ン、分離ゲル、濾紙の順番に置いた。10～15 Vで5.5 mA/cm^{2}の電流を30分間通電することでブロッティングを行った。ブロッティング終了後、メンブレンを1×Tris Buffered Saline with Tween20（以後TBSTと略記；pH7.6、25mM Tris、150 mM NaCl、0.1% Tween20）に5%量スキムミルク(Wako, Osaka, Japan)を添加した5%スキムミルクTBST溶液に浸し30分間ブロッキングした。

ブロッキングしていた溶液を除去後、5%スキムミルクTBST溶液を10 ml加えメンブレンを浸漬させ、一次抗体Anti-trx (FL-105) (Santa Cruz Biotechnology, California, USA)を10 μlおよびMonoclonal Anti-β-Actin Antibody (Sigma-Aldrich, St. Louis, MO, USA)を3.0 μl加え30分間室温でインキュベートした。その後、余分な一次抗体を除去するためTBSTを適量加え、5分間振とうさせた。この洗浄操作を5回行った。次に5%スキムミルクTBST溶液を10 ml加えメンブレンを浸漬させ、二次抗体Anti-Rabbit IgG (Jackson ImmunoRsearch, Philadelphia, USA)およびAnti-Mouse IgG (Jackson ImmunoRsearch, Philadelphia, USA)を各1.0 μl加え30分間室温でインキュベートした。その後、余分な二次抗体を除去するためTBSTを適量加え、5分間振とうさせた。この洗浄操作を5回行った。バンドの検出には、ImmunoStar LD (Wako, Osaka, Japan)を用いた。メンブレンにLuminescence Solution AおよびLuminescence Solution Bが同量含まれる混合液を滴下し、余分な溶液を除去した。二次抗体に結合しているペルオキシダーゼと基質の反応により生じた化学発光をLumiCube (Liponics, Inc., Tokyo, Japan)を用いてバンドの確認を行った。

統計学的処理

統計学的処理は、unpaired Student's *t*-testを用いて行った。

C. 研究結果

HepG2細胞内酸化度におよぼすDONとNIVの影響

ヒト肝ガン由来細胞株HepG2細胞を用いて、今回使用するDONとNIV各濃度における細胞毒性をMTTアッセイにより確認した。その結果、今回使用した濃度（1.25 - 10 μM）ではHepG2細胞は細胞毒性を示さないことが明らかとなった（Fig. 1）。同濃度域において、DCFHを用いてHepG2細胞内酸化度を測定した。DONにおいては同濃度依存的な細胞内酸化度の減少が確認された。特に10 μM存在下では顕著な減少が認められ

た。一方NIVにおいては1.25 μMで顕著な減少となることが明らかとなった（Fig. 2）。

HepG2細胞内GSH含量におよぼすDONとNIVの影響

次に、主要な細胞内還元物質の一つである細胞内GSH含量におよぼすDONとNIVの影響を検討した。DON処理されたHepG2細胞においては今回検討した濃度（1.25 - 10 μM）では少なくとも細胞内GSH含量の増加傾向は確認されなかった。10 μM存在下DON存在下では有意な減少が確認された。NIVにおいても減少傾向が認められ、細胞内GSH含量は2.5 μMから有意な減少が確認される結果となった（Fig. 3）。

HepG2細胞内Trx1発現量におよぼすDONの影響

GSHとともに細胞内に主要な還元力を提供するTrx1発現量におよぼすDONの影響について確認した。その結果、GSHとは異なり、濃度依存的な増加傾向が認められることが明らかとなった（Fig. 4）。

HepG2細胞におけるDON誘導性Trx1発現上昇におよぼす各種抗酸化剤の影響

HepG2細胞において認められたDON誘導性のTrx1発現量の上昇に対する各種還元剤の影響を検討した。その結果、同上昇は検討したGSH濃度（0.25 - 1.0 mM）において、濃度依存的に抑制されることが確認された（Fig. 5A）。さらにGSH以外の還元剤であるDTT（1.0 mM）およびNAC（1.0 mM）によっても同様にDON誘導性Trx1の発現上昇は阻害されることが明らかとなった（Fig. 5B）。

D. 考察

本分担研究では、ヒト肝ガン由来細胞HepG2を用いた細胞内レドックスにおよぼすトリコテセン系かび毒の影響について検討した。今回用いたトリコテセン系かび毒であるDONとNIVは細胞毒性を呈しない濃度（1.25 - 10 μM）でHEpG2細胞内の酸化度を減少させることが明らかとなった（Fig. 1, 2）。さらに、DONによる細胞内酸化度の減少は細胞内GSH含量に起因せず、Trx1のアップレギュレーションによる可能性が示唆された（Fig. 3, 4）。白血球細胞においてトリコテセン系かび毒は酸化的ストレスを誘発しアポトーシスを誘導することで細胞毒性を呈するとする報告がある。実際に、今回使用したDON濃度ではヒト白血球系細胞では顕著な細胞毒性が認められる。一方、ヒト肝ガン由来細胞HepG2においては、同濃度域において細胞毒性は認められず、むしろ細胞内の酸化度は減少傾向にあることが明らかとなった。本研究から、HepG2細胞においてはトリコテセン系かび毒による

細胞内酸化度の上昇を抑制する機序を有すること、また同機序が細胞内 GSH 含量ではなく Trx1 量のアップレギュレーションによる可能性が強く示唆された。HepG2 における DON 誘導性 Trx1 量の増大が各種還元剤により抑制されたことは（Fig. 5）、この推測を支持する。本研究結果は、トリコテセン系カビ毒に対する白血球系細胞の高感受性と、同毒に対して白血球系細胞と比較して低感受性となる肝臓細胞のそれぞれのメカニズムを合理的に説明する知見を提供したと言える。

E. 結論

ヒト肝ガン由来細胞 HepG2 を用いて、トリコテセン系かび毒が同細胞内レドックス状態におよぼす影響を細胞内の主要な還元力を提供する GSH と Trx1 含量を指標に検討した。その結果、細胞毒性を呈しないレベルのトリコテセン系かび毒存在下において HepG2 細胞は、Trx1 をアップレギュレーションすることにより細胞内酸化度を減少させている（上昇を抑制している）ことが示唆された。

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F. 健康危険情報

なし

G. 研究業績

【原著論文】

1. 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).

【学会発表】

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2. 杉山圭一、木下麻緒、薬袋裕二、鎌田洋一、谷　史人、小西良子：ヒト肝臓癌由来細胞株 HepG2 の細胞内レドックスにおよぼすデオキシニバレノールの影響、日本マイコトキシン学会第 70 回学術講演会講演要旨集　31 (2012, 1).

H. 知的所有権の取得状況

1．特許所得

なし

2．実用新案登録

なし

3．その他

なし

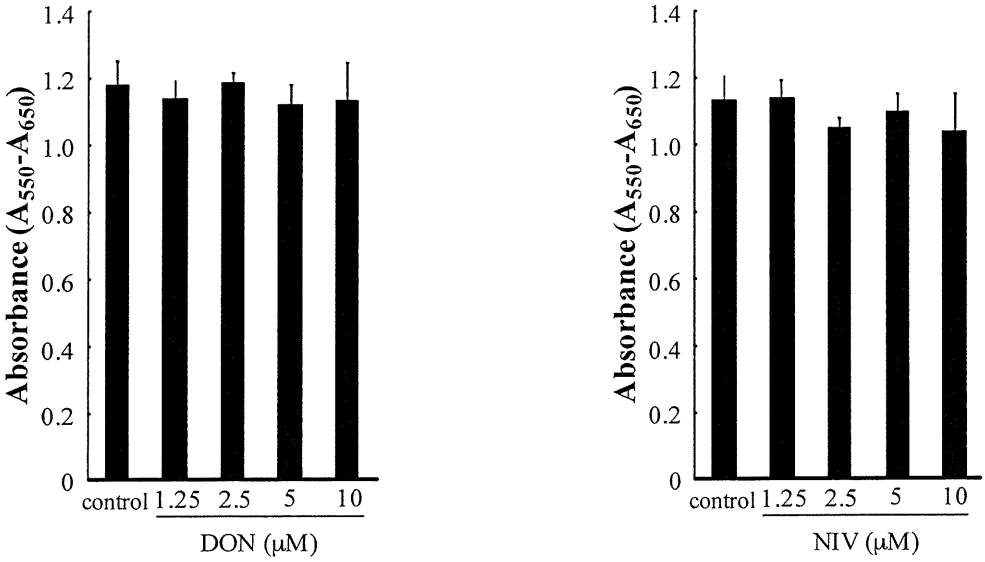

Fig. 1 Effect of DON and NIV on cell viability in HepG2. HepG2 cells were stimulated with DON and NIV for 24 h. A control was performed in DMEM. The results are expressed as mean value of absorbance that subtracted absorbance of 650 nm from absorbance of 550 nm. Results are shown as means ±SEM of three independent measurements.

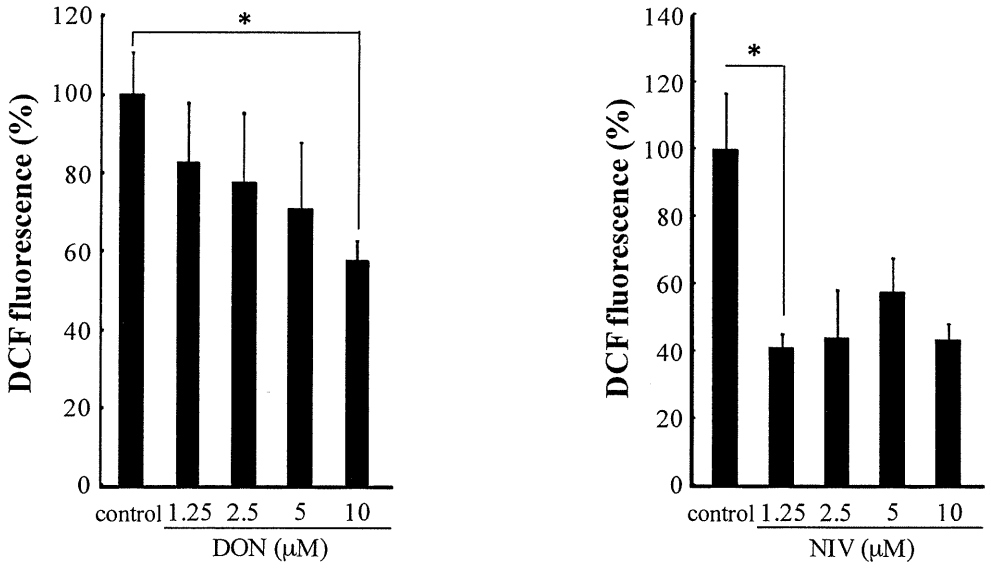

Fig. 2 Effect of DON and NIV on cellular oxidation in HepG2. HepG2 cells were stimulated with DON and NIV for 24 h and concentrations of ROS was then measured. A control was performed in DMEM. Values are means ±SEM from three independent experiments. A statistical analysis was performed using an unpaired Student's *t*-test. (*P<0.05, vs the control of each group)

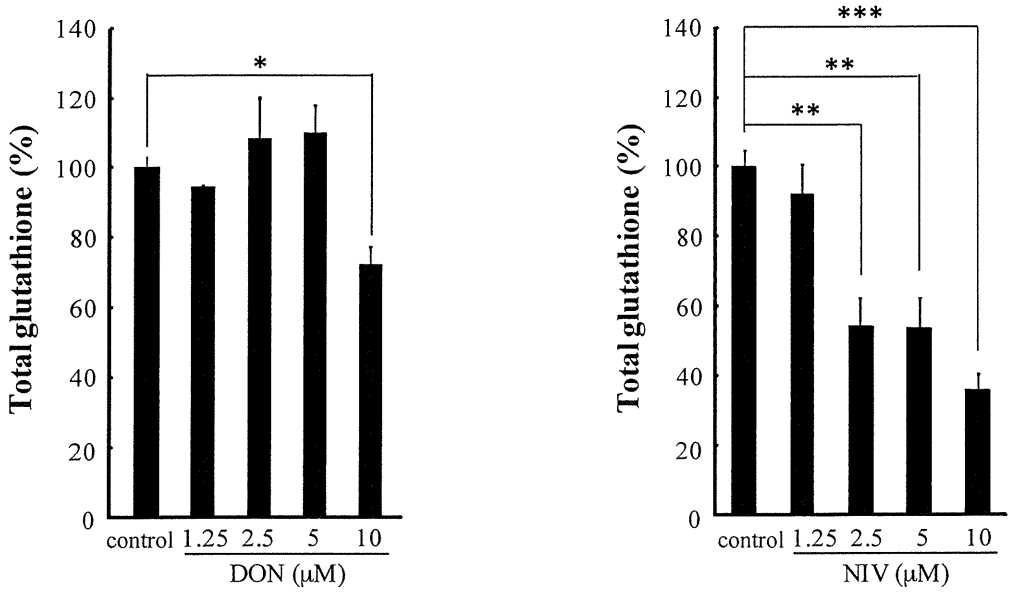

Fig. 3 Effect of DON and NIV on concentrations of total GSH in HepG2. HepG2 cells were stimulated with DON and NIV for 24 h and concentrations of total GSH was then measured. A control was performed in DMEM. Concentrations of total GSH was fixed to 100% at control. Values are means ±SEM from three independent 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)

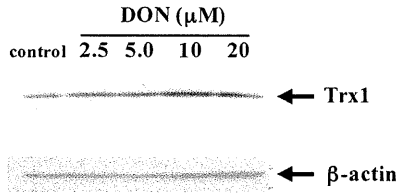

Fig. 4 Effect of DON on Trx1 expression in HepG2. HepG2 cells were stimulated with DON for 16 h. A control was performed in DMEM. Cell lysates were analyzed for Trx1 and β-actin proteins by western blotting. Shown is representative of three repeat experiments.

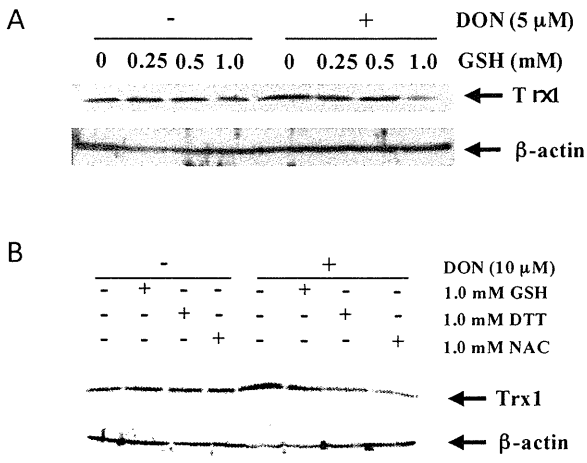

Fig. 5 Effect of DON with antioxidants on Trx1 expression in HepG2. A) HepG2 cells were stimulated with DON and GSH for 16 h. Cell lysates were analyzed for Trx1 and β-actin proteins by western blotting. Shown is representative of five repeat experiments. B) HepG2 cells were stimulated with DON and each 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., 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) 27:281–285
DOI 10.1007/s12550-011-0105-8

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

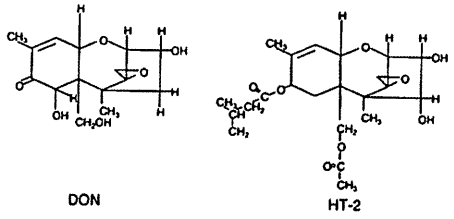


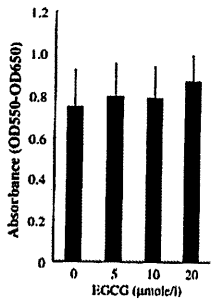
Fig. 1 The molecular structures of DON and HT-2

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

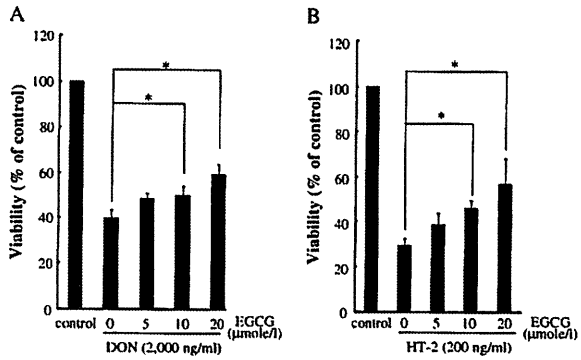


Fig. 3 The protective effects of EGCG on trichothecene mycotoxins-induced acute cytotoxicity to RAW264 cells. RAW264 cells were cultured in DMEM containing EGCG (0–20 μmole/l) in the presence of DON (a) or HT-2 (b) for 24 h. The results are expressed as the mean percentages of viable cells. These numbers 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

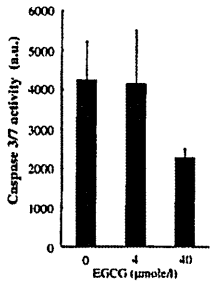


Fig. 4 The effects of EGCG on caspase 3/7 activity. RAW264 cells were treated with the indicated concentrations of EGCG for 24 h, and caspase 3/7 activity (in arbitrary units) was then measured using Caspase-Glo™ 3/7 assay. The values are the means ± SEM from three independent experiments

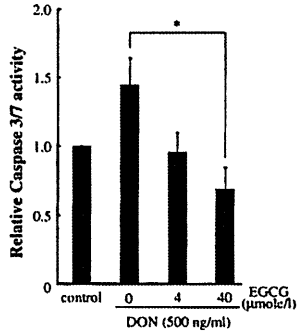


Fig. 5 The effects of EGCG on DON-induced caspase 3/7 activity. The RAW264 cells were treated with DON (500 ng/ml) in the presence of the indicated concentrations of EGCG for 24 h, and caspase-3/7 activity was then measured using the Caspase-Glo™ 3/7 assay. The value of the control (0.2% EtOH) in the 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 caaspase-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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ORIGINAL PAPER

Thioredoxin-1 contributes to protection against DON-induced oxidative damage in HepG2 cells

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Abstract Leucocytes are susceptible to the toxic effects of deoxynivalenol (DON), which is a trichothecene mycotoxin produced by a number of fungi including *Fusarium* species. One mechanism of action is mediated by reactive oxygen species (ROS). The liver is an important target for toxicity caused by foreign compounds including mycotoxins. On the other hand, little is known about the influence of the redox state on hepatocytes treated with DON. The present study investigated the effect of DON on the cytosolic redox state and antioxidative system in the human hepatoma cell line HepG2. The cell viability of human monocyte cell line THP-1 or leukemia cell line KU812 treated with 2.5 and 5 μmol/l DON were significantly reduced. However, HepG2 cells showed no toxic effects under the same conditions and did not exhibit an increased oxidative state. Further experiments showed that thioredoxin-1 (Trx-1) protein levels but not glutathione increased in the cells treated with 10 μmol/l DON. In addition, the enhancement of Trx-1 content was repressed by antioxidants. These results suggest that DON-induced accumulation of Trx-1 in HepG2 cells plays one of the key roles in protection against cytotoxicity caused by DON and that the mechanism may be mediated by the antioxidant properties of Trx-1.

Keywords Deoxynivalenol · Thioredoxin-1 · Cytotoxicity · Redox · HepG2 cells

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Introduction

Deoxynivalenol (DON) belongs to the trichothecene group of mycotoxins and is a secondary metabolite produced by several fungi, including *Fusarium*, *Mycothecium*, *Trichoderma*, *Trichothecium*, *Stachybotrys*, *Verticimonosporium*, and *Cephalosporium* species (Rocha et al. 2005). Although milling, boiling and alkaline cooking are effective in reducing DON (Kushiro 2008; Nowicki et al. 1988; Abbas et al. 1998), DON is not degraded by common cooking processes (Marzocco et al. 2009). Consumption of trichothecene mycotoxins including DON can cause vomiting and alimentary hemorrhage, and result in impairment of the immune response (Bennett and Klich 2003; Sugita-Konishi and Pestka 2001). In addition, it is known that trichothecenes inhibit translation by binding to the ribosome and disturb cytokine production. Taken together, leucocytes, which are the main cells in the immune system, are very sensitive to trichothecenes (Pestka 2008). Increased intracellular reactive oxygen species (ROS) level induced by trichothecene mycotoxins might be a key mechanism underlying the cytotoxic effect on some leucocytes (Sugiyama et al. 2011; Krishnaswamy et al. 2010; Braicu et al. 2009).

Many types of cells have developed antioxidant defense systems to reduce the harmful effects of ROS exposure. Glutathione (GSH), which is a tripeptide composed of

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glutamine–cysteine–glycine is considered to be a major component of the cellular antioxidant system (Sugiyama et al. 2000; Fath et al. 2011). ROS can be scavenged by GSH and GSH-related enzymes including GSH peroxidase and GSH *S*-transferase (GST) (Vincenzini et al. 1993), indicating that GSH plays an important role in reducing the effects of oxidative stress. Indeed, a GSH-deficient yeast strain is sensitive to oxidative stress caused by hydrogen peroxide in comparison to the wild-type strain (Grant et al. 1996). In addition to GSH, thioredoxin-1 (Trx-1), a ubiquitous 12-kDa cytosolic protein, is a key ROS-scavenging molecule (Tian et al. 2008; Ohashi et al. 2006). Trx-1 possesses scavenging activity for ROS including singlet oxygen, hydroxyl radicals, and hydrogen peroxide, and maintains cell redox homeostatic (Fath et al. 2011; Ohashi et al. 2006). Therefore, the intracellular content of GSH and Trx-1 lead to increased tolerance to ROS-induced cellular toxicity.

The objective of the present study was to assess the intracellular redox states and the content of endogenous antioxidants (GSH and Trx-1) in HepG2 cells treated with non-cytotoxic concentrations of DON. HepG2 cells are thought to be a model system for examining adaptive responses to xenobiotics. This study will provide promising evidence of the mechanisms needed for tolerance to DON toxicity in hepatocytes in comparison with leucocyte cell line of human monocytic leukemia THP-1 and human basophilic leukemia KU812. In addition, the findings of the present study might seem to provide a strategy for reducing trichothecene-induced toxicity.

Materials and methods

Cell culture and reagent

The human hepatoma cell line HepG2 and the human monocyte-like cell line THP-1 (both obtained from the Human Science Research Resources Bank, Tokyo, Japan) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Rockville, MD, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Filtron, Brooklyn, Australia), penicillin (100 U/ml), and streptomycin (100 μg/ml). A human leukemia cell line KU812 (obtained from DS Pharma Biomedical, Osaka, Japan) was grown in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Filtron). DON, GSH, dithiothreitol (DTT) and N-acetylcysteine (NAC) were purchased from Wako (Osaka, Japan). Dimethyl sulfoxide (DMSO) was purchased from Dojin (Kumamoto, Japan). 2'7'-dichlorofluorescin diacetate (DCFH-DA) was purchased from Lamba (Rainbach, Austria) and dissolved in DMSO to obtain a 1 mM stock solution. A solution of 0.25% Trypsin-EDTA was purchased from Gibco Invitrogen (Carlsbad, CA, USA). Anti-rabbit Trx antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-mouse β-actin antibody (AC-15) was purchased from Sigma-Aldrich. Horseradish peroxidase conjugated anti-rabbit and mouse secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

MTT assay for cell viability

Cell suspensions containing 1×10^4 cells of HepG2, 2.5×10^4 cells of THP-1 or 5×10^4 cells of KU812 were plated in 96-well plates and stimulated for 24 h on the following day. Cell viability was measured by the MTT assay (Roche Diagnostics, Mannheim, Germany) 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 overnight at 37°C. The absorbance was measured at 550 nm and the reference wavelength was measured at 650 nm.

ROS detection assay

HepG2 cells were plated (5×10^5 cells/well) in 6-well plates, and on the following day were exposed to DON for 24 h. Intracellular ROS formation was measured by using an oxidation sensitive fluorescent probe, DCFH-DA. Ten μl of 1 mmol/l DCFH-DA in DMSO was added to the final concentration of 10 μmol/l and the plate was incubated for 30 min at 37°C. The cells were washed with phosphate buffered saline (PBS), harvested in 500 μl Trypsin-EDTA and 500 μl DMEM and resuspended in 200 μl PBS. The fluorescence of 100 μl of cell suspension in PBS was measured using a microplate reader, Tristar LB 941 (Berthold Tech., Germany) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Measurement of intracellular GSH levels

HepG2 cells (5×10^5 cells/well) plated in 6-well plates were treated with DON for 24 h. Intracellular GSH levels were measured using the total glutathione quantification kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, harvested cells in 20 μl of 5% 5-sulfosalicylic acid was place on ice for 10 min and centrifuged at 8,000*g* for 10 min at 4°C. The resulting supernatant was used for the assay. The intracellular GSH levels in the sample were determined using a microplate reader, Tristar LB 941 at 405 nm.

Western blot analysis

HepG2 cells (5×10^5 cells/well) were plated in 6-well plates and treated with DON in the absence or presence of

antioxidants (NAC, DTT, and GSH) for 16 h. Cellular extracts were prepared as described (Sugiyama et al. 2010). The same amount of protein, determined by the Bradford method, was loaded onto each lane of a discontinuous SDS-12% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29:1) and then separated by electrophoresis by the method of Laemmli (1970). Proteins transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA) were subjected to western blotting with either rabbit anti-Trx (1:1,000) or mouse anti-β-actin (1:2,000,000) as the primary antibodies and subsequently detected with peroxidase conjugated species-specific IgG (1:10,000). The signals were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis

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

Results and discussion

HepG2 cells are more resistant to DON than THP-1 and KU812 cells

There are several reports that leucocytes are more sensitive to DON in comparison to the hepatoma HepG2 cells (Nielsen et al. 2009a, b; Schoettler et al. 2006).The cytotoxicity was analyzed by an MTT assay (Sugiyama et al. 2011) to confirm the cytotoxicity of DON on these cells. DON (1.25 to 10 μmol/l) showed no toxicity in HepG2 cells. In contrast, DON reduced the cell viability of leucocytes (THP-1 and KU812) in a concentration-dependent manner, and significant reductions in cell viability were observed at concentrations as low as 2.5 μmol/l (Fig. 1). These results suggest that HepG2 cells had a significantly higher level of resistance to DON-induced cytotoxicity than THP-1 and KU812 cells.

DON does not induce a more oxidative environment within the cytosol of HepG2 cells

It is considered that DON induces ROS-mediated apoptosis in immune component cells (Sugiyama et al. 2011; Krishnaswamy et al. 2010; Braicu et al. 2009; Pestka et al. 2005).Therefore, the effect of DON on the cellular redox status was examined in HepG2 cells, which were treated with DON (1.25-10 μmol/l), and the cellular oxidation level was subsequently evaluated using an oxidation-sensitive fluorescent probe DCFH-DA. The intensity of DCF fluorescence had a tendency to decrease in a dose dependent manner (Fig. 2). In contrast, 60 μmol/l DON induces intracellular ROS production in HepG2 cells (Zhang et al. 2009). In addition, the oxidative stress induced by trichothecene mycotoxins including DON has been explained by the ability of these toxins to provoke generation of ROS (Krishnaswamy et al. 2010; Braicu et al. 2009; Zhang et al. 2009; Bouaziz et al. 2006, 2008). Together, these findings suggest that non-cytotoxic concentrations of DON might reduce the intracellular redox state in HepG2 cells and not provoke the generation of ROS in HepG2 cells. In fact, it seemed that reducing intracellular redox states were enhanced by DON concentrations which exert no cytotoxic effect on HepG2 cells (Fig. 1).

HepG2 cells treated with non-cytotoxic levels of DON increase Trx-1 but not GSH levels

GSH and Trx-1 play an important role in the retention of a reducing the intracellular redox status (Go et al. 2011; Dröge 2002). GSH is the tripeptide including a cysteine residue

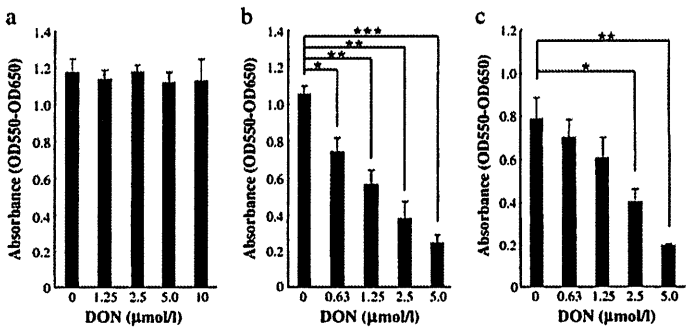

Fig. 1 Effects of DON on cell viability in HepG2, THP-1 and KU812. HepG2 (a), THP-1 (b) or KU812 (c) cells were treated with the indicated concentrations of DON for 24 h. The results are expressed as the mean value of absorbance that subtracted absorbance of 650 nm from absorbance of 550 nm. Results are expressed as the means ± SEM of three independent measurements. A statistical analysis was performed using unpaired Student's *t* test (*$P<.05$; **$P<.01$; ***$P<.001$, vs. control)