CHAPTER I

INTRODUCTION

The fungus *Stachybotrys* species and their associated trichothecene mycotoxins have been recognized as one of potential etiologic agents in outbreaks of sick building syndromes with clinical signs including nasal drainage and congestion, allergies, watering eyes, airway infection, headaches, cough, fatigue, and dizziness (Cooley *et al.*, 1998; Mahmoudi and Gershwin, 2000). Recently, it was reported that people in Japan got poisoning and some died 1-2 days after ingestion of the toxic mushroom, *Podostroma cornu-damae*. Some of the following symptoms were observed in these poisoning such as gastrointestinal disorder, decrease in the number of leukocytes and thrombocytes, and atrophy of the cerebellum which brought about a speech impediment and voluntary movement disorders (Saikawa *et al.*, 2001). The toxic constituents of the mushroom which contributed to the fungus *Stachybotrys atra* poisoning are satratoxin H. Satratoxin H potently inhibited protein synthesis and thymocyte proliferation (Ehrlich and Daigle, 1987; Sorenson *et al.*, 1987). *Stachybotrys atra* containing satratoxin H also caused diseases such as an immune dysfunction (Johanning *et al.*, 1996) and idiopathic pulmonary hemorrhage in infants (Mahmoudi and Gershwin, 2000). In addition to the potent toxicity on the immune system, recent reports suggested a possible relationship between trichothecenes and disorders of central nervous system. There was a severe loss of neurons of the brain in the cerebral hemisphere, the brain stem, Purkinje cells of the cerebellum, and the dorsal and ventral nervous tissue of the spinal cord (Croft *et al.*, 2002; Johanning *et al.*, 1999). Despite of the possible harmfulness, the mechanisms of action of trichothecene mycotoxins, satratoxin H, on central nervous system still remain unclear.

Rationale of the study

Trichothecenes have been found to cause toxicity to many organs lead to several symptoms including diarrhea, weight loss, neuronal changes, skin toxicity, and immune disorders (Kuhn and Ghannoum, 2003). Macrocyclic trichothecenes were shown to be 10 to 100 times more toxic than type A and type B trichothecenes in various toxicity testing systems such as toxicity studies in mice, HeLa cell cytotoxicity test, and rabbit reticulocyte protein synthesis inhibition assay (Ueno,

1989). In addition, macrocyclic trichothecenes including satratoxin H had more potent immunomodulatory effect than 8-keto trichothecene, and vomitoxin, a common food contaminant (Lee and Li, 1999).

Trichothecene mycotoxins, lipophilic chemicals, entered the cells by moving across plasma membrane, and interact with ribosomes (Middlebrook and Leatherman, 1989) and mitochondria (Pace *et al.*, 1988). Trichothecenes, such as T-2 toxin, nivalenol, have been shown to induce apoptosis via inhibition of protein synthesis by binding to the ribosomal peptidyl transferase site following by activation of p38 mitogen-activated protein kinases and c-Jun NH_2-terminal kinase (JNK) in the Jurkat human T-lymphoid cell line (Shifrin and Anderson, 1999), and also extracellular signal-regulated protein kinase (ERK1/2) in RAW 264.7 murine macrophages (Yang *et al.*, 2000). Trichothecenes including satratoxin H, inhibitors of protein synthesis (Sorensen *et al.*, 1987), have been shown to induce apoptosis by activation of JNK in RAW 264.7 murine macrophages (Yang *et al.*, 2000). Additionally, the stimulation of JNK and p38 kinases has been shown to be prerequisited for stress-induced apoptosis (Verheij *et al.*, 1996; Xia *et al.*, 1995). Oral administration of T-2 toxin in rats caused an increase of lipid peroxidation in liver, this suggested some alteration in membrane structure (Suneja *et al.*, 1989). Moreover, 4-acetyl-12, 12-epoxyl-9-trichothecene-3, 15-diol (AETD), a trichothecene mycotoxin from the fruiting bodies of *Isaria japonica* Yasuda, have been reported to induce apoptosis in human leukemia HL-60 cells via the generation of intracellular reactive oxygen species (ROS) concurrently caused depletion of intracellular reduced glutathione (GSH) which can be protected by *N*-acetyl cysteine (NAC) (Pae *et al.*, 2003). In addition, T-2 toxin stimulated lipid peroxidation and consequently decreased hepatic GSH content which can be protected by vitamin E (Rizzo *et al.*, 1994). Several lines of evidences showed that free radicals could activate JNK and p38 kinase following by cytochrome *c* release from mitochondria and caspase activation (Junn and Mouradian, 2001; Zang *et al.*, 2003). Interestingly, there is no evidence in satratoxin H-induced apoptosis using other cellular models except the immune cells.

The PC12 culture, a rat phreochromocytoma cell line, is widely used as a model system for neurobiologic and neurochemical studies including apoptosis. For example, PC12 cells have been used to investigate the protective effect of the oligosaccharides, extracted from *Morinda officinalis* as the mechanism of the

antidepressant action, against the corticosterone-induced apoptosis (Li *et al.*, 2003). Since satratoxin H can cause neurotoxicity, PC12 cells will be used as a cellular model to study for satratoxin H-induced apoptosis. The mechanism underlying satratoxin H-induced apoptosis such as generation of ROS and lipid peroxidation following by activation of mitogen-activated protein kinases (MAPKs) leading to apoptosis will be elucidated. In addition, antioxidants such as vitamin E, NAC, or GSH are proposed whether they could protect satratoxin H-induced apoptosis in PC12 cells.

Conceptual framework

Satratoxins belong to a macrocyclic trichothecene possessing 9, 10 double bond, a 12, 13 epoxide group and a cyclic diester or triester ring linking C-4 to C-15. Trichothecenes are toxic to various important tissues including immune system via stimulation or suppression of mediators in immune system such as cytokines, gastrointestinal system and central nervous system.

T-2 toxin caused lipid peroxidation by increasing lipid peroxides and toxic aldehydes in rat liver (Rizzo *et al.*, 1994), spleen and brain (Atroshi *et al.*, 1995) concurrently decreased GSH content can be protected by α-tocopherol. Study in immune cells showed that trichothecenes including T-2 toxin, verrucarin A and satratoxin G stimulated apoptosis via MAPKs (Shifrin and Anderson, 1999; Yang *et al.*, 2000). The trichothecene mycotoxin AETD induced apoptosis via the generation of ROS following by stimulation of mitochondrial pathway such as caspase 3, caspase 8, caspase 9 and DNA fragmentation factor 40 (DFF-40) protected by NAC (Pae *et al.*, 2003).

Satratoxin H, a fungal metabolite in macrocyclic trichothecene which was more toxic than the others, at a concentration of 10 nM has been shown to induce apoptosis via stimulation of JNK in RAW 264.7 murine macrophage and U937 human leukemic cells (Yang *et al.*, 2000). Up to date the molecular mechanism of trichothecenes especially satratoxin H-induced apoptosis on nervous system has not been investigated. PC12 cells, a dopaminergic model system, are used in the present study. The previous studies strongly suggested that satratoxin H might induce apoptosis in PC12 cells via the generation of ROS and/or lipid peroxidation following by

activation of MAPKs. ROS and lipid peroxidation induced by satratoxin H might be potentially protected by antioxidants such as vitamin E, NAC or GSH.

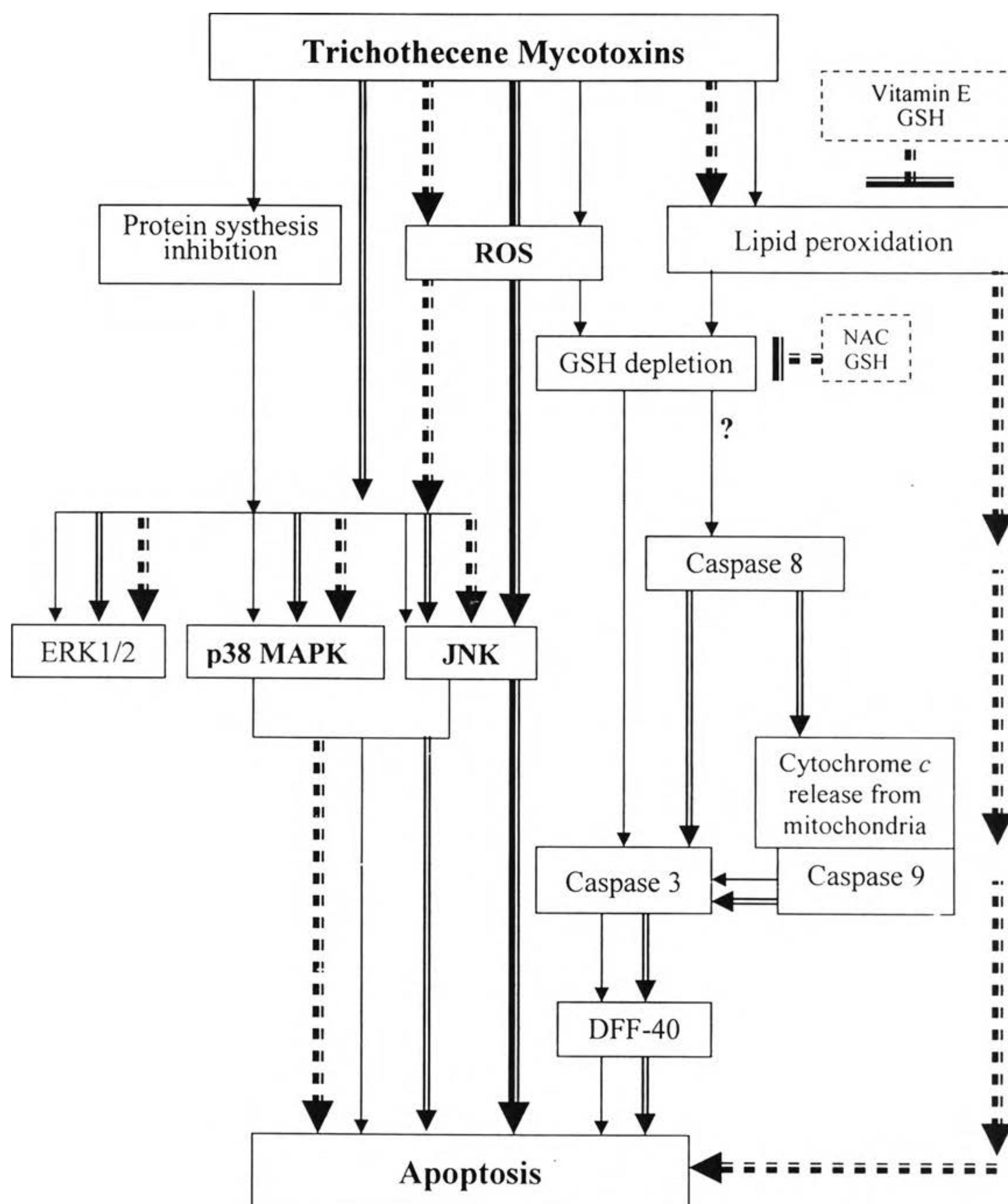

Figure 1 Proposed diagram of signaling pathways of trichothecene-induced apoptosis

- (thin arrow) Type A and B trichothecenes
- (double arrow) Satratoxin G
- (thick arrow) Satratoxin H
- (dashed arrow) No experimental evidence in satratoxin H-induced apoptosis

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Objectives

1. To clarify the molecular mechanism of satratoxin H-induced apoptosis in PC12 cells
2. To determine the protective effect of antioxidant(s) in satratoxin H-treated PC12 cells

Scope of study

The present study will cover the *in vitro* assay for satratoxin H-induced apoptosis using rat phreochromocytoma PC12 cells as a cellular model. The signaling pathway involved apoptosis via MAPKs, the generation of ROS and lipid peroxidation will be evaluated.

Experimental design

To investigate the molecular mechanism of satratoxin H-induced apoptosis, PC12 cells are used. The methods are designed as the followings. Cell viability is measured by MTT assay. Apoptotic cell death is detected by fluorescence microscopic assay staining with Hoechst 33342, DNA fragmentation analysis measuring by agarose gel electrophoresis and flow cytometry staining with propidium iodide (PI). Mechanistic study of the MAPKs activation using MAPKs inhibitors is determined by MTT assay and Western blot analysis. Satratoxin H-generated ROS and lipid peroxide production is investigated by flow cytometry using 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) and by thiobarbituric acid-reactive substances (TBARS), respectively. The protective effect of antioxidant(s) on satratoxin H-treated PC12 cells is also performed.

Contributions of the study

1. Understanding the role of satratoxin H in PC12 cells, which might explain the pathology of cell death.
2. Information for the medical treatment of satratoxin H, which might be protected by antioxidant(s).
3. A novel finding and technical experiences in cellular and molecular biology