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Activation of ERK and JNK signaling pathways by mycotoxin citrinin in human cells

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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 both the mRNA and protein levels of *egr-1*, *c-fos* and *c-jun* genes; additionally, the ERK1/2 pathway contributed to the upregulation of Egr-1 and c-Fos protein expression. CTN treatment also induced the transcription activity of Egr-1 and AP-1 proteins, as evidenced by luciferase reporter assays. Bioinformatic analyses indicated two genes *Gadd45β* and *MMP3* have Egr-1 and AP-1 response elements in their promoters, respectively. Furthermore, co-exposure of HEK293 cells to CTN and MAPK pathway inhibitors demonstrated that CTN increased the levels of *Gadd45β* mRNA through ERK1/2 signaling pathway and up-regulated the *MMP3* transcripts majorly via JNK pathway. Finally, CTN-triggered caspase 3 activity was significantly reduced in the presence of MAPK inhibitors. Our results suggest that CTN positively regulates ERK1/2 and JNK pathways as well as their downstream effectors in human cells; activated MAPK pathways are also involved in CTN-induced apoptosis.

Introduction

Mycotoxin citrinin (CTN) is a fungal secondary metabolite that was originally isolated from *Penicillium citrinum* (Fig. 1). 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 results in the formation of renal adenoma in 70% of the fed rats (Arai and Hibino, 1983). Furthermore, CTN 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 phosphorylated 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 transcription 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

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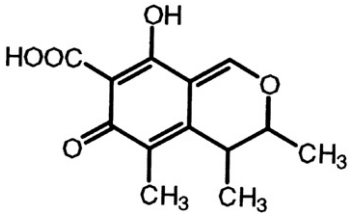

Fig. 1. Chemical structure of citrinin (CTN).

CTN-induced toxicity in human cells. This work established that CTN activates both the ERK1/2 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, phospho-JNK/SAPK (Thr183/Tyr185) and JNK/SAPK were purchased from Cell Signaling (Beverly, MA). Rabbit polyclonal antibodies against Egr-1, c-Jun and goat polyclonal antibody against c-Fos were purchased 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-(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 in 0.01 M phosphate buffered saline (PBS) 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 minimum essential 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 (1×10^4 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 and nuclear protein extracts. Cells (1×10^6 in a 6 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. Whole cell protein extracts were prepared according to Liu et al. (2006). For nuclear protein extraction, cells were rinsed with 0.01 M PBS and lysed by addition of extraction buffer A (10 mM HEPES, pH7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) and protease inhibitor solution (0.8 μM aprotinin, 1 mM AEBSF, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin A, 14 mM E-64, and 0.2 mM phenylmethylsulfonyl fluoride). The cell lysate was kept on ice for 10 min, and then centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant fluid was removed and the left pellet was added with extraction buffer B (10 mM HEPES, pH7.4, 0.75 mM $MgCl_2$, 210 mM NaCl, 0.1 mM EDTA, 12.5% glycerol, 0.5 mM DTT and protease inhibitor as described above). The cell lysate was kept on ice for 30 min, and then centrifuged at 13,000 rpm for 10 min at 4 °C again. The supernatant solution was collected and the protein concentration 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™ 1st strand synthesis kit (ABgene, Surrey, UK). Briefly, 2 μg of RNA was reverse transcribed at 42 °C for 1 h in a mixture containing 0.5 μg of Oligo$(dT)_{12-18}$, 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 2 U of Taq polymerase. The reactions were conducted at 94 °C for 5 min first and then went through the following procedures: denaturation at 94 °C for 60 s, annealing at specific temperatures for 30 s depending on genes, elongation at 72 °C for 90 s, and with a final extension step at 72 °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′-GCATGAGGAACCGCATTGCCGCCT-CCAAGT-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