ストレス応答MAPキナーゼのHL60細胞におけるニバレノール誘導性の細胞毒性とインターロイキン－8分泌への寄与

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Contribution of stress-activated MAP kinases to nivalenol-caused cytotoxicity and interleukin-8 secretion in HL60 cells

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Abstract

To elucidate the molecular mechanism underlying the toxicity of the *Fusarium* mycotoxin nivalenol, we investigated the involvement of stress-activated MAP kinases (SAPKs; c-Jun N-terminal kinases (JNKs) and p38s) in nivalenol-caused cytotoxicity and interleukin (IL) -8 secretion in human promyelocytic leukemia HL60 cells. Nivalenol treatment for 24 h increased the phosphorylated (i.e., the active-form) JNKs with maximum effect at 1 μg/mL; when nivalenol was given at this concentration in a time-series experiment, phosphorylated JNK quantity peaked at 12 h and then decreased. Essentially the same results were obtained for phosphorylated (active-form) p38s. To elucidate the functions of SAPKs, we investigated the effects of the JNK-specific inhibitor SP600125 and the p38-specific inhibitor SB203580 on nivalenol-caused cytotoxicity and IL-8 secretion. Nivalenol hindered cell proliferation regardless of the presence or absence of SAPK-specific inhibitors. However, co-treatment with SAPK inhibitors reduced this effect, indicating that JNKs and p38s play roles in nivalenol-associated retardation of cell proliferation. SP600125 significantly reduced nivalenol-induced IL-8 secretion, indicating that JNKs contribute to this phenomenon. SB203580 moderately lessened nivalenol-elicited IL-8 secretion, however, the contribution of p38s to nivalenol-induced IL-8 secretion appears to be meaningful, because SB203580 alone markedly increased IL-8 secretion.

Key words : cell proliferation, HL60 cell, interleukin-8, SAPK

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Introduction

A variety of *Fusarium* fungi produce a number of different mycotoxins of the class of trichothecenes and some other mycotoxins (zearalenone and fumonisins). *Fusarium* fungi are commonly found on cereals grown in the temperate regions of America, Europe, and Asia. There are more than 60 known trichothecene mycotoxins, one of which is nivalenol. Trichothecene mycotoxins are extremely toxic to rapidly dividing cells, including leukocytes, and one of the leading symptoms of trichothecene toxicosis is the leukopenia known as alimentary toxic aleukia[1]. We reported

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previously that nivalenol hinders cell proliferation[2] and induces apoptosis[3] and interleukin (IL) -8 secretion[2] in the human promyelocytic leukemia cell line HL60. In addition, intracellular calcium ion plays an important role in the exertion of nivalenol-associated toxicity[2,3].

Recently, mitogen-activated protein (MAP) kinases have drawn the attention of scientists, because they are important signal-transducing enzymes that are involved in numerous facets of cellular regulation, such as cell proliferation and differentiation, apoptosis, inflammation, and cytokine secretion[4,5]. In mammals, four groups of MAP kinases — extracellular signal-regulated kinases (ERK1 and 2), c-Jun N-terminal kinases (JNK1, 2, and 3), p38 proteins (p38 α, β and γ), and ERK5 — have been identified[4,5]. Two of these groups (JNKs and p38s) are categorized as stress-activated MAP kinases (SAPKs). In response to external stressors, including UV light, toxic agents, and inflammatory cytokines, SAPKs are converted into their active, phosphorylated forms through kinase cascades and mediate numerous biological phenomena. In this study, to elucidate the molecular mechanism underlying the toxicity of nivalenol, we investigated the involvement of SAPKs in nivalenol-caused cytotoxicity and IL-8 secretion in HL60 cells.

Materials and Methods

Chemicals and cells Nivalenol and SB203580[6] were purchased from Sigma-Aldrich (St. Louis, MO, USA). SP600125[7] was purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA, USA). The above three chemicals were dissolved in dimethyl sulfoxide. Anti-phospho-JNK (Thr 183/Tyr 185) (catalog # 9251) and anti-phospho-p38 (Thr 180/Tyr 182) (#9211) rabbit polyclonal antibodies and anti-rabbit IgG horseradish peroxidase-linked second antibody (#7074) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Cell proliferation ELISA, BrdU (Colorimetric) was purchased from Roche Diagnostics GmbH (Penzberg, Germany). The human promyelocytic leukemia cell line HL60 was purchased from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in RPMI 1640 medium containing 10 % fetal calf serum.

Protein preparation and western blotting HL60 cells (2×10^6 cells) in 10 mL of medium containing nivalenol were cultured in 10-cm plates. Whole-cell proteins were extracted with mammalian protein extraction reagent (M-PER) (Pierce Biotechnology, Rockford, IL, USA). The proteins (8 μg) were resolved on SDS-12 % polyacrylamide gel at 100 V for 80 min and then transferred to a PVDF membrane (Hybond-P; GE Healthcare Ltd., Little Chalfont, UK) at 100 V for 1 h. The membrane blots were blocked in TBST buffer (20 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.1 % Tween 20) containing 5 % skim milk for 1 h and then incubated with anti-phospho-JNK or anti-phospho-p38 antibodies in TBST containing 5 % bovine serum albumin at 4 °C overnight. After being washed with TBST buffer, the membrane was incubated with anti-rabbit-IgG horseradish peroxides-linked second antibody in TBST buffer containing 5 % skim milk for 1 h. After the membrane had been washed again with TBST buffer, the immuno-complexes were visualized with ECL Plus Western Blotting Detection Reagents (GE Healthcare Ltd.).

Cell proliferation Cell proliferation was measured by 5-bromo-2-deoxyuridine (BrdU) incorporation during DNA synthesis, as described previously[8]. HL60 cells (1×10^4 cells) in 100 μL of medium containing chemical(s) were placed in each well of a 96-well microtiter plate for BrdU

incorporation, and proliferation was examined after 24 h of culture.

Determination of IL-8 levels Approximately 1×10^5 cells in 0.5 mL of medium containing chemical(s) were cultured in each well of a 24-well culture plate for 24 h; the media were then collected. Cells were treated with 1 μg/mL nivalenol, because IL-8 secretion was peaked at this concentration in HL60 cells[2]. Collected media were centrifuged at 5,000 × *g* for 5 min to remove cells and cell debris, and the supernatants were assayed. Levels of IL-8 were quantified with a Quantikine Human IL-8 Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA) in accordance with the manufacturer's recommended procedure.

Statistics Data were expressed as means ± standard deviation. Differences between groups were analyzed by Tukey's test. A P value of < 0.05 was considered to indicate significance.

Results

We quantified phosphorylated SAPKs after 24 h of treatment with nivalenol at various concentrations, because the phosphorylated forms are known to be active forms[4,5]. Vehicle-treated HL60 cells contained little phosphorylated JNKs (Fig. 1A). Nivalenol increased the quantity of active forms; the quantity peaked at a nivalenol concentration of around 1 μg/mL (Fig. 1A). Essentially the same trend was observed with phosphorylated p38s (Fig. 1B).

The amounts of phosphorylated SAPKs were peaked at around 1 μg/mL (Fig. 1), we therefore used this concentration in our time-course experiments. Exposure to nivalenol increased phosphorylated JNK levels in HL60 cells with time until 12 h, after which the levels decreased (Fig. 2A); at 24 h, phosphorylated JNKs were less abundant than at 6 h. The phosphorylated p38 assessment gave almost the same results as for phosphorylated JNKs as a function of time (Fig. 2B).

These results led us to hypothesize that nivalenol exerts its toxicity through SAPK signal-transduction pathways. To elucidate the functions of SAPKs, we investigated the effects of specific SAPK inhibitors on nivalenol-caused cytotoxicity and IL-8 secretion.

We investigated the effects of the JNK-specific inhibitor SP600125[7] and the p38-specific inhibitor SB203580[6] on cell proliferation. In our hands, cell proliferation was the most sensitive measure of cell viability, and the 50 % inhibitory concentration was about 0.16 μg/mL[2], therefore, we performed these experiments at a concentration of nivalenol of 0.3 μg/mL. We chose the lowest effectual concentrations of 20 and 5 μmol/L as the final concentrations of SP600125 and SB203580, respectively. As reported previously[2], nivalenol alone significantly hindered cell proliferation (30.2 % and 26.9 % of vehicle-treated controls; Table 1). Likewise, with SP600125 alone, cell proliferation was retarded (44.3 %; Table 1). Nivalenol hindered cell proliferation in the presence of SP600125, however, the effect of nivalenol is much less (39 % of treatment with SP600125 alone) than treatment with nivalenol alone (30.2 %; Table 1), indicating that JNKs occupy one of the important positions in nivalenol-associated retardation of cell proliferation in HL60 cells. Unlike the case with SP600125, SB203580 alone modestly stimulated cell proliferation (124.3 %; Table 1). The result of concomitant treatment with nivalenol and SB203580 (36 % of treatment with SB203580 alone; Table 1) revealed that SB203580 attenuated the inhibitory effect of nivalenol. This result indicates that p38s also play considerable roles in nivalenol-associated retardation of cell proliferation in HL60 cells.

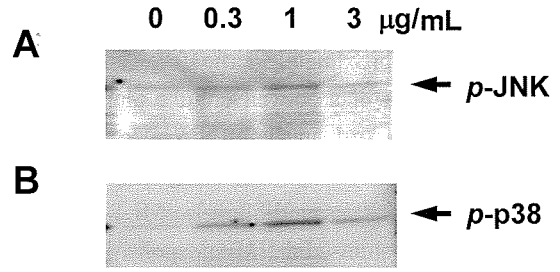

Fig. 1. Nivalenol causes SAPK phosphorylation.
HL60 cells were treated for 24 h with nivalenol at the concentrations indicated. Western blots of whole-cell protein were reacted with (A) anti-phospho-JNK, and (B) anti-phospho-p38 antibodies.

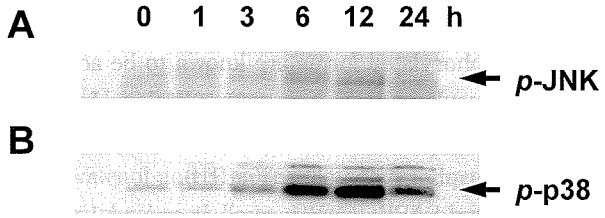

Fig. 2. Kinetics of SAPK phosphorylation by nivalenol.
HL60 cells were treated with nivalenol at 1 μg/mL for the periods indicated. Western blots of whole-cell protein were reacted with (A) anti-phospho-JNK, and (B) anti-phospho-p38 antibodies.

As shown in Table 2, nivalenol elicited IL-8 secretion in HL60 cells. This is consistent with our previous result[2]. SP600125 significantly reduced nivalenol-induced IL-8 secretion (Table 2). Since the effect of SP600125 alone was trivial (92.2 % of the value for IL-8 secretion in vehicle-treated samples; Table 2), JNKs appear important for nivalenol-induced IL-8 secretion. SB203580 lessened nivalenol-induced IL-8 secretion (Table 2). Although this effect was not very substantial, taking the marked increase in IL-8 secretion induced by SB203580 alone (1,027.5 %; Table 2) into consideration, it is conceivable that p38s contribute to nivalenol-induced IL-8 secretion.

Discussion

To our knowledge, no one had demonstrated the involvement of SAPKs in nivalenol toxicity, although the involvement of SAPKs in the signal transduction pathway associated with the toxicity of deoxynivalenol, another trichothecene mycotoxin, has been reported[9-11]. Here, we showed the importance of these enzymes in the exertion of nivalenol toxicity in HL60 cells.

According to Pestka *et al.*, deoxynivalenol elicits IL-8 secretion in Jurkat human T cells, but nivalenol does not[10]. Furthermore, in differentiated human U-937 macrophage cells, deoxynivalenol induces marked secretion of tumor necrosis factor-α and IL-6, but the degree of elevation of these

Table 1. Effects of SAPK inhibitors on cell proliferation

	Nivalenol (0.3 μg/mL)	
	−	+
None	100 ± 9.4 *,‡	30.2 ± 10.5 *
SP600125 (20 μmol/L)	44.3 ± 8.8 †,‡	17.3 ± 3.6 †
None	100 ± 8.6 §,#	26.9 ± 4.1 §
SB203580 (5 μmol/L)	124.3 ± 11.4 ¶,#	44.6 ± 7.0 ¶

HL60 cells were treated with chemicals as indicated for 24 h. Results are means ± standard deviation (n = 6). Proliferation in vehicle-treated samples was defined as 100 %. Differences were analyzed by Tukey's test. Differences between values labeled with the same superscript symbols (*, †, ‡, §, ¶, and #) are statistically significant ($P < 0.05$).

Table 2. Effects of SAPK inhibitors on nivalenol-induced IL-8 secretion

	Nivalenol (1 μg/mL)	
	−	+
None	100 ± 4.3 *,‡	431.2 ± 22.8 *,§,¶
SP600125 (20 μmol/L)	92.2 ± 7.2	126.0 ± 9.5 §
SB203580 (5 μmol/L)	1,027.5 ± 48.8 †,‡	326.3 ± 30.1 †,¶

HL60 cells were treated with chemicals as indicated for 24 h. Results are means ± standard deviation (n = 4). IL-8 secretion in vehicle-treated samples was defined as 100 %. Differences were analyzed by Tukey's test. Differences between values labeled with the same superscript symbols (*, †, ‡, §, and ¶) are statistically significant ($P < 0.05$).

cytokines by nivalenol is modest[12]. Although nivalenol and deoxynivalenol are structurally very much alike, these results indicate that nivalenol toxicity is different from deoxynivalenol toxicity. Moon and Pestka[13] and Zhou *et al.*[14] reported that deoxynivalenol immediately stimulated phosphorylated SAPKs within 15 min; the stimulation then tapered off, and no SAPKs were detected at the end of 8 h of treatment in murine macrophage RAW 264.7 cells. The difference in the time course of phosphorylated SAPK production between nivalenol (Fig. 2) and deoxynivalenol[13,14] might account for the difference in the toxicities of these two compounds.

The amounts of phosphorylated SAPKs produced with 3 μg/mL nivalenol were smaller than those at 1 and 0.3 μg/mL (Fig. 1). This phenomenon can be accounted for by cell damage, because nivalenol at 3 μg/mL or higher induces apoptosis in HL60 cells[3], and morphologic damage is also apparent at this concentration[2].

As reported by Hobbie *et al.*[15], Oltmanns *et al.*[16], and Nagashima *et al.*[17], it is common that SAPKs contribute to IL-8 secretion. Islam *et al.* reported that deoxynivalenol-elicited IL-8 secretion is p38-dependent but not JNK-dependent in human U937 monocyte cells and human primary blood mononuclear cells[11]. The discrepancy between their results and ours (Table 2) suggests that nivalenol and deoxynivalenol induce IL-8 secretion differently. Treatment with SB203580 alone enhanced IL-8 secretion (Table 2), suggesting that p38s repress IL-8 secretion in HL60 cells. It is likely that SB203580 and nivalenol interfere and cancel each other's effects (Table 2). From the viewpoint of signal transduction study, this phenomenon is not very common and worthwhile

elucidating. To our knowledge, no one has addressed the involvement of SAPKs in deoxynivalenol-associated retardation of cell proliferation.

We successfully showed here the importance of SAPKs in the exertion of nivalenol toxicity in HL60 cells. However, further studies are needed to elucidate the detailed mechanism of this toxicity.

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ストレス応答 MAP キナーゼの HL60 細胞におけるニバレノール誘導性の細胞毒性とインターロイキン-8 分泌への寄与

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ニバレノール（NIV）の毒性発現機構を解明するために，JNK と p38 の 2 種のストレス応答 MAP キナーゼ（SAPK）の HL60 細胞における NIV 誘導性の細胞毒性とインターロイキン（IL)-8 分泌への関与を検討した．NIV24 時間処理でリン酸化（活性型）JNK は増加し，その量は 1 μg/mL の時に最も多かった．この濃度で処理したタイムコース実験によりリン酸化 JNK は 12 時間でピークに達した．リン酸化 p38 でも同様の結果だった．次に JNK 特異的阻害剤 SP600125 と p38 特異的阻害剤 SB203580 が NIV による細胞毒性と IL-8 分泌に与える影響について検討した．NIV と SAPK 特異的阻害剤で同時処理すると NIV 単独処理と比べて細胞増殖阻害は減じたので，SAPK は NIV による細胞増殖阻害に関与すると考えられた．SP600125 が NIV 誘導性の IL-8 分泌を顕著に減じたことは，JNK がこの分泌に寄与していることを示している．SB203580 が NIV 誘導性の IL-8 分泌を減じた．この効果は穏やかであったが，SB203580 単独処理で顕著に IL-8 分泌が増加したことを考え合わせると，p38 の NIV 誘導性の IL-8 分泌への寄与は大きいと考えられた．

キーワード：細胞増殖，HL60 細胞，インターロイキン-8，ストレス応答 MAP キナーゼ