報　文

Ryanodine receptor inhibitor dantrolene alleviates nivalenol-induced cytotoxicity 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 ryanodine receptor 1 (RyR1), an intracellular calcium ion channel, in nivalenol-induced cytotoxicity in human promyelocytic leukemia HL60 cells by using an RyR1-specific inhibitor dantrolene. Cell proliferation and cytokine levels were investigated after 24-h treatment with the chemicals. Nivalenol markedly hindered cell proliferation, while dantrolene retarded moderately. If RyR1 is not concerned with the nivalenol signal-transduction pathway(s), dantrolene should magnify the inhibitory effect of nivalenol. However, dantrolene did not strengthen the effect of nivalenol, therefore, RyR1 might play a role in the nivalenol-associated retardation of cell proliferation. Nivalenol significantly elicited interleukin (IL)-8 secretion, while dantrolene did not. Dantrolene substantially attenuated the effect of nivalenol, indicating that RyR1 is important for nivalenol-induced IL-8 secretion. Nivalenol reduced monocyte chemotactic protein (MCP)-1 secretion, while dantrolene decreased moderately. Dantrolene clearly alleviated the effect of nivalenol, indicating that RyR1 mediates the nivalenol-induced inhibition of MCP-1 secretion. Taken together the above results, the involvement of RyR1 in nivalenol-induced toxicity in HL60 cells was deduced.

Keywords: dantrolene, interleukin-8, monocyte chemotactic protein-1, nivalenol, ryanodine receptor

Introduction

A variety of *Fusarium* fungi produce a number of different mycotoxins of the class of trichothecenes and some other mycotoxins (zearalenone and fumonisins). More than 60 trichothecene mycotoxins are known; nivalenol is one such trichothecene. Trichothecene mycotoxins are extremely toxic to rapidly dividing cells, including leukocytes, and alimentary toxic aleukia, a type of leucopenia, is one of the leading symptoms of trichothecene toxicosis[1]. We have previously reported that nivalenol hinders cell proliferation[2], induces apoptosis[3] and inter leukin-8 (IL-8)[2] secretion, and decreases monocyte chemotactic protein-1 (MCP-1)[4] secretion in the human promyelocytic leukemia cell line HL60. In addition, intracellular calcium ion[2, 3] and stress-activated mitogen-activated protein (MAP) kinases[5] occupy crucial positions in the exertion of nivalenol-associated toxicity.

Since intracellular calcium ion contributes to the niva lenol-induced cytotoxicity[2, 3], we hypothesized that calcium ion is discharged from the intracellular calcium ion

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stores into the cytoplasm by the action of nivalenol. Ryanodine receptors[6)]—RyR1, RyR2, and RyR3—are localized in the endoplasmic reticulum (ER) membrane and are specialized intracellular calcium ion channels for the rapid and extensive release of calcium ion. Upon external stimulation, RyRs mediate the release of calcium ion from the ER calcium store to the cytoplasm. RyRs are mainly expressed in excitable cells and play pivotal roles in muscle contraction and various neuronal activities. In this study, to elucidate the molecular mechanism underlying the toxicity of nivalenol, we focused on RyR1 and investigated the effects of dantrolene, an RyR1-specific inhibitor[7)], on nivalenol-induced cytotoxicity in HL60 cells.

Materials and Methods

Chemicals and cells

Nivalenol was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide. Dantrolene was purchased from Merck KGaA (Darmstadt, Germany) and dissolved in water. Cell proliferation ELISA, BrdU (Colorimetric) was purchased from Roche Diagnostics GmbH (Penzberg, Germany). The human promyelocytic leukemia cell line HL60 was purchased from RIKEN Cell Bank (Tsukuba, Japan) and cultured in RPMI (Roswell Park Memorial Institute) 1640 medium (Invitrogen Corp., Carlsbad, CA USA) containing 10 % fetal calf serum (JRH Biosciences Inc., Lenexa, KS, USA).

Cell proliferation

Cell proliferation was investigated by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation during DNA synthesis as described by a previous study[8)]. HL60 cells (1.2×10^4 cells) in 100 μl of RPMI 1640 medium containing chemical(s) were placed in each well of a 96-well microtiter plate for BrdU incorporation, and cell proliferation was assessed after 24 h of culture.

Determination of cytokine levels

HL60 cells (1×10^5 cells) in 0.5 ml of RPMI 1640 medium containing chemical(s) were cultured in each well of a 24-well culture plate for 24 h and then the media were collected. To achieve the maximum IL-8 concentration, HL60 cells were treated with 1 μg/ml of nivalenol, because IL-8 secretion was peaked at this concentration in HL60 cells[2)]. The morphology of the cells[2)] and the extent of DNA laddering[3)] suggested that the cells were still viable in 1 μg/ml of nivalenol. The collected media were centrifuged at $5,000 \times g$ for 5 min to obtain the supernatants and to remove cells and cell debris. Further, the supernatants were analyzed to determine the levels of IL-8 and MCP-1 using Quantikine Human IL-8 Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA) and Quantikine Human CCL2/MCP-1 Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA), respectively, according to the manufacturer's recommended procedures.

Statistics

Data were expressed in terms of mean ± standard deviation. Intergroup differences were analyzed by Tukey's test. A P value less than 0.05 was considered to be statistically significant.

Results and Discussion

To our knowledge, there are no studies that report the role of RyRs in nivalenol toxicity. Here, therefore, we examined the importance of RyR1 in the exertion of nivalenol toxicity in HL60 cells.

We investigated the effects of the RyR1-specific inhibitor dantrolene[7)] on cell proliferation. In our hands, cell proliferation was the most sensitive measure of cell viability[2)]. The 50 % inhibitory concentration of nivalenol was about 0.16 μg/ml[2)]; therefore, we performed our experiments with 0.3 μg/ml of nivalenol. We chose the lowest effective concentration of dantrolene—40 μM—as the final concentration. As we reported previously[2)], nivalenol alone markedly hindered cell proliferation (10.1 % of vehi cle-treated control value; Table 1). On the other hand, treatment with dantrolene alone led to moderate inhibition of cell proliferation (76.5 %; Table 1). We found that regardless of the presence of dantrolene, the values in niva lenol-treated samples were almost the same (10.1 % versus 9.9 %; Table 1). If RyR1 is indifferent to the nivalenol signal transduction pathway(s), the effects of nivalenol and dantrolene should be additive, therefore, our results suggest that RyR1 may play a role in the nivalenol-associated retardation of cell proliferation in HL60 cells. It has been reported that dantrolene inhibited cell prolif-

Table 1. Effect of dantrolene on cell proliferation

	Nivalenol (0.3 μg/ml)	
	−	+
None	100 ± 8.1*,‡	10.1 ± 3.7*
Dantrolene (40 μM)	76.5 ± 9.5†,‡	9.9 ± 2.5†

HL60 cells were treated with the chemicals, as indicated, for 24 h. Results are expressed in terms of mean ± standard deviation (n = 6). Cell proliferation in the vehicle-treated samples is defined as 100 %. Intergroup differences were analyzed by Tukey's test. Intergroup differences between the values labeled with the same superscript symbols (*, †, and ‡) are statistically significant (P < 0.05).

eration stimulated by leukotriene B4[9] and growth factors[10]. Conversely, this chemical antagonized morphine-caused inhibition of cell proliferation[11], thereby indicating that the contribution of RyR1 to cell proliferation is Janus-faced. Our results confirm the validity of this concept.

Nivalenol significantly elicited IL-8 secretion in HL 60 cells (319.6 % of the value in the vehicle-treated samples), alternatively, dantrolene alone did not alter IL-8 secretion (98.2 %; Table 2). The secretion of IL-8 in cells concomitantly treated with nivalenol and dantrolene was much lower (256.2 %) than IL-8 secretion in cells treated with nivalenol alone (319.6 %; Table 2). Thus, it is likely that RyR1 is important for nivalenol-induced IL-8 secretion. Hisatsune *et al.*[12] and Goyal *et al.*[13] reported that dantrolene inhibited IL-8 secretion induced by vacuolating cytotoxin A (VacA)[12], a *Helicobactor pylori*-produced protein, and the IL-8 secretion induced by an adhesin produced by enteroaggregative *Escherichia coli*[13], respectively. Since our results are consistent with these results, nivalenol might share signal transduction pathway(s) with these proteins.

Nivalenol reduced MCP-1 secretion (60.0 % of the value in the vehicle-treated samples; Table 3), which has also been reported by previous study[4]. Similarly, dantrolene moderately reduced MCP-1 secretion (85.0 %; Table 3). MCP-1 secretion in cells concomitantly treated with nivalenol and dantrolene (65.7 %) was higher than that in the cells treated with nivalenol alone (60.0 %), indicating that dantrolene alleviates the effect of nivalenol on HL60 cells. To our knowledge, the relationship between RyR1 and MCP-1 secretion has not been previously reported, and this is the first study that reports this association.

Table 2. Effect of dantrolene on nivalenol-induced interleukin-8 secretion

	Nivalenol (1 μg/ml)	
	−	+
None	100 ± 7.5*	319.6 ± 9.5*,‡
Dantrolene (40 μM)	98.2 ± 2.8†	256.2 ± 16.2†,‡

HL60 cells were treated with the chemicals, as indicated, for 24 h. Results are expressed in terms of mean ± standard deviation (n = 4). Interleukin (IL)-8 secretion in the vehicle-treated samples is defined as 100 %. Intergroup differences were analyzed by Tukey's test. Intergroup differences between the values labeled with the same superscript symbols (*, †, and ‡) are statistically significant (P < 0.05).

Table 3. Effect of dantrolene on nivalenol-induced decrease in monocyte chemotactic protein-1 secretion

	Nivalenol (1 μg/ml)	
	−	+
None	100 ± 4.3*,‡	60.0 ± 3.1*
Dantrolene (40 μM)	85.0 ± 2.6†,‡	65.7 ± 1.5†

HL60 cells were treated with the chemicals, as indicated, for 24 h. Results are expressed in terms of mean ± standard deviation (n = 4). Monocyte chemotactic protein (MCP)-1 secretion in the vehicle-treated samples is defined as 100%. Intergroup differences were analyzed by Tukey's test. Intergroup differences between the values labeled with the same superscript symbols (*, †, and ‡) are statistically significant (P < 0.05).

We showed that RyR1 plays a role in nivalenol-induced toxicity in HL60 cells. Because RyRs activation, in general, is considered to be triggered by an increase in the concentration of intracellular calcium ion[6], further studies are required to elucidate the detailed mechanism of nivalenol-induced activation of RyR1.

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リアノジン受容体の阻害剤ダントロレンは HL60細胞における ニバレノール誘導性の細胞毒性を軽減する

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要　約

ニバレノール（NIV）の毒性発現機構を解明するために，細胞内カルシウムチャンネルであるリアノジン受容体１（RyR1）の HL60細胞における NIV 誘導性の細胞毒性への関与を，RyR1特異的阻害剤のダントロレンを用いて検討した。それぞれのアッセイは薬剤の24時間処理後に行った。NIV は顕著に細胞増殖を阻害したが，ダントロレンによる阻害は穏やかであった。もし RyR1が NIV のシグナル伝達経路に関係ないのであれば，ダントロレンが NIV による阻害作用を増強することが期待される。ダントロレンは NIV の作用を増強しなかったことから，RyR1は NIV による細胞増殖阻害に何らかの役割を果たしているのかもしれない。NIV は顕著にインターロイキン（IL）-8の分泌を誘導したが，ダントロレンは IL-8分泌量を変化させなかった。ダントロレンは NIV の影響を十分に減じたので，RyR1が NIV 誘導性の IL-8分泌に重要であると考えられた。NIV とダントロレンは単球走化性タンパク質（MCP）-1の分泌を減少させたが，ダントロレンによる減少は穏やかだった。ダントロレンは明らかに NIV の作用を和らげたので，RyR1は NIV による MCP-1分泌阻害に関与していると考えられた。以上の結果から，NIV の HL60細胞に対する細胞毒性の発現には RyR1が関与していると考えられた。