ガスにて濃縮乾固し、DMEM 培地に溶解し使用した。

Escherichia coli 0111:B4 株由来 LPS (Sigma-Aldrich, St. Louis, MO, USA) は注射用水に溶解し 4°C で保存した。使用前にソニケーションを行った。

【NF-κB 依存性レポーター活性の測定】

マクロファージ様に分化させた THP-1 細胞の上清を除去し、LPS 100 ng/ml 存在下、T-2 (10, 20, 40, 80 ng/ml) 、または HT-2 (1.0, 2.0, 4.0, 8.0 ng/ml) を含む DMEM にて培養を継続した。6 時間後に培養液を除去、その後、PBS で洗浄した。Passive Lysis Buffer (Promega, Madison, WI, USA) を 50 μl/well を加えセルスクレイパーで細胞を掻き取った。掻き取った細胞をマイクロチューブに取り、on ice と vortex をそれぞれ 30 sec、計 10 分処理後、4°C、4,000 rpm、5 分遠心を行い、上清を試料として用いた。

試料 5.0 μl を 96-well flat bottom white polystyrene plate (Croning Costar, Kennebunk, ME, USA) に添加し、基質には Luciferase Assay Reagent II (Promega, Madison, WI, USA) を用いてルシフェラーゼ活性を測定した。測定にはマルチプレートリーダーTristar LB 941 (Berthold Technologies, Germany) を用いた。なお、測定結果はタンパク質量で補正した。

C. 研究結果

ヒト単球由来 THP-1 細胞をマクロファージ様細胞に分化させたのち、同細胞を用いて LPS 誘導性 NF-κB の活性化に及ぼす T-2 の影響を NF-κB 依存性レポーター活性化に対する作用を指標に検討した。LPS 単独刺激により NF-κB 依存性レポーター活性はコントロールと比較し、10 倍以上の上昇を認めた。本条件下において、T-2 による TLR4 を介した LPS 誘導性 NF-κB 依存性レポーター活性への影響を検討したところ、濃度依存的な抑制作用を示すことが明らかとなった。また、その作用は今回検討した最も低い濃度となる 10 ng/ml においても顕著に認められ、LPS 誘導性 NF-κB の活性化を約 50%にまで阻害する作用を示した (Fig. 3)。

同様に、LPS 誘導性 NF-κB 依存性レポーター活性を T-2 と同様に HT-2 も濃度依存的に抑制することが確認された (Fig. 4)。

D. 考察

本分担研究では、ヒトマクロファージ様細胞 THP-1 を用いて、LPS 誘導性 NF-κB に及ぼす T-2 と HT-2 の影響について検討し

た。今回、T-2 と HT-2 の両毒素とも LPS 誘導性 NF-κB の活性化を濃度依存的に抑制することが明らかとなった（Fig. 3, 4)。今回認められた作用は、T-2 においては 10 ng/ml 存在下において LPS 誘導性 NF-κB の活性化を約 50%に、HT-2 においては 1.0 ng/ml においても同活性化に対して顕著な抑制作用を示すレベルの阻害効果であった。

今回得られた結果は、TLR4 から転写因子 NF-κB の活性化に至る経路において T-2 ならびに HT-2 が抑制的に作用するシグナル伝達分子が存在することを示唆する。TLR4 シグナルに対する阻害作用は、本研究から、トリコテセン系かび毒の Type A および B ともに確認されたこととなる。従って、少なくとも THP-1 細胞に対してはトリコテセン系かび毒の Type A および B は TLR4 シグナル伝達系をともに抑制することが強く示唆された。このことは、これらかび毒に汚染された食品を摂取した場合、正常な免疫応答が誘導されないことが危惧されるため、感染症等に罹患する危険性が高まることが予想される。また、今回得られたデータから、これまでに指摘されていたとおり、Type A においてより低濃度で毒性が認められる可能性が示された。加えて、HT-2 と T-2 において TLR4 シグナルに対する阻害作用が HT-2 で約 10 倍高いことが示された事は、即ち、今後 Type A におけるトリコテセン系かび毒のリスクアセスメントを行うにあたり、HT-2 の毒性について十分留意する必要性を示している。つまり T-2 が生体内で HT-2 に代謝されることを考慮した場合、少なくとも TLR4 シグナルに対する毒性を基準にそのリスクアナリシスする場合には、HT-2 の毒性を基準に T-2 の毒性も評価する必要性があると言える。

E. 結論

ヒトマクロファージ様細胞を用いて LPS により惹起される TLR4 シグナルに対する T-2 と HT-2 の影響を、同シグナルの下流に存在する NF-κB の活性化を指標に検討した。その結果、T-2 と HT-2 ともに同シグナルを阻害する可能性が認められた。従って、本研究より Type A トリコテセン系かび毒は Type B と同様に自然免疫系の抑制を介して免疫毒性を呈する可能性が強く示唆された。

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F. 健康危険情報

なし

G. 研究業績

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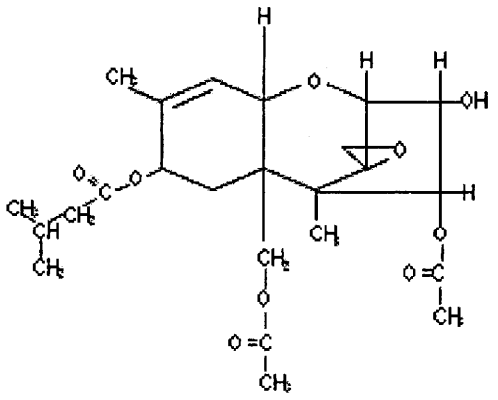

Fig. 1 T-2 toxin

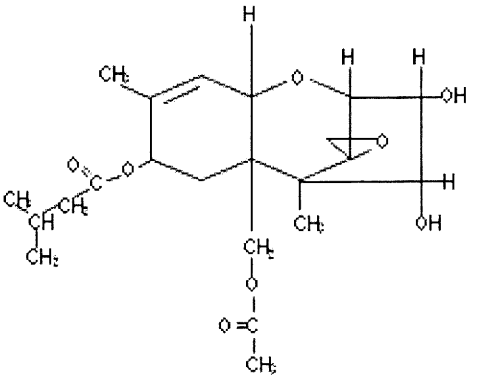

Fig. 2 HT-2 toxin

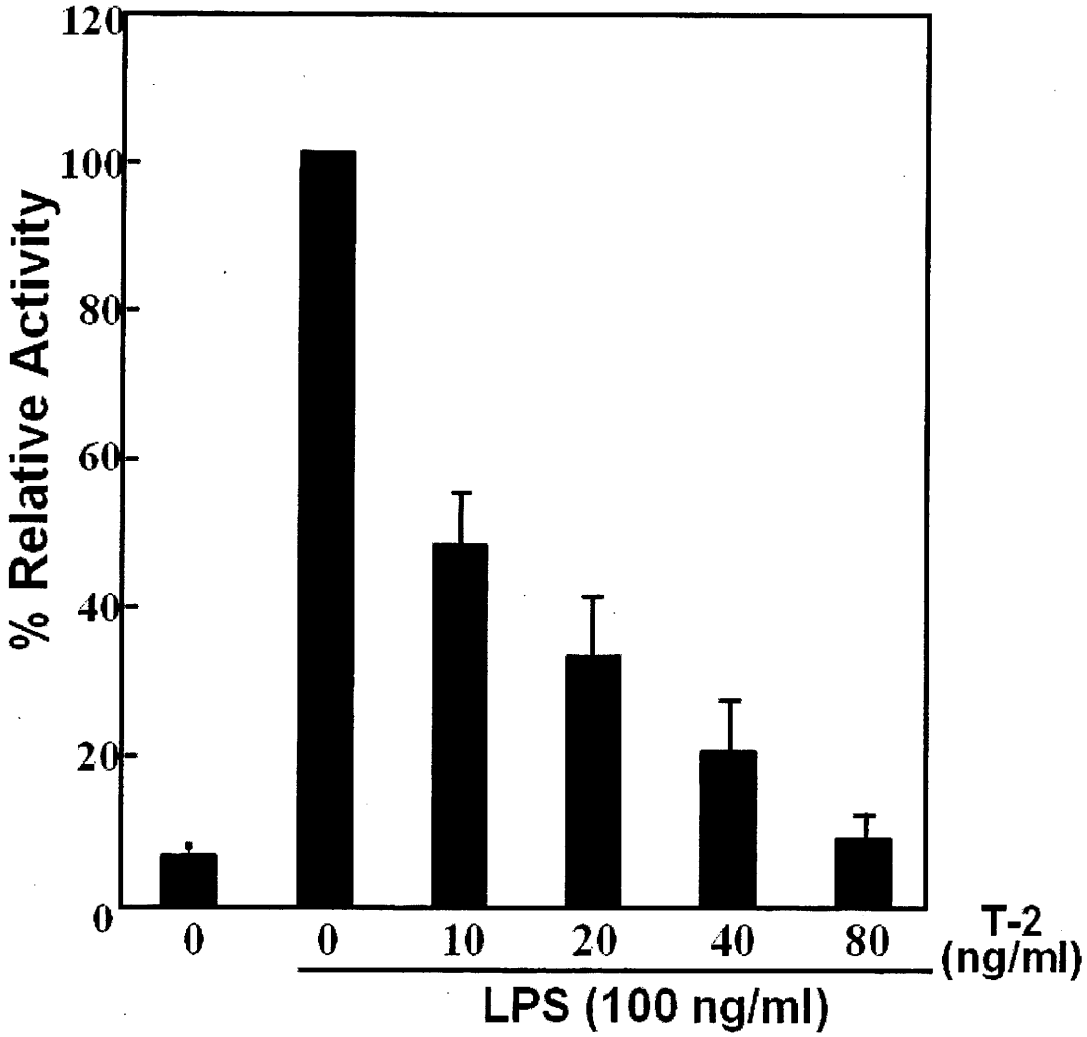

Fig. 3 Effects of T-2 on LPS-induced NF-κB dependent repoeter activity in differentiated THP-1 cells.

Differentiated THP-1 cells were stimulated with T-2 (10-80 ng/ml) and LPS (100 ng/ml) for 6 h and luciferase activity was then measured. The reporter activity in response to LPS alone is expressed as 100%. Values are means ± SEM from three independent experiments.

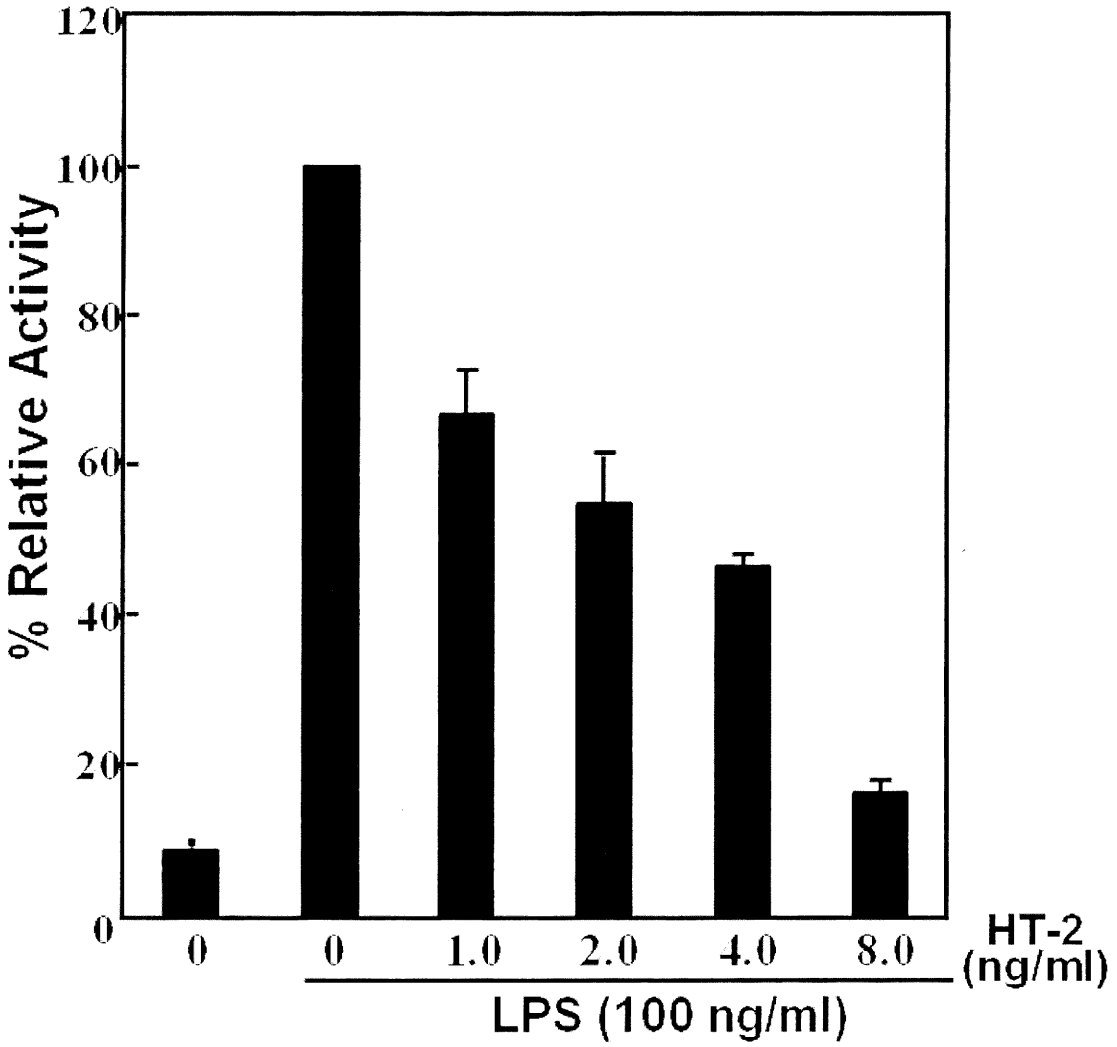

Fig. 4 Effects of HT-2 on LPS-induced NF-κB dependent reporter activity in differentiated THP-1 cells.

Differentiated THP-1 cells were stimulated with HT-2 (1.0-8.0 ng/ml) and LPS (100 ng/ml) for 6 h and luciferase activity was then measured. The reporter activity in response to LPS alone is expressed as 100%. Values are means ±SEM from three independent experiments.

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
Sugiyama, K., Kawakami, H., Kamata, Y. and Sugita-Konishi, Y	Effect of a combination of deoxynivalenol and nivalenol on lipopolisaccharide-induced nitric oxide production by mouse macrophages	*Mycotoxin Res.*	**27**	57-62	2011

研究成果の刊行物

Mycotox Res (2011) 27:57–62
DOI 10.1007/s12550-010-0076-1

ORIGINAL PAPER

Effect of a combination of deoxynivalenol and nivalenol on lipopolisaccharide-induced nitric oxide production by mouse macrophages

Kei-ichi Sugiyama · Hiroshi Kawakami · Yoichi Kamata · Yoshiko Sugita-Konishi

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Abstract Deoxynivalenol (DON) and nivalenol (NIV) are trichothecene mycotoxins produced by *Fusarium* fungi as secondary metabolites. Both compounds have the immunotoxic effects that the productions of inflammatory mediators by activated macrophages is disturbed. Co-contamination with DON and NIV can occur; however, the effects of simultaneous contamination are not well known. The present study investigated the combined effects of DON and NIV on nitric oxide (NO) production by mouse macrophages stimulated with lipopolisaccharide (LPS). The inhibitory effect of DON and NIV on NO release from activated macrophages has already been reported as an appropriate indicator of immunotoxic effect of the both compounds. LPS-induced NO production in macrophages was inhibited by both of these toxins individually in a dose-dependent manner, and toxin mixtures at the same concentration inhibited NO production in the same manner. In addition, there were no unique inhibitory effects on LPS-induced NO production in macrophages in the presence of mixtures of various molar ratios. These results suggest that the combined effects of DON and NIV can be predicted based on addition of each compound alone.

Keywords Combined toxicity · Deoxynivalenol · Nivalenol · Nitric oxide · Macrophage

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Introduction

Mycotoxicosis of human and various animals has been documented following consumption of fungus-infected cereal grains (Meky et al. 2001). Although more than 400 mycotoxins are known, the contamination of grains by trichothecene mycotoxins has been one of the most serious public health threats (Marzocco et al. 2009; Ueno 1985). The trichothecene mycotoxins are a structurally diverse group of secondary metabolites produced by several fungi, including *Fusarium*, *Mycothecium*, *Trichoderma*, *Trichothecium*, *Stachybotrys*, *Verticimonosporium*, and *Cephalosporium* (Ueno 1985), and have been found to be food contaminants in grain crops such as wheat and corn, and products derived from these grains (Marzocco et al. 2009; Rotter et al. 1996; Sugiyama et al. 2010). Especially common are deoxynivalenol (DON) and nivalenol (NIV), type B trichothecenes, whose contamination of foodstuffs occurs frequently worldwide (Fig. 1). It is reported that about 60% of the food in the European Union (EU) is contaminated by DON (Schothorst and van Egmond 2004). Contamination by NIV is commonly detected on cereals cultivated in temperature regions, including Japan (Schlatter 2004; Sugiura et al. 1993; Yoshizawa et al. 2004), and also found in food in the EU (Schothorst and van Egmond 2004). In addition, it is known that DON and NIV are not degraded by the usual cooking processes (Marzocco et al. 2009); however milling, boiling and alkaline cooking are effective in reducing DON (Kushiro 2008; Nowicki et al. 1988; Abbas et al. 1998).

Trichothecene mycotoxins have been implicated in vomiting and alimentary hemorrhage, and have been demonstrated to affect immune cells and impair the immune response (Bennett and Klich 2003; Sugita-Konishi and

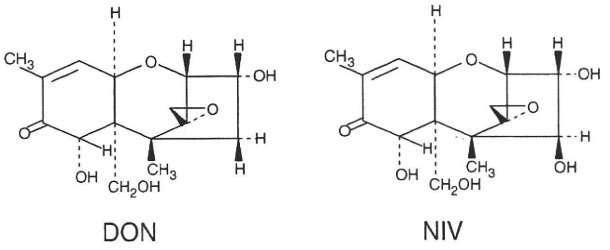

Fig. 1 Chemical structures of DON and NIV

Pestka 2001). In particular, toxicity studies revealed DON-induced cytotoxicity against immune cells, including macrophages, and dysregulation of the immune response (Instanes and Hetland 2004; Pestka 2008; Pestka and Smolinski 2005). These reports suggest that the data of immune dysfunction as well as cytotoxicity are important in risk analysis for trichothecene mycotoxins and immune cells are the primary target for this mycotoxins (Bondy and Pestka 2000). Therefore, susceptibility to infections caused by other food-borne pathogens, such as *Listeria monocytogenes*, is enhanced by DON (Tryphonas et al. 1986).

Nitric oxide (NO) production by activated macrophages is of particular importance for protection against intracellular microbiostasis, including that of *L. monocytogenes* (Boockvar et al. 1994). NO synthesis from specific stimuli-activated macrophage is catalyzed by inducible NO synthase (iNOS) whose expression is induced by lipopolysaccharide (LPS) (Shimomura-Shimizu et al. 2005). Our previous study demonstrated that both DON and NIV repress LPS-induced NO production from a macrophage-like cells by inhibiting LPS-induced iNOS expression (Sugiyama et al. 2010). These reports strongly suggest that the inhibitory effects of these trichothecene mycotoxins on LPS-induced NO production are related to their toxicity.

A number of mycotoxins produced by one mold species have been found in food commodities, but there is little information about the combined effect of mycotoxins (Speijers and Speijers 2004). The objective of the present study was to assess whether the combinations of DON and NIV cause additive, synergistic or antagonistic effects on the NO production.

Materials and methods

Cell culture and reagents

A mouse macrophage cell line, RAW264, was obtained from the Riken Cell Bank (Tsukuba, Japan) and cells were cultured in DMEM (Gibco-BRL, Rockville, MD, UA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco-BRL), penicillin (100 U/ml) and streptomycin (100 μg/ml). DON and NIV were purchased from Wako Purechemical Industries (Osaka, Japan) and biopure Referenzsubstanzen (Tulln, Austria), and LPS from *Escherichia coli* O111:B4 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

NO production

RAW264 cells were plated (2×10^5 cells/well) in 96-well plates, and on the following day were exposed to each toxin in the presence or absence of LPS for 24 h. Determination of NO production was performed by measuring the concentration of nitrite in culture supernatants using the Griess method with the NO_2/NO_3 Assay Kit-C II (Dojin; Chm. Lab. Institute, Kumamoto, Japan). Briefly, the mixture consisting of 80 μl aliquot of culture supernatant, 10 μl of nitrate reductase and 10 μl of enzyme cofactor was added to 50 μL of sulfanilamide and 50 μL of N-naphthyl ethylenediamine at room temperature for 15 min. The absorbance of the solution was determined at 550 nm using a micro plate reader, TriStar LB 941 (Berthold Tech., Germany). The NO_2 concentration was calculated from a $NaNO_2$ standard curve.

Statistical analysis

Statistical comparisons of multiple groups were done using one-way ANOVA followed by Dunnett's post hoc test. The values shown in the figures are expressed as the means ± SEM.

Results

DON and NIV affect the immune system (Sugita-Konishi and Pestka 2001; Pestka and Smolinski 2005), and it was recently reported that LPS-induced NO production by mouse macrophages is inhibited by these toxins in a concentration-dependent manner (Sugiyama et al. 2010). Therefore, to examine the toxicity of a DON and NIV in combination, LPS-induced NO production by a mouse macrophage cell line, RAW264, in the presence of these toxins was measured. As shown in Fig. 2, LPS-induced NO production was repressed by both toxins in a concentration-dependent manner. A significant inhibitory effect of each toxin on LPS-induced NO production was observed at concentrations greater than 500 ng/ml. These results suggest that there was no significant difference in the repression of LPS-induced NO production between DON and NIV. In nonstimulated RAW264, NO productions remained below approximately 10% of stimulated cells.

It has been reported that DON and NIV naturally co-exist in cereals in some countries, including Japan

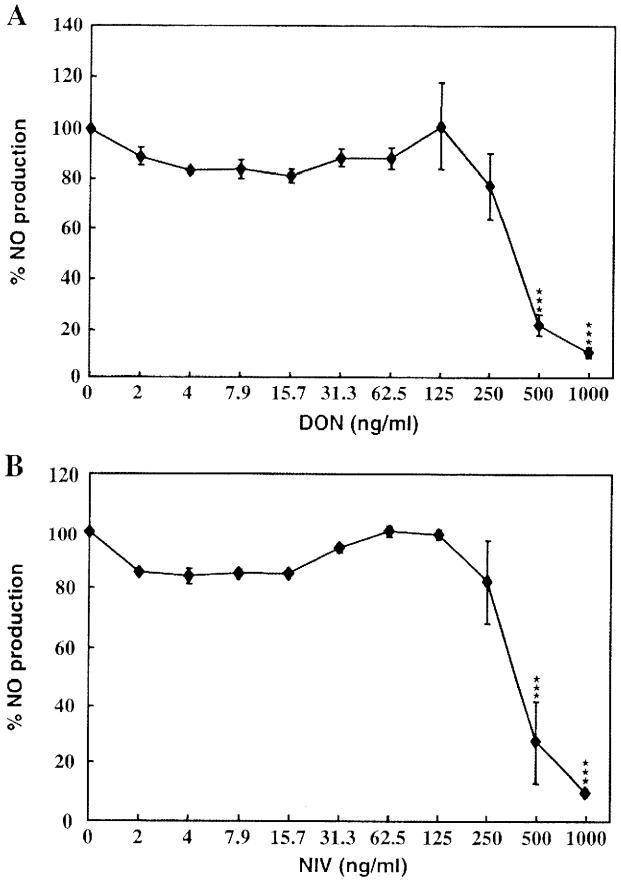

Fig. 2 Concentration-dependent effects of DON and NIV on LPS-induced NO production by RAW264 Cells RAW264 cells were stimulated with LPS (100 ng/ml) in the presence of the indicated concentrations of *DON* (**a**) or *NIV* (**b**) for 24 h. The culture supernatants were analyzed for NO levels. The NO induced by LPS treatment alone is expressed as 100%. Values are presented as the means ± SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (***$P < .001$, vs control)

(Yoshizawa et al. 2004; Sugiura et al. 1990). Therefore, to estimate the real risk to mammalian health, analysis of the combined toxicity of DON and NIV is of particular importance. To investigate the combined effects of DON and NIV on LPS-induced NO production by mouse macrophages, we also measured NO production in RAW264 stimulated with LPS in the presence of a mixture of DON and NIV at a ratio of 1:1. As shown in Fig. 3, the inhibitory effect of the mixture of DON and NIV (1:1) on LPS-induced NO production became significant above 500 ng/ml (total concentration of mycotoxins), thus suggesting that there was no synergistic or antagonistic toxic effect of the DON and NIV mixture.

To compare the effect of the DON and NIV molar ratio on the inhibitory effect of NO production by LPS-activated macrophages, we first examined the effect of DON in the presence of NIV on LPS-induced NO production by macrophages. There was no effect on LPS-induced NO production by macrophages at 600 nmol/l NIV (187.4 ng/ml). A significant inhibitory effect on LPS-induced NO production by macrophages exposed to 600 nmol/l of NIV was observed at a concentration as low as 1,500 nmol/l of DON (Fig. 4). Similar results were obtained when LPS-induced NO production by macrophages treated with 600 nmol/l (177.6 ng/ml) of DON was measured in the presence of NIV (Fig. 5).

Discussion

Trichothecene mycotoxins are implicated in toxicosis in humans and animals, and the simultaneous contamination of DON and NIV has been detected in cereals (Schlatter 2004; Sugiura et al. 1993; Yoshizawa et al. 2004). It has been reported that exposing trichothecene mycotoxins such as DON and NIV altered the immune response by lymphocytes (Sugita-Konishi and Pestka 2001). Moreover, we have previously reported that NO release from activated macrophages was repressed by trichothecene mycotoxins (Sugiyama et al. 2010). NO produced by activated macrophages plays an important role in protection against intracellular bacterial infections (Boockvar et al. 1994; Sakai et al. 2006). In fact, it is reported that the recruitment of iNOS to mycobacterial phagosomes is repressed by *Mycobacterium tuberculosis* which is an intracellular pathogen located within macrophages, and the immunotoxic

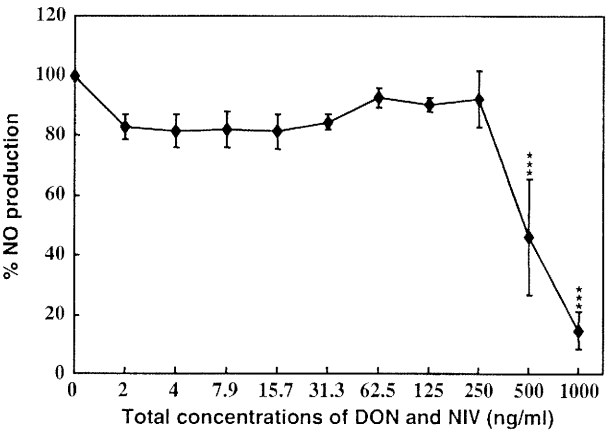

Fig. 3 Combined effects of DON and NIV on LPS-induced NO production by RAW264 Cells RAW264 cells were stimulated with LPS (100 ng/ml) in the presence of the indicated total concentrations of DON and NIV (1:1) for 24 h. The culture supernatants were analyzed for NO levels. NO induced by LPS treatment alone is expressed as 100%. Values are presented as the means ± SEM from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (***$P < .001$, vs control)

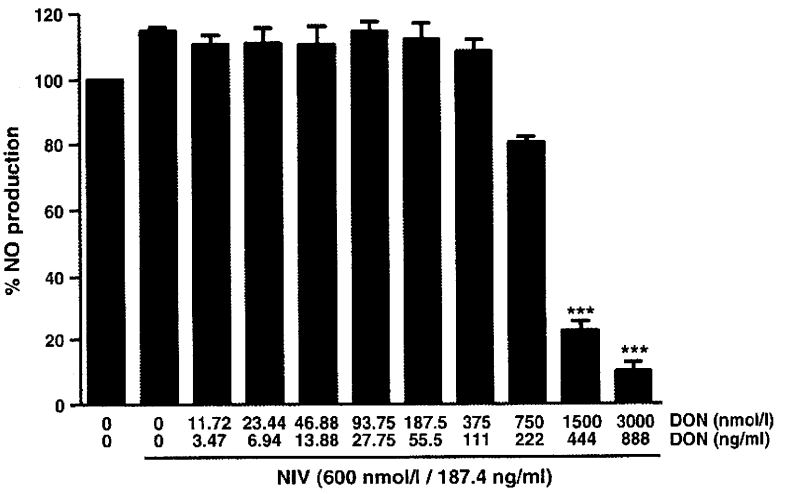

Fig. 4 Concentration-dependent effects of DON on LPS-induced NO production by RAW264 Cells in the Presence of NIV RAW264 cells were stimulated with DON (0–3,000 nmol/l) in the presence of LPS (100 ng/ml) and NIV (600 nmol/l) for 24 h. The culture supernatants were analyzed for NO levels. Values are the means ± SEM from three independent experiments. NO induced by LPS treatment alone is expressed as 100%. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (***$P < .001$, vs LPS treated control)

effects of DON reduce resistance of *L. monocytogenes* infection (Miller et al. 2004; Tryphonas et al. 1986). Therefore, the effect of DON and NIV on NO production by activated macrophages is considered to be a suitable toxicity marker of these toxins. The objective of this study is to estimate the toxicity of combined DON and NIV on the amount of NO produced by LPS-stimulated macrophages.

This study clearly demonstrated that both DON and NIV inhibited LPS-induced NO production by macrophages in the same manner (Fig. 2). Moreover, it seemed that the combined effect of DON and NIV on NO production by macrophages stimulated with LPS is equal to the sum of the effect of each individual toxin (Fig. 3). Our studies provide additional support for neither toxin having a unique inhibitory effect on LPS-induced NO production by macrophages, regardless of the molar ratios of the individual toxins (Figs. 4 and 5). Our findings are supported by Thuvander et al. (1999), who described that combination treatment of human lymphocytes with DON and NIV resulted in additive cytotoxicity (Thuvander et al. 1999). It has also been reported that NIV is considered to have toxicological profiles similar to DON (Schlatter 2004). However, the synergistic inhibition of growth of brewing yeast by the combination of DON and NIV has been demonstrated (Madhyastha et al. 1994). This discrepancy may account for the differences in membrane permeability for both DON and NIV and of intracellular components interacted with these toxins. Taken together, the toxicological risk of mixtures of these trichothcenes can be estimated to be equal to the sum of the effects of the individual DON and NIV toxins. In addition, this present study suggests that DON and NIV have no different sites or modes of action leading to inhibition of LPS-induced NO production through upregulation of iNOS in macrophages (Sugiyama et al. 2010).

It is reported that both DON and NIV have been found in wheat and barley (Ichinoe et al. 1983; Yoshizawa et al. 1979; Sydenham et al. 1991) and co-contaminated the same maize (Okoye 1993), leading to research of the risk

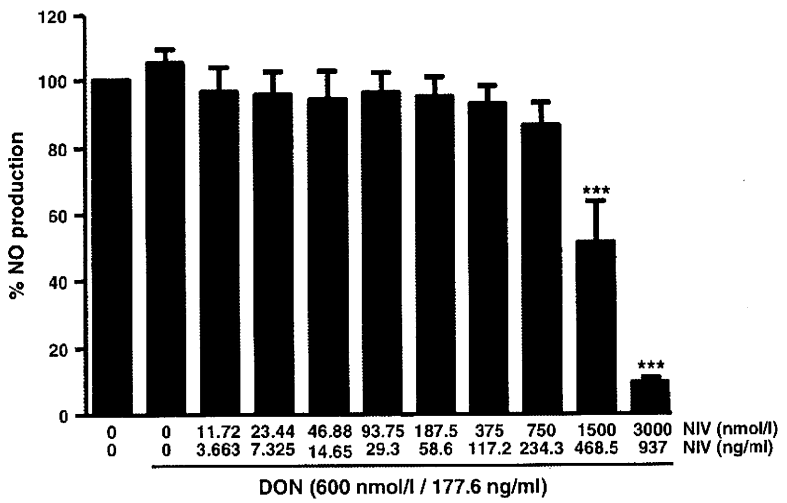

Fig. 5 Concentration-dependent effects of NIV on LPS-induced NO production by RAW264 cells in the presence of DON RAW264 cells were stimulated with NIV (0–3,000 nmol/l) in the presence of LPS (100 ng/ml) and DON (600 nmol/l) for 24 h. The culture supernatants were analyzed for NO levels. Values are the means ± SEM from four independent experiments. NO induced by LPS treatment alone is expressed as 100%. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (***$P < .001$, vs LPS treated control)

assessment of thecombination of DON and NIV. However, there is not enough information to understand the combined effect and to estimate the health risk at certain intakes of both toxins (Speijers and Speijers 2004). Dietary intake of trichothecene mycotoxins at levels below those that induce mycotoxicosis may reduce immune responses and increase susceptibility to infection (Corrier 1991). Considering inadequate toxicological data on the combination effects of DON and NIV, the findings of the present research evaluated the effect of DON and NIV on innate immune response and seem to support the evaluation of health risks from a mixture of DON and NIV. Hence, it will be of interest to investigate the possibility of estimating the combined effect of other trichothecenes using this model.

Conclusion

In the present study, it was found that DON and NIV, which have the ability to suppress NO production by activated macrophages, produced additive inhibitory effects that increased with increasing concentrations of toxin. Therefore, we conclude that the combination of these mycotoxins results in an additive effect on LPS-induced NO production by macrophages. It is well known that the immune system is a primary target of these toxins, and therefore risk assessments of the combination of DON and NIV should take the total amount of intake into account.

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