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Protective Effects of Metallothionein-III on Oxidative Stress-Induced Neuronal Cell Death

조 선 대 학 교 대 학 원

약 학 과

황 용 필

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조 선 대 학 교 대 학 원

약 학 과

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지도교수 정 혜 광

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황 용 필

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List of Abbreviations

6-OHDA	6-hydroxydopamine
AD	Alzheimer's disease
ARE	Antioxidant response element
BSA	Bovine serum albumin
DMEM	Dulbecco's modified Eagle's Medium
DMSO	Dimethylsulfoxide
Dox	Doxorubicin
EMSA	Electrophoretic mobility shift analysis
ERK1/2	Extracellular signal-related kinase1/2
ESI-TOF MS	Electrospray Ionization Time-of-flight Mass Spectrometry
FBS	Fetal bovine serum
GIF	Growth inhibitory factor
GST	Glutathione-S transferase
HO-1	Heme oxygenase-1
HPLC	High performance liquid chromatography
ICP- AES	Inductively Coupled Plasma-Atomic Emisssion Spectrometer
IKK1	IκB kinase 1
IκB	Inhibitor κB
JNK1/2	c-Jun N-terminal kinase1/2

MAPK	Mitogen-activated protein kinase
MT-III	Metallothionein-III
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NF-κB	Nuclear factor κB
Nrf2	NF-E2-related factor 2
PBS	Phosphate-buffered saline
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PMA	Phorbolmyristate acetate
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacryamide gel electrophoresis
TFA	Trifluoroacetic acid
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling

ABSTRACT

Protective Effects of Metallothionein-III on Oxidative Stress-Induced Neuronal Cell Death

Hwang Yong Pil

Advisor: Prof. Jeong Hye Gwang, Ph.D.

Department of Pharmacy,

Graduate School of Chosun University

Neuroprotecion by metallothionein-III involves activation of nuclear factor-κB through phosphatidylinositol-3 kinase signaling pathway

Metallothionein (MT) gene family consists of several members (MT-I, -II, -III, IV) that are tightly regulated during development. Human MT-I and MT-II are expressed in many tissues, including the brain, whereas human MT-III is expressed mainly in the central nervous system. Human MTs are thought to play roles both in the intracellular regulation of the essential trace elements (zinc or copper) and in the protection from a variety of stress conditions. The zinc-binding protein MT-III is associated with resistance to neuronal injury. However, the underlying mechanism for its effects is unclear. The present study investigated the mechanisms of how MT-III protects neuronal cells from hypoxia- or DNA damage-induced cell death. MT-III reduced the hydrogen peroxide- or DNA damage-induced effects on

neuronal cell including cell death, the activation of caspase-3 and -9, and the release of mitochondrial cytochrome c to the cytoplasm in a does-dependent manner. In the meantime, MT-III increased the activation of Akt, the phosphorylation and degradation of IκB, the nuclear translocation/accumulation and the transcriptional activity of NF-κB in neuronal cells in a dose-dependent manner. The MT-III-induced anti-apoptotic effects and increase in NF-κB activity were blocked by specific inhibitors of PI3K, Akt, or NF-κB, indicating that MT-III provides neuronal protection by activating NF-κB through PI3K/Akt signaling pathway.

Metallothionein-III protects against 6-hydroxydopamine-induced oxidative stress by increasing expression of heme oxygenase-1 in a PI3K and ERK/Nrf2 dependent manner

Parkinson's disease (PD) is a neurodegenerative movement disorder characterized by cell loss in the substantia nigra resulting in striatal dopamine depletion. Enhanced oxidative stress is implicated in the pathogenesis of PD. One mechanism by which cells defend themselves against oxidative stress is through the transcriptional upregulation of cytoprotective genes. Under oxidative stress conditions, the transcription factor NF-E2-related factor (Nrf2) binds to the antioxidant response element (ARE) to induce antioxidant and phase II detoxification enzymes, such as heme oxygenase-1 (HO-1). The catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) induces the production of reactive oxygen species (ROS), leading to neuronal cell death. Metallothioneins (MTs) are low molecular weight, metal-binding proteins with established antioxidant capabilities. The expression and induction of MTs have been associated with protection against DNA damage, oxidative stress and apoptosis. However, the underlying mechanism for their effects is unclear. The present study demonstrated that MT-III prevents the accumulation of ROS, caspase-3 activation, and subsequent cell death in dopaminergic SH-SY5Y cells challenged with the PD-related neurotoxin 6-OHDA. The mechanism involves phosphatidylinositol 3-

kinase (PI3K)/Akt and ERK1/2-dependent induction of the stress response protein HO-1. Pretreatment of SH-SY5Y cells with MT-III significantly reduced 6-OHDA-induced generation of ROS, caspase-3 activation, and subsequent cell death. MT-III up-regulates HO-1 expression and this expression confers neuroprotection against oxidative injury induced by 6-OHDA. Moreover, MT-III induces Nrf2 nuclear translocation, which is upstream of MT-III-induced HO-1 expression, and PI3K/Akt and ERK1/2 activation, a pathway that is involved in induced Nrf2 nuclear translocation, HO-1 expression and neuroprotection. Taken together, these results suggest that MT-III augments the cellular antioxidant defense capacity by inducing HO-1 via the PI3K and ERK/Nrf2 signaling pathway, thereby protecting cells from oxidative stress. Collectively, these data provide evidence that MT-III prevents the degeneration of dopaminergic neurons by 6-OHDA and may prove useful in the treatment of PD.

I. Introduction

Neuroprotecion by metallothionein-III involves activation of nuclear factor-κB through phosphatidylinositol 3-kinase signaling pathway

Metallothioneins (MT) are a group of low molecular weight (6-7 kDa), intracellular metal-binding proteins that are expressed in a wide range of eukaryotic species from yeast to mammals (Kagi and Schaffer, 1988; Kagi, 1991; Vallee, 1991; Vasak and Hasler, 2000). MT-I and MT-II are ubiquitously expressed, whereas MT-III and MT-IV are expressed mainly in the central nervous system and the squamous epithelia, respectively (Ebadi *et al*., 1995; Aschner, 1996; Hozumi *et al*., 1998; Ghoshal and Jacob, 2001). Like other members of the MT family, MT-III contains 20 highly conserved cysteine residues and binds copper and zinc. However, unlike the predominant MT-I or -II isoforms, the expression of MT-III is not induced by typical MT inducers such as zinc, kainic acid, glucocorticoids, interferons, and lipopolysaccharides (Hidalgo and Carrasco, 1998; Samson and Gedamu, 1998; Jacob *et al*., 1999; Hernandez *et al*., 2000; Haq *et al*., 2003). Furthermore, MT-III is unique in regard to its biological activities. MT-III was originally isolated from the brain and named growth inhibitory factor (GIF) based on its ability to inhibit the tropic effects of Alzheimer's disease brain extract on rat embryonic cortical neurons in culture (Hidalgo *et al*., 2001, 2006; Carrasco *et al*., 2006) Structural and

functional studies have shown that a unique sequence (Cys6ProCysPro9) at the N-terminal domain of MT-III is necessary and sufficient for its growth inhibitory activity (Irie and Keung, 2003). However, studies on the regulation of MT-III activity in several animal brain injury models suggested that this isoform is involved in reparative and/or protective processes in the brain (Hozumi *et al*., 2006). Moreover, it has been recently reported that MT-III deficient mice are more susceptible to kainic acid-induced seizures and suffer more severe neuronal injury than normal mice, and that MT-III transgenic mice are more resistant to neuronal injury (Erickson *et al*., 1995). Although accumulating evidences suggest that MT-III could play neuroprotective roles, the underlying mechanism remains unclear.

Nuclear factor κB (NF-κB) transcription factors play important roles in regulating cell survival and differentiation as well as inflammatory and immune responses (Karin and Lin, 2002). In most cell types, NF-κB is present as a heterodimer comprising p50 and p65 subunits and it is sequestered in the cytoplasm by tightly bound inhibitory proteins IκBs. NF-κB translocation to the nucleus occurs only after stimuli-induced degradation of IκB. This process requires the activation of a kinase cascade that converges on IκB kinase 1 (IKK1) and IKK2, which form a complex with NF-κB/IκB. IKKs subsequently phosphorylate IκB proteins and target IκBs for ubiquitination and proteosomal degradation (Zandi *et al*., 1998Schmitz *et al*., 2001; Sun and Andersson, 2002; Delhase *et al*., 1999; Zandi *et*

al., 1998). Upon activation, NF-κB induces transcription of a number of anti-apoptotic genes which interrupt the apoptotic cascade at multiple levels (Shishodia and Aggarwal, 2002; Monks *et al*., 2004).

Akt has been also shown to enhance the degradation of IκBs and to cooperate with other factors to increase the transcriptional activity of NF-κB (Kane *et al*., 1999; Madrid *et al*., 2000, 2001). Akt is necessary and sufficient for platelet-derived growth factor- or tumor necrosis factor-induced increase of NF-κB transcriptional activity (Reddy *et al*., 2000; Habib *et al*., 2001). It has been proposed that Akt may regulate the activity of NF-κB through direct interaction with IKKs, as Akt can associate with the IKK complex and phosphorylate and activate IKKα at a critical regulatory site Thr-23 (Ozes *et al*., 1999). Although the molecular details of how Akt regulates the IKK complex remain to be characterized, these results implicate Akt as a part of the survival signaling pathway that induces NF-κB activation. Typically, Akt is activated by phosphatidylinositol 3-kinase (PI3K) signaling pathway that enhances cell survival, cell growth, and glucose metabolism. In neuronal cells, Akt has been shown to prevent apoptosis and promote cell survival through the phosphorylation of proapoptotic Bad (Datta *et al*., 1997), procaspase-9 (Cardone *et al*., 1998), and eNOS (Luo *et al*., 2000) and through the activation of NF-κB (Ozes *et al*., 1999).

In the present study, the mechanism of how MT-III regulates neuronal cell survival was investigated. The results clearly indicated that MT-III protects Neuro2A cells from H_2O_2- and doxorubicin-induced neurotoxicity by activating Akt through the PI3K signaling pathway and subsequently up-regulating NF-κB transcriptional activity and cell survival.

Metallothionein-III protects against 6-hydroxydopamine-induced oxidative stress by increasing expression of heme oxygenase-1 in the PI3K and ERK/Nrf2 dependent manner

Metallothionein (MT)-III is a metal binding protein (Uchida *et al*., 1991; Uchida, 1994), also known as a growth inhibitory factor, and is a brain specific isomer of MT (Uchida *et al*., 1991). MT-III resembles other members of the MT family as it is small (68 amino acids), contains 20 cysteine residues in conserved positions, and binds to zinc and copper (Uchida *et al*., 1991; Palmiter *et al*., 1992). In previous studies, MT-III exhibited free radical scavenging activity and regulatory control of zinc metabolism in the brain to protect neurons against toxic metals (Masters *et al*., 1994; Hussain *et al*., 1996). Although MT-III was originally discovered as a growth inhibitory substance related to Alzheimer's disease (AD) (Uchida *et al*., 1991) and is abundantly present in the normal brain, the level of MT-III is markedly reduced in the brains of patients with AD (Uchida *et al*., 1991; Tsuji *et al*., 1992). Several reports suggest that MT-III is related to neuronal loss in neurodegenerative diseases (Uchida, 1994; Aoki *et al*., 1998; Hozumi *et al*., 1998). Based on these reports, there is a growing interest in its relationship to neurodegenerative diseases. However, the function and regulation of MT-III are poorly elucidated in Parkinson's disease.

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by progressive degeneration and subsequent selective loss of dopaminergic neurons in the substantia nigra. Although the etiology of PD remains obscure, previous studies have shown an increase of lipid peroxide and iron levels and a decrease of reduced glutathione in the substantia nigra of PD patients (Sian *et al*., 1994). In addition, it has been reported that the auto-oxidation and enzymatic oxidation of dopamine lead to the generation of reactive oxygen species (ROS) (Cohen and Heikkila, 1974; Graham, 1978; Hastings, 1995). 6-Hydroxydopamine (6-OHDA), a selective catecholaminergic neurotoxin, is widely used to produce animal models of PD. 6-OHDA is also used to study mechanisms of cell death in dopaminergic neurons. Nonenzymatic auto-oxidation of 6-OHDA generates several toxic oxidative species including quinines (Saner and Thoenen, 1971), superoxide radicals, hydrogen peroxide and the highly reactive hydroxyl radical (Cohen and Heikkila, 1974). These products cause lipid peroxidation (Kumar *et al*., 1995), DNA damage (Bruchelt *et al*., 1991), disorganization of the cytoskeleton (Davison *et al*., 1986), mitochondrial dysfunction (Glinka and Youdim, 1995) and eventually cell death. Several studies have shown that the catecholaminergic human neuroblastoma cell line SH-SY5Y is quite sensitive to 6-OHDA (Tiffany-Castiglioni and Perez-Polo, 1981; Spina *et al*., 1992). *In vivo* and *in vitro* experiments have revealed that antioxidants prevent the loss of dopaminergic

neurons caused by 6-hydroxydopamine (6-OHDA), which has been used to establish the experimental PD model. Based on these facts, enhanced oxidative stress is thought to be responsible for the progression of dopaminergic neurodegeneration.

ROS formation is a naturally occurring process. Mammalian cells have developed several protective mechanisms to prevent ROS formation or to detoxify ROS. These mechanisms employ molecules called antioxidants as well as protective enzymes (Chen and Kunsch, 2004a). Among the various cytoprotective enzymes, heme oxygenase-1 (HO-1) has recently been highlighted by virtue of its hepatoprotective roles (Guo *et al*., 2001; Selzner *et al*., 2003; Kapitulnik, 2004; Takahashi *et al*., 2004). HO-1 is a novel enzyme with potent anti-inflammatory, antioxidant, and anti-proliferative effects (Maines *et al*., 1998; Yamada *et al*., 2000; Tulis *et al*., 2001). HO-1 is the rate-limiting enzyme in the conversion of heme into biliverdin, releasing free iron and carbon monoxide. Biliverdin is rapidly metabolized to bilirubin, which is a potent antioxidant. There is a large body of evidence suggesting that HO-1 plays a key role in maintaining antioxidant homeostasis during cellular stress (Alam and Cook, 2003; Choi *et al*., 2003; Otterbein *et al*., 2003). The induction of the HO-1 gene is primarily regulated at the transcriptional level, and its inducibility by various inducers is linked to the transcription factor NF-E2-related factor 2 (Nrf2) (Alam and Cook, 2003). Under

normal conditions, Nrf2 is sequestered in the cytoplasm by binding to Keap1, an actin-binding protein (Itoh *et al*., 2004). Several electrophilic antioxidants cause the disruption of this complex, freeing Nrf2 for translocation to the nucleus. Nrf2, in turn, binds to antioxidant response element (ARE) sequences in the HO-1 promoter (Kong *et al*., 2001; Owuor *et al*., 2002). The mechanism by which Nrf2 is liberated from the Keap1-Nrf2 complex remains to be established. However, recent studies have suggested that Nrf2 nuclear translocation requires the activation of several signal transduction pathways, including mitogen-activated protein kinases (MAPKs) (Kong *et al*., 2001), protein kinase C (PKC) (Huang *et al*., 2000; Numazawa *et al*., 2003), and phosphatidylinositol 3-kinase (PI3K) pathways (Nakaso *et al*., 2003). In view of the growing evidence that HO-1 provides hepatoprotection (Jaeschke, 2000; Selzner *et al*., 2003; Takahashi *et al*., 2004), HO-1 expression by a pharmacological modulator may represent a novel target for therapeutic intervention. In particular, the identification of a non-cytotoxic inducer of HO-1 may maximize the intrinsic antioxidant potential of cells.

This study investigated the neuroprotective effects of MT-III against the pro-parkinsonian neurotoxin 6-OHDA-induced neurotoxicity in human dopaminergic neuroblastoma SH-SY5Y cells. The results indicated that MT-III protects SH-SY5Y cells from 6-OHDA-induced neurotoxicity through the PI3K/Akt and

ERK1/2 signaling pathway, subsequently up-regulating Nrf2 transcriptional activity and cell survival.

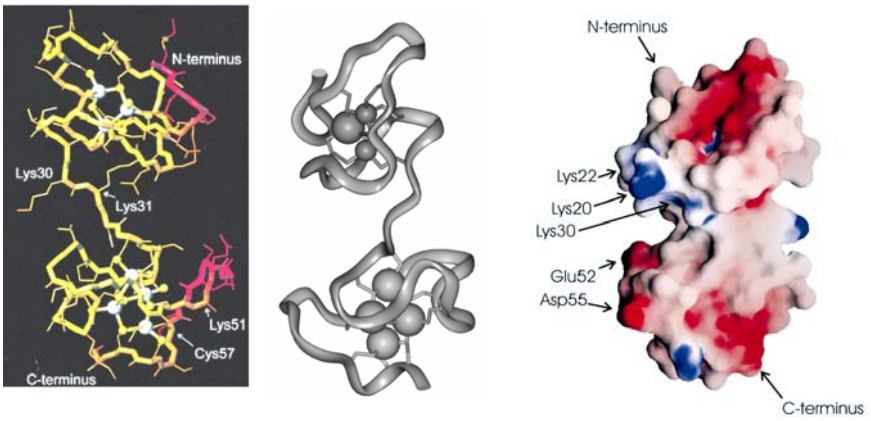

Fig. 1. Stereo drawing of the metallothionein crystal structure and electrostatic potential map of the surface of metallothionein. Red; negative charged. blue; positively charged (Zangger and Armitage, 2002: Vasak and Hasler, 2000).

II. Materials & Methods

1. Materials

Reagents used in this study were purchased from the following sources: doxorubicin, H_2O_2, 6-hydroxydopamine (6-OHDA), and zinc protoporphyrin (ZnPP) from Sigma (St. Louis, MO); MTT-based colorimetric assay kit from Roche (Indianapolis, IN); LipofectAMINE Plus, RPMI 1640, fetal bovine serum, and penicillin-streptomycin solution from Gibco-BRL/Life Technologies (Grand Island, NY); pGL3-4κB-Luc, pCMV-β-gal, and the luciferase assay system from Promega (Madison, WI); antibodies for phospho-Akt (ser473), phospho-MAPK (ERKl/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185) and HRP-linked anti-rabbit IgG from Cell Signaling Technology (Beverly, MA); antibodies for β-actin and HO-1 was obtained from Calbiochem (La Jolla, CA); antibodies for α-tubulin, lamin-B1, IKKβ, IκBα, p65, p53, Nrf2, cytochrome c, and phospho-IκBα (Ser32) from Santa Cruz Biotechnology (Santa Cruz, CA); Western blotting detection reagents (ECL) from Amersham Pharmacia Biotech (Piscataway, NJ). HO-1-ARE-luciferase reporter gene was kindly provided by Dr. J. Alam (Tulane University School of Medicine, New Orleans. LA). All other chemicals were of the highest commercial grade available.

2. Expression and purification of human MT-III

The method has been described previously (You *et al*., 2002b). Briefly, MT-III cDNA was constructed in pGEX-4T and was expressed in *E. coli* BL21. MT-III was purified by affinity chromatography on glutathione-Sepharose 4B. The Zn content in the MT-III was about 5% as determined by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP- AES, Perkin Elmer). Apo-MT-III was prepared from crude Zn-MT-III by cation-exchange and reverse-phase chromatographies. The crude Zn-MT-III fractions were then supplemented with 1 mM DTT, acidified to pH 2.5 with HCl, and applied to a 6-ml Resource S column pre-equilibrated with 20mM Na_3PO_4 (pH 2.5) and 20% acetonitrile (eluent A). Proteins were eluted with a gradient of 0–20% eluent B (2M NaCl in eluent A) over 30 column volumes (CV). The material in the peak fractions was collected, analyzed by ESI-TOF MS, and further purified. The final purification of apo-MT-III was carried out on a reverse-phase HPLC column (Vydac C18, 22_250 mm; the Separation Group, Hesperia, CA). Eluent A was 0.1% TFA, eluent B was 0.1% TFA/95% acetonitrile, and the gradient was 22–32% B in 7 CV. MT-III eluted between 27.7 and 28.3% acetonitrile. The molecular mass and the purity of the product were determined by ESI-TOF MS. Apo-MT-III was lyophilized and reconstituted for experiments.

3. Cell culture and cell viability assay

The mouse neuroblastoma Neuro2A and human neuroblastoma SH-SY5Y cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine

serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. MT-III was dissolved in H_2O. Primary hippocampal neurons were cultured as described before with some modifications (Chen and Bazan, 1999). Briefly, hippocampi were dissected from the brains of embryonic day 18 (E18 d) ICR mouse fetuses which were rapidly decapitated, and hippocampi were immediately dissected out from the brain. The tissue was incubated in oxygenated trypsin for 10 min at 37°C and then mechanically triturated. Cells were spun down and resuspended in Neurobasal-B27 medium (Invitrogen) supplemented with 0.5 mM L-glutamine, penicillin-streptomycin and 25 μM glutamate. Cells (1 x 10^6) were loaded into poly D-lysine-coated 24-well culture plates (BD Biosciences). One-third to one-half of the culture medium without glutamate was changed every 2-3 days. One-week-old hippocampal neuronal cultures pretreated with varying concentrations of MT-III for 6 hr were exposed to 30 μM H_2O_2 in HBSS for 24 hr at 37°C. Cell viability was examined using MTT-based assays according to the manufacturer's instructions.

4. ROS production

ROS production in SH-SY5Y cells was measured using the redox-sensitive fluorescent dye H_2DCFDA. After treatment with 75 μM 6-OHDA or vehicle for 7–8 hr, cells were incubated with 25 μM H_2DCFDA for 20 min. The cells were rinsed

twice with phenol-red-free DMEM containing 1% FBS, and fluorescence was detected on a FL600 fluorescence spectrophotometer (GeminiXS, Molecular Devices) by measuring the emission at 530 nm after excitation at 485 nm.

5. Electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described (Xie *et al*., 1993). Briefly, treated and untreated Neuro2A cells or SH-SY5Y were lysed in a hypotonic buffer [10 mM Hepes (pH 7.5), 1.5 mM $MgCl_2$] and the nuclei were pelleted by centrifugation at 3,000 × g for 5 min. Nuclear lysates were prepared using a hypertonic buffer [30 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 450 mM KCl, 0.3 mM EDTA, and 1 mM PMSF, and 1 μg/ml each of aprotinin and leupeptin]. The lysates were centrifuged at 14,500 × *g* for 15 min, and the supernatants were used for the DNA binding assay. A double-stranded DNA containing the NF-κB binding site (5′-GGGGACTTTCC-3′) (Ghosh *et al*., 1998) or Nrf2 binding site (5′-TTTTATGCTGTGTCATGGTT-3′) (Martin *et al*., 2004) were end-labeled with γ-^{32}[P]-dATP. Nuclear extracts (5 μg) were incubated with 2 μg of poly(dI–dC) and the ^{32}[P]-labeled DNA probe in a binding buffer [30 mM Hepes (pH 7.5), 100 mM NaCl, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml each of aprotinin and leupeptin] for 10 min on ice. DNA binding reactions

were subjected to a 4.8% polyacrylamide/0.5× TBE gel electrophoresis. Gels were dried and subjected to autoradiography.

6. Luciferase and β-galactosidase assays

Neuro2A or SH-SY5Y cells were plated to 24-well plates for 12 hr and then transiently co-transfected with the plasmids pGL3-4κB-Luc (0.5 μg) or HO-1-ARE-Luc (0.5 μg) and pCMV-β-gal (0.2 μg) using LipofectAMINE Plus according to the manufacturer's protocol. After 18 hr, the cells were treated with MT-III for 24 hr and lysed. Luciferase and β-galactosidase activities were determined as described previously (Choi *et al*., 2001). Luciferase activities were normalized using β-galactosidase activities and expressed relative to the luciferase activity of control cells.

7. Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from SH-SY5Y cells using Trizol. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed following standard techniques. PCR conditions for HO-1 and HO-2 genes and for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were as follows: 25 cycles of 94°C for 30 s; 56°C for 30 s; and 72°C for 45 s. Amplified products were

resolved by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

8. HO-1 assay

HO-1 activity was measured at the end of each treatment as described previously (Kutty and Maines, 1982). Cells were homogenized in 0.5 ml of ice-cold 0.25 M sucrose solution containing 50 mM potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 200 × g for 10 min. The supernatants were centrifuged at 9,000 × g for 20 min, and further centrifuged at 30,000 × g for 60 min. The pellets were then resuspended in 50 mM potassium phosphate buffer (pH 7.4) and the amount of protein was determined using the BCA protein assay kit. The reaction mixtures (200 μl), which contained 0.2 mM of the substrate hemin, 500 μg/ml of cell lysate, 0.5 mg/ml rat liver cytosol as a source of biliveridin reductase, 0.2 mM $MgCl_2$, 2 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, 1 mM NADPH and 50 mM potassium phosphate buffer (pH 7.4), were incubated at 37^{o}C for 2 hr. The reaction was terminated with 0.6 ml of chloroform. After extraction, the absorbance of the chloroform layer was measured spectrophotometrically. Bilirubin formation was calculated from the difference in absorption at 464 nm and 530 nm.

9. Nuclear and cytosolic lysate preparation

Nuclear extracts were prepared with a commercial kit according to the

manufacturer's instructions (Active Motif, Carlsbad, CA). All steps were carried out on ice or at 4°C unless stated otherwise. Protease inhibitors (10 μg/ml aprotonin, 10 μg/ml leupeptin) and a reducing agent (1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) were added to each buffer just prior to use. Briefly, cells were incubated in 5 vol of hypotonic buffer A [20 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl] on ice for 15 min and homogenized. Nuclei were recovered by centrifugation at 3,000 × *g* for 15 min, and the supernatant was kept as the cytoplasmic extract. The nuclei were washed once using nuclei wash buffer [10 mM HEPES (pH 7.9), 0.2 mM $MgCl_2$, 10 mM KCl] and extracted using buffer C [20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$] for 30 min on ice. Insoluble material was removed by centrifugation at 21,000 × *g* for 10 min. The supernatant was used as the nuclear extract.

10. Western immunoblot analysis

Nuclear extracts were prepared according to a commercial kit and following the manufacturer's instructions (Active Motif, Carlsbad, CA). All steps were carried out on ice or at 4°C unless otherwise stated. Protease inhibitors (10 μg/ml aprotonin, 10 μg/ml leupeptin) and a reducing agent (1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) were added to each buffer just prior to the use. Briefly, cells were incubated in 5 vol of hypotonic buffer A [20 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl] on ice for 15 min and homogenized. Nuclei were

recovered by centrifugation at 3,000 × g for 15 min, and the supernatant was kept as the cytoplasmic extract. The nuclei were washed once using nuclei wash buffer [10 mM HEPES(pH 7.9), 0.2 mM $MgCl_2$, and 10 mM KCl] and extracted using buffer C [20 mM HEPES(pH 7.9), 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, and 1.5 mM $MgCl_2$] for 30 min on ice. Insoluble material was removed by centrifugation at 21,000 × g for 10 min. The supernatant was used as the nuclear extract.

11. Immunocytochemistry

Cells were fixed with methanol for 20 min at 20°C. After washing with ice-cold phosphate-buffered saline (PBS), cells were incubated with a blocking buffer containing 2% BSA and 10% normal goat serum for 1 hr at 37°C. Rabbit anti-p65/NF-κB or Nrf2 antibody was applied to cells and incubated at 4°C overnight. For negative control, cells were incubated with the blocking buffer without the primary antibody. After washing the cells with PBS, biotin-conjugated anti-rabbit IgG were added to the cells followed by incubation with fluorescein avidin D. The cells were finally mounted with a mounting medium (Dako, Hamburg, Germany) and analyzed by fluorescence microscope (Axiovert 200M; Carl Zeiss, Germany).

12. Determination of caspase-3 activity

A modification of the method described by Wang *et al.* (2003) was used to examine caspase-3 activation (Datta *et al*., 1997). Cells treated with the indicated combinations of MT-III and doxorubicin (Dox) were lysed in hypotonic buffer [20 mM Tris-HCl (pH7.5), 1 mM EDTA, 100 μM PMSF, and 2 μg/ml each of aprotinin, pepstatin, and leupeptin]. The supernatants were collected and incubated with 100 μM DEVD-pNA as a substrate at 37°C. The change in absorbance was measured at 405 nm using a plate reader.

13. TUNEL assay

Apoptosis was detected by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis using the DeadEnd^{TM} Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. SH-SY5Y cells were seeded on sterile glass coverslips in 12-well plates overnight and pretreated with MT-III for 1 hr before stimulation with H_2O_2 for 24 hr. Cells were then washed twice with PBS, fixed with 4% methanol-free paraformaldehyde for 10 min, washed twice with PBS, and permeabilized with 0.2% Triton X-100 for 5 min. After two more washes, each glass coverslip was covered with equilibration buffer for 10 min. The buffer was then aspirated, and the glass coverslips were incubated with TdT buffer at 37°C for 1 hr. Chromosomal DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI), and stained cells were mounted on glass slides and analyzed by confocal laser scanning microscopy.

14. Statistical analysis

All experiments were repeated at least three times. Means ± SD were calculated for each group and Dunnet's t-test was used to assess statistical significance. Differences were considered statistically significant when $p < 0.01$.

III. Results

Neuroprotecion by metallothionein-III involves activation of nuclear factor-κB through phosphatidylinositol-3 kinase signaling pathway

1. Expression and purification of MT-III

The human MT-III (MT-III) coding region was amplified from human cerebral cortical cDNA (Clontech, USA) by PCR. A sense primer including the *Eco*RI restriction site 5′-AGCAGAATTCATGGACCCTGAGACCTGCCCCTGC-3′ and a reverse primer with the *Not*I restriction site 5′-GCCGCGGCCGCTCACTGGCAGCAGCTGCACTTCTC-3′ were designed to amplify 207 bp of MT-III cDNA (Palmiter *et al*., 1992). MT-III cDNA was constructed using vector pGEX-4T in *Eco*RI and *Not*I sites, and was expressed in *E. coli* BL21. First, the *E. coli* BL21 strains containing expression plasmid pGEX4T-hMT-III (Fig. 2) and pGEX4T-1 were cultivated in small scale and induced with IPTG, then the supernatant of the cell lysate was examined on 15% SDS-PAGE (Fig. 3A). The BL21 containing the expression vector pGEX-4T-1 expressed a 26-kDa GST protein while the plasmids with insertion of the human MT-III expressed a 33-kDa fusion protein, respectively. The yields of the fusion proteins are more

than 50% of the soluble protein. Then expression was performed in large scale in 2YT peptone- rich culture. MT-III was purified by affinity chromatography on glutathione-Sepharose 4B as described previously (Zhou *et al*., 2000). To purify recombinant human MT-III protein, during the digestion of GST fusion protein on column, $ZnCl_2$ and mercaptoethanol were added to the thrombin solution to assist the proper folding of the MT-III, instead of adding the cadmium to the culture medium which will be toxic to the host cells and might lead to the failure of expressing the MT-III (Fig. 3B and C)

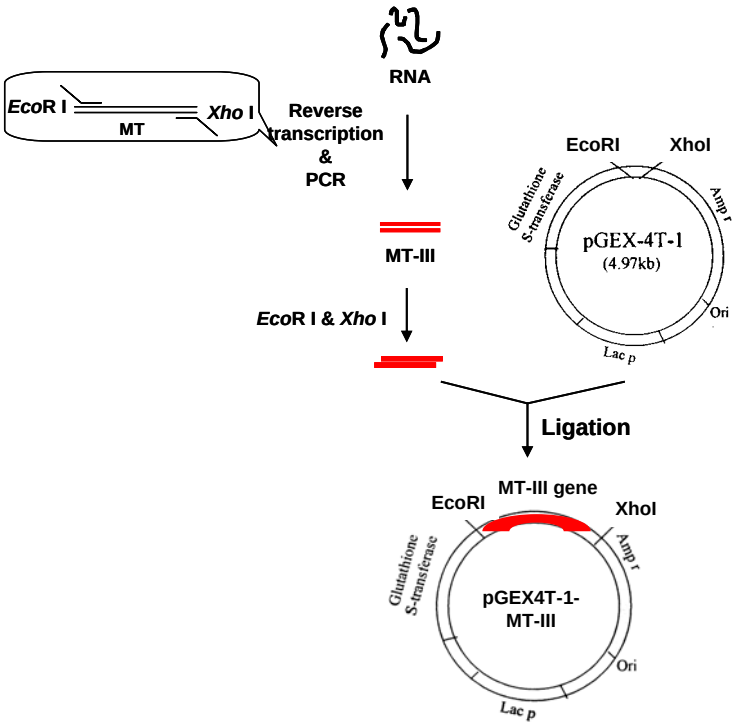

Fig. 2. Construct of human MT-III expression vector, pGEX4T-hMT-III.

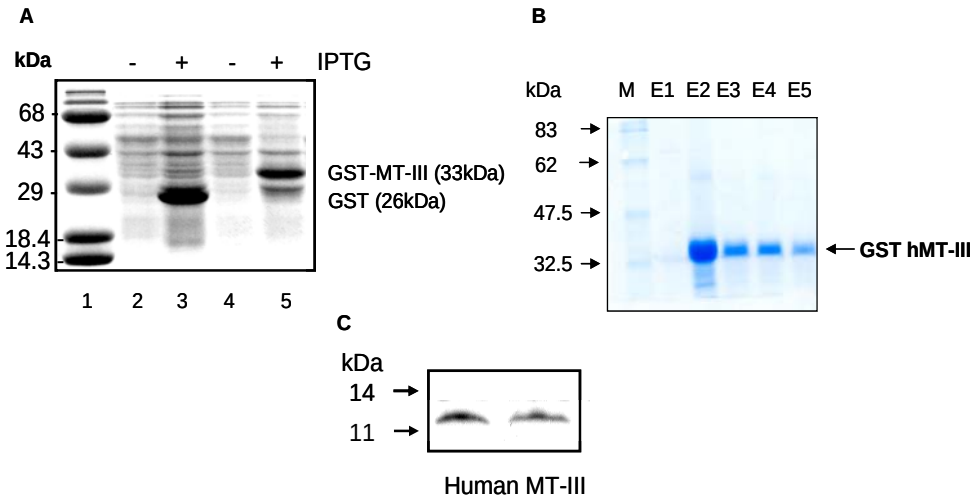

Fig. 3. Purification of recombinant human MT-III protein. (A) Identification the supernatant of the *E. coli* cell lysate on 15% SDS-PAGE. Lanes: 1, low range protein marker; 2, lysate with pGEX-4T-1 without inducing; 3, lysate with pGEX-4T-1 with IPTG; 4, lysate with pGEX-4T-MT-III without inducing; 5, lysate with pGEX-4T-MT-III with IPTG. (B) Purification of recombinant human MT-III protein. The GST-fused human MT-III protein was purified from the transformed *E. coli* cells. (C) After the treatment of thrombin at 37℃ for 12 hr, recombinant human MT-III protein could be obtained.

2. MT-III enhanced neuronal survival

H_2O_2 and Dox can induce cell death in neuronal cells. First, it was studied to examine if MT-III prevents H_2O_2 or Dox-induced neuronal cell death in the present study. H_2O_2 induced cell death in Neuro2A cells and MT-III reduced H_2O_2-induced cell death in a dose-dependent manner (Fig. 4). MT-III had a similar effect to Dox-induced Neuro2A cell death (Fig. 5). Metal binding is one of the properties of MT-III protein. In order to determine if the MT-III-bound metal ions exert the anti-apoptotic effects, effects of a metal ion-deprived form of MT-III, apo-MT-III on Dox-induced cell death were investigated in Neuro2A cells. Apo-MT-III reduced Dox-induced cell death in a dose dependent manner similar to the metal-bound form of MT-III (Fig. 6). Next, it was studied to examine whether MT-III protects against H_2O_2-induced apoptosis using TUNEL assay. Treatment of Neuro2A cells for 24 hr with H_2O_2 increased TUNEL staining, whereas MT-III significantly reduced H_2O_2-induced TUNEL staining (Fig. 7). These results indicate that MT-III protects neuronal cells from chemotherapy-induced and H_2O_2-induced apoptosis.

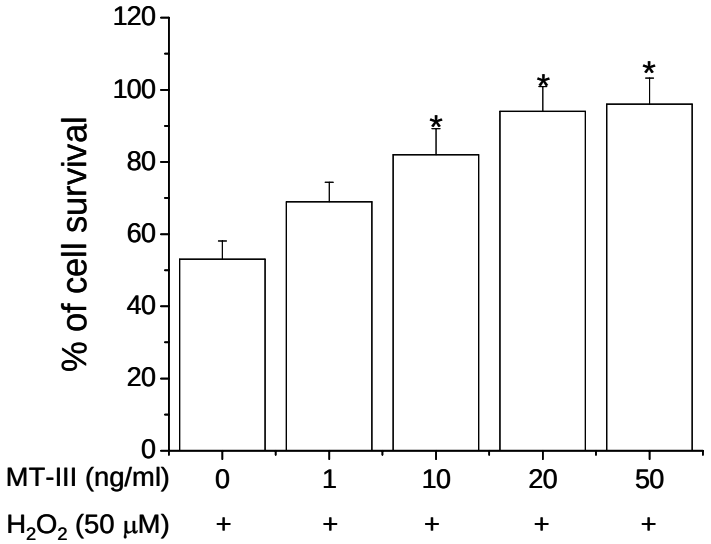

Fig. 4. MT-III prevented H_2O_2-induced cell death.

Neuro2A cells were cultured with various concentrations of MT-III. After 24 hr, cells were challenged for 24 hr with 50 μM H_2O_2. MTT assays were performed and MTT reduction, compared to MT-III-untreated control cells, was expressed as percentage of cell survival. Experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated control cells ($P < 0.01$, by t-test).

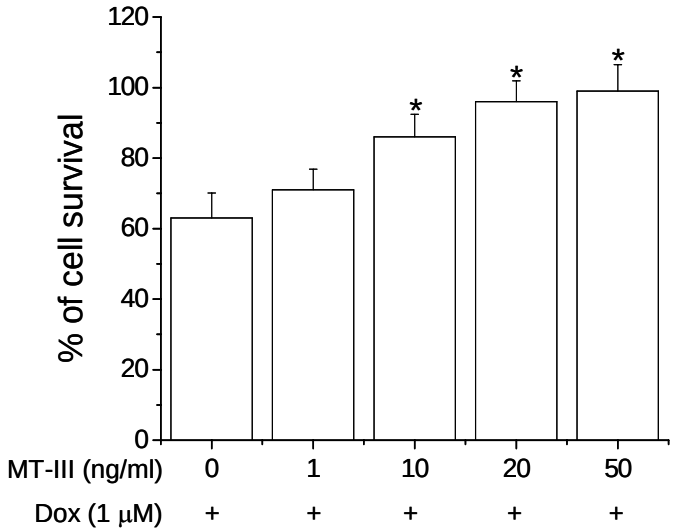

Fig. 5. MT-III prevented doxorubicin-induced cell death.

Neuro2A cells were cultured with various concentrations of MT-III. After 24 hr, cells were challenged for 24 hr with 1 μM of Dox. MTT assays were performed and MTT reduction, compared to MT-III-untreated control cells, was expressed as percentage of cell survival. Experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated control cells ($P < 0.01$, by t-test).

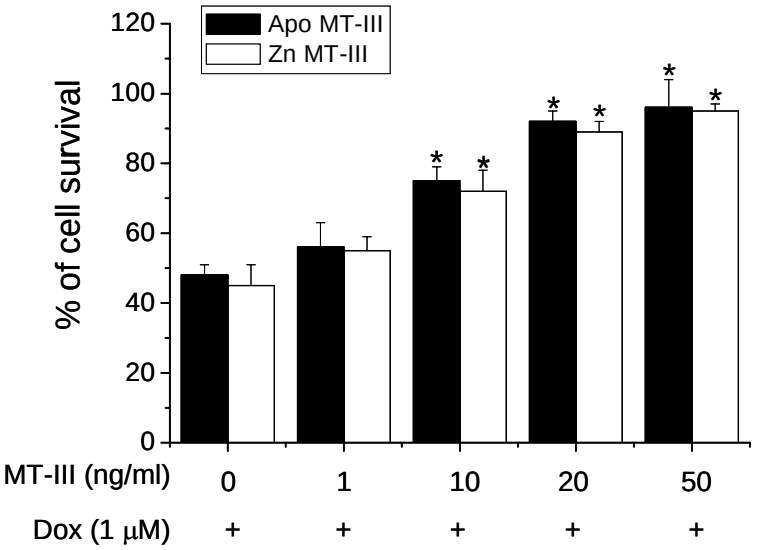

Fig. 6. Apo- and Zn-MT-III prevented Dox-induced cell death.

Neuro2A cells were cultured with various concentrations of Apo-MT-III or Zn-MT-III. After 24 hr, cells were challenged for 24 hr with 1 μM of Dox. MTT assays were performed and MTT reduction, compared to MT-III-untreated control cells, was expressed as percentage of cell survival. Experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated control cells ($P < 0.01$, by t-test).

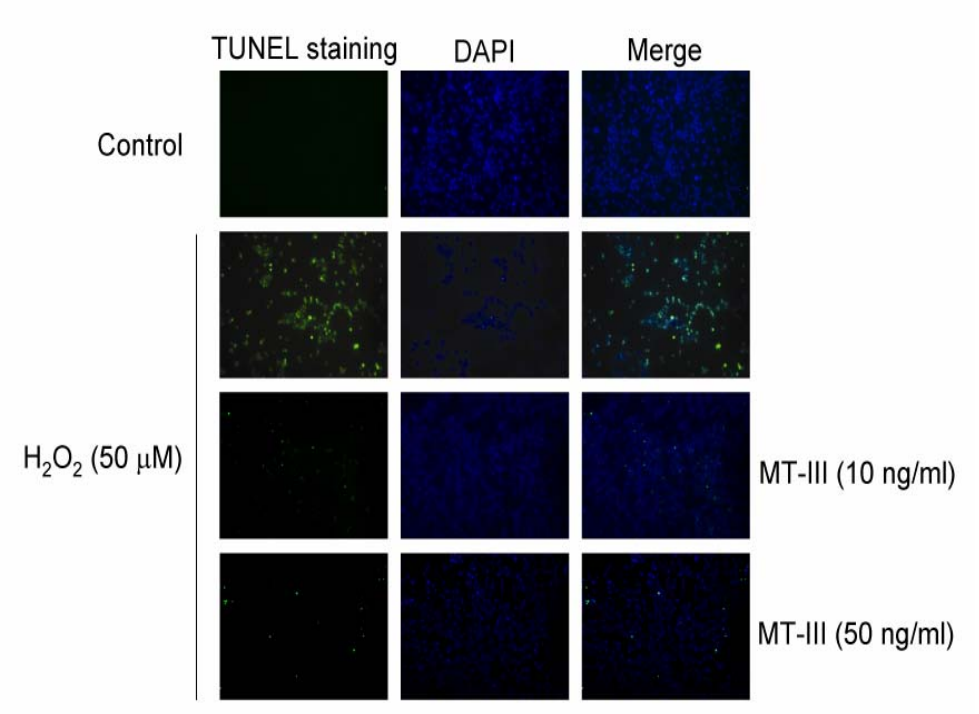

Fig. 7. MT-III inhibited H_2O_2-induced apoptosis.

Neuro2A cells were seeded on sterile slide cover slips in 12-well plates overnight and pretreated with indicated amounts of MT-III for 1 hr before stimulating with H_2O_2 for 24 hr. TUNEL assays were performed according to the manufacturer's instruction. All of the experiments were repeated at least three times and representative results are shown.

3. MT-III reduces the activation of caspase-3

Caspases-3 or -9 plays a pivotal role in the terminal execution phase of apoptosis induced by diverse stimuli. The effects of MT-III on Dox-induced activation of caspase-3 were measured using caspase-3 specific colorimetric peptide substrate DEVD-pNA. Dox increased in caspase-3 activity and MT-III reduced Dox-induced caspase-3 activation in Neuro2A cells in a dose-dependent manner (Fig. 8). Similar results were obtained when the effects of MT-III on Dox-induced caspase-9 activation were measured using a caspase-9-specific LEHD-pNA substrate (Fig. 9). Subsequently, the effects of MT-III on the protein levels of Bcl-2 were investigated. MT-III significantly increased the levels of Bcl-2 over 24 hr period (Fig. 10). When the effect of MT-III on cytochrome c release from the mitochondria to the cytoplasm was tested, Dox gradually increased the levels of cytoplasmic cytochrome c over 24 hr period and MT-III reduced Dox-induced release of cytochrome c to the cytoplasm (Fig. 11). These results suggest that MT-III prevents Dox-induced neuronal cell death by inhibiting caspase-3 activation.

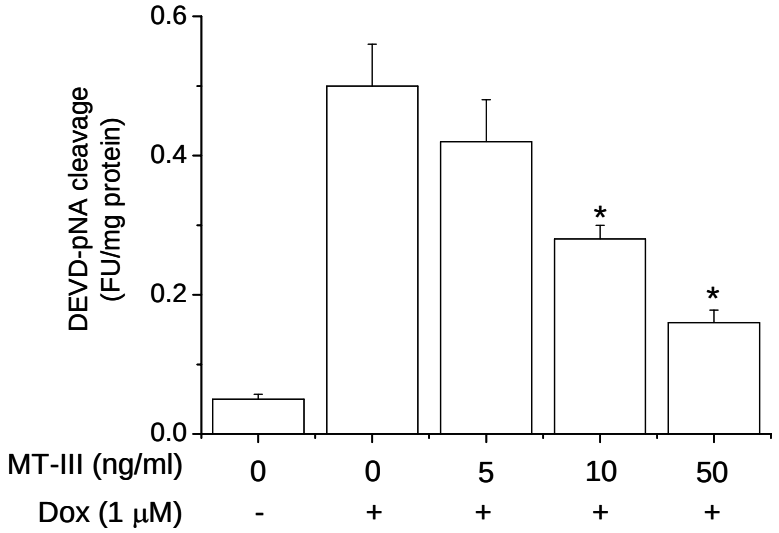

Fig. 8. MT-III reduced on Dox-induced the activation of caspase-3.

Neuro2A cells were treated as indicated. The catalytic activities of caspase-3 in cell lysates were assayed using a specific substrate DEVD-pNA. Experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated/Dox-treated cells ($P < 0.01$, by t-test).

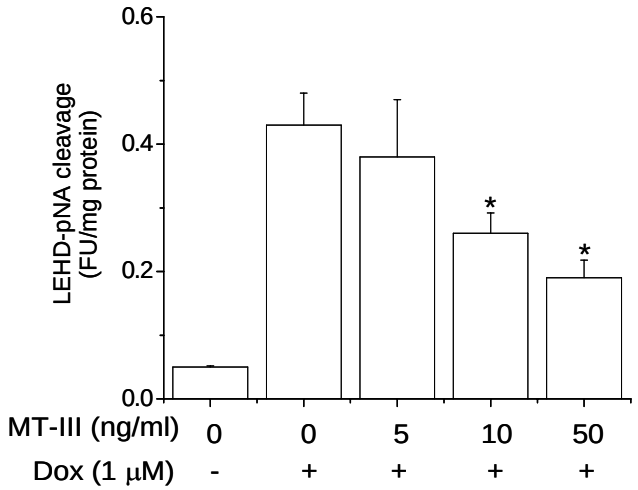

Fig. 9. MT-III reduced on Dox-induced the activation of caspase-9.

Neuro2A cells were treated as indicated. The catalytic activities of caspase-9 in cell lysates were assayed using a specific substrate LEHD-pNA. Experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated/Dox-treated cells ($P < 0.01$, by t-test).

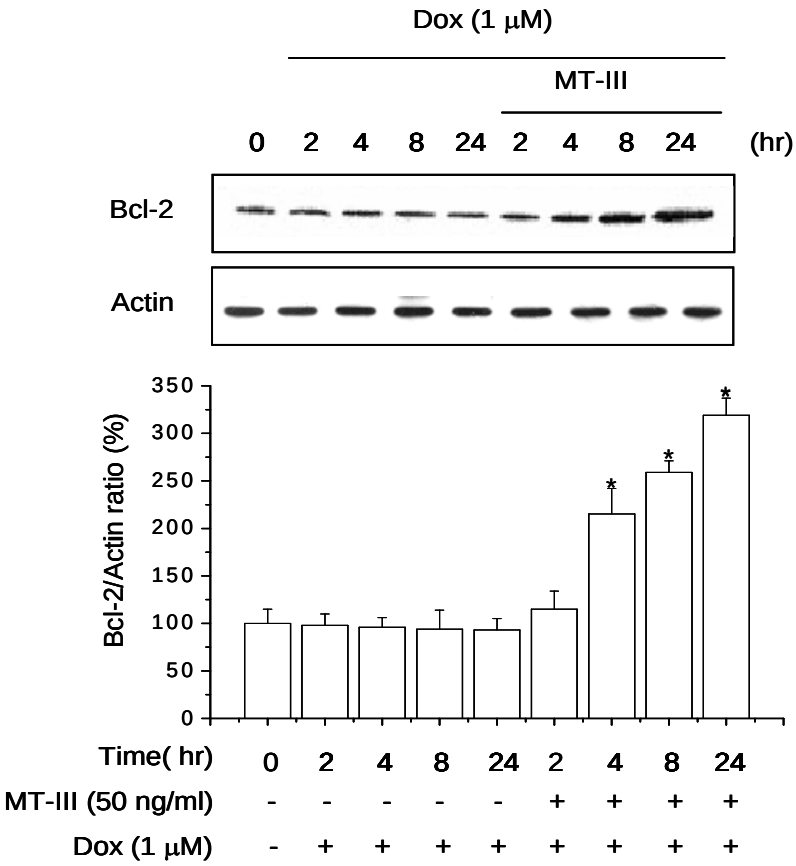

Fig. 10. MT-III up-regulated the expression of Bcl-2.

Neuro2A cells were cultured with indicated combinations of Dox and 50 ng/ml of MT-III. Top panel: At the indicated times, cells were assayed for Bcl-2 levels. Actin was used as a loading control. Bottom panel: The intensities of bands were measured by densitometry. Experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated cells ($P < 0.01$, by t-test).

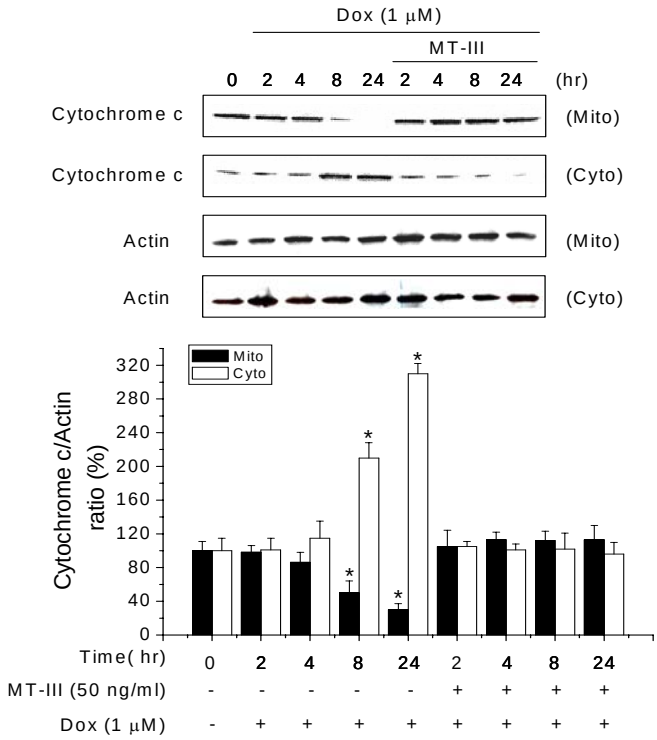

Fig. 11. Exogenous MT-III regulates the release of cytochrome c from the mitochondria.

Neuro2A cells were cultured with indicated combinations of Dox and 50 ng/ml of MT-III. Top panel: At the indicated times, cells were assayed for cytochrome c levels in the mitochondria (Mito) and the cytosol (Cyto). Actin was used as a loading control. Bottom panel: The intensities of bands were measured by densitometry. Experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the Dox-untreated cells ($P < 0.01$, by t-test).

4. Activation of NF-κB contributed to the anti-apoptotic effect of MT-III

The expression of MT and the activation of NF-κB activity can be triggered by stress. These processes are parts of a survival mechanism that mediates anti-apoptotic processes to protect cells from ionizing radiation and to provide resistance to anticancer drugs (Wang and Cassidy, 2003; Chuang *et al*., 2002). However, the role of MT proteins on NF-κB activation remains controversial: MT proteins have been reported to both positively and negatively regulate NF-κB (Abdel-Mageed and Agrawal, 1998; Papouli *et al*., 2002; Butcher *et al*., 2004). To determine the effect of MT-III on NF-κB activation in our culture system, the transcriptional activity of NF-κB by MT-III was determined using luciferase assays. MT-III increased the transcriptional activity of NF-κB in a dose-dependent manner in Neuro2A and SH-SY5Y cells (Fig. 12 and 13). In addition, when the DNA binding activity of NF-κB by MT-III was determined using electrophoretic mobility shift assays, MT-III increased the DNA binding of NF-κB in a dose-dependent manner and the increased DNA binding was sustained up to 30 min upon MT-III treatment (Fig. 14 and 15). Consistently, the amount of the nuclear p65 subunit of NF-κB was significantly increased. Taken together, these results indicated that MT-III induces the nuclear translocation and activation of NF-κB in our culture system (Fig. 16 and 17).

Next, how MT-III induces the activation of NF-κB in neuronal cells was investigated. Phosphorylation and the subsequent degradation of IκB are thought to be key steps in NF-κB activation. In fact, the results clearly showed that the levels of IκBα were decreased for the first 20 min upon MT-III treatment while the levels of IκBβ did not change up to 8 hr (Fig. 17). These results suggested that MT-III induces the activation of NF-κB through the phosphorylation and degradation of IκBα.

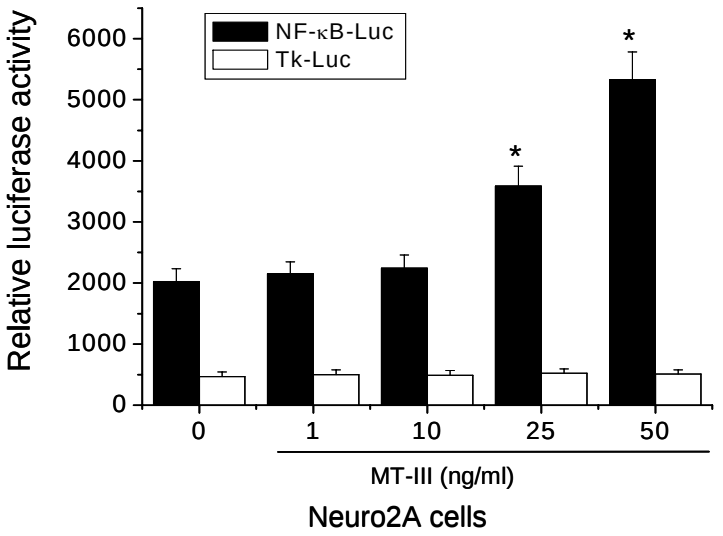

Fig. 12. MT-III increased the transcriptional activity of NF-κB in Neuro2A cells in a dose dependent manner.

Neuro2A cells were transiently transfected with either a NF-κB-responsive luciferase reporter (NF-κB-Luc) or a control luciferase reporter (TK-Luc). Cells were then treated with indicated amounts (ng/ml) of MT-III for 24 hr and assayed for luciferase activities. Experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated cells ($P < 0.01$, by t-test).

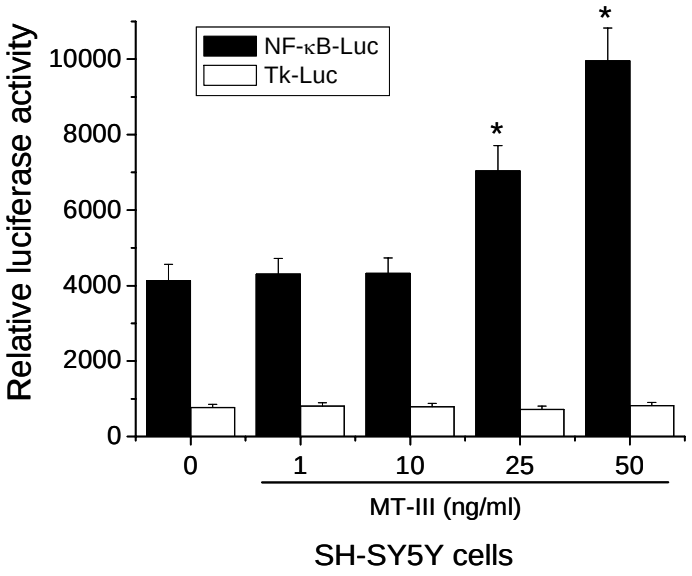

Fig. 13. MT-III increased the transcriptional activity of NF-κB in SH-SY5Y cells in a dose dependent manner.

SH-SY5Y cells were transiently transfected with either a NF-κB-responsive luciferase reporter (NF-κB-Luc) or a control luciferase reporter (TK-Luc). Cells were then treated with indicated amounts (ng/ml) of MT-III for 24 hr and assayed for luciferase activities. Experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated cells ($P < 0.01$, by t-test).

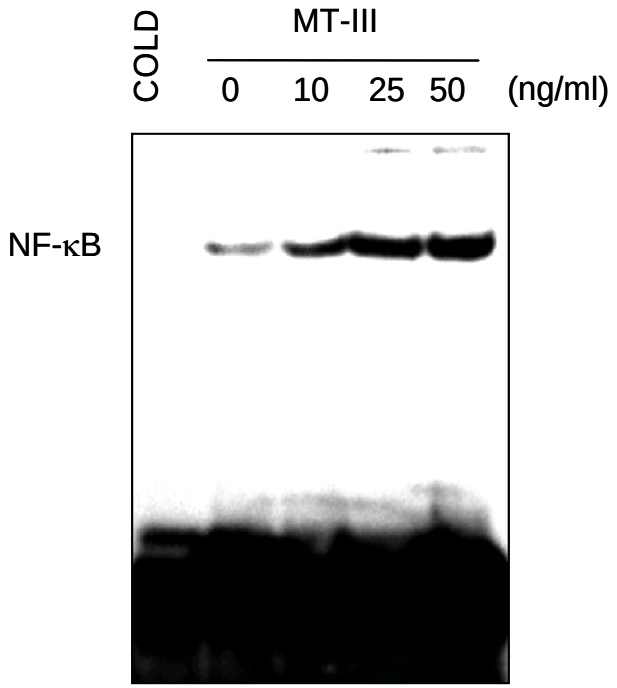

Fig. 14. MT-III increased the DNA binding activity of NF-κB: Dose response. Neuro2A cells were incubated with indicated amounts of MT-III, and nuclear extracts were subjected to EMSA using a radiolabeled NF-κB-specific oligonucleotide probe. All of the experiments were repeated at least three times and representative results are shown.

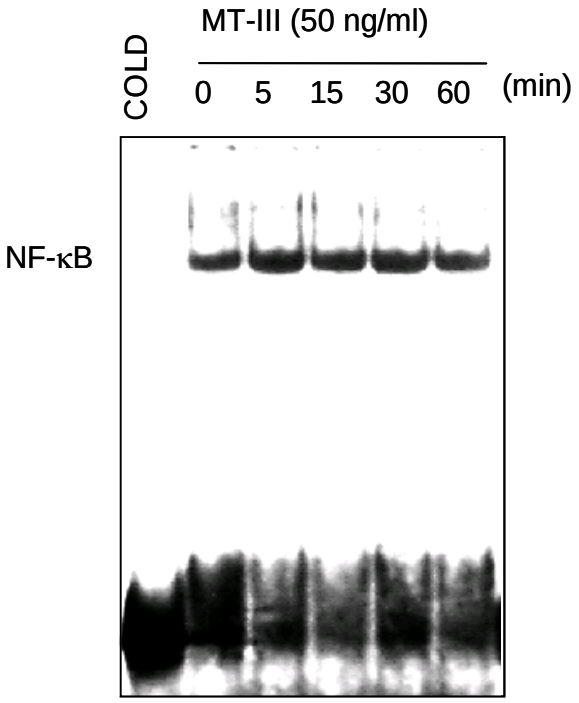

Fig. 15. MT-III increased the DNA binding activity of NF-κB: Time course. Neuro2A cells were incubated with 50 ng/ml of MT-III for 5 min to 1 hr and the nuclear extracts were subjected to EMSA. All of the experiments were repeated at least three times and representative results are shown.

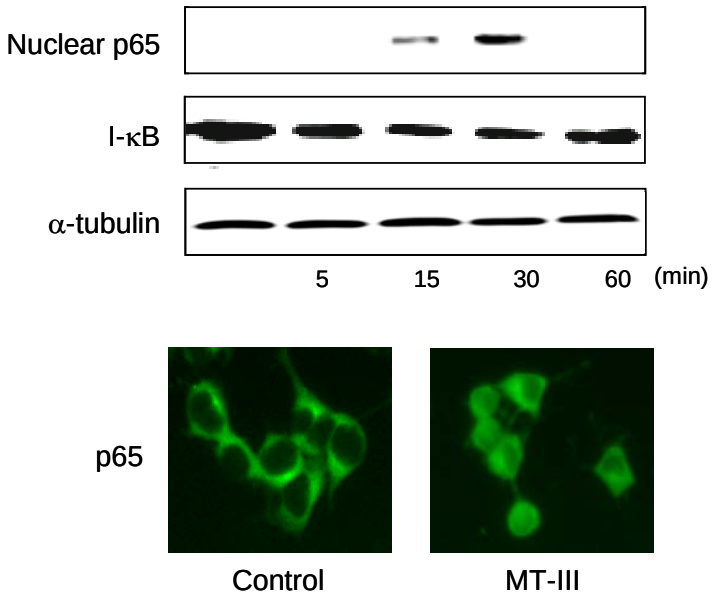

Fig. 16. MT-III increased the nuclear translocation of NF-κB.

Neuro2A cells were incubated for indicated times with 50 ng/ml of MT-III, and the levels of p65 NF-κB and IκB were examined by Western blot analysis. α-tubulin was used as a loading control. Neuro2A cells were treated with 50 ng/ml of MT-III for 30 min and immunostained with an anti-p65 antibody. All of the experiments were repeated at least three times and representative results are shown.

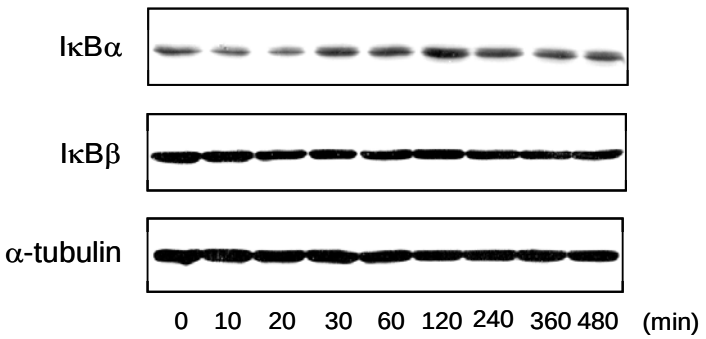

Fig. 17. MT-III induced IκB degradation.

Neuro2A cells were incubated with 50 ng/ml of MT-III for indicated times. Post-nuclear supernatants were examined by Western blot analysis using anti-IκB antibodies. All of the experiments were repeated at least three times and representative results are shown.

5. MT-III activated NF-κB through PI3K signaling pathway

Multiple signaling pathways can induce phosphorylation of IκB and subsequent activation of NF-κB. To study the possible role of PI3K signaling pathway in MT-III-induced activation of NF-κB, it was investigated whether MAPK and PI3K/Akt pathways are involved in neuronal cells. The transcriptional activity of NF-κB in MT-III-treated neuronal cells in the presence or absence of specific inhibitors of MAPK, PI3K, or Akt was measured by luciferase assays. Specific inhibitors of PI3K, such as wortmannin and LY294002, and a specific inhibitor of Akt decreased the MT-III-induced NF-κB transcriptional activity, while specific inhibitors of the MAPK pathway such as PD98059 and SB23058 had no effect (Fig. 18). A specific inhibitor of NF-κB, TPCK, was used as a positive control and TPCK also significantly reduced the MT-III-induced transcriptional activity of NF-κB. To determine whether MT-III activates PI3K/Akt signaling pathway, effects MT-III on Akt activation was investigated. MT-III dramatically increased Akt phosphorylation in a dose-dependent manner without altering the levels of total Akt in Neuro2A cells, and the MT-III-induced Akt phosphorylation was completely abolished by LY294002 (Fig. 19). These results supported the notion that MT-III activates NF-κB through the PI3K/Akt signaling pathway in neuronal cells.

Effects of MT-III on the regulation of the phosphorylation of IκB and the nuclear translocation of p65 subunit of NF-κB through the PI3K/Akt signaling pathway

were also studied in the present study. MT-III increased the nuclear translocation of p65 subunit of NF-κB as previously shown, and this was abolished in the presence of wortmannin or LY294002 (Fig. 20 and 21). These results also suggested that MT-III induces the phosphorylation of IκB and the subsequent nuclear translocation and activation of NF-κB through the PI3K/Akt signaling pathway.

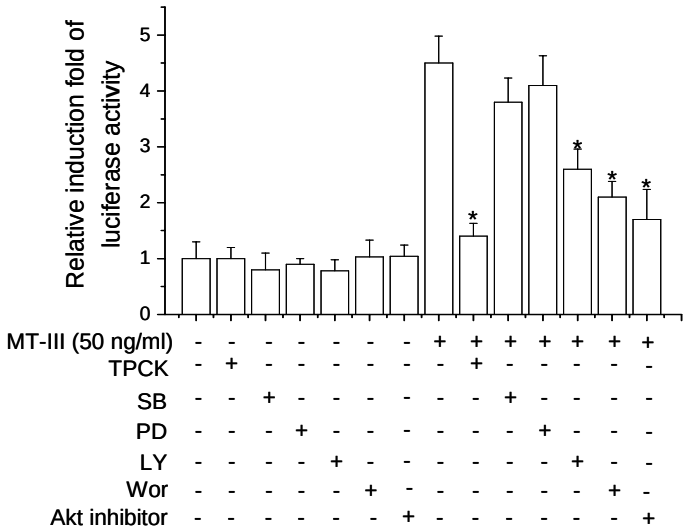

Fig. 18. MT-III-mediated activation of NF-κB was inhibited by PI3K inhibitors, LY294002 and Wortmannin, or Akt inhibitor.

Neuro2A cells were transfected with NF-κB-Luc and pCMV-β-gal plasmids. After 24 hr, cells were treated with 50 ng/ml of MT-III in the presence or absence of specific inhibitors of NF-κB, TPCK (50 μM) or MAPK, SB23058 (SB, 30 μM) and PD98059 (PD, 30 μM), or PI3K, LY294002 (LY, 20 μM) and Wortmannin (Wor, 100 nM), or Akt (25 μM) and cultured for additional 24 hr. Cells were then harvested and assayed for luciferase activities. Luciferase activities were normalized with β-galactosidase expression, determined by β-galactosidase assay. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III-treated cells ($P < 0.01$, by t-test).

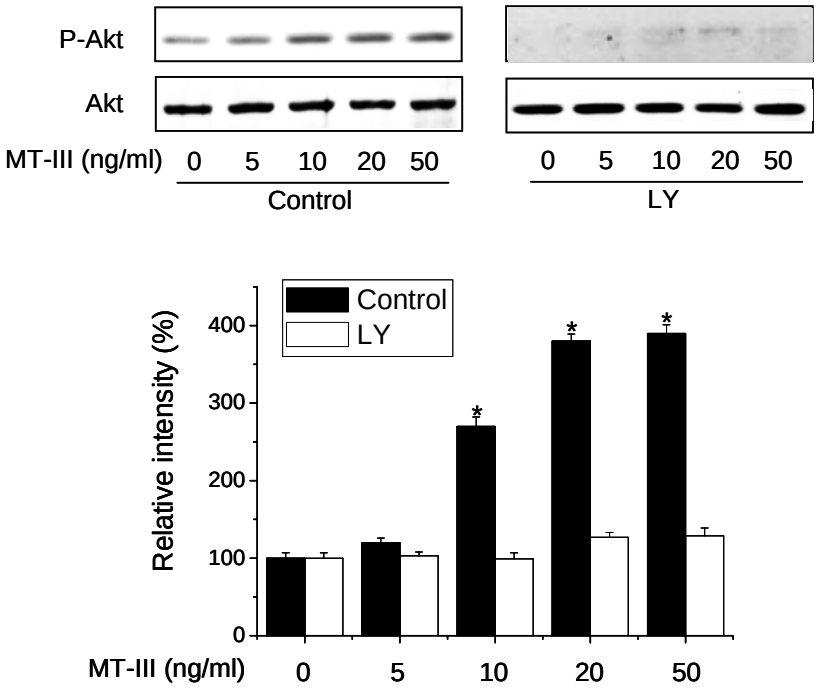

Fig. 19. MT-III increased the phosphorylation of Akt.

Neuro2A cells were incubated with indicated amounts of MT-III in the presence or absence of 20 μM of an inhibitor of PI3K, LY294002 (LY). Cell lysates were assayed by Western blot analysis using antibodies against phospho-Akt (P-Akt) and total Akt (Akt). Relative levels of phosphorylated Akt were calculated by densitometry. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated cells ($P < 0.01$, by t-test).

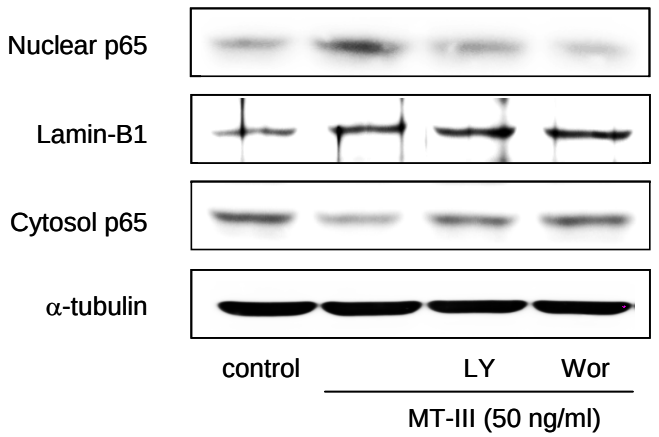

Fig. 20. PI3K inhibitors inhibited MT-III-induced nuclear translocation of NF-κB: Western immunoblot analysis.

Neuro2A cells were treated with 50 ng/ml of MT-III in presence or absence of LY294003 (LY, 20 μM) or Wortmannin (Wor, 100 nM). Cytoplasmic extracts and post-nuclear supernatants were subjected to Western blot analysis using an anti-p65 NF-κB antibody. Lamin-B1 and α-tubulin were used as nuclear and cytoplasmic markers. All of the experiments were repeated at least three times and representative results are shown.

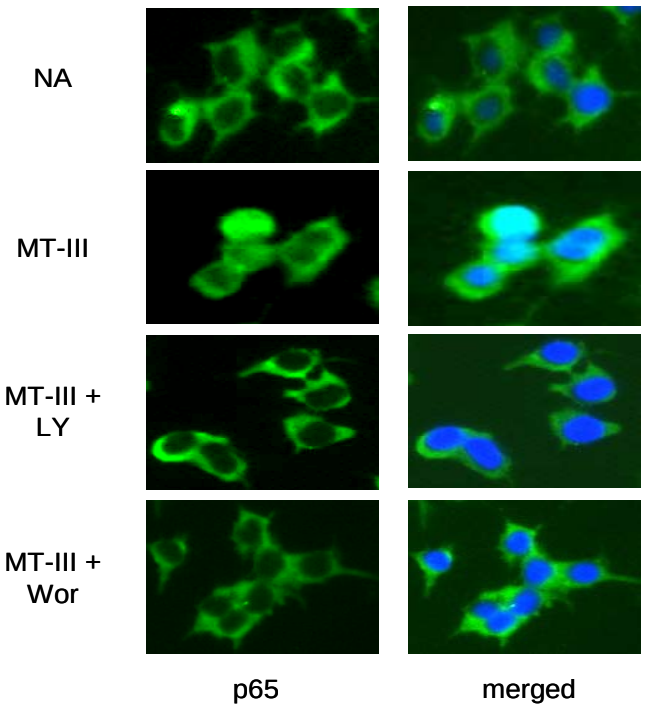

Fig. 21. PI3K inhibitors inhibited MT-III-induced nuclear translocation of NF-κB: Immunocytochemistry.

Cells were fixed and subjected to immunocytochemistry using an anti-p65 antibody (left column) and the images were superimposed with DAPI stained images (right column). All of the experiments were repeated at least three times and representative results are shown.

6. Inhibition of PI3K/Akt signaling pathway reduced the anti-apoptotic effect of MT-III

It was demonstrated that PI3K/Akt signaling pathway plays a central role in the MT-III-induced activation of NF-κB in the present study. These findings also suggested that active PI3K/Akt and NF-κB signaling pathways may be important for the anti-apoptotic effect of MT-III in neuronal cells. To confirm the results the effects of inhibiting PI3K/Akt signaling pathway or NF-κB on the anti-apoptotic effect of MT-III was further examined. MT-III protected Neuro2A cells from H_2O_2- or Dox-induced apoptosis, and this protective effect was almost completely abolished in the presence of LY294002 or TPCK (Fig. 22A and B). The effects of dominant negative of Akt (AKT-DN) and dominant negative of p65 NF-κB (p65-DN) were examined on the anti-apoptotic effect of MT-III using MTT and TUNEL assays. AKT-DN and p65-DN completely blocked the anti-apoptotic effect of MT-III (Fig. 23 and 24). Taken together, the present results has provided evidences that MT-III protects mouse neuroblastoma Neuro2A cells from H_2O_2- or Dox-induced apoptosis by activating NF-κB through the PI3K/Akt signaling pathway. Subsequently, the protective effect of MT-III was also examined in primary neuronal cells. MT-III protected primary neuronal cells from H_2O_2-induced apoptosis, and these effects were blocked in the present of LY294002 (Fig. 25 and 26). Furthermore, the nuclear translocation of p65 subunit of NF-κB through the

PI3K/Akt signaling pathway by MT-III was examined in primary neuron cells. MT-III increased the nuclear translocation of p65 subunit of NF-κB in primary neuronal cells, and this was abolished in the presence of LY294002 (Fig. 27) Taken together, these results clearly suggested that the PI3K/Akt signaling pathway plays the major role for the neuroprotective effect of MT-III.

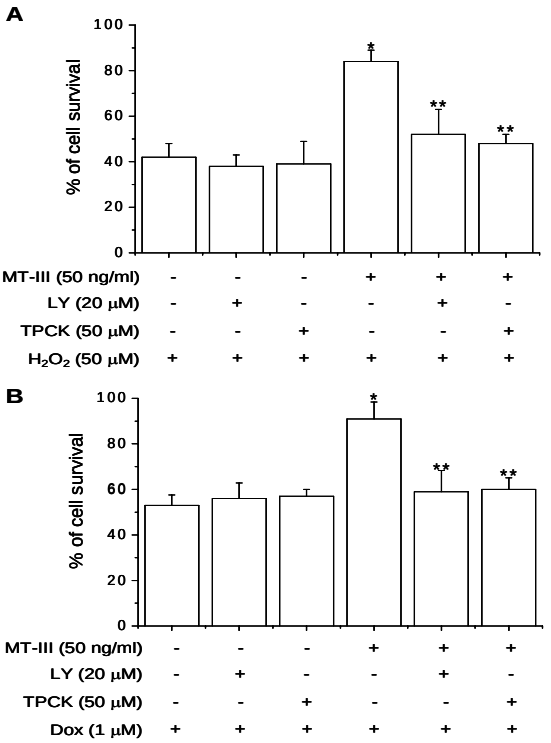

Fig. 22. PI3K and NF-κB inhibitors diminished the anti-apoptotic effect of MT-III. Neuro2A were cultured with 50 ng/ml of MT-III in the presence or absence of LY294002 (LY, 20 μM) or TPCK (20 μM). After 24 hr, cells were challenged for another 24 hr with 50 μM H_2O_2 (A) or 1 μM Dox (B). MTT assays were performed and MTT reduction, compared to the untreated control cells, was expressed as percentage of cell survival. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated cells ($P < 0.01$, by t-test). Asterisks (**) denote that the differences were significant compared to the MT-III plus H_2O_2/Dox-treated cells ($P < 0.05$, by t-test).

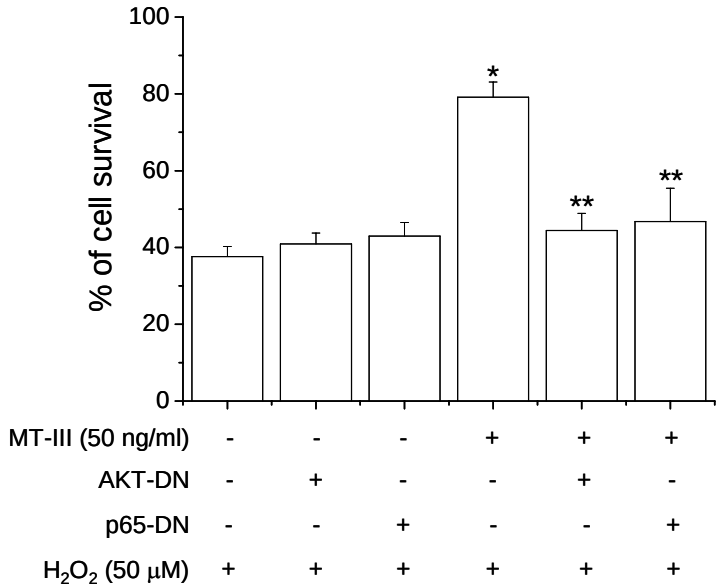

Fig. 23 Akt and p65 inhibitors diminished the anti-apoptotic effect of MT-III: Cell viability.

Neuro2A cells were transiently transfected with either a p65-DN expression vector or an AKT-DN expression vector. MTT assays were performed and MTT reduction, compared to the untreated control cells, was expressed as percentage of cell survival. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated cells ($P < 0.01$, by t-test). Asterisks (**) denote that the differences were significant compared to the MT-III plus H_2O_2-treated cells ($P < 0.05$, by t-test).

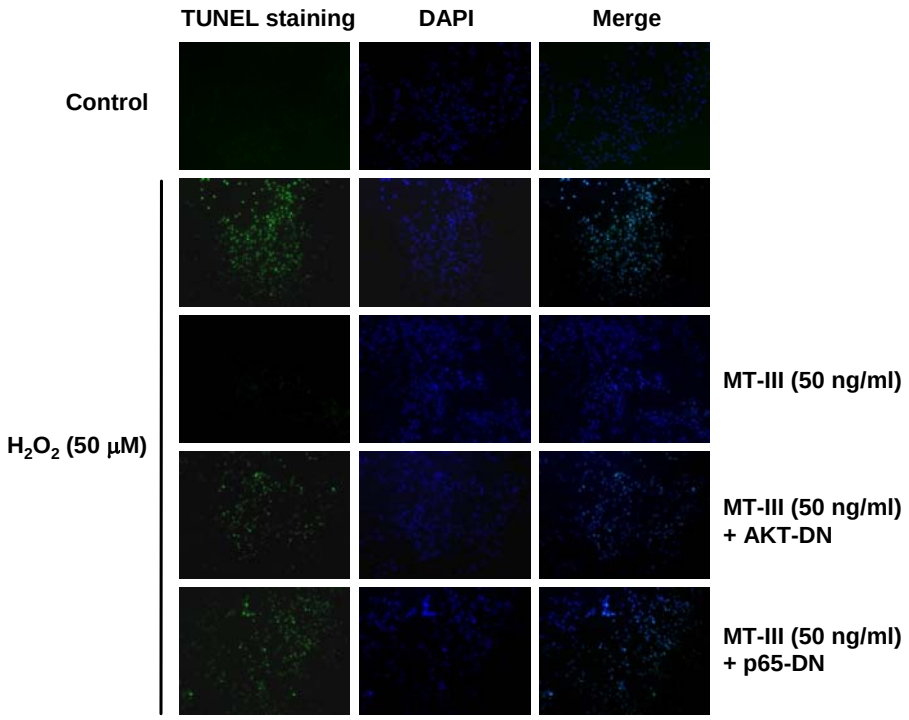

Fig. 24. Akt and p65 inhibitors diminished the anti-apoptotic effect of MT-III: TUNEL assay.

Neuro2A cells were seeded on sterile slide cover slips in 12-well plates overnight and transiently transfected with either a p65-DN expression vector or AKT-DN expression vector. Cells were then pretreated with indicated amounts of MT-III for 1 hr before stimulating with H_2O_2 for 24 hr. TUNEL assays were performed according to the manufacturer's instruction. All of the experiments were repeated at least three times and representative results are shown.

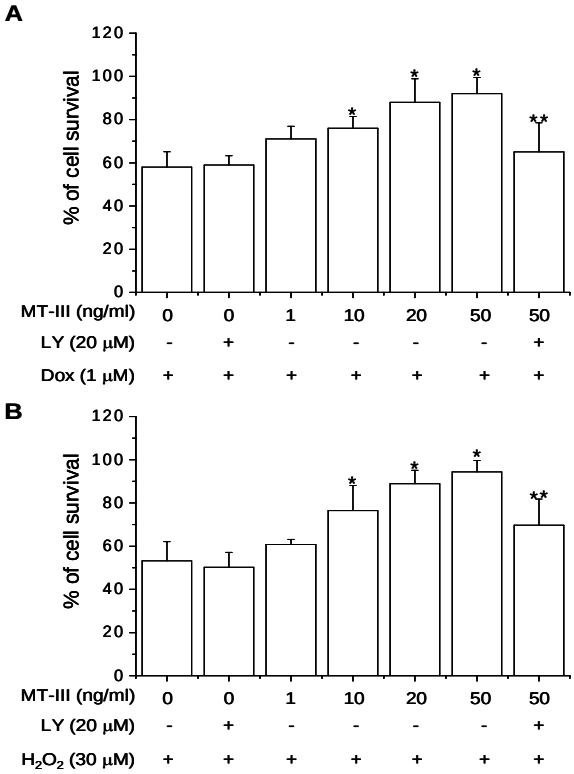

Fig. 25. PI3K inhibitor reduced the anti-apoptotic effect of MT-III.

Primary neuronal cells were cultured with various doses of MT-III in the presence or absence of LY294002 (LY, 20 μM). After 24 hr, cells were challenged for 24 hr with 1 μM of Dox (A) or 50 μM H_2O_2 (B). MTT assays were performed and MTT reduction, compared to untreated control cells, was expressed as percentage of cell survival. Asterisks (*) denote that the differences were significant compared to the MT-III-untreated cells ($P < 0.01$, by t-test). Asterisks (**) denote that the differences were significant compared to the MT-III plus Dox-treated cells ($P < 0.05$, by t-test).

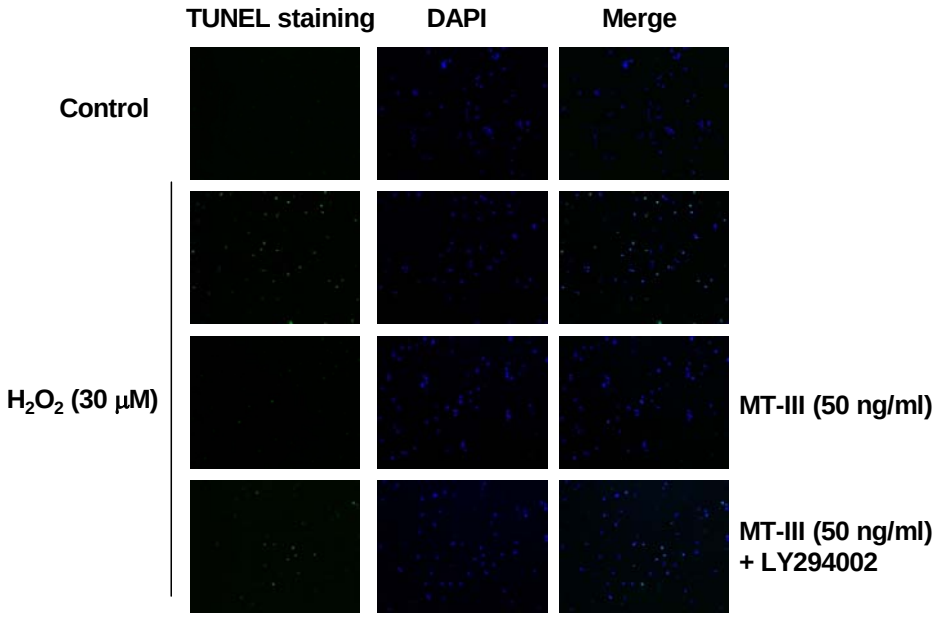

Fig. 26. MT-III inhibited H_2O_2-induced apoptosis in the primary neuronal cells. Cells were seeded on sterile slide cover slips in 12-well plates overnight. Primary neuronal cells were treated with 50 ng/ml of MT-III in presence or absence of LY294003 (LY, 20 μM). After 24 hr, cells were challenged for 24 hr with 30 μM H_2O_2. TUNEL assays were performed according to the manufacturer's instruction. All of the experiments were repeated at least three times and representative results are shown.

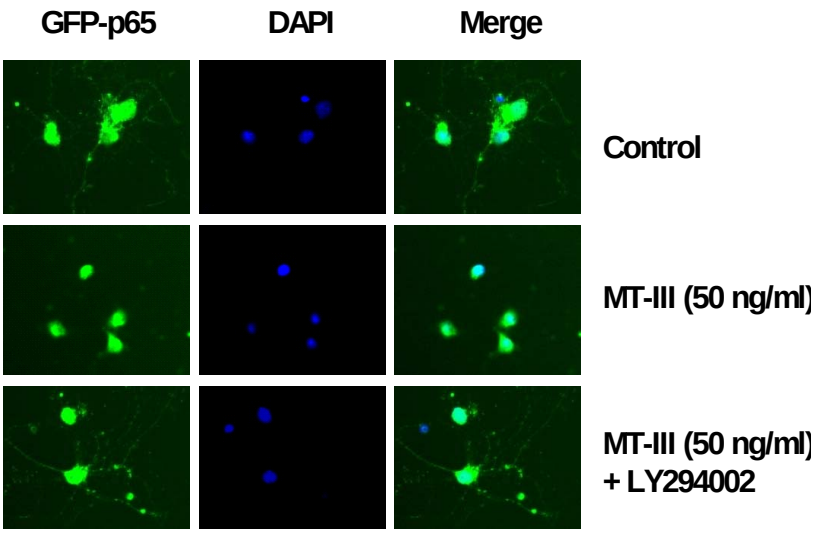

Fig. 27. MT-III increased the nuclear translocation of NF-κB in the primary neuronal cells.

Cells were treated with 50 ng/ml of MT-III in presence or absence of LY294003 (LY, 20 μM) for 30 min and immunostained with an anti-p65 antibody. All of the experiments were repeated at least three times and representative results are shown.

Metallothionein-III protects against 6-hydroxydopamine-induced oxidative stress by increasing expression of heme oxygenase-1 in the PI3K and ERK/Nrf2 dependent manner

7. MT-III protected against 6-OHDA-induced cell death

To determine the effect of MT-III on 6-OHDA-induced cell death, SH-SY5Y cells were treated with 50 ng/ml MT-III for 24 hr prior to exposure to 50 μM 6-OHDA for additional 24 hr (Fig. 28). Morphological analysis under light microscopy showed that 6-OHDA (50 μM) induced severe cell damage as evidenced by cell size and loss of the bright refringent halo around cell bodies. Pre-treatment with MT-III (50 ng/ml) for 24 hr blocked the morphological changes caused by 6-OHDA (Fig. 29). When effects of MT-III on 6-OHDA-induced apoptosis were determined using TUNEL assays, treatment of SH-SY5Y cells for 24 hr with 6-OHDA clearly increased TUNEL staining, whereas MT-III significantly reduced 6-OHDA-induced TUNEL staining (Fig. 30). Rupture of the plasma membrane during necrosis allows the release of cytosolic proteins such as LDH into the culture medium. LDH release due to 6-OHDA treatment was also significantly reduced in cells pre-treated with MT-III (Fig. 30). Taken together, these data showed that MT-III inhibits 6-OHDA-induced apoptosis and necrosis in SH-SY5Y cells.

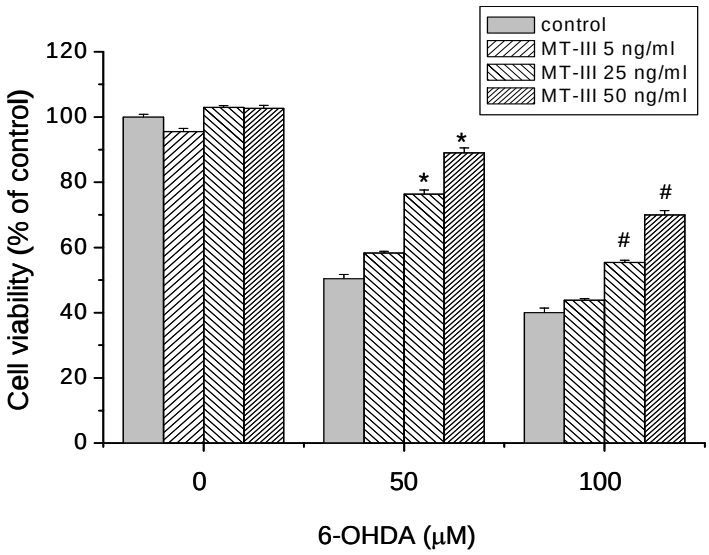

Fig. 28. MT-III prevented 6-OHDA-induced cell death in SH-SY5Y cells: Cell viability.

SH-SY5Y cells were treated with MT-III (5, 25 and 50 ng/ml) for 24 hr , and then incubated with 6-OHDA (50 or 100 μM) for additional 24 hr. After the treatment, cell viability was measured with an MTT assay. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*, #) denote that the differences were significant compared to 6-OHDA-treated cells ($P < 0.01$ and $P < 0.05$, by t-test).

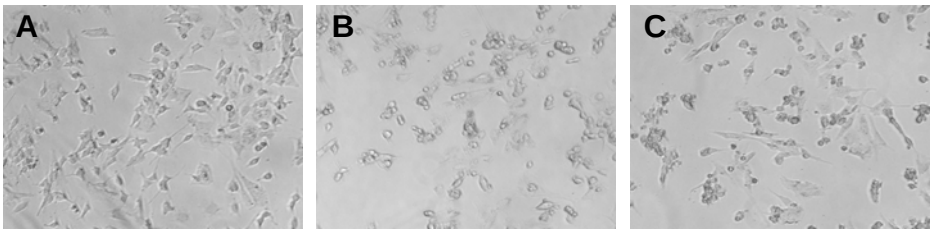

Fig. 29. MT-III prevented 6-OHDA-induced cell death in SH-SY5Y cells: Morphological analysis.

SH-SY5Y cells were treated with MT-III (50 ng/ml) for 24 hr followed by 6-OHDA (100 μM) incubation for additional 24 hr. After the treatment, morphological changes were measured under light microscopy; (a-c) phase contrast. Control cultures (a) or cells challenged for 24 hr with 100 μM 6-OHDA after pre-treatment (c) or not (b) for 24 hr with 50 ng/ml MT-III. All of the experiments were repeated at least three times and representative results are shown.

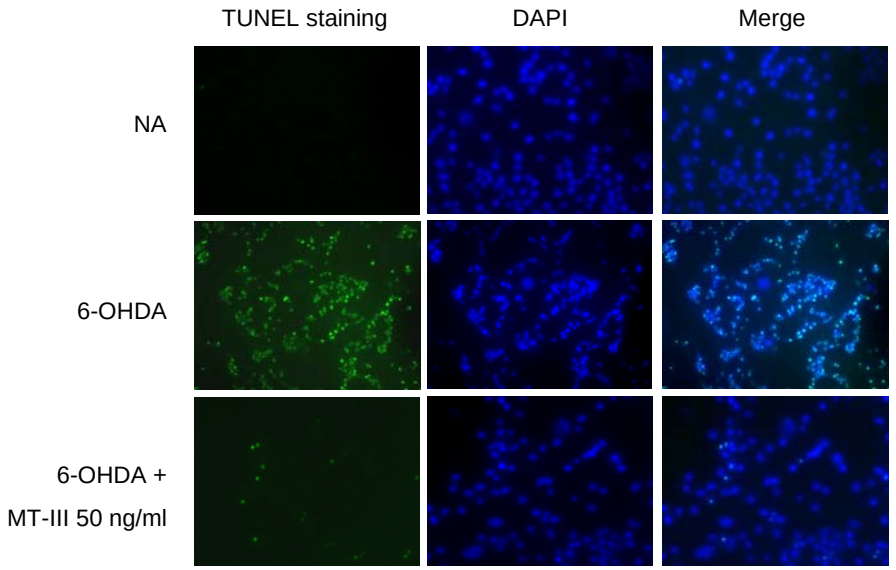

Fig. 30. MT-III prevented 6-OHDA-induced cell death in SH-SY5Y cells: TUNEL assays.

SH-SY5Y cells were treated with MT-III (50 ng/ml) for 24hr followed by 6-OHDA (50 μM) incubation for a further 24 hr. After the treatment, TUNEL assays were performed according to the manufacturer's instruction. All of the experiments were repeated at least three times and representative results are shown.

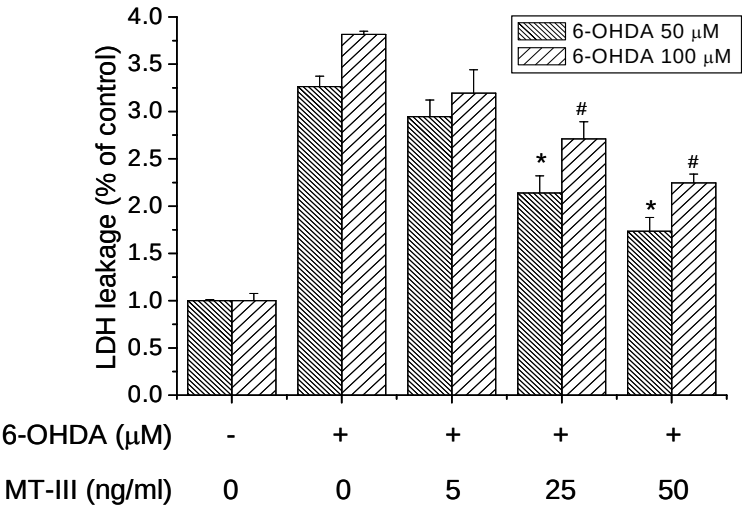

Fig. 31. MT-III prevented 6-OHDA-induced cell death in SH-SY5Y cells: LDH leakage assay.

SH-SY5Y cells were treated with MT-III (5, 25 and 50 ng/ml) for 24hr followed by 6-OHDA (50 or 100 μM) incubation for additional 24 hr. After the treatment, cell viability was measured with an LDH leakage assay. All of the experiments were done in triplicates and the means and standard deviations are shown Asterisks (*, #) denote that the differences were significant compared to 6-OHDA-treated cells ($P < 0.01$ and $P < 0.05$, by t-test).

8. MT-III reduces the activation of caspase-3

Caspase-3 plays a pivotal role in the terminal execution phase of apoptosis induced by diverse stimuli. To examine the effect of MT-III on caspase-3-like activity, cells were pretreated with MT-III for 24 hr followed by additional 24 hr 6-OHDA exposure, and then DEVDase activity was determined. The level of 6-OHDA-induced DEVDase activity was significantly reduced with prior MT-III treatment (Fig. 32). Similar results were obtained when the effects of MT-III on 6-OHDA-induced caspase-9 activation were measured using a caspase-9-specific substrate LEHD-pNA (Fig. 32). These results suggested that MT-III prevents 6-OHDA-induced neuronal cell death by inhibiting caspase-3 activation.

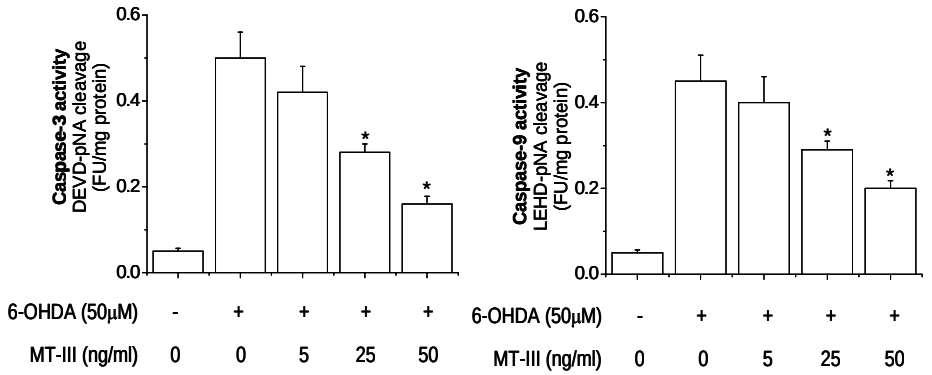

Fig. 32. MT-III reduced on 6-OHDA-induced the activation of caspase-3 and -9. SH-SY5Y cells were treated as indicated. The catalytic activities of caspase-3 and -9 in cell lysates were assayed using the specific substrates DEVD-pNA and LEHD-pNA. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to MT-III-untreated cells ($P < 0.01$, by t-test).

9. MT-III reduces 6-OHDA-induced ROS generation

6-OHDA auto-oxidation yields ROS, mainly H_2O_2, and quinones that might react with different proteins to produce quinoproteins (Tiffany-Castiglioni *et al*., 1982). To determine the effect of MT-III on 6-OHDA-induced ROS generation in SH-SY5Y cells, cells were pretreated with MT-III (5-50 ng/ml) for 24 hr, and then 6-OHDA and H_2DCFHDA were added for the last 6 and 1 hr, respectively. As shown in Fig. 33, the 24-hr pretreatment with 50 ng/ml MT-III significantly reduced the 6-OHDA-induced ROS generation, indicating that MT-III attenuates ROS production. Moreover, as shown in Fig. 34, this effect was dependent on the duration of MT-III pretreatment. Thus, attenuation of ROS release by MT-III would require the presence of MT-III at least 3 hr prior to the addition of 6-OHDA. These results suggested that MT-III induces the expression of gene(s) essential to ROS antagonism.

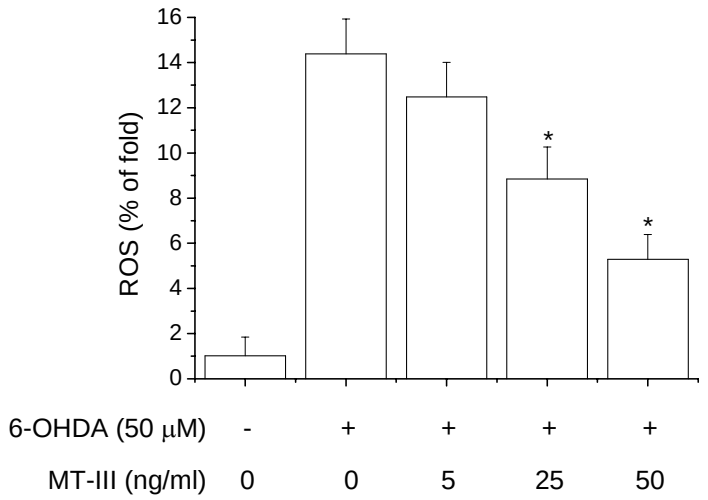

Fig. 33. MT-III reduced on 6-OHDA-induced cellular ROS production.

Cells were pretreated with MT-III (5-50ng/ml) or vehicle for 24 h. After the medium was removed, SH-SY5Y cells were exposed to 6-OHDA (50 μM) for 6 hr and fluorescence was then measured using FL600 fluorescence spectrophotometer with excitation at 485 nm and emission at 530 nm. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to MT-III-untreated cells ($P < 0.01$, by t-test).

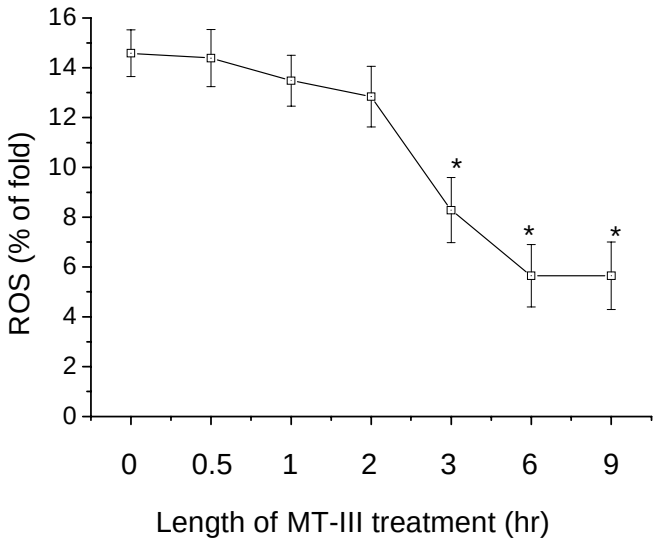

Fig. 34. Prevention of 6-OHDA-induced ROS by MT-III depended on the length of the MT-III pretreatment.

Prevention of 6-OHDA-induced ROS by MT-III depends on the duration of MT-III pretreatment. SH-SY5Y cells were incubated with MT-III (50 ng/ml) for the indicated time. They were then treated with 50 μM 6-OHDA for 9 hr and with H_2DCFHDA for 1 hr. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the 0. hr control cells ($P < 0.01$, by t-test).

10. MT-III up-regulates HO-1 expression

HO-1 is an important component of the cellular defense against oxidative stress. To assess the effect of MT-III on HO-1 expression in relation to its antioxidant activity, neuronal cells were treated with MT-III for 12 hr. The results showed a MT-III concentration-dependent increase in HO-1 mRNA and protein expression (Fig. 35A and B). Treatment of cells with MT-III (50 ng/ml) also resulted in a time-dependent increase in HO-1 mRNA and protein expression (Fig. 36A and B). The enhanced HO-1 expression correlated with the increased HO-1 activity (Fig. 37). This increase was sensitive to pretreatment of cells with actinomycin D or cycloheximide, suggesting that MT-III would enhance the expression of the inducible heme oxygenase isoform, HO-1 (Fig. 38). Moreover, the increase in mRNA was also sensitive to cycloheximide, suggesting, in agreement with other studies (Chen *et al*., 2000; Hill-Kapturczak *et al*., 2000), that the induction of HO-1 transcription involves *de novo* protein synthesis. MT-III failed to stimulate HO-2 expression (Fig, 35A and 36B), further indicating that MT-III specifically up-regulates the HO-1 isoform.

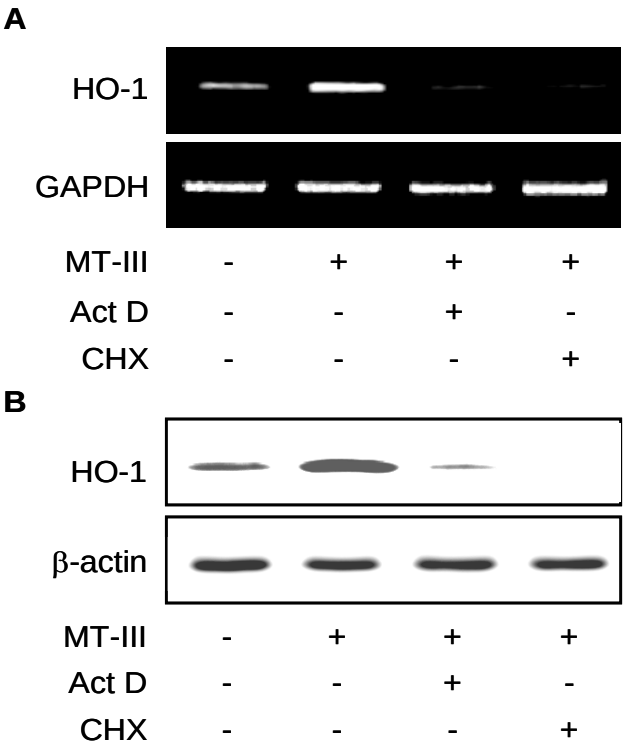

Fig. 35. MT-III induced HO-1 expression: Dose-dependent induction of HO-1.

(A) Semi-quantitative reverse transcription-PCR shows a dose-dependent induction of HO-1 mRNA by MT-III. (B) Dose-dependent induction of HO-1 protein by MT-III. For (A) and (B), SH-SY5Y cells were treated with the indicated MT-III concentrations for 12 hr and then analyzed for HO-1 mRNA and protein levels. All of the experiments were repeated at least three times and representative results are shown.

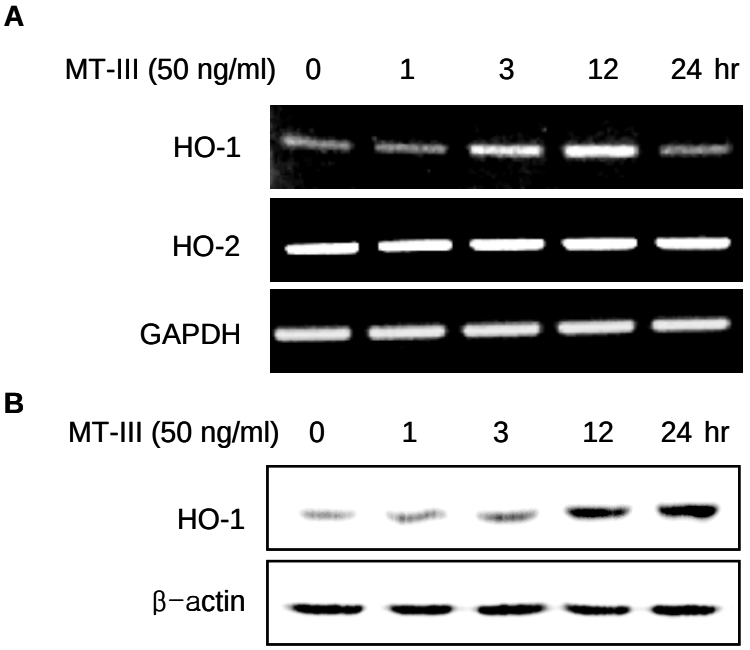

Fig. 36. MT-III induced HO-1 expression: Time-dependent induction of HO-1.

(A) Time course of HO-1 mRNA expression by MT-III. (B) Time course of HO-1 protein expression by MT-III. For (A) and (B), SH-SY5Y cells were treated with 50 ng/ml MT-III and then analyzed for HO-1 mRNA expression and protein at the indicated times. All of the experiments were repeated at least three times and representative results are shown.

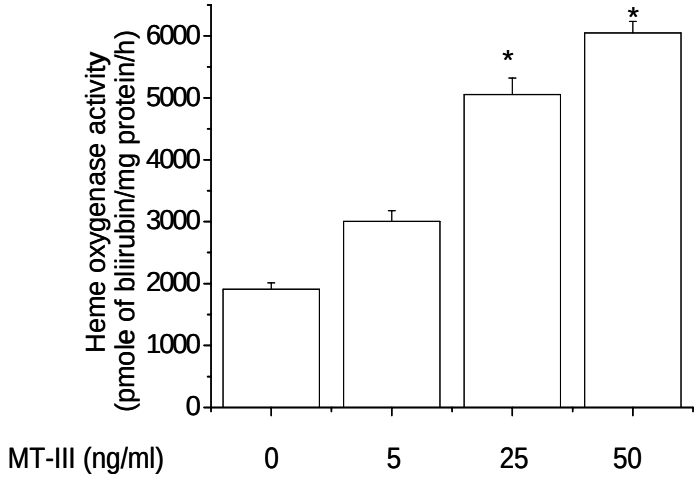

Fig. 37. MT-III increased HO-1 activity.

HO-1 activity was measured 12 hr after exposure to various concentrations of MT-III. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to MT-III-untreated cells ($P < 0.01$, by t-test).

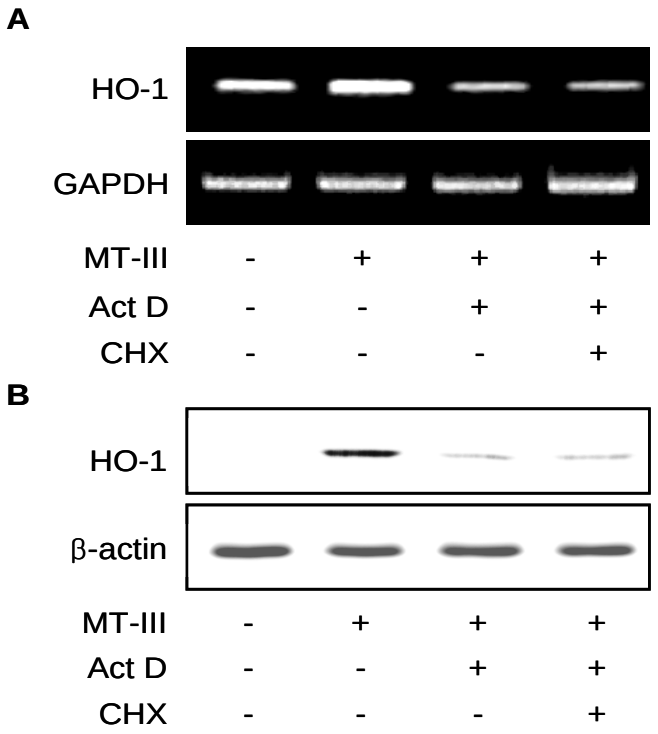

Fig. 38. MT-III induced HO-1 expression.

(A) Semiquantitative reverse transcriptase-PCR showing induction of HO-1 mRNA. (B) Immunoblot showing induction of HO-1 protein by MT-III. For (A) and (B), SH-SY5Ycells were untreated or pretreated with 50 μM cycloheximide (CHX) or 10 μg/ml actinomycin D (Act D) for 2 hr prior to the addition of 50 ng/ml MT-III for an additional 6 hr. All of the experiments were repeated at least three times and representative results are shown.

11. MT-III induces Nrf2 nuclear translocation

Most of the genes encoding phase II detoxifying and antioxidant enzymes have an ARE sequence in their promoter region. Nrf2 is an important transcription factor that regulates ARE-driven HO-1 gene expression. To examine whether MT-III could activate Nrf2 in association with HO-1 upregulation, SH-SY5Y cells were treated with 50 ng/ml MT-III for the indicated times, and nuclear fractions were extracted for Western blotting analysis. MT-III treatment increased Nrf2 accumulation in the nucleus (Fig. 39A and B). Confocal image of FITC-conjugated secondary antibody staining indicated the location of Nrf2 (green) by anti-Nrf2 antibody. The DAPI staining indicated the location of the nucleus (blue), and the merged image of MT-III-treated cells indicated the nuclear location of Nrf2 protein. Also, MT-III increased the transcriptional activity of HO-1 (Fig. 40A). To test whether MT-III activates Nrf2, electrophoretic mobility shift assays to determine Nrf2 DNA binding activity were performed using nuclear extracts prepared from SH-SY5Y cells treated with MT-III. MT-III time-dependently increased Nrf2 binding compared with untreated controls (Fig. 40B). This results suggested that the induction of HO-1 by MT-III is related with Nrf2-mediated ARE activation.

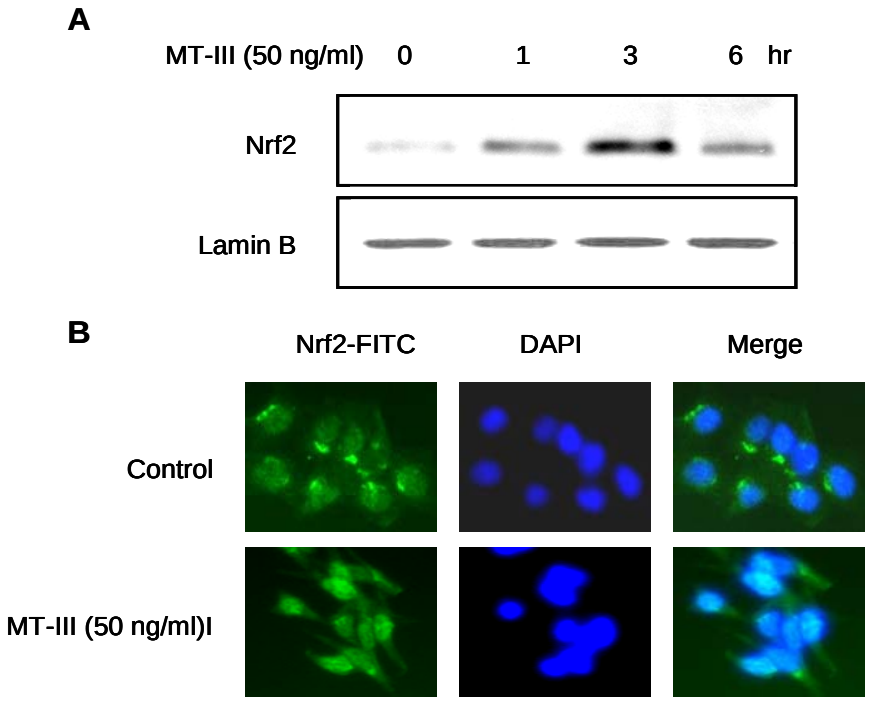

Fig. 39. MT-III increased Nrf2 translocation.

(A) SH-SY5Y cells were treated with MT-III (50 ng/ml) for the indicated time and then nuclear extracts were prepared for western blotting. (B) Effect of MT-III on nuclear localization of Nrf2. All of the experiments were repeated at least three times and representative results are shown.

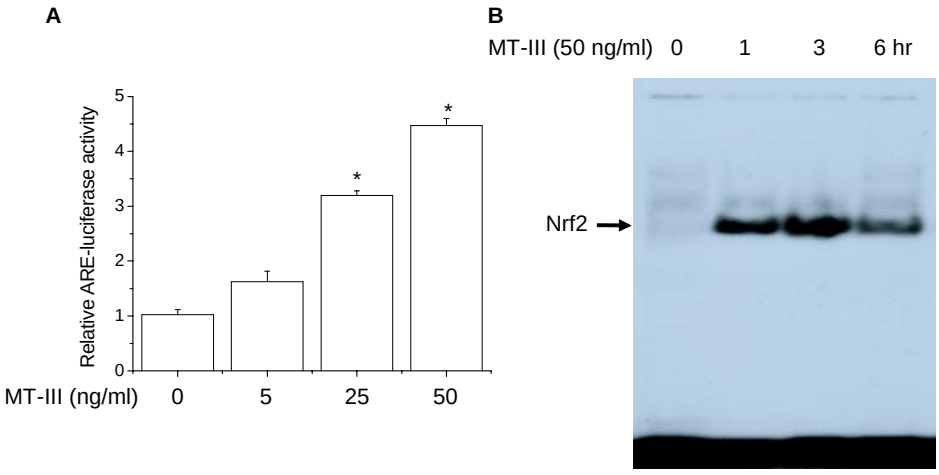

Fig. 40. MT-III increased the ARE-driven luciferase activities and ARE-binding activity of Nrf2.

(A) SH-SY5Y cells were transfected with the HO-1-ARE reporter plasmid and treated with MT-III (5-50 ng/ml). After 18 hr of treatment, the cells were harvested, and luciferase activities were determined. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to MT-III-untreated cells ($P < 0.01$, by t-test). (B) Effect of MT-III on ARE-binding activity of Nrf2 in SH-SY5Y cells. Nuclear extracts prepared from SH-SY5Y cells were treated with 50 ng/ml of MT-III for the indicated times. All of the experiments were repeated at least three times and representative results are shown.

12. Involvement of PI3K and MAPK pathway in HO-1 expression by MT-III

Many studies have demonstrated that several MAPKs, including JNK, p38, ERK and PI3K, are involved in regulating the phosphorylation of Nrf2 and ARE-mediated phase II gene expression (Zipper and Mulcahy, 2000; Martin *et al*., 2004). Meanwhile, the link between Nrf2 phosphorylation and activation and kinases remains unresolved. To further elucidate the upstream signaling pathway involved in MT-III mediated Nrf2 activation and/or induction of HO-1, the activation of PI3K/Akt and MAPKs were examined in SH-SY5Y cells. Interestingly, PI3K and ERK, but not other kinases such as p38 and JNK, were phosphorylated by MT-III (Fig. 41). Specifically, the HO-1 expression was reduced by LY294002; a PI3K inhibitor, and PD98059; an ERK inhibitor (Fig. 42). Likewise, nuclear accumulation of Nrf2 (Fig. 44), ARE binding of Nrf2 (Fig. 45), and transcriptional activity of HO-1 (Fig. 43B) was effectively blocked by LY294002 and PD98059. The inhibitor for PI3K or MAPK alone had no significant toxicity under our experimental conditions (data not shown). These results reflect a partial role for PI3K and ERK signaling in MT-III-mediated HO-1 induction through nuclear translocation of Nrf2.

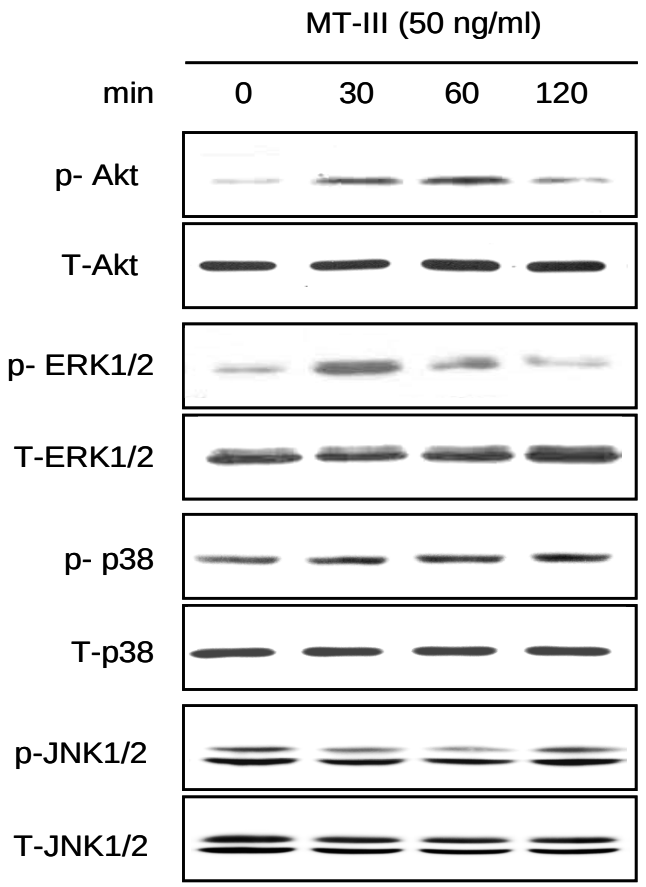

Fig. 41. MT-III activated the PI3K and ERK1/2 pathways.

SH-SY5Y cells were stimulated with 50 ng/ml MT-III for the indicated times and then immunoblotted with activation-specific antibodies that recognize p-Akt, p-ERK1/3, p-p38 and p-JNK1/2. Parallel immunoblots were analyzed for total kinase levels with anti-Akt, ERK1/3, p38 and JNK1/2 antibodies. All of the experiments were repeated at least three times and representative results are shown.

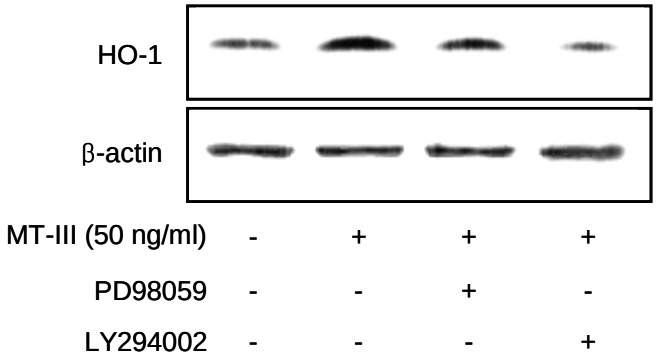

Fig. 42. PI3K and MAPK inhibitors attenuated the MT-III-induced HO-1 expression.

SH-SY5Y cells were preincubated with 30 μM LY294002 or 30 μM PD98059 for 30 min and then incubated with 50 ng/ml MT-III for 6 h. Cells were lysed and lysates were used for immunoblot analysis. All of the experiments were repeated at least three times and representative results are shown.

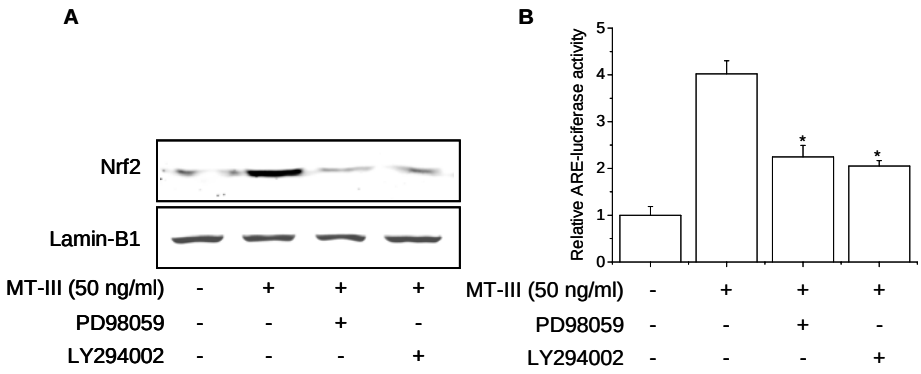

Fig. 43. PI3K and MAPK inhibitors attenuated the MT-III-induced HO-1-ARE-luciferase activity.

(A) SH-SY5Y cells were incubated with MT-III (50 ng/ml) for 3 hr in the presence or absence of 30 μM LY294002 or 30 μM PD98059 and nuclear extracts were harvested for Western blotting analysis with anti-Nrf2 antibody. All of the experiments were repeated at least three times and representative results are shown. (B) SH-SY5Y cells were transfected with the HO-1-ARE-luciferase plasmid construct. After overnight incubation, cells were treated with MT-III (50 ng/ml) for 18 hr in the presence or absence of 30 μM LY294002 or 30 μM PD98059, and cell lysates were mixed with luciferase substrate. The luciferase activity was measured by a luminometer. Asterisks (*) denote that the differences were significant compared to the MT-III-treated cells ($P < 0.01$, by t-test).

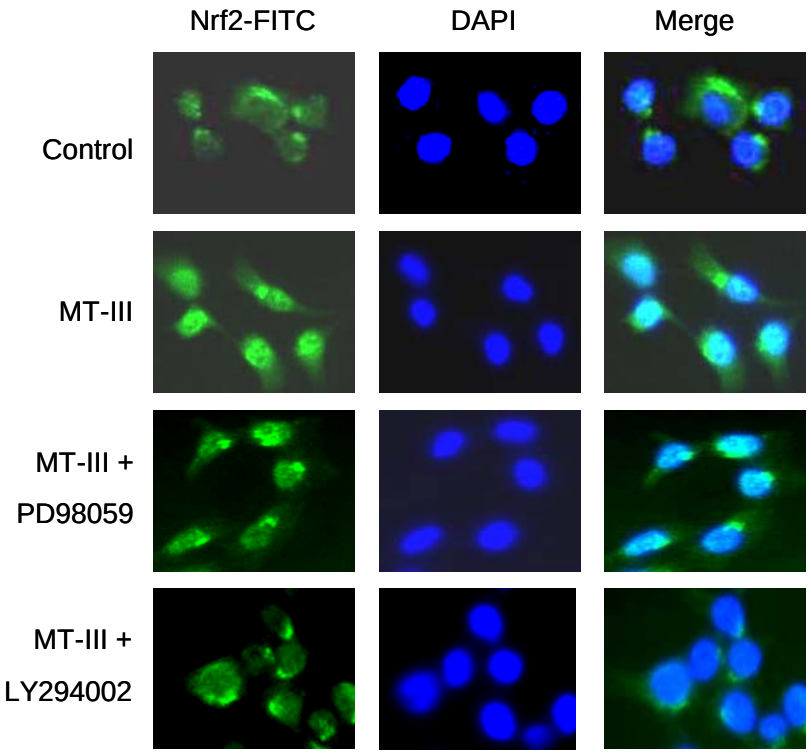

Fig. 44 PI3K and MAPK inhibitors attenuated the MT-III-induced nuclear translocation of Nrf2.

SH-SY5Y cells were treated with MT-III (50 ng/ml) in the absence or presence of PD98059 or LY294002 for 3 hr, and then immunostained to detect the nuclear localization of Nrf2. All of the experiments were repeated at least three times and representative results are shown.

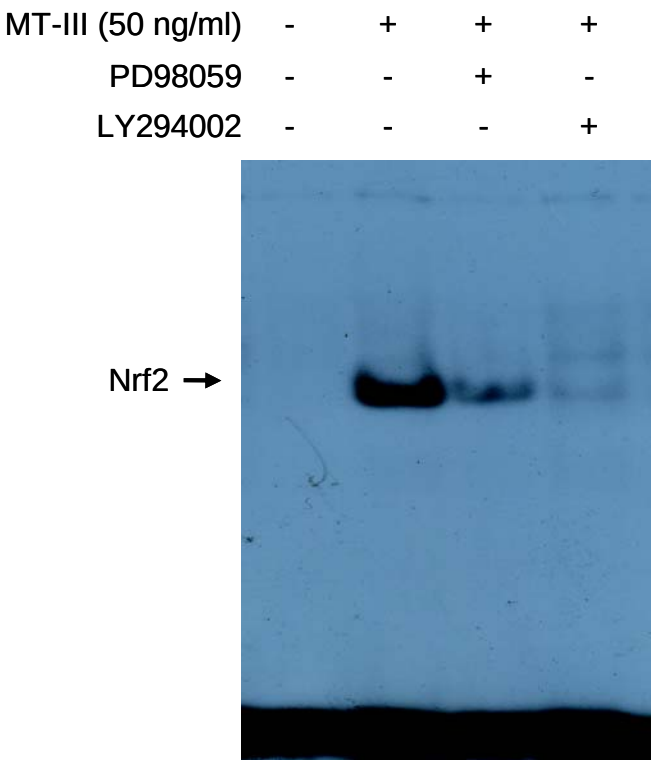

Fig. 45. PI3K and MAPK inhibitors attenuated the MT-III-induced the ARE-binding activity of Nrf2.

SH-SY5Y cells were treated with MT-III (50 ng/ml) for 3 hr in the absence or presence of PD98059 or LY294002 and then EMSA was performed. All of the experiments were repeated at least three times and representative results are shown.

13. HO-1 inhibitor suppresses the protective effect of MT-III against 6-OHDA-induced cell death

Zinc protoporphyrin (ZnPP) has been used as an HO inhibitor. In order to delineate if HO-1 induction participates in MT-III protection of SH-SY5Y cells against 6-OHDA-induced cytotoxicity, cells were treated with MT-III (50 ng/ml) for 24 hr to induce HO-1 protein expression then incubated with 5 μM ZnPP for 30 min and finally incubated with 6-OHDA for additional 24 hr. The MTT assay revealed that the protective effect of MT-III against 6-OHDA-induced cytotoxicity was reversed by the addition of ZnPP (Fig. 46). Data of the morphological observations indicated that condensed cells reappear in ZnPP-treated cells in the presence of MT-III and 6-OHDA (Fig. 47). These results suggested that the neuroprotective effect of MT-III is mediated through HO-1 induction.

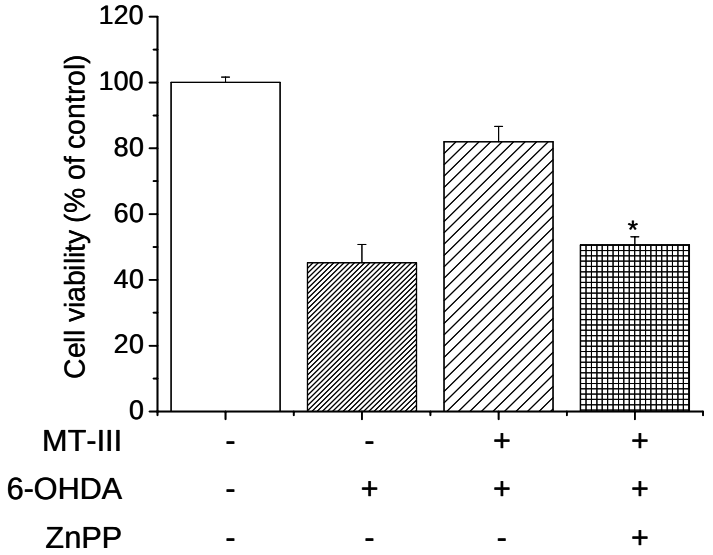

Fig. 46. A chemical HO-1 enzyme inhibitor ZnPP reversed the protective effect of MT-III against 6-OHDA-induced cell death: Cell viability.

SH-SY5Y cells were treated with MT-III (50 ng/ml) for 24 hr and then incubated with ZnPP (5 μM) for 30 min and addition of 6-OHDA (50 μM) into the cells for additional 24 hr. Cell viability was determined by the MTT assay. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to MT-III plus 6-OHDA-treated cells ($P < 0.01$, by t-test).

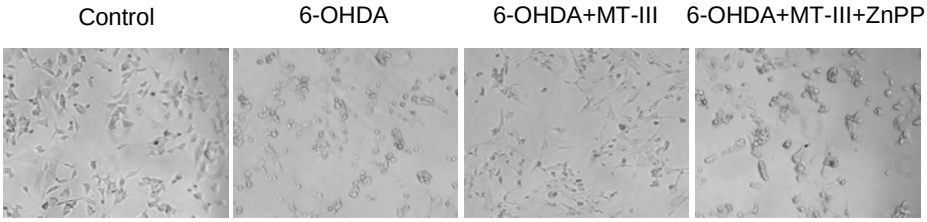

Fig. 47. ZnPP reverses the protective effect of MT-III against 6-OHDA-induced cell death: Morphological analysis.

SH-SY5Y cells were treated with MT-III (50 ng/ml) for 12 hr followed by incubating with ZnPP (5 μM) for 30 min and addition of 6-OHDA (50 μM) into the cells for additional 12 hr. Morphological changes in the condition with or without MT-III (50 ng/ml) or ZnPP (5 μM) followed by 6-OHDA stimulation for 12 hr were observed microscopically. All of the experiments were repeated at least three times and representative results are shown.

14. PI3K and ERK1/2 pathways are necessary and sufficient to attenuate 6-OHDA-induced cell death

To examine the effects of PI3K/Akt and three MAPK inhibitors on MT-III-induced neuroprotection against 6-OHDA, SH-SY5Y cells were pretreated for 30 min with LY294002, PD98059, SB203580 and SP600125 prior to addition of MT-III. Following 24-hr incubation with MT-III, cells were treated with 6-OHDA for additional 24 hr. As shown in Fig. 48-50, LY294002 and PD98059 reversed the protective effect of MT-III against 6-OHDA-induced cell death, further supporting the concept that the PI3K and ERK1/2 survival pathways control cell death, at least in part, by inducing HO-1 expression (Fig. 51).

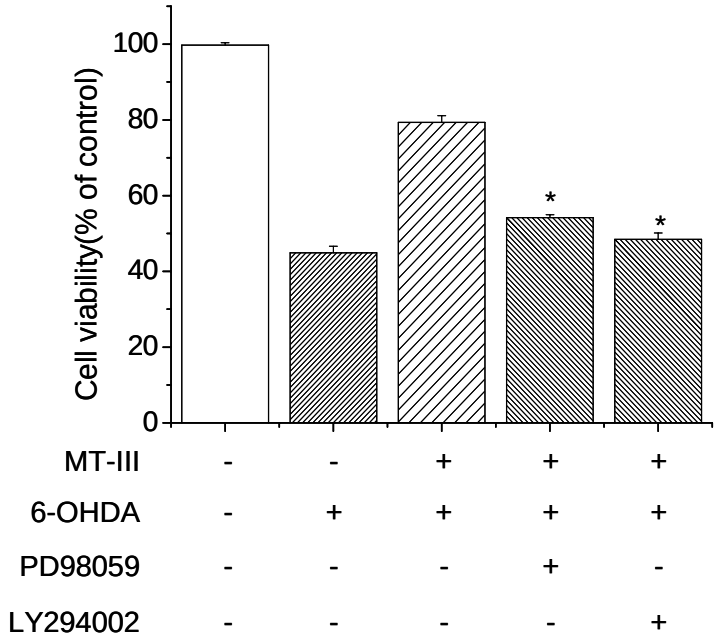

Fig. 48. PI3K and ERK1/2 inhibitors attenuated the anti-apoptotic effect of MT-III: cell viability.

SH-SY5Y cells were pretreated for 30 min with LY294002 and PD98059 prior to the addition of MT-III. Following a 24 hr incubation with MT-III, cells were treated with 6-OHDA for additional 24 hr. Cell viability was assessed by the MTT assay after 6-OHDA treatment. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III plus 6-OHDA treated cells ($P < 0.01$, by t-test).

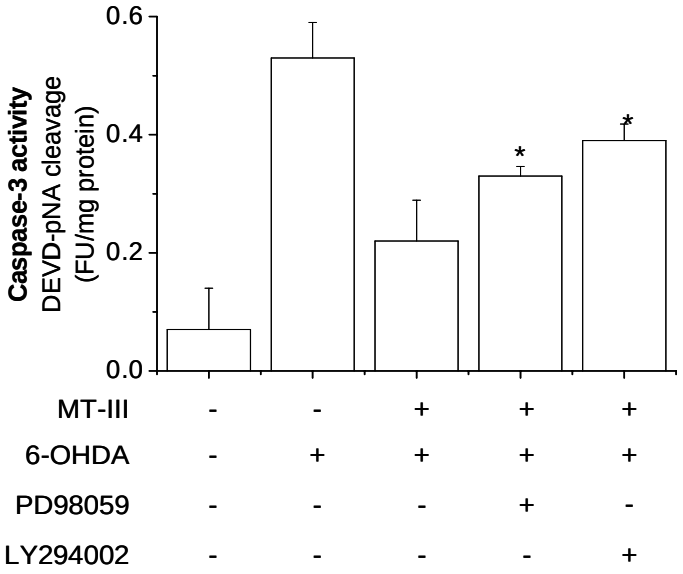

Fig. 49. PI3K and ERK1/2 inhibitors attenuated the anti-apoptotic effect of MT-III: Caspase-3 activity.

SH-SY5Y cells were pretreated for 30 min with LY294002 and PD98059 prior to the addition of MT-III. Following 24-hr incubation with MT-III, cells were treated with 6-OHDA for additional 24 hr. The catalytic activity of caspase-3 in cell lysates was assayed using the specific substrate DEVD-pNA. All of the experiments were done in triplicates and the means and standard deviations are shown. Asterisks (*) denote that the differences were significant compared to the MT-III plus 6-OHDA-treated cells ($P < 0.01$, by t-test).

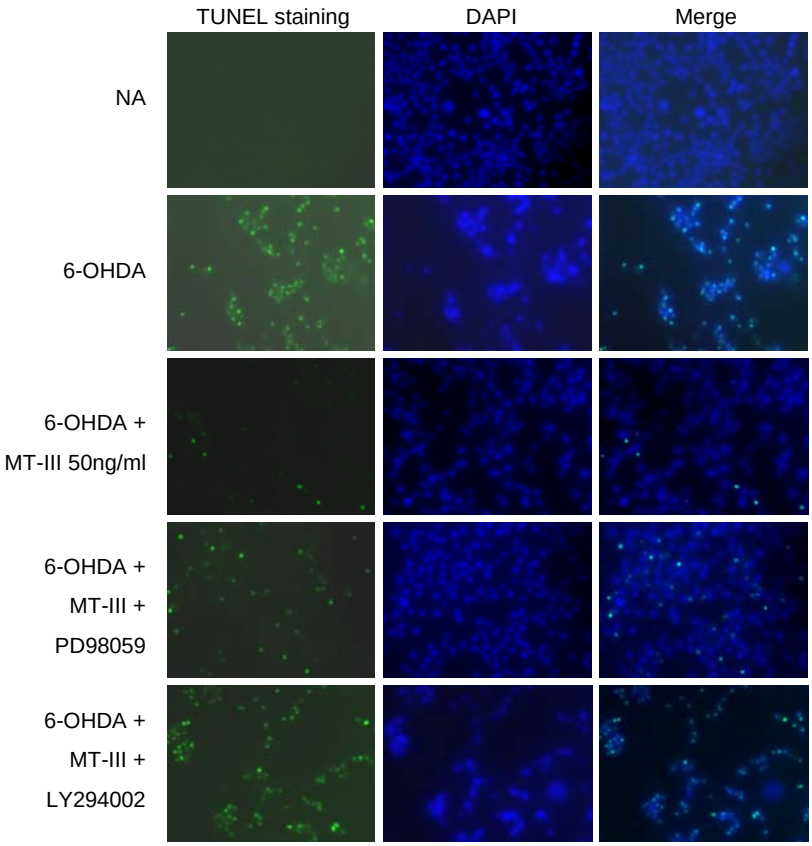

Fig. 50. PI3K and ERK1/2 inhibitor attenuated the anti-apoptotic effect of MT-III: TUNEL assay.

SH-SY5Y cells were cultured with 50 ng/ml of MT-III in the presence or absence of PD98059 or LY294002. After 24 hr, cells were challenged for additional 24 hr with 6-OHDA of 50 μM. After the treatment, TUNEL assays were performed according to the manufacturer's instruction. All of the experiments were repeated at least three times and representative results are shown.

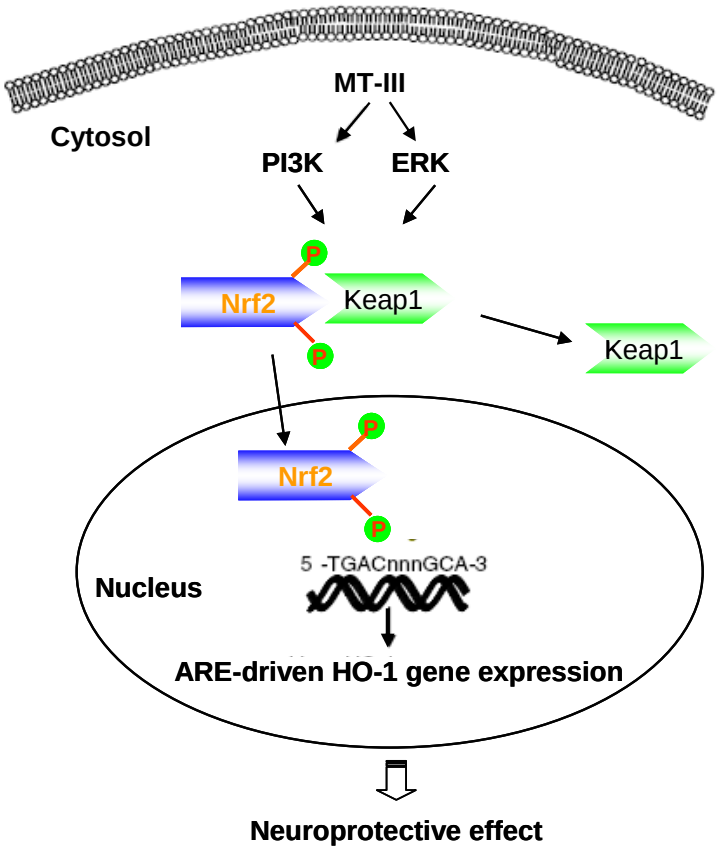

Fig. 51. A proposed pathway for MT-III-induced HO-1 expression via upregulation of PI3K, ERK1/2 and Nrf2, which explains neuroprotection of MT-III against oxidative stress in dopaminergic SH-SY5Y cells.

IV. Discussion

Neuroprotecion by metallothionein-III involves activation of nuclear factor-κB through phosphatidylinositol-3 kinase signaling pathway

In the previous reports, the overexpression of human MT-III reduces H_2O_2-induced oxidative stress and DNA damage in human fibroblasts (Jeong *et al*., 2004; You *et al*., 2002a). The present study reports the molecular mechanism underlying the neuroprotective effect of MT-III against apoptotic stimuli. The anti-apoptotic effect of MT-III was exerted by inducing the phosphorylation of IκB and the subsequent nuclear translocation and activation of NF-κB through the PI3K/Akt signaling pathway. The neuroprotective role of MT-III was proposed based on the following evidences. First, MT-III was discovered as an inhibitor of neuronal growth factors that appeared at lower levels in Alzheimer's disease brains. The role of MT-III has been studied in a number of animal brain damage models and these studies suggested that this isoform is involved in reparative and protective processes in the brain (Aschner, 1996; Hidalgo *et al*., 2006; Carrasco *et al*., 2006; You *et al*., 2002b). Second, it has been recently reported that MT-III-deficient mice are more susceptible to kainic acid-induced seizures and neuronal injury than normal mice, and that MT-III transgenic mice are more resistant to neuronal injury (Erickson *et*

al., 1995, 1997; Kim *et al*., 2003).

The major function of MT is to bind and regulate intracellular concentration of metal ions. MT proteins can donate or sequester metal ions from proteins including transcription factors, enzymes, and receptors, and then regulate the function of these proteins. Metal ion-deprived Apo-MT forms do occur in several tissues, including brain. MT-III resists proteolytic degradation in zinc-depleted cellular conditions where other MT proteins are degraded (Palmiter, 1995). In this regard, the present study was could provide evidence that apo-MT-III prevents Dox-induced cell death as similar as the like metal ion-bound form of MT-III in neuronal cells (Fig. 6). These results suggested that binding and sequestering of metal ions may not be important for neuroprotective effect of MT-III. Both the expression of MT and the activation of NF-κB are induced by stress. These processes are mediated through anti-apoptotic processes to protect cell from ionizing radiation and provide resistance to anticancer drugs (Choi *et al*., 2001; Wang and Cassidy, 2003). Several studies suggested a role for MT in modulating NF-κB activity, but the mode of action has remained controversial: some reports suggested positive effects of MT-III on NF-κB activity while others suggested negative effects (Abdel-Mageed and Agrawal, 1998; Butcher *et al*., 2004; Papouli *et al*., 2002; Kanekiyo *et al*., 2002; Crowthers *et al*., 2000; Sakurai *et al*., 1999). Overexpression or induction of MT in MCF7 cells increased the DNA binding, reporter activation and target

gene expression of NF-κB (Chuang *et al*., 2002). Also, the amount of cellular NF-κB p65 subunit in MT-knockout cells was less than 20% and the activation of a NF-κB reporter was than 50% compared to those in MT-wild type cells, consistent with the increased sensitivity of MT-knockout cells to apoptosis (Abdel-Mageed and Agrawal, 1998). On the other hand, MT has been also reported to inhibit TGF-ß induced degradation of IκB and the expression of NF-κB-dependent genes (Kanekiyo *et al*., 2002). In addition, MT-II antisense partially restores the NF-κB activity in a mitomycin C-resistant hamster cell line in response to mitomycin C (Butcher *et al*., 2004). However, none of these studies quantitatively assessed the role of MT-III for modulating NF-κB activity in the context of the neuroprotective effect of MT-III. The present study was provided clean evidences that MT-III increases the nuclear translocation of p65 subunit of NF-κB and the transcriptional activity of NF-κB in a dose-dependent manner, and that the activation of NF-κB is an important step for the anti-apoptotic effect of MT-III in neuronal cells (Fig. 12-18).

The IκB-dependent modulation of NF-κB activity in response to various stimuli has been well characterized in numerous cell types (Sun and Andersson, 2002). In this pathway, NF-κB activation is achieved by the phosphorylation of IκB, the binding of a specified ubiquitin ligase complex to phosphorylated IκB, and the polyubiquitination and subsequent proteasome-mediated degradation of IκB. The

phosphorylation of IκB is the only regulated step in this cascade and the enzymes involved in IκB polyubiquitination and degradation is constitutively active (Delhase *et al*., 1999; Zandi *et al*., 1998). The results that MT-III induces the phosphorylation and degradation of IκB suggested that NF-κB activation by MT-III in neuronal cells involves IκB phosphorylation and degradation (Fig. 17). The present study also characterized the upstream signaling pathway that mediates the MT-III-induced anti-apoptotic effect. Several groups have reported that PI3K and Akt are involved in the activation of NF-κB in response to TNF, interleukin 1, phorbolmyristate acetate, and PDGF signaling. These results indicated that IKK activity is involved for enhancing NF-κB nuclear translocation (Kane *et al*., 1999; Reddy *et al*., 2000; Habib *et al*., 2001). Using specific inhibitors of PI3K/Akt signaling pathway and NF-κB, the present study demonstrated that the MT-III-induced activation of Akt, phosphorylation and degradation of IκB increase in nuclear accumulation and transcriptional activity of NF-κB, and that anti-apoptotic effect in neuronal cells were all dependent on the activity of PI3K/Akt signaling pathway and NF-κB (Fig. 19-27). These observations suggested that MT-III activates PI3K/Akt signal pathway. Although the precise mechanisms by how MT-III prevents Dox- or H_2O_2-induced cell death via activation of PI3K/Akt signal pathway need to be elucidated, these findings may provide clues to understand the new role of MT-III in brain. Taken together, the present results suggested that MT-III exerts neuroprotective

effect by activating NF-κB through the PI3K/Akt signaling pathway in neuronal cells.

Metallothionein-III protects against 6-hydroxydopamine-induced oxidative stress by increasing expression of heme oxygenase-1 in the PI3K and ERK/Nrf2 dependent manner

The present study demonstratesd a new physiological role for the PI3K/Akt and ERK1/2 survival pathway activated by MT-III: control of intracellular levels of oxygen free radicals by regulating the expression of HO-1 through Nrf2 activation. Previous studies have indicated that overexpression of human MT-III reduces H_2O_2-induced oxidative stress and DNA damage in human fibroblasts (Jeong *et al*., 2004; You *et al*., 2002a). In addition, a previous study demonstrated that MT-III can scavenge $OH^{\cdot}$ radicals formed during radiolysis and $O_2^{\cdot-}$ radicals in a riboflavine-methionine-nitrobluetetrazolium (NBT) radical producing system (Shi *et al*., 2003). It suggested that MT-III could act as an efficient scavenger against free radicals *in vivo* (Fig. 33 and 34). It has also been shown that MT-III may modulate the canonical antioxidant machinery of ROS by increasing the expression of superoxide dismutase activities (Shi *et al*., 2003). However, the underlying mechanism for its effects is unclear. The present investigation further demonstrated that MT-III regulates the expression of the novel antioxidant mechanism including the stress protein HO-1. The results presented in this study are consistent with the hypothesis that MT-III affords neuroprotection through an antioxidant mechanism. The present study further demonstrated that MT-III regulates the expression of the antioxidant

mechanism involving the stress protein HO-1 (Fig. 35-38). Moreover, enhanced HO-1 expression is essential for MT-III's function in neuroprotection. Although PI3K/Akt is a well-documented pathway involved in protecting against apoptotic insults, including oxidative stress, this is the first report linking the survival pathway with a specific enzyme involved in ROS detoxification by mammalian cells. The present results showed that the PI3K/Akt and ERK1/2 pathway are both necessary and sufficient for MT-III-dependent attenuation of cell death in SH-SY5Y cells exposed to 6-OHDA (Fig. 48-50).

The cytoprotective properties of antioxidants have been partially attributed to their abilities to induce cytoprotective enzymes. Among the various cytoprotective enzymes, HO-1 expression has been considered an adaptive and beneficial response to oxidative stress in a wide variety of cells (Alam and Cook, 2003; Choi *et al*., 2003; Otterbein *et al*., 2003). Recent reports have shown that transgenic mice overexpressing moderate levels of HO-1 display augmented resistance to neural damage in animal models of cerebral ischemia (Panahian *et al*., 1999) or after H_2O_2- and glutamate-induced oxidant stress *in vitro* (Chen *et al*., 2000). Here, we demonstrated that MT-III increases HO-1 mRNA and protein expressions as well as HO-1 activity in SH-SY5Y cells (Fig. 35-38). The increase in HO-1 expression by MT-III conferred cytoprotection against 6-OHDA-induced oxidative stress. These results suggested that MT-III-induced HO-1 expression might serve an important

mechanism for the cytoprotective effects of MT-III. Accordingly, this study showed that constitutive but moderate overexpression of HO-1 attenuated 6-OHDA-induced ROS generation. The diversity of stimuli that induce HO-1 suggests that the molecular mechanisms for the regulation of HO-1 are complex. Several studies have described the regulatory sites and transcription factors required for activation of the HO-1 promoter (Gong *et al*., 2002; Hill-Kapturczak *et al*., 2003). Recent evidence has implicated the role of Nrf2 in the inducer-dependent activation of the HO-1 gene (Alam and Cook, 2003; Alam *et al*., 1999). The transcription factor, Nrf2, positively regulates the ARE-mediated expression of antioxidant enzyme genes, including HO-1 (Zhang and Gordon, 2004). In this regard, the present study demonstrated that MT-III might increase the expression of HO-1 genes through Nrf2-mediated ARE activation (Fig. 39 and 40).

PI3K/Akt and MAPK pathways have been reported to be involved in HO-1 expression (Chen *et al*. 2004b; Balogun *et al*., 2003; Gong *et al*., 2004) and also in Nrf2-dependent transcription (Zipper and Mulcahy, 2003; Shen *et al*., 2004). The present studies were designed to determine a possible role for PI3K/Akt and MAPK pathways in MT-III-induced HO-1 expression. The results showed that MT-III activates PI3K/Akt and ERK1/2 pathways (Fig. 41). Additionally, the use of specific inhibitors for PI3K/Akt and MAPK pathways confirmed the involvement of PI3K/Akt and ERK1/2, but not of p38 and JNK1/2, in MT-III-induced HO-1

expression (Fig. 42), as well as MT-III-modulated transcriptional activity of HO-1 (Fig. 43) and neuroprotection (Fig. 48-50). Moreover, PI3K/Akt inhibitor blocked MT-III-induced Nrf2 nuclear translocation (Fig. 44 and 45). These results suggest that PI3K/Akt and ERK1/2 pathways might be critical for MT-III-induced HO-1 expression and Nrf2 nuclear translocation.

In summary, MT-III induces HO-1 expression in dopaminergic SH-SY5Y neuronal cells, which confers neuroprotection against oxidative injury. MT-III also induces Nrf2 nuclear translocation, an upstream of MT-III-induced HO-1 expression, and activates PI3K/Akt and ERK1/2 phosphorylation. The PI3K/Akt and ERK1/2 pathways are involved in MT-III-induced Nrf2 nuclear translocation, HO-1 expression, and neuroprotection in neuronal cells (Fig. 51).

V. References

Abdel-Mageed, A. B. and Agrawal, K. C. (1998) Activation of nuclear factor kappaB: potential role in metallothionein-mediated mitogenic response. Cancer Res. 58, 2335-2338.

Alam, J. and Cook, J. L. (2003) Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. Curr. Pharm. Des. 9, 2499-2511.

Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A. M., and Cook, J. L., (1999) Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J. Biol. Chem. 274, 26071-26078.

Aoki, C., Nakanishi, T., Sogawa, N., Ishii, K. Ogawa, N., Takigawa, M., and Furuta, H. (1998) Stimulatory effects of 4-methylcatechol, dopamine and levodopa on the expression of metallothionein-III (GIF) mRNA in immortalized mouse brain glial cells (VR-2g). Brain Res. 792, 335-339.

Aschner, M. (1996) The functional significance of brain metallothioneins. FASEB J. 10, 1129-1136.

Balogun, E., Hoque, M., Gong, P., Killeen, E., Green, C. J., Foresti, R., Alam, J., and Motterlini, R. (2003) Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. Biochem. J. 371, 888-895.

Bruchelt, G., Schraufstatter, I. U., Niethammer, D., and Cochrane, C. G. (1991) Ascorbic acid enhances the effects of 6-hydroxydopamine and H_2O_2 on iron-dependent DNA strand breaks and related processes in the neuroblastoma cell line SK-N-SH. Cancer Res. 51, 6066-6072.

Butcher, H. L., Kennette, W. A., Collins, O., Zalups, R. K., and Koropatnick, J. (2004) Metallothionein mediates the level and activity of nuclear factor kappa B in murine fibroblasts. J. Pharmacol. Exp. Ther. 310, 589-598.

Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. Science 282, 1318-1321.

Carrasco, J., Adlard, P., Cotman, C., Quintana, A., Penkowa, M., Xu, F., Van Nostrand, W. E., and Hidalgo, J. (2006) Metallothionein-I and -III expression in animal models of Alzheimer disease. Neuroscience 143, 911-922.

Chen, C. and Bazan, N. G. (1999) Platelet-activating factor inhibits ionotropic GABA receptor activity in cultured hippocampal neurons. Neuroreport 10, 3831-3835.

Chen, C., Pung, D., Leong, V., Hebbar, V., Shen, G., Nair, S., Li, W., and Kong, A. N. (2004b) Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2: effect ofchemical structure and stress signals. Free Radic. Biol. Med. 37, 1578-1590.

Chen, K. and Maines, M. D. (2000) Nitric oxide induces heme oxygenase-1 via mitogen-activated protein kinases ERK and p38. Cell Mol. Biol. 46, 609-617.

Chen, K., Gunter, K., and Maines, M. D. (2000) Neurons overexpressing heme oxygenase-1 resist oxidative stress-mediated cell death. J. Neurochem. 75, 304-313.

Chen, X. L. and Kunsch, C. (2004a) Induction of cytoprotective genes through

Nrf2/antioxidant response element pathway: a new therapeutic approach for the treatment of inflammatory diseases. Curr. Pharm. Des. 10, 879-891.

Choi, B. M., Pae, H. O., Kim, Y. M., and Chung, H. T. (2003) Nitric oxide-mediated cytoprotection of hepatocytes from glucose deprivation-induced cytotoxicity: involvement of heme oxygenase-1. Hepatology 37, 810-823.

Choi, C. Y., Kim, J. Y., Kim, Y. S., Chung, Y. C., Hahm, K. S., and Jeong, H. G. (2001) Augmentation of macrophage functions by an aqueous extract isolated from *Platycodon grandiflorum*. Cancer Lett. 166, 17-25.

Chuang, S. E., Yeh, P. Y., Lu, Y. S., Lai, G. M., Liao, C. M., Gao, M., and Cheng, A. L. (2002) Basal levels and patterns of anticancer drug-induced activation of nuclear factor-kappaB (NF-kappaB), and its attenuation by tamoxifen, dexamethasone, and curcumin in carcinoma cells. Biochem. Pharmacol. 63, 1709-1716.

Cohen, G. and Heikkila, R. E. (1974) The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. J. Biol. Chem. 249, 2447-2452.

Crowthers, K. C., Kline, V., Giardina, C., and Lynes, M. A. (2000) Augmented humoral immune function in metallothionein-null mice. Toxicol. Appl. Pharmacol. 166, 161-172.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91, 231-241.

Davison, A. J., Legault, N. A., and Steele, D. W. (1986) Effect of 6-

hydroxydopamine on polymerization of tubulin. Protection by superoxide dismutase, catalase, or anaerobic conditions. Biochem. Pharmacol. 35, 1411-1417.

Delhase, M., Hayakawa, M., Chen, Y., and Karin, M. (1999) Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. Science 284, 309-313.

Ebadi, M., Iversen, P. L., Hao, R., Cerutis, D. R., Rojas, P., Happe, H. K., Murrin, L. C., and Pfeiffer, R. F. (1995) Expression and regulation of brain metallothionein. Neurochem. Int. 27, 1-22.

Erickson, J. C., Hollopeter, G., Thomas, S. A., Froelick, G. J., and Palmiter, R. D. (1997) Disruption of the metallothionein-III gene in mice: analysis of brain zinc, behavior, and neuron vulnerability to metals, aging, and seizures. J. Neurosci. 17, 1271-1281.

Erickson, J. C., Masters, B. A., Kelly, E. J., Brinster, R. L., and Palmiter, R. D. (1995) Expression of human metallothionein-III in transgenic mice. Neurochem. Int. 27, 35-41.

Ghosh, S., May, M. J., and Kopp, E. B. (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16, 225-260.

Ghoshal, K. and Jacob, S. T. (2001) Regulation of metallothionein gene expression. Prog. Nucleic Acid Res. Mol. Biol. 66, 357-384.

Glinka, Y. Y. and Youdim, M. B. (1995) Inhibition of mitochondrial complexes I and IV by 6-hydroxydopamine. Eur. J. Pharmacol. 292, 329-332.

Gong, P., Hu, B., and Cederbaum, A. I. (2004) Diallyl sulfide induces heme oxygenase-1 through MAPK pathway. Arch. Biochem. Biophys. 432, 252-260.

Gong, P., Stewart, D., Hu, B., Li, N., Cook, J., Nel, A., and Alam, J. (2002) Activation of the mouse heme oxygenase-1 gene by 15-deoxy-D(12,14)-prostaglandin J(2) is mediated by the stress response elements and transcription factor Nrf2. Antioxid. Redox Signal. 4, 249-257.

Graham, D. G. (1978) Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinines. Mol. Pharmacol. 14, 633-643.

Guo, X. Shin, V. Y., and Cho, C. H. (2001) Modulation of heme oxygenase in tissue injury and its implication in protection against gastrointestinal diseases. Life Sci. 69, 3113-3119.

Habib, A. A., Chatterjee, S., Park, S. K., Ratan, R. R., Lefebvre, S., and Vartanian, T. (2001) The epidermal growth factor receptor engages receptor interacting protein and nuclear factor-kappa B (NF-kappa B)-inducing kinase to activate NF-kappa B. Identification of a novel receptor-tyrosine kinase signalosome. J. Biol. Chem. 276, 8865-8874.

Haq, F., Mahoney, M., and Koropatnick, J. (2003) Signaling events for metallothionein induction. Mutat. Res. 533, 211-226.

Hastings, T. G. (1995) Enzymatic oxidation of dopamine: the role of prostaglandin H synthase. J. Neurochem. 64, 919-924.

Hernandez, J., Carrasco, J., Belloso, E., Giralt, M., Bluethmann, H., Kee, L. D., Andrews, G. K., and Hidalgo, J. (2000) Metallothionein induction by restraint stress: role of glucocorticoids and IL-6. Cytokine 12, 791-796.

Hidalgo, J. and Carrasco, J. (1998) Regulation of the synthesis of brain metallothioneins. Neurotoxicology 19, 661-666.

Hidalgo, J., Aschner, M., Zatta, P., and Vasak, M. (2001) Roles of the metallothionein family of proteins in the central nervous system. Brain Res. Bull. 55, 133-145.

Hidalgo, J., Penkowa, M., Espejo, C., Martinez-Caceres, E. M., Carrasco, J., Quintana, A., Molinero, A., Florit, S., Giralt, M., and Ortega-Aznar, A. (2006) Expression of metallothionein-I, -II, and -III in Alzheimer disease and animal models of neuroinflammation. Exp. Biol. Med. (Maywood.) 231, 1450-1458.

Hill-Kapturczak, N., Sikorski, E., Voakes, C., Garcia, J., Nick, H. S., and Agarwal, A. (2003) An internal enhancer regulates heme- and cadmium-mediated induction of human heme oxygenase-1. Am. J. Physiol. Renal Physiol. 285, 515-523.

Hill-Kapturczak, N., Truong, L., Thamilselvan, V., Visner, G.. A, Nick, H. S., and Agarwal, A. (2000) Smad7-dependent regulation of heme oxygenase-1 by transforming growth factor-beta in human renal epithelial cells. J. Biol. Chem. 275, 40904-40909.

Hozumi, I., Inuzuka, T., and Tsuji, S. (1998) Brain injury and growth inhibitory factor (GIF) - a mini review. Neurochem. Res. 23, 319-328.

Hozumi, I., Uchida, Y., Watabe, K., Sakamoto, T., and Inuzuka, T. (2006) Growth inhibitory factor (GIF) can protect from brain damage due to stab wounds in rat brain. Neurosci. Lett. 395, 220-223.

Huang, H. C., Nguyen, T., and Pickett, C. B. (2000) Regulation of the antioxidant

response element by protein kinase C-mediated phosphorylation of NFE2- related factor 2. Proc. Natl. Acad. Sci. U. S. A. 97, 12475-12480.

Hussain, S., Slikker, W., and Ali, S. F. (1996) Role of metallothionein and other antioxidants in scavenging superoxide radicals and their possible role in neuroprotection. Neurochem. Int. 29, 145-152.

Irie, Y. and Keung, W. M. (2003) Anti-amyloid beta activity of metallothionein-III is different from its neuronal growth inhibitory activity: structure-activity studies. Brain Res. 960, 228-234.

Itoh, K., Tong, K. I., and Yamamoto, M. (2004) Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. Free Radic. Biol. Med. 36, 1208-1213.

Jacob, S. T., Ghoshal, K., and Sheridan, J. F. (1999) Induction of metallothionein by stress and its molecular mechanisms. Gene Expr. 7, 301-310.

Jaeschke, H. (2000) Reactive oxygen and mechanisms of inflammatory liver injury. J. Gastroenterol. Hepato.15, 8718-724.

Jeong, H. G., Youn, C. K., Cho, H. J., Kim, S. H., Kim, M. H., Kim, H. B., Chang, I. Y., Lee, Y. S., Chung, M. H., and You, H. J. (2004) Metallothionein-III prevents gamma-ray-induced 8-oxoguanine accumulation in normal and hOGG1-depleted cells. J. Biol. Chem. 279, 34138-34149.

Kagi, J. H. (1991) Overview of metallothionein. Methods Enzymol. 205, 613-626.

Kagi, J. H. and Schaffer, A. (1988) Biochemistry of metallothionein. Biochemistry 27, 8509-8515.

Kane, L. P., Shapiro, V. S., Stokoe, D., and Weiss, A. (1999) Induction of NF-kappaB by the Akt/PKB kinase. Curr. Biol. 9, 601-604.

Kanekiyo, M., Itoh, N., Kawasaki, A., Matsuyama, A., Matsuda, K., Nakanishi, T., and Tanaka, K. (2002) Metallothionein modulates lipopolysaccharide-stimulated tumour necrosis factor expression in mouse peritoneal macrophages. Biochem. J. 361, 363-369.

Kapitulnik, J. (2004) Bilirubin: an endogenous product of heme degradation with both cytotoxic and cytoprotective properties. Mol. Pharmacol. 66, 773-779.

Karin, M. and Lin, A. (2002) NF-kappaB at the crossroads of life and death. Nat. Immunol. 3, 221-227.

Kim, D., Kim, E. H., Kim, C., Sun, W., Kim, H. J., Uhm, C. S., Park, S. H., and Kim, H. (2003) Differential regulation of metallothionein-I, II, and III mRNA expression in the rat brain following kainic acid treatment. Neuroreport 14, 679-682.

Kong, A. N., Owuor, E., Yu, R., Hebbar, V., Chen, C., Hu, R., and Mandlekar, S. (2001) Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). Drug Metab. Rev. 33, 255-271.

Kumar, R., Agarwal, M. L., and Seth, P. K. (1995) Free radical-generated neurotoxicity of 6-hydroxydopamine. J. Neurochem. 64, 1703-1707.

Kutty, R. K. and Maines, M. O. (1982) Oxidation of heme c derivatives by purified heme oxygenase in the rat liver. J. Biol. Chem. 257, 9944-9952.

Luo, Z., Fujio, Y., Kureishi, Y., Rudic, R. D., Daumerie, G., Fulton, D., Sessa, W. C., and Walsh, K. (2000) Acute modulation of endothelial Akt/PKB activity alters nitric oxide-dependent vasomotor activity in vivo. J. Clin. Invest. 106, 493-499.

Madrid, L. V., Mayo, M. W., Reuther, J. Y., and Baldwin, A. S., Jr. (2001) Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. J. Biol. Chem. 276, 18934-18940.

Madrid, L. V., Wang, C. Y., Guttridge, D. C., Schottelius, A. J., Baldwin, A. S., Jr., and Mayo, M. W. (2000) Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. Mol. Cell Biol. 20, 1626-1638.

Maines, M. D. (1998) Heme oxygenase: function, multiplicity, regulatory mechanisms and clinical application. FASEB J. 2, 2557-2568.

Martin, D., Rojo, A. I, Salinas, M., Diaz, R., Gallardo, G., Alam, J., Galarreta, C. M., Cuadrado, A., (2004) Regulation of heme oxygenase-1 expression through the phosphatidylinositol 3-Kinase/Akt pathway and the Nrf2 transcription factor in response to the antioxidant phytochemical carnosol. J. Biol. Chem. 279, 8919-8929.

Masters, B. A., Quaife, C. J., Erickson, J. C., Kelly, E. J., Froelick, G.. J., Zambrowicz, B. P., Brinster, R. L., and Palmiter, R. D. (1994) Metallothionein III is expressed in neurons that sequester zinc in synaptic vesicles. J. Neurosci. 14, 5844-5857.

Monks, N. R., Biswas, D. K., and Pardee, A. B. (2004) Blocking anti-apoptosis as a

strategy for cancer chemotherapy: NF-kappaB as a target. J. Cell Biochem. 92, 646-650.

Nakaso, K., Yano, H., Fukuhara, Y., Takeshima, T., Wada-Isoe, K., and Nakashima, K. (2003) PI3K is a key molecule in the Nrf2-mediated regulation of antioxidative proteins by hemin in human neuroblastoma cells. FEBS Lett. 546,181-184.

Numazawa, S., Ishikawa, M., Yoshida, A., Tanaka, S., and Yoshida, T. (2003) A typical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress. Am. J. Physiol. Cell Physiol. 285, 334-342.

Otterbein, L. E., Soares, M. P., Yamashita, K., and Bach, F. H., (2003) Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol. 24, 449-455.

Owuor, E. D. and Kong, A. N. (2002) Antioxidants and oxidants regulated signal transduction pathways. Biochem. Pharmacol. 64, 765-770.

Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 401, 82-85.

Palmiter, R. D. (1995) Constitutive expression of metallothionein-III (MT-III), but not MT-I, inhibits growth when cells become zinc deficient. Toxicol. Appl. Pharmacol. 135, 139-146.

Palmiter, R. D., Findley, S. D., Whitmore, T. E., and Durnam, D. M. (1992) MT-III, a brain-specific member of the metallothionein gene family. Proc. Natl. Acad. Sci. U.S.A. 89, 6333-6337.

Panahian, N., Yoshiura, M., and Maines, M. D. (1999) Overexpression of heme oxygenase-1 is neuroprotective in a model of permanent middle cerebral artery occlusion in transgenic mice. J. Neurochem. 72, 1187-1203.

Papouli, E., Defais, M., and Larminat, F. (2002) Overexpression of metallothionein-II sensitizes rodent cells to apoptosis induced by DNA cross-linking agent through inhibition of NF-kappa B activation. J. Biol. Chem. 277, 4764-4769.

Reddy, S. A., Huang, J. H., and Liao, W. S. (2000) Phosphatidylinositol 3-kinase as a mediator of TNF-induced NF-kappa B activation. J. Immunol. 164, 1355-1363.

Sakurai, A., Hara, S., Okano, N., Kondo, Y., Inoue, J., and Imura, N. (1999) Regulatory role of metallothionein in NF-kappaB activation. FEBS Lett. 455, 55-58.

Samson, S. L. and Gedamu, L. (1998) Molecular analyses of metallothionein gene regulation. Prog. Nucleic Acid Res. Mol. Biol. 59, 257-288.

Saner, A. and Thoenen, H. (1971) Model experiments on the molecular mechanism of action of 6-hydroxydopamine. Mol. Pharmacol. 7, 147-154.

Schmitz, M. L., Bacher, S., and Kracht, M. (2001) I kappa B-independent control of NF-kappa B activity by modulatory phosphorylations. Trends Biochem. Sci. 26, 186-190.

Selzner, N., Rudiger, H., Graf, R., and Clavien, P. A. (2003) Protective strategies against ischemic injury of the liver. Gastroenterology 125, 917-936.

Shen, G., Hebbar, V., Nair, S., Xu, C., Li, W., Lin, W., Keum, Y. S., Han, J., Gallo,

M.A., and Kong, A. N. (2004) Regulation of Nrf2 transactivation domain activity. The differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein. J. Biol. Chem. 279, 23052-23060.

Shishodia, S. and Aggarwal, B. B. (2002) Nuclear factor-kappaB activation: a question of life or death. J. Biochem. Mol. Biol. 35, 28-40.

Shi, Y., Wang, W., Mo, J., Du, L., Yao, S., and Tang, W. (2003) Interactions of growth inhibitory factor with hydroxyl and superoxide radicals. Biometals 16, 383-389.

Sian, J., Dexter, D.T., Lees, A.J., Daniel, S., Agid, Y., Javoy-Agid, F., Jenner, P., Marsden, C.D. (1994) Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. Ann. Neurol. 36, 48-355.

Sian, J., Dexter, D. T., Lees, A. J., Daniel, S., Agid, Y., Javoy-Agid, F., Jenner, P., Sogawa, C. A., Asanuma, M., Sogawa, N., Miyazaki, I., Nakanishi, T., Furuta, H., and Ogawa, N. (2001) Localization, regulation, and function of metallothionein-III/growth inhibitory factor in the brain. Acta Med. Okayama 55, 1-9.

Spina, M. B., Squinto, S. P., Miller, J., Lindsay, R. M., and Hyman, C. (1992) Brainderived neurotrophic factor protects dopamine neurons against 6-hydroxydopamine and N-methyl-4-phenylpyridinium ion toxicity: involvement of the glutathione system. J. Neurochem. 59, 99-106.

Sun, Z. and Andersson, R. (2002) NF-kappaB activation and inhibition: a review. Shock 18, 99-106.

Takahashi, T., Morita, K., Akagi, R., and Sassa, S. (2004) Heme oxygenase-1: a novel therapeutic target in oxidative tissue injuries. Curr. Med. Chem. 11, 1545-1561.

Tiffany-Castiglioni, E. and Perez-Polo, J. R. (1981) Stimulation of resistance to 6-dydroxydopamine in a human neuroblastoma cell line by nerve growth factor. Neurosci. Lett. 26, 157-161.

Tiffany-Castiglioni, E., Saneto, R. P., Proctor, P. H., and Perez-Polo, J. R. (1982) Participation of active oxygen species in 6-hydroxydopamine toxicity to a human neuroblastoma cell line. Biochem. Pharmacol. 15, 181-188.

Tsuji, S., Kobayashi, H., Uchida, Y., Ihara, Y., and Miyatake, T. (1992) Molecular cloning of human growth inhibitory factor cDNA and its down-regulation in Alzheimer's disease. EMBO J. 11, 4843-4850.

Tulis, D. A., Durante, W., Peyton, K. J., Evans, A. J., and Schafer, A. I., (2001) Heme oxygenase-1 attenuates vascular remodeling following balloon injury in rat carotid arteries. Atherosclerosis 155, 113-122.

Uchida, Y. (1994) Growth inhibitory factor, metallothionein-like protein, and neurodegenerative diseases. Biol. Signals 3, 211-215.

Uchida, Y., Takio, K., Titani, K., Ihara Y., and Tomonaga, M. (1991) The growth inhibitory factor that is deficient in the Alzheimer's disease brain is a 68 amino acid metallothionein-like protein. Neuron 7, 337-347.

Vallee, B. L. (1991) Introduction to metallothionein. Methods Enzymol. 205, 3-7.

Vasak, M. and Hasler, D. W. (2000) Metallothioneins: new functional and structural

insights. Curr. Opin. Chem. Biol. 4, 177-183.

Wang, W. and Cassidy, J. (2003) Constitutive nuclear factor-kappa B mRNA, protein overexpression and enhanced DNA-binding activity in thymidylate synthase inhibitor-resistant tumour cells. Br. J. Cancer 88, 624-629.

Xie, H., Chiles, T. C., and Rothstein, T. L. (1993) Induction of CREB activity via the surface Ig receptor of B cells. J. Immunol. 151, 880-889.

Yamada, N., Yamaya, M., Okinaga, S., Nakayama, K., Shibahara, S., and Sasaki, H. (2000) Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. Am. J. Hum. Genet. 66, 187-195.

You, H. J., Lee, K. J., and Jeong, H. G. (2002b) Overexpression of human metallothionein-III prevents hydrogen peroxide-induced oxidative stress in human fibroblasts. FEBS Lett. 521, 175-179.

You, H. J., Oh, D. H., Choi, C. Y., Lee, D. G., Hahm, K. S., Moon, A. R., and Jeong, H. G. (2002a) Protective effect of metallothionein-III on DNA damage in response to reactive oxygen species. Biochim. Biophys. Acta. 1573, 33-38.

Zandi, E., Chen, Y., and Karin, M. (1998) Direct phosphorylation of IkappaB by IKKalpha and IKKbeta: discrimination between free and NF-kappaB-bound substrate. Science 281, 1360-1363.

Zangger, K. and Armitage, I. M. (2002) Dynamics of interdomain and intermolecular interactions in mammalian metallothioneins. J. Inorg. Biochem. 88, 135-143.

Zhang, Y. and Gordon, G. B. (2004) A strategy for cancer prevention: stimulation of

the Nrf2-ARE signaling pathway. Mol. Cancer Ther. 3, 885-893.

Zhou, Y., Li, L., and Ru, B. (2000) Expression, purification and characterization of beta domain and beta domain dimer of metallothionein Biochim. Biophys. Acta 1524, 87-93.

Zipper, L. M. and Mulcahy, R. T. (2000) Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes. Biochem. Biophys. Res. Commun. 278, 484-492.

Zipper, L. M. and Mulcahy, R. T. (2003) Erk activation is required for Nrf2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase modulatory gene expression in HepG2 cells. Toxicol. Sci. 73, 124-134.

[국문 초록]

산화적 손상으로 유도된 신경세포 사멸에 대한 메탈로치오네인-III 의 신경세포 보호효과

황용필

약학과

조선대학교 대학원

(지도교수: 정혜광)

본 연구에서는 신경세포에서만 특이적으로 발현되는 메탈로티오네인-III (Metallothionein-III: MT-III)의 신경세포 손상에 대한 보호효과와 그 작용기전을 조사하였다. MT는 최초로 말의 신장에서 Cd와 Zn을 함유하는 단백질로서 발견된 이 후, 현재 박테리아, 균류, 식물 및 사람을 포함한 동물 등에서 존재하고 있음이 밝혀졌다. MT의 효소 기능은 아직 알려져 있지 않기 때문에 그 구조에 따라서 MT는 흔히 유형 I, II, III, IV로 분류된다. 체내 거의 모든 조직에서 발현되는 MT-I 및 MT-II와는 달리 MT-III는 뇌조직 특이적으로 신경세포에서만 발현되며, MT-IV은 일부

상피세포에서만 발현되는 것으로 알려져 있다. 체내 가장 풍부한 MT-I과 MT-II는 체내에서 다양한 금속 (Zn, Cu, Cd, Hg)과 결합하여 금속이온의 항상성 유지에 매우 중요한 역할을 하고 있는 것으로 알려져 있고 그 외 생리적 기능으로 금속 독성에 대한 방어 및 항산화제 역할 등이 있다. 그 동안 MT에 관한 연구는 주로 MT-I 및 MT-II에 대하여 이루어진 연구들로서 간이나 신장에서 중금속에 대한 보호효과 및 금속이온들의 항상성 유지에 대한 MT의 역할에 초점이 맞추어져 연구가 진행되어 왔다. 그러나 최근에 신경세포에서 과도한 신경돌기 (neurofibril tangle)의 형성을 억제하는 성장 억제인자 (growth inhibitory factor; GIF)가 MT-III임이 밝혀지면서 MT-III의 신경세포 내 역할에 대한 관심이 증가되고 있다. 특히 흥미로운 것은 GIF가 부족한 경우 과도한 신경 돌기가 정상적인 신경세포 주위에 생성되어 신경세포가 결국 사멸하게 되는데, 이런 과도한 신경 돌기형성이 퇴행성 뇌 질환인 알쯔하이머 병의 대표적인 병리학적인 소견이라는 사실이다. MT-III가 부족할 경우 알쯔하이머 병에서 나타나는 병리학적인 소견인 과도한 신경돌기 형성이 관찰되며, 또한 알쯔하이머 환자에서 MT-III 발현이 감소된다는 사실은 MT-III가 알쯔하이머 질환의 발병을 억제하는 중요한 물질로 작용할 가능성이 높음을 시사한다. 따라서 MT-III의 발현 정도가 신경돌기 형성에 영향을 주어 신경돌기 형성에 의한 신경세포 손상에 관여할 가능성이 매우 높기 때문에 MT-III의 발현 저하는 뇌 질환의 발병과

밀접한 관련이 있을 것이라는 추론이 강력하게 대두되고 있다. 또한 신경 손상에 대한 MT-III 의 보호효과가 알려져 있지만 아직까지 MT-III 의 신경보호 작용 기작은 잘 알려져 있지 않다. 본 연구는 아직 기능에 대한 연구가 매우 부족한MT-III 의 신경 보호효과와 그 작용기전을 연구하고자 하였다. MT-III 는 신경세포에서 H_2O_2 나 DNA 손상을 유도하는 화학물질에 의해 증가한 caspase-3 활성 및 미토콘드리아로부터 세포질로의 cytochrome c 의 방출 등으로 인한 신경세포 사멸을 억제하였다. 이러한 MT-III 의 신경세포 보호 효과는 MT-III 가 세포 성장과 생존에 관여하는 인자인 Akt 의 활성화를 유도함으로써 나타남을 알 수 있었다. 또한 IκB 의 인산화 및 분해가 증가하였으며 NF-κB 의 활성화 및 핵 안으로의 축적이 증가하였다. MT-III 에 의한 NF-κB 의 활성과 anti-apoptotic 효과는 PI3K, Akt, NF-κB 등의 저해제에 의해서 감소하였다. 이는 MT-III 가 PI3K/Akt 신호전달을 통해 NF-κB를 활성화 시켜 신경세포 보호효과가 있음을 알 수 있었다.

파킨슨 병 (Parkinson's disease)은 신경전달물질인 도파민의 감소로 인해 뇌의 흑색질 (substantia nigra) 부위의 신경세포가 줄어드는 중추신경계 질환이다. 세포 내 증가된 산화적 손상은 파킨슨 병의 중요한 발병원인으로 알려져 있다. 세포는 이러한 산화적 손상에 대한 방어기전으로 세포보호 유전자의 발현을 증가시킨다. 대표적인 예로

항산화 효소나 phase II 무독화 효소가 있다. 위의 유전자발현에는 Nrf2 라는 전사 조절인자의 조절을 받는다. 6-OHDA 는 활성산소를 생성하는 신경독소로 파킨슨 병의 유도에 사용하는 화학물질이다. 본 연구에서는 6-OHDA 으로 인해 증가되는 신경손상에 대한 MT-III 의 보호효과 및 그 작용기전에 대해 연구하였다. 신경세포에서 6-OHDA 처리에 의해 증가한 caspase 활성, 활성산소 및 세포독성은 MT-III 처리에 의해서 감소하였다. 또한 MT-III 는 항산화 효소인 heme oxygenase-1 (HO-1) 의 발현 및 NF-E2-related factor 2 (Nrf2) 활성을 증가 시켰으며, 이는 Akt 및 ERK1/2 의 인산화 증가를 통해 이루어짐을 알 수 있었다. PI3K 와 ERK1/2 저해제 실험 결과 MT-III 에 의한 신경세포 보호 기전은 PI3K 와 ERK1/2 의 활성이 필요함을 알 수 있었다. 이러한 결과를 종합하여 볼 때, MT-III 는 PI3K 와 ERK1/2/Nrf2 신호전달을 경유 하여 산화적 손상에 대한 방어 역할을 하는 HO-1 의 발현을 증가 시킴으로써 6-OHDA 와 같은 신경세포손상 유도 물질에 대한 세포 내 저항성을 증가 시켰을 것으로 사료되었다. 이로써 MT-III 는 6-OHDA 에 의한 도파민 유래 신경퇴화를 억제 할 수 있으며, 파킨슨 병의 치료에 유용하게 사용할 수 있을 것이다.

저작물 이용 허락서

학 과	약학과	학 번	20057394	과 정	박 사
성 명	한글 : 황 용 필 한문 : 黃 龍 必 영문 : Hwang Yong Pil				
주 소	광주 광역시 동구 산수동 563-6 번지 101 호				
연락처	E-mail : protoplast@hanmail.net				
논문제목	한글 : 산화적 손상으로 유도된 신경세포 사멸에 대한메탈로치오네인-III의 신경세포 보호효과				
	영문 : Protective Effects of Metallothionein-III on Oxidative Stress-Induced Neuronal Cell Death				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의 여부 : 동의 (○) 반대 ( )

2008년 2 월 일

저작자: 황 용 필 (인)

조선대학교 총장 귀하