figure 2.7. Phosphorylation changes of proteins involved in the MAPK (A), NFκB (B), AKT and AMPK pathways (C) during DON-induced RSR. The heat map depicts the log 2 transformed relative abundance (red to green) of the protein phosphorylation.
Concomitant with impacting transcriptional and other processes, DON altered the phosphorylation of proteins mediating MAPK (Figure 2.7A), NFκB (Figure 2.7B), and AKT/AMPK (Figure 2.7C) signaling, suggesting likely roles for these master regulators.

Consistent with our findings, DON has previously been shown to alter RNA processing, as well as ribosome structure and function, during prolonged RSR in human lymphoid cells (Katika et al., 2012b). During early DON-induced RSR, fine-tuning of the transcriptional activity is a hallmark of cells in responding to the stress, with concurrent translational activity as an assisting measure. If stress persists, such balance likely shifts towards regulation of translation for systematic shutdown, via mechanisms such as phosphorylation of eIF2α, damage of the peptidyl transferase center and cleavage of rRNA (Pestka, 2010a).

**Integration of phosphoproteomic data with prior studies - transcriptional regulation**

The extent of gene expression generally reflects the availability of mRNA for translation through modulation of transcription and mRNA stability. Prolonged exposure to DON has been previously shown to activate transcription factors (e.g., c-JUN, NF-κB, CREB, AP-1 and C/EBP) in RAW 264.7 at 2 hr (Wong et al., 2002). Consistent with these findings, we confirmed c-JUN phosphorylation at Ser63, and further identified phosphorylation sites on other transcription factors not previously known to be modulated by DON (Figure 2.6A). For example, n-MYC (V-myc myelocytomatosis viral related oncogene, neuroblastoma derived, MYCN) targets and enhances the expression of genes functioning in ribosome biogenesis and protein synthesis, and thus modulates the abundance of the translation machinery (Boon et al., 2001). Since n-MYC also
regulates global chromatin structure (Murphy et al., 2009), modification of its phosphorylation might simultaneously function in epigenetic regulation.

Transcription cofactors were similarly impacted during DON-induced RSR (Figure 2.6B). For example, MYBBP1A, found to be rapidly dephosphorylated at 30 min, is known to interact with and activate several transcription factors such as c-JUN, while repressing others such as PPARγ co-activator 1α (PGC-1α), NFκB, and c-MYB (Fan et al., 2004; Owen et al., 2007; Yamauchi et al., 2008). This protein was reported to be upregulated at the protein level by DON (150 ng/mL) at 6 h in a prior proteomic study in EL4 mouse thymoma cells (Osman et al., 2010b). Another interacting protein and repressor of c-MYB, tripartite motif protein 28 (TRIM28), was altered via phosphorylation. TRIM28 is a universal co-repressor that induces cell differentiation in the monocytic cell line U937 via its interaction and activation of C/EBP beta (Rooney and Calame, 2001), a transcription factor also activated by DON (Wong et al., 2002). Overall, phosphorylation alterations of these two MYB-binding proteins as observed in our analysis suggest that MYB transcription factor complex could be a possible hub for transcriptional regulation during DON-induced RSR.

DON might also affect gene expression via epigenetic processes (Figure 2.6C). Altered phosphoproteins included histone deacetylases 1 and 2 (HDAC1 and HDAC2), GATA zinc finger domain-containing protein 2A (GATAD2A), metastasis-associated protein 3 (MTA3) - all four proteins function as core components of the nucleosome remodeling and deacetylase (NuRD) complex, an ATP-dependent chromatin remodeling entity (Lai and Wade, 2011). Phosphorylation at Ser421 and Ser423 (significantly regulated by DON) of HDAC1 promotes enzymatic activity and interactions
with the NuRD complex (Pflum et al., 2001). Similarly, HDAC2 interaction with co-repressor proteins and transcription factors is further dependent on phosphorylation at Ser422 and Ser424 (also significantly regulated by DON) (Adenuga and Rahman, 2010). Phosphorylation changes of HDAC1 and HDAC2 at these sites could impact NuRD assembly and enzymatic activity (Adenuga and Rahman, 2010) and further modulate gene expression (Kurdistani et al., 2004). Another phosphoprotein altered by DON treatment, promyelocytic leukaemia (PML), is part of an oncogenic transcription factor that recruits the NuRD complex to target genes through direct protein interaction (Lai and Wade, 2011). Thus, DON impacted several critical regulators of chromatin remodeling.

Besides nucleosome remodeling, DON also altered phosphorylation status of key regulators in DNA methylation and microRNA biogenesis (Figure 2.6C). Most notably, differential phosphorylation of DNA methyltransferase 1 (DNMT1) at multiple sites that dictate its DNA-binding activity (Sugiyama et al., 2010; Esteve et al., 2011) was identified. Also affected was SNIP1, which interacts with Drosha and is involved in the biogenesis of miRNAs (Yu et al., 2008). Consistent with this observation, DON has been shown to alter miRNA profile in RAW 264.7 (He and Pestka, 2010). We further identified other mediators of epigenetic regulation. Ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1) recruits DNMT1 to hemi-methylated DNA (Sharif et al., 2007) and binds to the tail of histone H3 in a methylation sensitive manner, bridging DNA methylation and histone modification (Sharif et al., 2007). Besides being a transcriptional co-repressor, the aforementioned TRIM28 is recruited to DNA and subsequently initiates epigenetic silencing by recruiting histone deacetylase, histone
methyltransferase, and heterochromatin protein 1 (HP1) family members (Ellis et al., 2007). This process could be inhibited by phosphorylation of TRIM28 at Ser473 (Hu et al., 2012). Taken together, in early DON-induced RSR, regulation of transcription is the main target as reflected on the large scale of phosphorylation changes of proteins in the transcription factor/cofactor paradigm, as well as epigenetic regulation of transcription.

Although the potential for DON to evoke epigenetic effects has been indicated previously in a human intestinal cell line in which DON treatment increased overall genomic DNA methylation (Kouadio et al., 2007), the mechanisms for such effects were not understood. Our analysis provides a plausible explanation as evidenced by altered phosphorylation of DNA methyltransferase DNMT1 at phosphosites important for regulating its activity, and reveals further possible epigenetic effects linked to DON-induced RSR such as nucleosome remodeling and miRNA biogenesis. Effects on nucleosome remodeling by macrophage-stimulating non-ribotoxic agents have been reported previously. For example, lipopolysaccharide (LPS) induces NuRD component MTA1 depletion (Pakala et al., 2010) and selective recruitment of Mi-2β-containing NuRD complex (Ramirez-Carrozzi et al., 2006). However, post-translational modification across multiple components of the NuRD complex as shown here is novel suggesting that these latter epigenetic effects might be a specific ribotoxin-linked response. Additional studies are needed to elucidate how epigenetic regulation coordinates the balance between induction of stress related proteins and the global system shutdown during RSR caused by DON.
Integration of phosphoproteomic data with prior studies - kinase signaling

DON might broadly impact kinase signaling pathways (Figure 2.7, 2.8). Activation of MAPK cascades is considered a central feature of RSR (Iordanov et al., 1997), and indeed, we were not only able to recapitulate the activation of p38, ERK, and c-JUN, but, in addition, identified other potential mediators upstream and downstream to MAPK (Figure 2.7A, 2.8A). These included B-RAF which activates ERK via MEK1/2 directing cell proliferation, as well as MLK-like mitogen-activated protein triple kinase (MLTK) which can activate JNK-mediated pathway regulating actin organization, and has been shown to be an upstream MAP3K in stress induced by the ribotoxins anisomycin, ricin and Shiga toxin (Gotoh et al., 2001; Jandhyala et al., 2008).

Potential downstream effectors of the activated MAPKs were also identified that are capable of modulating both cell structure and function during DON-induced RSR. One of these, stathmin (STMN1), is a small binding protein that regulates the rates of microtubules polymerization and disassembly. ERK-driven phosphorylation of STMN1 at four serine residues including Ser25 (also significantly regulated by DON) leads to cell cycle arrest and apoptosis (Nakamura et al., 2006). Another was cytosolic phospholipase A2 (cPLA2), the phosphorylation of which is required for the release of arachidonic acid, and has been implicated in the initiation of inflammatory responses (Pavicevic et al., 2008). Activation of cPLA2 could set the stage for the later prostaglandin E2 (PGE2) production catalyzed by cyclooxygenase-2 (COX-2),
Figure 2.8. Signaling pathways mediating DON-induced RSR, including the MAPK (A, blue), NFκB (B, red), AKT and AMPK pathways (C, green). Dark-filled boxes indicate novel mediators based on this study, light-filled boxes indicate known mediators identified here, open boxes with solid outline indicate known mediators not identified here, and open boxes with dashed outline indicate known mediators in canonical pathway and not identified here.
Figure 2.8. (cont’d)

Modulation expression of cytokines (e.g. IL-1β, IL-6), chemokines (e.g. MIP-2, MCP-1) and other inflammatory-related genes (e.g. CD40, TLR2)

Modulation of energy metabolism (e.g. via GSK3β, PGC1α), translation regulation (e.g. via eIF4G), apoptosis (e.g. via p53, NFκB)
which is consistent with previous observations in DON-treated cells and animals (Moon and Pestka, 2002). Accordingly, these phosphoproteome data provide new insights into precursor events and functional consequences of MAPK pathways during DON-induced RSR.

Among other proteins identified, metadherin (LYRIC), programmed cell death protein 11 (PDCD11) and p38 are responsible for positive regulation of the I-kappaB kinase/NF-kappaB (IKK/NFkB) cascade (Saccani et al., 2002; Sweet et al., 2003; Emdad et al., 2006) (Figure 2.7B, 2.8B). Furthermore, Smad nuclear interacting protein (SNIP1) and Myb-binding protein 1A (MYBBP1A) function as repressors of NF-κB signaling pathway by competing with the NFκB transcription factor p65/RelA for binding to the transcriptional coactivator p300 (Kim et al., 2001; Owen et al., 2007). Collectively these data suggest that the NFκB pathway might be crucial transcription regulation in DON-induced RSR. Importantly, functions of some phosphosites identified here have not been previously reported, suggesting the need for extended investigation of NFκB function in DON toxicity.

AKT- and AMPK-related pathways are also likely impacted during DON-induced RSR (Figure 2.7C, 2.8C). AKT, an AGC kinase known to be activated in response to DON starting at 15 min (Zhou et al., 2005a), indirectly activates mammalian target of rapamycin complex 1 (mTORC1) by phosphorylation and inhibition of the tumor suppressor TSC2 which heterodimerizes with TSC1 (Memmott and Dennis, 2009). Interestingly, modulated phosphorylation of 5'-phosphatase SH2-containing inositol-5'-phosphatase (SHIP) was observed. SHIP regulates intracellular levels of phosphatidylinositol 3,4,5-trisphosphate (PIP3), an important second messenger
necessary for AKT activation (Rauh et al., 2004). In addition to the potential positive regulation of the mTOR pathway via AKT, we detected AMP-activated protein kinase (AMPK). AMPK at Ser108 at 15 min followed by rapid and robust phosphorylation dephosphorylation at 30 min. AMPK inhibits the mTOR pathway directly through phosphorylation of Raptor or indirectly through phosphorylation and activation of TSC2 (Memmott and Dennis, 2009). Autophosphorylation of AMPK at this site is associated with increased phosphorylation at Thr172 and increased AMPK activity (Sanders et al., 2007). This latter site can be phosphorylated by liver kinase B1 (LKB1) (Yang et al., 2010), the activity of which could be dampened via phosphorylation by ERK1. Interestingly, AMPK phosphorylation at Thr172, which boosts energy-producing catabolic processes in the cell (Schultze et al., 2012), can be impeded by PKA, also significantly affected by DON, by inducing AMPK phosphorylation at another site (Ser173) (Djouder et al., 2010). Overall, these results suggest that extensive crosstalk is likely among AKT, AMPK and MAPK pathways while mediating DON-induced RSR (Zheng et al., 2009).

Modulation of signaling within pathways involving MAPK, NF-κB, AKT and AMPK could enable cells to prepare for subsequent onset of proinflammatory gene expression and maintain an appropriate level of stress-related proteins during the ensuing response. In macrophages, AKT and AMPK have proinflammatory and anti-inflammatory effects, respectively, both of which are modulated via IKK in the NFκB pathway (Murakami and Ohigashi, 2006; Rajaram et al., 2006; Sag et al., 2008). As observed in LPS-treated macrophages (Kim et al.), AMPK is dephosphorylated and AKT is phosphorylated in DON-treated RAW 264.7 prior to the release of proinflammatory mediators. Modulation
of the NFκB pathway could regulate the expression of cytokines (e.g. IL-1β, IL-6), chemokines (e.g. MIP-2, MCP-1) and other inflammatory-related genes that are reported to be regulated on the transcription level (e.g. CD40, TLR2) (Kinser et al., 2005; He et al., 2012a). The AKT and AMPK pathways also converge and regulate the activity of mTOR pathway that controls translation via p70S6K and eIF4E, with the latter also being modulated by MAPK. As has been reported for another ribotoxin Shiga toxin 1 (Cherla et al., 2006), DON-induced expression of inflammatory proteins might reflect in part the rapid onset of translation initiation following eIF4E phosphorylation (He et al., 2012a).

**Categorization of DON-regulated phosphopeptides into distinct temporal profiles**

Fuzzy c-means clustering analysis was utilized to trace the temporal changes of protein phosphorylation observed. Based on the pattern of phosphorylation changes and the average probability of the clusters, six distinctive temporal profiles (rapid, intermediate, prolonged) of the regulated (phosphorylated, dephosphorylated) phosphopeptides were recognized (Figure 2.9A-F). DON-induced RSR initiated with rapid (≤ 5 min) dephosphorylation of ribosomal proteins (e.g. RPLP0, RPLP1, RPLP2) (Figure 2.9A), and phosphorylation of cell cycle proteins (e.g. MYH9, RCC1, Ki-67) (Figure 2.9B). The stress response proceeded with a cascade of phosphorylation events involving proteins in RNA splicing (e.g. USP39, SNW1, SRRM2, RBM17, SFRS1) (Figure 2.9C, 2.9D) up to approximately 15 min. Such proteins function in both constitutive pre-mRNA splicing and alternative splicing, suggesting the possible involvement of RNA splicing during DON-induced RSR, which could be important to modulating gene expression in the recovery of stressed cells (Biamonti and Caceres,
As RSR progressed, prolonged dephosphorylation of epigenetic regulators (e.g. HIST1H1B, DNMT1) occurred which could further fine-tune gene expression (Figure 2.9E) whereas prolonged phosphorylation of cytoskeleton proteins (e.g. STMN1) was evident, possibly affecting cell morphology, motility, and trafficking to adapt to ribotoxic stress posed by DON (Figure 2.9F).

**Limitations of phosphoproteomic approach**

It is critical to note that the phosphorylation changes observed here likely represent the most abundant phosphoproteins - a general characteristic of proteomics (Pandey and Mann, 2000). In addition, this study focuses on a time window representing acute DON exposure (0-30 min), while many studies published addressing protein phosphorylation of signaling molecules and transcription factors targeted prolonged exposures (2-6 hr) (Wong et al., 2002; Zhou et al., 2005a). As a result, it is not surprising that some of the phosphorylation targets that have been previously demonstrated immunochemically to be linked to DON-induced RSR, such as JNK and AKT, were not detected in our phosphoproteomic analysis. As demonstrated here, it was thus necessary to meld large-scale phosphoproteomic data with that of focused small-scale studies to comprehensively understand the molecular mechanisms of a complicated response such as DON-induced RSR.

It should also be emphasized that some of the phosphorylation sites affected here have not been previously reported in the literature, and the precise effects of these phosphosites on cell function are not understood. Phosphorylation of a protein could theoretically lead to activation or inhibition of its activity, altered protein-protein
Figure 2.9. DON-regulated phosphosites can be categorized into several temporal profiles. Based on the phosphorylation dynamics, Fuzzy c-means clustering analysis generated clusters representing early, intermediate and late responders with phosphorylation levels up- or down-regulated were selected from 40 stable clusters based on the average probability (A-F). Each trace depicts the natural log value of relative phosphorylation level as a function of time, and is color coded according to its membership value (i.e. the probabilities that a profile belongs to different clusters) for the respective cluster. Each cluster is designated by the function of prominent members. Examples of such members are given for each cluster.
Figure 2.9. (cont’d)
interaction, or degradation by the ATP-dependent ubiquitin/proteasome pathway (Jung and Jung, 2009). Thus further elucidation how DON-modulated phosphorylation patterns alter cell functions is necessary. This would involve characterization of how mutations of the phosphosite to another amino acid genetically would impact enzymatic activity or cell physiology and organization, which exceeds the scope of the present study. Nevertheless phosphoproteomic analysis serves as a hypothesis-generating tool and inventories proteins with changed phosphorylation status for future focused studies.

Conclusion

The global, quantitative and kinetic analysis of the early phosphoproteome changes described here demonstrates that DON-induced RSR is extremely complex and goes far beyond the simple capacity to activate the MAPK pathway and inhibit translation for which it has been long-recognized. These findings provide new insights into the processes by which DON impacts intracellular signaling and aberrantly modulate critical cell processes in a well-established macrophage model with high relevance to innate immune system activation in vivo. Given that a single regulatory protein can be targeted by many protein kinases and phosphatases, phosphorylation is critical to effectively integrating information carried by multiple signal pathways, thus providing opportunities for great versatility and flexibility in regulation (Jackson, 1992). As summarized in Figure 10, in response the ribosome damage caused by ribotoxic stressors, signaling pathways including MAPK, NFκB, AKT and AMPK pathways are activated which mediate key biological processes in the cytoplasm and the nucleus to adapt and respond to RSR.
Taken together, the phosphoproteome changes observed here suggest DON-induced RSR entails 1) a complex signaling network involving the MAPK, NF-κB, AKT and AMPK pathways; 2) delicate balance between stress response and quiescence achieved by transcription regulation via transcription factors/cofactors and epigenetic modulators as the main strategy, and 3) translation regulation by modulating of translation and ribosome biogenesis processes coordinating with it. These data provide a foundation for future exploration of phosphoprotein functions in DON-induced RSR.

Elucidation of cellular-response networks on the basis of large-scale proteomic analyses such as that observed here will enhance mechanistic understanding of toxicity pathways leading to the adverse effects of DON and other ribotoxic stressors that is a prerequisite for human health risk assessment within the “toxicity testing in the 21st century” paradigm (Andersen and Krewski, 2009). Given that the DON concentration used here is toxicologically relevant, mechanistic understanding of DON-induced RSR derived from this in vitro mouse macrophage cell model should have value for prediction of the molecular events taking place in vivo. Therefore this study could ultimately contribute to designing strategies for the treatment and prevention of the adverse effects of DON and other ribotoxic stressors.
Figure 2.10. Summary of pathways and biological processes involved in DON-induced RSR. In response the ribosome damage caused by ribotoxic stressors, signaling pathways including MAPK, NFκB, AKT and AMPK pathways are activated which mediate key biological processes in the cytoplasm and the nucleus to adapt and respond to RSR. Proteins in bold indicate novel mediators identified in the present study, proteins in bold and italics indicate previously known mediators confirmed here, and proteins in italics indicate previously known mediators but not identified here.
CHAPTER 3: Dynamic changes in ribosome-associated proteome and phosphoproteome during deoxynivalenol-induced translation inhibition and ribotoxic stress

Data in this chapter will be submitted to Toxicological Sciences.

Abstract

Deoxynivalenol (DON), a trichothecene mycotoxin produced by *Fusarium* that commonly contaminates food, interacts with the ribosome to cause translation inhibition and activates stress kinases in mononuclear phagocytes via a process known as the ribotoxic stress response (RSR). To further understand the role of the ribosome in the spatiotemporal regulation of translation inhibition and RSR, we employed stable isotope labeling of amino acids in cell culture (SILAC)-based proteomics to quantify the early (≤ 30 min) DON-induced changes in ribosome-associated proteins in RAW 264.7 murine macrophage. Specific changes in the proteome and phosphoproteome were determined using off-gel electric focusing and titanium dioxide chromatography, respectively. Following exposure to a toxicologically relevant concentration of DON (250 ng/mL), we observed an overall decrease in translation-related proteins interacting with the ribosome, concurrently with a compensatory increase in proteins that mediate protein folding, biosynthetic pathways, and cellular organization. Alterations in the ribosome-associated phosphoproteome reflected proteins recognized to modulate translational and transcriptional regulation, as well as others that converged with known signaling pathways that overlapped with phosphorylation events previously characterized in intact RAW 264.7 cells. These results suggest that the ribosome plays
a central role as a platform for association and phosphorylation of proteins involved in the coordination of the early translation inhibition as well as recruitment and maintenance of stress-related proteins, all of which enable cells to adapt and respond to RSR. This study provides a template for the elucidating the molecular mechanisms of DON and other ribosome-targeting agents.

Introduction

The ribotoxic stress response (RSR) is a process in which mitogen-activated protein kinases (MAPKs) are activated by natural ribosome-targeting translational inhibitors produced by fungi (e.g. deoxynivalenol [DON], T-2 toxin), bacteria (e.g. anisomycin, Shiga toxin) and plants (e.g. ricin) (Iordanov et al., 1997; Laskin et al., 2002). The trichothecene DON, one such translational inhibitor, has been widely used as a model to study the molecular mechanisms of RSR (Pestka, 2010b). This mycotoxin is of public health significance because it is a common contaminant in human and animal foods.

DON targets the mononuclear phagocytes of the innate immune system (Pestka and Smolinski, 2005), stimulating proinflammatory gene expression at low or modest doses, but causing translation inhibition and leukocyte apoptosis at higher doses (Pestka, 2010a). The immunomodulatory effects of DON are believed to be mediated through RSR, primarily via the activation of kinases associated with the ribosome, the primary cellular target of DON (Zhou et al., 2003a). Following binding to the ribosome, DON activates the ribosome-associated MAPK p38 (Bae and Pestka, 2008; Bae et al., 2009). Two kinases upstream of MAPK activation are double-stranded RNA-dependent protein kinase (PKR) and hemopoietic cell kinase (Hck) (Zhou et al., 2003b; Zhou et al.,
which constitutively associate with the ribosome, and are rapidly phosphorylated (≤ 5 min), thus serving as early sentinels for DON exposure (Bae and Pestka, 2008; Bae et al., 2010). In addition, DON causes the ribosome mobilization and phosphorylation of other upstream components in the MAPK cascade, ASK1 (a MAPKKK) and MKK6 (Bae et al., 2010).

DON-induced translation inhibition has been well documented in vivo and in vitro (Azcona-Olivera et al., 1995; Zhou et al., 2003b). Such translational inhibition occurs in at least three ways. First, DON is known to cause the degradation of 18S and 28S rRNA (He et al., 2012c), and interfere with peptidyl transferase function on the ribosome, with consequent impairment of initiation and elongation (Shifrin and Anderson, 1999). Second, DON can induce the activation of ribosome-associated kinase PKR, which when activated, phosphorylates eIF2α, thereby inhibiting translation (Zhou et al., 2003b). Finally, DON can upregulate a large number of microRNAs (miRNAs) which can target mRNA for translational inhibition, most notably for ribosomal proteins (He et al., 2010).

Although the ribosome is considered to be an immensely complex molecular machine dedicated to protein translation, it has been proposed that the composition and post-translational modifications of ribosomal proteins might impart specific regulatory capacities in cells of different phenotypes or developmental stages (Xue and Barna, 2012). For example, ribosomal protein S3 (RPS3) is a p65-binding component of NF-κB complex and is essential for the function of the complex as a transcription factor (Wan et al., 2007). In addition, there is growing recognition that the ribosome can orchestrate other fundamental aspects of cell function by interacting with myriad of non-ribosomal proteins (Xue and Barna, 2012). For example, some members of the Akt/mTOR
pathway, including 3-phosphoinositide-dependent protein kinase-1 (PDK1), AKT, mammalian target of rapamycin (mTOR) and p70 S6 kinase (p70S6K), bind to the ribosomes (Ruggero and Sonenberg, 2005; Lee et al., 2010; Zinzalla et al., 2011). Non-ribosomal proteins can potentially interact with 40S and 60S subunits, monosomes or actively translating polysomes. Identity and function of such proteins that interact on the ribosome landscape can provide significant insight about their cellular roles during the response to environmental stimuli.

Many questions remain regarding DON-induced translational inhibition and RSR in terms of its impact on the ribosome, the identity of the other proteins that are recruited to the ribosome and/or activated, and how they might impact overall cell function. The goal of this study was to test the hypothesis that the ribosome functions as a platform for spatiotemporal coordination of DON-induced translational inhibition and RSR. Specifically, stable isotope labeling with amino acids in cell culture (SILAC)-based quantitative proteomics was utilized to analyze the alterations in ribosome-associated proteins and phosphoproteins during early RSR (≤30 min) induced by DON at a toxicologically relevant concentration (250 ng/mL) known to partially inhibit translation and evoke robust RSR (Azcona-Olivera et al., 1995; Moon et al., 2003; Zhou et al., 2003b). The results revealed that DON broadly affected both composition and phosphorylation status of ribosomal and associated non-ribosomal proteins involved in translational and other biological processes. These findings further provide a foundation for further studies of the ribosome’s role in coordinating partial translational arrest and the activation of stress-related proteins for repair/recovery.
Materials and Methods

Experimental design

Changes in the ribosome-associated proteome and phosphoproteome during DON-induced RSR were measured in RAW 264.7 cells (American Type Tissue Collection, Rockville, MD) by a multitiered approach exploiting SILAC for quantification, isoelectric focusing (IEF) for peptide fractionation for ribosome-associate proteome analysis, TiO2 chromatography for phosphopeptide enrichment for ribosome-associate phosphoproteome analysis, and high-accuracy mass spectrometric characterization (Olsen et al., 2006) (Figure 3.1). Briefly, RAW 264.7 cells were labeled with L-arginine and L-lysine (R0K0), L-arginine-U-\(^{13}\)C\(_6\)\(^{14}\)N\(_4\) and L-lysine-\(^2\)H\(_4\) (R6K4), or L-arginine-U-\(^{13}\)C\(_6\)-\(^{15}\)N\(_4\) and L-lysine-U-\(^{13}\)C\(_6\)-\(^{15}\)N\(_2\) (R10K8) (Cambridge Isotope Laboratories, Andover, MA) (12 plates of 80% confluent cells per condition) in Dulbecco's Modified Eagle Medium (DMEM, from SILAC™ Phosphoprotein ID and Quantitation Kit, Invitrogen, Grand Island, NY). Since SILAC requires sufficient proliferation for the full incorporation of labeled amino acids into the cellular proteome, metabolic labeling was performed for six cell doubling times (≈108 h). The defined mass increments introduced by SILAC among the three RAW 264.7 populations resulted in characteristic peptide triplets that enabled measurement of their relative abundances.

Labeled RAW 264.7 cells were treated with 250 ng/mL of DON for 0 min, 5 min, and 30 min (Experiment 1). A second, identically labeled set of cells were treated with DON for 1 min, 15 min, and 30 min (Experiment 2). Each time course set was repeated in three independent experiments (n=3) to account for biological and technical variability.
In addition, a separate unlabeled RAW 264.7 cells were treated with 250 ng/mL of DON for 0, 1, 5, 15 or 30 min for confirmatory Western blot analysis. This toxin concentration selected was toxicologically relevant because it is achieved in tissues during DON-induced proinflammatory gene activation in the mouse (Azcona-Olivera et al., 1995; Amuzie et al., 2008). This concentration partially (≈50%) inhibits translation in RAW 264.7 cells (Zhou et al., 2003b). The time window selected encompasses both initiation and peak of DON-induced RSR in RAW 264.7 cells as reflected by MAPK activation (Zhou et al., 2003b).

**Ribosome isolation**

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 500 μl ice-cold polysome extraction buffer (PEB) [50 mM KCl, 10 mM MgCl₂, 15 mM Tris–HCl [(pH 7.4), 1% (v/v) Triton X-100, 0.1 mg/mL cycloheximide, 1 mM DTT, protease inhibitor (Sigma, St. Louis, MO), and phosphatase inhibitor (Santa Cruz Biotechnology, Santa Cruz, CA)] (Bae et al., 2010). Sucrose solutions (10% and 50%, w/v) were prepared prior to use by dissolving sucrose into RNase-free water with 50 mM KCl, 10 mM MgCl₂, 15 mM Tris–HCl (pH 7.4), 0.1 mg/mL cycloheximide and protease inhibitor. Cell lysates were centrifuged at 16,000 × g, 4 °C, for 15 min to remove nuclei, mitochondria and cell debris.

SILAC-labeled ribosome extractions from three different time points within each replicate were pooled equally based on protein content as determined by BCA Protein Assay (Pierce, Rockford, IL). These resultant mixtures (4 mL) was layered on a 28 mL linear sucrose gradient solution (10–50%, w/v) prepared using an ISCO 160 Gradient
Former and held at 4 °C in an 36 mL Sorvall centrifuge tube and centrifuged at 28,000 × g, 4 °C for 16 h in Sorvall AH-629 rotor. 40S, 60S, monosome and polysome fractions were isolated by fractionating gradient at a rate of 1 mL per min into 2 mL tube by upward displacement using an ISCO Density Gradient Fraction Collector, consisting of a needle-piercing device with a syringe pump connected to an EM-1 UV monitor for continuous measurement of the absorbance at 254 nm (Teledyne ISCO, Lincoln, NE).

Proteins from each fraction were precipitated by slow addition of trichloroacetic acid to a final concentration of 10% (w/v) followed by overnight incubation at 4°C. Pellets were recovered by centrifugation (10,000 × g for 15 min), washed with cold acetone twice, and air dried. Proteins were suspended in 6 M urea, 2 M thiourea, 10 mM HEPES, pH 8.0 and digested in solution with trypsin (Olsen et al., 2006). Peptides were desalted with 100 mg reverse-phase tC18 SepPak solid-phase extraction cartridges (Waters, Milford, MA), and two equal aliquots were dried in a Speedvac separately for determination of ribosome-associated proteome and phosphoproteome, respectively.
Figure 3.1. Experimental design for SILAC-based quantitative proteomic of ribosome-associated proteome and phosphoproteome during DON-induced RSR.
Isolation of peptides for ribosome-associated proteome and phosphoproteome analysis

For isolation of the ribosome-associated proteome, one aliquot of peptides were resuspended to 200uL in IEF buffer (7M Urea, 2M Thiourea, 0.05M dithiothreitol (DTT), 2% 3-[3-cholamidopropyl]-dimethylammonio] propanesulfonate (CHAPS), 2% ASB-14, 0.2% Biolyte pH3-10, 0.1% bromophenol blue), applied to Bio-Rad IPG strips, 8 cm, pH 3-10 (Biorad, Hercules, CA) and allowed to re-hydrate overnight at 25 °C. The strips were then electrophoresed at 250 V for 15 min followed by rapidly ramping the voltage to 8000 V and holding for 30,000 VHrs. Strips were frozen at -20 °C until peptides could be harvested for analysis. Strips were sliced into 5 equal sections and placed into individual microfuge tubes. Each of these sections was further chopped into ~5mm pieces and sequentially extracted by sonication in 0.1% trifluoroacetic acid (TFA), 0.1% TFA/30% acetonitrile (ACN), 0.1% TFA/70% ACN and pooled.

For isolation of the ribosome-associated phosphoproteome, a second aliquot of peptides was enriched for phosphopeptide with Titansphere PHOS-TiO Kit (GL Sciences, Torrance, CA) following manufacturer’s instructions. For both the ribosome-associated proteome and phosphoproteome, extracted peptides from both preparations were then separately dried in a Speedvac. Dried samples were reconstituted in blank solution (2% ACN/0.1% TFA) to 100 µL and purified by solid phase extraction using OMIX tips (Varian, Palo Alto, CA) according to manufacturer’s instructions.

Mass spectrometry

Purified peptides were then dried in a Speedvac and resuspended in blank solution to 20 µL. From this, 10 µL were automatically injected by a Waters
nanoAcquity Sample Manager (Waters, Milford, MA) and loaded for 5 min onto a Waters Symmetry C18 peptide trap (5 µm, 180 µm x 20 mm) at 4 µL/min in 2% ACN/0.1% formic acid. The bound peptides were then eluted using a Waters nanoAcquity UPLC (Buffer A = 99.9% water/0.1% formic acid, Buffer B = 99.9% ACN/0.1% formic acid) onto a Michrom MAGIC C18AQ column (3µ, 200Å, 100U x 150mm, Michrom, Auburn, CA) and eluted over 120 min with a gradient of 5% B to 30% B in 106 min, ramping up to 90% B by 109 min, held there for 1 min, returned to 5% B at 110.1 min and kept there for the remainder of the run. Solvent flow was kept at a constant rate of 1 µl/min.

Eluted peptides were sprayed into a ThermoFisher LTQ-FT Ultra mass spectrometer (Thermo, Hudson, NH) using a Michrom ADVANCE nanospray source with an ionization voltage of 2.0 kV. Survey scans were taken in the FT (50000 resolution determined at m/z 400) and the top five ions in each survey scan were then subjected to automatic low energy collision induced dissociation (CID) in the LTQ. The resulting data files were processed into peak lists using MaxQuant (Cox and Mann, 2008), v1.2.2.5, and searched against the IPI rat database v3.78 using the Andromeda (Cox et al., 2011) search algorithm within the MaxQuant environment. All SILAC quantitation was performed using MaxQuant and the data exported to the program Perseus, v1.2.0.16 (www.maxquant.org) for statistical analysis.

**Data analysis**

Peak list generation, protein quantitation based upon SILAC, extracted ion chromatograms (XIC) and estimation of false discovery rate (FDR) were all performed using MaxQuant (Cox and Mann, 2008), v1.2.2.5. MS/MS spectra were searched against the IPI mouse database, v3.72, appended with common environmental
contaminants using the Andromeda searching algorithm (Cox et al., 2011). Further statistical analysis of the SILAC labeled protein and peptide ratio significance was performed with Perseus (www.maxquant.org). MaxQuant parameters were protein, peptide and modification site maximum FDR = 1%; minimum number of peptides = 1; minimum peptide length = 6; minimum ratio count = 1; and protein quantitation was done using all modified and unmodified razor and unique peptides. Andromeda parameters were triplex SILAC labeling: light condition (no modification), medium condition (Arg6, Lys4), heavy condition (Arg10, Lys8); fixed modification of Carbamidomethylation (C), variable modifications of Oxidation (M), Phosphorylation (STY) and Acetyl (Protein N-term); Maximum number of modifications per peptide = 5; enzyme trypsin max missed cleavage = 2; parent ion tolerance = 6ppm; fragment ion tolerance = 0.6 Da and reverse database search was included. For each identified SILAC triplet, MaxQuant calculated the three extracted ion chromatogram (XIC) values, and XICs for the light and medium member of the triplet were normalized with respect to the heavy common 30 min time point of DON treatment, which was scaled to one. A significance value, Significance B, was calculated for each SILAC ratio and corrected by the method of Benjamini and Hochberg using Perseus (Cox and Mann, 2008). Significance B values of a peptide in Experiment 1 and Experiment 2 were calculated independently, and the significance cutoff was set as Significance B value <0.05 in at least one set of time course (Cox et al., 2009). Median values of multiple peptides originating from the same protein or multiple measurements of the same phosphopeptide were used to represent the relative abundance of proteins or
phosphopeptides, respectively (Ong and Mann, 2006). Phosphosites were mapped with PhosphoSitePlus (Hornbeck et al., 2012).

**DAVID analysis**

Analyses for gene ontology (GO) annotation terms were performed with the functional annotation tool DAVID (http://david.abcc.ncifcrf.gov/). Uniprot accession identifiers of significantly regulated phosphoproteins were submitted for analysis of GO biological processes and KEGG pathways, with a maximal DAVID EASE score of 0.05 for categories represented by at least two proteins.

**Western blot**

Immunoblotting analysis was performed on selected proteins as described previously (Bae and Pestka, 2008) to verify phosphoproteome quantitation. Antibodies against the following proteins were used: p38, phospho-p38 MAPK (Thr180/Tyr182), p42/p44 MAP kinase, phospho-p42/p44 MAP kinase (Thr202/Tyr204), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling, Danvers, MA), RPL7, RPS6 (Bethyl Labs, Montgomery, TX). Blots were scanned on the Odyssey IR imager (LICOR, Lincoln, NE) and quantification was performed using LICOR software v.3.0.

**Results and Discussion**

**Proteomic profile of ribosome-associated proteins**

SILAC-labeled RAW 264.7 cell extracts were fractionated into 40S, 60S subunits, monosome and polysome fractions and further analyzed for the associating proteome and phosphoproteome (Figure 3.2). The average mammalian ribosomal protein is small
(average molecular weight 18.5 kD) and very basic (average isoelectric point 11.05) (Wool et al., 1995). Consequently in order to resolve less abundant non-ribosomal proteins from the highly abundant ribosomal proteins, we employed off-gel IEF to further fractionate samples so peptides from ribosomal proteins with high pI could be separated from other peptides with lower pI. Off-gel IEF reduces sample complexity and prevents the peptides originating from ribosomal proteins from overwhelming the mass spectrometer thus facilitating identification of ribosome-interacting proteins.

Using the approach above, the ribosome-associated proteome was found to contain many proteins involved in other biological functions that extended beyond canonical ribosomal, translation- and ribosome biogenesis-related proteins (Figure 3.2A). It should be emphasized that this figure reflects the counts of proteins in the ribosomal fractions, as opposed to the relative abundance. At an accepted FDR of 1%, hundreds of unique proteins (Figure 3.2B), corresponding to the unique peptides identified in the LC-MS/MS (Figure 3.2C), were found in each ribosomal fraction. Approximately 70% of those proteins were identified with two or more independent peptide measurements. Among the unique protein identified, ninety-one percent of the known murine ribosomal proteins (http://ribosome.med.miyazaki-u.ac.jp/) were identified, including 29 of the 32 small subunit and 43 of the 47 large subunit proteins. Translation-related proteins included initiation/elongation factors, tRNA synthetases, and ribosome biogenesis-related proteins that drive ribosome nuclear transport and function, such as ribosomal RNA
Figure 3.2. Impact of DON on the ribosome-associated proteome. (A) The ribosome-associated proteome includes extensive non-ribosomal proteins. Count of ribosomal proteins, translation-, ribosome biogenesis-related proteins, and other proteins found in different fractions of the ribosome. Number of unique proteins (B) and peptides (C) associated with the ribosome (light + dark blue) that were significantly affected by DON exposure (FDR<5%, light blue).
Figure 3.2. (cont'd)

C

Number of unique peptides

40S 60S 80S Polysome

0 1000 2000 3000 4000 5000
methyltransferase. Other ribosome-interacting proteins that do not directly function in translation may play alternative roles such as serving as recruitment sites for proteins involved within one metabolic or signaling pathway. With Benjamini-Hochberg corrected FDR<5% as the significance cutoff, 17% and 10% of the unique proteins and peptides for all four fractions of the ribosome identified were significantly altered, respectively, suggesting the effects of DON on protein association with the ribosome (Figure 3.2B).

**DON-induced changes in ribosome-associated proteins – impact on translation**

To discern the potential impact of DON on ribosome-associated proteome, proteins with significant differential association with the ribosome were related to specific biological processes using Gene Ontology annotation in DAVID. Based on changes in protein association with the ribosome, early DON-induced effects mainly involved modulation of translation, biosynthetic pathways, and macromolecular complex assembly (Figure 3.3). Of these, the process most affected for all four ribosomal fractions was translation, consisting 20% or more of the ribosome-associated proteins impacted by DON across all four fractions of the ribosome (Figs. 3.4-3.7). During the first 30 min of DON exposure, there was a general depletion of ribosomal and translation-related proteins associated with the ribosome. Although the total protein amount in each respective ribosomal fraction for different time points remained constant, there was a global reduction in the levels of ribosomal proteins across the fractions (Figs. 3.4-3.7), suggesting increases in ribosome degradation/disassembly and/or decreased in ribosome biogenesis/assembly. Consistent with the latter, we have previously observed in RAW 264.7 that DON exposure alters phosphorylation of proteins involved in ribosome biogenesis, including NOP56, NOP58, NPM1 and
Among the ribosomal fractions, there was a transient increase of translation-related proteins in the 80S fractions, which might indicate polysome disassembly. Such reduction in the polysome/monosome ratio has been observed in several mutants defective in translation initiation (Wong et al., 2004; Wilhelm et al., 2006), and could support the inhibition of translation initiation by DON as evidenced by PKR and eIF2α phosphorylation as early as 1 min (Zhou et al., 2003b). Ultimately, ribosomal protein depletion likely contributes to DON-induced translational inhibition.

Multiple subunits of eukaryotic initiation factor 3 (eIF3) (A, B, C, E, F, L), eIF4A, and eIF4G had reduced association with 40S ribosomal subunit (Figure 3.4). eIF3 plays a key role in recruiting the preinitiation complex to the mRNA and also forms a protein bridge to the mRNA by interacting with eIF4G. eIF3 and eIF4G are likely retained on the ribosome during elongation of small uORFs to make them available for renewed scanning following termination. eIF3 also stimulates reinitiation after translation of a long uORF (Sonenberg and Hinnebusch, 2009). Cap-dependent initiation requires interaction of eIF4E with the mRNA 5’ cap structure, which forms the eIF4F helicase complex together with RNA eIF4A helicase and eIF4G. In addition to the ribosome-association alteration shown here, eIF4G has been shown to be differentially phosphorylated in the whole cell (Pan et al., 2013) and in the ribosomal fractions. Accordingly, displacement of translation-related factors very likely contributes to translation arrest induced by DON.

Discrepancies observed between transcriptome and proteome data underlines the importance of translational control of gene expression in addition to the better
understood transcriptional regulation (Sonenberg and Hinnebusch, 2009). DON. impairment of translation possibly liberates translational machinery from pre-existing translation-competent transcripts thus enabling newly made mRNAs to more effectively compete for the translational machinery. This shift in translation, known as “translational reprogramming” (Ron and Walter, 2007), may allow for a bias in the synthesis of proteins that enable the macrophage to combat the ribotoxic stress and respond to danger by evoking an innate immune response. Such a biased translatome, as compared to transcriptome, has been recently documented by our laboratory for several proinflammatory genes induced by DON in RAW 264.7 cells (He et al., 2013).

**DON-induced changes in ribosome-associated proteins – impact on protein folding**

Disruption of normally well-coordinated translation might generate improperly folded proteins and therefore impact protein folding. Thus it was notable that DON exposure altered HSC70, HSP90 and GRP78 among the ribosome interacting proteins (Figs. 3.4-3.7).

Ribosome-associated complex (RAC), which consists of heat shock protein Hsp40 and Hsp70 proteins, supports de novo folding pathways (Wegrzyn and Deuerling, 2005). Heat-shock cognate Hsc70 serves as a chaperone with RAC or independent of RAC with its intrinsic ATPase activity (Jaiswal et al., 2011). There was an increase in HSC70 and other chaperones in protein folding associated with the ribosome (Figs. 3.4, 3.6, 3.7), which could boost co-translation folding (Craig et al., 2003). HSP90 is known
Figure 3.3. Gene Ontology analysis of ribosome-interacting proteins significantly regulated affected by DON exposure by DAVID Biological Process terms (DAVID EASE score < 0.05). Data are presented as a histogram of the relevant biological processes identified and shown as a percentage of the total identified proteins that fall within each category. Numbers in parentheses represents the number of proteins in the ribosome fraction significantly altered by DON treatment.
<table>
<thead>
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<th>Process</th>
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<td></td>
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<tr>
<td>Generation of precursor metabolites and energy</td>
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Figure 3. (cont’d)
Figure 3.4. Changes in the levels of proteins associated with 40S ribosome during DON-induced RSR. The heat map depicts the log 2 transformed relative protein abundance (red to green), and grey represents missing values.
Figure 3.4. (cont’d)

C

Generation of precursor metabolites and energy

Nitrogen compound biosynthetic process

D

Macromolecular complex assembly

Cytoskeleton organization
Figure 3.5. Changes in the levels of proteins associated with 60S ribosome during DON-induced RSR. The heat map depicts the log 2 transformed relative protein abundance (red to green), and grey represents missing values.
Figure 3.6. Changes in the levels of proteins associated with 80S ribosome during DON-induced RSR. The heat map depicts the log 2 transformed relative protein abundance (red to green), and grey represents missing values.
Figure 3.7. Changes in the levels of proteins associated with polysome during DON-induced RSR. The heat map depicts the log 2 transformed relative protein abundance (red to green), and grey represents missing values.
to interact with RPS3 and RPS6, and such interaction protects the two ribosomal proteins from ubiquitination and proteasomal degradation (Kim et al., 2006). HSP90 exhibited a transient increase association with 40S, 80S and polysome (Figs. 3.4, 3.6, 3.7), thereby possibly protecting of ribosomal proteins RPS3 and RPS6. Ribosome association of HSP90 decreased at 30 min, which could possibly coordinate the global reduction of ribosomal proteins in response to DON. In addition to the induction of chaperones, weakened translation, as suggested by reduced ribosomal availability in early DON-induced RSR, might decrease the pool of chaperone substrates and thereby increasing the capacity of the folding system (Meriin et al., 2012). The degradation of the ER chaperone and signaling regulator glucose-regulated protein 78 (GRP78) showed increased association with the polysome within 30 min of DON treatment (Figure 3.7), which could be mediated via ERj1 (ER-resident J-domain protein 1) (Benedix et al., 2010), and might further enhance with protein folding.

**DON-induced changes in ribosome-associated proteins – impact on biosynthesis and cellular organization**

Other proteins altered in their ribosome interaction following DON exposure were capable of impacting biosynthesis and cellular organization processes (Figs. 3.4-3.7). Proteins involved in glucose, ATP and nucleotide biosynthesis showed an overall increased association with the ribosome. On the cellular level, enhanced energy metabolism in early DON-induced RSR has been previously implicated in RAW 264.7 by the phosphorylation of AMP-activated protein kinase (AMPK) (Pan et al., 2013). There are approximately 15,000 ribosomes in a single cell, and they make up about 25% of the dry weight of cells (Luisi and Stano, 2011). Accordingly, translation uses a
significant proportion of the cell’s energy which needs to be replenished to compensate for the partially inhibited translation and to restore translation of stress-related proteins in DON-induced RSR.

Dysregulation of cellular organization, including cytoskeleton organization and chromatin assembly processes, has been suggested to occur at the protein phosphorylation level during early DON-induced RSR in RAW 264.7 (Pan et al., 2013). Multiple proteins involved in cellular organization showed increased association with the ribosome, including lymphocyte cytosolic protein 1 (LCP1) and multiple chains of tubulin (TUBs) across different ribosomal fractions (Figs. 3.4-3.7). It has been proposed that upon translation perturbation, stressed ribosome complex aggregation and dissolution requires anchorage of cytoskeleton to the ribosomes and the activity of motor molecules for active transport (Kwon et al., 2007; Anderson and Kedersha, 2008). Thus enhanced association of proteins in cell organization might facilitate transportation of the aforementioned proteins to associate or dissociate from the ribosome.

**Proteomic profile of ribosome-associated phosphoproteins**

A pilot immunoblot experiment was performed to test 1) if the RSR is induced in the SILAC-labeled RAW 264.7, 2) if previously reported ribosome association and phosphorylation of p38 is reproducible under the ribosome isolation conditions used, and 3) if other MAPKs characteristic of DON-induced RSR are also associated with the ribosome. MAPKs, including p38, ERK, JNK, were found to constitutively bind to the ribosome, with RPS6 and RPL7 as markers of the ribosome (Figure 3.8). At 30 min, which represents the peak of DON-induced RSR, MAPKs were moderately recruited to the ribosome fractions, with overall levels of phosphorylation markedly increased. It has
been previously shown that DON induces p38 mobilization to the ribosome and its subsequent phosphorylation under more stringent ribosome isolation conditions (Bae and Pestka, 2008), indicating the potential role of the ribosome as a platform for signaling proteins. The results presented here further extend the ribosome association of MAPKs from p38 to ERK and JNK.

For the proteomic analysis, the ribosome-associated phosphoproteome was enriched using TiO2 chromatography after sucrose density fractionation of the ribosome and protein digestion due to the low abundance of phosphoproteins. More than 92% of identified peptides were found to harbor at least one phosphorylation residue, indicating that phosphopeptide enrichment by TiO2 was highly efficient. The distribution of phosphorylated serine, threonine, and tyrosine sites was 77%, 18% and 5%, respectively (Figure 3.9A). Fifteen of all phosphoproteins identified were ribosomal (e.g. RPS6, RPLP2), translation- (e.g. eIF3, eIF4G1) and ribosome biogenesis-related proteins (e.g. NPM1, BYSL) with known and novel phosphorylation events. However, like the ribosome-associated proteome, the majority of phosphoproteins interacting with the ribosome were involved in non-ribosomal processes (Figure 3.9B).

Using the same set of criteria for protein identification and significance (FDR<5%), approximately 16% of the total phosphopeptides identified were considered significantly altered by DON, with total and significantly altered unique phosphoproteins and phosphopeptides identified in each ribosomal fraction depicted in Figure 3.9C,D. Based on PhosphoSitePlus annotation, the ribosome-associated phosphoproteome changes occurring in the macrophage after DON exposure encompass known and yet-to-be functionally characterized phosphosites.
DON-induced changes in ribosome-associated phosphoproteins – impact on translation

GO analysis revealed that ribosome-interacting phosphoproteins with phosphorylated status significantly altered by DON were primarily involved in translation, regulation of transcription, RNA processing and cell morphogenesis (Figure 3.10).

Eukaryotic protein translation is mainly controlled at the level of initiation, a process of elongation-competent 80S ribosome assembly from 40S and 60S subunits. Translation initiation involves multiple protein phosphorylation events (Jackson et al., 2010). Following DON exposure, ribosome-associated phosphorylation events observed include those on ribosomal proteins (e.g. RPS6), as well as translation initiation (e.g. eIF4G) and elongation factors (eEF1D) (Figure 3.11-3.14).

RPS6 interacts with the 5’ cap complex required for translation initiation. RPS6 undergoes inducible phosphorylation in response to mitogenic and cell growth stimuli ((Ruvinsky and Meyuhas, 2006)), and such phosphorylation occurs on a cluster of five serine residues at the carboxyl terminus of RPS6, i.e., Ser-235, Ser-236, Ser-240, Ser-244, and Ser-247. Differential phosphorylation of RPS6 at the first three of these residues was detected during DON-induced RSR, which might modulate RPS6’s affinity for the cap and mRNA translation initiation (Hutchinson et al., 2011). Besides the effect on translation, RPS6 phosphorylation has been implicated in attenuating DNA damage (Khalaileh et al., 2013).

Platform protein eIF4G is the binding partner for the cap-binding factor during
Figure 3.8. Western blot analysis of p38, ERK and JNK in RAW 264.7 with total and phospho-specific antibodies, with RPL7 and RPS6 as markers of the large and small ribosomal subunits. MAPKs were constitutively found in the ribosomal fractions and phosphorylated during DON-induced RSR. Results shown are representative of 3 replicate experiments.
translation initiation. Increased phosphorylation of eIF4G1 at Ser187 and Ser1189, as found transiently in the 60S ribosome at 15 min during DON-induced RSR, enhances interaction with MNK, an eIF4E kinase implicated in DON-induced RSR in vivo (Chapter 3). Binding to eIF4G approximates MNK to its substrate eIF4E and facilitates eIF4E phosphorylation and translation initiation (Dobrikov et al., 2011).

**DON-induced changes in ribosome-associated phosphoproteins – impact on transcriptional regulation**

Transcriptional regulation has been shown to be the primary target during early DON-induced RSR in RAW 264.7 cells on the level of protein phosphorylation (Wong et al., 2002; Kirat et al., 2009; Pan et al., 2013). Ribosome-associating phosphoproteins involved in transcriptional regulation were mainly found to interact with the 40S, 60S and 80S (Figure 3.11-3.13), and overlapped with those previously described in intact RAW 264.7 (e.g. epigenetic regulators TRIM28 and DNMT1, and transcription cofactors LYRIC and MYBBP1A) (Pan et al., 2013), as well as uncovered new ones not previously demonstrated in DON-induced RSR (e.g. PRKRA).

Tripartite motif protein 28 (TRIM28) phosphorylation at Ser473, has been shown to compromise its interaction with heterochromatin protein 1 (HP1) and activate cell cycle regulatory genes (Chang et al., 2008). Phosphorylation of TRIM28 at this site increased in 40S and 60S ribosome at 30 min (Figure 3.11, 3.12), following the same pattern in the whole cell (Pan et al., 2013). Relatedly, TRIM28 is a universal co-its repressor that induces cell differentiation in the monocytic cell line U937 via its interaction and activation of C/EBP beta (Rooney and Calame, 2001), a transcription factor also activated by DON (Shi and Pestka, 2009).
Figure 3.9. Impact of DON on the ribosome-associated phosphoproteome. (A) Distribution of phospho-Serine, Threonine and Tyrosine identified in peptides from all ribosomal fractions. (B) The ribosome-associated phosphoproteome includes extensive non-ribosomal proteins. Count of ribosomal proteins, translation-, ribosome biogenesis-related proteins, and other proteins found in different fractions of the ribosome. Number of unique phosphoproteins (C) and phosphopeptides (D) associated with the ribosome (light + dark blue) that were significantly affected by DON exposure (FDR<5%, light blue).
Figure 3.9. (cont’d)
Figure 3.10. Gene Ontology analysis of ribosome-interacting phosphoproteins significantly regulated affected by DON exposure by DAVID Biological Process terms (DAVID EASE score < 0.05). Data are presented as a histogram of the relevant biological processes identified and shown as a percentage of the total identified proteins that fall within each category.
Figure 3.10. (cont'd)

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- Negative regulation of transcription
- Mesenchymal cell development
- Leukocyte proliferation
- Translation
- RNA processing
- Ribosome biogenesis
- Leukocyte proliferation

% total
Figure 3.11. Phosphorylation changes of proteins associated with 40S ribosome during DON-induced RSR. The heat map depicts the log 2 transformed relative abundance (red to green) of the protein phosphorylation, and grey represents missing values.
Figure 3.12. Phosphorylation changes of proteins associated with 60S ribosome during DON-induced RSR. The heat map depicts the log 2 transformed relative abundance (red to green) of the protein phosphorylation, and grey represents missing values.
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Figure 3.13. Phosphorylation changes of proteins associated with 80S ribosome during DON-induced RSR. The heat map depicts the log 2 transformed relative abundance (red to green) of the protein phosphorylation, and grey represents missing values.
Figure 3.14. Phosphorylation changes of proteins associated with polysome during DON-induced RSR. The heat map depicts the log 2 transformed relative abundance (red to green) of the protein phosphorylation, and grey represents missing values.
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<tbody>
<tr>
<td>RPS6</td>
<td>40S ribosomal protein S6</td>
<td>Translation; RNA processing; Ribosome biogenesis; Leukocyte proliferation</td>
<td>Ser235</td>
</tr>
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<td>Ribosome biogenesis</td>
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<td>Translation</td>
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<td>60S acidic ribosomal protein P0</td>
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<tr>
<td>SFRS1</td>
<td>ASF/SF2;Splicing factor, arginine/serine-rich 1</td>
<td>RNA processing</td>
<td>Ser199</td>
</tr>
<tr>
<td>U2AF2</td>
<td>Splicing factor U2AF 65 kDa subunit</td>
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<tr>
<td>RPLP1</td>
<td>60S acidic ribosomal protein P1</td>
<td>Translation</td>
<td>Ser104</td>
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</table>
Myb-binding protein 1A (MYBBP1A), found to be rapidly dephosphorylated at 30 min, is known to interact with and activate several transcription factors such as c-JUN, while repressing others such as PPARγ co-activator 1α (PGC-1α), NFκB, and c-MYB (Fan et al., 2004; Owen et al., 2007; Yamauchi et al., 2008). MYBBP1A associated with 60S ribosome dephosphorylated at Ser6 at 30 min after DON treatment (Figure 3.12). This protein was observed to be upregulated at the protein level by DON (150 ng/mL) at 6 h in a prior proteomic study in EL4 mouse thymoma cells (Osman et al., 2010a).

DON has been indicated to alter transcription via miRNA-based mechanisms (He et al., 2010). PKR-associated protein X (PRKRA) is required for miRNA production by Dcr-1 homolog (DICER1) and for subsequent miRNA-mediated post-transcriptional gene silencing (Patel and Sen, 1998). It could also regulate gene expression on the translation level. PRKRA could activate PKR in the absence of double stranded RNA (dsRNA), leading to eIF2α phosphorylation and inhibition of translation, such effect requires phosphorylation of PRKRA at Ser18 (Bennett et al., 2004), which was observed to be increased in 60S at 30 min after DON treatment (Figure 3.12). However, in DON-induced RSR, PKR phosphorylation is observed before phosphorylation of PRKRA (between 1 and 5 min) during DON-induced RSR, suggesting that PRKRA mainly participates in the transcriptional regulation via modulation of miRNA expression.

**DON-induced changes in ribosome-associated phosphoproteins – impact on stress-related signaling**

Ribosome-associated phosphoproteins could link to the stress signaling network that consists of MAPK, AKT and NFκB pathways.
Besides the aforementioned MAPKs including p38, ERK and JNK, a downstream player of the MAPK pathway cytosolic phospholipase A2 (cPLA2), which associated the 40S (Figure 3.11), was dephosphorylated at Ser437 over time. Phosphorylation of cPLA2 has been reported during DON-induced RSR in RAW 264.7 (Pan et al., 2013) is required for the release of arachidonic acid, and has been implicated in the initiation of inflammatory responses (Pavicevic et al., 2008).

RPS6 represents a point of regulatory convergence for MAPK- and AKT-linked pathways known to mediate DON-induced RSR (Pan et al., 2013). p70 ribosomal protein S6 kinase (S6K) via the AKT-mTOR pathway and p90 ribosomal protein S6 kinases (RSK) via the MAPK pathway (Ruvinsky and Meyuhas, 2006). In 40S, 80S and polysome fractions, RPS6 was differentially phosphorylated at Ser235, Ser236 or Ser240, possibly due to the different activities of RSKs on these fractions. Thus, the phosphorylation status of RPS6 is determined by the two pathways, and could control translation efficiency in response to environmental perturbations such as DON-induced RSR.

MYBBP1A associated with 60S (Figure 3.12) and LYRIC associated with 80S (Figure 3.13) are two inhibitors of p65 and negative regulators of the NFκB signaling, a pathway also known to be evoked by DON (Pan et al., 2013). These two phosphoproteins also link the RSR with transcriptional regulation during DON-induced RSR. These results suggest that the ribosome serves as a platform for stress-related signaling in addition to that for translational and transcriptional regulation.
Limitations of the methodology

Previously, proteins identified in the ribosomal fractions without a known indicated role in ribosome-related function have been dismissed as contaminating proteins from complexes that comigrate with ribosomal fractions in sucrose gradient, which is a common pitfall for the sucrose density fractionation approach in ribosome purification. Basic techniques to isolate ribosomes were developed in the 1960s and 1970s, and are still widely used with relatively minor modifications (Mehta et al., 2012). Most of these techniques require differential or density gradient ultracentrifugation of cell lysates to yield ribosomes or ribosomal subunits.

Factors that interact with functional ribosomes may interact transiently with a wide range of affinities. Protein–ribosome interactions exhibit a range of salt sensitivities. Higher stringency approaches might cause the dissociation of specific ribosome-surface associated factors. While low stringency salt conditions allow most specific ribosome-binding proteins to remain associated with the ribosome, it could result in higher background signal (Mehta et al., 2012). Accordingly, after comparing different published studies (Blobel, 1971; Thiebeauld et al., 2009; Tsai et al., 2012), we chose moderate salt conditions in this study and evaluated the ribosome association of MAPKs using immunoblot as a pilot study (Figure 3.8). The present proteomic study resolved proteins with altered association with the ribosome upon DON treatment that function in processes other than protein synthesis and folding at moderate stringency. However, this does not exclude the possibility of the inclusion of contaminating proteins in our discussion. Nevertheless, if these proteins are contaminating proteins comigrated with the ribosomal fractions in sucrose gradient centrifugation, they should remain constant.
regardless of DON treatment because different treatment groups with different SILAC labels were combined before sucrose gradient centrifugation and fractionation.

While affinity tagging and immunoprecipitation might be an alternative to confirm the interactions identified in the study (Inada et al., 2002; Zanetti et al., 2005), it would require verification that 1) the tag does not interfere with the biological function, and 2) over-expression does not lead to non-specific interactions that can be misleading. We have attempted to express affinity-tagged ribosomal proteins in RAW 264.7 macrophages. However, expression and ribosomal incorporation of these tagged proteins were inefficient, possibly due to the nature of the macrophages to combat exogenous material.

Another caveat with regards to the ribosome purification method used was the overlap among the four ribosomal fractions, which is inevitable in sucrose density gradient fractionation. In addition, it is also possible that proteins found are nascent peptide chains produced by the actively translating polysome. This could add to the complexity of the polysome-associated proteome. Finally there is very likely some sample loss during the ribosome isolation, fractionation and digestion procedures, which might have removed some less abundant proteins, and reduced the sensitivity of the method. This could explain why this study failed to detect some of the ribosome-associated proteins and phosphoproteins identified by Western blot analysis (e.g. PKR, p38, ERK) (Figure 3.8), an approach known to be more sensitive than mass spectrometry-based proteomics used here (Mann, 2008).
Conclusion

Taken together, the quantitative proteomic analysis presented suggests that upon exposure to DON at a toxicologically relevant concentration that causes partial translation inhibition, proteins critical in mediating translation inhibition and the RSR showed altered interaction and/or phosphorylation in the ribosome. Proteomics identified large numbers of proteins and phosphoproteins not directly function in translation- or ribosome biogenesis-related protein in the ribosome fractions, which sets a foundation for the versatile extraribosomal functions of the ribosome (summarized in Figure 3.15).

The key findings here were that after DON exposure, 1) there was an overall decrease in translation-related proteins interacting with the ribosome, concurrent with compensatory increase in protein folding, biosynthetic pathways, and cellular organization; 2) alterations in ribosome-associated phosphoproteome involved proteins that could finetune regulation of translation and transcription, and those that also converged with known signaling pathways. Changes in ribosome-associated proteome and phosphoproteome correspond to composition and post-translational modifications of ribosomal and its interacting proteins, which could offer additional layers of regulation. Therefore, in addition to its role in protein synthesis, the ribosome could function as a platform for translation-related and other processes. Such a structural role for the ribosome could facilitate spatiotemporal coordination of cell behaviors to respond and adapt to the ribotoxic stress caused by DON.

This study could contribute to the understanding of the role of the ribosome under stress conditions posed by agents like DON that directly impact the ribosome. It
could be used to understand the molecular mechanisms, at the subcellular level, of DON and other ribotoxic agents, many of which are public health threats or chemothapeutic agents.
Figure 3.15. Summary of ribosome-associated proteome (blue) and phosphoproteome (red) changes in the four ribosomal fractions during DON-induced RSR.
CHAPTER 4: Early Phosphoproteomic Changes in the Mouse Spleen during Deoxynivalenol-Induced Ribotoxic Stress


Abstract

The trichothecene mycotoxin deoxynivalenol (DON) targets the innate immune system and is of public health significance because of its frequent presence in human and animal food. DON-induced proinflammatory gene expression and apoptosis in the lymphoid tissue have been associated with a ribotoxic stress response (RSR) that involves rapid phosphorylation of mitogen-activated protein kinases (MAPKs). To better understand the relationship between protein phosphorylation and DON’s immunotoxic effects, stable isotope dimethyl labeling-based proteomics in conjunction with titanium dioxide chromatography was employed to quantitatively profile the immediate (≤30 min) phosphoproteome changes in the spleens of mice orally exposed to 5 mg/kg body weight DON. A total of 90 phosphoproteins indicative of novel phosphorylation events were significantly modulated by DON. In addition to critical branches and scaffolds of MAPK signaling being affected, DON exposure also altered phosphorylation of proteins that mediate PI3K/AKT pathways. Gene ontology analysis revealed that DON exposure affected biological processes such as cytoskeleton organization, regulation of apoptosis, and lymphocyte activation and development, which likely contribute to immune
dysregulation associated with DON-induced RSR. Consistent with these findings, DON impacted phosphorylation of proteins within diverse immune cell populations, including monocytes, macrophages, T cells, B cells, dendritic cells and mast cells. Fuzzy c-means clustering analysis further indicated that DON evoked several distinctive temporal profiles of regulated phosphopeptides. Overall, the findings from this investigation can serve as a template for future focused exploration and modeling of cellular responses associated with the immunotoxicity evoked by DON and other ribotoxins.

Introduction

The trichothecene deoxynivalenol (DON), a ribotoxic mycotoxin produced by toxigenic *Fusarium* species, commonly contaminates cereal-based foods and has the potential to adversely affect human and animal health (Pestka, 2010a). A primary target of this mycotoxin is the innate immune system, as has been demonstrated *in vivo* in mouse systemic and mucosal immune organs (Zhou *et al.*, 1999; Zhou *et al.*, 2003a), as well as *in vitro* in primary and cloned cell cultures derived from mice and humans (Zhou *et al.*, 2003b; Zhou *et al.*, 2005a; Islam *et al.*, 2006). Low dose DON exposures are immunostimulatory as evidenced by robust upregulation of mRNAs and proteins for a diverse array of cytokine, chemokine, and other inflammation-related genes (Kinser *et al.*, 2004; He *et al.*, 2012b). High dose DON exposures evoke apoptosis in actively dividing immune tissues including spleen, thymus and bone marrow and in myeloid cell models, which could contribute to immunosuppression (Yang *et al.*, 2000; Zhou *et al.*, 2000).
DON-induced proinflammatory gene activation and apoptosis are mediated by the activation of mitogen-activated protein kinases (MAPKs) via a process known as the ribotoxic stress response (RSR) (Pestka, 2010a). Besides MAPKs and their substrates, DON-induced RSR involves the rapid and transient activation of at least two upstream ribosome-associated kinases in vitro, double-stranded RNA-dependent protein kinase (PKR) and hematopoietic cell kinase (HCK), suggesting the pivotal role of protein phosphorylation in DON-induced RSR (Zhou et al., 2003; Zhou et al., 2005b; Bae et al., 2010). Phosphoproteomic changes in cloned immune cell lines, including mouse macrophage (Pan et al., 2013), and human T and B cell lines (Nogueira da Costa et al., 2011a) have been performed to model molecular mechanisms of DON-induced RSR in the responsive cell populations, as well as to identify biomarkers of effect for DON. However, DON’s effects of protein phosphorylation during RSR have not yet been comprehensively characterized within the immune system of an intact animal.

Resolving the complexity of DON-induced RSR in vivo requires an integrative approach to map the signaling and molecular events occurring at the cellular and subcellular levels. Due to the naturally low stoichiometry of phosphorylation, assessment of its dynamics requires specific affinity enrichment for identification and metabolic or chemical labeling in conjunction with high accuracy proteomics for relative quantification (Mann et al., 2002). While in vitro metabolic labeling using stable isotope labeling of amino acids in cell culture (SILAC) is commonly used for in vitro proteomic studies and has been recently successfully employed to track DON-induced phosphorylation changes in macrophages (Pan et al., 2013), this approach is not readily amenable to animal studies. Two alternative strategies for in vivo studies are chemical
labeling by isobaric tag for relative and absolute quantitation (iTRAQ) and by stable isotope dimethyl labeling (Kovanich et al., 2012). The latter method entails rapid and complete reductive amination of peptides, which makes it a relatively simple and economic strategy for large-scale quantitative analyses. It involves chemically labeling primary amine groups (lysine and amino termini) of peptides with different isotopically labeled formaldehyde through reductive amination (Kovanich et al., 2012). Since this approach has been used for comprehensive quantitative proteome analyses for assessing protein expression and post-translational modifications, such as phosphorylation, glycosylation and proteolysis (Kovanich et al., 2012), it should be ideal for the measurement of DON-induced phosphoproteome changes in vivo.

The overall goal of this investigation was to identify early protein phosphorylation changes in the immune system of DON-exposed mice and relate these to the toxin's downstream immunomodulatory effects. Specifically, we identified and quantitated phosphoproteomic changes in the spleens of mice orally exposed to a dose of DON (5 mg/kg body weight) previously found to elicit robust MAPK activation and proinflammatory gene expression (Zhou et al., 1997; Zhou et al., 2003a; Amuzie et al., 2008). We employed a 30 min time window to view events in the spleen during DON’s fast pharmacokinetic turnover prior to proinflammatory gene activation (1-3 h) and apoptosis (≥6 h). Stable isotope dimethyl labeling in conjunction with titanium dioxide (TiO₂) chromatography and LC-MS/MS was utilized to profile phosphoproteomic changes (Figure 4.1). The results revealed the involvement of an extremely complex cell signaling network in vivo that was consistent with the induction of resultant immune cell activation and apoptosis by DON.
Materials and Methods

Experimental Design

All animal studies followed NIH guidelines and were approved by the Michigan State University Committee on Animal Care. Eight- to ten-week old male B6C3F1 mice (Charles River, Portage, MI) were used in the study. Prior to the experiment, animals were held for a one-week acclimation period in a room with a 12-h light/dark cycle, 70% humidity, and a temperature of 22–23°C.

DON (>98% purity by elemental analysis) was obtained from Dr. Tony Durst (University of Ottawa). An initial pilot study was conducted to measure DON tissue levels in the spleen of mice (n=6) at 0, 5, 15, and 30 min after exposure to the toxin at 5 mg/kg body weight. Mice were sacrificed by cervical dislocation and spleens removed. Spleens (≤200 mg) were homogenized in PBS (1:10 [w/v]), and the homogenates centrifuged at 15,000 × g for 10 min. The supernatant fraction first heated to 100 °C for 5 min and then centrifuged at 15,000 × g for 10 min. DON concentrations in the supernatant were measured using a Veratox High Sensitivity (HS) ELISA (Neogen, Lansing, MI) (Amuzie et al., 2008).

For the proteomic study, two groups of mice were used (Figure 1). This first group included mice that were 1) untreated, 2) treated with vehicle and held for 15 min, or 3) treated with 5 mg/kg b.w. DON via oral gavage and held for 30 min. The second group of mice were orally gavaged with 5 mg/kg DON and held for 1) 5 min, 2) 15 min or 3) 30 min. Mice were sacrificed by cervical dislocation, and spleens were removed for extraction of proteins to be used in Western blot and proteomic analyses. Each group was repeated in three independent experiments (n=3).
Figure 4.1. Experimental design for stable isotope dimethyl labeling-based quantitative phosphoproteomic analysis of DON-induced RSR in the spleen. Mice were orally gavaged with vehicle or 5 mg/kg b.w. DON for different time periods in two independent groups. After nuclear and cytoplasmic proteins were extracted, equal amount of proteins from different treatment groups were trypsin digested, dimethyl labeled and equally mixed. Phosphopeptides were enriched by titanium dioxide chromatography and analyzed by high-resolution mass spectrometry. Each set was repeated in three independent experiments (n=3).
Western blot

Nuclear and cytoplasmic fractions were extracted from spleen using a Nuclear Extract Kit (Active Motif, Carlsbad, CA). Fractions from three treatment types within a group were pooled equally based on protein content as determined by BCA Protein Assay (Pierce, Rockford, IL). Immunoblotting analysis was performed as described (Bae and Pestka, 2008). Antibodies against the following proteins were used: p38, phospho-p38 MAPK (Thr180/Tyr182), p42/p44 MAP kinase, phospho-p42/p44 MAP kinase (Thr202/Tyr204), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), beta-actin, stathmin, phospho-stathmin (Ser38), filamin A, phospho-filamin A (Ser2152), myosin 9, phospho-myosin 9 (Ser1943), lamin A (Cell Signaling, Danvers, MA), and phospho-lamin A (Ser392) (Abcam, Cambridge, MA). Blots were scanned on the Odyssey IR imager (LICOR, Lincoln, NE) and quantification was performed using LICOR software v.3.0.

Phosphopeptide measurement

Protein phosphorylation changes in the spleens were analyzed by proteomics analysis using stable isotope dimethyl labeling coupled with TiO₂ chromatography for phosphopeptide enrichment and high-accuracy mass spectrometric characterization (Figure 1). Cytoplasmic (3.3 mg) and nuclear (500 µg) protein were digested with trypsin using the FASP protocol (Wisniewski et al., 2009) using spin ultrafiltration units of nominal molecular weight cut of 30,000 (Millipore, Billerica, MA). Employing a triplex dimethyl labeling strategy, peptides originating from nuclear and cytoplasmic proteins of three treatment types within a group were labeled on-column with light, intermediate or heavy dimethyl reagents as described previously (Boersema et al., 2009). The defined
mass increments introduced among the three groups resulted in characteristic peptide triplets that enabled relative quantification of peptide abundance. Eluted labeled peptides within each set were mixed equally and further enriched for phosphopeptide with Titansphere PHOS-TiO Kit (GL Sciences, Torrance, CA) following manufacturer's instructions. Enriched peptides were desalted using 100 mg reverse-phase tC18 SepPak solid-phase extraction cartridges (Waters, Milford, MA). The phosphoproteomic study comprised three independent biological replicates to account for the biological and technical variability.

Peptides were resuspended in 2% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) (v/v) to final concentration of 20 µg/µL. From this, 10 µL (200 µg) were loaded for 5 min onto a Waters Symmetry C18 peptide trap (5 µm, 180 µm x 20 mm) using a Waters nanoAcquity Sample Manager (www.waters.com). The column was washed at 4 µL/min with 2% ACN/0.1% Formic Acid (v/v). Bound peptides were eluted using a Waters nanoAcquity UPLC (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 99.9% ACN/0.1% Formic Acid [v/v]) onto a Michrom MAGIC C18AQ column (3 u, 200 A, 100 U x 150 mm, www.michrom.com) and eluted over 240 min with a gradient of 5% B to 30% B in 225 min, ramping to 90%B at 227 min and back to 5% B at 228.1 min at a constant flow rate of 0.8 µL/min. Eluted peptides were sprayed into a ThermoFisher LTQ-FT Ultra mass spectrometer (www.thermo.com) using a Michrom ADVANCE nanospray source. Survey scans were taken in the FT (25000 resolution determined at m/z 400) and the top ten ions in each survey scan are then subjected to automatic low energy collision induced dissociation (CID) in the LTQ.
**Data analysis**

Peak list generation, protein quantitation based upon dimethyl labeling, extracted ion chromatograms (XIC) and estimation of false discovery rate (FDR) were all performed using MaxQuant (Cox and Mann, 2008), v1.2.2.5. MS/MS spectra were searched against the IPI mouse database, v3.72, appended with common environmental contaminants using the Andromeda searching algorithm (Cox *et al.*, 2011). Further statistical analysis of the dimethyl labeled protein and peptide ratio significance was done using Perseus, v1.2.0.17 (www.maxquant.org). MaxQuant parameters were protein, peptide and modification site maximum FDR = 0.01; minimum number of peptides = 1; minimum peptide length = 6; minimum ratio count = 1; and protein quantitation was done using all modified and unmodified razor and unique peptides. Andromeda parameters were triplex dimethyl labeling: light condition (DimethylLys0, DimethylNter0), medium condition (DimethylLys4, DimethylNter4), heavy condition (DimethylLys8, DimethylNter8); fixed modification of Carbamidomethylation (C), variable modifications of Oxidation (M), Phosphorylation (STY) and Acetyl (Protein N-term); Maximum number of modifications per peptide = 5; enzyme trypsin max missed cleavage = 2; parent Ion tolerance = 6ppm; fragment Ion tolerance = 0.6 Da and reverse database search was included. For each identified dimethyl triplet, MaxQuant calculated the three extracted ion chromatogram (XIC) values, and XICs for the light and medium member of the triplet were normalized with respect to the heavy common 30 min time point of DON treatment, which was scaled to one. A significance value, Significance B, was calculated for each dimethyl relative quantification ratio and corrected by the method of Benjamini and Hochberg using the
statistical program Perseus in the MaxQuant environment (Cox and Mann, 2008). Significance B values of a phosphopeptide in the two groups are calculated independently. Significant protein ratio cutoff was set as at least one set of time course has a Significance B value ≤0.05 as calculated by MaxQuant (Cox et al., 2009). Phosphosites were mapped with PhosphoSitePlus (Hornbeck et al., 2012).

**DAVID analysis**

Analyses for gene ontology (GO) annotation terms were performed with the functional annotation tool DAVID (http://david.abcc.ncifcrf.gov/). Uniprot accession identifiers of significantly regulated phosphoproteins were submitted for analysis of GO biological processes and KEGG pathways, with a maximal DAVID EASE score of 0.05 for categories represented by at least two proteins.

**Fuzzy c-means clustering**

Fuzzy c-means (FCM) clustering (Olsen et al., 2006) in MATLAB (MathWorks) was used to search for patterns in the time profile patterns among all identified phosphopeptides. To robustly represent the ratio of a peptide being quantified multiple times, the median values of the three biological replicates were used for clustering. The phosphoproteomic ratios of peptides quantified over all four time points were included, natural log transformed and normalized. From the 20 clusters, we selected 6 based on the average probability of each cluster to represent early, intermediate and late responders with phosphorylation levels up- or down-regulated.
Statistics

Data were analyzed using SigmaStat for Windows (Jandel Scientific, San Rafael, CA). A Kruskal-Wallis One Way Analysis of Variance was performed. Data sets were significantly different when p<0.05.

Results and Discussion

Relation of DON distribution to RSR onset in the spleen

Following oral administration, DON was detectable in the spleen within 5 min and reached peak concentration within 15 min (Figure 4.2A). The toxin was rapidly cleared to about half the peak concentration around 30 min, which is consistent with previous observations (Pestka et al., 2008). DON-induced phosphorylation of MAPKs, including p38, ERK1/2, and JNK2, was evident at 15 min (Figure 4.2B). These results confirm that the time window used in this investigation included both peak DON concentration and the initiation of RSR.

DON-induced changes in splenic phosphoproteomic profile

We reproducibly identified 500 and 516 unique phosphopeptides corresponding to 226 and 302 proteins in the nuclear and cytoplasmic fractions, respectively, at an accepted false discovery rate (FDR) of 1%. Approximately 22% of the total phosphopeptides identified were considered significant (significance B with a Benjamini-Hochberg corrected FDR<5%) (Figure 4.3A, B). A total of 90 proteins possessed significantly altered phosphorylation status, with 43 in the nucleus and 53 in the cytoplasm. More than 96% of identified peptides harbored at least one phosphosite, indicating that there was highly efficient phosphopeptide enrichment by TiO2.
chromatography. The distribution of all phosphorylated serine, threonine, and tyrosine sites identified was 83.0%, 14.8% and 2.1%, respectively (Figure 4.3C, D).

The potential for handling stress-induced changes in phosphorylation was evaluated by comparing no treatment and vehicle groups. There were 15 proteins that were affected by animal handling, and these were excluded for functional analysis. Overall, extensive phosphoproteome changes occurred in the spleen following DON exposure that encompass known and yet-to-be functionally characterized phosphosites.

**Confirmation of proteomic changes**

To verify the quantification of phosphorylation in stable isotope dimethyl labeling-based proteomics, the phosphorylation kinetics of four representative proteins, stathmin 1 (STMN1), filamin A (FLNA), myosin 9 (MYH9) and lamin A (LMNA) were measured by Western blotting using phosphosite-specific antibodies. Detection of relative up- and down-regulation of protein phosphorylation by proteomics and immunoblot were in agreement (Figure 4), supporting the reliability of the proteomics approach in measuring the dynamic phosphorylation during DON-induced RSR.

**Biological analysis of altered phosphoproteins**

To discern the potential impact of DON-induced phosphorylation, changes in the prevalence of differentially phosphorylated proteins were related to specific biological processes using GO annotation in DAVID. GO analysis indicated that 14 percent of all significantly regulated phosphoproteins were associated with intracellular signaling cascades (Figure 5). Thus, DON-induced RSR in the spleen was extraordinarily complex involving the inter-related MAPK and PI3K/AKT pathways (Table 4.1) that
Figure 4.2. Relationship between of DON tissue concentrations and RSR onset in the spleen. (A) DON concentrations in the spleen after oral exposure to 5 mg/kg b.w. DON. Data are presented as mean ± SEM. (n=6). Different letters indicate a statistically significant difference between time points (p<0.05). (B) Representative Western blot of p38, ERK and JNK and their phosphorylation status in the spleen of mice exposed to DON using total and phospho-specific antibodies, with beta-actin as internal control.
Figure 4.3. Summary of protein phosphorylation changes during early DON-induced RSR in the mouse spleen. Numbers of significantly regulated (A) phosphopeptides and (B) phosphoproteins (grey) among all unique proteins and peptides identified (grey + black). Significance was determined by Significance B values corrected for false discovery rate (FDR) ≤ 0.05. Overall distribution of phospho-serine, threonine and tyrosine detected in the study (C) and significantly regulated by DON (D) in nuclear and cytoplasmic fractions combined.
Figure 4.3. (cont’d)

C

- pSer: 83.0%
- pThr: 14.8%
- pTyr: 2.1%

D

- pSer: 82.8%
- pThr: 13.8%
- pTyr: 3.4%
Figure 4.4. Confirmation of phosphoproteome quantification. Phosphorylation levels of STMN1, FLNA, MYH9 and LMNA were measured using Western blot by normalizing the signaling intensity of the bands from phosphosite-specific antibody with those from total antibody. Normalized intensities (red, shown as mean ± SEM) were then correlated with the phosphoproteomic ratios (blue, shown as median).
Figure 4.4.(cont’d)
linked early cellular events to later proinflammatory gene activation, apoptosis and other immunotoxic effects of DON. In addition, DON exposure affected other biological processes such as cytoskeleton organization, regulation of apoptosis and lymphocyte activation and development (Figure 4.5).

It is important to note that phosphorylation changes described in this study represent the average among many cell types in the spleen. However, the phosphorylation of proteins could be differentially regulated within individual cells. Some of the phosphorylation changes might cross-compete, and therefore have little or no impact on cell functions. In addition, it is possible that a temporal gap exists in the onset of DON’s effects in cell signaling, proinflammatory gene activation and apoptosis. Consequently, it is important to meld the in vivo findings herein with prior in vitro and in vivo studies to dissect the differential effects of DON within various cell populations and the spleen as a whole.

Integration of phosphoproteomic data with prior research: MAPK signaling

The MAPK pathway is central to DON-induced RSR, and the phosphorylation of p38, ERK1/2 and JNK1/2 have been demonstrated in the mouse spleen here (Figure 4.2B) and previously (Zhou et al., 2003a). Consistent with these findings, phosphoproteomic analysis identified kinases that could function as upstream and downstream mediators of the activated MAPKs (Figure 4.6A). These included astrocytic phosphoprotein PEA15 (upstream of ERK), STMN1 and MAP kinase-interacting serine/threonine-protein kinase 2 (MNK2) (downstream of ERK), and filamin A (FLNA) which is a scaffold in MAPK signaling.
Table 4.1. Splenic phosphosites associated with proteins involved in MAPK and PI3K/AKT pathways in the mouse spleen

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Protein description</th>
<th>MAPK pathway</th>
<th>PI3K/AKT pathway</th>
</tr>
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<tbody>
<tr>
<td>PEA15</td>
<td>Astrocytic phosphoprotein PEA15</td>
<td>S116</td>
<td></td>
</tr>
<tr>
<td>STMN1</td>
<td>Stathmin</td>
<td>S38</td>
<td></td>
</tr>
<tr>
<td>MNK2</td>
<td>MAP kinase-interacting serine/threonine-protein kinase 2</td>
<td>S270, T272</td>
<td></td>
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<tr>
<td>FLNA</td>
<td>Filamin A</td>
<td>S2152</td>
<td></td>
</tr>
<tr>
<td>PI4K</td>
<td>Phosphatidylinositol 4-kinase</td>
<td>T292</td>
<td></td>
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<tr>
<td></td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma</td>
<td>S257, S262, S264</td>
<td></td>
</tr>
<tr>
<td>PIK3CG</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NHERF1</td>
<td>Sodium-hydrogen exchanger regulatory factor 1</td>
<td>S275</td>
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<tr>
<td>PKD2</td>
<td>Protein kinase D2</td>
<td>S197</td>
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<tr>
<td>CTNNB1</td>
<td>β-catenin</td>
<td>T556</td>
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<tr>
<td>DNMT1</td>
<td>DNA methyltransferase 1</td>
<td>S125</td>
<td></td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukaemia</td>
<td>T527</td>
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</table>
The upstream mediator PEA15 binds to ERK1/2 and inhibits its nuclear localization, thus blocking cell proliferation (Krueger et al., 2005). Such binding and inhibitory effects are abolished when PEA15 is phosphorylated at Ser116, which was observed at 15 min during DON-induced RSR. The downstream mediator STMN1 is a small microtubule binding protein that regulates the rates of polymerization and disassembly of microtubules (Nakamura et al., 2006). ERK-driven phosphorylation of STMN1 at four serine residues including Ser38, which is significantly regulated in DON-induced RSR, leads to cell cycle arrest and apoptosis.

Dual phosphorylation of another downstream mediator, MNK2, at previously unreported sites Ser270, Tyr272 was decreased at 30 min. With ERK and serine/threonine-protein phosphatase 2A (PP2A) controlling its phosphorylation status, MNK1/2 could also regulate the release of arachidonic acid and proinflammatory gene activation by phosphorylating cytosolic phospholipase A2 (cPLA2) (Hefner et al., 2000), which was recently shown to be phosphorylated in DON-treated RAW 264.7 cells (Pan et al., 2013).

Phosphorylation of a scaffold protein that organizes MAPK signaling, FLNA, was also altered during DON-induced RSR. FLNA integrates the JNK pathway via interaction with MKK4, the p38 pathway via interaction with TAK1 and p38, and the ERK pathway via interaction with MAPK6, and transcription factors cJUN, p65 and STAT1 (Morrison and Davis, 2003; Bandyopadhyay et al., 2010). Over the 30 min of DON treatment period, FLNA phosphorylation at Ser2152 gradually increased, which could protect it against proteolytic cleavage by calpain (Chen and Stracher, 1989), thereby
Figure 4.5. Gene Ontology associated with significantly regulated phosphoproteins as determined by DAVID Biological Process terms (DAVID EASE score<0.05). Data are presented as a histogram of the relevant biological processes identified and shown as a percentage of the total identified proteins that fall within each category.
Figure 4.6. MAPK (A, blue) and PI3K/AKT (B, red) signaling pathways linked to DON-induced RSR in the spleen.
strengthening the structural framework and enhancing MAPK signaling in the RSR. Accordingly phosphoproteins affected by DON exposure might not only be involved in directly mediating the MAPK signaling, but also in the regulation of MAPK signaling organization.

Integration of phosphoproteomic data with prior studies: PI3K/AKT signaling

The PI3K/AKT pathway was also potentially targeted in DON-induced RSR (Figure 4.6B), with affected proteins being involved in the regulation of phosphoinositide turnover (via PI4K, PI3K, NHERF1), as well as transcriptional regulation (via DNMT1, PML, PKD2).

Phosphoinositide turnover involves a series of reversible lipid phosphorylation reactions. The catalytic domain of phosphatidylinositol 4-kinase (PI4K) had decreased phosphorylation at T292 in the PI3K/PI4K domain at 30 min after DON treatment. This kinase mediates the production of P(4,5)P2, which could be further phosphorylated to P(3,4,5)P3 (PIP3) by phosphatidylinositol 3-kinase (PI3K), or hydrolyzed to inositol triphosphate (IP3) and diacylglycerol (DAG) by phospholipase C (PLC). All of these lipids are important secondary messengers in cellular signaling.

PI3K, which positively catalyzes phosphorylation of second messenger PIP3, was differentially phosphorylated by DON in the spleen. PIP3 is negatively regulated by two lipid phosphatases, namely SH2-containing inositol-5'-phosphatase (SHIP) that was shown to be affected by DON in the RAW 264.7 (Pan et al., 2013), and phosphatase and tensin homolog (PTEN). Triple phosphorylation of the catalytic subunit of PI3K (PIK3CG) at novel phosphosites Ser257, Ser272, Ser274 within the RAS-binding
domain (RBD) was increased at 15 min but rapidly decreased at 30 min, which might indicate a transient modulation of PI3K interaction with and activation by RAS.

The sodium-hydrogen exchanger regulatory factor 1 (NHERF1) is known to recruit PTEN and restrict PI3K activation (Takahashi et al., 2006). Dephosphorylation of NHERF1 at Ser275 occurred at 30 min in DON-induced RSR, which could weaken the association of NHERF1 with PTEN, and facilitate the activation of PI3K pathway (Voltz et al., 2007).

Transcription is potentially regulated during DON-induced RSR via transcription factor and epigenetic mechanisms. AKT1 is a kinase activated by PIP3 that was previously demonstrated to be rapidly phosphorylated and activated by DON in RAW 264.7 cells and peritoneal macrophages (Zhou et al., 2005a; Shi and Pestka, 2009). This results in phosphorylation and inhibition of glycogen synthase kinase-3 beta (GSK3B), which phosphorylates β-catenin (CTNNB1) and targets it for degradation. As shown here, DON induced CTNNB1 dephosphorylation at 15 min. This protein has been previously shown to translocate to the nucleus, where it acts as a coactivator for transcription factors of the TCF/LEF family, c-JUN, c-MYC and n-MYC (Staal et al., 2008). AKT1 is also a kinase for DNA methyltransferase 1 (DNMT1) (Esteve et al., 2011), which was phosphorylated here at Ser125. The possible effect of DON on DNA methylation has been previously suggested in RAW 264.7 macrophages (Pan et al., 2013) and Caco-2 intestinal epithelial model (Kouadio et al., 2007).

Also significantly altered in both mouse spleen and RAW 264.7 was promyelocytic leukaemia (PML), a protein that induces gene hypermethylation and
silencing by recruiting DNMTs to target promoters (Di Croce et al., 2002). Decreased phosphorylation of PML at Ser528 at 30 min stabilizes it from being targeted for degradation via the ubiquitination pathway (Yuan et al., 2011). Notably protein kinase D2 (PKD2) was significantly phosphorylated at 30 min in DON-induced RSR. Activated PKD2 could translocate to the nucleus where it phosphorylates and inhibits Class IIa HDACs (Vega et al., 2004). Similarly, DON was previously shown to modulate the phosphorylation of HDAC1 and HDAC2 in RAW 264.7 (Pan et al., 2013). Overall, phosphoproteins impacted during DON-induced RSR likely affect phosphoinositide signaling and transcriptional regulation via the PI3K/AKT pathway.

**Integration of phosphoproteomic data with prior research – leukocyte targets of DON**

Phosphorylation of proteins involved in lymphocyte activation and development was significantly altered, suggesting that diverse immune cell populations, including monocytes, macrophages, T cells, B cells, dendritic cells and mast cells, could be impacted by DON exposure (Figure 4.7). Two of the identified proteins, lymphocyte cytosolic protein 1 (LCP1) and myosin, heavy polypeptide 9 (MYH9), are involved in the activation and development of monocytes and T cells. LCP1 is an actin-binding protein expressed exclusively in hemopoietic cell lineages. Phosphorylation of LCP1 at Ser5, as observed in DON-induced RSR at 5 min, plays a role in the activation of T cells in response to co-stimulation through T cell receptor (TCR) /CD3 and CD2 or CD28 (Wabnitz et al., 2007). This phosphosite has also been shown to be phosphorylated in response to lipopolysaccharide (LPS) (Shinomiya et al., 1995). MYH9 is a myosin protein involved in cell adhesion and is required for the establishment of T cell polarity.
and monocyte differentiation (Jacobelli et al., 2004). It is phosphorylated at Ser1943 transiently at 5 min in DON-induced RSR. Apoptotic chromatin condensation inducer 1 (ACIN1) plays a part in positive regulation of monocyte differentiation (Sordet et al., 2002), and erythrocyte differentiation (Zermati et al., 2001).

T cells and B cells have been implicated previously in DON-induced RSR. It has been proposed that, based on transcriptomic studies in the mouse thymus (van Kol et al., 2011) and Jurkat human T cells (Katika et al., 2012a), DON induces cellular events that contribute to T cell activation. Furthermore, B cell activation has been implicated in mediating DON-induced IgA nephropathy (Pestka and Dong, 1994). The phosphoproteomic results suggested that early signaling is likely to precede these events. Relative to T cell activation, minor histocompatibility antigen HA-1 (HMHA1) was differentially phosphorylated significantly in DON-induced RSR. When complexed with MHC, this protein is known to drive an immune response following recognition by TCR complex on T cells (Lin and Weiss, 2001). Finally, SH2 domain containing 3C (SH2D3C), which exhibited significantly altered phosphorylation status here, enhances T cell activation by interacting with tyrosine kinase PYK2 (Sakakibara et al., 2003).

Among the shared components of T cell and B cell activation, CD45 is a surface antigen and tyrosine phosphatase. It enhances the activation T cells and B cells by dephosphorylating the C-terminal inhibitory tyrosine and activating the SRC proteins LCK and FYN (Lin and Weiss, 2001). Phosphorylation of CD45 at Ser962 within its tyrosine-protein phosphatase domain 2 was significantly increased at 30 min, which might suggest increased CD45 phosphatase activity and enhanced T cell and B cell activation. T cell and B cell activation both involve the phosphorylation of caspase
Figure 4.7. Phosphorylation changes of proteins associated with lymphocyte activation and development during DON-induced RSR in the spleen. The heat map depicts the log 2 transformed relative abundance (red to green) of the protein phosphorylation, grey indicates missing values in the phosphoproteomic analysis.
<table>
<thead>
<tr>
<th>Protein name</th>
<th>Protein description</th>
<th>Regulatory function involvement</th>
<th>Phosphosite</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>Protein tyrosine phosphatase, receptor type, C</td>
<td>T cell, B cell, MAPK cascade, antigen receptor-mediated signaling pathway, response to cytokine stimulus, defense response to virus</td>
<td>S962</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CARD11</td>
<td>Caspase recruitment domain family, member 11</td>
<td>T cell, B cell, NF-κB pathway, response to cytokine stimulus</td>
<td>Y493</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTNNB1</td>
<td>Catenin (cadherin associated protein), beta 1</td>
<td>T cell, dendritic cell, NF-κB pathway, Wnt receptor signaling pathway, MAPK cascade, response to cytokine stimulus</td>
<td>T556</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>UNC13D</td>
<td>Protein unc-13 homolog D</td>
<td>T cell, mast cell</td>
<td>S149</td>
<td></td>
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</table>
recruitment domain family member 11 (CARD11). This protein, transiently phosphorylated at 15 min in DON-induced RSR, could contribute to the activation of NF-κB (Sagaert et al., 2010), which has been demonstrated in RAW 264.7 macrophages under DON-induced ribotoxic stress (Wong et al., 2002; Pan et al., 2013).

In addition to its involvement in PI3K/AKT signaling as discussed above, CTNNB1 is a regulator for T cell and dendritic cell function. It is a critical factor in CD8-positive T-cell differentiation and memory development (Gattinoni et al., 2009). In intestinal dendritic cells, CTNNB1 is required for the expression of anti-inflammatory mediators such as retinoic acid metabolizing enzymes, IL-10, and transforming growth factor-beta (TGFβ) (Manicassamy et al., 2010). Unc-13 homolog D (UNC13D), another protein with significantly regulated phosphorylation status, plays a role in cytotoxic granule exocytosis in lymphocytes, and is required for both granule maturation, granule docking and priming at the immunologic synapse (Menager et al., 2007). UNC13D also regulates Ca(2+)-dependent secretory lysosome exocytosis in mast cells (Neeft et al., 2005). In summary, DON-induced RSR in the spleen entails activation and regulation of multiple leukocyte targets including monocytes, macrophages, T cells, B cells, dendritic cells and mast cells.

Large-scale phosphoproteomic studies have been employed previously to elucidate molecular mechanisms of DON-induced RSR in cloned immune cell lines, including mouse macrophage (Pan et al., 2013), and human T and B cell lines (Nogueira da Costa et al., 2011a), as well as to identify biomarkers of effect for DON. While valuable for mechanistic exploration of DON-induced RSR in specific immune cell types, it is recognized that alterations in signaling pathways and biological processes
derived from these *in vitro* models cannot always be extrapolated to *in vivo* systems, due to the complexity relative in 1) diversity of cell types, 2) pharmacokinetic distribution of DON, and 3) intercellular communication in the immune system of an intact animal. Nevertheless, among the early phosphoproteome alterations identified *in vivo*, a total of 30 significantly regulated phosphoproteins, including STMN1, LMNA, PML and BCLAF1, overlap with those previously detected *in vitro* in DON-exposed RAW 264.7 macrophages, suggesting that the innate immune system is a critical target.

**Integration of phosphoproteomic data with prior research – regulation of apoptosis**

DON-induced apoptosis has been demonstrated both *in vivo* and *in vitro*. *In vivo* administration of high doses of DON to mice causes apoptosis in spleen, thymus, Peyer's patches, and bone marrow (Zhou *et al.*, 1999). Similarly, high concentrations of DON can evoke rapid onset of apoptosis *in vitro*, such as in RAW 264.7 murine macrophages, T cells and B cells (Pestka *et al.*, 1994; Zhou *et al.*, 2005a) potentially leading to suppression of immune function. DON induces apoptosis by both intrinsic and extrinsic pathways (Zhou *et al.*, 2005a; He *et al.*, 2012c). As shown here, prior to apoptosis onset, regulators of both survival and apoptotic pathways are differentially phosphorylated in the spleen of mice treated with DON (Figure 4.8).

One protein with altered phosphorylation status during DON-induced RSR was Bcl-2-associated transcription factor 1 (BCLAF1), a Bcl-2 family protein, is proapoptotic (Kasof *et al.*, 1999), and encodes a transcriptional repressor that interacts with several members of the Bcl-2 family of proteins. The Bcl-2 family includes both proapoptotic and anti-apoptotic regulators that regulate the intrinsic apoptosis pathway. CD45 mediates
apoptosis via both the intrinsic and extrinsic pathways and is involved in the DNA fragmentation and DNA condensation process, playing a role in the proapoptotic pathway activation (Dupere-Minier et al., 2010). Another protein impacted in DON-induced RSR also identified in the phosphoproteomic study, PML, mediates apoptosis in a p53-dependent manner by stabilizing p53 and phosphorylation/activation of p53 at Ser20 (Guo et al., 2000). p53 phosphorylation and binding activity are induced by DON in RAW 264.7 macrophages (Zhou et al., 2005a). Alternatively, PML could stimulate apoptosis in a p53-independent manner via the ATM and checkpoint kinase-2 (CHEK2) in U937 human monocytes, in which overexpression of PML led to apoptosis (Yang et al., 2002).

Multiple caspase substrates showed altered phosphorylation during DON-induced RSR, including apoptotic chromatin condensation inducer 1 (ACIN1), DNA fragmentation factor alpha subunit (DFFA), lamin A (LMNA) and lamin B1 (LMNB1). ACIN1 induces apoptotic chromatin condensation after activation by caspase 3 (Sahara et al., 1999). DNA fragmentation factor (DFF) is composed of heterodimer of DFFA (significantly regulated during DON-induced RSR) and DFFB subunits, and is one of the major endonucleases responsible for internucleosomal DNA cleavage during apoptosis. Upon cleavage of DFFA by caspase-3, DFFB triggers DNA fragmentation and chromatin condensation (Zhang et al., 1999). In addition, caspase 6-dependent degradation of lamins, including LMNA and LMNB1, facilitates the nuclear events of apoptosis by inducing nuclear breakdown as a prelude to nuclear destruction (Burke, 2001).
Figure 4.8. Phosphorylation changes of proteins associated with regulation of apoptosis during DON-induced RSR in the spleen. The heat map depicts the log 2 transformed relative abundance (red to green) of the protein phosphorylation, and grey indicates missing values in the phosphoproteomic analysis.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Protein description</th>
<th>Role in apoptosis</th>
<th>Phosphosite</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCLAF1</td>
<td>BCL2-associated transcription factor 1</td>
<td>Pro-apoptotic</td>
<td>S494</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CD45</td>
<td>Protein tyrosine phosphatase, receptor type, C</td>
<td>Pro-apoptotic</td>
<td>S962</td>
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<tr>
<td>PML</td>
<td>Promyelocytic leukemia</td>
<td>Pro-apoptotic</td>
<td>T527</td>
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<td></td>
<td></td>
<td></td>
<td>S528</td>
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<tr>
<td>ACIN1</td>
<td>Apoptotic chromatin condensation inducer 1</td>
<td>Pro-apoptotic</td>
<td>S479</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>S1003</td>
<td></td>
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<tr>
<td>DFFA</td>
<td>DNA fragmentation factor, alpha subunit</td>
<td>Pro-apoptotic</td>
<td>S314</td>
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<td></td>
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<tr>
<td>Protein name</td>
<td>Protein description</td>
<td>Role in apoptosis</td>
<td>Phosphosite</td>
<td>0</td>
<td>5</td>
<td>15</td>
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<tr>
<td>LMNA</td>
<td>Lamin A</td>
<td>Pro-apoptotic</td>
<td>S392</td>
<td></td>
<td></td>
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<tr>
<td>LMNB1</td>
<td>Lamin B1</td>
<td>Pro-apoptotic</td>
<td>S24</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td>S392</td>
<td></td>
<td></td>
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<tr>
<td>CARD11</td>
<td>Caspase recruitment domain family, member 11</td>
<td>Anti-apoptotic</td>
<td>Y493</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PEA15</td>
<td>Phosphoprotein enriched in astrocytes 15A</td>
<td>Anti-apoptotic</td>
<td>S116</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol-3-kinase, catalytic, gamma polypeptide</td>
<td>Anti-apoptotic</td>
<td>S257, S262, S264</td>
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In addition to proapoptotic proteins, regulators of anti-apoptotic pathways were also impacted during early DON-induced RSR. CARD11 might contribute to NF-κB activation, which could further activate members of the inhibitor of apoptosis protein (IAP) family (Ryan et al., 2000). PEA15 contains death effector domain (DED), and binds other DED-containing proteins, preventing the formation of the death-inducing signaling complex and inhibiting activation of the caspase cascade (Trencia et al., 2004). Finally, the PI3K/AKT activated pathway during DON-induced RSR is one of the strongest intracellular pro-survival signaling systems. It can phosphorylate and inactivate BAD (a proapoptotic mitochondrial Bcl-2 family protein) or phosphorylates mTOR that then activates p70S6K to phosphorylate BAD. Other downstream targets of AKT such as IκBα/NF-κB and GSK3 are involved in regulating cell survival as well (Fresno Vara et al., 2004).

Accordingly, the diverse phosphoproteomic changes in early DON-induced RSR associated with regulation of apoptosis discovered here recapitulate previous in vitro observations that this toxin evokes competing apoptotic and survival pathways (Zhou et al., 2005a). Before apoptosis onset, phosphorylation changes occur on proteins in the proapoptotic pathways, such as PML and BCLAF1 that regulate p53 and Bcl-2 family, which are previously known to be phosphorylated in DON-induced RSR, as well as downstream caspase substrates that have directly lead to physiological and morphological alterations characteristic of apoptosis. Proteins modifying the activity of the anti-apoptotic pathway, PI3K and PEA15, were also differentially phosphorylated. These early phosphorylation changes likely fine-tune the balance between apoptotic and survival signaling.
Categorization of DON-regulated phosphopeptides into distinct temporal profiles

Fuzzy c-means clustering analysis was employed to trace and categorize the temporal changes of protein phosphorylation observed. Based on the pattern of phosphorylation changes and the average probability of the clusters, six distinctive temporal profiles (rapid, intermediate, prolonged) of the regulated (phosphorylated, dephosphorylated) phosphopeptides were recognized (Figure 4.9A-F). DON-induced RSR initiated with rapid (≤ 5 min) dephosphorylation of RNA processing proteins (e.g. SFRS1, SFRS9, DKC1) (Figure 4.9A), and phosphorylation of apoptosis regulatory proteins (e.g. STMN1, PDCD4, SLTM, STEAP3) (Figure 4.9B). The stress response proceeded with a cascade of phosphorylation events involving proteins in transcriptional regulation that are phosphorylated (e.g. PML, TCOF1, HDGF, TRP53BP1) (Figure 4.9C) and RNA processing that are dephosphorylated (e.g. RBM39, SRRM2, SF1) (Figure 4.9D) up to approximately 15 min. As RSR progressed, prolonged dephosphorylation of cellular protein complex assembly-related proteins (e.g. FLNA, MYH11) occurred to organize intracellular and intercellular signaling (Figure 4.9E) whereas prolonged phosphorylation of cell cycle proteins (e.g. NIPBL, EEF1D) was evident, possibly arresting the cell cycle under DON-induced RSR (Figure 4.9F).

Conclusions

The global, quantitative and kinetic phosphoproteomic analysis presented here expands our understanding of signaling events leading to DON-elicited immunotoxicity from simply MAPK activation to an intricate finely-tuned signaling network involving the MAPK and PI3K/AKT signaling cascades. Phosphoproteome changes were also
Figure 4.9. Temporal profiles of DON-regulated phosphosites. Based on the phosphorylation dynamics, Fuzzy c-means clustering analysis generated clusters representing early, intermediate and late responders with phosphorylation levels up- or down-regulated were selected from 20 clusters based on the average probability (A-F). Each trace depicts the natural log value of relative phosphorylation level as a function of time, and is color coded according to its membership value (i.e. the probabilities that a profile belongs to different clusters) for the respective cluster. Each cluster is designated by the function of prominent members, with examples of such members given for each cluster.
Figure 4.9. (cont’d)

Phosphorylation increase
- PML(T527), TCOF1(S1191), HDGF(S165), TRP53BP1(S261)

Phosphorylation decrease
- RBM39(S136), SRRM2(S349), SF1(S80)

Graphs showing changes in phosphorylation over time after DON treatment. The x-axis represents time after DON treatment (min), and the y-axis represents phosphorylation change.
reflected in proteins associated with downstream regulation of the immunostimulatory and immunosuppressive sequelae. Multiple immune cell populations were targeted as indicated by the identification of altered phosphoproteins associated with immune system activation and development. Competing apoptotic and survival pathways were both fired in early in DON-induced RSR before the balance shifts towards apoptosis in case of prolonged stress. Extensive protein phosphorylation changes take place in lymphoid tissue very early within 30 min, preceding previously reported immunotoxic effects of DON such as proinflammatory gene activation and apoptosis that occur later after 1 to 3 h and 6 h, respectively (Figure 4.10).

It must be recognized that the spleen is comprised of T cells, B cells, macrophages, dendritic cells, mast cells, natural killer cells, red blood cells, as well as cells associated with splenic vasculature and supporting tissues. While it would be desirable to dissect the contribution to total phosphoproteome changes by cell type and to understand the different impact of DON on different cell populations in vivo, it is not possible to dissociate and sort the diverse cell types in the spleen without disturbing protein phosphorylation. In addition, DON concentration in the spleen rises and falls during absorption, distribution, metabolism and excretion, whereas in the cell culture, cells as a single layer are exposed to toxin treatment instantaneously. Finally, analysis of homogenous cell cultures neglects the importance of cell communication across cell types in mounting an immune response. DON affects proteins, such as claudin (Pinton et al., 2010), ZO-1 (Diesing et al., 2011), SRC, ZAK (Pan et al., 2013), AKT as previously reported, and myosins (MYH9, MYH11), CTNNB1, EPB4.1 as identified in this study, that are involved in tight junctions. They allow intercellular communication
and interaction by metabolically and electrically coupling cells together (Denker and Nigam, 1998), which could not be otherwise captured in an in vitro model.

As compared to metabolic labeling, dimethyl labeling, as employed in this study, involves an addition chemical derivatization step, and therefore, could introduce additional sample handling and concomitant sample loss, which might compromise the coverage of the phosphorylation events. It should be further emphasized that, except for those proteins that are discussed above, the functions of many phosphosites identified here have not yet been functionally characterized, which prevents further functional interpretation of the effect of the phosphorylation changes on immunotoxicity. Functional annotation of the phosphoproteome awaits further studies, and characterization of phosphosites is often confined to protein location without understanding of their role in relation to cell functions. Full characterization of phosphosite function will require genetic mutation of these sites to other amino acids, however, it exceeds the scope of the present study. Nevertheless, our research provides a hypothesis-generating tool and inventories proteins with changed phosphorylation status for future focused studies.

Taken together, this investigation provides a foundation for future exploration of the function of phosphoproteins identified and their associated pathways in DON-induced RSR in the spleen. This study advances the knowledge in the molecular mechanisms of the early development of immunotoxicity elicited by an immunotoxicant frequently encountered in human and animal food. Over the long term, these findings could ultimately contribute to designing strategies for the treatment and prevention of the adverse effects of DON and other ribotoxic stressors.
Figure 4.10. Summary of pathways and biological processes potentially leading to the immunotoxicity caused by DON. In response the ribosome damage caused by DON, signaling pathways including MAPK and PI3K/AKT pathways are activated which mediate key biological processes regulating different aspects of immunotoxicity elicited by DON. Proteins in bold indicate novel mediators identified in the present study, proteins in bold and italics indicate previously known mediators confirmed here, and proteins in italics indicate mediators previously determined in other studies but not identified here.
CHAPTER 5: Conclusions and future directions

DON, a well-established translational inhibitor, causes immunotoxicity via the RSR. It has been reported that DON binds to the ribosome and the RSR induced by DON involves phosphorylation of stress-related kinases associated with the ribosome. However, critical gaps exist in 1) the global phosphorylation changes leading to DON-induced RSR and immunotoxicity and 2) the potential role of the ribosome as a platform for protein association and phosphorylation. Therefore, quantitative and kinetic proteomic analyses were performed to address these gaps in \textit{in vitro} and \textit{in vivo} models.

First, the dynamic phosphoproteome changes during early DON-induced RSR (≤30 min) in SILAC-labeled RAW 264.7, a well-established macrophage model, were identified and quantified (Chapter 2). The results suggest DON-induced RSR entails 1) a complex signaling network involving the MAPK, NF-κB, AKT and AMPK pathways; 2) delicate balance between stress response and quiescence achieved by transcription regulation via transcription factors/cofactors and epigenetic modulators as the main strategy; and 3) translation regulation by coordinating translation control and ribosome biogenesis processes.

Zooming in from the entire cell to the subcellular target of DON, the dynamics of ribosome-associated proteins and their phosphorylation were evaluated to understand on the function of the ribosome in DON-induced RSR (Chapter 4). Large numbers of proteins and phosphoproteins not directly function in translation- or ribosome biogenesis-related protein in the ribosome fractions were identified, which sets a foundation for the versatile extraribosomal functions of the ribosome. During DON-induced RSR, there was an overall decrease in translation-related proteins interacting
with the ribosome, concurrent with compensatory increase in protein folding, biosynthetic pathways, and cellular organization. Alterations in ribosome-associated phosphoproteome mainly involved proteins that could finetune regulation of translation and transcription. These phosphoproteins also converged with known signaling pathways and overlapped with phosphorylation events previously characterized in the whole cell in Chapter 2. Therefore, these results may indicate that in addition to its role in protein synthesis, the ribosome could function as a platform for translation-related and other processes and facilitate spatiotemporal coordination of cell behaviors during DON-induced translational inhibition and RSR. The mechanisms of DON-induced RSR as revealed by the proteomics analyses are summarized in Figure 5.1.

While RAW 264.7 serves as an extensively studied model for DON-induced RSR, the response of the different cell types in the innate immune system to DON, the intercellular communication, and the pharmacokinetic distribution of this toxin could not be represented in this in vitro model. Therefore, phosphoproteome alterations in the spleen of mice exposed to a dose of DON known to induce a robust proinflammatory response were investigated using stable isotope dimethyl labeling (Chapter 3). Similar to the macrophage cell culture model, extensive protein phosphorylation changes take place in lymphoid tissue very early within 30 min, preceding previously reported immunotoxic effects of DON such as proinflammatory gene activation and apoptosis that occur later after 1 to 3 h and 6 h, respectively. Signaling events leading to DON-elicited immunotoxicity entails an intricate finely-tuned signaling network involving the MAPK and PI3K/AKT signaling cascades. Phosphoproteome changes were also reflected in proteins associated with downstream regulation of the immunostimulatory
and immunosuppressive sequelae. Multiple immune cell populations were targeted as indicated by the identification of altered phosphoproteins associated with immune system activation and development. Competing apoptotic and survival pathways were both fired in early in DON-induced RSR before the balance shifts towards apoptosis in case of prolonged stress.

Given that a single regulatory protein can be targeted by many protein kinases and phosphatases, phosphorylation is critical to effectively integrating information carried by multiple signal pathways, thus providing opportunities for great versatility and flexibility in regulation. Signal transduction and modulation of essential biological processes via protein phosphorylation allow the innate immune system to promptly adapt and respond to early DON-induced RSR. These data provide a foundation for future exploration of phosphoprotein functions in DON-induced RSR.

Based on the discoveries in this dissertation, some future research could be performed:

1) Phosphoproteomics results in vitro and in vivo (Chapters 2 and 4) suggested that DON might regulate transcriptional via epigenetic mechanisms, including DNA methylation and histone modification, which could be evaluated with DNA methylation disulfide sequencing and anti-PTM antibodies coupled with Western blot or chromatin immunoprecipitation (ChIP).
Figure 5.1. Suggested model of DON-induced RSR. Upon binding to the ribosome, DON causes ribosome perturbation and alterations in proteins (blue) and phosphoproteins (red) associated with the ribosome. Concurrently in the entire cell, DON induces phosphorylation of key regulators in various biological processes (red) mediated by a signaling network that consist of MAPK-, AKT-, AMPK- and NFκB-linked pathways (green).
2) Ribosome-associated and total proteome alteration (Chapter 3 and Appendix A) suggest that translational reprogramming in the context of global translational inhibition. Therefore, profiling global translatome (i.e. mRNA associated with actively translating polysomes) could facilitate the understanding of the translational regulation/reprogramming in the mouse macrophage after DON exposure and discerning the effect of translational regulation from that of protein localization changes in the total proteome (Appendix A)

3) Recent research on another ribotoxin, ricin, suggest that the ribosomal stalk proteins (RPLP0, RPLP1 and RPLP2) are essential to ricin’s ability to bind to the ribosome and damage rRNA. These stalk protein were differentially phosphorylated in the ribosomal fractions and inhibitors of their phosphorylation may serve as candidates for inhibitors of ribotoxin–ribosome interactions and the development of novel therapeutics

This research could advance our understanding of the molecular mechanisms of DON-induced RSR at the cellular and subcellular level. While serving as a hypothesis generator of the candidate mediators of DON-induced RSR and immunotoxicity, proteomics require further validation and specific inhibitor or siRNA knockdown studies to fully establish their role in this stress response. The results indicate the role of the ribosome other than as the translation machinery, proteins and phosphoproteins with altered association with the ribosome might facilitate future research of the ribosome functions in stress responses. The methodology employed in this research could be applied to other subcellular targets of DON, such as the mitochondria, or other ribosome-targeting agents. Knowledge gained from these studies might contribute to
mechanism-based intervention and prevention of the adverse health consequences of DON and other ribotoxins, many of which are public health threats or chemotherapeutic agents.
APPENDIX
Appendix A: Contribution of overall proteome changes to overall phosphoproteome and ribosome-associated proteome

Abstract

Trichothecene mycotoxin deoxynivalenol (DON) is a public health threat due its common contamination of human and animal food and its ability to cause immunotoxicity. It has been reported that DON induced immunotoxicity is mediated by ribotoxic stress response, a process that involves extensive phosphorylation changes, and altered ribosome association of various proteins. However, DON is a translational inhibitor, and the contribution of overall proteome changes to these phosphorylation and ribosome interaction changes was not understood. In the present study, a quantitative, dynamic proteomic analysis was performed in SILAC-labeled RAW 264.7 exposed to a toxicologically relevant concentration of DON. Extensive protein level alterations were found within 30 min of DON treatment. DON’s ability to induce partial translational inhibition and its impact on protein localization process suggest that these changes might be due to translational arrest and protein transport between nuclear and cytoplasmic compartments. The most impacted processes in the nucleus and the cytoplasm were regulation of transcription and translation, respectively, which is consistent with the previous findings in DON’s impact on total phosphoproteome and ribosome-associated proteome (Chapters 2 and 3). Phosphorylation and subcellular stoichiometry of representative proteins revealed that DON-induced changes in total proteome were not the main driving force of total phosphoproteome and ribosome-associated proteome changes, which should be a result of differential phosphorylation and ribosome interaction.
Introduction

Deoxynivalenol (DON), a trichothecene mycotoxin produced by *Fusarium* that commonly contaminates food, is capable of activating mononuclear phagocytes of the innate immune system via a process termed ribotoxic stress response (RSR). It has been shown that DON-induced RSR involves extensive alterations in the phosphoproteome (Pan *et al.*, 2013) (Chapter 2), and at the specific subcellular target of DON, it affects ribosome-associated proteome (Chapter 3) in RAW 264.7 after exposure to a toxicologically relevant concentration of DON (250 ng/mL). However, DON is known to induce partial translational inhibition (≈50%) (Zhou *et al.*, 2003b) and therefore possibly affecting the total protein levels of the ones that were identified to have altered phosphorylation status or ribosome association. Different contribution of the total proteome could lead to different interpretations of the phosphoproteome and ribosome-associated proteome changes.

Conventionally, biochemical methods, such as Western blotting, have been used to measure phosphorylation and subcellular stoichiometry. For phosphorylation stoichiometry, phosphoproteins and non-phosphoproteins are separated physically via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and their quantities are estimated using antibodies (Stukenberg *et al.*, 1997). This method is time consuming and requires a phosphorylation-induced migration difference in the gel for phosphorylation stoichiometry.

Proteomics could be used to measure protein stoichiometry, and phosphorylation stoichiometry is more systematically studied (Steen *et al.*, 2005; Steen *et al.*, 2008; Jin *et al.*, 2010). For example, phosphorylation stoichiometry can be measured from the
ratios of ion signals of phosphopeptides and the corresponding non-phosphopeptides using a label-free approach (Steen et al., 2005; Steen et al., 2008). The premise of this method is the assumption that differences in the ionization and detection efficiencies of a peptide’s phosphorylated and nonphosphorylated forms are negligible. To overcome this shortcoming, a recently reported method determined the response ratios of phosphopeptides and non-phosphopeptides using synthetic peptide standards, and was applied to measure the stoichiometries of two tyrosine residues in the Lyn protein (Jin et al., 2010). Recently, a method was reported to measure site stoichiometry at a large scale by integrating phosphatase treatment and stable isotope labeling with amino acids in cell culture (SILAC) to determine site stoichiometries of protein phosphorylations on a large-scale (Wu et al., 2011).

Here to understand the contrition of overall proteome changes to overall phosphoproteome and ribosome-associated proteome, we identified the total proteome changes in SILAC-labeled RAW 264.7 treated with 250 ng/mL DON within 30 min, and relate these alterations to those identified in total phosphoproteome and ribosome-associated proteome. The results suggest that DON-induced RSR involves extensive protein level and DON-induced changes in total proteome were not the main driving force of total phosphoproteome and ribosome-associated proteome changes, which should be a result of differential phosphorylation and ribosome interaction.

Materials and Methods

Experimental design
As outlined in Figure A.1, RAW 264.7 cells were labeled with L-arginine and L-lysine (R0K0), L-arginine-U-$^{13}$C$_6$-$^{14}$N$_4$ and L-lysine-$^2$H$_4$ (R6K4), or L-arginine-U-$^{13}$C$_6$-$^{15}$N$_4$ and L-lysine-U-$^{13}$C$_6$-$^{15}$N$_2$ (R10K8) (Cambridge Isotope Laboratories, Andover, MA) (6 plates of 80% confluent cells per condition) in Dulbecco's Modified Eagle Medium (DMEM, from SILAC™ Phosphoprotein ID and Quantitation Kit, Invitrogen, Grand Island, NY). Since SILAC requires sufficient proliferation for the full incorporation of labeled amino acids into the cellular proteome, metabolic labeling was performed for six cell doubling times (≈108 h). The defined mass increments introduced by SILAC among the three RAW 264.7 populations resulted in characteristic peptide triplets that enabled relative quantification of peptide abundance.

Labeled RAW 264.7 cells were treated with 250 ng/mL of DON for 0 min, 5 min, and 30 min. A second, identically labeled set of cells were treated with DON for 1 min, 15 min, and 30 min. Nuclear and cytoplasmic fractions were extracted using a Nuclear Extract Kit (Active Motif, Carlsbad, CA). Each fraction of three treatment types were pooled equally based on protein content as determined by BCA Protein Assay (Pierce, Rockford, IL). These resultant mixtures were then trichloroacetic acid to a final concentration of 10% (w/v) followed by overnight incubation at 4°C. Pellets were recovered by centrifugation (10,000 × g for 15 min), washed with cold acetone twice, and air dried. Proteins were suspended in 8 M urea, 20 mM HEPES, pH 8.0 and ran on 4-20% gradient gel (BioRad, Hercules, CA). The gel was cut into 10 equal slices (6 mm wide) and the proteins in each slice was in-gel digested with trypsin (Promega, Madison, WI) (Jensen et al., 1999). Each time course set was repeated in three independent experiments (n=3) to account for biological and technical variability.
Figure A.1. Experimental design for SILAC-based quantitative proteomic analysis of DON-induced RSR. RAW 264.7 cultured in media supplemented with normal lysine and arginine (Lys0, Arg0), medium-labeled lysine and arginine (Lys4, Arg6), and heavy-labeled lysine and arginine (Lys8, Arg10) were subjected different DON treatments in two sets of experiments. Nuclear and cytoplasmic proteins were extracted, mixed equally among different groups based on protein amount and separated on a 4-20% gradient gel. Proteins in 10 equal gel slices were digested in gel with trypsin and analyzed by high-resolution mass spectrometry. Each set was repeated in three independent experiments (n=3).
Mass spectrometry

Purified peptides were then dried in a Speedvac and resuspended in blank solution to 20 µL. From this, 10 µL were automatically injected by a Waters nanoAcquity Sample Manager (Waters, Milford, MA) and loaded for 5 min onto a Waters Symmetry C18 peptide trap (5 µm, 180 µm x 20 mm) at 4 µL/min in 2% ACN/0.1% formic acid. The bound peptides were then eluted using a Waters nanoAcquity UPLC (Buffer A = 99.9% water/0.1% formic acid, Buffer B = 99.9% ACN/0.1% formic acid) onto a Michrom MAGIC C18AQ column (3µ, 200Å, 100U x 150mm, Michrom, Auburn, CA) and eluted over 120 min with a gradient of 5% B to 30% B in 106 min, ramping up to 90% B by 109 min, held there for 1 min, returned to 5% B at 110.1 min and kept there for the remainder of the run. Solvent flow was kept at a constant rate of 1 µl/min.

Eluted peptides were sprayed into a ThermoFisher LTQ-FT Ultra mass spectrometer (Thermo, Hudson, NH) using a Michrom ADVANCE nanospray source with an ionization voltage of 2.0 kV. Survey scans were taken in the FT (50000 resolution determined at m/z 400) and the top five ions in each survey scan were then subjected to automatic low energy collision induced dissociation (CID) in the LTQ. The resulting data files were processed into peak lists using MaxQuant (Cox and Mann, 2008), v1.2.2.5, and searched against the IPI rat database v3.78 using the Andromeda (Cox et al., 2011) search algorithm within the MaxQuant environment. All SILAC quantitation was performed using MaxQuant and the data exported to the program Perseus, v1.2.0.16 (www.maxquant.org) for statistical analysis.

Data analysis
Peak list generation, protein quantitation based upon SILAC, extracted ion chromatograms (XIC) and estimation of false discovery rate (FDR) were all performed using MaxQuant (Cox and Mann, 2008), v1.2.2.5. MS/MS spectra were searched against the IPI mouse database, v3.72, appended with common environmental contaminants using the Andromeda searching algorithm (Cox et al., 2011). Further statistical analysis of the SILAC labeled protein and peptide ratio significance was performed with Perseus (www.maxquant.org). MaxQuant parameters were protein, peptide and modification site maximum FDR = 0.01; minimum number of peptides = 1; minimum peptide length = 6; minimum ratio count = 1; and protein quantitation was done using all modified and unmodified razor and unique peptides. Andromeda parameters were triplex SILAC labeling: light condition (no modification), medium condition (Arg6, Lys4), heavy condition (Arg10, Lys8); fixed modification of Carbamidomethylation (C), variable modifications of Oxidation (M), Phosphorylation (STY) and Acetyl (Protein N-term); Maximum number of modifications per peptide = 5; enzyme trypsin max missed cleavage = 2; parent Ion tolerance = 6ppm; fragment Ion tolerance = 0.6 Da and reverse database search was included. For each identified SILAC triplet, MaxQuant calculated the three extracted ion chromatogram (XIC) values, and XICs for the light and medium member of the triplet were normalized with respect to the heavy common 30 min time point of DON treatment, which was scaled to one. A significance value, Significance B, was calculated for each SILAC ratio and corrected by the method of Benjamini and Hochberg using the statistical program Perseus in the MaxQuant environment (Cox and Mann, 2008). Significance B values of a peptide in the two sets of time courses are calculated independently. Significant protein ratio cutoff
was set as at least one set of time course has a Significance B value <0.05 as calculated by MaxQuant (Cox et al., 2009).

**Results and Discussion**

*DON-induced changes in phosphoproteomic profile*

Using SILAC in conjunction with LC-MS/MS, we reproducibly identified 10507 and 4521 unique peptides corresponding to 1233 and 829 proteins in the nuclear and cytoplasmic fractions, respectively, at an accepted FDR of 1% (Figure A.2). Approximately 17% of the total phosphopeptides identified were considered significant (FDR<0.05).

*Biological analysis of altered phosphoproteins*

To discern the potential impact of DON-induced protein changes, prevalence of differentially expressed proteins were related to specific biological processes using GO annotation in DAVID (Figure A.3). In the nucleus (Figure A.3A), the most affected biological process was regulation of translation, constitute more than 18 percent of all significantly regulated proteins in the nucleus. These included transcription factors [e.g. BCL2-associated transcription factor 1 (BCLAF1), transcription factor 4 (TCF4), basic transcription factor 3 (BTF3)], transcription cofactors [e.g. MYB binding protein 1a (MYBBP1A), GATA zinc finger domain-containing protein 2A]; GATA zinc finger domain containing 2A (GATAD2A), programmed cell death 11 (PDCD11)], as well as epigenetic regulators [e.g. DNA
Figure A.2. DON-induced RSR involves extensive protein level changes. The numbers of significantly regulated (light blue) phosphorylated proteins (A) and peptides (B) as determined by Significance B corrected for FDR<0.05 among all unique proteins and peptides identified (light + dark blue).
Figure A.3. Gene Ontology associated with significantly regulated proteins as determined by DAVID Biological Process terms (DAVID EASE score < 0.05). Data are presented as a histogram of the relevant biological processes identified in the nuclear (A) and cytoplasmic (B) fractions and shown as a percentage of the total identified proteins that fall within each category.
methyltransferase 1 (DNMT1), lysine-specific demethylase 1 (KDM1A), histone deacetylase 2 (HDAC2)]. These discoveries are consistent with the observation in total phosphoproteome that regulation of transcription is the main target in early DON-induced RSR.

In the cytoplasmic (Figure A.3B), the process most affected by DON was translation, which was also significantly altered in both total phosphoproteome and ribosome-associated proteome. Affected proteins involved in translation included ribosomal proteins, translation- or ribosome biogenesis-related proteins.

Another process impacted in both the nuclear and the cytoplasmic fractions was protein localization, suggesting that besides the altered protein expression as a result of transcriptional and translational regulation, protein transportation between the two fractions could also lead to the changes observed. Among the 174 proteins that overlapped between the nuclear and cytoplasmic proteins significantly altered by DON, 40 were involved in translation and 16 were involved in macromolecular complex assembly. Together with results in ribosome-associated proteome, this indicates that early DON-induced translational inhibition, transport of nascent ribosomal proteins synthesized in the nucleus might be interfered by DON treatment, to coordinate with the translational arrest.

**Contribution of total proteome changes to those observed in total phosphoproteome and ribosome-associated proteome**

Proteome and phosphoproteome at the whole cell and ribosome levels overlapped, suggesting that proteome alterations could drive phosphoproteome
changes, and the overall proteome and phosphoproteome changes could also have impacted the alterations associated with the ribosome (Figure A.4).

There were 64 proteins that overlapped in total proteome and phosphoproteome significantly affected by DON. Among these, most (16) were involved in regulation of transcription, including transcription factors/cofactors, and epigenetic regulators. Phosphorylation (Chapter 2), protein levels, and normalized phosphorylation (phosphorylation stoichiometry) of representative phosphosites were shown in Figure A. 5.

There were 222 proteins that overlapped in total and ribosome-associated proteome significantly affected by DON. Among these, most (59) were involved in translation, including ribosomal proteins, translation- and ribosome biogenesis-related proteins. Ribosome-associated (Chapter 3), total protein levels, and normalized ribosome levels (subcellular stoichiometry) of representative proteins were shown in Figure A. 6.

For both total phosphoproteome and ribosome-associated proteome, total proteome did contribute moderately to the alterations in the net phosphorylation and ribosome interaction, but they were not the main determinant. Therefore, the phosphoproteome changes (Chapter 2) were mainly driven by differential phosphorylation or dephosphorylation by the corresponding kinases or phosphatases; and the ribosome-associated proteome changes (Chapter 3) were mainly driven by differential interaction with the ribosome as opposed to a secondary effect of overall protein level changes.
Figure A.4. Venn diagram representing the overlap among total and ribosome-associated proteome and phosphoproteome.
Limitations

In the current analysis, only representative phosphorylation and ribosome-association events were evaluated on a small-scale, correlation analyses assessing the stoichiometry of all these events are desirable for obtain a comprehensive view of the contribution of total proteome. In addition, sample loss was inevitable in the sample preparation process which involved protein precipitation and in-gel tryptic digestion. Consequently, this could have compromised the sensitivity of the detection, and some DON-induced alterations were not identified.

Conclusions

In the present study, a quantitative, dynamic proteomic analysis was performed in SILAC-labeled RAW 264.7 exposed to a toxicologically relevant concentration of DON. Extensive protein level alterations were found within 30 min of DON treatment. DON’s ability to induce partial translational inhibition (Zhou et al., 2003b) and its impact on protein localization process suggest that these changes might be due to translational arrest and protein transport between nuclear and cytoplasmic compartments. The most impacted processes in the nucleus and the cytoplasm were regulation of transcription and translation, respectively, which is consistent with the previous findings in DON’s impact on total phosphoproteome and ribosome-associated proteome (Chapters 2 and 3). Phosphorylation and subcellular stoichiometry of representative proteins revealed that DON-induced changes in total proteome were not the main driving force of total phosphoproteome and ribosome-associated proteome changes, which should be a result of differential phosphorylation and ribosome interaction.
Figure A.5. Phosphorylation, protein levels, and normalized phosphorylation (phosphorylation stoichiometry) of representative phosphosites involved in transcriptional regulation.
Figure A.6. Ribosome-associated, total protein levels, and normalized ribosome levels (subcellular stoichiometry) of representative proteins.


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