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Protective mechanisms involving enhanced mitochondrial functions and mitophagy against T-2 toxin-induced toxicities in GH3 cells

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ABSTRACT

T-2 toxin is the most toxic member of trichothecene mycotoxin. So far, the mechanism of mitochondrial toxicity and protective mechanism in mammalian cells against T-2 toxin are not fully understood. In this study, we aimed to investigate the cellular and mitochondrial toxicity of T-2 toxin, and the cellular protective mechanisms in rat pituitary GH3 cells. We showed that T-2 toxin significantly increased reactive oxygen species (ROS) and DNA damage and caused apoptosis in GH3 cells. T-2 toxin induced abnormal cell morphology, cytoplasm and nuclear shrinkage, nuclear fragmentation and formation of apoptotic bodies and autophagosomes. The mitochondrial degradative morphologies included local or total cristae collapse and small condensed mitochondria. T-2 toxin decreased the mitochondrial membrane potential. However, T-2 toxin significantly increased the superoxide dismutase (SOD) activity and expression of antioxidant genes glutathione peroxidase 1 (GPx-1), catalase (CAT), mitochondria-specific SOD-2 and mitochondrial uncoupling protein-1, -2 and -3 (UCP-1, 2 and 3). Interestingly, T-2 toxin increased adenosine triphosphate (ATP) levels and mitochondrial complex I activity, and increased the expression of most of mitochondrial electron transport chain subunits tested and critical transcription factors controlling mitochondrial biogenesis and mitochondrial DNA transcription and replication. T-2 toxin increased mitophagic activity by increasing the expression of mitophagy-specific proteins NIP-like protein X (NIX), PTEN-induced putative kinase protein 1 (PINK1) and E3 ubiquitin ligase Parkin. T-2 toxin activated the protective protein kinase A (PKA) signaling pathway, which activated the nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/PINK1/Parkin pathway to mediate mitophagy. Taken together, our results suggested that the mammalian cells could increase their resistance against T-2 toxin by increasing the antioxidant activity, mitophagy and mitochondrial function.

1. Introduction

T-2 toxin is a highly toxic secondary metabolite. It belongs to type A trichothecenes which are produced by different *Fusarium* species (Rocha et al., 2005). T-2 toxin has been detected as contaminates in cereal grains like wheat, barley, oats, maize worldwide and drinking water in endemic areas of China, posing a health hazard to humans and animals (Marin et al., 2013; Sun et al., 2012). Historically, T-2 toxin was identified as the major etiogenic agent of alimentary toxic aleukia (ATA) characterized by nausea, vomiting, diarrhea, leukopenia, hemorrhaging, and sometimes death in many parts of the former Soviet Union during World War II (Bouaziz et al., 2006). T-2 toxin was also implicated with Kashin-Beck disease (KBD), a chronic, endemic type of osteochondropathy that was mainly distributed from northeastern to southwestern China (Lei et al., 2017).

Many studies during the past decades suggested that T-2 toxin was immunosuppressive or immunostimulatory which was dependent on the toxin dose (Wu et al., 2017; Arunachalam and Doohan, 2013). Notably, low T-2 toxin doses promoted the immunostimulatory expression of a diverse array of cytokines *in vitro* and *in vivo* (Wu et al., 2017). Another study also confirmed that T-2 toxin increased the resistance of mice to the bacteria of *Listeria monocytogenes* (*L. monocytogenes*) when mice were treated orally with T-2 toxin and were then infected with *L. monocytogenes* by intraperitoneal injection (Ziprin and McMurray, 1988). Thus, lower T-2 toxin dose increase the host defenses against pathogens.

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A recent report also indicated that T-2 toxin could be effective anti-cancer agent when conjugated to a cancer-specific monoclonal antibody (T-2-mAb), as it effectively and specifically killed the tumor cells both *in vitro* and *in vivo* (Wu et al., 2017). In a study, when T-2 toxin was conjugated to a mAb specific for murine thymoma EL-4 cells, the resulting T-2-mAb markedly inhibited the proliferation of EL-4 cells without inducing cytotoxic effects in the control SP2/0 cells (de Carvalho et al., 2016). All of this suggest that T-2 toxin-mAb conjugates may be useful as selective immunotoxins for cancer immunotherapy, because it is likely to possess fewer serious side effects than free T-2 toxin.

A major toxicity of T-2 toxin is the mitochondrial toxicity, which however, is lack of comprehensive understanding. As the "power house", mitochondria are responsible for oxidative phosphorylation to produce the ATP. This process is executed by five multi-subunits complexes I–V of mitochondrial electron transport chain (ETC) (Distelmaier et al., 2009). The deficiencies in mitochondrial complex I, III and IV have been implicated with many human diseases such as encephalopathy, tubulopathy, liver and heart failure, aging and neurodegenerative disorders (Emerit et al., 2004; Ogilvie et al., 2005). Mitochondria also play significant roles in ROS generation and Ca^{2+} homeostasis, and coordinate extrinsic and intrinsic signals for apoptosis. Available data suggested that T-2 toxin could induce the collapse of mitochondrial membrane potential (Δψm), promote the production of mitochondrial ROS and induce release of apoptotic factors from the mitochondria to cytoplasm (Fang et al., 2012; Wu et al., 2011; Yang et al., 2017). However, the effects of T-2 toxin on mitochondrial biogenesis and mitochondrial genes expression, especially the mitochondrial ETC core subunits, has not been investigated. Previous study has suggested that the alterations in mitochondrial gene expression could alter the coupling efficiency of mitochondrial oxidative phosphorylation, leading to ROS generation and decreased ATP levels (Mu et al., 2013). The side effects of T-2 toxin on mitochondrial ETC core subunits could be a cause of mitochondrial ROS generation.

The cellular protective mechanisms against T-2 toxin remain unclear as well. The enzymatic (SOD, CAT, Gpx) and non-enzymatic (glutathione, GSH) antioxidant systems play major defense roles against oxidative stress (Wu et al., 2013; Yang et al., 2016). However, the protective mechanism involving mitochondria turnover against T-2 toxin in mammalian cells is not known. Mitophagy is a kind of cargo-specific autophagy, which mediates the selective removal of mitochondria. This process is critical for maintenance of mitochondrial quality control and prevention of mitochondrial ROS (Ding and Yin, 2012). It was reported that mitophagy played a critical role in cellular protection against toxicity of ochratoxin A in human embryonic kidney 293 cells (Shen et al., 2014). Three proteins, including the outer mitochondrial membrane (OMM) protein NIP-like protein X (NIX), the OMM kinase PINK1 and the Parkin, play essential roles in the mitophagy (Bingol and Sheng, 2016). PINK1, together with Parkin, work in the same pathway to prevent the pathology by eliminating damaged mitochondria during mitophagy (Bingol and Sheng, 2016). NIX was suggested to be involved in the removal of mitochondria during the maturation of immature red blood cells (Ding and Yin, 2012).

In this study, we aimed to investigate the cellular and mitochondrial toxicity of T-2 toxin, as well as the cellular protective mechanisms in mammalian rat pituitary GH3 cells. We showed that T-2 toxin caused the toxicities characterized by increased ROS, DNA damage, apoptosis, loss of Δψm and a variety of cellular and mitochondrial ultra-structural alterations. However, the GH3 cells increased the cytoplasmic and mitochondrial specific antioxidant activity and increased the mitophagic activities to reduce the mitochondrial ROS generation. The PKA/Nrf2/PINK1/Parkin pathway plays an important role in T-2 toxin-induced mitophagy in GH3 cells. We also showed that T-2 toxin increased the mitochondrial biogenesis, mitochondrial ETC activity, mitochondrial ETC gene expression and ATP levels in GH3 cell.

2. Materials and methods

2.1. Materials

T-2 toxin (CAS NO. 21259-20-1), H89, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and Rhodamine 123 (Rh 123) were purchased from Sigma (Aldrich, Fluka). T-2 toxin was dissolved in dimethyl sulfoxide (DMSO). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Hyclone (Logan, USA). Fetal bovine serum (FBS), antibiotics (penicillin, streptomycin), trypsin-EDTA solution and Opti-MEM reduced serum medium were purchased from Gibco-BRL Life Technologies (Logan, UT). The Annexin V-FITC apoptosis assay kit (abs50001) was obtained from Absin Bioscience Inc. (Shanghai, P.R. China). The rat 8-hydroxy-desoxyguanosine (8 − OHdG) ELISA kit, superoxide dismutase activity assay kit and GSH Assay Kit were acquired from Jiancheng-Bioengineering Institute (Nanjing, P.R. China). The enhanced ATP assay kit (S0027), radio immuno precipitation assay (RIPA) lysis buffer and BCA protein assay kit were purchased from Beyotime Institute of Biotechnology (Nantong, P.R. China). The PrimeScript RT Reagent Kit with gDNA Eraser and SYBR Premix Ex Taq RT-PCR Kit were from TaKaRa (Japan). Mitochondrial complex I activity assay kit was from GENMED Scientifics Inc. (USA). The anti-PINK1 antibody (ab23707), anti-Parkin (ab179812) and anti-Nrf2 antibody (ab31163) were purchased from Abcam (Cambridge, Massachusetts). Anti-LC3A/B rabbit monoclonal antibody (#4108) and anti-Atg3 rabbit polyclonal antibody (#3415) were purchased from Cell Signaling Technology (Beverly, CA). Anti − COX IV mouse monoclonal antibody (#A01060) and anti-β-actin mouse monoclonal antibody were purchased from Abbkine (California, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) antibody and anti-rabbit IgG (H + L) antibody were purchased from Thermo Scientific™ Pierce (USA).

2.2. Cell culture

GH3 cells (Cell Bank of Academy of Sciences, Shanghai, P.R. China) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. After inoculation in cell culture plate for about 24 h, the cells were incubated with or without (control) T-2 toxin for 24 h. GH3 cells passaged 5–15 times were used in the experiments. All experiments were performed at least in triplicate on three separate occasions.

2.3. Dose selection

In the present study, the rat pituitary GH3 cells were used as the experimental system *in vitro* to test the oxidative stress, apoptosis, cellular and mitochondrial alterations and autophagy/mitophagy induced by T-2 toxin. The cytotoxicity of GH3 cells caused by T-2 toxin was evaluated in our previous experiments and the results indicated that T-2 toxin induced a dose- and time-dependent decrease in cell viability and a dose-dependent lactate dehydrogenase leakage in GH3 cells (Liu et al., 2017). The IC_{50} value after 24 h of T-2 toxin exposure was approximately 46.78 nM. To ensure the toxic effects of T-2 toxin while ensuring the cell viability, low and high doses of 10 and 40 nM of T-2 toxin were chosen in this study. The selected doses were comparable to the circulating levels that could be achieved in animals according to the toxicokinetic studies (Osselaere et al., 2013; Sun et al., 2015, 2014), given that the maximal levels of T-2 toxin in barley, oat and oat-based feed, wheat-based bread and pasta, feedstuff samples in Croatia and Bosnia (Pleadin et al., 2017), Italy (Morcia et al., 2016), Spain (Gonzalez-Osnaya et al., 2011) and China (Wang et al., 2013) could reach 304.2, 787, 68.37 and 735 μg/kg, respectively.