

Full Paper

Schizandrin Protects Primary Cultures of Rat Cortical Cells From Glutamate-Induced Excitotoxicity

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Abstract. The neuroprotective effect of schizandrin on the glutamate (Glu)-induced neuronal excitotoxicity and its potential mechanisms were investigated using primary cultures of rat cortical cells. After exposure of primary cultures of rat cortical cells to 10 μ M Glu for 24 h, cortical cell cultures exhibited remarkable apoptotic death. Pretreatment of the cortical cell cultures with schizandrin (10, 100 μ M) for 2 h significantly protected cortical neurons against Glu-induced excitotoxicity. The neuroprotective activity of schizandrin was the most potent at the concentration of 100 μ M. Schizandrin reduced apoptotic characteristics by DAPI staining in Glu-injured cortical cell cultures. In addition, schizandrin diminished the intracellular Ca^{2+} influx, inhibited the subsequent overproduction of nitric oxide (NO), reactive oxygen species (ROS), and cytochrome c, and preserved the mitochondrial membrane potential. Furthermore, schizandrin also increased the cellular level of glutathione (GSH) and inhibited the membrane lipid peroxidation malondialdehyde (MDA). As indicated by Western blotting, schizandrin attenuated the protein level changes of procaspase-9, caspase-9, and caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP). Taken together, these results suggest that schizandrin protected primary cultures of rat cortical cells against Glu-induced apoptosis through a mitochondria-mediated pathway and oxidative stress.

Keywords: schizandrin, glutamate, cortical cell culture, apoptosis, neuroprotection

Introduction

Glutamate (Glu) receptor-mediated toxicity is an important mechanism of neuronal death in various pathologic conditions including ischemia (1), trauma (2), epileptic seizures (3), and neurodegeneration (4). Glu-induced excitotoxicity, mainly associated with excessive release of Glu and subsequent influx Ca^{2+} via the *N*-methyl-D-aspartate (NMDA)-subtype receptor of Glu, leads to an intracellular cascade of cytotoxic events. Excessive activation of Glu receptors can evoke neuronal dysfunction and even damage or death (5). Intracellular Ca^{2+} overload activates neuronal NOS

(nNOS), resulting in excessive production of NO and formation of reactive oxygen species (ROS) and lipid peroxidation (6). On the other hand, mitochondria are involved in Glu-induced excitotoxicity (7) because it possesses a large capacity for calcium uptake (through the potential-driven calcium uniporter) (8). Ca^{2+} -dependent depolarization of mitochondria contributes to oxidative stress in neuronal injury (7).

The excitotoxic neuronal death induced by Glu has been shown to occur through both necrosis and apoptosis depending on the intensity and duration of Glu exposure. The apoptosis-induced by Glu predominates after exposure to low concentrations in a delayed time course, whereas an acute exposure to high concentration of Glu leads to necrosis (9). Apoptosis plays an important homeostatic role in several cellular processes in the development of the immune and nervous systems

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(10). Pathological features of apoptosis include various morphological and biochemical characteristics that distinguish the process from necrosis. The morphological features of apoptosis are implicated with a variety of biochemical changes in the cell death signaling pathway such as increases of Ca^{2+} , free radicals, NO, cytochrome c, and the decrease of mitochondrial potential.

Apoptosis can be induced by two major pathways, the surface death receptor pathway and the mitochondrion-initiated pathway, both of which are regulated by caspases (11). Caspase, a family of cysteine proteases, is the common executor of apoptosis induced by various stimuli. Caspases exist as inactive proenzymes and are activated after cleavage at specific aspartate residues upon apoptotic signals. In the mitochondrial pathway, caspase-9 and caspase-3, in particular, are believed to be most commonly involved in the execution of apoptosis in various cell types (12) because it cleaves most of caspase-related substrates, including key proteins such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP) (13). PARP is one of the earliest proteins targeted by caspase-3 during apoptosis.

Schizandrin possesses many biological properties (14), including hepatoprotective (15), anti-inflammatory (16), antitumor (17), and anti-amnesic effects (18), and a suppressive effect against lipid peroxidation and a potentiating effect on glutathione mediated antioxidation (19). To date, there has been less information concerning its neuroprotective activity against Glu-induced neurotoxicity. Therefore, the present study was intended to evaluate the neuroprotective effect of schizandrin on the Glu-induced neuronal apoptosis in primary cultures of rat cortical cells. We further tried to elucidate the anti-apoptotic mechanism of schizandrin. MK801, a well known non-competitive NMDA antagonist (20), has been reported to inhibit Glu-induced neurotoxicity in primary cultures of rat cortical cells (21). It was used as a positive control in this study.

Materials and Methods

Materials and reagents

Schizandrin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). L-Glutamic acid, 2,7-dichloro-fluorescein diacetate (DCF-DA), Fura 2-AM, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), poly-D-lysine, cytosine arabinoside, 4',6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) DNase I, papain, bovine serum albumin (BSA), and cysteine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Neurobasal medium,

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, B-27 supplement, and trypan blue were purchased from Gibco (Grand Island, NY, USA). Hanks' balanced salt solution (HBSS) was purchased from Hyclone (Logan, UT, USA). MK-801 maleate was purchased from TOCRIS (Ellisville, MO, USA). The kit for assessing NO was purchased from Cayman (Ann Arbor, MI, USA). The kit for assessing cytochrome c release was purchased from Assay Designs' Products (Ann Arbor, MI, USA). Antibodies for α -tubulin, caspase-3, and caspase-9 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Purified mouse anti-PARP antibody was obtained from BD Biosciences (San Diego, CA, USA).

Primary cultures of rat cortical cell

Primary neuronal cultures of cerebral cortex were obtained from rat embryos (E16-18) according to Nishikawa (22) with modifications as described before (23). The cerebral cortex of rat embryos were dissected and placed in cold HBSS. After removal of meninges, the tissues were minced and incubated at 37°C for 15 min in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS containing 0.25% trypsin and 0.2 mg/ml DNase I, and then the cell suspensions were centrifuged ($300 \times g$ for 10 min). The resulting pellets were resuspended in the 1:1 mixture of DMEM and F12 supplemented with 20% heat-inactivated FBS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells were plated into 96-well poly-D-lysine-coated dishes at a density of $1.5 \times 10^5/\text{ml}$. Cytosine arabinoside (10 μM) was added to the culture at 24 h after plating to prevent nonneuronal cell proliferation. Cortical cell cultures were incubated at 37°C in 5% CO_2 . After 48 h, the medium was replaced with neurobasal medium supplemented with B-27 and penicillin (without L-Glu, Gibco). Only mature cultures (12–14 days in vitro) were used for experiments. Immunohistochemical staining with anti-microtubule associated protein-2 (MAP-2) antibody and anti-glial fibrillary acidic protein (GFAP) antibody revealed that the culture method used in this study provided cell cultures containing about 90% neurons as described previously (23).

Drug treatment and measurement of neurotoxicity

L-Glutamic acid was dissolved in distilled water at a concentration of 100 μM (final concentration of 10 μM in the incubation medium). Schizandrin and MK-801 were dissolved in methanol and distilled water and diluted with medium. On the day of the experiment, schizandrin and MK-801 were added to culture medium for 2 h. Then the cells were exposed to 10 μM Glu and maintained for 24 h. After the incubation, the cells were assessed for viability by MTT assay. Cell viability was

measured by MTT reduction essentially as described (24). The absorbance was read at 570 nm on a microplate reader. To assess cell viability, cell counts using trypan blue exclusion were also performed.

DAPI staining of apoptotic cells

To detect morphological evidence of apoptosis, cell nuclei were visualized following DNA staining with the fluorescent dye DAPI. Cells were rinsed with phosphate-buffered saline (PBS), fixed for 20 min in formalin, rinsed with PBS, and then incubated for 10 min with DAPI (1 μ g/ml) (25). After washing with PBS, cultures were examined using fluorescent microscopy (Microphot FX; Nikon, Tokyo) and the percentage of apoptotic nuclei with condensed or fragmented chromatin was evaluated.

Measurement of cellular peroxide and mitochondrial membrane potential

The relative levels of free radicals in primary rat cortical neurons were measured with the oxidation-sensitive fluorescent dye DCF-DA (26). Cortical cell cultures were pretreated with different concentrations of schizandrin or MK-801 for 2 h. Then the cells were exposed to 10 μ M Glu and maintained for 24 h. DCF-DA fluorescence was then determined with a spectrofluorometer. Mitochondrial membrane potential change was examined using the mitochondrial membrane potential-sensitive fluorescent dye JC-1 (27). Fluorescence was viewed at 527 and 590 nm with excitation at 480 nm.

Measurement of nitrite and calcium content

The level of NO was determined by measuring the content of nitrate released into the medium by the method of Dawson and Dawson (28) using Griess reagent 24 h after Glu administration. The absorbance was measured at 540 nm. The content of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) was determined with the fluorescent dye Fura 2-AM by ratio fluorometry (29). Cortical cell cultures were pretreated with schizandrin or MK-801 2 h before exposure to 10 μ M Glu. The changes of $[\text{Ca}^{2+}]_i$ were measured with a spectrofluorometer. Calcium concentration was calculated according to the method of Grynkiewicz et al. (30).

Measurement of cytochrome c release

Cytochrome c release was measured with the cytochrome c enzyme immunometric assay (EIA) kit. The kit uses a monoclonal antibody to cytochrome c immobilized on a microtiter plate to bind the cytochrome c in the standards or sample. After a short incubation, the excess sample or standards are washed out and a biotinylated monoclonal antibody to cyto-

chrome c is added. This antibody binds to the cytochrome c captured on the plate. After a short incubation, the excess antibody is washed out and streptavidin conjugated to alkaline phosphatase is added, which binds to the biotinylated monoclonal cytochrome c antibody. Excess conjugate is washed out and substrate is added. The optical density of each well was then measured at 405 nm using a microplate reader. The concentration of cytochrome c was calibrated from a standard curve based on reference standards (31).

Assays of glutathione, MDA, and protein

Cells from three culture plates were pooled in 2 ml of 0.1 M phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged for 30 min at $3000 \times g$ at 4°C and the supernatant, consisting of the cytosolic and mitochondrial fractions, was used for enzyme assays. Glutathione (GSH) level was determined spectrophotometrically using the DTNB-GSH reductase recycling method (32). To measure oxidized glutathione (GSSG), GSH was first removed from the supernatant by reacting it with 2-vinylpyridine.

The levels of malondialdehyde (MDA), an intermediate product of lipid peroxidation, were determined by the thiobarbituric acid (TBA) reaction (33). The absorbance of the supernatant was determined at 535 nm against a blank that contained all the reagents except the cells. TBA-reactive substances were expressed as nmole of MDA equivalents per 10^6 cells. Protein content was measured by the method of Lowry et al. (34) with bovine serum albumin as a standard.

Western blotting

Cell extracts were prepared as described by Mesmer and Brune (35). Protein concentration in the resultant supernatant was determined by using a Bio-Rad Protein Assay kit according to the manufacturer's protocol. For Western blotting, total cellular proteins were resolved by SDS-PAGE (12% (w/v) gel) under reducing conditions. Gels were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane, and the immobilized proteins were then incubated with the polyclonal antibody against cleaved caspase-9 or monoclonal antibodies against caspase-3 or PARP. Antibody binding was detected with a horseradish peroxidase-coupled anti-mouse or anti-rabbit secondary antibody (36) and ECL plus Western blotting detection reagents (minipore). The Western blotting systems were detected with a cooled CCD camera (LAS-3000mini; Fujifilm, Kanagawa).

Statistical analyses

Data are each expressed as a mean \pm S.E.M. Statistical

evaluation was carried out by Oneway analysis of variance (One-way ANOVA) followed by Scheffe's multiple range test. A *P* value less than 0.05 were considered to indicate a significant difference.

Results

Schizandrin attenuated Glu-induced excitotoxicity in primary cultures of rat cortical cells

Exposure of the cortical cell cultures to 10 μ M of Glu markedly reduced cell viability about 75% compared with control cells. A dose of schizandrin (10, 100 μ M) and MK-801 (15 μ M) showed significant neuroprotective activities against Glu-induced excitotoxicity (Fig. 1). It was noteworthy that cellular viability was maintained at 50%, 70%, or 85% in Glu-induced excitotoxicity by pretreatment with 10 μ M schizandrin, 100 μ M schizandrin, or 15 μ M MK-801, respectively. These results indicate that schizandrin and MK-801 prevent Glu-induced excitotoxicity in primary cultured rat cortical cells.

Schizandrin improved apoptotic cells of DAPI staining

As shown in Fig. 2, DAPI nuclear staining was observed by fluorescent microscopy. DAPI is a specific DNA stain and the DAPI-DNA complex fluoresces bright blue when excited with light at a wavelength of 365 nm. Cortical cell cultures with condensed chromatin or fragmented nuclei were considered apoptotic. In this study, Glu-treated cells demonstrate significant nuclear rounding and shrinkage compared to control cells as

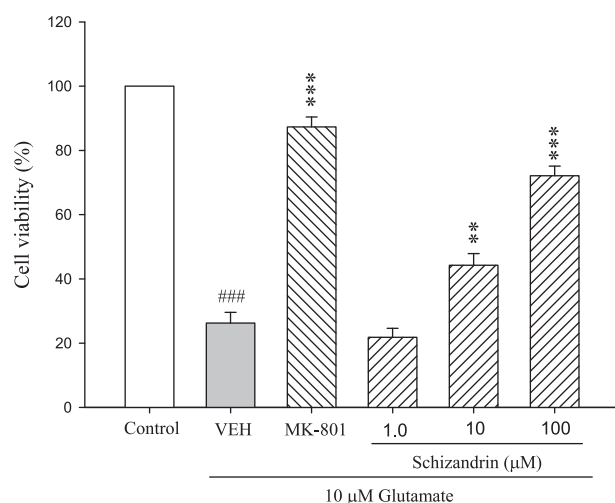


Fig. 1. Neuroprotective activities of schizandrin against Glu-induced neurotoxicity. Cortical cell cultures were pretreated with schizandrin (1, 10, and 100 μ M) or MK-801 (15 μ M) 2 h before exposure to 10 μ M Glu and then maintained for 24 h. Cell viability was measured by MTT. Data were each expressed as a mean \pm S.E.M. (*n* = 3). ###*P* < 0.001, compared with control cells; ***P* < 0.01, ****P* < 0.001, compared with Glu-treated cells.

indicated by DAPI staining. Pretreatment with schizandrin (10, 100 μ M) or MK-801 (15 μ M) attenuated the fluorescence of nuclei as compared to Glu-treated cells. Typical photographs of DAPI staining showing inhibitory effects of schizandrin and MK-801 on Glu-induced apoptotic-like cell death are shown in Fig. 2.

Schizandrin reduced cellular peroxide and restored the mitochondrial membrane potential

To elucidate the neuroprotective mechanisms of schizandrin, cellular metabolic markers- Ca^{2+} influx, cellular peroxide levels-GSH levels, and the activities of antioxidative enzymes were examined. Glutaminergic receptor activation induced by Glu causes a significant change in Ca^{2+} influx, followed by a five to tenfold increase in levels of cellular peroxide (29). Moreover, superoxide anion radical, a major ROS generated in the mitochondria, can interact with NO to form peroxynitrite (another detrimental free radical). Thus, we evaluated the effect of schizandrin and MK-801 on cellular ROS using specific fluorescence dyes, DCF-DA. Under our experimental condition, exposure of cortical cell cultures to Glu increased the cellular ROS levels compared to control cells (Fig. 3). Pretreatment with 10 μ M schizandrin, 100 μ M schizandrin, or 15 μ M MK-801 significantly reduced ROS production up to 15%, 25%, or 35% of that in Glu-injured cortical cell cultures, respectively.

One of the characteristic features of Glu-induced neurotoxicity is a decrease of mitochondrial membrane potential representing the collapse of mitochondrial membrane function (37). In our cortical cell cultures, the mitochondrial membrane potential of Glu-injured cells was reduced to almost 50% of that of control cells. Pretreatment with schizandrin (10, 100 μ M) or MK-801 (15 μ M) restored the mitochondrial membrane potential up to 80%–85% that of control cells (Fig. 4). These results indicated that the schizandrin and MK-801 may prevent Glu-mediated neurotoxicity partially through a reduction of ROS production and thereby restored the mitochondrial membrane potential.

Schizandrin inhibited NO overproduction and Ca^{2+} influx

Glu-receptor activation elicited by an exposure to Glu causes a significant change in Ca^{2+} influx, followed by the activation of NOS and subsequent overproduction of NO (6). Thus, the effect of schizandrin and MK-801 on the content of NO induced by Glu was determined. Pretreatment with schizandrin (10, 100 μ M) or MK-801 (15 μ M) reduced the NO concentration provoked by Glu (Fig. 5). Schizandrin (100 μ M) significantly reduced NO production up to 40% of that in Glu-injured cells. In

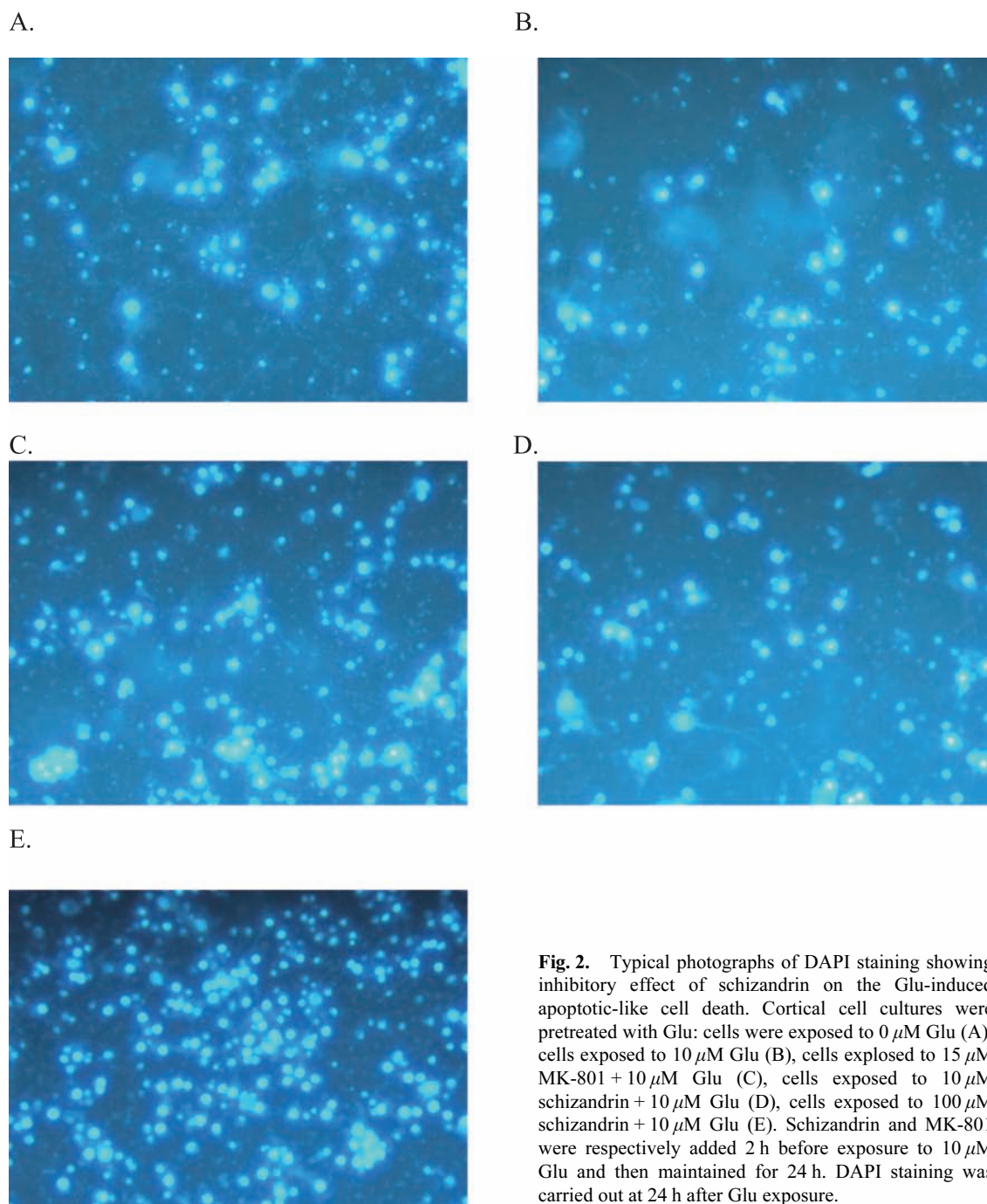


Fig. 2. Typical photographs of DAPI staining showing inhibitory effect of schizandrin on the Glu-induced apoptotic-like cell death. Cortical cell cultures were pretreated with Glu: cells were exposed to 0 μM Glu (A), cells exposed to 10 μM Glu (B), cells exposed to 15 μM MK-801 + 10 μM Glu (C), cells exposed to 10 μM schizandrin + 10 μM Glu (D), cells exposed to 100 μM schizandrin + 10 μM Glu (E). Schizandrin and MK-801 were respectively added 2 h before exposure to 10 μM Glu and then maintained for 24 h. DAPI staining was carried out at 24 h after Glu exposure.

addition, the effect of schizandrin on the increases in the $[\text{Ca}^{2+}]_i$ induced by Glu was evaluated by using Fura 2-AM, a calcium-specific indicator dye. As shown in Fig. 6, Ca^{2+} influx in Glu-treated cells was increased approximately threefold compared to that of control cells. Pretreatment with schizandrin (10, 100 μM) or MK-801 (15 μM) significantly blocked the increases in the $[\text{Ca}^{2+}]_i$ induced by Glu (Fig. 6). Schizandrin (100 μM) and MK-801 (15 μM) blocked the increases in the $[\text{Ca}^{2+}]_i$ to a degree of 50% as compared with that of

Glu-injured cells. In our experimental condition, both schizandrin (10, 100 μM) and MK-801 (15 μM) decreased the NO concentration and inhibited Ca^{2+} influx in Glu-injured cells. These results suggested that the neuroprotective effect of schizandrin correlates with a reduction of $[\text{Ca}^{2+}]_i$ and NO increase in the Glu-induced apoptotic pathway.

Schizandrin decreased cytochrome c release

The elevation of $[\text{Ca}^{2+}]_i$ and ROS production sub-

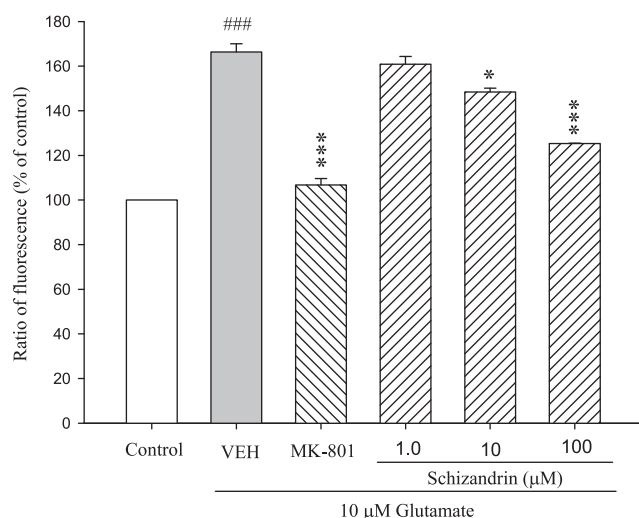


Fig. 3. Effect of schizandrin on cellular peroxide level in Glu-induced neurotoxicity. Cortical cell cultures were pretreated with schizandrin (1, 10, and 100 μ M) or MK-801 (15 μ M) 2 h before exposure to 10 μ M Glu and then maintained for 24 h. Data were each expressed as a mean \pm S.E.M. ($n = 3$). ^{###} $P < 0.001$, compared with control cells; ^{*} $P < 0.05$, ^{***} $P < 0.001$, compared with Glu-treated cells.

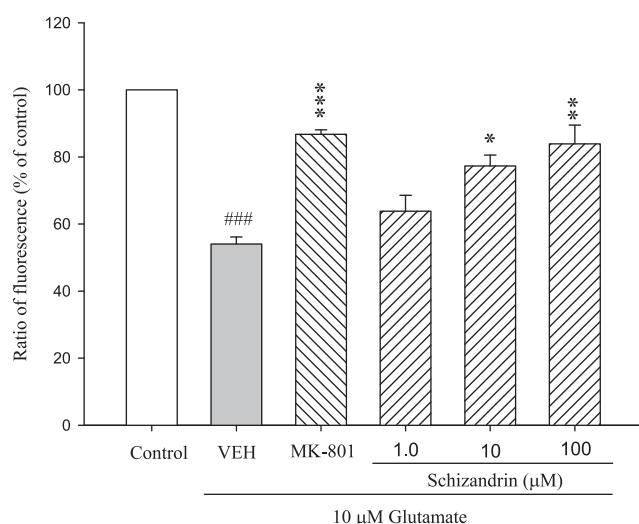


Fig. 4. Effect of schizandrin on mitochondrial potential in Glu-induced neurotoxicity. Cortical cell cultures were pretreated with schizandrin (1, 10, and 100 μ M) or MK-801 (15 μ M) 2 h before exposure to 10 μ M Glu and then maintained for 24 h. Data were each expressed as a mean \pm S.E.M. ($n = 3$). ^{###} $P < 0.001$, compared with control cells; ^{*} $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$, compared with Glu-treated cells.

sequently results in the release of cytochrome c from mitochondria into cytosol during apoptotic cell death (38). Therefore, we examined the effect of schizandrin on the cytochrome c release induced by Glu. Pretreatment of cortical cells with schizandrin (100 μ M) and MK-801 (15 μ M) significantly decreased the cytochrome c release

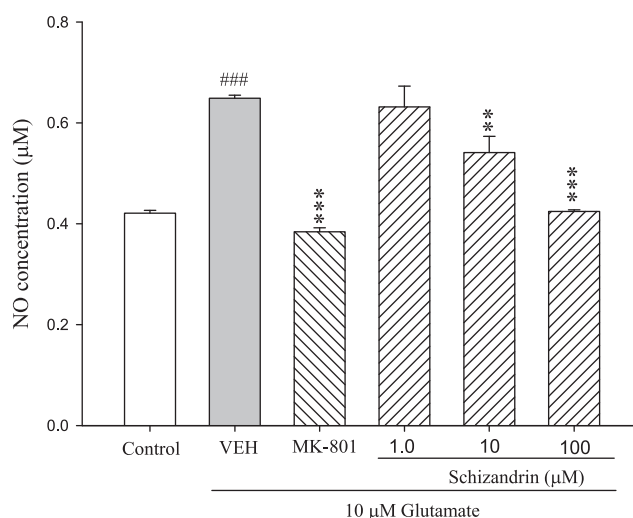


Fig. 5. Effect of schizandrin on the NO production in Glu-induced neurotoxicity. Cortical cell cultures were pretreated with schizandrin (1, 10, and 100 μ M) or MK-801 (15 μ M) 2 h before exposure to 10 μ M Glu and then maintained for 24 h. Data were each expressed as a mean \pm S.E.M. ($n = 3$). ^{###} $P < 0.001$, compared with control cells; ^{**} $P < 0.01$, ^{***} $P < 0.001$, compared with Glu-treated cells.

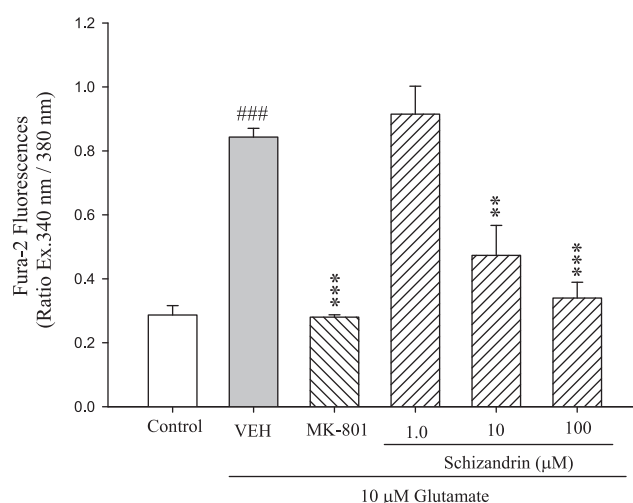


Fig. 6. Effect of schizandrin on $[Ca^{2+}]_i$ level in Glu-induced neurotoxicity. Cortical cell cultures were pretreated with schizandrin (1, 10, and 100 μ M) or MK-801 (15 μ M) 2 h before exposure to 10 μ M Glu and then maintained for 24 h. Data were each expressed as a mean \pm S.E.M. ($n = 3$). ^{###} $P < 0.001$, compared with control cells; ^{**} $P < 0.01$, ^{***} $P < 0.001$, compared with Glu-treated cells.

increased by Glu insult (Fig. 7).

Schizandrin attenuated the activities of procaspase-9, caspase-9, caspase-3, and cleaved PARP

Among the members of caspase family, caspase-9 and caspase-3 have been suggested to play an important

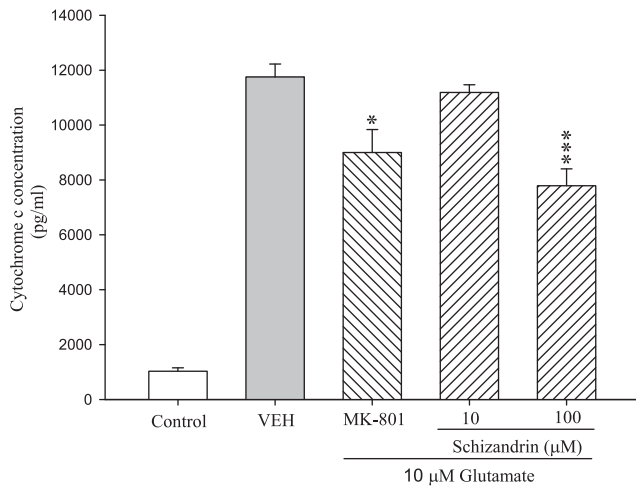


Fig. 7. Effect of schizandrin on cytochrome c release in Glu-induced apoptosis. The cytochrome c release was measured with the Cytochrome c ELISA kit. Cortical cell cultures were pretreated with schizandrin (10 and 100 μ M) or MK-801 (15 μ M) 2 h before exposure to 10 μ M Glu and then maintained for 24 h. The concentration of cytochrome c is calibrated from a standard curve based on reference standards. Data were each expressed as a mean \pm S.E.M. * P <0.05, *** P <0.001, compared with Glu-treated cells.

role in several models of apoptosis. To elucidate the apoptotic neuronal cell death, caspase-9, caspase-3, and cleaved PARP immunoreactivities were measured after the treatment with Glu. The activated procaspase-9, caspase-9, caspase-3, and cleaved PARP were also found to be induced by Glu. Schizandrin (100 μ M) or MK-801 (15 μ M) significantly blocked the Glu-induced increases in the procaspase-9, caspase-9, caspase-3, and cleaved PARP activities. Therefore, our results indicate that schizandrin agonist Glu-induced toxicity is able to induce the activations of procaspase-9, caspase-9, caspase-3, and cleaved PARP. The results suggest that schizandrin inhibited Glu-induced apoptosis through the mitochondrial apoptotic pathway.

Schizandrin improved the GSH defense system

GSH is a major endogenous antioxidant in cells. GSH, of course, plays important roles in protection from oxidative stress in the brain and is present in high concentrations in astrocytes (39). We further investigated the effect of schizandrin and MK-801 on GSH level in cortical cell cultures. The pretreatment of the cells with schizandrin (10, 100 μ M) or MK-801 (15 μ M) also increased the glutathione content and the GSH/GSSG ratio (Table 1). These results indicated that schizandrin (10, 100 μ M) and MK-801 (15 μ M) significantly prevented the depletion of GSH and reduced the level of MDA in the Glu-induced excitotoxicity (Table 1).

Discussion

The aim of this study was to evaluate the neuroprotective mechanisms of schizandrin against Glu-induced neuronal apoptosis in primary cultures of rat cortical cells. We used immunocytochemistry to determine the neuronal marker, MAP-2, and the glial marker, GFAP to assess the viability of the cell culture. This modified culture method produces a viable and essentially pure neuronal population (40). We found that the primary cultures of rat cortical cells were more than 90% neurons (data not shown). Therefore, based on our findings, it is possible to dissect temporally different mechanisms of Glu-induced apoptosis.

A variety of biochemical changes are implicated in apoptotic signaling pathway in a time-dependent manner: increases in the levels of $[Ca^{2+}]_i$, oxygen radicals and NO and decreases in the mitochondrial membrane potential, followed by the release of cytochrome c, which result in typical apoptotic morphology, including plasma membrane blebbing, nuclear condensation, cellular shrinkage, and formation of apoptotic bodies (41). For the MTT assay, schizandrin and MK-801 significantly protected primary cultures of

Table 1. The effect of schizandrin on the GSH level and lipid peroxidation in glutamate-injured cortical cell cultures

	Concentration (μ M)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH/GSSG (ratio)	MDA (nmol/ 10^7 cells)
Control		42.11 \pm 1.09	5.67 \pm 0.78	7.43	2.10 \pm 0.09
Glu-injured	10	25.34 \pm 1.09###	14.18 \pm 0.23###	1.79	2.55 \pm 0.12 [#]
MK-801 + Glu	15	30.27 \pm 0.49***	10.17 \pm 0.49***	2.98	1.94 \pm 0.01**
Schizandrin + Glu	1	26.76 \pm 0.87	13.21 \pm 0.33	2.03	2.32 \pm 0.14
	10	28.45 \pm 0.67*	10.36 \pm 0.43**	2.75	2.09 \pm 0.09*
	100	34.15 \pm 0.4***	10.09 \pm 0.39***	3.38	2.07 \pm 0.03*

Cortical cell cultures were pretreated with schizandrin and MK-801 2 h before exposure to 10 μ M Glu and maintained for 24 h. Data were expressed as mean \pm S.E.M. (n = 3). [#] P <0.05, ### P <0.001 compared with control cells, * P <0.05, ** P <0.01, *** P <0.001 compared with Glu-treated cells.

rat cortical cells from Glu excitotoxicity (Fig. 1). In addition, DAPI is a blue nucleic acid fluorescence dyeing agent; it can concentrate and attach to the DNA strand spiral gutter. When the cell weakly perishes, chromosome agglutination and the DNA breakage will happen. In our present study, exposure of the cortical cell cultures to 10 μ M Glu induced cell apoptosis. Typical photographs of DAPI staining showing inhibitory effects of schizandrin and MK-801 on Glu-induced apoptotic-like cell death are shown in Fig. 2.

It has been postulated that reactive oxygen species participate in Glu-induced neurotoxicity (42). The overproduction of NO by NOS activation is involved in Glu-induced neurotoxicity though the increased formation of ROS (43). Annunziato et al. pointed out that during oxidative stress in neuronal cells, the production of ROS triggers a mechanism that, leads to apoptosis (44). Therefore, we examined the possible role of NO caused by exposure of cortical cell cultures to Glu. Excessive Ca^{2+} influx is subsequently followed by the activation of various enzymes including NOS (6). At the concentration of 10 μ M, Glu also tended to increase the level of NO. Pretreatment with schizandrin or MK-801 reduced the level of NO in Glu-injured cells (Fig. 5). Moreover, schizandrin and MK-801 also significantly reduced the formation of cellular peroxide in cortical cell cultures exposed to excess Glu as measured by 2,7-DCF-DA, a cellular peroxide specific fluorescent dye (Fig. 3). The present study demonstrated that schizandrin prevented NO-mediated Glu neurotoxicity in cortical cell cultures. Therefore, schizandrin and MK-801 may prevent neuronal death by interacting with functional molecules that act in key stages of NO-mediated Glu neurotoxicity and promote neuronal survival by augmenting the resistance to radical stress.

On the other hand, during Glu receptor activation, excessive Ca^{2+} is accumulated within the mitochondrial matrix. Accumulation of Ca^{2+} could dissipate membrane potentials across the mitochondria inner membrane to open the mitochondrial permeability transition pores that are inner membrane channels allowing the free passage of various solutes such as cytochrome c, apoptosis-inducing factor, and procaspases (45). These findings have placed the mitochondria at the focus of apoptosis research and further underlined the important function of these organelles in cell life and death (46). In order to study whether Glu-induced mitochondrial depolarization might be associated with Ca^{2+} influx, we measured the mitochondria membrane potential and Ca^{2+} level in cortical cell cultures exposed to Glu. Glu significantly increased the fura-2 fluorescence (ratio of Ex. 340 nm /380 nm) and decreased the mitochondria membrane potential. Both schizandrin and MK-801 effectively

attenuated the excessive Ca^{2+} influx (Fig. 6) and restored mitochondria membrane potential in Glu-induced apoptosis (Fig. 4). Schizandrin and MK-801 seem to maintain the mitochondrial potential by reducing Glu-induced Ca^{2+} influx. Activation of Glu receptors leads to mitochondrial dysfunction as a consequence of mitochondrial depolarization elicited by a loss of Ca^{2+} homeostasis (37).

The lignans of *Schizandra chinensis*, such as schizandrin, are proven to be useful in protecting the liver from free radical attack (19) and enhancing liver functions though the stimulation of glutathione-related enzymes. CNS cells are usually vulnerable to oxidative stress because of their higher consumption of oxygen and the high content of unsaturated fatty acid (47). Thus, mammalian brain has an antioxidative system, such as GSH and GSSG antioxidative enzymes, to defend itself against oxidative stress. GSH is an important intracellular defense against no mediated mitochondrial damage in cells (48). Andrew et al. reported that apoptosis is typically accompanied by a depletion of intracellular reduced GSH (49). Schizandrin and MK-801 partially restored the level of GSH and reduced the level of a GSH metabolite (GSSG) (Table 1). Furthermore, MDA, formed from the breakdown of polyunsaturated fatty acid, serves as a convenient index for determining the extent of the lipid peroxidation. The determination of MDA is an excellent index of cell damage induced by oxygen free radicals. As shown in Table 1, schizandrin and MK-801 eventually reduced the level of MDA. These results illustrated that schizandrin and MK-801 reduced the formation of ROS in cortical cell cultures by enhancing the antioxidative defense system and free radical scavenging activity. We suggest that schizandrin decreases the membrane lipid peroxidation via enhancing the level of the GSH defense system.

Cytochrome c is found in the mitochondria and released into the cytoplasm shortly after Glu-induced apoptosis. Increased mitochondrial Ca^{2+} accumulation is a trigger for the release of cytochrome c from the mitochondrial permeability transition (PT) pore into the cytosol where it can activate caspases and lead to apoptosis (50, 51). The elevation of $[\text{Ca}^{2+}]_i$ and ROS production can result in the release of cytochrome c from mitochondria and the activation of the caspase cascade (52). Apoptosis can be induced by two major pathways, the surface death receptor pathway and the mitochondrion-initiated pathway, both of which are regulated by caspases. Subsequently, cytochrome c binds to Apaf-1, which in turn self-associates and binds procaspase-9, resulting in an apoptosome transactivation of the complexes procaspase-9 to active caspase-9, and the caspase then cleaves and activates downstream

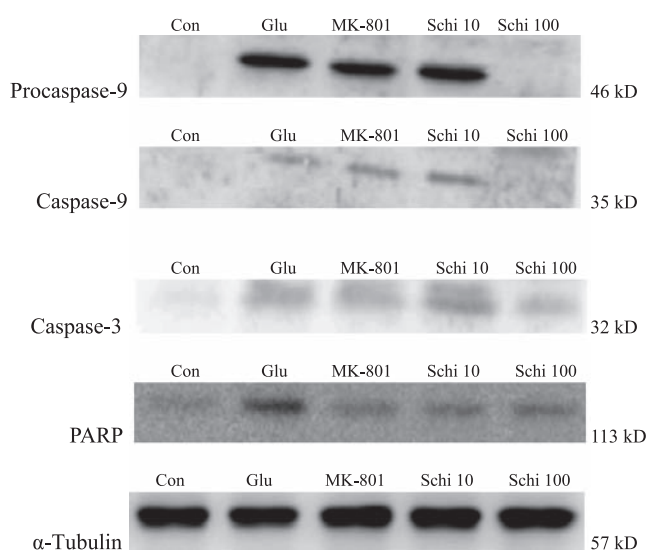


Fig. 8. Western blot analysis of schizandrin on Glu-induced protein levels changes of procaspase-9, caspase-9, caspase-3, and PARP cleavage in rat cortical cells. Cortical cell cultures were pretreated with schizandrin (10 and 100 μ M) or MK-801 (15 μ M) 2 h before exposure to 10 μ M Glu and then maintained for 24 h. α -Tubulin was used as an internal loading control.

caspases 3. Furthermore, recent data show that AIF is released from mitochondria by a mechanism distinct from that of cytochrome c, but probably mediated by PARP (53). In our studies, schizandrin significantly decreased the cytochrome c release increased by Glu insult (Fig. 7). Therefore, inhibition of $[Ca^{2+}]_i$ and ROS might be achieved as a result of reduction in cytochrome c release. Similarly, as shown in Fig. 8, the procaspase-9, caspase-9, caspase-3, and PARP cleavage played a critical role in the induction of apoptosis by Glu. In the present study, our results indicated that schizandrin protected primary cultures of rat cortical cells against Glu-induced apoptosis through a mitochondria-mediated pathway.

Chiu et al. showed that schizandrin B decreases the sensitivity of mitochondria to calcium ion-induced permeability transition (PT) and protects against ischemia reperfusion injury in rat hearts (54). Furthermore, glutamate and its receptors have long been recognized to play key roles in the pathology of ischemia, leading to the glutamate-calcium overload hypothesis (55). In addition, schizandrin B pretreatment against CCl_4 toxicity was paralleled by the decrease in the sensitivity of hepatic mitochondria to Ca^{2+} stimulated PT as well as the attenuations of mitochondrial Ca^{2+} loading, ROS production, and cytochrome c release under the CCl_4 -intoxicated condition (56). This is in agreement with our results indicating that the ability for schizandrin to protect primary cultures of rat cortical cells against

Glu-induced apoptosis may be related to the resistance of cellular mitochondria to Ca^{2+} -stimulated PT.

Our results suggest that schizandrin protected primary cultures of rat cortical cells against Glu-induced apoptosis. In order to determine whether schizandrin acts in the same manner as MK-801, which has the NMDA-receptor antagonist effect, we employed a docking technology experiment to analyze the structures of schizandrin and the NR₁ and NR₂ subunits of the NMDA receptor (57). Our results show that the molecular structure of schizandrin is bigger than the binding site of the NR₁ and NR₂ subunit. Therefore, schizandrin cannot bind with the NR₁ and NR₂ subunits (Schizandrin volume: 351.4 \AA^3 , NR₁ binding site volume: 88.5 \AA^3 , NR₂ binding site volume: 33.5 \AA^3) (data not shown). We speculate that schizandrin does not exert its action through the NMDA receptors. Although MK-801 is a non-competitive NMDA antagonist, it possesses a neuroprotective effect against AMPA and kainite-induced neurotoxicity (58). Therefore, we speculate that schizandrin is probably involved with the AMPA or kainate receptor, or even with metabotropic receptors. We will confirm the detailed mechanism in the future.

In conclusion, our results demonstrate that schizandrin possesses significant neuroprotective effect on cultured cortical neurons at least in part by inhibiting the mitochondrial pathway of the apoptotic process and oxidative stress. On the basis of the present study, the anti-apoptotic effect of schizandrin may provide a potential therapeutic approach for preventing and/or treating neurodegenerative diseases. Further studies must be carried out to explore this possibility.

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