# The role of mitochondria-mediated intrinsic death pathway in gingerdione derivative I6-induced neuronal apoptosis

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*Abbreviations*: I6, 1-(3,4-dimethoxyphenyl)-3,5-dodecenedione; Vit E, vitamin E; NAC, N-acetylcysteine; ROS, reactive oxygen species; PARP, poly(ADP-ribose)polymerase; $H_2$DCFDA, 2′, 7′-dichlorodihydrofluorescein diacetate; FCCP, Carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; MMP, mitochondria membrane potential; JC-1, 5,5`-6,6`-tetrachloro-1,1`-3,3`-tetraethyl benzimidazolylcarbocyanine iodide; MTT, (3-(4,5-dimethylthianol-2-yl)-2,5 diphenyl tetrazolium bromide).

## Abstract

Gingerdione, an active component from ginger, and its derivative I6 have been demonstrated to inhibit HL-60 cell proliferation. Neuronal death induced by I6 displayed apoptotic characteristics but the precise mechanism has not been fully elucidated. In the present studies, I6 at 24 h after intraperitoneal administration significantly decreased the density of surviving neurons and increased caspase-3 activity in frontal cortex, suggesting that peripherally administered I6 may cross BBB to induce CNS toxicity. In rat embryonic primary cortical cells, I6-induced reduction of mitochondrial viability and neuronal apoptosis was inhibited by vitamin E. In addition, I6-induced reactive oxygen species (ROS) caused the disruption of mitochondria membrane potential (MMP), the release of cytochrome c, the activation of caspase-9 and caspase-3, and cleavage of poly(ADP-ribose) polymerase (PARP), resulting in activation of mitochondrial-mediated intrinsic death pathway. Pre-treatment with antioxidant vitamin E or N-acetylcysteine (NAC) completely abolished the I6-induced generation of ROS, loss of MMP, release of cytochrome c, activation of caspase-9 and caspase-3, and cleavage of PARP. Carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), a mitochondrial uncoupler, significantly reduced I6-induced neuronal death as well as caspase-3 activation and PARP cleavage. These results suggest that I6 induces neuronal death by promoting intracellular ROS production to cause a loss of MMP that result in release of cytochrome c and activation of mitochondria-mediated intrinsic death pathway.


*Keyword:* Reactive oxygen species; Poly(ADP-ribose)polymerase; Apoptosis; Antioxidant; Gingerdione

## Introduction

Gingers (*Zingiber officinale*) are used for culinary purposes. They also ameliorate symptoms such as inflammation, arthritis, and gastrointestinal discomforts. Plants of ginger family can induce cell cycle arrest and cell death of pancreatic cancer cell (Park et al., 2006) and inhibit proliferation of human endothelial cells induced by vescular endothelial growth factor (VEGF) through down-regulation of cyclin D1 (Kim et al., 2005). Furthermore, [6]-gingerol inhibits expression of phorbol 12-myristate 13-acetate (PMA)-induced cyclooxygenase-2 (COX 2) and activation of nuclear factor-kappa B (NF-κB) and p38 mitogen-activated protein kinase (p38 MAPK) in mouse skin (Kim et al., 2004). However, the effects of ginger on neuronal cells have not been fully explored.

The major active ingredients of ginger include gingerol, zingerone, shagoal, and gingerdione. Gingerdione and its derivatives possess wide range of biological activities including modulation of macrophage phagocytosis, regulation of neutrophil biological function (Koh et al., 2010; Flynn et al., 1986), and inhibition of human hepatoblastoma growth (Chen et al., 2010). In additional, gingerdione is a serotonin 5-HT1A receptor partial agonist and possesses anxiolytic activity (Nievergelt et al., 2010).

I6, a synthetic gingerdione derivative, exhibits anti-tumor activity in human leukemia cell at a concentration of approximately 25 $\mu$M. However, at high concentration (78 $\mu$M), I6 can cause significant cytotoxicity of human normal leukocytes (Hsu et al., 2005). In a previous study, we showed that I6-induced neuronal death displays apoptotic characteristics. The concentrations for eliciting neurotoxicity is approximately 60 $\mu$M. Moreover, I6-induced activation of caspase-3 precedes induction of neuronal apoptosis (Lin et al., 2006). In the present studies, we further clarify the mechanism of action of I6. Our results suggest that I6-induced

production of ROS reduces mitochondria membrane potential, thus resulting in the activation of mitochondria-mediated intrinsic death signaling and neuronal apoptosis. Furthermore, both activation of caspases and induction of neuronal death can be prevented by antioxidants. The gingerdione derivatives are considered to be anti-cancer agents, which are promising in leukemia and hepatoblastoma (Hsu et al., 2005; Chen et al., 2010). However, it is important to take potential neuronal damage into consideration while using gingerdione derivatives as an anti-tumor agent for therapeutic purpose in the future.

## 1. Materials and Methods

### 1.1. Chemicals and Reagents

Vitamin E、N-acetylcysteine、MTT、Hoechst33342 and FCCP were purchased from Sigma (St. Louis, MO). $H_2$DCFDA and JC-1 were purchased from Molecular Probe (Eugene, OR). Cytochrome c, caspase-3, caspase-9, and PARP antibodies were obtained from Cell Signaling Technology (Beverly, MA). I6 was supplied by Dr. Li-Jiau Huang (China Medical University).

### 1.2. Primary cortical cultures

Primary neuronal cultures of cerebral cortex were obtained from embryos (E16-17) of Sprague-Dawley rats. Cerebral cortex was dissected, meninges were removed and cells were dissociated by mild trypsination and trituration as described by Culmsee et

al. (2002). Cortical cells were then seeded onto poly-D-lysine-coated 96-well plate culture dishes at a density of $5 \times 10^4$ (for survival analysis) or 60-mm culture dishes at a density of $3 \times 10^6$ (for immunoblot analysis). The neurons were cultured in neurobasal medium supplement with 2% B27, 0.5 mM l-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen, San Diego, CA). All experimental treatments were performed on 9-day-old cultures, at which time they contained less than 2% astrocytes as determined by GFAP-immunocytochemistry.

### 1.3. MTT (3-(4,5-dimethylthianol-2-yl)-2,5 diphenyl tetrazolium bromide) Assay

The colorimetric MTT reduction assay that quantified cell viability was carried out as described previously (Sheng et al., 1991). This method assessed mitochondrial activity by measuring the ability of cultured cells to convert yellow MTT to the purple formazan dye. Cortical neurons in 96-well plate were treated with I6 for 24 h and then were incubated with MTT (125 μg/ml) in growth medium (without phenol red) for 4 h at 37℃. The precipitated formazan was solubilized with SDS (25 mg/ml) and quantified spectrophotometrically at a wavelength of 570 nm. Data were expressed as the percentage of viable cell in I6-treated neurons compared with control neurons.

### 2.4. Measurement of intracellular ROS by microscopy and fluorescent plate reader

To measure the production of intracellular ROS, oxidant-sensitive dyes $H_2$DCFDA was used. $H_2$DCFDA passively diffuses into cells, where the acetate is cleaved by intracellular esterases to form 2`,7`-dichlorodihydrofluorescein ($H_2$DCF). The latter is trapped within the cell and reacts with ROS to emit fluorescence. After treatment of

cells with I6 (80 $\mu$M) for 0-12 h, the cells were loaded with $H_2$DCF-DA (10 μM) and incubated for 10 min at 37 °C. After washing twice with PBS, the accumulation of ROS was visualized using the fluorescence microscope with a constant exposure time (100 ms) from the CCD camera. The fluorescence intensity of $H_2$DCF was measured using the fluorescent-plate reader at 490 nm excitation (Ex)/526 nm emission (Em). The relative fluorescence intensity in I6-treated neurons is expressed graphically as a percentage of the control neurons.

### 1.4. Measurement of apoptotic neuronal death

The bisbenzimide H 33342 (Hoechst 33342) penetrates the plasma membrane and stains DNA in cells without permeabilization. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by Hoechst 33342. These morphological changes in the nuclei of apoptotic cells may be visualized by fluorescence microscope. Cortical neurons on covers lips were exposed to I6 (40~120 μM) for 24 h as described in MTT assay, were fixed in 3% paraformaldehyde at the room temperature for 20 min, and then stained with Hoechst 33342 dye at the concentration of 1μg/ml in the incubation buffer for 15 min. The morphological change was examined under UV illumination using a fluorescence microscope (Olympus IX71, Tokyo, Japan). The dye was excited at 340 nm, and emission was filtered with a 510 nm barrier filter. To quantify the I6-induced apoptotic extent, neurons with fragmented or condensed DNA and normal DNA were counted. Apoptotic neurons as a percentage of total neurons were calculated.

### 1.5. Mitochondrial membrane potential

Since oxidative stress and neuronal apoptosis may be associated with the perturbation

of mitochondrial membrane potentials. To investigate whether mitochondria membrane potentials were altered in response to I6 treatment, we used a mitochondrial membrane potentials (MMP) indicator JC-1 with dual emission characteristics. At lower MMP, it exists in the cytoplasm as a green-fluorescent monomer, whereas at higher MMP, it accumulates in the mitochondria and forms red-fluorescent aggregates. Drop in mitochondrial membrane potential were indicated by a decrease in the ratio of the red/green fluorescent signal. Cortical neurons were treated with I6 (80 μM) for 0-12 h, followed by incubation with JC-1 (10 μM) at 37 °C for 10 min. The fluorescent alteration of JC-1 was visualized using the fluorescence microscope with a constant exposure time (100 ms) from the CCD camera. The red/green fluorescence ratio of JC-1 was measured using the fluorescent-plate reader at 529 nm emission (Green)/590 nm emission (Red). The relative red/green fluorescence ratio in I6-treated neurons is expressed graphically as a percentage of red/green fluorescence ratio value of the control neurons.

### 1.6. Isolation of cytosolic fractions for analysis of cytochrome c release

To measure cytochrome c release from mitochondria following treatment with I6, cortical neurons were treated with I6 (40~120 μM) for 18 h, harvested by gentle scraping and washed once with ice cold PBS. Cell pellets were resuspended in 100 μl extraction buffer (250 mM sucrose, 70 mM KCl, 0.5 mM DTT, 2.5 μg/ml pepstatin in PBS) containing 50 μg/ml digitonin and allowed to swell on ice for 5 min. The cell suspension was centrifuged at 20,000 × *g* for 20 min at 4℃. For preparation of cytosolic extracts lacking mitochondria, the supernatant constituted the cytosolic fraction (lacking mitochondria) and the pellet was resuspended in 100 μl extraction buffers as the mitochondrial and nuclear fraction. Samples were stored at –20℃ until

further analysis by Western blotting.

### 1.7. Western blot Assay

Cells were lysed in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM phenyl methyl sulfonyl fluoride, and 100 μg/ml leupeptin. Lysates were centrifuged at 19,720 g for 10 min. Supernatants were collected, subjected to electrophoresis on 8.5% or 14% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The blot was incubated in 5% nonfat dry milk for 60 min, reacted with primary antibodies overnight at 4℃, and then incubated with HRP-conjugated secondary antibodies for 1 hr at room temperature. Immunoreactivity was detected by using the Western blot chemiluminescence reagent system (Perkin-Elmer, Boston, MA). Films were exposed at different time points to ensure the optimum density, but not saturated.

### 2.8. Experimental procedure for animals

Adult male Sprague–Dawley rats (200–250 g, 8 weeks old) were obtained from National Animal Center, and were housed in groups of five per cage in standard metal cages at 22 ± 2℃on light/dark cycle of 12:12 h. All procedures adhered to the Guidelines for Care and Use of Experimental Animals of the Tzu Chi University (Hualien, Taiwan).

#### 2.8.1. I6 treatment and determination of the neurotoxicity in animals

The animals were divided into five groups including control、vehicle and various dose

of I6 (including 2.1、4.2 and 3.6 mg/kg). The corn oil was used for vehicle of I6. Each group contained six rats. The animals were treated with the I6 by ip injection for 24 h and then they were determined the neurotoxicity using the density of survival neurons cortex and caspase-3 activity in frontal cortex as indices. The neurotoxicity was assayed after 24 h of I6 treatment in order to compare with in vitro studies. The dose of I6 for animals is calculated approximately according to the concentration of I6 in the culture medium (80 μM). If 2.1 mg/kg is injected ip and completely absorbed into the blood and it is distributed evenly in the body. A 250 g rat should have plasma volume about 19.2 cc. the concentration in plasma should be roughly 2.1 mg/19.2 cc (the molecular weight of I6 is 332 g/mol, thus 2.1 mg/19.2 cc is near 80 μM).

**2.8.2. Histological Procedures and Nissl Staining**

For examination of neuronal damage, rats were killed 24 h after I6 ip injection and studied by Nissl staining. Following anesthesia with sodium pentobarbital (60 mg/kg BW), fixation of the brain was carried out by transcardial perfusion with fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3. The brains were removed after perfusion and stored over night in a fixative solution that used for perfusion. Then, they were infiltrated with 30% sucrose solution for approximately 4℃. The specimens were frozen rapidly and 30 μM thick sections were cut on cryostat. They were rinsed in the phosphate buffer and picked up on slides coated with 0.01% of aqueous solution of a high molecular weight poly L-lysine. Thereafter, Duplicate coronal sections of the brains with frontal cortex were stained with 0.75% cresyl violet, dehydrated through graded alcohols (70, 95, 100%

2·), placed in xylene and coverslipped using DPX mountant.

### 2.8.3. Morphological analysis and quantification of surviving neurons

Five coronal sections from each rat in each group were studied quantitatively. Neuronal counts were performed by eye using a 40× objective with final field 252μm$^2$ and bregma coordination according to the stereotaxic co-ordinate such as frontal cortex is AP 0.2 mm, lateral ± 1–4 mm, depth 1–3 mm. The tissue section after Nissl Staining viable stained neurons were identified on the basis of a stained soma with at least two visible processes. Counts were made in five adjacent fields and the mean number extrapolated to give total number of neurons per 252 μm$^2$. All data are represented as number of neurons per 252 μm$^2$.

### 2.8.4. Casepase-3 activity assay

The rats were divided into five groups including control、vehicle and various dose of I6. Each group contained six rats. After treatment with vehicle or I6 for 24 h, rats were sacrificed by decapitation. The frontal cortex was microdissected and frozen on dry ice. Caspase-3 activity assay was performed according to the instructions of the caspase-3 assay kit (Calbiochem, San Diego, CA). Frontal cortex areas were homogenized in ice-cold buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM EGTA, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 20 $\mu$g/ml leupeptin, and 4 $\mu$g/ml aprotinin) and centrifuged at 100,000 rpm for 1 h to remove particular matter. Supernatants containing 50 μg of protein were added to 100 μM final concentration of Ac-DEVD-AFC substrate in a 50 μl volume of assay and were incubated at 37 °C for 2 h. The fluorescence intensity of cleavage of the ACF fluorophore was measured using the fluorescent plate reader at ~400 nm

excitation (Ex)/~505 nm emission (Em). Data are expressed as the percentage of control caspase-3 activity.

## 2.9. Statistical Analysis

The Kruskal-Wallis test was conducted to compare the difference between groups. The post-hoc test using Mann-Whitney U tests based on the bonferroni correction method for controlling overall type-I error was performed if p-value of Kruskal-Wallis test < 0.05

## 3. Results

### 3.1. I6 induces apoptotic cell death in cultured cortical neurons

We have shown previously that I6 can reduce mitochondrial viability and induce apoptosis in cortical neuronal cultures. In that study, cortical cells were taken from postnatal (P0~P2) pup containing about 10~20% of glia cells that may interfere with the results. In the present study, we harvested cortical cells from the cerebral cortices of embryonic day 16 or 17 that contain about 95~98% of neurons. At day 9 in vitro, I6 (40~120 μM) was added to the culture medium for 24 h at 37 ℃ and apoptotic cell death was examined by Hoechst 33342 staining as described in the material and methods. As shown in figure 1A, I6 triggered nuclear morphological changes and condensation of nuclear chromatin in neuronal cell. Quantification of condensed nuclei further revealed that I6 induced neuronal apoptosis in a concentration-dependent manner (Fig. 1B). The vehicle (0.6% DMSO) alone had no effect on nuclear condensation.

### 3.2. Antioxidants prevent neuronal apoptosis induced by I6

Cultured neurons were treated with I6 (80 μM) either alone or in combination with vit E (100~300 μg/ml) for 24 hr and cell viability was assessed. We found that treatment with Vit E completely blocked I6-induced neuronal death (Fig. 2A). One hundred and three hundred μg/ml of Vit E increased the survival rate from 47.3±1.7% to 100.1±1.9% and 102±2.2%, respectively. We further examined whether Vit E and NAC rescued cortical neurons from apoptosis using Hoechst 33342 staining. As shown in figures 2B and 2C, NAC and Vit E reduced apoptotic nuclei in a dose-dependent manner (Kriskal-wallis test was performed by comparing I6 alone and I6 with NAC or vit E in different concentration; $p<0.001$). These results suggest that

oxidative stress is the cause of I6-induced neuronal apoptosis.

### 3.3. I6-induced oxidative stress results in collapse of mitochondria membrane potentials

Cortical neurons were stained with JC-1 at 0~12 h after I6 (80 μM) treatment. As shown in figure 3A, the microscopic image showed a decrease in red-fluorescent aggregates and an increase in green-fluorescent monomer following the addition of I6 in a time dependent manner. Quantitative analysis and time course of red/green fluorescence ratio revealed that the loss of mitochondrial membrane potentials began at 6 hr after I6 treatment (Figure 3B). Furthermore, I6-induced disruption of mitochondrial membrane potentials can be prevented efficiently by pretreatment with antioxidants Vit E and NAC (Figure 3C). These results indicate that the disruption of mitochondria membrane potentials is closely correlated with I6-induced oxidative stress.

### 3.4. I6-induced ROS surge precede the loss of mitochondria membrane potential

Whether I6-induced ROS production occurs upstream or downstream of mitochondria membrane potential loss is not known, thus, we further characterized the ROS surge. Cortical neurons were loaded with the ROS-sensitive dye $H_2$DCF and exposed to I6 (80 μM) for 0~12 h. The intracellular DCF fluorescence resulting from interaction of $H_2$DCF with ROS was evaluated by microscopy and fluorescent plate reader. As shown in figure 4A, DCF fluorescence can be clearly seen after exposure to I6. Quantitative analysis and time course of fluorescence intensity accumulated by cortical neurons revealed that production of ROS was transient, which started to appear at 2 hr after I6 treatment and subsided within 9 hr (Figure 4B). Moreover, pre-treatment with NAC or Vit E effectively blocked the effect of I6 (Figure 4C).

These results suggest that I6-induced ROS surge is an important upstream trigger to induce disruption of mitochondrial membrane potential.

### 3.5. Activation of intrinsic death signaling is induced by I6

ROS production can trigger many biochemical processes including cytochrome c release which induces activation of intrinsic death pathway (Kluck et al. 1997; Higuchi et al. 1998; Atlante et al. 1999). We therefore investigated whether I6 could induce cytochrome c release. Cortical neurons were treated with I6 (40~120 μM) for 18 hr and cytosolic extracts, lacking mitochondria, were prepared and analyzed by Western blotting. Figure 5A shows that I6 significantly and dose-dependently induces cytochrome c release. We further investigated whether intrinsic caspase cascades were involved in I6-induced neuronal apoptosis. Cortical neurons were treated with I6 at various doses for 18 hr and cell lysates were harvested. The activation of caspase 9 and caspase 3 was assessed by Western blotting with specific polyclonal antibodies that recognize only the active (cleaved) forms of the enzymes. As shown in Figures 5B, I6 significantly and dose-dependently enhances expression of the active form of caspase 9 and caspase 3.

Poly-(ADP-ribose) polymerase (PARP), a nuclear enzyme that is involved in DNA repair following DNA nicks, is a death substrate of caspase-3 (Tewari et al. 1995). The cleavage of PARP abolishes its enzymatic activity (Lazebnik et al. 1994). Figure 5C shows that I6 exposure causes cleavage of PARP and the levels of cleaved PARP are closely correlated with the concentrations of I6. These results suggest that I6 induces apoptosis in cultured cortical neurons possibly via a mitochondria-mediated intrinsic death signaling cascade.

### 3.6. Antioxidants inhibit I6-induced activation of intrinsic death signaling

We further investigated whether activation of intrinsic death signaling induced by I6 is dependent on the generation of ROS. Cortical neurons were treated with I6 (80 μM) for 18 h in the presence and absence of antioxidants. The cytosolic extract, lacking mitochondria, was blotted with cytochrome c antibody. Figure 5D shows that Vit E and NAC prevent I6-induced cytochrome c release. We further tested whether antioxidants also affect I6-induced activation of caspase-3, -9 and cleavage of PARP. As shown in Figure 5E and 5F, I6-induced activation of caspase-9, -3 and cleavage of PARP were not seen after NAC or Vit E treatment. These results indicate that I6 induces neuronal apoptosis possibly via generation of ROS which causes collapse of mitochondrial membrane potential and activation of intrinsic death signaling.

### 3.7. Mitochondrial uncoupling agent FCCP inhibits I6-induced activation of caspase-3 and neuronal death

The mitochondria uncoupling agent carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) in low doses (1~2 μM) has been shown to prevent glutamate-induced cytochrome c release and generation of ROS, (Reynolds et al. 1995; Pivovarova et al. 2004; Perez-Ortiz et al. 2007). Therefore, we examined whether FCCP inhibits I6-induced activation of caspase-3 and cleavage of PARP. Cortical neurons were treated with I6 (80 μM) for 18 hr in the presence and absence of FCCP (0.5~2 μM) and cellular extract was blotted with antibodies directly against the active forms of caspase-3 and cleaved PARP. Figure 6A and 6B shows that FCCP dose-dependently inhibits I6-induced activation of caspase-3 and cleavage of PARP. We further examined the effect of FCCP on I6-induced neuronal death. Cortical neurons were pre-treated with this drug for 30 min before I6 (80 μM) was added. The results revealed that FCCP was able to prevent neuronal death caused by I6 (Fig. 6C). Concentrations of 0.5, 1, 1.5, and 2 μM of FCCP increased the survival rate from

44.1±1.1% to 67±1.7%, 77.6±0.9%, 83.4±0.8%, and 82.9±1.8%, respectively (Kriskal-wallis test was performed by comparing I6 alone and I6 with FCCP in different concentration; $p<0.001$). The FCCP (2μM) alone had no effect on the neuronal viability. These results suggest that FCCP blocks I6-induced generation of ROS and cytochrome c release, and subsequently prevents the activation of caspase-3 and cleavage of PARP. Thus, FCCP exhibits neuroprotective effect on I6-induced neuronal death.

### 3.8. I6 produces cell loss and increases caspase-3 enzymatic activity in frontal cortex of rat brain

To further test whether peripherally administered I6 can also result in neurotoxicity in the rat brain, we use the density of survival neuron and caspase-3 enzymatic activity in frontal cortex as indices of CNS toxicity after I6 (2.1 、 4.2 and 6.3 mg/kg) administration. As shown in Fig. 7A, I6 significantly decreased the density of surviving neurons in a dose-dependent manner after the administration of I6 for 24h in frontal cortex. Moreover, the enzymatic activity assay showed that I6 significantly enhanced caspase-3 activity at 24h later (Fig. 7B). These results suggest that peripherally administered I6 may cross BBB and induce CNS toxicity.

## 4. Discussion

I6 is a synthetic gingerdione derivative and we have demonstrated that it induces cell apoptosis of cultured cortical neurons in a dose-dependent manner (Lin et al., 2006). In the present studies, we further revealed that I6-induced neuronal apoptosis required production of ROS and loss of MMP in primary neuronal culture. Furthermore, in animal studies we found that peripherally administered I6 can also result in neuronal cell loss and increase caspase-3 enzymatic activity in the frontal cortex of rat brain.

These results demonstrated that the synthetic gingerdione derivative I6 can cross BBB and result in neurotoxicity in CNS.

Two apoptotic pathways have been well established. Activation of the extrinsic death pathway via ligand binding to death receptors results in the recruitment and activation of initiator caspases that further activate effector caspases leading to apoptosis (Ashkenazi et al., 1996; Pandey et al., 2003). In the intrinsic death pathway, apoptotic signals stimulate mitochondria resulting in the release of apoptogenic factor such as cytochrome c that binds to pro-caspase-9 and adaptor protein apaf-1 to form the apoptosome. Consequently, apoptosome leads to the activation of effector caspases such as caspase-3, which activates downstream events ultimately causing apoptosis. In addition, apoptotic mechanisms include production of ROS (Lotem et al. 1996; Tan et al. 1998; Um et al. 1996), release of apoptosis inducing factor (AIF) (Susin et al. 1999), and collapse of mitochondria membrane potential (MMP) (Zamzami et al. 1995). In previous studies, we found that I6-induced neuronal apoptosis is prevented by N-acetylcysteine (NAC) (Lin et al. 2006). Although NAC is an antioxidant, it has a broad range of actions which may potentially relate to its protective effect. These include antioxidant properties (Aruoma et al., 1989), enhancement of intracellular glutathione levels, stimulation of Ras-ERK signaling pathway (Yan and Greene, 1998), and regulation of transcription (Cotgreave et al., 1997). Thus, to evaluate whether oxidative stress indeed is involved in the neurotoxicity of I6, we simultaneously use another antioxidant vitamin E (Vit E) to clarify the effects of I6. Our data revealed that both Vit E and NAC completely inhibited I6-induced neuronal apoptosis as well as generation of ROS. It is likely that ROS is the critical mediator for I6-induced neurotoxicity.

We performed the time course analysis of ROS production under I6 treatment. The results showed that I6 induced a transient increase in intracellular ROS within 12

h. The ROS surge could first be observed after 2 h and was most pronounced 6 h after exposure to I6 (Figure 4B). Moreover, pretreatment with antioxidants vit E or NAC can effectively block I6-induced ROS surge. Similar results were observed in other active ingredients of ginger such as 6-shogoal and 6-dehydrogingerdione, which can induce apoptosis of human colorectal carcinoma cell and hepatoblastoma by oxidative stress and caspases activation (Pan et al., 2008; Chen et al., 2010). These results further suggest that oxidative stress is involved in I6-induced neuronal apoptosis.

Since mitochondria play an important role in oxidative stress-induced apoptosis, we focus our attention on the intrinsic death pathway. Collapse of mitochondria membrane potential is a sensitive indicator of mitochondrial damage induced by several toxins. We performed a time course assessment of mitochondrial membrane potential using a specific and sensitive fluorescent dye JC-1. Our results showed that I6 induced loss of MMP in a time-dependent manner and the time point of loss of MMP started at 6 h after I6 treatment (Figure 3B). This result revealed that I6-induced ROS surge preceded loss of MMP. Due to the ROS surge is transient and disappear within 9 h, but the MMP loss is sustained after I6 treatment 6 h. This result suggested that the MMP loss induced by I6-induced oxidative stress was irreversible, even the ROS surge at these time point 9、10、11and 12 h no longer was seen. In addition, both Vit E and NAC prevented I6-induced loss of MMP, suggesting that production of I6-induced ROS plays an important role in mediating the mitochondrial toxicity of I6.

ROS generation has been recognized as a mediator of apoptotic signaling cascades (Cai et al., 1998; Esteve et al., 1999; Valencia et al., 2001; Curtin et al., 2002). Consistent with this notion, we found that I6 caused cytochrome c release from mitochondria, activation of caspase-3 and -9, and cleavage of PARP. Importantly, the activation of mitochondria-mediated intrinsic death signaling accompanied by neuronal apoptosis was completely blocked by antioxidants. These results suggest that

I6 induces the production of ROS which causes collapse of mitochondria membrane potential and triggers activation of mitochondria-mediated death signaling.

Although mitochondria uncoupling agent FCCP induced loss of mitochondria membrane potential, in low doses (0.5~2 μM), FCCP has been shown to reduce glutamate-induced production of ROS and cytochrome c release, and to exhibit neuroprotective effect (Reynolds et al., 1995; Tirosh et al., 2000; Pivovarova et al.,2004). In the present study, low doses FCCP (1~2 μM) reduced I6-induced activation of caspase-3 and cleavage of PARP, and prevented I6-induced neuronal apoptosis. These results suggest that mitochondria are a likely target of I6 action. In agreement with this notion, generation of ROS and cytochrome c release from mitochondria are required for I6-induced neuronal apoptosis.

Many reports indicated that induction of neuronal apoptosis may be mediated by altering intracellular signaling process such as phosphorylation of ERK and p38 (Willaime et al., 2001; Kim et al., 2011), and these protein kinase inhibitors can prevent neuronal apoptosis. In previous studies, however, we show that I6 has no effect on phosphorylation of ERK and p38 (Lin et al., 2006), and both ERK and p38 inhibitor can not prevent I6-induced neuronal apoptosis (data not shown). Thus, we can rule out the possibility that ERK and p38 pathway is involved in I6-induced neuronal apoptosis.

The mechanisms by which I6 induced generation of ROS and the exact location of ROS production were not clear. We can not rule out the possibility that I6-induced ROS production occurred in the extramitochondria. I6 is a lipid-soluble compound that could affect lipid component of cell membrane and cause membrane lipid peroxidation resulting in oxidative stress. It will be important in the future study to determine the exact location of I6-induced ROS production and to determine whether I6 by its own is an oxidative compound.

## 5. **Conflicts of interest statement**

The authors declare no conflicts of interest.

## **Acknowledgement**

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## Figure Legends

**Figure 1. I6 induces concentration-dependently apoptotic death of cultured cortical neurons.**

(A) Hoechst staining of apoptotic cells. Cortical neurons were treated with I6 at

different concentrations for 24 hr. (B) Summary of data. Apoptotic cells were counted in three microscopic fields with a minimum of 100 cells per field and expressed as percentage of apoptotic cells over the total number of cells. ** $p$<0.01, *** $p$<0.001 vs. control. All data were represented as the mean±SEM from six independent experiments and statistical analysis was performed by using Kriskal-wallis test.

**Figure 2. Antioxidants completely inhibit I6-induced neuronal apoptosis.**

(A) The effects of antioxidants Vit E on I6-induced neuronal death. Viability was quantified using MTT assay. ***$P$<0.001 vs. I6 alone. (B) and (C) The effects of antioxidants Vit E and NAC in I6-induced neuronal apoptosis examined with Hoechst staining. Cortical neurons were pretreated with 100 or 300 μg/ml of vitamin E, or with NAC 5 or 10 mM for 30 min, followed with I6 (80 μM) for 24 hr. ***$P$<0.001 vs. I6 alone. All data were represented as the mean±SEM from six independent experiments and statistical analysis was performed by using Kriskal-wallis test.

**Figure 3. I6 induces loss of mitochondrial membrane potential (MMP) in a time - dependent manner.**

Cortical neurons were treated with I6 (80 μM) for different time periods (0, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 h) and loaded with MMP indicator JC-1. The ratio of red/green fluorescence at each time point examined under a fluorescent microscope was considered as a MMP indicator. (A) A typical experiment (B) Quantification of JC-1 red/green ratio. **$P$<0.01, ***$P$<0.001 vs. control. (C) Effects of antioxidants in I6-induced loss of MMP. Cortical neurons were pretreated with vitamin E (300 μg/ml) or NAC (10 mM) for 30 min, followed with I6 (80 μM) for 12 hr, and thereafter loaded with JC-1 for examination of MMP. ***$P$<0.001 vs. I6 alone. All data were represented as the mean±SEM from six independent experiments and

statistical analysis was performed by using Kriskal-wallis test.

**Figure4. I6 induces time-dependent formation of ROS.**

Cortical neurons were treated with I6 (80 μM) for 0-12 h and loaded with ROS-sensitive dye $H_2$DCF. At each time point, the $H_2$DCF fluorescence was examined under a fluorescent microscope. (A) A typical experiment. (B) Quantitative analysis of ROS production ($H_2$DCF fluorescence). *$P$<0.05, **$P$<0.01, ***$P$<0.001 vs. control. (C) Effects of antioxidants in I6-induced ROS production. Cortical neurons were pretreated with vitamin E (300 $\mu$g/ml) or NAC (10 mM) for 30 min, followed with I6 for 6 hr and thereafter loaded with $H_2$DCF for examination of ROS surge. ***$P$<0.001 vs. I6 alone. All data were represented as the mean±SEM from six independent experiments and statistical analysis was performed by using Kriskal-wallis test.

**Figure 5. The effects of antioxidants in I6-induced activation of mitochondria-mediated intrinsic death signaling**

Effects of I6 in cytochrome c release, **$P$<0.01, ***$P$<0.001 vs. control. (A), effects of I6 in caspase activations (cleaved caspases), **$P$<0.01, ***$P$<0.001 vs. control. (B), and in cleaved PARP, **$P$<0.01, ***$P$<0.001 vs. control (C); Effects of antioxidants (Vit E; 100 μg/ml and NAC; 10 mM) in I6-induced cytochrome c release, **$P$<0.01 vs. I6 (D), in I6-induced caspase activations, **$P$<0.01 vs. I6 (E), and I6-induced cleavage of PARP, **$P$<0.01 vs. I6 (F). Cytosolic fraction was prepared for cytochrome c release from cortical neurons treated with I6 for 18 h. Cleaved caspases were prepared from cell lysate and cleaved PARP from nuclei. All protein levels were analyzed by Western blotting.

$\beta$-actin (A, B, D, E) and histone 3 (C, F) were used as internal control. All data were

represented as the mean±SEM from six independent experiments and statistical analysis was performed by using Kriskal-wallis test.

**Figure 6. Effects of mitochondria uncoupling agent FCCP on I6-induced activation of caspase-3, cleavage of PARP, and survival rate.**

Effects of FCCP in I6-induced cleaved caspase-3, ***$P$<0.001 vs. I6 alone (A), in cleaved PARP, ***$P$<0.001 vs. I6 alone (B), and survival rate, **$P$<0.01, ***$P$<0.001 vs. I6 (C). Cortical neurons were treated with I6 (80 μM) in the presence and absence of FCCP (0.5~2 μM). Cleaved caspase-3 was extracted from cell lysates and Cleaved PARP from nuclei. The proteins were determined with Western blotting assay. Survival rate was assessed using MTT assay. All data were represented as the mean±SEM from six independent experiments. Statistical analysis was performed by using Kriskal-wallis test.

**Figure7. I6 induces neuronal loss and enhances caspase-3 enzymatic activity in the frontal cortex of rats.**

(A) Photographs of Nissl staining in frozen section (magnification, 400×) of frontal cortex of rats treated with I6 at various doses, intraperitoneally. The rat was killed at 24h after the injection. (B) Quantification of density of surviving neurons in frontal cortex after ip administration of I6 at various doses. *$P$<0.05, **$P$<0.01 vs. control. (C) Caspase-3 activity induced by I6. Caspase-3 activity was assayed using Ac-DEDV-ACF (100 μM) as the substrate, and the data are expressed as the percentage of control caspase-3 activity. *$P$<0.05, **$P$<0.01 vs. control. All data were represented as the mean±SEM from six independent experiments. Statistical analysis was performed by using Kriskal-wallis test.