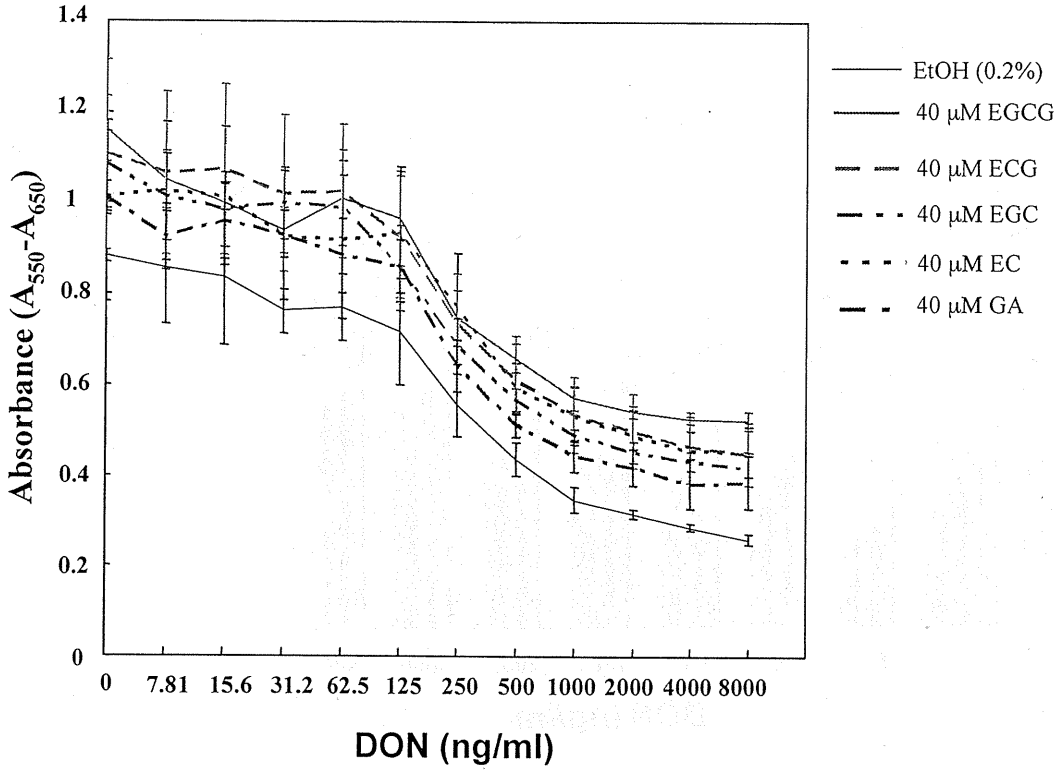

Fig. 18. Protective effect of Catechins on DON-induced acute damage to RAW264 cells. RAW264 cells were cultured in DMEM containing 40 μM Catechin in the presence of DON for 24 h. These are based on the RAW264 cells were cultured with EtOH (0.2%). The results are expressed as mean value of absorbance that subtracted absorbance of 650 nm from absorbance of 550 nm. Results are shown as means ±S.D. of three independent measurements.

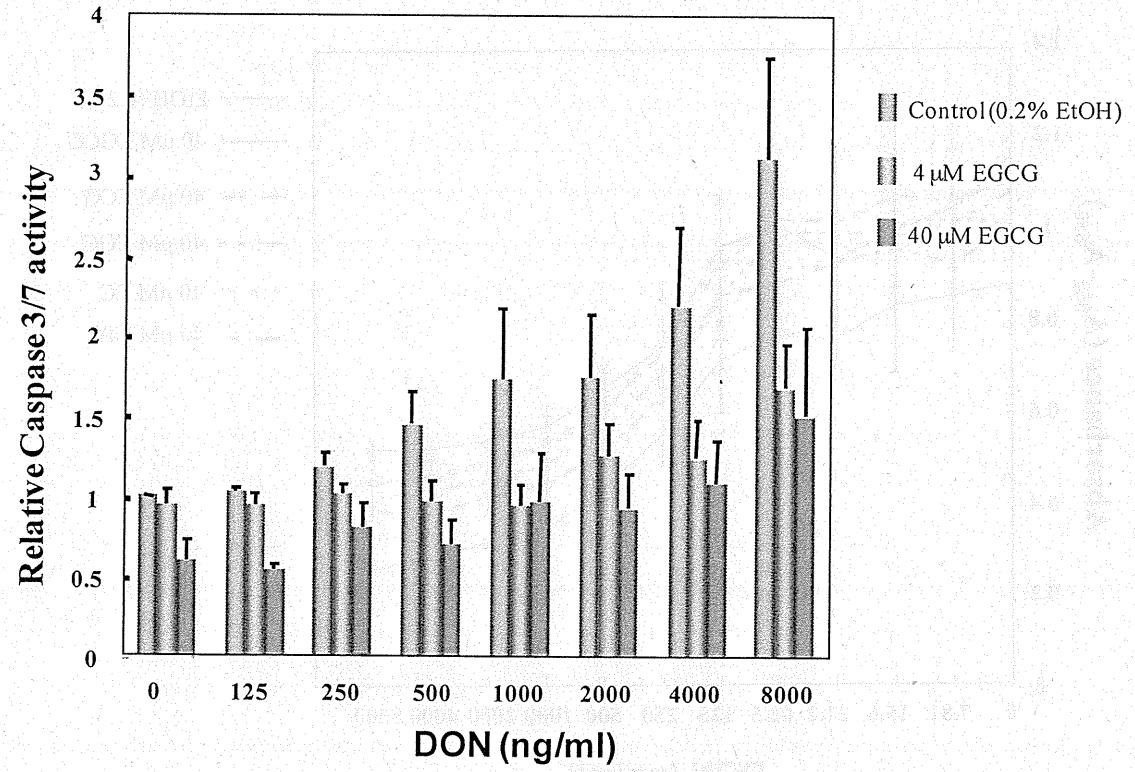

Fig.19. Effects of EGCG on DON-induced caspase 3/7 activity. RAW264 cells were treated with DON (125 – 8,000 ng/ml) in the presence of indicated concentrations of EGCG for 24 h, and caspase 3/7 activity was then measured using Caspase-Glo™ 3/7 assay. The value of control (0.2% EtOH) in the absence of DON was fixed at 1, and the rest of the values are compared relative to that. Values are means ± SEM from three independent experiments.

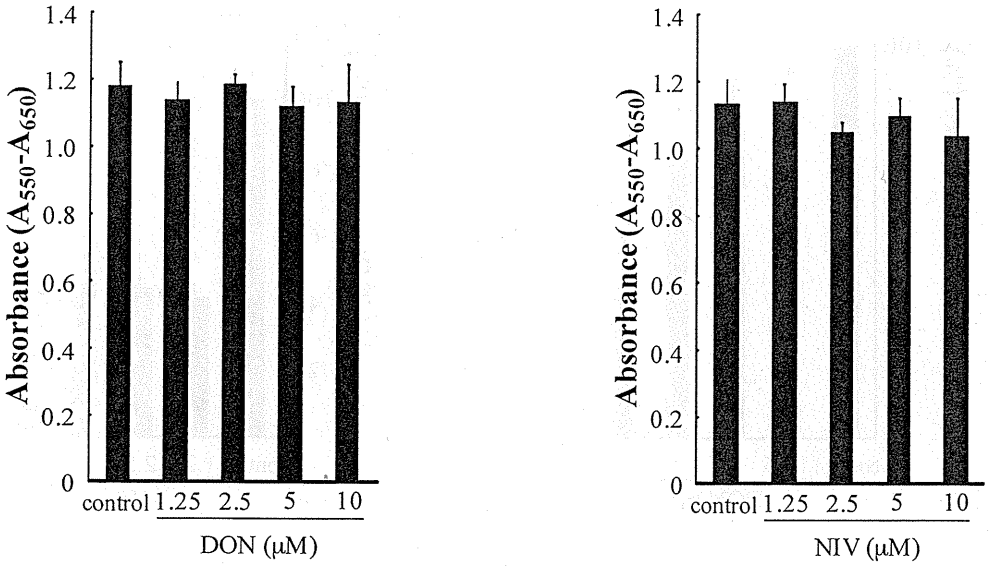

Fig. 20 Effect of DON and NIV on cell viability in HepG2. HepG2 cells were stimulated with DON and NIV for 24 h. A control was performed in DMEM. The results are expressed as mean value of absorbance that subtracted absorbance of 650 nm from absorbance of 550 nm. Results are shown as means ±SEM of three independent measurements.

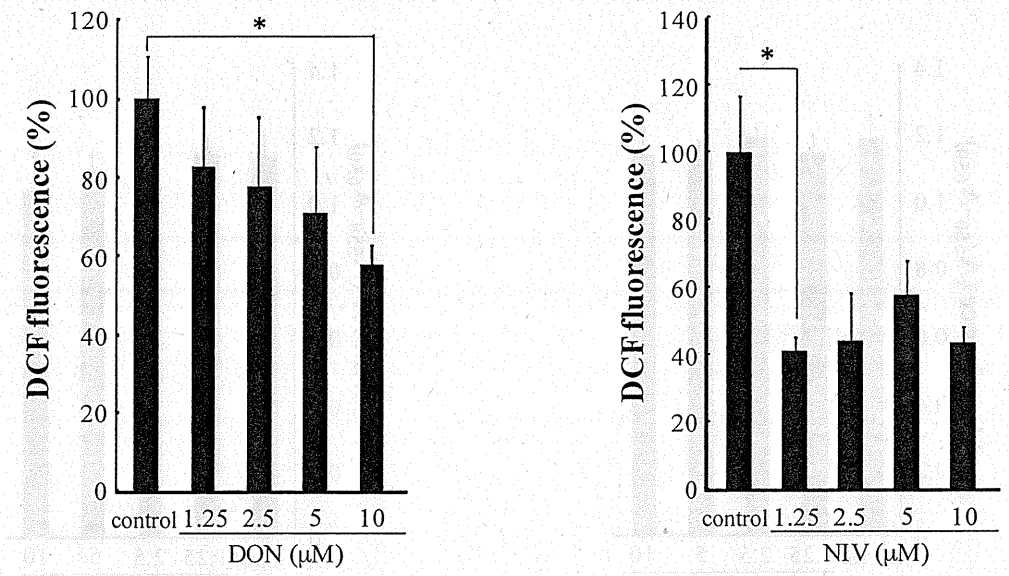

Fig. 21 Effect of DON and NIV on cellular oxidation in HepG2. HepG2 cells were stimulated with DON and NIV for 24 h and concentrations of ROS was then measured. A control was performed in DMEM. Values are means ±SEM from three independent experiments. A statistical analysis was performed using an unpaired Student's *t*-test. (*P<0.05, vs the control of each group)

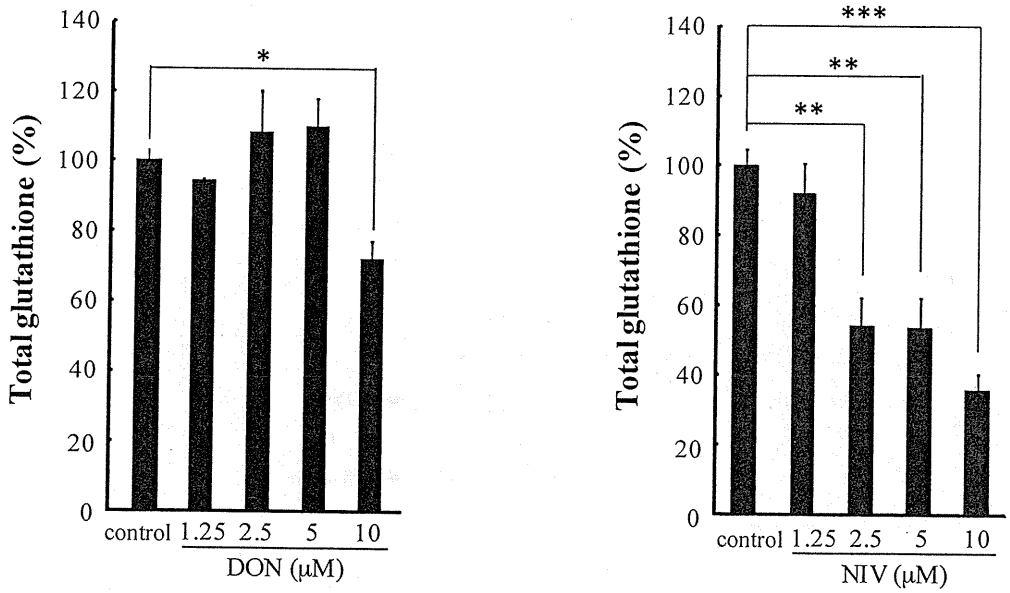

Fig. 22 Effect of DON and NIV on concentrations of total GSH in HepG2. HepG2 cells were stimulated with DON and NIV for 24 h and concentrations of total GSH was then measured. A control was performed in DMEM. Concentrations of total GSH was fixed to 100% at control. Values are means ±SEM from three independent experiments. A statistical analysis was performed using an unpaired Student's *t*-test. (**P*<0.05, ***P*<0.01, ****P*<0.001 vs the control of each group)

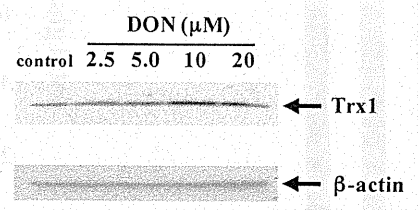

Fig. 23 Effect of DON on Trx1 expression in HepG2. HepG2 cells were stimulated with DON for 16 h. A control was performed in DMEM. Cell lysates were analyzed for Trx1 and β-actin proteins by western blotting. Shown is representative of three repeat experiments.

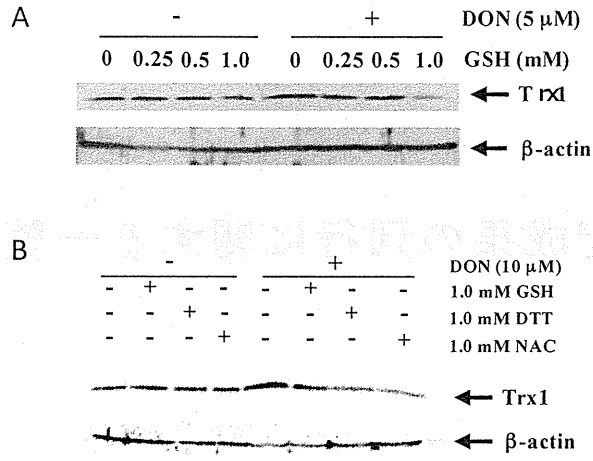

Fig. 24 Effect of DON with antioxidants on Trx1 expression in HepG2. A) HepG2 cells were stimulated with DON and GSH for 16 h. Cell lysates were analyzed for Trx1 and β-actin proteins by western blotting. Shown is representative of five repeat experiments. B) HepG2 cells were stimulated with DON and each antioxidant for 16 h. Cell lysates were analyzed for Trx1 and β-actin proteins by western blotting. Shown is representative of seven repeat 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
Sugiyama, K., Kinoshita, M., Kamata, Y., Minai, Y. and Sugita-Konishi, Y	(-)-Epigallocatechin gallate suppresses the cytotoxicity induced by trichothecene mycotoxins in mouse cultural macrophages	*Mycotoxin Res.*	**27**	281-285	2011
Sugiyama, K., Kinoshita, M., Kamata, Y., Minai, Y., Tani, F. and Sugita-Konishi, Y	Thioredoxin-1 contributes to protection against DON-induced oxidative damage in HepG2 cells	*Mycotoxin Res.*			in press
Sugiyama, K., Kinoshita, M., Minai, Y., Muroi, M., Tanamoto, K. and Sugita-Konishi, Y	Trichothecene mycotoxins inhibit MyD88-independent pathways of Toll-like receptors	*Cytokine*	**56**	39	2011

研究成果の刊行物

Mycotox Res (2011) 27:57–62
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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

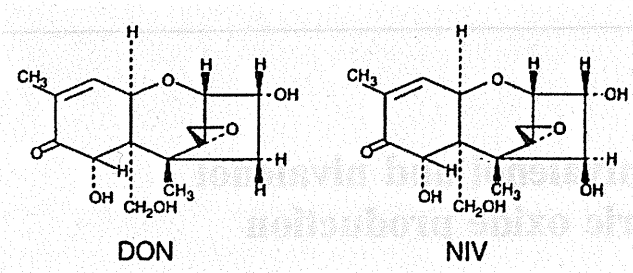

Fig. 1 Chemical structures of DON and NIV

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

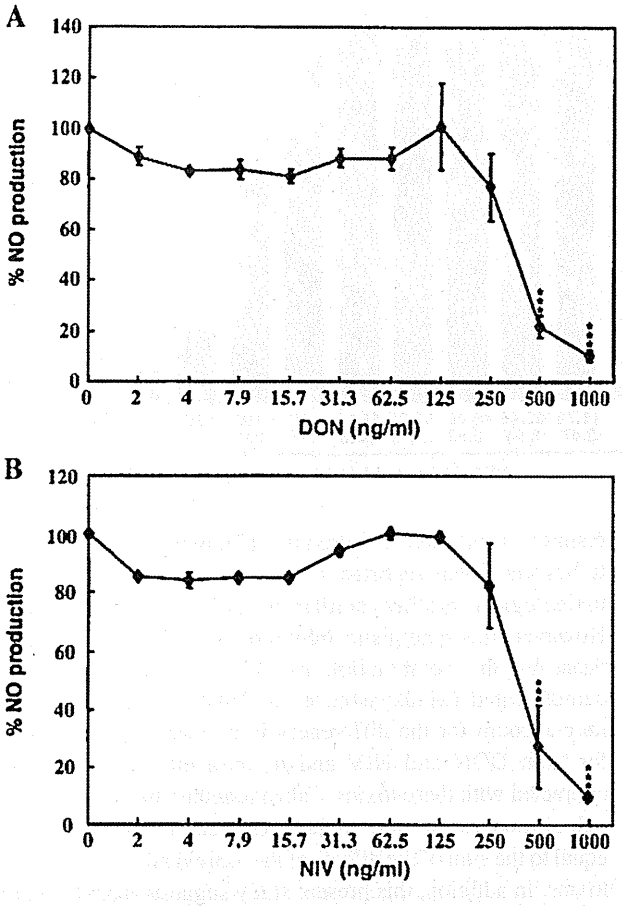

Fig. 2 Concentration-dependent effects of DON and NIV on LPS-induced NO production by RAW264 Cells RAW264 cells were stimulated with LPS (100 ng/ml) in the presence of the indicated concentrations of *DON* (a) or *NIV* (b) for 24 h. The culture supernatants were analyzed for NO levels. The NO induced 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

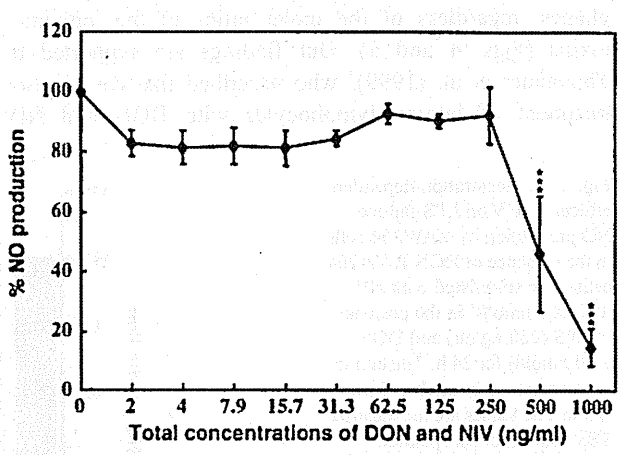

Fig. 3 Combined effects of DON and NIV on LPS-induced NO production by RAW264 Cells RAW264 cells were stimulated with LPS (100 ng/ml) in the presence of the indicated total concentrations of DON and NIV (1:1) for 24 h. The culture supernatants were analyzed for NO levels. NO induced by LPS treatment 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)

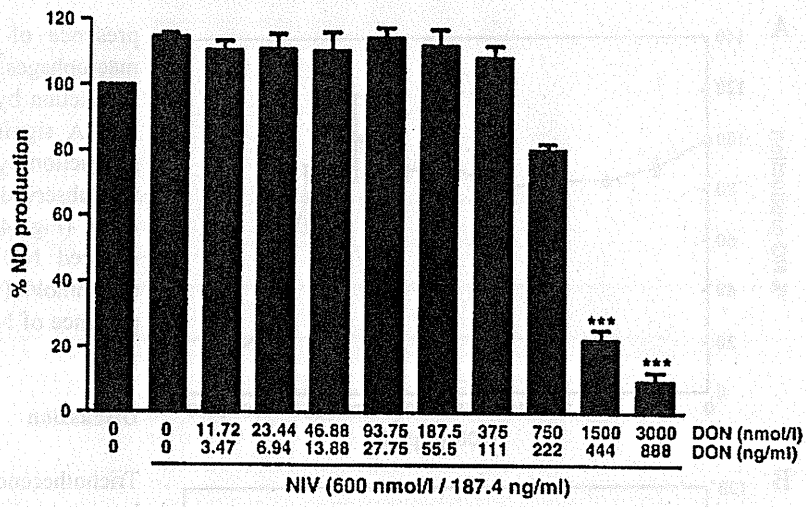

Fig. 4 Concentration-dependent effects of DON on LPS-induced NO production by RAW264 Cells in the Presence of NIV RAW264 cells were stimulated with DON (0–3,000 nmol/l) in the presence of LPS (100 ng/ml) and NIV (600 nmol/l) for 24 h. The culture supernatants were analyzed for NO levels. Values are the 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

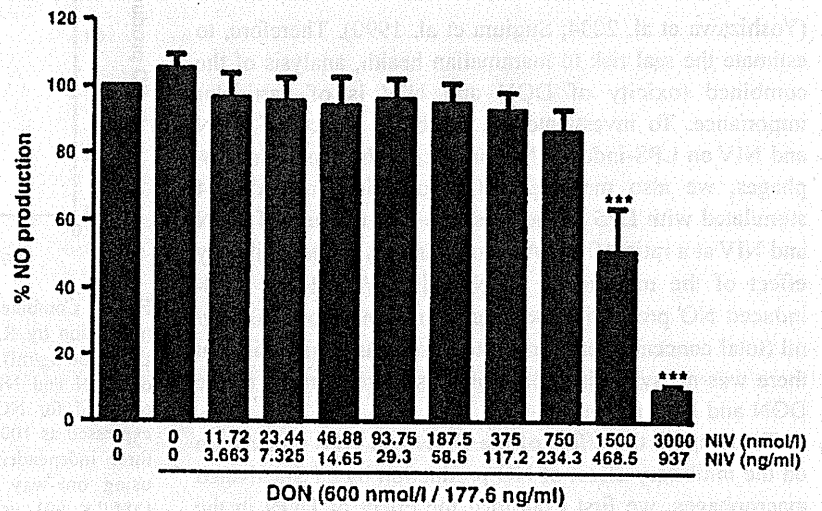

Fig. 5 Concentration-dependent effects of NIV on LPS-induced NO production by RAW264 cells in the presence of DON RAW264 cells were stimulated with NIV (0–3,000 nmol/l) in the presence of LPS (100 ng/ml) and DON (600 nmol/l) for 24 h. The culture supernatants were analyzed for NO levels. Values are the 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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ORIGINAL PAPER

(−)-Epigallocatechin gallate suppresses the cytotoxicity induced by trichothecene mycotoxins in mouse cultural macrophages

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Abstract Trichothecene mycotoxins are toxic secondary metabolites produced by a number of fungi including *Fusarium* species, which adversely affect lymphocytes. Deoxynivalenol (DON) and HT-2 toxin (HT-2) belong to the trichothecene group of mycotoxins and the occurrence of cereals and foodstuffs with these compounds are serious health problems. The aim of this study was to examine the effect of (−)-epigallocatechin gallate (EGCG), one of the main components in green tea catechins, on DON- or HT-2-induced cytotoxicity in mouse macrophages. EGCG had protective effects against the trichothecene-induced cytotoxicities of both mycotoxins. Additionally, EGCG suppressed the DON-induced activation of caspase-3/7, which is an indicator of apoptosis. These results indicate that EGCG might be useful in protection against DON- or HT-2-induced cell death, suggesting that EGCG could contribute to reducing the toxicities of trichothecenes.

Keywords Deoxynivalenol · HT-2 toxin · (−)-epigallocatechin gallate · Cytotoxicity · Macrophage

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Introduction

Trichothecene mycotoxins are secondary metabolites produced by several fungi, including *Fusarium*, *Mycothecium*, *Trichoderma*, *Trichothecium*, *Stachybotrys*, *Verticimonosporium*, and *Cephalosporium* species (Rocha et al. 2005). More than 150 trichothecenes and their derivatives have been identified (Yazar and Omurtag 2008). Trichothecene mycotoxins are characterized by a double bond between C-9 and C-10 and an epoxide at C-12 and C-13 which is considered to be responsible for their high toxicity (Desjardins et al. 1993). Trichothecene mycotoxins are relatively stable when subjected to the usual cooking processes (Rocha et al. 2005), indicating that the occurrence of trichothecene mycotoxins in animal feed and human food is a potentially serious problem related to food safety.

Trichothecene mycotoxins can be classified into four different types (types A–D) based on their chemical properties and the type of fungus that produces them (Rocha et al. 2005; Yazar and Omurtag 2008). Types A and B are the major trichothecenes which are detected in food and animal feed (Krska and Josephs 2001). Deoxynivalenol (DON) belongs to the type B class, which has a carbonyl functionality at the C8 position (Yazar and Omurtag 2008), and has frequently been found in foods worldwide (Fig. 1) (Pestka and Smolinski 2005). DON is frequently detected in cereals including wheat, barley, corn and rice (Yazar and Omurtag 2008). Therefore, contamination of foodstuff with DON has been considered to be a serious public health threat (Ueno 1985; Marzocco et al. 2009). HT-2 toxin (HT-2), which is a type A trichothecene, is more acutely toxic than type B

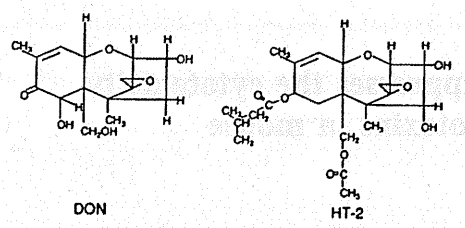


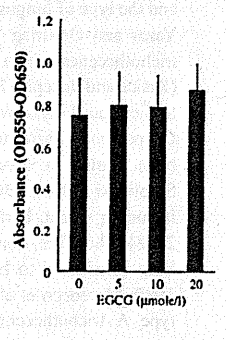
Fig. 1 The molecular structures of DON and HT-2

trichothecenes (Fig. 1) (Yazar and Omurtag 2008). The T-2 toxin (T-2) is also included in the type A trichothecenes, and it has been reported that T-2 is predominantly converted into HT-2 by hydrolysis of the acetate in C4 during metabolism. It could therefore be considered that the toxicity of HT-2 in vivo is analogous to that of T-2. Given their prevalence in the environment, a temporary tolerable daily intake for HT-2 and T-2 (alone or in combination) was established by the European Commission Scientific Committee on Food (Schlatter 2004).

The trichothecene mycotoxins induce the inhibition of protein synthesis, alimentary hemorrhage, vomiting and immunotoxicity (Ueno et al. 1973; Parent-Massin and Thouvenot 1995; Sugiyama et al. 2010). B and T cells, NK cells and macrophages are all sensitive to DON (Instanes and Hetland 2004; Pestka 2008), and it is known that leukocyte apoptosis (programmed cell death) is promoted by trichothecene mycotoxins, including DON and HT-2 (Holme et al. 2003; Pestka 2008).

The tea catechin (−)-epigallocatechin gallate (EGCG), which is a major polyphenol in green tea, was previously shown to supress rubratoxin B-induced apoptosis in hepatocytes (Kagaya et al. 2002). Rubratoxin B is a mycotoxin produced by some *Penicillium* fungi (Natori et al. 1970), which increases the frequency of apoptotic HepG2 hepatoma cells (Nagashima et al. 2005). In addition, increased cytokine production by human hepatoma cells treated with rubratoxin B has been observed (Nagashima et al. 2003). It is known that DON also induces the release of inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α in macrophages (Sugita-Konishi and Pestka 2001). Based on these reports, it has been proposed that there is a similarity between the trichothecene mycotoxins and rubratoxin B. Therefore, we hypothesized that EGCG would protect against trichothecene mycotoxin-induced cell death. To examine this hypothesis, we determined the effects of EGCG on the cell toxicity provoked by DON or HT-2.

Fig. 2 The effects of EGCG on cell viability of RAW264 cells. RAW264 cells were cultured in DMEM containing EGCG (0–20 μmole/l) for 24 h. The results are expressed as the mean value of absorbance which measured at 550 nm (reference at 650 nm). The results are the means ± SEM of three independent measurements



Materials and methods

Cell culture and reagents

The mouse macrophage cell line, RAW264, was cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sugiyama et al. 2010). DON, HT-2 and EGCG were purchased from Wako (Osaka, Japan). EGCG was dissolved in ethanol and the stock solution of EGCG was kept at −30°C and diluted in culture medium before use.

MTT assay for cell viability

RAW264 cells were plated in 96-well plates at a density of 1×10^4 cells/well for 24 h. The cells were cultured in DMEM containing trichothecene mycotoxins and EGCG for 24 h, and control cells were cultured in DMEM containing 0.2% ethanol for 24 h. Cell viability measured by the MTT assay (Roche Diagnostics, Mannheim, Germany), which was performed according to the manufacturer's protocol. Briefly, the MTT labeling reagent was added to each well, and the plate was incubated for 4 h at 37°C. Solubilization buffer was then added to each well and the plate was incubated for 24 h at 37°C. The absorbance was measured at 550 nm and the reference wavelength was measured at 650 nm.

Apoptosis detection assay

Apoptosis was determined by measuring caspase-3/7 activity in RAW264 cells. RAW264 cells were plated in 96-well plates at a density of 5×10^4 cells/well for 24 h. The cells were cultured in DMEM containing DON and EGCG for 24 h, and control cells were cultured in DMEM containing 0.2% ethanol for 24 h. The cellular caspase-3/7 activity was measured with the Caspase-Glo 3/7 assay kit (Promega, Madison, WI, USA) according to the manufacturer's

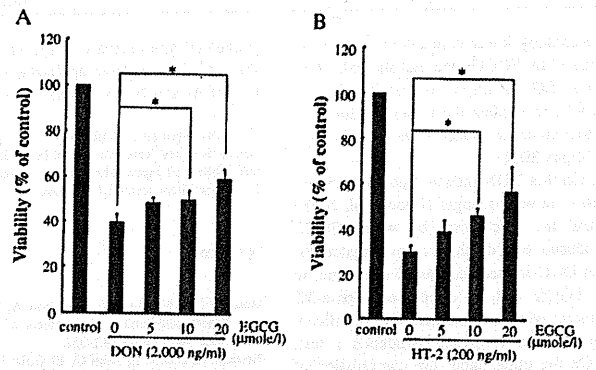


Fig. 3 The protective effects of EGCG on trichothecene mycotoxins-induced acute cytotoxicity to RAW264 cells. RAW264 cells were cultured in DMEM containing EGCG (0–20 μmole/l) in the presence of DON (a) or HT-2 (b) for 24 h. The results are expressed as the mean percentages of viable cells. These numbers were calculated considering the RAW264 cells that were cultured with EtOH (0.2%) as 100% viable. The results are the means ± SEM of three independent measurements. A statistical analysis was performed using an unpaired Student's *t* test (**P*<0.05, vs the DON or HT-2 groups)

protocol. Briefly, Caspase-Glo 3/7 reagent was added to each well and the plate was incubated for 1.5 h at 37°C. After 1.5-h incubation, the supernatant was added to 96-well white plates, and luminescence was measured using a multiplate reader (TriStar LB 941; Berthold Technologies, Bad Wildbad, Germany).

Statistical analysis

Statistical comparisons between two groups were done using an unpaired Student's *t* test. The values shown in the figures are expressed as means ± SEM.

Results and discussion

We first examined the EGCG cytotoxicity in RAW264 cells, and differences between means were assessed using an unpaired Student's *t* test and were regarded statistically significant at $P<0.05$. The result indicated that EGCG had no effect on cell viability (Fig. 2). Figure 3 shows the protective effects of EGCG against trichothecene-induced cell death. EGCG (10 and 20 μmole/l) significantly suppressed the cell death caused by the type B trichothecene, DON (2,000 ng/ml). In addition, the cell toxicity induced by 200 ng/ml of the type A trichothecene, HT-2, was also inhibited by EGCG. Likewise, it is confirmed that

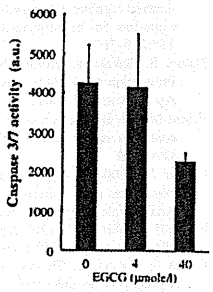


Fig. 4 The effects of EGCG on caspase 3/7 activity. RAW264 cells were treated with the indicated concentrations of EGCG for 24 h, and caspase 3/7 activity (in arbitrary units) was then measured using Caspase-Glo™ 3/7 assay. The values are the means ± SEM from three independent experiments

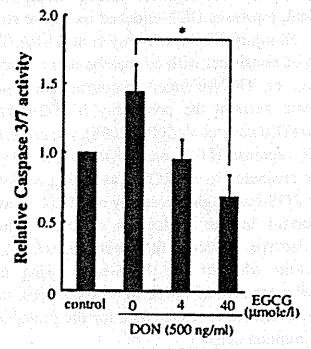


Fig. 5 The effects of EGCG on DON-induced caspase 3/7 activity. The RAW264 cells were treated with DON (500 ng/ml) in the presence of the indicated concentrations of EGCG for 24 h, and caspase-3/7 activity was then measured using the Caspase-Glo™ 3/7 assay. The value of the control (0.2% EtOH) in the absence of DON was fixed at 1, and the values are compared relative to this control. The values are the means ± SEM from three independent experiments. A statistical analysis was performed using an unpaired Student's *t* test (**P*<0.05, vs the DON group)

the cytotoxicities caused by lower concentrations of DON or HT-2 were attenuated by EGCG (data not shown). These results suggest that EGCG might be useful for the protection of macrophages against the toxicity induced by the A- and B-type trichothecenes which have been found in food (Krska and Josephs 2001).

It has been reported that DON induces apoptosis via the activation of caspase-3 in macrophages (Zhou et al. 2005). To better understand the mechanism by which EGCG suppresses DON-induced cell death, we investigated the effects of EGCG on DON-induced caspase-3 activation. In the present study, EGCG had no effect on caspase-3/7 activity because there were no statistically significant differences between means (unpaired Student's *t* test, $P<0.05$) (Fig. 4). On the other hand, the upregulation of caspase-3/7 activity in macrophages stimulated by 500 ng/ml of DON was significantly decreased in the presence of EGCG (40 μmole/l) (Fig. 5). This result indicates that the DON-induced apoptosis could be inhibited by EGCG. However, it has been reported that rubratoxin B, a mycotoxin, increased caspase-3 activity more than 2-fold compared to the control in hepatocytes (Kagaya et al. 2002). Taken together, the present study implies that DON-induced cell death in RAW264 cells is partly due to apoptotic cell death.

EGCG is a well-known major green tea polyphenol which has strong anti-oxidant activities (Kagaya et al. 2002). It has been reported that DON leads to oxidative stress by producing intracellular reactive oxygen species (ROS). Indeed, the carotenoid lutein, which is a natural antioxidant, represses DON-induced oxidative stress which leads to cell injury (Krishnaswamy et al. 2010). This report seems to be consistent with our results of a protective effect of EGCG on DON-induced apoptosis and cytotoxicity. Taking into account the possibility of T-2-induced ROS generation (Bouaziz et al. 2006, 2008), the protective effect of EGCG against HT-2- and DON-induced cytotoxicity might be attributed to its ROS-scavenging activity. Therefore, the ROS-scavenging property of EGCG is assumed to be important in the protection against trichothecene-induced damage. However, further studies will be necessary to ascertain whether the ROS-scavenging ability of antioxidants are also effective in macrophages, or whether another mechanism is responsible for the protective effects in these immune cells.

Conclusion

The cytotoxicity caused by DON or HT-2 was repressed by EGCG in mouse macrophage-like RAW264 cells. In addition, caspase-3/7 activation by DON was inhibited by EGCG. These results indicate that EGCG can prevent trichothecene-induced damage in macrophages, suggesting that EGCG may have applications as a tool to reduce the risk of A- and B-type trichothecene toxicity of food.

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