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橘黴素 Citrinin 所誘發之訊號傳遞與細胞毒性的相關性
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行政院國家科學委員會補助專題研究計畫 成果報告

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Abstract

Mycotoxin citrinin (CTN) is commonly found in foods and feeds that are contaminated/inoculated with *Penicillium*, *Aspergillus* and *Monascus* species. The exposure of human embryonic kidney (HEK293) and HeLa cells to CTN resulted in a dose-dependent increase in the phosphorylation of two major mitogen-activated protein kinases (MAPKs), ERK1/2 and JNK. In HEK293 cultures, the administering of CTN increased the levels of *egr-1*, *c-fos*, *fosB*, *c-jun* and *junB* mRNA; additionally, the ERK1/2 pathway contributed to the upregulation of Egr-1, c-Fos and c-Jun protein expression, but JNK was only involved in the expression of c-Jun protein. CTN treatment also induced the activity and binding capacity of Egr-1 and AP-1 proteins, as evidenced by luciferase reporter assays. Two genes *Gadd45β* and *MMP3* with Egr-1 and AP-1 binding sites in their promoters, respectively, were transcriptionally upregulated following the treatment of HEK293 and HeLa cells with CTN. Finally, the presence of ERK1/2 inhibitor, U0126, and the JNK inhibitor, SP600125 significantly reduced the caspase 3 activity, mediated by CTN. However, neither ERK nor JNK pathway played a role in the CTN-induced plasma membrane damage. Our results demonstrate that CTN activates ERK1/2 and JNK as well as their downstream effectors in human cells; activated signaling pathways are also involved in CTN-induced apoptosis.

Key words: mycotoxin, citrinin, ERK1/2, JNK, immediate-early gene, *MMP3*, *Gadd45β*

1. Introduction

Mycotoxin citrinin (CTN) is a fungal secondary metabolite that was originally isolated from *Penicillium citrinum*. CTN is regarded as a contaminant in foods and feeds (Bennett and Klich, 2003). However, a variety of fungi that are adopted in the food industry, such as cheese, sake, red pigment and dietary supplement, have been found to generate this natural occurring toxin (Manabe, 2001).

CTN acts as a nephortoxin or hepatotoxin in various experimental species, including rabbits, poultry, dogs and rats (Bennett and Klich, 2003; Kogika et al., 1993; Kumar et al., 2007). CTN also has teratogenic effects in rats and causes early developmental injury in mice (Chan, 2007; Singh et al., 2007). The oral administering of CTN to male F344 rats resultes in the formation of renal adenoma in 70 % of the

fed rats (Arai and Hibino, 1983). CTN also has been associated with mycotoxic nephropathy in porcine and Balkan endemic nephropathy in humans (Hald, 1991; Chernozemsky, 1991). From a cellular perspective, a possible toxic mode of CTN is to interfere with the electron transport systems of mitochondria; CTN is known to alter the permeability of a mitochondrial membrane and the calcium ion efflux in isolated kidney cortex and liver mitochondria (Chagas et al., 1995; Da Lozzo et al., 1998). Additionally, treatment with CTN induces apoptosis and micronuclei formation, an indicator of DNA damage, in specific cells (Chan, 2007; Donmez-Altuntas et al., 2007; Yu et al., 2006).

The members of mitogen-activated protein kinases (MAPKs) have been associated with a broad spectrum of cellular behaviors in response to extracellular signals (Chang and Karin, 2001). The extracellular signal-related kinases (ERK) cascade is typically a response to mitogenic stimuli, such as epidermal growth factors. Sequential activation of Ras, Raf-1 and the MAPK kinases (MEK) leads to the phosphorylation of ERK1/2 (Cobb and Goldsmith, 1995), and the phosporylated ERK1/2 is then translocated into the nucleus to upregulate the transcriptional expression of some immediate-early genes, such as *egr-1*, *c-fos*, and *junB* (Chai and Tarnawski, 2002; Hodge et al., 1998).

Mammalian c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), encoded by three genes, responds primarily to cellular stress signals such as UV irradiation, heat shock, and protein synthesis inhibitors. Two upstream kinases MKK4/7 are known to activate JNK, and several transcriptional factors, including c-Jun, activating transcription factor-2 (ATF-2) and Elk-1, have been identified as the phosphorylated substrates of JNK（Weston and Davis, 2007）. Transcription factor AP-1 proteins that are composed of various Jun/Fos family also seem to be regulated by JNK (Ip and Davis, 1998; Yang et al., 1997). In multicellular organisms, the activation of JNK isoforms is associated with inflammation, apoptosis and cell growth (Weston and Davis, 2007).

Many mycotoxins, including trichothecene, ochratoxin A, and patulin, have been demonstrated to activate MAPK pathways in various cellular models (Liu et al., 2006; Moon and Pestka, 2002; Schramek et al., 1997; Shifrin and Anderson, 1999), but few studies have demonstrated the association of cell signaling pathways with CTN-induced toxicity in human cells. This work established that CTN activates both the ERK and the JNK signaling pathways in two human cell lines, HEK293 and HeLa, and further elucidated the biological consequences of their activation.

Materials and Methods

Reagents. Cell culture medium and serum were obtained from Life Technologies (Grand Island, NY). Rabbit polyclonal antibodies against phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phosphor-JNK/SAPK (Thr183/ Tyr185) and JNK/SAPK were purchased from Cell Signaling (Beverly, MA). Rabbit polyclonal antibodies against Egr-1, c-Jun and goat polyclinal antibody against c-Fos were purchase from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies against α-tubulin and β-actin were purchased from Sigma Chemical Co (St. Louis, MO). PD98059 (MEK1 Inhibitor) [-(2'-amino-3'-metho xyphenyl)-oxanaphthalen-4-one] and U0126 (MEK1/2 Inhibitor) [1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene] were purchased from Cell Signaling (Beverly, MA). SP600125 [anthra(1,9-*cd*)pyrazol-6(2*H*)-one] was purchased from Calbiochem (La Jolla, CA). Horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG secondary antibodies were obtained from Pierce (Rockford, IL). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO). CTN was dissolved in 25% ethanol at a concentration of 10 mM and stored at -20°C.

Cell cultures. Human embryonic kidney cell lines (HEK293) and human cervical cancer cell lines (HeLa) were obtained from Bioresources Collection and Research Center in Taiwan. HEK293 cells were cultured in minimal Eagle's medium supplemented with 10% horse serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator.

Cell viability assay. Either HEK293 or HeLa cells ($1x10^4$ cells) were seeded in 96-well plates, treated with vehicle alone (25 % ethanol in PBS) or various concentrations (final concentration 0-100 μM) of CTN at the designated times. MTT （3-(4,5- Dimethy- lthiazol-2-yl)- 2,5- diphenyltetrazolium bromide, a tetrazole） assay, a method applying the mitochondrial metabolic enzyme activity as an indicator of cell viability, was conducted following the protocol described in the report of Liu et al. (2006).

Preparation of whole cell extracts. Cells (3×10^5 in a 3.5 cm tissue culture plate) were cultured for 72 h in medium containing 10% serum, and then serum-starved by transferring to 1% serum for 18 h to maintain the minimal basal levels of phospho-ERK and phospho-JNK in cells. Cells were rinsed with 0.01M PBS and lysed by addition of extraction buffer (0.01M PBS containing 5% glycerol, 1 mM

dithiothriotol, 1 mM EDTA, pH 8.0, 0.5% Triton X-100, 0.8 μM aprotinin, 1 mM AEBSF, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstain A, 14 mM E-64, and 1 mM phenylmethylsulfonyl fluoride). The cell lysate was kept on ice for 10 min, and then centrifuged at 16,000 g for 20 min at 4°C. The protein concentration of the supernatant solution was determined using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

Western blot analysis. Extracted total proteins were incubated with Laemmli buffer and separated by 10 % SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), and reacted with primary antibodies specific to MAPKs (phospho-ERK1/2, ERK1/2, phospho-JNK, and JNK) or the products of immediate early genes (Egr-1, c-Fos, and c-Jun), and then anti-rabbit and anti-mouse secondary antibodies conjugated to horseradish peroxidase. Bound antibodies on the membrane were detected using an enhanced chemiluminescence detection system according to the manufacturer's manual (Amersham Pharmacia Biotech, Amersham, UK). The intensities of bands on blots were quantitated using the ImageGauge program Ver. 3.46 (Fuji Photo Film, Tokyo).

RNA isolation and reverse transcription. RNAs were isolated from solvent or CTN-treated cells with RNeasy mini kit (Quiagen). Reverse transcription was conducted with Reverse-iT^{TM} 1st strand synthesis kit (ABgene, Surrey, UK). Briefly, 2 μg of RNA was reverse transcribed at 42 ^{o}C for 1 h in a mixture containing 0.5 μg of Oligo(dT)$_{12\text{-}18}$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 1 mM each dNTP and 40 U of M-MLV Reverse transcriptase. Negative controls including all the above components except the reverse transcriptase were run in parallel.

Polymerase chain reaction (PCR). For semi-quantitative RT-PCRs, the reaction solution contained the cDNA template, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of sense and anti-sense primers and 2U of Taq polymerase. The reactions were conducted at 94^{o}C for 5 min first and then went through the following procedures: denaturation at 94^{o}C for 60 s, annealing at specific temperatures for 30 s depending on genes, elongation at 72^{o}C for 90 s, and with a final extension step at 72^{o}C for 10 min. The PCR products were resolved by 1% agarose gel electrophoresis and stained with ethidium bromide. The primer sets used in semi-quantitative PCRs are as followings.

egr-1 (201 bp) forward: 5'-CAGCACCTTCAACCCTCAG-3'
reverse: 5'-CACAAGGTGTTGCCACTGTT-3'

c-fos (247 bp) forward: 5'-AGGAGAATCCGAAGGGAAAG-3'
reverse: 5'-CAAGGGAAGCCACAGACATC-3'
fosB (249 bp) forward: 5'-TTCTGACTGTCCCTGCCAAT-3'
reverse: 5'-CGGGGTCAGATGCAAAATAC-3'
c-jun (409 bp) forward: 5'-GCATGAGGAACCGCATTGCCGCCTCCAAGT-3'
reverse: 5'-GCGACCAAGTCCTTCCCACTCGTGCACACT-3'
junB (214 bp) forward: 5'-CACCAAGTGCCGGAAGCGGA-3'
reverse: 5'-AGGGGCAGGGGAGGTTCAGA-3'
MMP-3 (160 bp) forward: 5'-GCATAGAGACAACATAGAGCT-3'
reverse: 5'-TTCTAGATATTTCTGAACAAGG-3'

*Gadd45β (*247 bp) forward: 5'-AACATGACGCTGGAAGAGCT-3'
reverse: 5'-AGAAGGACTGGATGAGCGTG-3'
gapdh (287 bp) forward: 5'-GCCAAAAGGGTCATCATCTC-3'
reverse: 5'-GTAGAGGCAGGGATGATGTTC-3'

For real-time RT-PCR analysis, the reaction solution contained cDNA template, 125 nM forward and reverse primers, and SYBR Green I Master Mix (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's instructions. Primer pairs were designed using PrimerExpress software. Reactions were conducted in the cycler (ABI Prism 7700, PerkinElmer Life Sciences) with a condition set as following: polymerase activation 10 min at 95^{o}C, 40 cycles at 95^{o}C for 15 sec, and 60^{o}C for 1 min. The relative amount of each gene to GAPDH internal control and the fold-induction was calculated by using the cycle threshold (Ct) methods as following: $\triangle\triangle Ct = (Ct_{Target} - Ct_{Housekeeping})$ treatment - $(Ct_{Target} - Ct_{Housekeeping})$ non-treatment, and the final data were derived from $2^{-\triangle\triangle Ct}$. The primer sets used in real-time RT-PCRs are as followings.

MMP-3 forward*:* 5'-CATAGAGCTAAGTAAAGCCTGTGGAA -3'
reverse: 5'-TGCCACGCACAGCAACA-3'
Gadd45β forward: 5'-CACGCTCATCCAGTCCTTCTG-3'
reverse: 5'-CCGACACCCGCACGAT-3'
gapdh forward: 5'-TGTTCGACAGTCAGCCGC-3'
reverse: 5'-GGTGTCTGAGCGATGTGGC-3'

Plasmid construction and transfection. The vector pLuc-MCS (Stratagene, La Jolla, CA) contained a minimal promoter with a TATA box linked to the luciferase gene, so constructs were created by ligating synthetic oligos into the *Hin*dIII/*Xho*I sites immediately upstream of the TATA box. For pEgr-Luc construction, the synthetic oligo with one Egr-1 binding site was 5'-AGCTTC**GCGGGGGCG**AGGAAG-3'; for

pAP-Luc, the synthetic oligo with one AP-1 binding site was 5'-AGCTTCGCTT**GATGAGTCA**GCCGGAAC- 3'. All the above constructed plasmids were verified by DNA sequencing.

Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacture's recommendations. HEK293 or HeLa cells, grown in 3.5 cm-culture plates with serum free medium, were 80-90% confluence at the time of transfection. Cells were co-transfected with 2 μg of constructed plasmid and 1 μg of pSV-β-galactosidase control vector (Promega, Madison, WI), and then incubated in the CO_2 incubator for 16 h prior to replacement with fresh medium containing 1% serum and antibiotics. Twenty-four hours after medium replacement, the transfected cells were treated with various concentrations of CTN for 24 h before determination of intracellular luciferase activity.

Luciferase assay. HEK293 cells which had been co-transfected with pEgr-Luc /pAp-Luc and pSV-β-galactosidase were treated with solvent or CTN for 24 h. The cell-free extracts were prepared and luciferase activities were determined using luciferase assay kit (Stratagene, La Jolla, CA) and Tropix TR717 luminometer (Applied Biosystems, Foster city, CA). The activity of β-galactosidase was measured by adding a diluted extract sample to an equal volume of 2 x assay buffer that contains the substrate ONPG (O-nitrophenyl-D-galactopyranoside) 1.33 mg/ml, 200 mM sodium phosphate buffer (pH 7.3), 2 mM $MgCl_2$ and 100 mM β-mercaptoethanol. Samples are incubated at 37^{o}C for 30 minutes. The reaction was terminated by addition of 1 M sodium carbonate, and the absorbance was read at 420 nm with an Optimax microplate reader (Molecular Device, CA).

Statistical analysis of data. All statistical analyses were conducted using the software GraphPad Prism Version 4.0 (GraphPad Software, San Diego, CA). Experimental data grouped by one variable were analyzed by unpaired two-tailed t-test or one-way ANOVA followed by Tukey post test. Experiments with two variables were analyzed by two-way ANOVA in combination with Tukey post test.

Results

Effects of CTN on cell viability

To evaluate the effects of CTN on cell viability, HEK293 and HeLa cells were treated with 0-100 μM CTN for 24 h and then the survival rates of cells were determined by the MTT assay. As presented in Table 1, CTN reduced the cell viability of both cell lines in a dose-dependent manner. The cell viability of HEK293 or HeLa was not significantly lower than that of vehicle-treated cultures until 100 μM of CTN

had been administered.

CTN activated ERK1/2 and JNK pathways in human cells

HEK293 cultures were exposed to various concentrations of CTN for designated times and the whole cell protein extracts were subjected to Western blotting using specific antibodies. As shown in Fig. 2A, the exposure of cells to CTN for 6 h caused a dose-dependent increase in ERK phosphorylation. CTN at concentrations from 50 to 100 μM increased the phosphorylated ERK1/2 levels 5.2 to 16.4 fold over that of solvent-treated control. CTN also activated the JNK pathway in HEK293 cells, as evidenced by the increased levels of phosphorylated JNK1 (46 kDa) and JNK2 (54 kDa) after 25 μM treatment (Fig. 2B). Similarly, the effect of toxin on human HeLa cells was examined. The incubation of HeLa cells with 100 μM CTN for 6 h substantially increased both phospho-ERK1/2 and phospho-JNK levels (Fig. 2C). CTN also activated the MAPKs in a time-dependent manner (Fig. 3). When HEK293 cells were incubated with 75 μM CTN for various periods, the signals of phospho-ERK1/2 and phospho-JNK were initially detected in 6 h and 3 h, respectively, and remained detectable for at least 24 h.

Induction of immediate-early genes by CTN treatment

The activation of the MAPK signaling pathway can modulate the transcriptional expression of various immediate-early genes in numerous cell models. To identify the downstream target genes that are activated by the CTN-induced MAPK pathways, RNAs prepared from CTN-treated HEK293 and HeLa were subjected to RT-PCR analysis. Exposure to CTN increased the mRNA levels of *egr-1*, *c-fos*, *fosB*, *c-jun* and *junB* in HEK293 cells (Fig. 4A). Western blotting also demonstrated that CTN treatment increased the protein levels of Egr-1, c-Fos and c-Jun (Fig. 4 B). Pre-treatment of H293 cultures with U0126, a specific ERK pathway inhibitor, greatly reduced the Egr-1, c-Fos and c-Jun signals induced by CTN. Exposure of cells to SP600125, an inhibitor of JNK pathway, clearly down-regulated the c-Jun signal, but did not affect Egr-1 and c-Fos levels. These data indicate that CTN mediates the expression of examined immediate-early genes via ERK and JNK activation.

CTN increased DNA binding ability of Egr-1 and AP-1 proteins

Egr-1 protein and Jun/Fos complex (also known as AP-1) are transcription factors that can recognize their specific DNA binding domains, 5'-GCGG/TGGGCG-3' and 5'-TGAG/CTCA-3', respectively, in the promoter regions of numerous genes (Christy and Nathans, 1989; Lee et al., 1987). Therefore, we investigated whether the CTN-induced Egr-1 and AP-1 proteins exhibit DNA binding

ability *in vivo*, a biological function of transcription factor. Luciferase activities in CTN-treated and untreated cells that had been transfected with pEgr-Luc or pAP-Luc were examined; both pEgr-Luc and pAP-Luc have one copy of typical Egr-1 and AP-1 binding sequences, respectively. As presented in Fig. 5A, a clear dose-dependent increase in luciferase activity was observed in the pEgr-Luc transfectant that was treated with CTN; a similar effect was also observed in the pAP-Luc transfectant (Fig. 5B). After the transfectants were exposed to 75 μM CTN, the presence of Egr or AP-1 binding sites significantly upregulated the TATA promoter activity by factors of 2.4±0.2 and 3.8±0.6, respectively.

CTN induced Gadd45β and MMP3 gene expression in HEK293 and HeLa cells

CTN enhanced the DNA binding ability of Egr-1 and AP-1 proteins *in vivo* (Fig. 5), so we hypothesized that the administering of CTN also affects the expression of specific genes that have Egr- or AP-1 binding sequences in their promoters. This possibility was initially investigated by performing a computer search of the eukaryotic promoter database (EPD) (Perier, et al., 1998). Two toxicology-related genes, matrix metalloproteinase (*MMP*)-3 (stromelysin 1) and growth arrest DNA damage-inducible gene 45 β (*Gadd45β*), were screened out. *MMP3* with AP-1 binding site in its promoter region has recently emerged as a candidate for a mammary tumor accelerator (Johansson et al., 2000; Sternlicht et al., 2000). *Gadd45β,* containing an Egr-1 binding site within the promoter, participates in controlling the cell cycle, DNA repair and apoptosis (Abdollahi et al., 1991). Accordingly, the effects of CTN on the expression of *Gadd45β* and *MMP3* in HEK293 were analyzed using PCR. The transcriptional induction of *Gadd45β* and *MMP3* genes, measured by semi-quantitative RT-PCR, was observed following treatment with 100 μM CTN (Fig. 6A). Similar results were obtained from real-time PCR; 100 μM CTN increased *Gadd45β* and *MMP3* mRNA in HEK293 by factors of 12.0 and 2.6, respectively (Fig. 6B). Furthermore, when HeLa cells were exposed to 50 μM CTN, the transcripts of *Gadd45β* and *MMP3* increased to 7.1- and 2.7-folds, respectively, over those of the vehicle-exposed group (Fig. 6C), indicating that CTN induces *Gadd45β* and *MMP3* gene expression in both human normal cells and cancer cells.

CTN-directed caspase 3 activation involves MAPK signaling pathways

To elucidate the relationship between MAPK signaling and CTN-mediated apoptosis, HEK293 cells were left untreated or pretreated with PD98059, a specific inhibitor of the ERK pathway, or with SP600125, a JNK inhibitor, for 1 h before co-exposure to 75 μM CTN, and then the caspase 3 activity was evaluated. As shown in Fig. 7A, CTN treatment resulted in a 7.5-fold activation of caspase 3, but the

presence of either MAPK inhibitor significantly inhibited caspase 3 activation, suggesting the contribution of MAPK signaling pathways to CTN-induced apoptosis. Furthermore, the levels of extracellular LDH activity were determined to study the involvement of MAPKs in CTN-mediated membrane leakage. The LDH activity in 100 μM CTN-treated HEK293 cells was 2.5 times higher than that of the solvent-treated group; the presence of PD98059, U0126 or SP600125 did not substantially affect this increase (Fig. 7B). Therefore, the data herein indicates that neither ERK1/2 nor JNK pathway is involved in CTN-triggered membrane leakage.

Discussion

CTN is frequently found in fungi-contaminated foodstuffs, as well as in fermented foods, which are intentionally inoculated with fungi. These fermented foods, such as *Monascus* products (red yeast rice extracts), not only are a dietary staple in many Asian countries, but also are used in popular dietary supplements in the West to reduce cholesterol levels （Liu et al., 2005; Wei et al., 2003）. Although exposure to massive amounts of CTN from food is rare today in developing countries, the long-term consumption of food that is contaminated with low levels of lipophilic CTN remains a serious issue.

In this work, CTN treatment activated ERK and JNK pathways in both HEK293 and HeLa cultures (Fig. 2). HEK293 cells derived from normal embryonic kidney were used herein because CTN is known to be nephrotoxic; moreover, HeLa, a human cancer cell line, was adopted to confirm that the effect of CTN is not specific to normal cells. The peripheral blood mononuclear cells (PBMC) were also collected from the blood of several individuals and treated with CTN. Although substantial basal levels of phospho-ERK1/2 and JNK were present in some of the PBMC samples, the administering of CTN still slightly strengthened p-ERK1/2 and p-JNK signals in PBMC (data not shown).

CTN, like some other mycotoxins (Schramek et al., 1997; Wu et al., 2005), upregulated the phopho-ERK1/2 in various cell types at low serum levels, but a recent study has demonstrated that the exposure of serum-stimulated mouse embryonic stem cells to CTN for 24 h leads to the degradation of Ras and Raf-1 and the subsequent inhibition of ERK1/2 phosphorylation (Chan, 2007). CTN seems to mediate the functions of ERK cascade according to the cell types and culturing conditions (Lu and Xu, 2006). The mechanism by which CTN activates MAPKs is unclear. CTN is a hydrophobic compound of low molecular weight (250 Dalton), which enters the cells by permeating the plasma membrane or interacting with membrane-bound organic anion transporters (Tachampa et al., 2008). The intracellular CTN may induce the generation of reactive oxygen species (ROS) (Chan, 2007; Ribeiro et al., 1997) and

oxidative stress is known to be an inducer of MAPK activation (Kamata et al., 2005; Wu et al., 2006).

The stimulation of HEK293 cells with CTN up-regulated the RNA and protein levels of immediate-early genes, such as *egr-1*, *c-fos* and *c-jun* (Figs. 4 A and B). Immediate-early genes are activated rapidly and transiently upon cellular stimulation and regarded as early regulators of cell growth and differentiation. The examined genes, e*gr-1*, *c-fos*, *fos B* and *jun B,* are known to have a serum-responsive element (SRE) located within their promoter regions (Chai and Tarnawaski, 2002). This element is a binding site for the transcription factor called the serum responsive factor (SRF), and the activation of ERK pathway is a major mechanism by which the SRF regulates SRE activity (Sharrocks, 1995; Treisman, 1992). Therefore, the presence of U0126, an ERK pathway inhibitor, effectively weakened the signals of Egr-1 and c-Fos, which were induced by CTN (Fig. 4B). We also found that the levels of CTN-mediated c-Jun declined when either U0126 or SP600125 were added to the HEK 293 culture, revealing that both ERK and JNK pathways contribute to the CTN-activated *c-jun* expression. Several predicted c-Jun binding site were found in the promoter region of *c-jun* gene; therefore, we suppose that CTN-activated JNK first phosphorylated c-Jun protein, and the phosphorylated c-Jun activated the transcription of itself. Additionally, although, unlike the *egr-1* and *junB* genes, *c-jun* promoter does not contain any SRE domain, predicted Elk-1 (Ets-like transcrption factor-1) binding sites are found, and Elk-1 protein is the down-stream target of both ERK1/2 and JNK (Janknecht et al., 1993；Cavigelli et al., 1995). Altogether immediate-early gene expression is induced by CTN through the coordination and cooperation of MAPK signaling pathways.

To investigate whether CTN can modulate the expression of toxicity-related genes via ERK/Egr-1 or MAPK/AP-1 pathways, the core sequences of Egr-1/AP-1 binding sites were employed to search the EPD database. EPD is an annotated non-redundant collection of eukaryotic POL II promoters, which are experimentally defined to have biological functions in a higher eukaryote (Perier et al., 1998). Among numerous genes with Egr-1 binding sites in their promoters, *Gadd45β* was selected to study the effect of CTN because this gene product is a positive mediator of apoptosis following genotoxic stress; CTN is known as an inducer of ROS and apoptosis in specific cell types (Chan, 2007; Yu et al., 2006). A previous report has demonstrated that a sequential activation of NF-κB/Egr-1/Gadd45 in epidermal cells is responsible for UVB-mediated cell death (Thyss et al., 2005). The role of GADD45β in CTN-mediated cell death is under investigation.

CTN was found dramatically to induce *MMP3* gene transcripts in both HEK293 and HeLa cells (Figs. 6B and 6C). MMP-3 (also known as stromelysin-1), a member

of the MMP family, is known to degrade various extracellular matrix and cell-surface molecules, and also to activate other MMPs (Chakraborti et al., 2003). Increased MMP3 expression has been implicated in tumor initiation in mammary glands and epithelial cells of mice (Lochter et al., 1997; Sternlicht et al., 1999). Two major regulatory elements, AP-1 and polyomavirus enhancer A-binding protein 3 (PEA3) binding sites, are located in the promoter region of human *MMP-3* gene (Buttice and Kurkinen, 1993). The regulation of AP-1 transcriptional activity is mediated by MAPKs via multiple mechanisms (Whitmarsh et al., 1996). It has been reported that both ERK and JNK cascades are independently involved in the regulation of PEA3 activity in COS cells (O'Hagan et al., 1996). Moreover, the interaction between AP-1 and PEA3 contributes to the mitogenic induction of *MMP-3* gene in NIH3T3 cells (Kirstein et al., 1996). This information suggests that the CTN induction of *MMP3* expression proceeds by the activation of ERK and JNK pathways and the cooperation of their downstream effectors AP-1/PEA3.

CTN has been demonstrated to be an apoptosis inducer both *in vivo* and *in vitro* (Chan, 2007; Yu et al., 2006). In the presence of MAPK inhibitors in HEK293 cultures, we found that both ERK and JNK pathways contributed to the caspase 3 activity that was induced by CTN (Fig. 7A). JNK cascade is generally considered to be associated with the activation of apoptosis, but ERK1/2 may have dual roles in the apoptotic process in which it either promotes cell survival or shows pro-apoptotic functions, depending on the conditions. (Lu and Xu, 2006; Verheij et al., 1996). However, blocking the ERK/JNK pathways did not alter the LDH activity that occurred upon treatment with CTN (Fig. 7B). The release of LDH from cells is an indicator of increased plasma membrane permeability, which may be caused by necrosis (Krysko et al., 2008).

In conclusion, we have demonstrated that CTN, a mycotoxin frequently found in dietary staple and supplements, caused rapid and persistent phosphorylation of ERK1/2 and JNK in human cells. The activation of MAPKs increased not only the biological function of transcriptional factors Egr-1/AP-1, but also their down stream effectors GADD45β/ MMP-3. Since the induction of GADD45β and MMP-3 are associated with DNA damage and tumor initiation, an understanding of the signaling cascades driven by CTN in human cells will provide useful information for evaluating the exposure risk and the toxicological mechanism of mycotoxins.

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Legend of figures

Fig. 1. Chemical structure of citrinin (CTN)

Fig. 2. Phosphorylation of MAPK by CTN in HEK293 and HeLa cells. Subconfluent HEK293 cells (A and B) and HeLa cells (C) were rendered quiescent by incubation for 18 h in medium containing 1% serum, and then incubated for 6 h with vehicle or various CTN concentrations. Whole cell extracts were prepared and MAPK activation was estimated by Western blotting using antibodies that recognized phosphorylated or unphosphorylated forms of ERK1/2 or JNK. The relative phospho-ERK1/2 levels in the lower panel of (A) and the phospho-JNK levels in (B) were densitometric analyses of three independent experiments and expressed as the mean ± SEM. The fold inductions correspond to the ratio between vehicle- and CTN-treated cells. * Significant difference compared to the vehicle-treated group (** P<0.01, ***P<0.001).

Fig 3. Time-dependent induction of ERK/JNK phosphorylation by CTN. Subconfluent HEK293 in medium containing 1% serum were incubated with 75 μM CTN for up to 24 h. Whole cell extracts were prepared and subjected to Western blotting in which phospho-ERK/JNK and parent ERK/JNK antibodies were used as probes. Results are representatives of three independent experiments.

Fig. 4. CTN treatment induced the expression of immediate early genes in HEK293. (A) HEK293 cultures in 1% serum were treated with vehicle or CTN for 6 h. Total RNAs were prepared and analyzed for induction by semi-quantitative RT-PCR as described in Material and Methods. (B) HEK293 cells were exposed to various concentrations of CTN for 24 h or exposed to U0126/SP600125 (20 μM) for 1 h before co-incubated with 100 μM CTN for another 24 h. Samples of nuclear protein were extracted and subjected to Western blotting with antibodies specific to Egr-1, c-Fos, c-Jun, and actin. Results are representatives of three independent experiments.

Fig. 5. CTN enhanced the DNA binding ability of Egr-1 and AP-1 in HEK 293. The pEgr-Luc (A) or pAP-Luc construct (B) was transiently transfected into HEK293, and then the transfectants were stimulated or not with CTN for 24 h. Whole cellular protein preparations were applied to luciferase activity assays according to Material and Methods. The data are given as the mean ± SEM (n=5). (* P<0.05, ***P<0.001).

Fig. 6. CTN induced *Gadd45β* and *MMP3* mRNA expression in HEK293 and HeLa cells. HEK293 (A and B) and HeLa (C) cells were exposed to vehicle or CTN for 24 h before total RNA preparation. *Gadd45β* and *MMP3* mRNA expression were assessed

by semi-quantitative RT-PCR (A) or real-time RT-PCR (B and C). The Results in (A) are representatives of three independent experiments. The data in (B) and (C) are given as the mean ± SEM (n=4). The fold inductions correspond to the ratio between vehicle- and CTN-treated cells. * Significant difference compared to the vehicle-treated group

Fig. 7. Effects of ERK1/2 and JNK inhibitors on CTN-induced apoptosis and LDH leakage. HEK293 cells were left untreated or treated with U0126, PD98059 or SP600125 (20 μM) for 1 h and then co-incubated with vehicle or CTN for 24 h. Caspase 3 activity (A) and extracellular LDH activity (B) were determined as described in Materials and Methods. The data from five or three independent experiments are expressed as the mean ± SEM.

Figure 1.

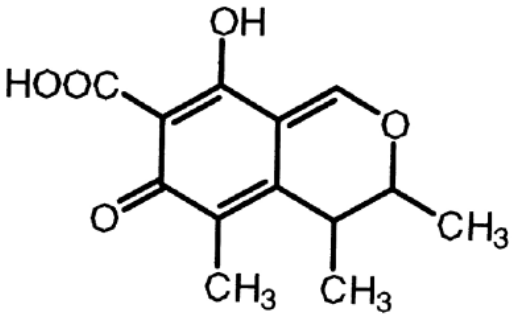

Figure 2

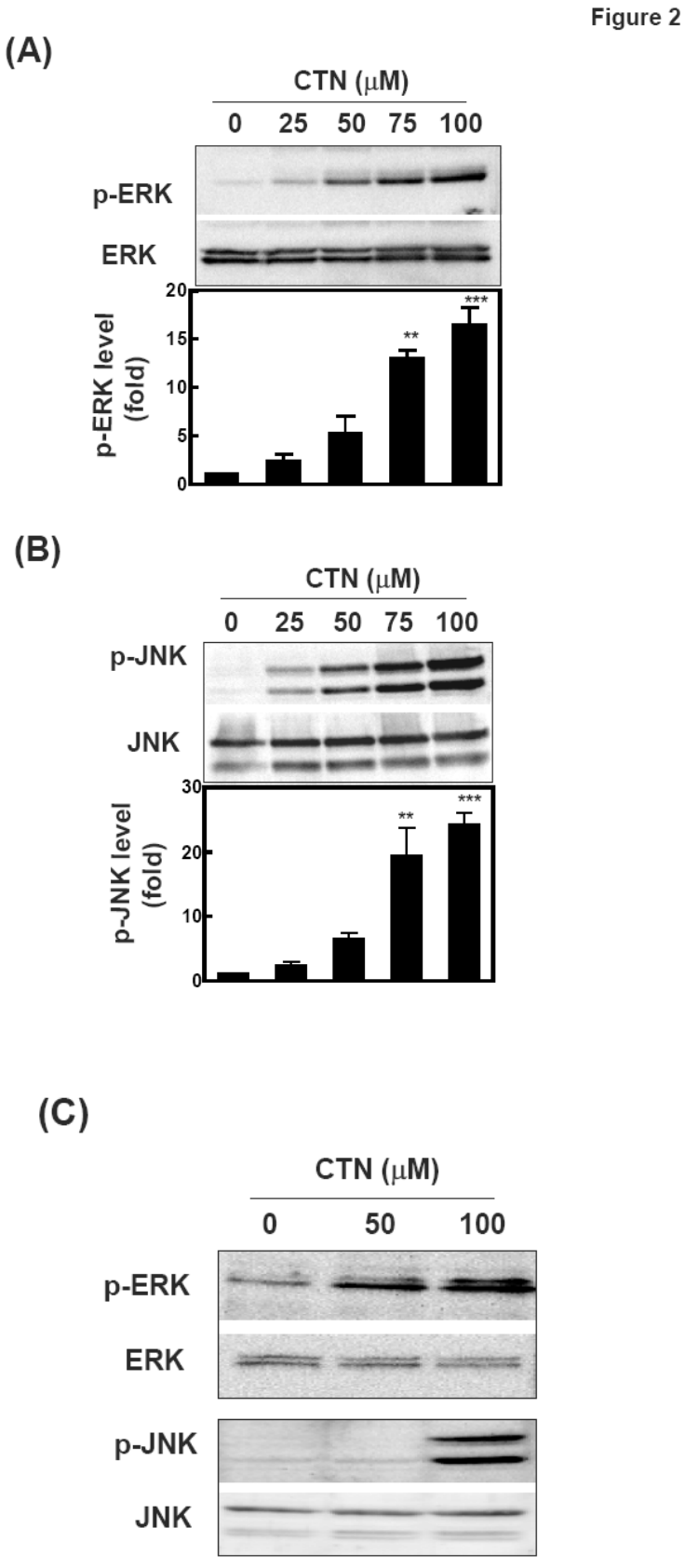
Figure 2
(A)
CTN (μM)
0 25 50 75 100
p-ERK
ERK
p-ERK level (fold)
20
15
10
5
0
**
***
(B)
CTN (μM)
0 25 50 75 100
p-JNK
JNK
p-JNK level (fold)
30
20
10
0
**
***
(C)
CTN (μM)
0 50 100
p-ERK
ERK
p-JNK
JNK

Figure 3.

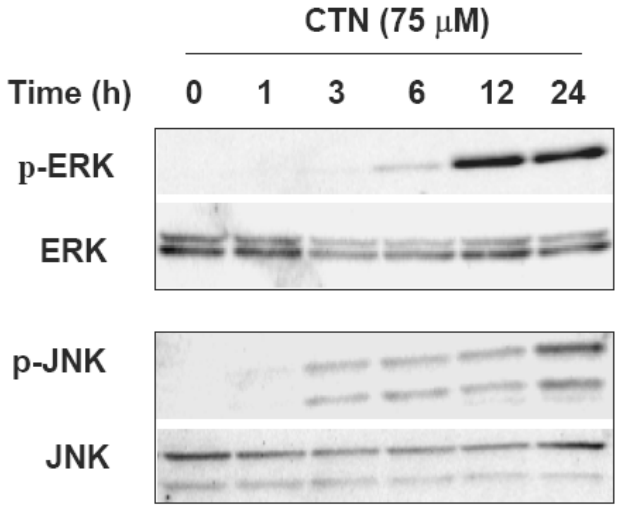

Figure 4

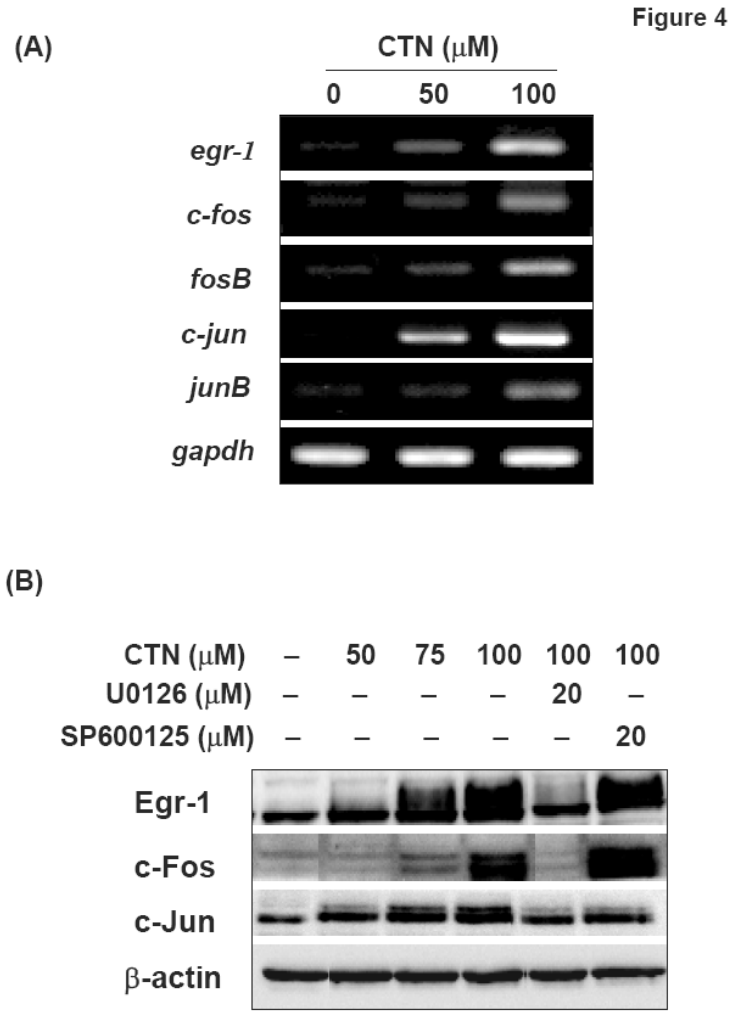

Figure 5

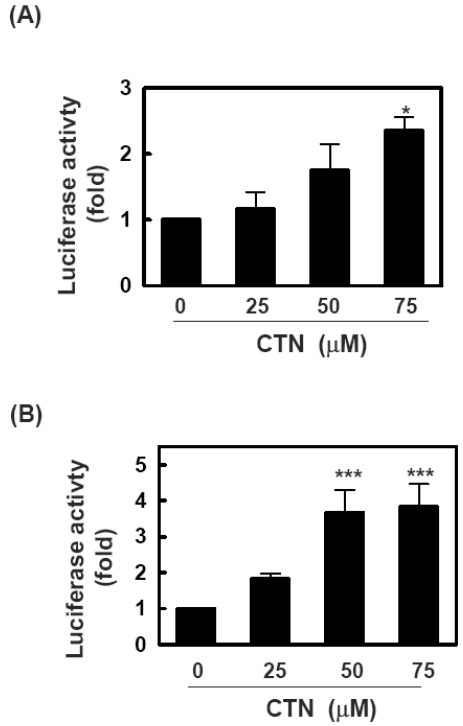

Figure 6

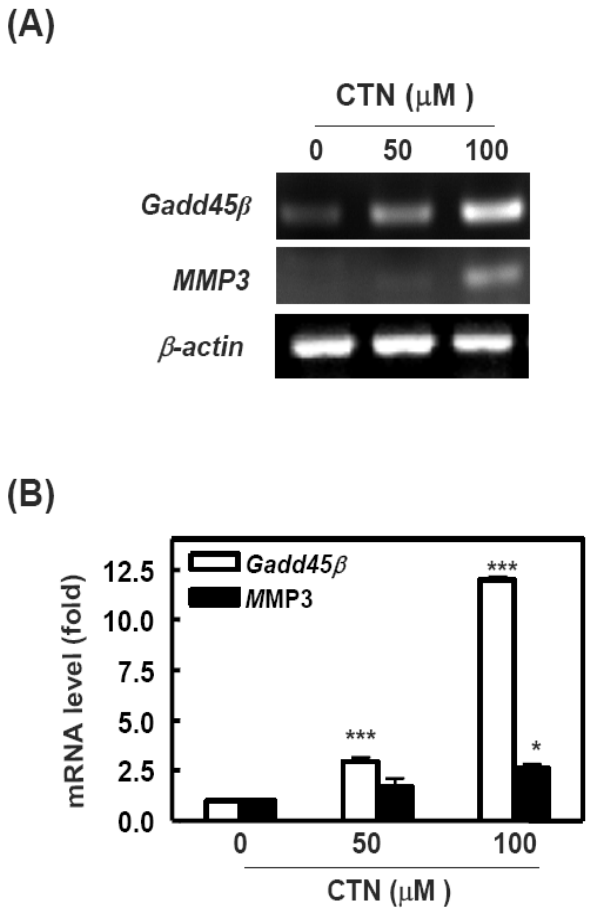

Figure 7

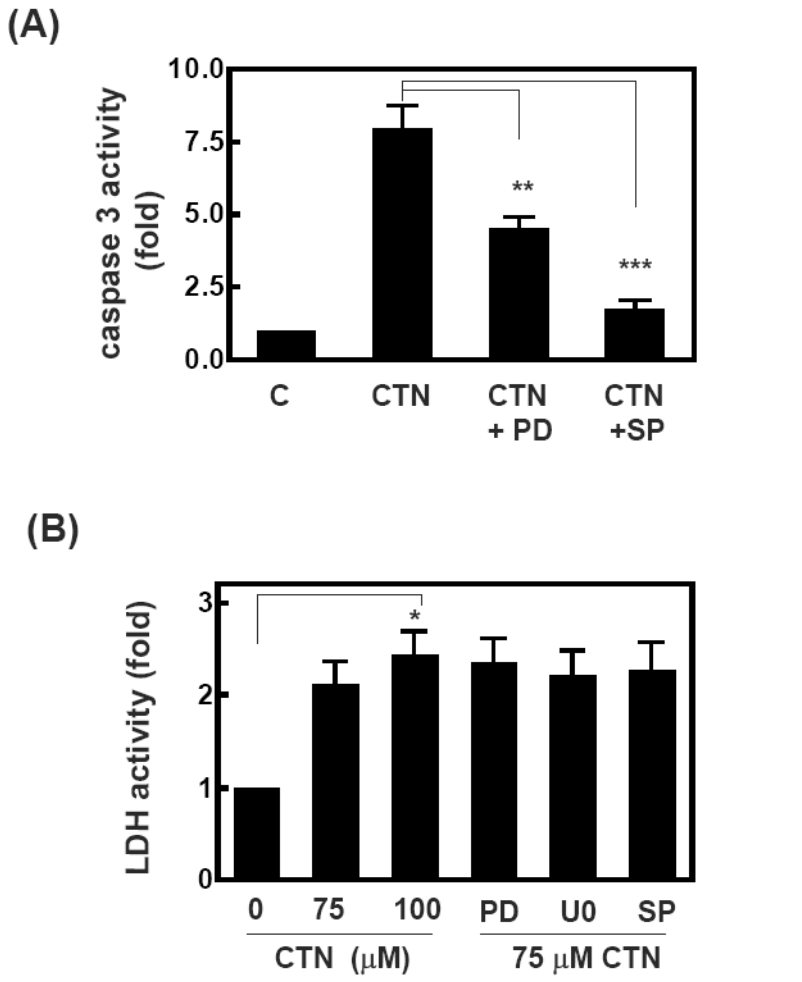
Figure 7
(A)
caspase 3 activity (fold)
10.0
7.5
5.0
2.5
0.0
**
***
C
CTN
CTN + PD
CTN +SP
(B)
LDH activity (fold)
3
2
1
0
*
0
75
100
PD
U0
SP
CTN (μM)
75 μM CTN