Mechanisms of cell death induction by food-borne mycotoxins

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Abbreviations

AFs aflatoxins, ALT alanine aminotransferase, AMPK adenosine 5'-monophosphate (AMP)-activated protein kinase, AST aspartate aminotransferase, ATG autophagy related protein, CHOP C/EBP-homologous protein, CTN citrinin, CIT citreoviridin, CYP450 cytochrome P450, DON deoxynivalenol, ERs endoplasmic reticulum stress, FNs fumonisins, FB1 fumonisin B1, FGF-2 fibroblast growth factor-2, GADD34 (growth arrest DNA damage inducible gene 34), IKK inhibitor of nuclear factor kappa-B kinase, IRE1α Inositol-requiring enzyme-1α, LC3 microtubule-associated protein 1A/1B-light chain 3, MAPK mitogen activated

protein kinase, mTOR mammalian target of rapamycin, OTA ochratoxin A, PAT patulin, PERK RNA-dependent protein serine/threonine kinage (PKR) like kinase, ROS reactive oxidative specials, SOD superoxide dismutase, VEGF vascular endothelial growth factor, XBP1S X-box binding protein 1 spliced, ZEA zearalenone.

Abstract

Mycotoxins are secondary metabolites of fungi that contaminate a wide range of foods and feeds. Mycotoxin contamination is considered to be an important risk factor for food safety which poses serious threat to human and animal health. Cell death induction is suggested to be the key cellular event contributed to the pleiotropically toxic effects of mycotoxins. During the past decades, substantial progress has been made in uncovering the mechanisms of cell death induction by mycotoxins. Understanding of the mechanisms underlying mycotoxin-induced cytotoxicity will benefit the development of effective strategies for the management of mycotoxin-associated health issues. The current review will discuss the types of cell death induced by mycotoxins and summarize the present understanding of signaling pathways involved in mycotoxin-mediated cytotoxicities.

Key words

mycotoxins, apoptosis, necrotic cell death, autophagic cell death, p53, MAPKs, endoplasmic reticulum stress, reactive oxygen species

Introduction

Mycotoxins are secondary metabolites of fungi and are widely present in foods. Mycotoxins contamination is considered to be an important risk factor for food safety which poses serious threat to human and animal health (Turner et al., 2012). The most common mycotoxins found in foods include aflatoxins (AFs), fumonisins (FNs), ochratoxins (OTs), zearalenone (ZEA), deoxynivalenol (DON), T-2 toxin, patulin (PAT), citrinin (CTN), citreoviridin (CIT), alternariol and certain ergot alkaloids (Bennett and Klich, 2003). These food-borne mycotoxins can cause various toxic effects including nephrotoxicity, hepatotoxicity, neurotoxicity, immunotoxicity, developmental and reproductive toxicity, teratogenicity and carcinogenicity for animals (Dai et al., 2014; Kim et al., 2007; Doi et al., 2011; Abbès et al., 2016; Han et al., 2016; Patil et al., 2006; De et al., 2015). Given the frequent nonspecific effects of mycotoxin involvement, the animal-based findings are useful for understanding of potential toxicities of mycotoxins for humans (Peraica et al., 1999). Cell death induction is suggested to be a key event that contributes to mycotoxin-induced multiple toxicities. Understanding of the mechanisms underlying mycotoxin-induced cytotoxicity is essential for developing mechanism-based approaches to counteract mycotoxin-associated health issues.

1. Modes of cell death induction by mycotoxins

Apoptotic cell death induced by mycotoxins

Apoptosis is the most common type of programmed cell death and is involved in numerous physiological and pathological processes (Kerr et al., 2002). Most of these mycotoxins can induce apoptosis in both cell culture and animal models. Different types of mycotoxins exhibit differential capacity of apoptosis induction, whereas different types of organ sites reveal differential sensitivity to apoptosis induction by these mycotoxins. The studies related to mycotoxin-induced apoptotic cell death *in vitro* and *in vivo* conditions are summarized in Tables 1 and 2 respectively.

Apoptosis induction by these mycotoxins is generally accompanied by activation of multiple caspases, such as caspase -9, -8 and -3. Activation of mitochondrial pathway (intrinsic pathway) is suggested to play an important role in mycotoxin-induced caspase-dependent apoptosis. Disruption of mitochondrial membrane potential (MMP) is a hallmark of mitochondrial pathway activation. Mitochondrial membrane potential is tightly controlled by Bcl-2 family proteins including pro-apoptotic proteins Bax, Bak, Bok, Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma, Blk, etc and anti-apoptotic proteins Bcl-2, Bcl-xL, Bcl-w, A1, Mcl-1, etc. The balance between pro-apoptotic and anti-apoptotic proteins is essential to keep mitochondrial membrane potential at normal levels. Mycotoxins such as AFB1 (Peng et al., 2016; Yuan et al., 2016), AFG1 (Shen et al., 2013), ZEA (Banjerdpongchai et al., 2010; Hu et al., 2016), PAT (Wu et al., 2008; Jin et al., 2016) and OTA (Bouaziz et al., 2011) have been reported to up-regulate pro-apoptotic proteins and/or to decrease anti-apoptotic proteins, leading to imbalance of Bcl-2

family pro-apoptotic and anti-apoptotic proteins, followed by loss of MMP, release of cytochrome c from mitochondria, formation of the apoptosome, and consequently caspase-mediated apoptosis. A number of upstream signals that contribute to mycotixins-mediated mitochondrial activation have been identified, such as p53, MAPKs, ER stress, ROS etc. We will discuss these signaling pathways in more details below.

Autophagy or autophagic cell death induced by mycotoxins

Autophagy is the process by which cells break down their own long-lived proteins and damaged organelles, such as mitochondria (Wang and Klionsky 2011). Activation of autophagy can function either as pro-survival or pro-death signaling (Guo et al., 2013; Solhaug et al., 2014; Yin et al., 2016). Induction of autophagy serves in most cases to promote cell survival. However, increasing evidence has indicated that autophagy induction may trigger an alternative, caspase-independent form of programmed cell death, named autophagic cell death. Autophagy was activated in response to a number of mycotoxins and the functional role of autophagy in mycotoxin-mediated cytotoxicity varies depending on types of mycotoxins (Wang et al., 2014; Tang et al., 2015; Yin et al., 2016; Zhu et al., 2016).

Studies by us or others demonstrated that autophagy induction by fumonisin B1 (FB1) or Citreoviridin (CIT) promotes their cytotoxicity (Yin et al., 2016; Liu et al., 2015). Using MARC-145 monkey kidney cell culture model, we (Yin et al., 2016) found that autophagy is

activated by FB1 at concentrations of 5-20 μM evidenced by increased LC-3 I/II conversion, autophagosome formation and autophagic flux. Inhibition of autophagy by either RNAi or chemical inhibitors led to a significant attenuated cell death induction by FB1. Moreover, we demonstrated that autophagy induction by FB1 is attributed to its ability to disruption of sphingolipid metabolism and activation of IRE1-JNK axis. A study by Liu et al (Liu et al., 2015) using human liver HepG2 cell culture model showed that treatment with CIT at concentrations of 1-5 μM induces an elevated autophagosome numbers and LC-3 II levels accompanied by a reduction of p62, a substrate of autophagy, indicating autophagy was induced by CIT. Suppression of autophagy by knockdown of ATG5 resulted in an ameliorated cell death induction by CIT, suggesting involvement of autophagy-dependent cell death in CIT-induced cytotoxicity.

Apoptosis and autophagy are two important cellular processes. These two events can occur simultaneously in response to some mycotoxins. Regarding the relationship between apoptosis and autophagy induced by mycotoxins, a number of studies have demonstrated that autophagy induction can exert cytoprotective effect to suppress mycotoxin-mediated apoptosis. For examples, Wang et al (Wang et al., 2014) reported that treatment with ZEA at concentrations of 5 to 20 μM results in an increased LC-3I/II conversion accompanied by up-regulation of beclin-1, suggesting that autophagy was activated by ZEA exposure in rat Leydig cells. Inhibition of autophagy by its inhibitor chloroquine led to an enhanced apoptosis, whereas

induction of autophagy by its inducer rapamycin caused a reduction of apoptosis in response to ZEA exposure. The authors therefore concluded that autophagy induction protects rat Leydig cells from ZEA-induced apoptosis. The pro-survival function of autophagy induction has been also found in alternariol-treated RAW264.7 macrophages (Solhaug et al., 2014) and DON-treated pig intestinal epithelial IPEC-J2 cells (Tang et al., 2015). Alternariol treatment induced autophagy in RAW264.7 macrophages through activation of p53 and the Sestrin2-AMPK-mTOR-S6K signaling pathway. Autophagy inhibitor chloroquine significantly enhanced the cytotoxic effects of alternariol compared to the effects of these two compounds given separately. Treatment with DON at concentrations of 1 μg/ml induced a complete autophagy induction in IPEC-J2 cells through regulation of the IKK, AMPK, and mTOR signaling pathways. Compared with the parental IPEC-J2 cells, knockout of the ATG5 gene using double-nicking CRISPR-Cas9 approach increased the DON-induced cell apoptosis or death, suggesting that autophagy plays a protective role in DON-induced cytotoxicity in pig intestinal cells. In addition, it has been also shown that autophagy can promote mycotoxin-induced apoptosis (Wang et al., 2015). Treatment HepG2 cells with CIT led to apoptosis accompanied by autophagy activation. Inhibition of autophagy by knockdown of ATG5 significantly attenuated CIT-induced apoptosis of HepG2 cells, suggesting that autophagy activation contributed to CIT-induced apoptosis in HepG2 cells.

Collectively, the results from these studies indicate that autophagy induction plays an either a protective or cytotoxic role in certain types of mycotoxins-mediated cytotoxicity. The determinants of the functional role (pro-survival vs pro-death) of autophagy in the regulation of cell death remain elusive. However, it is generally believed that excessive autophagy leads to cell death and contributes to the development of pathological conditions. Further studies are needed to clarify the factors that affect the functional role (pro-survival vs pro-death) of autophagy induction in response to mycotoxins exposure and decipher the mechanisms underlying the pro-survival or pro-death action of autophagy induced by mycotoxins.

Necrotic cell death induced by mycotoxins

Previously, necrosis is considered to be a passive cell death and uncontrolled process. However, recent studies have suggested that necrosis can be processed in a programmed mode in certain conditions (Hitomi et al., 2008). In response to mycotoxins exposure, necrotic cell death was commonly observed (Yu et al., 2011; Zhu et al., 2012). Osuchowski et al (Osuchowski and Sharma, 2005) showed that exposure to FB1 at 50 μM for up to 8 days causes necrotic but not apoptotic cell death in both murine microglial BV-2 cell line and primary astrocytes. Yu et al (Yu et al., 2011) reported that in RAW264.7 cells, the cell populations showing high-PI and low-Annexin V/FITC signals are significantly increased from 3.5% to 28% after 50 μM ZEA treatment for 24 h. These results suggest that ZEA-induced cell death may be predominantly necrosis rather than apoptosis. The authors linked the necrotic cell death with AIF relocation and

ROS induction, in which p53 and JNK/p38 MAPK played vital roles as upstream effectors. In the same cell line, another group (Chen et al., 2015) later found that both late apoptotic and necrotic cells are observed after exposure to 30 and 50 μM ZEA for 24 h, which was associated with induction of ER stress. In a similar manner, treatment with ZEA at concentrations of 60 to 120 μM induced both apoptotic and necrotic cell death dose-dependently in procine granulosa cells (Zhu et al., 2012). In animal models, induction of necrotic cell death by mycotoxin in different types of organ has also been reported (Kumagai et al., 1998; Corcuera LA, 2015; Korn et al., 2014). For example, in an acute toxicity study (Kumagai et al., 1998) , oral administration of 1.0 mg/kgBW of AFB1 for 72 h caused an obvious liver necrosis. Apoptosis and necrosis can be induced simultaneously by certain mycotoxins. The predominant type of the cell death in this situation is affected by several factors including cell types, exposure time or dose levels. For example, treatment with ZEA at concentrations of 30 μM for 3 or 7 d induced a predominantly necrotic cell death rather than apoptosis in primary human peripheral blood mononuclear cells (PBMC) (Vlata et al, 2006). However, exposure of ZEA at concentrations of 30-50μM for 12 h resulted in mainly apoptotic cell death in murine RAW 264.7 macrophages (Chen et al, 2015). The present data suggested that mycotoxins can induce necrotic cell death in different cell lines and types of organs apart from apoptosis and autophagic cell death. Further study is needed to determine whether this necrotic cell death induced by mycotoxins is a primary event or a second event of the other programmed cell death.

2. Signaling pathways involved in cell death induction by mycotoxins

p53 signaling pathway

The role of p53 in the regulation of apoptosis is well established (Green and Kroemer, 2009). Activation of p53 generally serves as cytotoxic signal to trigger apoptosis. However, a growing body of evidence has suggested that p53 is also involved in cellular pro-survival response in certain conditions. p53 was activated in response to certain of these food-borne mycotoxins, including aflatoxins (Mulder et al., 2015), OTA (Li et al., 2011; Kuroda et al., 2015), ZEA (Yu et al., 2011), T2 (Fang et al., 2012) and PAT (Saxena et al., 2009; Jin et al., 2016; Boussabbeh et al., 2016a), evidenced by increased p53 phosphorylation and up-regulation of its transcriptional targets such as p21, Bax and PIG3, which is possible attributed to their genotoxicity. Inactivation of p53 by either RNAi approach or chemical inhibitors led to either enhanced or attenuated cell death induction depending on type of cells or types of mycotoxins (Yu et al., 2011; Li et al., 2011; Kuroda et al., 2015).

It has been shown that ZEA or PAT can activate p53 and induce p53-mediated cell death in a number of cell types. Moreover, it seems like the pro-death function of p53 is associated with its pro-oxidant activity. Study by Yu et al (Yu et al., 2011) showed that p53 phosphorylation and ROS are increased by ZEA at concentrations of 10-50 μM followed by cell death induction in RAW264.7 cells. Inhibition of p53 by its inhibitor pifithrin-α led to a significant reduction of

ROS generation and cell death induction by ZEA. Consistent with this notion, our recent study (Jin et al., 2016) revealed that blockade of p53 activation by either chemical inhibitor or genetic approach results in a decreased ROS and cell death induced by PAT. Moreover, we found that the pro-oxidant role of p53 in response to PAT is attributed to its ability to suppress catalase activity through transcriptional up-regulation of PIG3. These data further support a link between the pro-death function of p53 and its pro-oxidant activity.

However, the cytoprotective function of p53 has also been found in response to mycotoxins exposure. Our study on OTA showed that p53 is activated in MARC-145, Vero monkey kidney cells and HEK293 human kidney cells (Li et al., 2011). Inhibition of p53 by knockdown or a chemical inhibitor pifithrin-α resulted in a significant enhanced apoptosis induction, indicating that p53 activation by OTA exerted pro-survival function to inhibit apoptosis induction. Furthermore, we uncovered that the pro-survival activity of p53 is attributed to its ability to suppress JNK activation and JNK-mediated mitochondrial apoptotic signaling (regulation of Bcl-2 family proteins). Consistent with our *in vitro* findings, the pro-survival function of p53 in response to OTA was further confirmed by an *in vivo* study, in which apoptosis induction of renal tubular cells by OTA was significant higher in p53-knockdown mice than that found in p53- proficient mice (Kuroda et al., 2015). As mentioned above, the pro-death function of p53 induced by PAT is associated with its pro-oxidant activity. We speculate that the protective

action of p53 in response to OTA is likely correlated with its anti-oxidant function. This hypothesis is being tested.

Taken together, p53 activation in response to mycotoxins can exert either pro-death or opposite activity (Fig 1). Most of mycotoxins are genotoxic agents. We therefore hypothesize that the levels of DNA damage in response to mycotoxins exposure may be the key determinant for the dual functions of p53 in mycotoxin-induced cytotoxicity. If the DNA damage occurs at low levels, activation of p53 prefers to protect the cell from the death via inducing cell cycle arrest, promoting DNA repair or activating cytoprotective autophagy (Saxena et al., 2009; Chang et al., 2011; Solhaug et al., 2014). Indeed, p53-dependent p21 up-regulation was involved in PAT-induced G1 or S-phase arrest in skin of mice (Saxena et al., 2009) and in CTN induced G2/M phase arrest in HEK293 cells through inhibiting the activity of cdc2/cyclin B1 complex (Chang et al., 2011). If high levels of DNA damage occur, activation of p53 favors to kill the cells rather than protecting them through p53-transcriptional dependent or -independent activation of mitochondrial pathway. These hypotheses need to be tested in the follow-up studies. Understanding of how p53 decides which of these two activities to implement has important practical and clinical significance.

MAPK signaling pathways

The mitogen-activated protein kinase (MAPK) cascades are evolutionary conserved intracellular signal transduction pathways that transmit cell-surface signals to regulatory targets and control diverse cellular processes including proliferation, differentiation and apoptosis (Yang et al., 2013). To date, at least four distinct mammalian MAPK cascades have been identified: extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38, and ERK5. Each one consists of three enzymes, MAPK, MAPK kinase (MAPKK) and MAPK kinase kinase (MAPKKK) that are sequentially activated through phosphorylation.

Activation of MAPKs has been implicated in cytotoxicity induced by several food-borne mycotoxins (Fig 2) (Sharma et al., 2005; Sauvant et al., 2005; Liu et al., 2006; Rumora et al., 2014; Özcan et al., 2015; Agrawal et al., 2015; Yin et al., 2016). JNK and p38, the two stress-activated kinases, have been reported to be activated in response to most of the food-borne mycotoxins such as FB1, OTA, PAT, DON and CTN. Activation of these kinases was shown to play a critical role in these mycotoxins-induced apoptosis in various types of cells. For instance, a study by Sharma et al (Sharma et al., 2005) showed that JNK and TNFα are activated in response to FB1 exposure in murine primary hepatocytes. Inhibition of JNK activation by its specific inhibitor SP600125 resulted in a significant reduction of apoptosis by FB1, suggesting involvement of JNK activation in FB1-mediated cytotoxicity. Our recent (Yin et al., 2016) study also revealed that JNK activation plays an important role in FB1-induced autophagic cell death

in MARC-145 monkey kidney cells. Using rat model, Rumora et al (Rumora et al., 2007) demonstrated that JNK is activated in the livers of rat treated with 0.5 mg FB1/kg b.w. (i.p) for 2 days, followed by activation of ERK and p38 after the treatment for 7days treatment. Similar activation of MAPKs was also found in FB1-treated kidney samples. However, the functional role of MAPKs in FB1-induced toxicity has not been addressed in this *in vivo* study. Similar to the functional role of JNK, cytotoxic role of p38 has also been implicated in mycotoxin PAT, DON or T-2-induced cell death of various types of cells (Liu et al., 2006; He et al., 2012a; Agrawal et al., 2015).

Mechanistic investigation on MAPKs-mediated cell death in response to mycotoxins has demonstrated that activation of mitochondrial pathway is common downstream event in mycotoxin-activated JNK/p38 MAPKs (Yu et al., 2011; Li et al., 2011; Lu et al., 2013；). In addition, activation of p53, PKR, Hck, cathepsin L and increase of the intracellular calcium concentrations have been also reported to contribute to JNK/p38-mediated cell death in response to certain mycotoxins (He et al., 2012a; He et al., 2012b; Rumora et al., 2014).

ERK1/2 also could be activated in response to certain types of mycotoxins, such as OTA (Sauvant et al., 2005; Wang et al., 2012; Özcan et al., 2015), PAT (Liu et al., 2007), citrinin (Rumora et al., 2014). Activation of ERK1/2 has been reported to be involved in either mycotoxin-induced cell cycle arrest or apoptosis in various cell models (Chang et al., 2009; Sauvant et al., 2005; Wang et al., 2012). To date, the functional role of ERK1/2 activation in

mycotoxin-induced cytotoxicity remains controversial. For example, inhibition of ERK1/2 activation led to enhanced cytotoxicity of OTA in opossum kidney (OK) proximal tubule epithelial cells (Sauvant et al., 2005), suggesting that activation of ERK1/2 exerted a protective effect on OTA-induced opossum proximal tubular toxicity. In contrast, a recent study by Özcan et al (Özcan et al., 2015) revealed that ERK1/2 activation by OTA promotes its apoptotic effect on human proximal tubule epithelial-originated cells HK-2 cells, indicating a pro-apoptotic function of ERK1/2 activation in OTA-mediated human proximal tubular toxicity. The possible explanations for this discrepancy may include cell types (opossum kidney (OK) proximal tubule epithelial cells vs human proximal tubule epithelial-originated cells) and dosage levels (1 μM vs 10 μM) used in these two studies. It is assumed that sustained and aberrant subcellular localization of ERK1/2 are the hallmarks of ERK1/2-mediated cell death (Cagnol and Chambard, 2010). Determination of ERK1/2 subcellular distribution may give us a better understanding of OTA-induced ERK1/2-dependent cytotoxic effect.

ER stress

The endoplasmic reticulum (ER) is an important organelle found in eukaryotic cells. The functions of ER mainly include lipid synthesis, calcium storage, protein folding and protein maturation. Perturbations in the ER function owing to increased protein synthesis or accumulation of misfolded proteins lead to a condition termed ER stress. Induction of ER stress activates an evolutionary conserved signaling pathway called the unfolded protein response

(UPR) or ER stress response. The final outcome of UPR is mitigation of ER stress and to re-establish homeostasis (Aguileta et al., 2016). However, prolonged activity of the UPR could lead to cell death that is involved in the pathogenesis of various diseases (Wang and Kaufman 2016). ER stress was induced in response to a number of food-borne mycotoxins such as ZEA (Chen et al., 2015), FB1 (Yin et al., 2016) and PAT (Guo et al., 2013; Boussabbeh, et al., 2015) in different cell types. Activation of ER stress can function as either pro-cell death or pro-survival signaling depending on types of the cells or types of mycotoxins.

A study by us showed that PAT can activate unfolded protein response (UPR) in human keratinocyte HaCaT cells (Guo et al., 2013). Inhibition of ER stress by knockdown of IRE1 or PERK led to an increased apoptosis, indicating that ER stress exerted cytoprotective effect against PAT-induced cytotoxicity in HaCaT cells. A recent study by Boussabbeh et al (Boussabbeh, et al., 2015) demonstrated that ER stress is also activated by PAT in human colon carcinoma HCT116 and human kidney HEK293 cells. Suppression of ER stress by a chemical inhibitor 4-phenylbutyric acid (PBA) offered a protection against PAT-induced apoptosis, suggesting that ER stress triggered pro-apoptotic signaling in these two cell lines tested. The obvious differences between these two studies include cell types, dose levels and the approach of ER stress inhibition. A more precise molecular understanding of differing roles of ER stress in different types of cells is clearly needed.

Reactive Oxygen Species (ROS)

Generation of ROS as an initial signal is involved in the cytotoxicity of a battery of food-borne mycotoxins including AFs (Paul et al., 2015; Liu and Wang 2016), OTA (Petrik et al., 2003), FNs (Stockmann-Juvala et al., 2003), ZEA (Yu et al., 2011), PAT (Zhang et al., 2015; Boussabbeh et al., 2015) and T2 toxin (Zhuang et al., 2013; Agrawal et al., 2015). Possible downstream cytotoxic signaling of ROS induced by these mycotoxins includes p53, MAPK and ER stress activation (Yu et al., 2011; Agrawal et al., 2015; Boussabbeh et al., 2015).

p53 is a redox-sensitive protein and numerous studies have revealed that ROS play an essential role in the regulation of p53 activity via modification of its cysteine residues (Maillet and Pervaiz, 2012). In certain conditions, ROS-mediated p53 activation promotes second round of ROS generation via a positive feedback loop. For example, our recent study (Jin et al., 2016) on PAT showed that ROS generation precedes p53 activation based on the time-course experiment using HEK293 human kidney cells. However, inhibition of p53 led to a decreased ROS level induced by PAT, suggesting involvement of ROS-p53-ROS feedback loop in PAT-induced ROS generation.

Activation of MAPKs is a common event in response to ROS generation induced by mycotoxins. The mechanisms involved in MPAKs activation by ROS are not well understood. The possible mechanisms may include inhibition of MAPK phosphatases and modification of MAPK signaling proteins. Activated MAPKs by ROS generally trigger activation of mitochondrial dependent apoptotic pathway. For example, Shen et al (Shen et al., 2013) found

that treatment with aflatoxin G1 at concentrations of 0.5-10 mg/ml results in a concentration-dependent ROS generation accompanied by activation of JNK and p38 MAPK in A549 cells. Inhibition of JNK or p38 activation by their specific inhibitor led to a significant attenuated apoptosis induction, whereas blockade of ROS by n-acetyl-l-cysteine (NAC), a ROS scavenger, caused a dramatically reduced phosphorylation of JNK and p38. The authors therefore concluded that aflatoxin G1 induces JNK/p38-mediated apoptosis via ROS generation in A549 cells. Similar results were also found in oxidative stress induced by AFB1 (Paul et al., 2015), ZEA (Yu et al., 2011), CTN (Huang et al., 2009) , PAT (Liu et al., 2007) or T-2 toxin (Agrawal et al., 2015). In addition, activation of MAPKs may promote ROS generation induced by some of the mycotoxins (Liu et al., 2007; Yu et al., 2011). For example, Both ROS production and ERK1/2 phosphorylation levels were increased in PAT-treated human embryonic kidney HEK293 cells. Treatment of the cells with Tiron, a free radical scavenger, decreased PAT-mediated ERK1/2 phosphorylation, while treatment of the cells with the ERK1/2 inhibitor, U0126, inhibited PAT-induced ROS (Liu et al., 2007). These results suggest that a positive feedback loop exists between ROS generation and ERK1/2 activation in response to PAT exposure. Similar role of JNK activation in the regulation of ROS has been also found in ZEA-treated RAW264.7 macrophages (Yu et al., 2011).

ROS generation is often linked to ER stress since protein folding is a highly redox-dependent cellular event (Santos et al., 2009). Redox disturbances can be upstream and

downstream of ER stress and a feedback loop can be formed depending on cell type or stimuli (Eletto et al., 2014). Studies have shown that PAT (Boussabbeh, et al., 2015) and ZEA (Ben et al., 2015) can induce ROS-mediated activation of ER stress in various types of cells. However, it has not yet been addressed whether activation of ER stress plays a role in oxidative stress induced by these mycotoxins. Addressing this issue may be helpful to define the functional role of ER stress in cytotoxicity elicited by these mycotoxins.

The mechanisms of ROS generation by these mycotoxins may include increased mitochondrial Ca(2+) loading, mitochondrial electron leakage (Paul et al., 2015), activation of NADPH-oxidase, the arachidonic acid metabolism, induction of CYP450 (Mary et al., 2012), and direct or indirect inhibition of anti-oxidant enzymes (Fliege and Metzler, 2000).

3. Targeting the key signaling pathways for fighting against mycotoxin-induced toxicities

As mentioned above, several signaling pathways have been identified as key players in mycotoxin-induced cytotoxicity. It is reasonable to hypothesize that targeting these signaling pathways maybe a useful approach to manage mycotoxin-induced cytotoxicity. Indeed, a number of studies demonstrated that certain bioactive agents exhibit a promising activity against the cytotoxicity posed by mycotoxins through modulation of the key signaling pathways (Table 3).

Curcumin, a naturally occurring plant phenolic compound isolated from turmeric (Curcuma longa), has been shown to inhibit JNK activation in certain model systems (Chen and Tan, 1998).

Given the established role of JNK activation in FB1-induced cytotoxicity, we (Yin et al., 2016) tested the possibility of curcumin as anti-FB1 agent to protect kidney cells from JNK-mediated cytotoxicity. As we expected, FB1-induced JNK activation was obviously inhibited by curcumin in MARC-145 monkey kidney cells. Consistent with JNK inactivation by curcumin, FB1-induced cell death was significantly decreased in the presence of curcumin. A similar result (Chen and Chan, 2009) was also observed in a study on resveratrol, a member of the phytoalexin family found in grapes and other dietary plants, in which resveratrol offered a protective effect against citrinin (CTN)-induced apoptosis in HepG2 cells via suppression of JNK activation that was required for CTN-induced activation of mitochondrial pathway.

A couple of studies reported that some compounds have protective effects on mycotoxin induced toxicity *in vivo* models. Liao et al (Liao et al., 2014) evaluated the protective role of selenium effect on AFB1 induced liver dysfunction-apoptosis in duckling. Compared to the control group, they found that AFB1 (0.1 mg/kgBW, i.g) significantly up-regulates serum ALT and AST, as well as p53 and caspase-3 protein levels after 14 to 28 days exposure, but sodium selenite (1 mg/kgBW, i.g) could reduce p53 and caspase-3 expression and protect the liver damage. A very recently study showed that curcumin nanoparticles loaded hydrogels can counteract AFB1-induced injury in rat liver and spleen, and suggested that hydrogels are excellent candidates as drug delivery system (Abdel-Wahhab et al., 2016).

Crocin is a natural carotenoid chemical compound that is found in the flowers crocus and gardenia. It has been reported that crocin can prevent ZEA-induced cardiotoxicity in Balb/c mice (Salem et al., 2015). The same group (Boussabbeh et al., 2016a) also showed that crocin has protective effect on PAT-induced oxidative damage in both liver and kidney in Balb/c mice. Silymarin is a natural polyphenolic flavonoid extracted from milk thistle (*Silybum marianum*). A study by Sozmen et al revealed that silymarin can ameliorate liver damage caused by FB1 in BALB/c mice through reduction of the increased VEGF and FGF-2 expression levels (Sozmen et al., 2014). The detailed mechanisms of these in vivo protective effects are not well elucidated. We speculated that the above mentioned signaling pathways are likely to be involved in the protection offered by these bioactive agents.

4. Potential application of mycotoxin-mediated cytotoxicity in cancer therapy

Given the cytotoxicity of mycotoxins, it is reasonable to question that whether mycotoxins are toxic to cancer cells. Indeed, a number of studies have shown that several mycotoxins are able to induce apoptosis in multiple types of cancer cells (Wätjen et al, 2009; Kwon et al, 2012; Boussabbeh et al, 2016b; Chang et al, 2016). For example, Kwon et al (Kwon et al, 2012) revealed that PAT induces caspase-dependent apoptosis in human colon cancer cell line HCT116. Moreover, a recent study by Boussabbeh et al (Boussabbeh et al, 2016b) demonstrated that intraperitoneally administration of PAT for 20 d significantly induces tumor reduction

without potential toxicity in B16F10 cell-implanted mouse model. These results suggested that these mycotoxins merit to further investigation as potential anti-cancer agents.

5. Discussion

Mycotoxins are highly toxic to humans and animals. Cytotoxicity is a major cellular mechanism behind mycotoxin-mediate toxicity. Multiple forms of programmed cell death such as apoptosis, autophagic cell death and necrosis have been found contributing to the cytotoxicity of mycotoxins. A number of signaling pathways including p53, MAPKs, ER stress, ROS have been identified to be involved in mycotoxin-induced programmed cell death. Targeting these key signaling pathways represents a practical and an effective approach against mycotoxin-mediated toxicities. A better understanding of the mechanisms that contribute to cytotoxic effect of mycotoxins is needed for more effectively reducing adverse health effects posed by mycotoxins. To this end, the following issues need to be addressed in the future:

1) The key *in vitro* findings need to be validated *in vivo*, such as autophagic cell death induction by mycotoxins.

2) To determine whether the necrosis induced by mycotoxins is a consequence of apoptosis or an independent programmed necrotic cell death.

3) To examine whether the pro-survival function of p53 associated with its anti-oxidant activity in response to mycotoxins.

4) The detailed mechanisms underlying ROS generation by mycotoxins need to be further defined.

5) The role of the gut microbiome in the cytotoxicity of mycotoxins needs to be investigated.

6) The combined cytotoxic effects of mycotoxins need to be further studied.

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Conflict of interest statement

The authors have no conflicts of interest to disclosure

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Table 1 In vitro mycotoxin-induced death studies

Mycotoxin	Cell line	Dose & Exposure time	Cytotoxicity endpoints	Ref
FB1	African green monkey kidney cells (CV-1)	5 μM, 24 h	DNA ladders, apoptotic bodies increased	Wang et al., 1996
FB1	Rabbit kidney RK13 cell	100-500 nM, 24-48 h	Apoptotic cell, LDH increased	Rumora et al., 2002
FB1	Murine primary hepatocytes	20 μM, 24 h	TNFα receptor, JNK phosphorylation expression increased	Sharma et al., 2005
FB1	Mouse GT1-7 hypothalamic, rat C6 glioblastoma, human U-118MG glioblastoma, and human SH-SY5Y neuroblastoma	0.1-100 μM, 0-144 h	c-aspase 3 and DNA fragmentation increased	Stockmann-Juval et al., 2007
OTA	Human peripheral blood mononuclear cells, human lymphoid cell line, Kit 225	5, 10 μM, 24-48 h	PBMC, caspase 9/3 activation, subG1 cells increased, Bcl-xL decreased	Assaf, et al., 2004
OTA	Primary rat hepatocytes	1 μM, 24 h	Apaf1, FAS, faslg, bad, caspase 8, 9 3/7, polb, p53 dependent apoptosis	Chopra et al., 2010
OTA	Human embryonic kidney cells (HEK293)	20 μM, 24 h	ROS, ASK1 increased, Δψm loss	Liang et al., 2015
T2	Murine embryonic stem cells	0.5, 1, 2 ng/ml	ROS, MMP lose, p53, Bax, c-caspase9/3 increased, Bcl-2 decreased	Fang et al., 2012
T2	Human chondrocytes	1-20 ng/ml, 5 days	ROS, ATP	Liu et al., 2014
HT2	Mouse oocytes	30 nM, 12 h	Bax, caspase-3 mRNA	Zhu et

			expression increased	al., 2016
PAT	HEK293	15 μM, 8 h	losses of SOD, CAT, GSH	Zhang et al., 2015
PAT	HCT116 HEK293	HCT116, 20 μM, HEK293, 15 μM, 24 h	ROS, ER stress, GRP78, GADD34, Bax protein, XBP1s and CHOP mRNA increased, and Bcl-2 protein decreased	Boussabbeh, et al., 2015
DON	mouse thymic epithelial cell line 1	500-2000 ng/ml 48-72 h	P53, ROS, Bax, c-caspase 9/3 expression increased, Bcl-2 expression decreased	Li et al., 2014
DON	Human umbilical vein endothelial cells (HUVEC)	0.84--3.37 μM, 48 h	ROS, c-caspase 9/3 expression, and Bax genes increased, Bcl-2 gene decreased	Deng et al., 2016
AFG1	Human bronchial epithelial cells stably express CYP2A13	80 nM, 24 h	c-PARP, c- caspase-3 increased, ATM, ATR, Chk2, p53, BRCA1, γH2AX activated	Zhang et al., 2013
AFB1	Primary broiler hepatocytes	0.5-5 μM, 6-24 h	Mitochondrial ROS, Nrf2 mRNA, cleaved caspase 9/3 increased	Liu and Wang, 2016
ZEA	RAW264.7 macrophages	10-50 μM 3-24 h	ROS, P53, MAPKs, AIF and Bax expression increased, Bcl-2 expression decreased	Yu et al., 2011
ZEA	Mouse Leydig cells MLTC-1	5-50 μg/ml, 24 h	GRP78, CHOP, cleaved caspase12/3 expression increased	Lin et al., (2015)
ZEA	Mouse endometrial stromal cells	25-125μM, 24 h	Bax/Bcl-2 ratio, c-caspase 9/3 increased	Hu et al., 2016
Ergot alkaloids	Renal proximal tubule epithelial cells, normal human astrocytes	1-20 μM 24-48 h	LDH release, caspase 3 activity, Hoechst 33258 dye positive cells increased	Mulac and Humpf,2011
CIT	HUVEC	0.1-0.2 mg/ml, 24-48 h	Annexinv V positive cells increased, Bax, c-caspase 9/3 increased, Bcl-2 decreased	Hou, et al., 2014

Table 2 In vivo mycotoxin-induced cell death studies

Mycotoxin	Animals/organ	Dose & Exposure time	Cytotoxicity endpoints	Ref
FB1	Sprague-Dawley rats, kidney	1.25 mg/kgBW, i.v. 5 days	Apoptosis and nephrosis	Lim et al., 1996
FB1	ICR mice, liver	10 mg/kgBW, i.p. 5 days	JNK, p38, ERK1/2 activated, c-caspase expression increased	Kim et al., 2007
FB1	Female mice, gastric mucosa	150 mg/kgBW diet, 16 wks	Bax and fragmentation of DNA increased, Bcl-2 decreased	Alizadeh et al., 2015
OTA	Male Wistar rats, kidney	0.12 mg/kgBW, i.g. 10, 30, 60 days	Compare with control group, apoptotic cells in kidney tissue increased values of 5, 6.4 and 12.8 times in 10, 30, 60 days group	Petrik, et al., 2003
DON	Female BALB/c mice, spleen, spleen and mesenteric lymph node, intra-epithelial lymphocytes	0.5, 5 mg/kgBW, i.g. 14 days	Bax expression increased, apoptosis, Bcl-2 expression decreased	Islam, et al., 2013
T2	Mice, thymus, spleen, and liver	2.5 mg/kgBW, i.p. 2 h	DNA fragmentation increased	Ihara et al., 1997
T2	Female BALB/c mice, thymus	0.35, 1.75, or 3.5 mg/kgBW, i.p. 8 h	DNA fragmentation increased	Islam et al., 1998
PAT	Female Swiss albino mice, skin	160 μg/100 μl acetone, Dermal administration,	p53, p21, cytoC, Bax, caspase 3, DNA damage increased	Saxena et al., 2009

		24-72 h		
PAT	BALB/c mice, liver and kidney	3.75 mg/kgBW, i.p. 24 h	p53, cytoC, Bax, caspase 3 expression increased, Bcl-2 decreased	Boussabbeh et al., 2016a
AFB1	SD rat, liver	0.05 mg/kgBW, i.g. 8wks	MDA, NO increased, GSH, Zn, enzyme activities of GSPx and GR decreased, caspase3 activity increased	Meki et al., 2001
AFB1	Broiler chickens,thymus & bursa of fabricius	0.3-0.6 mg/kgBW, formulate AFB diet, 7-21 days	Apoptotic cells, caspase3 in both organ increased; Bax increased, Bcl-2 decreased in the thymus; Bax, Bcl-2 ns. in the bursa of fabricius	Peng et al., 2015
AFB1	Chickens, thymus	0.6 mg/kgBW, AFB1 containing diet, 3wks	ROS, Bax, Bak, cytC ,FasL, Fas, FAAD increased, Bcl-2, Bcl-xL decreased	Peng et al., 2016
ZEA	SD rat, testicular germ cell	5 mg/kgBW, i.p. 3-48 h	TUNEL-labeled germ cells, Fas, Fas-L, and ERα expression increased	Jee et al., 2010
ZEA	Kunming mice, liver	40 mg/kgBW, i.g., 5 days, tissues were collected in the 7th day	ALT, AST, MDA increased, gene and protein levels of Nrf2, GSH-Px, HO-1, γ-GCS, and NQO1 decreased	Long et al., 2016

Table 3 Protective effects of bioactive agents against mycotoxin-induced toxicity in vitro and in vivo models

Mycotoxins	Dose of mycotoxins	In vitro or in vivo models	Bioactive agent	Dose of bioactive agents	Targeted detoxification	Ref
FB1	20 μM, 48 h	Marc145 cell line	Curcumin	20 μM, 48 h	JNK	Yin et al., 2016
FB1	1.5~4.5 mg/kgBW, i.p.21 days	BALB/c mice, liver	Silymarin	100mg/kgBW i.g. 21 days	TNFα, FGF, VEGF	Sozmen et al., 2014
FBs	200 mg FBs/kgBW (68% FB1, 21% FB2, and 11% FB3) diet, 3wks	SD rats, liver and kidney	Royal jelly	100-150 mg/kgBW i.g. 3 wks	GPX, SOD, ALT, AST, TG, cholesterol, HDL, LDL, createnine and uric acid levels	El-Nekeety et al., 2007
FB1 + AFB1	AFB_1 (80 μg /kg BW)+FB_1 (100 μg/kgBW) i.g. 2 wks	BALB/c mice, spleen	Lactic acid bacteria(Lactobacillus paracasei BEJ01)	~2 mg/kgBW, i.g. 2 wks	Caspase-3, GSH, GPx, SOD, IFNγ and TNFα mRNA	Abbès et al., 2016
AFB1	0.125 mg/kgBW i.g. 21 days	SD rats, liver and kidney	Curcumin-nanoparticles-hydrogels	200 mg/kgBW, i.g. 21 days	Body weight, Bax, Bcl-2	Abdel-Wahhab et al., 2016
AFB1	0.1 mg/kgBW	Duckling,	Selenium	1 mg/kgBW,	ALT, AST, Bcl-2, Bax,	Liao et al.,

	i.g. 2-4 wks	liver		i.g. 2-4 wks	caspase-3, p53	2014
AFB1	0.066 mg/kgBW i.g. 30-90 days	Swiss albino mice, kidney	Esculin	100 mg/kgBW i.g. 30-90 days	LPO, GSH, GPx, GR, CAT	Naaz et al., 2014
OTA	10 μM, 24 h, after quercetin treatment	Vero cells	Quercetin	10 μM, 24 h	Nrf2,Ca^{2+},GSH, ROS, caspase 9, caspase 3	Ramyaa and Padma, 2013
OTA	0.4mg/kgBw, i.g. 28 days	Wistar rats, kidneys, liver and heart	Chlorogenic acid	5 mg/kgBw, i.g. 28 days	MDA, SOD, serum creatinine and glucose, urine volume	Cariddi et al., 2016
PAT	3.75 mg/kgBw, 24 h i.p.	BALB/c mice, liver and kidney	Corcin	50, 100, 250 mg/kg BW, 3 h before the PAT administration, i.p.	lipid peroxidation, HSP70, SOD, GSH/GSSG ratio	Boussabbeh et al., 2016a
PAT	1 mg/kg, i.p. 8wks	Kunming mice, Liver and kidney	Selenomethio-nine, sodium selenite	0.2 mg Se/kg of diet	GSSG,ROS, TBARS, GPx, GR	Song et al., 2014
DON	4 μM, 12-24 h	IPEC-J2 intestinal cell line	Resveratrol	50 μM, 12-24 h	Intestinal barrier dysfunction, bacterial	Ling et al., 2016

					translocation	
ZEA	40 mg/kg, 24 h i.p.	BALB/c mice, liver and kidney	Corcin	50-250 mg/kgBW/day, 3 h before the ZEA treatment, i.p.	MDA, SOD, Hsp70	Salem et al., 2015
CTN	30 μM, 24 h	HepG2 cell line	Resveratrol	20 μM, 1 h before CTN treatment	JNK, ROS, PAK2, caspase-9	Chen et al., 2005

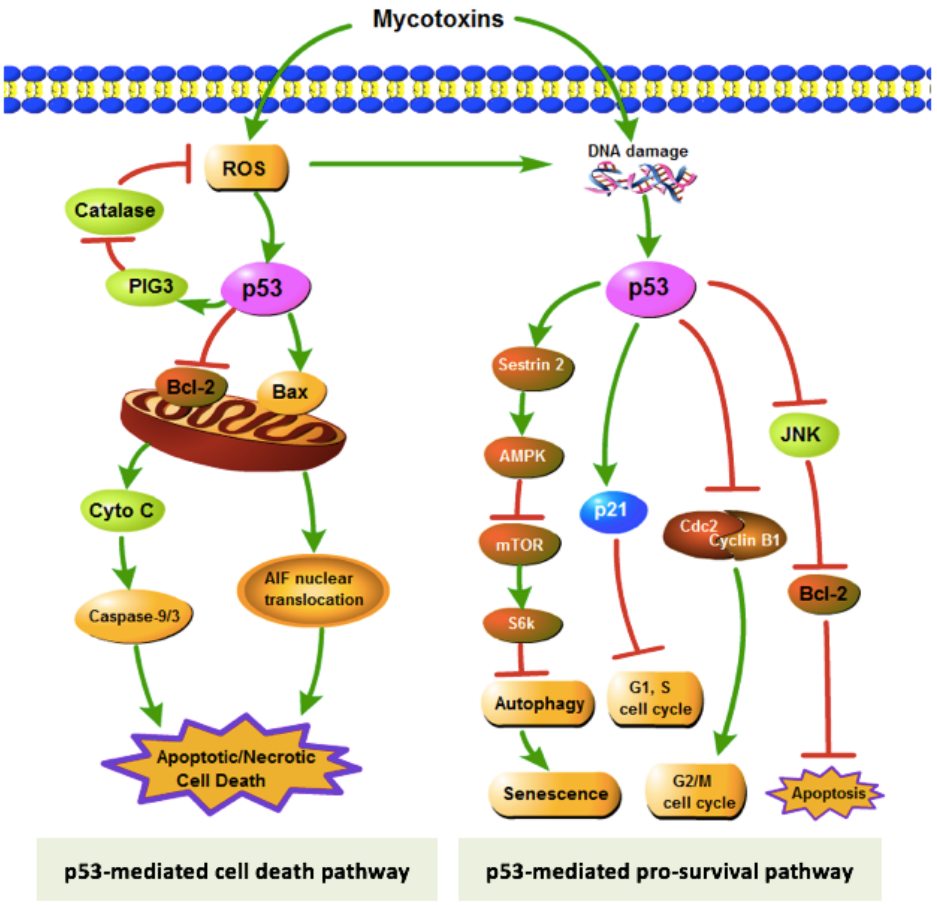

Fig.1 The role of p53 signaling pathways in cytotoxicity of food-borne mycotoxins (compiled from different cell types/lines and tissues). p53 activation in response to mycotoxins can exert either pro-death or pro-survival activity. Mycotoxins induce p53-mediated cytotoxicity through its transcriptional dependent or -independent activation of mitochondrial pathway, leading to caspase-dependent or -independent cell death induction. Mycotoxins cause p53-mediated cytoprotective effect through mechanisms involved in cell cycle arrest, protective autophagy induction or suppression of JNK-mediated pro-death signals.

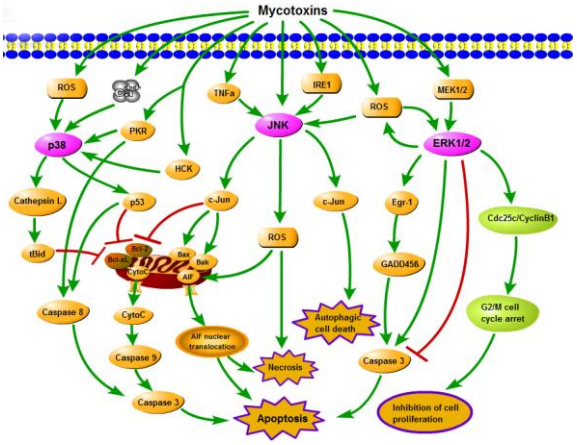

Fig.2 The role of MAPKs signaling pathways in the regulation of cytotoxic effects of food-borne mycotoxin. Activation of JNK, p38 and ERK is a common event in response to mycotoxins exposure. Activation of JNK and p38, the two stress-activated kinases MAPKs generally triggers pro-death signaling through mechanisms involved in induction of p53, activation of mitochondrial pathway and up-regulation of cathepsins; activation of ERK1/2 by mycotoxins can be either cytotoxic or protective possibly associated with duration of its activation and its subcellular localization.