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

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ABSTRACT

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.

1. 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 vesicular 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., 2009; 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 μM. However, at high concentration (78 μM), 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 μM. 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

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_2DCFDA, 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).

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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.

2. Materials and methods

2.1. Chemicals and reagents

Vitamin E, N-acetylcysteine, MTT, Hoechst33342 and FCCP were purchased from Sigma (St. Louis, MO). H_2DCFDA 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).

2.2. 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 5×10^4 (for survival analysis) or 60-mm culture dishes at a density of 3×10^6 (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.

2.3. 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 °C. 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_2DCFDA was used. H_2DCFDA passively diffuses into cells, where the acetate is cleaved by intracellular esterases to form 2′,7′-dichlorodihydrofluorescein (H_2DCF). The latter is trapped within the cell and reacts with ROS to emit fluorescence. After treatment of cells with I6 (80 μM) for 0–12 h, the cells were loaded with H_2DCFDA (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_2DCF 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.

2.5. 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 1 μg/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.

2.6. 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.

2.7. 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 °C. 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 °C until further analysis by Western blotting.

2.8. 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 °C, and then incubated with HRP-conjugated secondary antibodies for 1 h 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.

2.9. 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 °C 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).

2.9.1. 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).

2.9.2. 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 °C. 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.

2.9.3. 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 252 μm^2 and bregma coordination according to the stereotaxic co-ordinate such