

Original Article

Effect of caspase-9 inhibition on endoplasmic reticulum stress induced cortical neuronal injury in rats

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Received June 19, 2013; Accepted July 16, 2013; Epub August 1, 2013; Published August 15, 2013

Abstract: Our study investigated the apoptotic mechanism of rat cortical neurons following hypoxia/reperfusion induced endoplasmic reticulum stress (ERS) in vitro and to explore the effect of caspase-9 inhibition on ERS induced apoptosis. Cortical neurons were collected from neonatal rats and cultured in vitro. Immunohistochemistry and immunofluorescence staining for neuron-specific enolase (NSE) were performed to determine the purity of neurons. AnnexinV/PI staining followed by flow cytometry was employed to detect apoptosis rate. Fluorescein isothiocyanate (FITC) staining was done to measure the expression of caspase-3 and -9. Western blot assay was carried out to measure the protein expression of caspase-12, glucose-regulated protein (GRP) 78 and Cytochrome C. The cortical neurons from neonatal rats could be purified and cultured in vitro. In the in vitro hypoxia/reperfusion of cortical neurons (hypoxia for 6 h and reperfusion for 24 h and 48 h), the protein expression of GRP78, caspase-3, 9 and 12 was markedly increased ($P < 0.01$). Following pre-treatment with caspase-9 inhibitor, the number of apoptotic cells was significantly reduced following hypoxia for 6 and reperfusion for 24 h or 48 h ($P < 0.05$). Moreover, the expression of caspase-3 and 12 and GRP78 was also significantly reduced in the presence of caspase-9 inhibitor treatment ($P < 0.05$), but the release of Cytochrome C remained unchanged ($P > 0.05$). These results demonstrated that ERS is involved in the neuronal apoptosis following in vitro hypoxia/reperfusion, and caspase-9 inhibition can depress the ERS induced apoptosis of neurons.

Keywords: Cortical neuron, hypoxia/reperfusion, endoplasmic reticulum stress, caspase, apoptosis, immunoblotting

Introduction

Cumulative evidence suggests that apoptosis plays a pivotal role in neuronal cell death in ischemic cerebral injury. Apoptotic signaling involves both extrinsic and intrinsic/stress-induced pathways. Endoplasmic reticulum (ER) is an organelle that ensures the correct control of protein folding and assembly by expressing numerous molecular chaperones and using other quality control system of protein [1]. Under various conditions, unfolded proteins accumulate in the ER lumen, a process called ER stress. ER stress could also be elicited in the cell culture system by pharmacological agents including tunicamycin (Tun), a protein N-glycosylation inhibitor; brefeldin A (BFA), which blocks protein transport from ER to Golgi; and thapsigargin (TG), which blocks ER uptake of calcium by inhibiting the sarcoplasmic/endo-

plasmic Ca^{2+} -ATPase (SERCA) [2]. Excessive ER stress induces cell death [3]. Caspase-12, one of the caspase family of proteases, is localized to the ER and is specifically activated by ER stress [4]. Activation of ER resident caspase-12 causes activation of cytoplasmic caspase-3, and not mitochondria-related caspase-9, during ER stress-induced apoptosis [5]. Evidence is also emerging that there is cross-talk between the ER and the mitochondria. Nobuhiro et al [6] used a murine myoblast cell line C2C12 to study caspase-12, and they suggested that pro-caspase-9 was a substrate of caspase-12, and the proteolytic signals in the cascade were transmitted from caspase-12 to an effector caspase (caspase-3) via caspase-9. There is complex cross-talk among different apoptotic pathways in different organelles. After cerebral ischemia/reperfusion, the mechanisms underlying the neuronal apoptosis are still unclear,

especially the cross-talk between mitochondrial apoptotic pathway and ER stress. The present study aimed to investigate the relationship between ER stress and mitochondrial apoptosis and the effect of caspase-9 inhibition on ER stress in rat cortical neurons following hypoxia/reperfusion.

Materials and methods

Materials

Neonatal Sprague Dawley (SD) rats (< 24-h old) were purchased from the Experimental Animal Center of Shandong University. High glucose DMEM, Neurobasal medium, B27, fetal bovine serum (FBS), horse serum (GibcoBRL), trypsin, L-polylysine (Sigma), Annexin V-FITC (Bender MedSystems), anti-Caspase-12, anti-Grp78 polyclonal antibodies (Stressgen), active caspase-3, caspase-9, Z-LEHD-fmk (caspase-9 inhibitor) kits (BioVision), kit of immunohistochemistry for NSE (Beijing Zhongshan Biotech), Cytochrome C (Santa Cruz Biotechnology), and other analytically pure reagents were used in the present study. Incubator (Napco 5410-220, USA), inverted fluorescence microscope (Olympus, JAPAN), Automatic ELISA analyzer (Thermo electron corporation Multiskan MK3, USA), flow cytometer (FACSCLSR, Becton- Dickton, USA), and laser scanning confocal microscope were applied in the detection.

Primary culture of cerebral cortical cells

The cerebral cortex was isolated from of neonatal Sprague-Dawley rats. After the removal of meninges and blood vessels, cerebral cortex was cut, digested with trypsin at 37°C for 20 min, and filtered with a strainer to exclude tissue fragments. The filtrate was centrifuged at 300 × g for 5 min and its sediment was washed twice with Hank's medium. Then cells were resuspended with DMEM medium containing 10% FBS and 10% HBS (pH 7.2~7.4), adjusted to approximately 10⁶ cell/ml, and plated into polylysine-coated plastic culture flasks and maintained at 37°C in 5% CO₂ humid environment. At 24 hours after initial plating, plate medium was completely replaced with neurobasal medium containing B27 supplements. After 3-5 days, half of medium was exchanged for glutamate free neurobasal medium. All experiments were performed on neurons after 7-10 days in culture. Cortical neurons were reacted with primary antibody NSE at 7 days after the culture by immunohistochemistry and

immunofluorescence staining. And these positive cells of NSE expression were accounted of the total cells.

Establishment of neuronal *in vitro* models of hypoxia/reperfusion

To mimic cerebral ischemia *in vitro*, hypoxia/reperfusion was performed with primary neuronal cultures as described previously by Goldberg et al [7] and Doukas et al [8]. Medium was first removed from the cultures, and then rinsed twice with phosphate buffered solution (PBS) without Ca²⁺/Mg²⁺. Cultures were subjected to ischemia in an anoxia chamber for 6 hours by rinsing twice and covering with glucose-free Earles medium pre-equilibrated with the atmosphere in the chamber (95% N₂ and 5% CO₂). Control cells were incubated in the same solution with glucose under normoxic conditions (in a CO₂ incubator). After ischemia cultures 6h, glucose-free Earles medium was replaced by fresh neurobasal medium with B-27 supplement. Finally, cultures were maintained in a CO₂ incubator for the next 48 hours reperfusion, as mentioned above.

Detection of neuronal apoptosis by flow cytometry and fluorescence microscopy

The above cells were harvested by digestion in trypsin and centrifuged at 4°C for 5 min. The supernatant was removed and cells were washed in PBS twice and re-suspended in 195 μL of binding buffer. Then, these cells were mixed with 5 μL of Annexin V-F ITC followed by incubation in dark at room temperature for 10 min. After washing in binding buffer thrice, cells were re-suspended in 190 μL of binding buffer followed by addition of 10 μL of 20 μg/mL PI (final concentration 1 μg/mL). Apoptosis rate was measured by flow cytometry. Detection was repeated three times. For Hoechst 33258 staining, cells on coverslips were fixed in methanol: glacial acetic acid solution (3:1) at 4°C for 5 min. After rinsing in PBS, cells were stained with Hoechst 33258 at a final concentration of 5 mg/L for 10 min. After rinsing in PBS, mounting was done and cells were observed under a fluorescence microscope.

Detection of caspase-3 and -9 activities by flow cytometry

Cells (1 × 10⁶/mL) in above groups and negative control group (cells were treated with 1 μL/

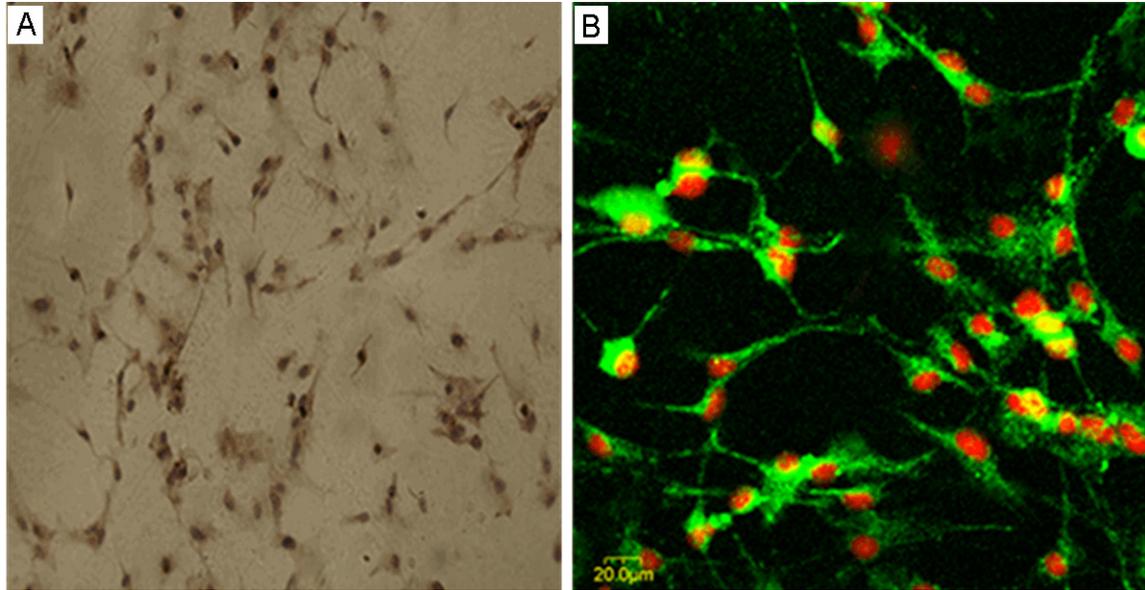


Figure 1. Identification of primary neurons. A: Immunohistochemistry for NSE of cells at 7 days after culture (X200); B: Immunofluorescence staining for NSE of cells at 7 days after culture (X100).

mL caspase inhibitors) were digested and re-suspended. Then, 300 μ L of cell suspension was added to the EP tubes followed by FITC conjugated caspase-3 and -9 (1 μ L per tube). Incubation was done at 37°C in an environment with 5% CO₂ for 1 h. Cells were centrifuged at 3000 rpm for 5 min and the supernatant was removed. Cells were re-suspended in 0.5 ml of buffer followed by centrifugation at 3000 rpm for 5 min. The supernatant was removed and cells were re-suspended in 300 μ L of buffer. Flow cytometry was performed and detection was done thrice.

Detection of caspase-12 and GRP-78 expressions by western blot assay

Total protein was extracted from cells in each group according to manufacturer's instructions followed by the determination of protein concentration. Western blot assay was performed according to the procedures described by Elyaman et al [9]. Detection was done at least three times. The optical density was measured to semi-quantitatively determine the protein expression of active caspase-12 and GRP-78.

Subcellular fractionation

Fractionation was performed as described previously [10, 11] with some modifications. Briefly, cells were resuspended and lysed in ice-cold

hypotonic extraction buffer (10 mmol/L Tris-HCl, pH 7.4, 50 mmol/L KCl) using a B-type pestle. The lysate was immediately adjusted to 250 mmol/L sucrose, 1 mmol/L MgCl₂, 0.5 mmol/L EGTA, 1 mmol/L DTT, 0.1 mmol/L PMSF, 5 μ g/mL pepstatin A, 10 μ g/mL leupeptin and aprotinin, and centrifuged at 750 \times g for 10 min at 4°C so as to remove nuclei and cell debris. The supernatant was further centrifuged at 10,000 \times g for 30 min at 4°C. The pellet containing mitochondrial fraction was resuspended in the buffer above. After re-centrifuged at 100,000 \times g for 60 min, the supernatant then contained soluble cytoplasmic fraction, and the pellet constituting ER-enriched microsomal fraction was rinsed and resuspended as above. The quality of the fractionation experiments was controlled by assessing the distribution of mtHSP70 for mitochondria, SREBP-1 for ER.

Statistical analysis

Statistical analysis was done with SPSS version 12.0, and data were expressed as mean \pm standard deviation (SD). Comparisons between two groups were done with t test and those among multiple groups with one way analysis of variance (ANOVA) followed by Dunnett test. A value of P < 0.05 was considered statistically significant.

Caspase-9 inhibition and cortical neuronal injury

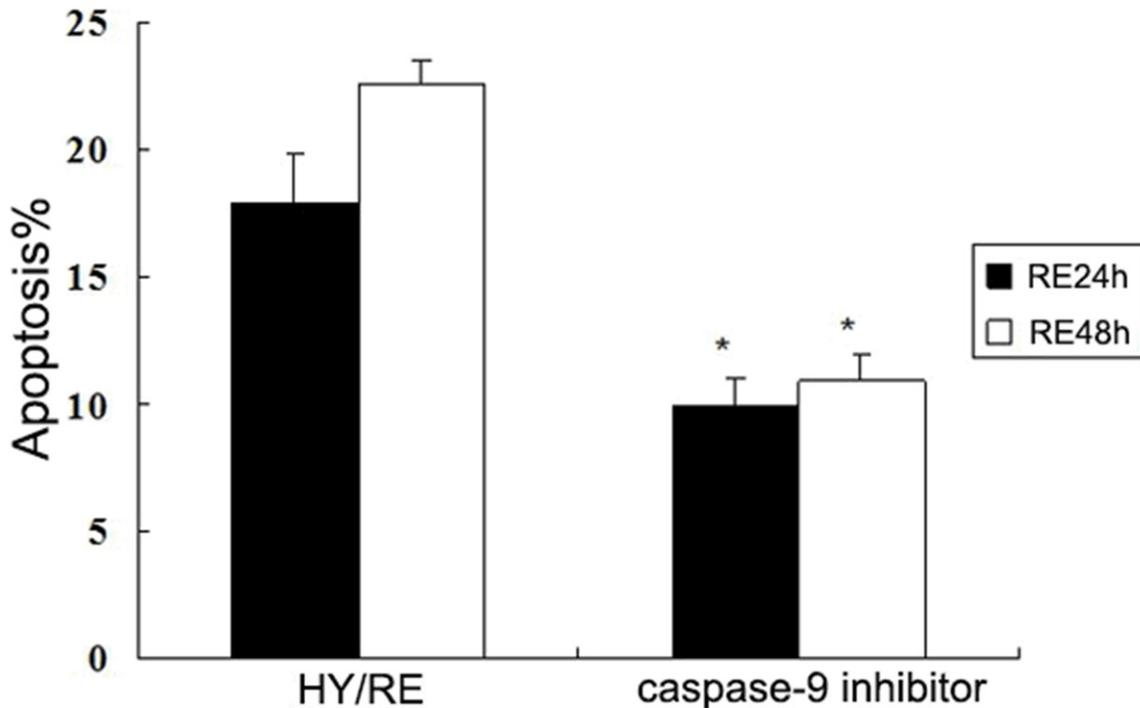


Figure 2. Percentage apoptosis as determined by flow cytometry was plotted. * $P < 0.05$.

Results

Culture of primary neurons

7 days after culture, cells were harvested and immunohistochemistry was done for NSE. The NSE positive cells were counted. Results showed the proportion of NSE positive cells was $92.69 \pm 4.1\%$ ($n = 5$). It was therefore deemed as pure neuron culture. (Figure 1A and 1B).

Mimic cerebral hypoxia/reperfusion in vitro induces apoptosis

To determine whether hypoxia/reperfusion resulted in apoptosis or not, we analyzed the apoptosis percentages by flow cytometry, which identifies the positive percentage of cells with annexin V-FITC without propidium iodide uptake. The apoptosis of normally-cultured cortical neurons of rats notably increased after analogous hypoxia/reperfusion *in vitro* (17.95% at 24 h and 22.62% at 48 h). The percentage of apoptotic cells was depressed after treatment with caspase-9 inhibitor for 1 h (9.87% at 24 h and 10.91% at 48 h) (Figure 2). Furthermore, with Hoechst 33258 stain, morphological alteration of apoptotic cells showed that the nucleus of living cells was uniformly stained blue while apoptotic cells were hyperchromatic.

Western blot assay of caspase-12, GRP78 and cytochrome C

Western blot assay showed the GRP78 expression was markedly increased after ischemia/reperfusion, and the GRP78 expression at 48 h was higher than that at 24 h. In addition, the caspase-12 expression at 24 h was also significantly elevated (anti-active caspase-12 antibody was used), but the caspase-12 expression was comparable to that before ischemia/reperfusion. After pre-treatment with caspase-9 inhibitor, the expression of GRP78 and caspase-12 was significantly reduced ($P < 0.05$), but the Cytochrome C expression remained unchanged ($P > 0.05$) (Figure 3).

Expression of active caspase-3

After ischemia for 6 h and reperfusion, flow cytometry showed the expression of active caspase-3 was significantly increased over time. However, in the presence of caspase-9 inhibitor pre-treatment, the expression of active caspase-3 was markedly reduced.

Discussion

It has been known that apoptotic morphological changes are observed in cell death caused

Caspase-9 inhibition and cortical neuronal injury

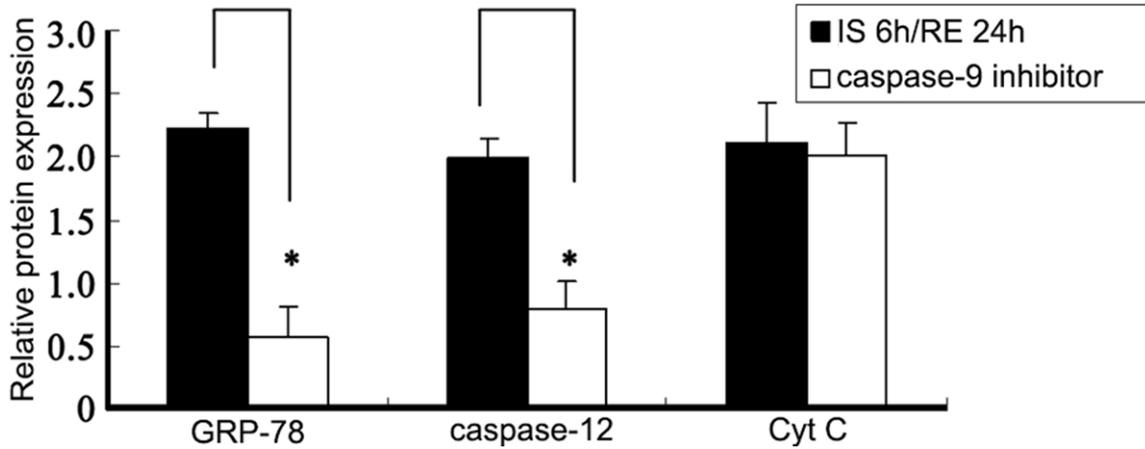


Figure 3. Western blot assay. After pre-treatment with caspase-9 inhibitor, the expression of GRP78 and caspase-12 was significantly reduced, but the Cyt C expression remained unchanged. *P < 0.05 between two groups.

by ER stress [12]. In recent years, increasing attention has been paid to the ER as a regulator of mitochondrial apoptosis pathway [13]. Currently, several pathways have been directly implicated in ER stress-induced apoptosis. The apoptosis signals of neuron caused by hypoxia/reperfusion are multiple. In past study we provided that cultured neurons undergo caspase-3/-8/-9/-12-dependent apoptosis under analogous hypoxia/reperfusion circumstance and its mechanism might involve mitochondrial dysfunction and ER stress. Our results indicated that there is cross-talk between the ER and the mitochondria during hypoxia/reperfusion, which as a result, induces neuronal apoptosis [14].

The expression of molecular chaperone GRP78 as a marker for ER stress is usually up-regulated in the presence of ER stress and has been regarded as an indicator of ER stress [15]. ER is very sensitive to oxidative indicated mean effective pressure [16]. Caspase-12, one of the caspase families, is localized in ER and specifically activated by ER stress [4]. In the present study, our findings showed, in rat cortical neurons following hypoxia/reperfusion, the GRP78 and Cytochrome C expression increased significantly, which was not found in the control group. We report here that ER stress response and mitochondrial dysfunction are involved in neuronal apoptotic pathways induced by hypoxia/reperfusion *in vitro*. Neurons showed typical apoptotic features, such as chromatin condensation, pyknosis and cell shrinkage. In addition, Annexin V-FITC and PI staining followed by flow

cytometry was performed to detect the apoptotic cells. Rao et al found *in vitro* study that the caspase-12 activation was dependent caspase-7 [17]. However, Shibata et al found that the caspase-12 activation was not associated with caspase-7 translocation [18]. Nobuhiro et al found caspase-12 activation triggers the caspase cascade in response to ER stress and caspase-9 activation can be achieved in cells without the release of cytochrome C from mitochondria [6]. To investigate the mechanism underlying the apoptosis of neurons following hypoxia/reperfusion, flow cytometry and western blot assay were employed to detect the expression of active caspase-3, -9, -7 and -12 and Cytochrome C. In spite of certain onset of caspase cascade initiated by hypoxia/reperfusion, we detected that caspase-9/-3/-12 were involved but not caspase-7 in neuronal apoptotic signals caused by hypoxia/reperfusion. In previous studies [19], our results showed silencing of Cytochrome C with siRNA could significantly reduce the GRP78 expression and the expression of caspase-12, -3 and -9 in neurons following hypoxia/reperfusion. In the present study the activities of caspase-3 and -9 are positively related to the apoptosis rate. In early apoptosis phase, caspase-12 is activated, and specific inhibition of caspase-9 expression failed to reduce the expression of Cytochrome C but caspase-3/-12 and GRP-78 expression was markedly inhibited. Therefore, specific protective effect of specific inhibition of caspase-9 on cultured neurons *in vitro* may have no relation to Cytochrome C.

This reveals, in the neuronal hypoxia/reperfusion, there is close relationship between ER and mitochondria, and stabilizing the mitochondrial function is helpful to attenuate ER stress induced injury. However, only the expression of one factor in the apoptosis pathway in neurons was detected, and the interaction among different apoptosis related factors is lack. The studies on multiple cascade reactions and their interaction will elucidate the mechanisms underlying the neuronal apoptosis and provide strategies for the treatment and prevention of cerebral ischemia/reperfusion.

Acknowledgment

The study was supported by the grant from Science and Technology Development Plan of Traditional Chinese Medicine of Shandong Province (2009-159).

Disclosure of conflict of interest

None declared.

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