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The Relationship Between the Expression of CIDE-B and the Neuronal Apoptosis Following Cerebral Ischemia Reperfusion in Rats

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Abstract: To explore the relationship between the cell-death-inducing DNA-fragmentation factor (DFF45)-1ike effecter-B (CIDE-B) and the neuronal apoptosis after cerebral ischemia reperfusion in rats. The middle cerebral artery occlusion and reperfusion (MCAO/R) models were established by inserting a monofilament suture from left external-internal carotid artery in adult healthy male Wistar rats. The neuronal apoptosis in hippocampus was detected by TUNEL assay, the expression of CIDE-B protein were determined by immunohistochemical staining and Western blot and CIDE-B mRNA by RT-PCR, respectively. After ischemia 2 h and reperfusion 6 h, 1, 3, 7 and 14 days, the No. of neuronal apoptosis was significantly increased ($p<0.01$) and the expressions of CIDE-B mRNA and protein were evaluated ($p<0.01$), while all above indexes reduced significantly than those in the control group at reperfusion 28 days ($p<0.01$). The time-phase of neuronal apoptosis was coincident with the expressions of CIDE-B gene. The neuronal apoptosis was timely dependent on the expression of CIDE-B gene after cerebral ischemia reperfusion in rats.

Key words: Cerebral ischemia, reperfusion injury, apoptosis, CIDE-B, rats

INTRODUCTION

It is known to all that apoptosis was a way of death under the control of their own genetic encoding with polygene-regulated. Cerebral ischemia could cause neuronal apoptosis in ischemia sensitive areas such as the hippocampus (Wang *et al*., 2012). Blood reperfusion was the basic condition for the recovery of neurological function after cerebral ischemia, but also an important factor in aggravating brain damage (Hu *et al*., 2012). Cerebral ischemia could initiate oxidative stress cascade and induce the expression of related genes and proteases, ultimately leading to apoptosis (Ramassamy, 2006). Cell death DNA fragmentation factor 45 was the subunit of DNA fragmentation factor, including the CIDE-A and CIDE-B (Liang *et al*., 2003). CIDE-B was located in mitochondria as two types of molecular forms, i.e., homodimers and heterodimers. The lacalization of CIDE-B in mitochondria and the dimerization of molecular structure were the essential conditions to play its important role (Sakahira *et al*., 2000; Sun *et al*., 2006). This experiment was designed to study the correlation between the expression of CIDE-B gene and neuronal apoptosis after cerebral ischemia/reperfusion injury, so as to provide a theoretical basis for the regulation of CIDE-B gene expression and effective inhibition of neuronal apoptosis.

MATERIALS AND METHODS

Animal models: Total of 110 adult healthy male Wistar rats, SPF grade, weighted 220-240 g, supplied by the Experiment Animal Center of Qingdao Drug Inspection Institute (SCXK (LU) 20090007). This experiment was approved by the Ethics Committee of Qingdao University Medical College (QUMC 2011-09). The local legislation for ethics of experiment on animals and guidelines for the care and use of laboratory animals were followed in all animal procedures. All animals were acclimatized for 7 days and allowed free access to food and water in a room temperature (23±2)°C and hunidity-controlled housing with natural illumination and fasting for 12 h before operation. Firstly, 48 rats were randomly selected as sham group and the rest rats were anesthetized by injecting intraperitoneally 4% chloral hydrate (300 mg kg^{-1}) and fixed in supine position to conduct aseptic operation and to establish Middle Cerebral Artery Occlusion (MCAO) models by inserting a monofilament suture from the left external-internal carotid artery (ECA-ICA) into the MCA

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(Longa *et al*., 1989). After ischemia 2 h, the monofilament suture was withdrawn from MCA to recover blood supply. The successful models showed left Horner's sign, right forelimb flexing and circling rightward as running. Fourteen animals which died or didn't waked 2 h after surgery were rejected out of the experiment, while the rest 48 cases of successful models were brought into statistical ranges which were randomly subdivided into reperfusion 6 h, 1, 3, 7, 14, 28 days after ischemia 2 h and 8 rats in each subgroup. The rats of sham group were experimented the same surgical procedure except of inserting suture. The core body temperature was keeping with a rectal probe and maintained at 36-37°C using a homeothermic blanket control unit during and after the surgery operation.

Preparation of paraffin section: Four rats were randomly chosen from each subgroup and anesthetized by injecting intraperitoneally 4% chloral hydrate (300 mg kg^{-1}). Full brain was taken out after cardiac perfusion by 0.1 mol L^{-1} phosphate buffer 200 mL and 4% paraformaldehyde solution 200 mL successively. Coronal sections with a thickness of 5 μm were continuously sectioned from the posterior of optic chiasma (CM2027, Leica Co. Ltd., Germany) after conventional gradient ethanol dehydration, xylene transparent, paraffin embedding. One section was selected interval of 5 sections and adhered on the slices processed with poly lysine and stored at 4°C.

TUNEL apoptotic cell detection: Five sections from each rat were chosen and operated according to specification of the TUNEL detection kit (Wuhan Boster Biotech. Co. Ltd.). After conventional de-waxing and hydration, developing by DAB, the nuclei of apoptotic cells appeared dark brown. Some slices were added with 0.01 mol L^{-1} PBS to replace terminal deoxynucleotidyl transferase (TdT) and no positive reaction appeared. Four slices were taken for each rat; five non-overlap visual fields in hippocampus were randomly observed under high power microscope (Olympus CK2, Japan) to count positive cells (positive cells/visual field) and then the data was calculated as the average (Mean±SD).

Immunohistochemisty: Rabbit anti-rat CIDE-B antibody was purchased from Santa Cruz Co. Ltd. USA. Five sections were chosen in each rat and performed according to the specification of horseradish peroxidase labeling goal anti-rabbit kits (Bio Vis, USA), developed by DAB. The cells with cytoplasm or nucleus containing brown particles observed under light microscope were considered as positive cells. Some slices were added with 0.01 mol L^{-1} PBS to replace the primary antibody and there was no positive reaction. Five non-overlap visual fields at each section of hippocampus were randomly observed under 400-fold light microscope to calculate the positive cells in each visual field (positive cells/visual field) and the positive reaction was expressed as average (Mean±SD).

Western blot: Four rats in each subgroup were randomly chosen and anesthetized by injecting intraperitoneally 4% chloral hydrate (300 mg kg^{-1}). The rats were sacrificed after anesthetized and the brain was collected on ice after cardiac perfusion by 0.1 mol L^{-1} phosphate buffer 200 mL and 4% paraformaldehyde solution 200 mL successively. Total of 100 mg hippocampal tissue in ischemic zone was taken and cell lysis buffer was added as the proportion of 1:4 (Beijing Gaining Biotech. Co. Ltd.) and then grinded fully and homogenized by ultrasonic at -4°C ice bath, centrifuged with 12000 r min^{-1} for 10 min at 4°C (Eppendorf 5801, Germany). The protein concentration in the supernate was determinate by BCA-100 protein quantitative kit (Shanghai Shenneng Biotech. Co. Ltd.). Total of 50 μg protein sample was collected for electrophoresis on 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred onto PVDF membrane (Millipore, Bedford, MA, USA) using a semi-dry electrophoretic transfer system. The PVDF membrane were blocked for 1 h at room temperature with 0.1% Tween-20 in Tris-buffered saline (TBST) containing 10% BSA and then incubated with the primary goal anti-rat primary CIDE-B antibody (diluted 1:300 in TBS) (Santa Cruz, USA) for 12 h at 4°C and continually incubated for 2 h using diluting with HRP labeled goat anti-rabbit second antibody (1:2000) at room temperature. Finally, the film was put into the X-ray film box, exposed 4 min, developed 40 sec, fixated 2 min and washed 5 min successively. After scanning by Bio-Rad-2000 gel-imaging system, the absorbance (A) value was determined by Quantity One software. In the same specimen, the value of β-actin, as an internal parameter was also detected to calibrate the concentration of the target protein. The relative A value was calculated as follows: the relative A value = the A value of CIDE-B / the A value β-actin. The experiment was repeated 3 times and the results were presented with Mean±standard deviation.

RT-PCR: Total of 100 mg hippocampal tissue in ischemic zone as above was collected and homogenized in Trizol reagent (Life Tech, USA) using 1ml of Trizol per 50 mg of tissue. Total RNA was extracted and the RNA was re-suspended in PCR grade water and calculated the abundance by A260/280 nm ultraviolet spectrophotometer (Beckman DU640, USA). The primers of CIDE-B was

designed and synthesed by Shanghai Shenyou Biotech. Co. Ltd. Sense primer: 5'GTG GGA TGT TGT CAT ACG G 3', anti-sense primer: 5'AGT CAG CTT GGT TAC CTA GG 3', the product length was 438 bp. Takara RNA reverse transcription kits (Santa Cruz, USA) was used to reverse transcribe RNA into cDNA. PCR circulation conditions: 95°C initial degeneration 3 min, 94 °C degeneration 30 sec, followed by 30 cycles at 56°C for 30 sec and 72°C 40 sec, extended 3 min at 72°C. The experiment was repeated 3 times with a 2% agarose gel electrophoresis (Sigma, USA) of PCR products and visualized under ultraviolet illumination by ethidium bromide (EB) staining. The A value of each mRNA band in the same gel was captured and determined through imaging analysis and analyzed by SPSS 11.0 software. The relative A value of CIDE-B mRNA was expressed as: the A value of CIDE-B mRNA/the A value of β-actin) and presented with Means±SD.

Statistical analysis: The data was expressed as the Means±SD, determination of statistical significance was carried out with Student's t-test between two groups, a $p<0.05$ was considered to be statistically significant. These analyses were performed using SPSS15.0 statistical software.

RESULTS

Neuronal apoptosis in hippocampus: The number of apoptotic cells was a few in hippocampus in control group and increased after ischemia reperfusion with shrinking of cells, pyknosis of nucleus and irregular shape. Compared with the control group, the number of apoptotic cells increased significantly at ischemia 2 h reperfusion 6, 1, 3, 7 and 14 days, peaked at the reperfusion 7 days and reduced significantly at reperfusion 28 days (Table 1 and Fig. 1).

The expression of CIDE-B in hippocampus: A few CIDE-B positive neurons were observed in hippocampus in control group. The number of CIDE-B positive neurons with hyperchromatic cytoplasm increased gradually after ischemia 2 h consistent with neuronal apoptosis, peaked at the reperfusion 7 d and reduced significantly at reperfusion 28 days (Table 2 and Fig. 2).

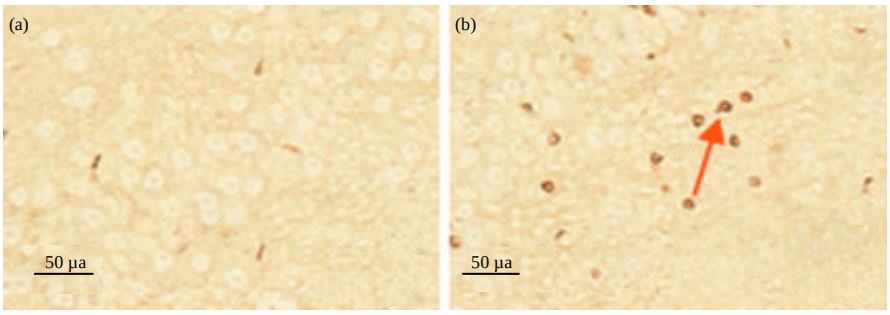

Fig. 1(a-b): Neuronal apoptosis in hippocampus CA3 area detected by TUNEL staining (a) Control group, (b) Ischemia reperfusion 7 days, Arrow. apoptotic cells

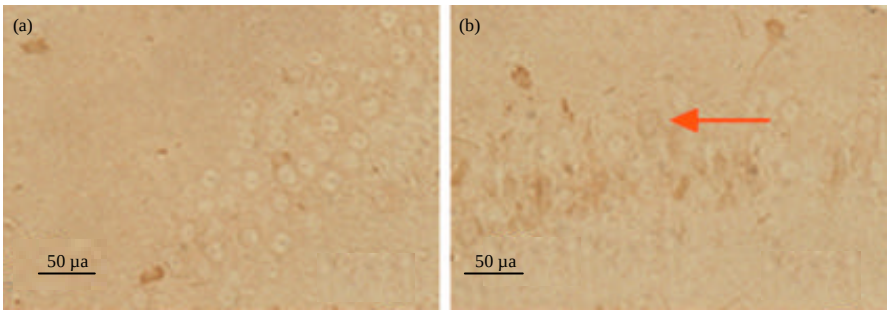

Fig. 2(a-b): The expression of CIDE-B in hippocampus CA3 area detected by immumohistochemistry (a) Control group and (b) Ischemia reperfusion 7 days, Arrow. CIDE-B positive cells

Table 1: TUNEL positive neurons in hippocampus after cerebral ischemia reper3fusion $(\bar{X} \pm S, n = 4)$

Groups	6 h	1 day	3 days	7 days	14 days	28 days
Control group	6.2±1.3	7.0±1.5	7.5±1.4	9.0±1.5	7.3±1.8	6.5±1.2
Model group	12.2±2.6[a]	21.5±4.5[a]	26.2±5.3[a]	33.8±6.2[a]	21.6±4.4[a]	9.6±1.5[ab]

[a]Compared with control group, t = 3.95-9.91, p<0.01; [b]Compared with reperfusion 14 days, t = 9.06, p<0.01

Table 2: The expression of CIDE-B in hippocampus after cerebral ischemia reperfusion $(\bar{X} \pm S, n = 4)$

Groups	6 h	1 day	3 days	7 days	14 days	28 days
Control group	4.2±1.2	5.3±1.3	5.5±1.5	6.5±1.7	5.3±1.5	4.0±1.4
Model group	8.6±2.4[b]	13.6±3.7[b]	19.3±3.3[b]	25.3±5.2[b]	15.5±3.8[b]	8.8±2.0

[a]Compared with control group, t = 4.02-9.32, p<0.01, [b]Compared with reperfusion 14 day, t = 3.82, p<0.01

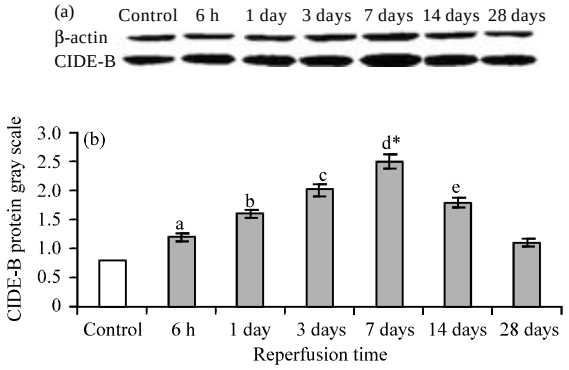

Fig. 3(a-b): The expression of CIDE-B detected by Western blot, compared with control group, a, b, c, d, e p<0.01; Compared with reperfusion 14 days, *p<0.01

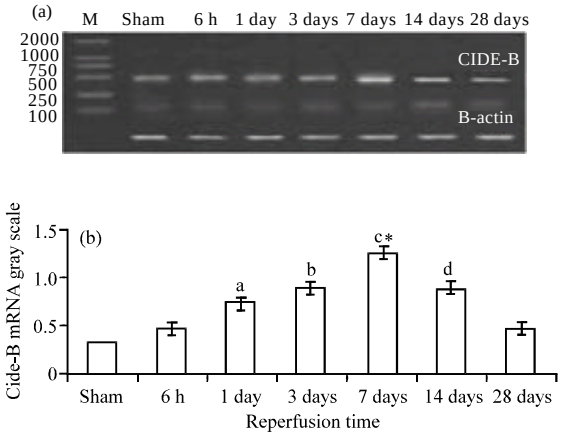

Fig. 4(a-b): The expression of CIDE-B mRNA detected by RT-PCR, compared with control group, a, b, c, d p<0.01; compared with reperfusion 14 days, *p<0.01

The expression of CIDE-B by Western blot: The Western blot results showed that compared with the control group (0.8±0.2), the expression of CIDE-B protein increased significantly in hippocampus after ischemia reperfusion (t = 4.56-7.23, p<0.01), peaked at the reperfusion 7 days(2.5±0.7) and reduced significantly to 1.1±0.3 at the reperfusion 28 days (t = 4.50, p<0.01) (Fig. 3).

Expression of CIDE-B mRNA by RT-PCR: The RT-PCR results indicated that compared with the control group (0.3±0.1), the expression of CIDE-B mRNA increased significantly in hippocampus after ischemia reperfusion (t = 4.35-6.82, p<0.01), reached the highest at the reperfusion 7 d (1.3±0.3) and significantly reduced to 0.4±0.1 at the reperfusion 28d (t = 6.97, p<0.01) (Fig. 4).

DISCUSSION

Acute cerebral ischemic injury involved many mechanisms, such as the production of free radicals (Niatsetskaya *et al*., 2012), intracellular calcium overload (Garcia-Dorado *et al*., 2012), neuronal apoptosis (Blank and Shiloh, 2007), toxicity of excitatory amino acid (Ramanathan *et al*., 2012), mitochondrial damage (Kim *et al*., 2012), the energy reduction and inflammation (Huttemann *et al*., 2012) and so on. There were two apoptotic pathways: externally and internally pathway (Crow *et al*., 2004). Externally pathway was also known as apoptosis receptor pathway which started with death ligends binding with cognate receptor on cell surface. Internally pathway was also called mitochondrial pathway which could be induced by many intracellular and extracellular stimulus. The hippocampus was a vulnerable brain region and extremely sensitive to ischemia and hypoxia (Busl and Greer, 2010). Wang *et al*. (2010) found that apoptosis was a transient intermediate link which usually required only 1 h to a few days from the occurrence of apoptosis to finally apoptotic cells cleared from body. Our experiment showed that neuronal apoptosis in hippocampus began to increase gradually from cerebral ischemia 2 h and reperfusion 6 h and reached the highest level at reperfusion 7 days. At the same time, the neurons in hippocampus degenerated seriously, cell layer arrange disorganized with irregular shape, karyopyknosis and chromatin condensation. After a short plateau, the neuronal apoptosis gradually decreased and reduced significantly to the level of control group at reperfusion 28 days. The results further

confirmed that neuronal apoptosis in hippocampus aggravated with ischemia and reperfusion severity within certain period.

CIDE-B could induce mitochondria to release cytochrome C and activate caspase-9 as well as caspase-3 further (Erdtmann *et al*., 2003). When the neuronal apoptosis induced by cerebral ischemia/reperfusion injury started, the apoptotic related gene could mutate and cause CIDE-B mRNA expressing actively. In this experiment, the results showed that the expression of CIDE-B mRNA and protein increased gradually from cerebral ischemia 2 and reperfusion 6 h-7 days. The increase of CIDE-B mRNA expression was the base of its functional protein translation and expression. During this period, neuronal apoptosis in hippocampus increased significantly and were consistent with the increase of CIDE-B gene and protein expression time window. It is suggested that the neuronal apoptosis and the expression of CIDE-B mRNA and protein were time-dependent and there were inherent links between the neuronal apoptosis and the expression of CIDE-B gene and protein.

CONCLUSION

It is suggested that neuronal apoptosis was timely dependent on the expression of CIDE-B gene after cerebral ischemia reperfusion in rats.

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