JAK/STAT Pathway Plays a Critical Role in the Proinflammatory Gene Expression and Apoptosis of RAW264.7 Cells Induced by Trichothecenes as DON and T-2 Toxin

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Deoxynivalenol (DON) and T-2 toxin commonly affect cells of the immune system and cause inflammation and apoptosis. Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is highly associated with inflammatory process and apoptosis and is worth investigating its role when cells were exposed to trichothecenes. The results showed that DON and T-2 upregulated the messenger RNA (mRNA) expressions of interleukin (IL)-6, IL-1β, tumor necrosis factor-α, JAK1–2, STAT1–3, and suppressors of cytokine signaling members and activated the tyrosine phosphorylation of STAT1 and STAT3 with a dose-dependent manner in RAW264.7 cells. AG490 and Stattic, the specific inhibitors of JAK/STAT pathway, blocked the STAT1 and STAT3 tyrosine phosphorylation and decreased the gene expressions of proinflammatory cytokines induced by trichothecenes. Interestingly, the time when the mRNA levels of STAT1 and STAT3 were significantly upregulated was at 12 h, which was much later than the time when mitogen-activated protein kinase protein kinase was activated, indicating that STATs might be the downstream targets of the trichothecenes. With the intervention of AG490 and Stattic, DON and T-2 toxin induced apoptosis in a strengthened way, with the loss of mitochondrial membrane potential and the decrease ratios of the B-cell leukemia/lymhophoma 2 (Bcl-2)/bcl-2-associated X (Bax) and B-cell lymphoma-extra large (Bcl-xL)/Bax. After exposing to DON and T-2 toxin, cells exhibited G2/M and G0/G1 phase arrest, respectively. The increased mRNA expressions of STAT target genes p21 and cyclin D1 for DON and the increases in p21 mRNA and the decreases in cyclin D1 for T-2 toxin were observed. These results demonstrated for the first time that the activation of JAK/STAT might be a critical mediator to induce the inflammatory response and apoptosis in macrophage in response to trichothecenes.

Key Words: d; deoxynivalenol; T-2 toxin; JAK/STAT; inflammatory cytokines; RAW264.7 cell; cell apoptosis.

The trichothecenes contaminate foods and feeds worldwide (Pestka and Smolinski, 2005). T-2 toxin and deoxynivalenol (DON) typically represented type A and type B of the trichothecenes. They have drawn much attention due to their high toxicity. They cause serious toxicities in the body (Pestka et al., 2004). The mononuclear phagocytes appear to be the cells most sensitive to trichothecenes (Pestka et al., 2004).

Expression of the proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α could be rapidly activated and might serve as the sensitive biomarkers of the DON’s effects (Pestka and Amuzie, 2008). DON could increase the binding activities of several transcription factors, including nuclear factor-kappa B (Krishnaswamy et al., 2010), activator protein-1, and CCAAT/enhancer binding protein (Li et al., 2000; Wong et al., 2002) in a number of leukocyte models. In addition, DON exposure also stabilized the messenger RNAs (mRNAs) of TNF-α, IL-6, and COX-2 in macrophages (Chung et al., 2003; Moon and Pestka, 2003) as well as IL-8 in cell line HCT-8 (Choi et al., 2009). The excessive mRNA expressions of proinflammatory cytokines further caused cell functional disorder and apoptosis (Pestka, 2010). The trichothecene-mediated apoptosis was closely correlated with the activation of mitogen-activated protein kinase (MAPK) pathway, which also highly contributed to upregulation of trichothecene-induced proinflammatory genes (Pestka, 2008).

It has been proven that DON and other trichothecenes can induce MAPK phosphorylation after only 15–30 min in a rapid and immediate way in vivo and in vitro (Zhou et al., 2003a). DON markedly induced the phosphorylation of p38, JNK, and extracellular signal-regulated kinase (ERK) as well as proinflammatory gene expression and apoptosis (Moon and Pestka, 2002; Pestka and Smolinski, 2005). Recent research found that p38 was responsible for mediating the
inflammatory properties of the T-2 toxin (Kruber et al., 2011). Double-stranded RNA-activated protein kinase (PKR) and hematopoietic cell kinase (Hck) were the possible upstream signal transducers for the MAPK activation (Zhou et al., 2003b). Inhibition of PKR and Hck in the DON-exposed macrophages would suppress the MAPK activation as well as the transcription factor activation, cytokine expression, and apoptosis (Pestka et al., 2004).

In the past few years, most of the attention was focused on the trichothecene-activated MAPK pathway. However, less is known about the role of other evolutionary conserved signaling pathways in the toxic effects of trichothecenes, such as the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. The activation of this essential pathway was critical for cell proliferation and apoptosis (Rawlings et al., 2004). In most cell types, STAT1 and STAT3 played important roles in directing cells toward cytokine responsiveness, gene expression, and apoptotic cell death (Murray, 2007). For example, STAT3 tyrosine phosphorylation was critical in IL-6 production in macrophages in response to lipopolysaccharides (Samavati et al., 2009), and the lack of IL-6 induction was observed in STAT1-deficient mice (Bottrel et al., 1999). In addition, suppressors of cytokine signaling (SOCS) proteins might act as the part of a negative feedback loop to attenuate signal transduction from cytokines that acted through the JAK/STAT pathway (Minamoto et al., 1997). Moreover, induction of SOCS expression was recently reported in a dose-dependent manner when mice were treated with DON (Amuzie et al., 2009), suggesting that JAK/STAT pathway might mediate a possible connection between trichothecenes and their diverse toxic effects. Specific inhibitors would be useful to further identify the role of JAK/STAT pathway in the toxic effects induced by DON and T-2 toxin. AG490, an inhibitor of JAK2, has been shown to predominantly inhibit the JAK2-STAT1 pathway (Gorina et al., 2005) and the JAK2-STAT3 pathway (Eriksen et al., 2001). Statistic, a newly identified STAT3 inhibitor, obviously inhibited tyrosine phosphorylation of STAT3 (Schust et al., 2006).

Based on the above studies, we hypothesize that JAK/STAT pathway may be involved in trichothecene-induced proinflammatory gene expression and apoptosis. One of the most sensitive target cells, RAW264.7 macrophage cell line, would be a better model to test the hypothesis. Components of JAK/STAT pathway and SOCS family were detected to be a better model to test the hypothesis. Six components as JAK1, JAK2, JAK3, STAT1, STAT2, and STAT3 of the JAK/STAT pathway, four members (SOCS1, SOCS2, SOCS3, and cytokine-induced SH2 protein [CIS]) of the SOCS gene family, inflammatory cytokines (IL-6, IL-1β, and TNF-α), Bax, Bcl-2, Bcl-xl, p21, and cyclin D1 were determined by quantitative real-time PCR (qRT-PCR), respectively. Briefly, total RNA was isolated from the cells using TRIzol Reagent (Takara, Dalian, PR China) according to the manufacturer’s instructions. The DNA was eluted and stored at −80°C until use. One microgram of RNA was reverse transcribed to complementary DNA (cDNA) with the use of ReverTra Ace First Strand cDNA Synthesis Kit (Takara). cDNA was amplified by qRT-PCR (BioRad, Hercules, CA) using SYBR Premix Ex Taq RT-PCR kit (Takara, Code QPK-201, China). Each 25 μl reaction mixture consisted of 12.5 μl SYBR Premix Ex Taq, 0.5 μl of each primer (10 μm), 2 μl of cDNA, and 9.5 μl RNase-free dH2O. Cycling conditions were as follows: step 1, 30 s at 95°C; step 2, 40 cycles at 95°C for 5 s, 60°C for 30 s; step 3, dissociation stage. Data from the reaction were collected and analyzed by the complementary computer software. Relative quantification of gene expression was calculated using the 2−ΔΔCt analysis method as previously described (Ihsan et al., 2011; Wang et al., 2011) and normalized to GAPDH in each sample. Primers used in this study were provided in Table 1.
TABLE 1
Primer Sequence (5′→3′) Amplified region

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Amplified region</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>GCCCAAGATGGCCTCCTCAGT</td>
<td>690–850</td>
</tr>
<tr>
<td>JAK1</td>
<td>CAGATGCCACACCTTACC</td>
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</tr>
<tr>
<td>JAK2</td>
<td>GGCAGCAGCAGAAGACCTAC</td>
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</tr>
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</tr>
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<tr>
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<tr>
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<td>SOCS2</td>
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<td>IL-6</td>
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<td>IL-1β</td>
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<td>TNF-α</td>
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<td>P38</td>
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<td>GATGGCCATCCACCAAGA</td>
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<tr>
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<tr>
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<td>352–600</td>
</tr>
<tr>
<td>p21</td>
<td>ACTCTCCTCTGGCTTGCTG</td>
<td>68–300</td>
</tr>
</tbody>
</table>

Note. The primers were manufactured by Shanghai Generay Biotech Co. Ltd. (Shanghai, PR China). p38, p38 mitogen-activated protein kinases; p21, p21WAF1.

Protein extraction and Western blot analysis. RAW264.7 cells in six-well plates were exposed to DON (2μM) or T-2 toxin (14nM) for 12 h, and the inhibitor groups were pretreated with AG490 (10μM for DON or 5μM for T-2 toxin) or Stattic (10μM for DON or 5μM for T-2 toxin) for 45 min. After incubation, the cells were washed with ice-cold PBS for three times. Total cellular proteins were extracted by adding radio immunoprecipitation assay lysis buffer (Beoytime, PR China) after addition of the protease inhibitor PMSF and antiphosphatases with the final concentrations of 1mM, respectively. Cells were disrupted by ultrasonication instrument (CV18, Xinchen, PR China) for two times (5 s/time) and placed on the ice for about 1 h. The lysate was centrifuged at 12,000 × g for 15 min at 4°C. The protein concentration of the supernatant was measured with the bicinchoninic acid protein assay (Beoytime, PR China). Equal amounts of proteins (about 50 μg) were mixed 4:1 with 5× loading sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromophenol blue, and 1.25M Tris-HCl, pH 6.8), boiled for 10 min, then loaded onto 10% SDS-polyacrylamide gel, and ran at 120 V for 2.5 h. Proteins were transferred to a polyvinylidene difluoride membrane for 1.5–2 h at 100 V on Trans-Blot Cells (Liuyi, Beijing, PR China). The membranes were blocked with 5% nonfat milk in TBST (Tris-buffered saline with 0.1% Tween-20) for 1 h, washed, and then incubated with the primary antibody (diluted 1:1000 in 5% nonfat milk in TBST) for an overnight at 4°C. The membranes were washed three times with TBST and then incubated for 1 h with the horseradish peroxidase-conjugated secondary anti-IgG antibody using a dilution rate of 1:2000 and 1:500 in 5% nonfat milk in TBST. The membranes were washed four times in TBST. Immunoreactive bands were visualized with a chemiluminescent substrate (ECL-Plus, Miniprepared) for 2–5 min. Images were captured with a LAS-4000 luminance image analyzer (FujiFilm, Tokyo, Japan).

Flow cytometry for apoptosis of Annexin V-FITC/PI binding. The apoptotic rate of RAW264.7 cells was detected by using flow cytometry with annexin V-FITC/PI double labeling method according to the vendor’s protocol (BD BioSciences, San Jose, CA). The cells were plated into six-well plates and exposed to DON (1–4μM) or T-2 toxin (7–28nM) for 12 h at 37°C. All results were presented as mean ± SD. Group differences were analyzed using one-way ANOVA followed by Least Significant Difference post hoc tests. A probability of p < 0.05 was considered significant.

RESULTS

The Effects of DON and T-2 Toxin on Cell Viability of RAW264.7 Cells

Concentration- and time-dependent increases in cytotoxicity occurred when RAW264.7 cells were exposed to DON and T-2...
toxin (Supplementary data 1). A significant decrease in cell viability was observed at all concentration levels when compared with control. After the cells were incubated with the toxins for 24 h, an almost complete reduction was observed at the highest concentrations. An apparent dose-dependent antiproliferative effect was found for T-2 toxin, which was more harmful than DON at each indicated time point. The dose levels of 1, 2, and 4 μM for DON, and 7, 14, and 28 nM for T-2 toxin, respectively, were selected in the future studies according to the MTT results.

The mRNA Expressions of IL-6, IL-1β, and TNF-α Induced by DON and T-2 Toxin

As shown in Figure 1, significant increases of IL-6, IL-1β, and TNF-α gene expressions induced by mycotoxins were in a dose-dependent way. IL-6 mRNA expressions were about 346 and 776 times, respectively, 4 μM of DON or 28 nM T-2 toxin for 12 h higher than those of the controls. Similarly, IL-1β mRNA expressions were about 303 and 1397 times, respectively, for DON and T-2 toxin with the same dose and same incubation time. Compared with DON, the gene expressions of TNF-α were higher than that when cells were exposed to T-2 toxin. The proinflammatory gene expressions indicated that T-2 toxin was more toxic than DON.

Gene Expressions of JAK/STAT Pathway and SOCS Family Members Induced by DON and T-2 Toxin

In the present study, JAK1 and JAK2 were both significantly activated when cells were treated with 1–4 μM of DON or 7–28 nM of T-2 toxin for 12 h (Fig. 2). Activation of JAK2 was more obvious than that of JAK1. However, the activation of JAK3 was not significant. Significant increases of mRNA levels of STAT1, STAT2, and STAT3 were also found. The expressions of four SOCS mRNAs, CIS, SOCS1, SOCS2, and SOCS3, were also found. The expressions of four SOCS mRNAs, CIS, SOCS1, SOCS2, and SOCS3, were also found.
FIG. 2. The mRNA expressions of JAKs, STATs, and SOCSs induced by DON and T-2 toxin in RAW264.7 cells. The cells were treated with 1, 2, and 4 μM of DON and 7, 14, and 28 nM of T-2 toxin for 12 h, respectively. Data were presented as mean ± SD (n = 6). Significant differences versus control were indicated by *p < 0.05 and **p < 0.01, respectively.
SOCS3 were significantly upregulated in response to the highest dose of trichothecenes for 12 h. Gene expressions of these four SOCS members were upregulated about 9.93–18.79 folds for 2 μM DON and 3.83 to 57.28 for 14 nM T-2 toxin, respectively.

**AG490 and Stattic Blocked DON- and T-2 Toxin–Induced Tyrosine Phosphorylation of STAT1 and STAT3 and the Gene Expressions of IL-6, IL-1β, and TNF-α**

As shown in Figure 3, AG490 and Stattic abrogated the mRNA expressions of JAK2, STAT1, STAT2, and STAT3 induced by DON and T-2 toxin. Both STAT1 and STAT3 tyrosine phosphorylation induced by DON (2 μM) or T-2 toxin (14 nM) were detected after 12 h. However, incubation with AG490 or Stattic before the stimulation with mycotoxins totally prevented the tyrosine 701 phosphorylation of STAT1 and partly prevented the tyrosine 705 phosphorylation of STAT3. The presence of both AG490 and Stattic did not have significant effects on total (nonphosphorylated) STAT1, STAT3, and GAPDH protein levels. The immunoblot analysis of STAT1 and STAT3 phosphorylation showed good correlations with gene expressions of STAT1 and STAT3.

It was found that AG490 partly inhibited gene expressions of IL-6, IL-1β, and TNF-α induced by DON or T-2 toxin. However, Stattic almost completely abrogated all of these three cytokine gene expressions. The results suggested that JAK/STAT pathway might play critical roles in the production of proinflammatory cytokines induced by DON or T-2 toxin, and STATs could be activated not only by JAKs but also by other pathways.

**The Different Time Course and the Potential Cross-talk Between STAT1, STAT3, and MAPK Pathway Induced by DON and T-2 Toxin**

As shown in Figure 4, the gene expressions of STAT1 and STAT3 induced by DON significantly increased at 8 h. This process was time dependent, and the highest mRNA levels of STAT1 and STAT3 were reached at 12 h. Both gene expressions of them significantly decreased at 24 h. Similarly, the mRNA level of STAT1 was continually expressed from 8 to 24 h during the T-2 toxin stimulation. In contrast, p38 as an important pathway of MAPK occurred much earlier after DON treatment. It was found that p38 could rapidly be activated by both mycotoxins at 0.5–1 h, which was in accordance with the previous report in vivo (Zhou et al., 2003a). The different activations of MAPK and JAK/STAT indicated that a spatio-temporal integration rule might exist when DON and T-2 toxin activated these two pathways.

Both SB253080 and PD98059 did not have inhibitory effects on the transcriptional activity of STAT1 in response to DON. With the intervention of SB253080, the mRNA expression of STAT3 induced by DON significantly decreased. However, both SB253080 and PD98059 inhibited STAT1 gene expression induced by T-2 toxin. The gene expression of STAT3 induced by T-2 toxin was suppressed by PD98059. However, SB253080
showed no significant effect on the gene expression of STAT3. These results indicated for the first time that JAK/STAT pathway might have different specificities in the toxicological mechanisms of DON and T-2 toxin.

The JAK/STAT-Dependent Apoptosis Induced by DON and T-2 Toxin

The extents of apoptosis induced by DON and T-2 toxin were shown in Figure 5. Exposure of cells to DON (1–4 μM) and T-2 toxin (7–28 nM) for 12 h resulted in a gradual increase in the apoptotic cell population. Clear increases in the number of early apoptotic cells as well as late apoptotic cells were observed. Mitochondrial membrane potential was also detected by flow cytometry with cationic lipophilic dye rhodamine 123. Results showed that MMP was significantly decreased when cells were exposed to mycotoxins for 12 h. It was found that toxins could induce apoptosis and MMP loss in a further strengthened way when cells were coincubated with mycotoxins and AG490 or Stattic.

The mRNA expressions of Bax, Bcl-xL, and Bcl-2 were detected, and then the mRNA expression ratios of Bcl-xL/Bax and Bcl-2/Bax were calculated with the mean values. Results showed that, with the intervention of inhibitor AG490 or Stattic, the ratios of Bcl-xL/Bax and Bcl-2/Bax significantly decreased, which is in agreement with the apoptosis and the loss of MMP.

The Regulations of p21 and Cyclin D1 in Different Cell Cycle Arrest Induced by Toxins

As shown in Figure 6, the percentage of G2/M phase period was sharply increased when cells were exposed to DON. In contrast, T-2 toxin mainly exhibited G0/G1 cell cycle arrest (Supplementary data 2). p21 and cyclin D1, the cell cycle related genes, were analyzed by qRT-PCR when cells were treated with DON and T-2 toxin for 12 h. The results showed that the increases in mRNA expressions of p21 and cyclin D1 were detected in a dose-dependent way, and the changes of p21 were more significant. The gene expressions of p21 and cyclin D1 were enhanced approximately five- and twofold in comparison to controls when cells were treated with 4 μM DON, respectively. Similarly, the increases in p21 mRNA were noted when cells were treated with T-2 toxin. However, the cyclin D1 was significantly suppressed when cells were treated with the high dose of T-2 toxin.

**DISCUSSION**

In the present study, we first reported that JAK/STAT pathway played a critical role in DON or T-2 toxin–mediated IL-6, IL-1β, and TNF-α gene upregulation in macrophages. Our data also suggested that JAK/STAT might be a new downstream molecular target to regulate apoptosis and cell proliferation induced by DON and T-2 toxin. Understanding the molecular mechanism of these mycotoxins will facilitate the development of novel preventive treatments.

The present study, firstly, showed that only the mRNA expression of STAT3 mediated by DON was significantly repressed by SB253080, whereas STAT1 was not affected by the two inhibitors, SB253080 and PD98059. It is suggested that STAT1 was p38 and ERK independent in response to DON, whereas STAT3 was p38 dependent and ERK independent. However, both SB253080 and PD98059 inhibited STAT1 gene expression induced by T-2 toxin. STAT3 induced by T-2 toxin was suppressed only by SB253080, suggesting that the activation of STAT1 induced by T-2 toxin might be p38 and ERK dependent, whereas STAT3 showed p38 independent and ERK dependent. These results indicated that there might be a potential cross talk between MAPK and JAK/STAT pathway in the macrophage in response to trichothecenes. Furthermore, the exact mode of this cross talk is probably different between DON and T-2 toxin.

As known, the activation of JAK/STAT pathway is usually rapid and transient, with activated STAT molecules evident in the nucleus within minutes mechanistically (Hoyt et al., 2007). Trichothecenes can activate MAPK in a rapid and immediate way in vivo (Zhou et al., 2003a). In contrast, our research showed that the gene expressions of STAT1 and STAT3 induced by DON or T-2 toxin were significantly increased at/after 8 h and reached the highest levels at 12 h. The activation of JAK/STAT induced by DON and T-2 toxin was much later than that of MAPK. Therefore, STAT1 and STAT3 might be downstream molecular targets of MAPK pathway mediated by DON and T-2 toxin. JAK/STAT pathway not only plays important roles in the regulation of inflammation gene expressions but also can be further activated by cytokine binding to receptors (Murray, 2007). JAK2/STAT3 was also proved to be activated by IL-6 (Murray, 2007). Considering the present results, we speculated that there were at least two mechanisms to regulate the activation of JAK/STAT pathway when cells were exposed to mycotoxins (Fig. 7). One potential mechanism was likely associated with the phosphorylation of MAPK. Another one might be associated with the release of proinflammatory cytokines from the cells, binding to the cytokine receptor, and then activating the associated JAK combination.

STAT1 and STAT3 have been proven to have opposite biological effects (Regis et al., 2008). STAT1 plays important roles in promoting apoptotic genes such as Bax and negatively
regulating antia apoptotic genes such as Bcl-xL (Kim and Lee, 2007). In contrast, STAT3 inhibits apoptosis by inducing antia apoptotic genes such as Bcl family (Stephanou and Latchman, 2005). The balance between Bax homodimers that favor death and Bcl-2/Bax or Bcl-xL/Bax heterodimers that inhibit cell death is critical (Kim et al., 1997; Oltvai et al., 1993). Furthermore, the induction of Bax was reported to cause a fall in the MMP (Xiang et al., 1996). The current study found that both DON and T-2 toxin downregulated Bcl-xL and Bcl-2 and upregulated Bax at the mRNA level, which was in consistent with the decreased MMP induced by DON and T-2 toxin. With the intervention of inhibitors AG490 or Stattic, the apoptotic effects mediated by DON or T-2 toxin were more aggravated than those in the cells treated only with toxins. Accompanied with the decreased mRNA expressions of Bcl-2 and Bcl-xL and the decreased mRNA expression ratios of Bcl-2/Bax and Bcl-xL/Bax, the inhibitory effects were obviously found in AG490- and Stattic-treated groups. These results implied that AG490 or Stattic might reduce the levels of Bcl-2 and Bcl-xL by decreasing the expressions of STAT3. Considering the fact that DON and T-2 toxin had more obvious advantages to induce STAT1 mRNA expression than STAT3, we presumed that the proapoptotic effects of STAT1 might play a leading role in mycotoxins-induced apoptosis. Caspase pathway could be activated by STAT1 (Kim and Lee, 2007), and caspase 3 was involved in proapoptotic effect of DON with Bax increase (Marzocco et al., 2009). Therefore, we speculated that the phosphorylation of STAT1 might also participate in the regulation of caspase-3 activation to a certain extent. However, further studies are required to validate this hypothesis.

The cell cycle analysis can give another insight into the toxicology mechanisms of DON and T-2 toxin on cell proliferation. In the present study, a significant increase in the G2/M phase was observed when RAW264.7 cells were in response to DON. The results are highly in accordance with previous studies that the cell cycle was blocked in the G2/M phase in human epithelial cells (Yang et al., 2008). Whereas T-2 toxin mainly exhibited a G0/G1 cell cycle arrest, which is different from DON. This suggests that the mechanisms of DON and T-2 causing cell proliferation inhibitions are different. As known, p21^{waf1/cip} (p21) is one of the critical CDK inhibitors to negatively modulate cell cycle progression (Brugarolas et al., 2002). In addition, among all the effective cyclin-dependent kinase inhibitors, only p21 is strongly regulated by STAT1 (Chin et al., 1996). As an important regulator, cyclin D1 mainly modulates cell cycle progress through G1 phase in a positive regulation way (Kim et al.,

![FIG. 6.](image_url) The effects of DON and T-2 toxin on the cell cycle of RAW264.7 cells (A). The cells were incubated with DON and T-2 for 12 h, respectively, for the analysis of the cell cycle. The percentage of cells in each cell cycle phase was calculated from three representative data. The gene expressions of p21 and cyclin D1 after the treatment with DON and T-2 for 12 h, respectively (B). Data were presented as mean ± SD (n = 6). Significant differences versus control were indicated by *p < 0.05 and **p < 0.01, respectively.

![FIG. 5.](image_url) The apoptosis of RAW264.7 cells induced by DON and T-2 toxin (A). After the treatment with AG490 and Stattic for 45 min, respectively, the cells were exposed to DON and T-2 toxin for 12 h, respectively. The analysis of MMP (B). Flow cytometry was used to detect the fluorescence of rhodamine 123 after the cells were treated with DON and T-2 toxin. The mRNA expression ratios of Bcl-2/Bax and Bcl-xL/Bax (C). The cells were exposed to DON and T-2 toxin for 12 h, respectively. Data were presented as mean ± SD (n = 6). Significant differences versus control were indicated by *p < 0.05 and **p < 0.01, respectively. Significant differences versus the same group without inhibitors were indicated by #p < 0.05 and ##p < 0.01, respectively.
The activation of cyclin D1 was regulated by STAT3 protein (Leslie et al., 2006), and the loss of cyclin D1 can result in the prevention of G1/S transition (Walker and Assoian, 2005). In the present study, the significant increases in cyclin D1 mRNA were observed, suggesting that the transcription of cyclin D1 was promoted by STAT3, which further successfully regulated the G1/S transition when cells were exposed to DON. Additionally, the significant increases in p21 mRNA induced by DON might result in the cell cycle arrest in G2/M, which was consistent with the previous report that the cell cycle arrest in G2/M was highly associated with the upregulation of p21 (Yang et al., 2008). In the present study, we first provided evidence that the upregulation of p21 might be affected by the activation of STAT1 induced by DON, which was likely a new molecular target for DON to suppress cell proliferation. Similarly, the significant increases in p21 mRNA and the significant decreases in cyclin D1 mRNA were noted when cells were treated with the high dose of T-2 toxin. The results of decreased gene expression of the cyclin D1 might be one important reason that T-2 toxin mainly exhibited a G0/G1 cell cycle arrest. However, the decreased cyclin D1 expression and the roles of STAT1 and STAT3 in cell cycle arrest when cells were exposed to T-2 toxin were confused and needed to be further investigated.

Chronic exposure to a low dose of trichothecenes usually caused growth retardation (Pestka, 2010). The present results suggested that this adverse effect could probably result from blocking cell proliferation, which was related to the JAK/STAT pathway. On the other hand, DON-induced growth retardation reported to have high correlations with the significant upregulation of SOCS3 with the impairment of growth hormone axis (Amuzie and Pestka, 2010; Amuzie et al., 2009). As the well-known negative feedback regulators of the JAK/STAT pathway, the SOCS family, including SOCS3, could be activated in macrophages by DON and T-2 toxin in the present study, which suggested that the SOCS family members not only played important roles in the expression of proinflammatory cytokines but also in the regulation of cell growth through regulating the JAK/STAT pathway.

In conclusion, this study demonstrated for the first time that JAK/STAT pathway played critical roles in the regulation of proinflammatory cytokines and apoptosis in response to trichothecenes. STAT1 and STAT3 might be the newly attractive

**FIG. 7.** The proposed activation of JAK/STAT signal pathway induced by DON and T-2 toxin in RAW264.7 cells. One proposed activation of JAK/STAT pathway might be associated with the phosphorylation of MAPK. Another proposed one of JAK/STAT pathway might be the proinflammatory cytokines released from the cells bind to the cytokine receptor associated with JAK combination and then activated JAK/STAT pathway.
downstream targets for the regulation of proinflammatory response, cell proliferation, and apoptosis induced by trichothecenes, which offered new opportunities to understand the different toxic mechanisms of type A and type B trichothecenes. Because trichothecenes are commonly found in food, this study may throw a new light on a new downstream molecular target to combat their toxicity from the angle of food safety. In the future, it will be necessary to identify the role of MAPKs in the downstream events of STATs activation and whether the ribosome participated in the activation of JAK/STAT pathway.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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