Ischemic preconditioning induces chaperone hsp70 expression and inhibits protein aggregation in the CA1 neurons of rats

Peng-Fei GE¹, Tian-Fei LUO², Ji-Zhou ZHANG², Da-Wei CHEN¹, Yong-Xin LUAN¹, Shuang-Lin FU¹

¹Department of Neurosurgery, First Hospital Affiliated to Jilin University, Changchun 130021, China

²Department of Biochemistry, Norman Bethune Medicine School, Jilin University, Changchun 130021, China

Abstract: Objective To investigate the effect of ischemic preconditioning on chaperone hsp70 expression and protein aggregation in the CA1 neurons of rats, and to further explore its potential neuroprotective mechanism. **Methods** Two-vessel-occluded transient global ischemia rat model was used. The rats were divided into sublethal 3-min ischemia group, lethal 10-min ischemia group and ischemic preconditioning group. Neuronal death in the CA1 region was observed by hematoxylin-eosin staining, and number of live neurons was assessed by cell counting under a light microscope. Immunohistochemistry and laser scanning confocal microscopy were used to observe the distribution of chaperone hsp70 in the CA1 neurons. Differential centrifuge was used to isolate cytosol, nucleus and protein aggregates fractions. Western blot was used to analyze the quantitative alterations of protein aggregates and inducible chaperone hsp70 in cellular fractions and in protein aggregates under different ischemic conditions. **Results** Histological examination showed that ischemic preconditioning significantly reduced delayed neuronal death in the hippocampus CA1 region ($P < 0.01$ vs 10-min ischemia group). Sublethal ischemic preconditioning induced chaperone hsp70 expression in the CA1 neurons after 24 h reperfusion following 10-min ischemia. Induced-hsp70 combined with the abnormal proteins produced during the secondary lethal 10-min ischemia and inhibited the formation of cytotoxic protein aggregates ($P < 0.01$ vs 10-min ischemia group). **Conclusion** Ischemic preconditioning induced chaperone hsp70 expression and inhibited protein aggregates formation in the CA1 neurons when suffered secondary lethal ischemia, which may protect neurons from death.

Keywords: ischemic preconditioning; protein aggregation; chaperone; hsp70

1 Introduction

In 1982, Kirino reported delayed neuronal death after transient cerebral ischemia^[1]. The neurons suffered lethal ischemia did not die immediately when ischemia ended, but generally died 3 d later after reperfusion. Again, in 1991, Kirino *et al.* reported that brief episode of ischemia could render the brain more resistant against subsequent longer ischemic events^[2]. This ischemic tolerance has been shown in numerous experimental models of cerebral ischemia. However, the molecular mechanisms of ischemic tolerance are uncertain.

In 2001, Hu *et al.* found that abnormal proteins accumulated in the neurons destined to die after transient cerebral ischemia^[3]. In recent years, scientists discovered that protein aggregates was an important pathological feature of brain ischemia^[4]. Ischemic stress can cause the proteins in neurons unfolded and lose its three dimensional conformation, thus the sticky hydrophobic residues buried in the proteins are exposed and tend to stick to one another, precipitate, and form protein aggregates^[5]. These denatured aggregated proteins cannot function normally but be toxic to cells^[6]. They stick to the intracellular membrane structures and result in cell losing its normal function and dying in the end^[7]. Therefore, the denatured and aggregated proteins should be rescued or eliminated in order to keep cells alive and function normally. Under normal physiological condition, chaperones and ubiquitin-proteasome system are responsible for clearing these abnor-

Corresponding author: Shuang-Lin FU
Tel: 86-431-85612466
E-mail: fu.shuanglin@yahoo.com.cn
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mal proteins from cells^[8]. Chaperones are a functionally related group of proteins that assist protein folding under physiological and stress conditions^[9]. In addition to their role in protein folding, chaperones help to assemble and disassemble protein complexes, to present substrates for degradation, and to suppress protein aggregation^[10,11]. Among the chaperones, hsp70 is a stress-induced protein located in cytoplasm and has the ability to recognize and bind nascent, partially folded and unfolded proteins, thus prevent protein aggregation and facilitate correct protein folding^[12,13].

In the present study, we investigated the influence of ischemic preconditioning on the formation of protein aggregates and on the expression and distribution of protein chaperone hsp70, and the significance of which in the cerebral protection mechanism of ischemic preconditioning was discussed.

2 Materials and methods

2.1 Materials Anti-hsp70 polyclonal antibody was from Cell Signaling Company (Beverly, MA, USA). Anti-ubiquitin monoclonal antibody was from Chemicon Company (Temecula, CA, USA). Propidium iodide and fluorescence-labeled anti-mouse and anti-rabbit IgG were from Jackson ImmunoResearch Laboratory (West Grove, PA, USA). Protein concentration assay kit was from Bio-rad Laboratory (Hercules, CA, USA). ECL Western blot detection reagents were from Amersham Company (Piscataway, NJ, USA). Actin- β and other reagents were from Sigma Company (St. Louis, MO, USA). PVDF membranes were from Millipore Company (Billerica, MA, USA). Light microscope was Olympus BX-51, laser scanning confocal microscope was Zeiss LSM 510, and vibrotome was Leica VT-1000S.

2.2 Experimental animals Adult male Wistar rats (weighing 280–320 g) were supplied by the Experimental Animal Center of Jilin University and housed in plastic cages (2 rats per cage) with soft bedding and free access to food and water under a 12:12 h day/night cycle. The rats were kept for 7 d before the experiments. All the rats were divided into 3-min ischemia group, 10-min ischemia group, and ischemic preconditioning group (rats were subjected to 3-min ischemia followed by 48-h recovery before suffering the secondary 10-min ischemia). Every group included the following subgroups (four rats in each subgroup): sham group, 4-h reperfusion group, 24-h reperfusion group, 48-h reperfusion

group, and 72-h reperfusion group. The sham rats in the ischemia groups only suffered sham operation without ischemia, while the sham rats in the ischemic preconditioning group were subjected to 3-min ischemia followed by 48-h reperfusion before sham surgery. All the experiments were approved by the Animal Care and Use Committee and in accordance with the guidelines for the Care and Use of Experiment Animals.

2.3 Ischemia model Brain ischemia was produced by using the 2-vessel occlusion (2VO) model^[7]. Male Wistar rats were fasted overnight and freely accessed to water. Anesthesia was induced with 5% halothane and maintained with 2% halothane in oxygen/nitrous oxide (30%/70%) gas mixture. Catheters were inserted into the femoral artery and tail artery to allow blood sampling, arterial blood pressure recording and drug infusion. Both of the common carotid arteries were encircled by loose ligatures. At 10 min prior to ischemia induction and 10 min post-ischemia, blood gases were measured and adjusted to $PaO_2 > 90$ mmHg, $PaCO_2$ 35–45 mmHg, and pH 7.35–7.45 by regulating the tidal volume of the respirator. Bipolar electroencephalograph was recorded before, during and after ischemia until rat recovery from anesthesia. Brain ischemia was induced by withdrawing blood via the femoral artery catheter to produce a mean artery blood pressure of 50 mmHg, followed by clamping both carotid arteries for 10 min or 3 min for different groups. Mean artery blood pressure was maintained at 50 mmHg during the ischemic period by withdrawing or infusing blood through the femoral catheter. At the end of the ischemia, the clamps were removed and the withdrawn blood was re-infused. Halothane was discontinued and all wounds were sutured. In all experiments, brain temperature was maintained at 37 °C before, during and after ischemia (15 min of reperfusion). For the ischemic preconditioning group, rats were pretreated with 3-min ischemia and 48-h reperfusion prior to receiving 10-min ischemia.

2.4 Brain tissue fixation After rat was anesthetized, the thorax was opened and the heart was disclosed. Heparin (0.1 mL, 300 IU/kg) was injected into the left ventricle before a catheter was inserted into the main artery via left atrium. Then, PBS was perfused into the vascular system at 4 °C for 3 min, and PBS with 4% paraformaldehyde was perfused at 4 °C for another 3 min. Subsequently, the brain tissue was taken out and put into the PBS fixation solution containing 4% paraformaldehyde at 4 °C. Twelve hours later, the 13- μ m and

50- μ m coronal brain slices were cut by a vibrotome, and the brain slices in similarity were selected for hematoxylin-eosin (HE) staining and immunohistochemistry labeling, respectively.

2.5 HE staining and histological examination The 13- μ m brain slices were mounted on slides and kept in dark room until dried. These dried slices were immersed in distilled water for 1 min prior to dehydration in gradient ethanol solution. Then, they were put into haematoxylin solution for 15 s, washed up by distilled water, immersed in Scott solution for 10 s, and washed again by distilled water. After immersed in Eosin solution for 10 s, these brain slices were rehydrated in gradient ethanol solution again, treated with dimethylbenzene and covered with coverslips. Under 40 $\times$ objective lens of light microscope, three view fields of CA1 region were randomly selected from each slice for live cell counting.

2.6 Immunohistochemical analysis The 50- μ m brain slices were put into 24 wells plate with 500 μ L citrate buffer (pH 6.0) and incubated in a 100 °C water bath for 10 min. After cooling down, the slices were washed with the TBS solution containing 0.2% TX-100 for 10 min and blocked in the TBS solution containing 3% BSA and 0.2% TX-100 for 1 h at room temperature. Then, they were incubated with 1:300 polyclonal anti-hsp70 antibody at 4 °C overnight. After washed up with TBS solution containing 0.1% TX-100 for three times (10 min each) at room temperature, the antibody-labeled brain slices were incubated with 1% BSA containing fluorescence labeled anti-rabbit IgG (1:200) and propidium iodide (1:1 000) for 1 h at room temperature. Again, the brain slices were washed with TBS solution containing 0.1% TX-100 for three times (10 min each), and then they were mounted on slides and dried in dark room. Mounting medium was added on the slides prior to be covered with coverslips for observing by a laser scanning confocal microscope.

2.7 Isolation of cytosolic fractions, nucleus fractions and protein aggregates^[14] After rats were anesthetized, the heads were cut. The brain tissues in the CA1 region were isolated on dry-ice, frozen in liquid nitrogen and then kept in -70 °C refrigerator. After weighting, the tissues were homogenized with Dounce homogenizer in 10 volumes of ice-cold homogenization buffer (1.5 mmol/L Tris base-HCl pH 7.6, 1 mmol/L DTT, 0.25 mol/L sucrose, 1 mmol/L MgCl₂, 1.25 μ g/mL pepstatin A, 10 μ g/mL leupeptin, 2.5 μ g/mL aproptonin, 0.5 mmol/L PMSF, 2.5 mmol/L EDTA, 1 mmol/L EGTA, 0.1 mol/L Na₃VO₄, 50 mmol/L NaF, and 2 mmol/L sodium pyrophosphate).

The homogenate was centrifuged at 800 g for 10 min at 4 °C to obtain the pellets and supernatants. The pellets were suspended in homogenization buffer and sonicated for 5 s for 3 times (nucleus fractions), and the supernatants were further centrifuged at 10 000 g for 20 min at 4 °C to obtain the second supernatants and second pellets. The second supernatants were centrifuged at 165 000 g for 1 h at 4 °C to obtain the final supernatants (cytosolic fraction). The second pellet fractions were suspended in the homogenization buffer containing 1% TX100 and 400 mmol/L KCl, sonicated for 5 s for 3 times, washed on a shaker for 1 h at 4 °C, and then centrifuged at 20 000 g for 10 min at 4 °C to obtain TX-insoluble fractions (protein aggregates). After the protein concentrations were measured, the cytosolic fraction, nucleus fractions and protein aggregates were kept in -20 °C refrigerator.

2.8 Western blot analysis Western blot analysis was conducted with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples for Western blot contained 50 μ g of cytosolic fractions, 40 μ g of nucleus proteins, or 20 μ g of protein aggregates. Four samples (from four different rats) in every experimental group were used for statistical analysis. After electrophoresis, proteins were transferred onto PVDF membranes. The membranes were probed with anti-ubiquitin (1:2 000) or anti-hsp70 (1:1 000) antibody, and then incubated with anti-mouse (1:20 000) or anti-rabbit (1:50 000) secondary antibody for 1 h at room temperature. The blots were developed with ECL detection method, and the optical density of protein band was quantified using Kodak 1D gel analysis software.

2.9 Data analysis Data are presented as mean $\pm$ SD. Differences between groups were analyzed by one-way ANOVA (Student-Newman-Keuls) using the SPSS software package (Version 10.0, Chicago, Illinois, USA). $P < 0.05$ was considered statistically significant.

3 Results

3.1 Ischemic preconditioning decreased delayed neuronal death in the CA1 region Histological examination by HE staining is an effective and easy way to evaluate cell viability, and it was used in this study to evaluate delayed neuronal death after different ischemic conditions. Generally, the dead or dying neurons present polyglonal condensed nucleus and pink cytosol^[15].

Our results showed that no neuron died in the CA1 re-

Fig. 1 HE staining images of CA1 neurons at 72-h reperfusion. Results shown are the representatives of four animals under each condition. A: Sham group (only received sham operation without ischemia); B: 3-min ischemia group, no dead neurons were found under light microscope; C: 10-min ischemia group, almost all the CA1 neurons was dead; D: ischemic preconditioning group, only few CA1 neurons was dead. The dead neurons presented shrunken polygonal nucleus and pink cytosol (yellow arrow). Scale bar, 50 μ m. E: Quantitative analysis showed that ischemic preconditioning significantly reduced cell death. ** $P < 0.01$ vs 10-min ischemia group.

Fig. 2 Confocal images of the distribution of chaperone hsp70 in the CA1 neurons at 48-h reperfusion. Red: nucleus labeled by propidium iodide; green: hsp70. Results shown are representatives of four animals under each condition. A: Sham group (only received sham operation without ischemia), almost no hsp70 were detected. B: 3-min ischemia group, induced chaperone hsp70 distributed relatively homogeneously in the cytosol and nucleus. C: 10-min ischemia group, hsp70 decreased significantly in the cytosol and nucleus, and the protein aggregates containing hsp70 were detected by anti-hsp70 in the cytosol (white arrowhead). D: Ischemic preconditioning group, hsp70 moderately decreased in the cytosol and the nucleus (vs 3-min ischemia), and no protein aggregates containing hsp70 were discerned in the cytosol. Scale bar, 30 μ m.

gion of hippocampus after 3-d reperfusion following 3-min ischemia (Fig. 1), and the neurons presented normal nucleus and cytosol as that in sham group; while at the same time, only $7.44\% \pm 1.65\%$ of the CA1 neurons were alive in the 10-min ischemia group. After 3-min ischemic preconditioning, the percentage of live neurons following the 10-min ischemia increased to $71.66\% \pm 8.94\%$ ($P < 0.01$ vs 10-min ischemia group, Fig. 1). Therefore, many neurons became resistant to the secondary lethal ischemia after 3-min ischemic preconditioning, and 3-min was set as the duration of the ischemic preconditioning in the subsequent experiments.

3.2 Ischemic preconditioning induced chaperone hsp70 expression in the CA1 neurons Hsp70 is an inducible chaperone, many factors such as traumatic injury and thermal injury could induce expression of hsp70 in the brain tissue and optic nerve^[16,17]. Lee *et al.*, by using hsp72 transgenic mouse, proved that over-expression of hsp72 could protect brain tissue against cerebral ischemia^[18]. Therefore, we first investigated whether hsp70 was induced in the CA1 neurons after 3-min sublethal ischemia.

As confocal images and Western blot analysis shown, in the 3-min or 10-min ischemia group, no chaperone hsp70 was observed exist in the neurons of sham subgroup (Fig. 2-4). However, Western blot analysis showed that the chaperone hsp70 was induced in the cytosol at 24 h reperfusion and maintained to 48 h reperfusion ($P < 0.01$ vs the sham, Fig. 3). Hsp70 was also detected to transfer into the nucleus at 24 h and 48 h reperfusion ($P < 0.01$ vs the sham, Fig. 4). It seemed that in both 3-min and 10-min ischemia groups, hsp70 in the nucleus dropped down slightly at 48 h reperfusion compared

with that at 24 h reperfusion, but this dropping-down only had statistical significance in the 10-min ischemia group ($P < 0.05$, Fig. 4). In the ischemic preconditioning group, as Fig. 3 and 4 shown, the high-level hsp70 induced by the previous 3-min preconditioning in the cytosol and nucleus dropped down temporarily at 4 h reperfusion ($P < 0.01$ vs the sham), then the hsp70 level increased at 24 h and 48 h reperfusion ($P < 0.01$ vs 4 h reperfusion). At each time point of reperfusion, the hsp70 levels in the cytosol and nucleus were both much higher than that in the contemporary 10-min ischemia group ($P < 0.05$ or $P < 0.01$, Fig. 3, 4). Moreover, confocal image showed that the distribution of induced chaperone hsp70 in the cytosol and nucleus was relatively homogeneous in the 3-min ischemia group, while remarkably lower in the 10-min ischemia group, but this reduction could be attenuated by 3-min ischemic preconditioning (Fig. 2).

3.3 Ischemic preconditioning depressed protein aggregates formation in the CA1 neurons Protein aggregation is an important pathological feature of cerebral ischemia and neurodegenerative disease. Toxic protein aggregates may directly results in cell death. Moreover, recent reports indicated that hsp70 reduced aggregation of polyalanine expansion mutant of poly(A)-binding protein, nuclear 1 (PABPN1) in Hela cells and reduced aggregation and toxicity of α -synuclein^[19,20]. Therefore, we investigated the effects of ischemic preconditioning on the protein aggregates formation.

After cellular fractions was isolated, the TX-100 insoluble fraction was regarded as protein aggregates^[14]. Anti-ubiquitin antibody was used in Western blot to analyze the formation of protein aggregates in the CA1 neurons. In the 3-min

Fig. 3 Changes of hsp70 expression in the cytosol detected by Western blot analysis. Results shown are mean $\pm$ SD of four animals under each condition. In the 3-min and 10-min ischemia group, hsp70 increased from 24 h reperfusion until 48 h reperfusion compared with the sham group. In the ischemic preconditioning group, the high-level hsp70 induced by previous 3-min preconditioning decreased at 4 h reperfusion, then hsp70 rose at 24 h and 48 h reperfusion compared with that at 4 h reperfusion. At each time point after reperfusion, the hsp70 level was significantly higher in the ischemic preconditioning group than that in the contemporary 10-min ischemia group. * $P < 0.01$ vs corresponding sham group, $^{\#}P < 0.01$ vs corresponding 4 h reperfusion group, $^{\#}P < 0.01$ vs contemporary 10-min ischemia group.

Fig. 4 Changes of hsp70 expression in the nucleus detected by Western blot analysis. Results shown are mean $\pm$ SD of four animals under each condition. In the 3-min and 10-min ischemia group, hsp70 appeared in the nucleus at 24 h and 48 h reperfusion. In the ischemic preconditioning group, hsp70 decreased in the nucleus at 4 h reperfusion, then rose at 24 h and 48 h reperfusion compared with that at 4 h reperfusion. At each time point after reperfusion, the hsp70 level was significantly higher in the ischemic preconditioning group than that in the contemporary 10-min ischemia group. * $P < 0.01$ vs corresponding sham group; § $P < 0.01$ vs corresponding 24 h reperfusion; # $P < 0.05$, ## $P < 0.01$ vs contemporary 10-min ischemia group.

Fig. 5 Protein aggregates formation detected by Western blot analysis using anti-ubiquitin antibody. A: 3-min ischemia group, B: 10-min ischemia group, C: ischemic preconditioning group. S: sham group. D: Quantitative analysis of the bands in Western blot. No protein aggregate formation was detected in 3-min ischemia group. In 10-min ischemia group, protein aggregate formation was detected at different time points of reperfusion. After 3-min ischemic preconditioning, the protein aggregate formation was decreased significantly at each time point of the reperfusion following the 10-min ischemia. * $P < 0.01$ vs contemporary 10-min ischemia group.

ischemia group, almost no protein aggregates formed at any reperfusion time. In the 10-min ischemia group, protein aggregates formed at 4 h reperfusion and maintained to 48 h reperfusion ($P < 0.01$ vs the sham subgroup). However, in the ischemic preconditioning group, the protein aggregates significantly reduced at each time point of reperfusion ($P < 0.01$ vs the contemporary 10-min ischemia group, Fig. 5).

These data suggested that after ischemic preconditioning, the protein aggregation induced by subsequent lethal ischemia was depressed.

3.4 Ischemic preconditioning induced combination of chaperone hsp70 with abnormal proteins in the CA1 neurons In the 10-min ischemia group, protein aggregates formed at 4 h reperfusion, but chaperone hsp70 was induced at 24 h reperfusion. Although confocal images and Western blot analysis showed that hsp70 deposited into protein aggregates as soon as it was induced at 24 h reperfusion and maintained to 48 h reperfusion (Fig. 2, 6), the influence of hsp70 expression on lethal ischemia-induced abnormal proteins was not clear. However, we found some clues in the ischemic preconditioning group.

The results of Western blot analysis showed that in the 3-min ischemia group, hsp70 was hardly found in the protein aggregates. In the 10-min ischemia group, the induced-hsp70 was significantly deposited into protein aggregates at 24 h and 48 h reperfusion ($P < 0.01$ vs the sham, Fig. 6). In the preconditioning group, since chaperone hsp70 had been induced by previous 3-min preconditioning before rats suffered from secondary lethal ischemia (Fig. 3, 4), hsp70 could be observed deposit into protein aggregates at 4 h reperfusion, which was higher than that in the contemporary 10-min ischemia group ($P < 0.01$). However, at 24 h and 48 h

reperfusion, the protein aggregate formation was decreased significantly at each time point of the reperfusion following the 10-min ischemia. * $P < 0.01$ vs contemporary 10-min ischemia group.

Fig. 6 Quantitative alterations of hsp70 in the protein aggregates detected by Western blot analysis. Results shown are mean $\pm$ SD of four animals under each condition. In the 3-min ischemia group, hsp70 was hardly found in the protein aggregates. In the 10-min ischemia group, the induced-hsp70 deposited significantly into protein aggregates at 24 h and 48 h reperfusion. In the ischemic preconditioning group, at reperfusion 4 h, the hsp70 deposited into the protein aggregates was higher than that in the contemporary 10-min ischemia group, due to the existence of hsp70 induced by 3-min preconditioning. However, at 24 h and 48 h reperfusion, the quantity of hsp70 in the protein aggregates was significantly lower than that in the contemporary 10-min ischemia group. * $P < 0.01$ vs contemporary 10-min ischemia group.

reperfusion, the quantity of hsp70 in the protein aggregates in the preconditioning group was significantly lower than that in the contemporary 10-min ischemia group ($P < 0.01$, Fig. 6). Meanwhile, as mentioned above, the hsp70 in the cytosol and nucleus of the ischemic preconditioning group was higher than that in the 10-min ischemia group (Fig. 3, 4).

These data suggested that the preconditioning-induced chaperone hsp70 might combine with the abnormal proteins induced by subsequent lethal ischemia and kept them soluble in the cytosol.

4 Discussion

Induction of stress response (heat shock) proteins (HSPs) is a highly conserved response to protect many cell types from diverse physiological and environmental stressors. After injury, HSPs are believed to facilitate restoration of protein normal function by assistance in refolding of dena-

tured proteins and degrading irreparably damaged proteins and toxic metabolites, limitation of aggregation of damaged peptides, and appropriate folding of newly synthesized essential polypeptides^[21].

In this study, we successfully established ischemic preconditioning rat model. As the histological study showed, no neuron died after 3-d reperfusion following 3-min ischemia, whereas almost 90% of the CA1 neurons died after 3-d reperfusion following 10-min ischemia, which indicates that 10-min ischemia is lethal but 3-min ischemia is not. In the preconditioning group, we found that the quantity of dead neurons decreased significantly: only small part of neurons presented the features of dead neurons such as polygonal shrunken nucleus and pink cytoplasm. Therefore, 3-min ischemic preconditioning may make the CA1 neurons become resistant to subsequent 10-min lethal ischemia and rescue the neurons destined to die from lethal ischemic stress.

Many studies have shown that protein aggregation is one of the most important pathological bases resulting in neuronal dysfunction and even neuronal death, especially in brain ischemia and neurodegenerative disease^[22-24]. Protein aggregates commonly have ubiquitin immunoreactivity, suggesting that the proteins targeted for degradation but failed to be degraded may end up in aggregates^[25]. Therefore, anti-ubiquitin antibody was selected to probe protein aggregates in the present study. The Western blot analysis indicated that following the 3-min ischemic preconditioning, the protein aggregates induced by 10-min ischemia decreased significantly after 4 h, 24 h and 48 h reperfusion. These data suggested that ischemic preconditioning might protect neurons by decreasing protein aggregates formation in the neurons suffered subsequent lethal ischemia.

When proteins denatured, unfolded or misfolded, the hydrophobic segments buried in the protein would be exposed and prone to aggregation. One of the ways to avoid protein aggregation is keeping abnormal proteins soluble by molecular chaperone^[26]. After lethal ischemia, misfolded or unfolded proteins were produced in the neurons and prone to aggregation^[27]. Chaperones could combine with these abnormal proteins and prevent them from sticking together and accumulating in the cells^[28]. Therefore, we investigated the expression of inducible cytoplasmic chaperone hsp70 and its effects on abnormal proteins in the neurons suffered lethal ischemia. Our data showed that hsp70 could be in-

duced in the cytosol and transferred into nucleus after 3-min ischemia or 10-min ischemia. TX-100 insoluble cellular fraction is often regarded as protein aggregate and it was isolated by differential centrifuge. Anti-hsp70 was used in Western blot analysis to detect hsp70 in the protein aggregates. Our results showed that the quantity of hsp70 in the protein aggregates was lower, while the quantity of hsp70 in the cytosol was higher in the preconditioning group than that in the 10-min ischemia group at different times of reperfusion. These data suggested the induced hsp70 may combine with abnormal proteins and kept them in soluble in the cytosol. Therefore, we speculated that under 10-min ischemia condition, the quantity of abnormal protein exceeded the ability of CA1 neurons to maintain them in soluble form or to degrade them, and thus these abnormal proteins formed protein aggregates. In the ischemic preconditioning group, however, the previously induced-hsp70 could combine with the abnormal proteins to make them in stable condition and not to form cytotoxic protein aggregates, which allowed the abnormal proteins be removed by the proteolytic machineries such as ubiquitin proteasome system^[29].

In conclusion, inhibiting protein aggregation and inducing chaperone hsp70 should be important factors underlying cerebral protective mechanism of ischemia preconditioning.

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References:

- [1] Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Res* 1982, 239: 57-69.
- [2] Kirino T, Tsujita Y, Tamura A. Induced tolerance to ischemia in gerbil hippocampal neurons. *J Cerebral Blood Flow Metab* 1991, 11: 299-307.
- [3] Hu BR, Janelidze S, Ginsberg MD, Busto R, Perez-Pinzon M, Sick TJ, *et al.* Protein aggregation after focal brain ischemia and reperfusion. *J Cereb Blood Flow Metab* 2001, 21: 865-875.
- [4] Mengesdorf T, Jensen PH, Mies G, Auenberg C, Paschen W. Down-regulation of parkin protein in transient focal cerebral ischemia: A link between stroke and degenerative disease? *Proc Natl Acad Sci U S A* 2002, 99: 15042-15047.
- [5] Macario AJ, Conway de Macario E. Sick chaperones, cellular stress, and disease. *N Engl J Med* 2005, 353: 1489-1501.
- [6] Stefani M, Dobson CM. Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. *J Mol Med* 2003, 81: 678-699.
- [7] Hu BR, Martone ME, Jones YZ, Liu CL. Protein aggregation after transient cerebral ischemia. *J Neurosci* 2000, 20: 3191-3199.
- [8] Miller J, Gordon C. The regulation of proteasome degradation by multi-ubiquitin chain binding proteins. *FEBS Lett* 2005, 579: 3224-3230.
- [9] Hartl FU. Molecular chaperones in cellular protein folding. *Nature* 1996, 381: 571-579.
- [10] Ohtsuka K, Hata M. Molecular chaperone function of mammalian Hsp70 and Hsp40 — a review. *Int J Hyperthermia* 2000, 16: 231-245.
- [11] Frydman J. Folding of newly translated proteins *in vivo*: the role of molecular chaperones. *Annu Rev Biochem* 2001, 70: 603-647.
- [12] Beissinger M, Buchner J. How chaperones fold proteins. *Biol Chem* 1998, 379: 245-259.
- [13] Fink AL. Chaperone-mediated protein folding. *Physiol Rev* 1999, 79: 425-449.
- [14] Liu CL, Ge PF, Zhang F, Hu BR. Co-translational protein aggregation after transient cerebral ischemia. *Neuroscience* 2005, 134: 1273-1284.
- [15] Ge P, Luo Y, Liu CL, Hu B. Protein aggregation and proteasome dysfunction after brain ischemia. *Stroke* 2007, 38: 3230-3236.
- [16] Truettner JS, Hu B, Alonso OF, Bramlett HM, Kokame K, Dietrich WD. Subcellular stress response after traumatic brain injury. *J Neurotrauma* 2007, 24: 599-612.
- [17] Kim JM, Park KH, Kim YJ, Park HJ, Kim DM. Thermal injury induces heat shock protein in the optic nerve head *in vivo*. *Invest Ophthalmol Vis Sci* 2006, 47: 4888-4894.
- [18] Lee WC, Wen HC, Chang CP, Chen MY, Lin MT. Heat shock protein 72 overexpression protects against hyperthermia, circulatory shock, and cerebral ischemia during heatstroke. *J Appl Physiol* 2006, 100: 2073-2082.
- [19] Wang Q, Mosser DD, Bag J. Induction of HSP70 expression and recruitment of HSC70 and HSP70 in the nucleus reduce aggregation of a polyalanine expansion mutant of PABPN1 in HeLa cells. *Hum Mol Genet* 2005, 14: 3673-3684.
- [20] Klucken J, Shin Y, Masliah E, Hyman BT, McLean PJ. Hsp70 reduces α -synuclein aggregation and toxicity. *J Biol Chem* 2004, 279: 25497-25502.
- [21] Kelly KJ. Heat shock (stress response) proteins and renal ischemia/reperfusion injury. *Contrib Nephrol* 2005, 148: 86-106.
- [22] Uversky VN. Neuropathology, biochemistry, and biophysics of α -synuclein aggregation. *J Neurochem* 2007, 103: 17-37.
- [23] Hasegawa M. Biochemistry and molecular biology of tauopathies. *Neuropathology* 2006, 26: 484-490.
- [24] Schindowski K, Bretteville A, Leroy K, Bégard S, Brion JP,

- Hamdane M, *et al.* Alzheimer's disease-like tau neuropathology leads to memory deficits and loss of functional synapses in a novel mutated tau transgenic mouse without any motor deficits. *Am J Pathol* 2006, 169: 599-616.
- [25] Liu CL, Martone ME, Hu BR. Protein ubiquitination in post-synaptic densities after transient cerebral ischemia. *J Cereb Blood Flow Metab* 2004, 24:1219-1225.
- [26] Giffard RG, Xu L, Zhao H, Carrico W, Ouyang Y, Qiao Y, *et al.* Chaperones, protein aggregation, and brain protection from hypoxic/ischemic injury. *J Exp Biol* 2004, 207 (Pt 18): 3213-3220.
- [27] DeGracia DJ, Rudolph J, Roberts GG, Rafols JA, Wang J. Convergence of stress granules and protein aggregates in hippocampal cornu ammonis 1 at later reperfusion following global brain ischemia. *Neuroscience* 2007, 146: 562-572.
- [28] Houry WA. Chaperone-assisted protein folding in the cell cytoplasm. *Curr Protein Pept Sci* 2001, 2: 227-244.
- [29] Esser C, Alberti S, Hahfeld J. Cooperation of molecular chaperones with the ubiquitin/proteasome system. *Biochim Biophys Acta* 2004, 1695: 171-188.

预缺血诱导大鼠 CA1 神经元中蛋白伴侣 hsp70 的表达并抑制蛋白聚集物的形成

葛鹏飞¹, 罗天飞², 张纪周², 陈大伟¹, 栾永新¹, 付双林¹

¹ 吉林大学附属第一医院神经外科, 长春 130021

² 吉林大学白求恩医学院生物化学系, 长春 130021

摘要: **目的** 研究预缺血对蛋白伴侣 hsp70 表达和蛋白聚集物形成的影响, 探讨其可能的脑保护机制。 **方法** 采用大鼠双侧颈总动脉暂时夹闭法建立全脑缺血模型。大鼠分为 3 min 缺血组, 10 min 缺血组以及预缺血组。苏木素-伊红染色, 光镜下随机计数分析预缺血后海马 CA1 区死亡神经元数量变化。免疫组织化学及激光扫描共聚焦显微镜法观察蛋白伴侣 hsp70 在 CA1 区神经元内的分布。差速离心分离细胞浆、细胞核及蛋白聚集物。蛋白印迹法检测不同缺血状态下海马 CA1 神经元内蛋白聚集物含量的变化, 以及胞浆、胞核及蛋白聚集物内蛋白伴侣 hsp70 含量的变化。 **结果** 组织学检查显示预缺血能够显著减少海马 CA1 区神经元死亡数量。预缺血诱导海马 CA1 区神经元内蛋白伴侣 hsp70 在再灌注后 24 h 表达。预缺血处理后, 海马 CA1 区神经元内蛋白聚集物显著减少。预缺血诱导的蛋白伴侣 hsp70 与再缺血形成的异常蛋白结合在一起并防止其聚集。 **结论** 预缺血可能通过诱导蛋白伴侣 hsp70 的表达和抑制再缺血后蛋白聚集物的形成, 减少再缺血引起的神经元死亡。

关键词: 预缺血; 蛋白聚集; 蛋白伴侣; hsp70