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The protection of Bcl-2 overexpression on rat cortical neuronal injury caused by analogous ischemia/reperfusion in vitro

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Abstract

Recent studies have suggested that neuronal apoptosis in cerebral ischemia could arise from dysfunction of endoplasmic reticulum (ER) and mitochondria. B-cell lymphoma/leukemia-2 gene (Bcl-2) has been described as an inhibitor both in programmed cell death (PCD) and ER dysfunction during apoptosis, and the Bcl-2 family play a key role in regulating the PCD, both locally at the ER and from a distance at the mitochondrial membrane. However, its signal pathways and concrete mechanisms in endoplasmic reticulum-initiated apoptosis remain incompletely understood. We therefore investigate whether ischemia/reperfusion (I/R) causes neuronal apoptosis in part via cross-talk between ER and mitochondria or not, and how the overexpression of Bcl-2 prevents this form of cell death. Here we show that analogous I/R-induced cell death occurs consequent to interactions of ER stress and mitochondrial death pathways. The participation of the mitochondrial pathway was demonstrated by the release of cytochrome *C* (cyt *C*) from mitochondrial into cytoplasmic fractions and caspase-9 cleavage. The involvement of ER stress was further supported by the observable increase of glucose-regulated protein 78(GRP78)/BiP expression and caspase-12 activity. Furthermore, prior to these changes, swelling of the ER lumen and dissociation of ribosomes from rough ER were detected by electron microscopy. Bcl-2 overexpression inhibits the release of cyt *C* and the activation of caspase-9/-8/-3 but not caspase-12 based on the results of Western blot. These suggest that cross-talk between ER and mitochondria participate in neuronal damage after ischemia/reperfusion. Bcl-2 overexpression could suppress I/R-induced neuronal apoptosis via influencing mitochondrial integrity.

Keywords: Cortical neuron; Ischemia/reperfusion; Endoplasmic reticulum stress; Caspases; Bcl-2

1. Introduction

Cumulative evidence suggested that apoptosis plays a pivotal role in neuronal death in cerebral ischemic injury. Apoptotic signal involves both extrinsic and intrinsic/stress-induced pathways. The endoplasmic reticulum (ER) is an organelle that ensures the correction of protein fold and assembly via the expression of numerous molecular chaperones and other control system of protein quality (Hammond and Helenius, 1994). Various conditions, such as glucose starvation, disturbance of intracellular calcium homeostasis, inhibition of protein glycosylation, as well as exposure to free radicals, unfolded proteins to accumulate in ER lumen, that is, a process named ER stress. ER stress could also be elicited in cell culture system by pharmacological agents, including tunicamycin (Tun), a protein inhibitor of *N*-glycosylation; brefeldin A (BFA), which blocks protein transportation from ER into Golgi; and thapsigargin (TG), which depresses ER uptake of calcium by inhibiting the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) (Lee, 2001). Excessive ER stress induces cell death (Kadowaki et al., 2004). Caspase-12, a protease of caspase family, is localized in ER and specifically activated by ER stress (Nakagawa et al., 2000). And the activity of ER resident caspase-12 can ignite cytoplasmic caspase-3, but not mitochondria-related caspase-9, during ER stress-induced apoptosis (Hitomi et al., 2003). Evidence also showed the cross-talk between ER and mitochondria. Bcl-2 family members have been considered as key mediators in either pro- or anti-apoptosis. Owing to the mitochondrial localization of many Bcl-2 family members, they are often assumed to play a direct biochemical effect in mitochondria. Recent work has

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demonstrated that in addition to maintaining mitochondrial integrity, pro- and anti-apoptotic members of Bcl-2 family also reside ER, where they regulate organelle homeostasis and cell death in response to signals impacting ER function (Oakes et al., 2006). Inhibition of I/R-induced cell death by anti-apoptotic Bcl-2 family protein in vivo has been examined (Chien et al., 2007; Miao et al., 2007), nevertheless, the certain mechanisms of neuronal apoptosis underlying cerebral ischemia/reperfusion damage remains elusive. In the present study, we examined the involvement of ER stress and mitochondria and the effect of overexpression Bcl-2 in rat neuronal apoptosis induced by analogous ischemia/reperfusion in vitro.

2. Materials and methods

2.1. Primary culture of cerebral cortical cells

Primary neuronal culture was performed as previously described (Wang et al., 2003). Briefly, cerebral cortex was isolated from 16 to 18 days old fetuses of 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% fetal bovine serum (FBS) and 10% horse serum (HS), adjusted to approximately 10^6 cell/ml, and plated into polylysine-coated plastic culture flasks and maintained at 37 °C in 5% CO_2 humid environment. At 24 h after initial plating, plate medium was completely replaced with neurobasal medium containing B27 supplements (Gibco, Rockville, MD, USA). After 4 days, half of medium was exchanged for glutamate free neurobasal medium. All experiments were performed on neurons after 7–10 days in culture.

2.2. Establishment of neuronal in vitro models of ischemia/reperfusion

To mimic cerebral ischemia in vitro, ischemia/reperfusion was performed with primary neuronal cultures as described previously (Meloni et al., 2001). Medium was first removed from the cultures, and then rinsed twice with phosphate buffered solution (PBS) without Ca^{2+}/Mg^{2+}. Cultures were subjected to ischemia in an anoxia chamber for 6 h by rinsing twice and covering with glucose-free Earles medium (143.8 mmol/L Na^+, 5.5 mmol/L K^+, 1.8 mmol/L Ca^{2+}, 0.8 mmol/L Mg^{2+}, 125.3 mmol/L Cl^-, 26.2 mmol/L HCO_3^-, 1.0 mmol/L $H_2PO_4^{2-}$, 0.8 mmol/L SO_4^{2-}, 0.01 mmol/L glycine, and 10 mmol/L HEPES at pH 7.4) pre-equilibrated with the atmosphere in the chamber (95% N_2 and 5% CO_2). Control cells were incubated in the same solution with glucose under normoxic conditions (in a CO_2 incubator). After ischemia cultures 6 h, glucose-free Earles medium was replaced by fresh neurobasal medium with B-27 supplement. Finally, cultures were maintained in a CO_2 incubator for the next 48 h reperfusion, as mentioned above.

2.3. Plasmid

The pcDNA3-hBcl-2 was granted from Dr. Karen S. Poksay (Buck Institute for Age Research, Novato, California 94945 and the University of California, San Francisco, California 94143).

2.4. Transfection

All procedures were performed with the lipidosome 2000 transfection reagent (Invitrogen) following the manufacturer's protocols. Briefly, cells were seeded in the culture plates and cultured to achieve 50% of confluence. The cells were then transiently transfected using a mixture of plasmid and lipidosome 2000 in Optimem (Invitrogen). A lipidosome 2000 to DNA ratio of 3 μl:2 μg was maintained for all experiments. Media was completely replaced with conditioned media 6–8 h after transfection, and neurons were undergone experiment 2–4 days after transfection.

2.5. Transmission electron microscopy (TEM)

The specimens were fixed in a mixture of 4% glutaraldehyde and 4% formaldehyde (freshly prepared from paraformaldehyde) buffered at pH 7.2 with 0.1 mol/L sodium cacodylate at room temperature for 16–20 h. After washings in 0.1 mol/L sodium cacodylate at pH 7.2, the specimens were transferred to cacodylate-buffered 1% osmium tetroxide at pH 7.2 for 1 h at room temperature. After treated with 0.5% uranyl acetate for 2 h, the specimens were dehydrated in gradient ethanol, rinsed in propylene oxide and then embedded in Araldite.

Semithin sections stained with 1% toluidine blue were examined under a light microscope, and suitable regions were carefully selected for trimming of the blocks. Ultrathin sections were collected on collodion-coated grids, stained in alcoholic 2% uranyl acetate and lead citrate and examined in a transmission electron microscope (Philips-CM 200).

2.6. Apoptosis assays

After subjected to ischemia/reperfusion, 10^6 cells were used to determine the translocation of phosphatidylserine to the outer surface of plasma membrane during apoptosis by using human phospholipids binding protein, annexin V, conjugated with fluorescein isothiocyanate, according to the manufacturer's instructions (Bender Med Systems). The percentage of apoptotic cells (annexin V positive and propidium iodide negative) was determined by flow cytometric analysis (FACSCLSR, Becton-Dickton, USA). For fluorescent microscope, cells were incubated with Hochest 33258 with a final concentration of 5 mg/L.

2.7. Western immunoblotting analysis

Total protein was extracted from cells in each group in accordance with kit operation of protein extraction, and