Time-course of μ-calpain activation, c-Fos, c-Jun, HSP70 and HSP27 expression in neonatal hypoxic-ischemic rat brain

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***Background*: The perinatal brain shows both an increased tolerance to hypoxic ischemic injury and a faster and more complete recovery than the adult one. It is, therefore, important to understand the sequence of events following hypoxia and ischemia in young animals. This study aims to clarify the time-course of μ-calpain activation, and expressions of c-fos protein (c-Fos), c-jun protein (c-Jun), HSP70 and HSP27 during hypoxic-ischemic brain injury (HIBI) in neonatal rats.**

***Methods*: The model of HIBI was made in 7-day-old SD rats by left carotid arterial ligation and hypoxia (8% oxygen). The protein concentration was determined using a modified Bradford assay. μ-calpain activation, and expressions of c-Fos, c-Jun, HSP70 and HSP27 were observed by Western blot in cortical and hippocampal samples at 0, 1, 2, 4, 12 and 24 hours after the development of lesion.**

***Results*: The cleavage of cytosolic μ-calpain was demonstrated in both cortical and hippocampal samples in neonatal rats after hypoxic-ischemia (HI). The ratio of 76/80 kD of μ-calpain was increased significantly after HI and reached a maximum at 24 hours after HI. Compared with that observed in the control group, the expression of nuclear c-Fos and c-Jun in cortical and hippocampal samples increased significantly at 1, 2, 4, 12 and 24 hours after HI ($P<0.05$). But significant expressions of cytosolic HSP70 and HSP27 could only be seen at 12 or 24 hours after HI ($P<0.05$). The significant differences between the cerebral cortex and the hippocampus were observed in c-Fos expression at 2 and 4 hours, and in HSP70 and HSP27 expressions at 24 hours after HI ($P<0.05$).**

***Conclusion*: The early activation of μ-calpain and increased expressions of c-Fos, c-Jun, HSP27 or HSP70 following HI may contribute to neuronal apoptosis as well as induction of a significant brain neuroprotection in neonatal hypoxic-ischemic rat brain.**

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***Key words*: cerebral anoxia; cerebral ischemia; μ-calpain; immediate-early genes; heat shock proteins**

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Introduction

The cascade of physiological events underlying hypoxic-ischemic brain injury (HIBI) remains to be fully established. Previous studies have supported the involvement of a sequence of biological events requiring activation of various signaling pathways as well as changes in gene expression.[1-5] One of the early events following hypoxic-ischemic cellular injury is the preferential synthesis of the inducible heat shock proteins (HSPs), such as HSP70 and HSP27. However, hypoxic-ischemia (HI)-induced synthesis of HSPs is often preceded by a rapid and transient activation of a number of immediate-early genes (IEGs) and their proteins, including c-fos, c-jun, c-fos protein (c-Fos) and c-jun protein (c-Jun). The functional significance of either HSPs or AP-1 complex protein expression is unclear, but it is evident that they may be linked to HI events that will either promote cellular recovery or lead to neuronal death and apoptosis.[6-9] Calpains, existing as a beterodimer composed of a small regulatory subunit and 1 of the 3 catalytic subunits designated μ-calpain, m-calpain and calpain p94, are a family of calcium activated neutral cysteine proteases that perform important intracellular signalling functions. Recent studies have shown that μ-calpain plays critical roles in mediating apoptosis, necrosis, as well as delayed cytotoxicity resulting from hypoxia and ischemia.[10,11]

The perinatal brain shows both an increased tolerance to HI injury and a faster and more complete recovery than the adult one. It is, therefore, important to un-

derstand the sequence of events following hypoxia and ischemia in young animals. In the present study, we examined the activation of μ-calpain and the expressions of c-Fos, c-Jun, HSP70 and HSP27 following severe HI (2h hypoxia) in order to determine whether these protein activations/expressions have similar time-course and anatomical distributions and how their inter-relationship will further relate to cell necrosis or apoptosis.

Methods

Animals and experiment protocols

Rats were obtained from the Experimental Animal Center of Zhejiang Academy of Medical Sciences, Hangzhou, China. The procedures involving the use of animals and their care were conducted according to the institutional guidelines that are consistent with international laws and policies (NIH Guidelines for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

A standardized animal model of HIBI used in the study, as a modification described by Rice,[12] was created by unilateral occlusion of the left carotid artery, followed by exposure to hypoxia in 7-day-old rats. In brief, after the rats were anesthetized with ether inhalation, the left carotid artery was isolated, doubly ligated, and transected. After recovery in an incubator (37℃, 2 hours), the rats were exposed to a gas mixture with 8% O_2 and 92% N_2 for 2 hours in a glass chamber (35 ± 1 ℃) equipped with an inlet and an outlet for gas at a flow rate of 5 L/min.

Forty-two postnatal 7-day-old Sprague-Dawley rats of either sex, weighing 11-12 g, were divided randomly into 7 groups (6 rats in each group; one control group and 6 time-point groups) and then exposed to HI insult except for the control group. The rats in the control group did not receive any procedure. Samples were collected in the 6 time-point groups, at 0, 1, 2, 4, 12 and 24 hours after HI. The rats in the control group were sacrificed at the same time as those in the 24 hours time-point group. The brains of the rats were removed quickly, and the ischemic cerebral cortex and hippocampus were carefully isolated within 2 minutes on ice, immediately frozen in liquid nitrogen, and then stored at −80℃ until assay.

Preparation of nuclear and cytosolic extracts

The brain samples from rats were mechanically homogenized in lytic buffer on ice. Lysis was prepared in cytosolic buffer containing 10 mmol HEPES (pH 7.9), 40 mmol KCl, 3 mmol $MgCl_2$, 1 mmol DTT, 5% glycerol, 0.2% nonidet P40, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mmol PMSF for 10 minutes. Centrifugations were done at 14 000 r/min for 20 seconds in a microcentrifuge (Sigma) to remove the nuclei, which were then lysed in hypertonic buffer containing 20 mmol HEPES (pH 7.9), 420 mmol KCl, 1.5 mmol $MgCl_2$, 0.2 mmol EDTA, 0.5 mmol DTT, 25% glycerol, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.5 mmol PMSF for 30 minutes on ice. Then centrifugations were performed for another 15 minutes at 14 000 r/min at 4℃ to remove the insoluble fractions. Cytosolic and nuclear extracts were frozen and stored at −80℃. Protein was determined using a modified Bradford assay (Sigma). Samples were normalized to an equal protein concentration.

Western blot analysis

Total proteins (50 μg) were resuspended to 2 × loading buffer, heated for 5 minutes at 95°C and separated using an electrophoresis system from Bio-Rad on 10% (HSP27 on 12%) polyacrylamide gel with 4% stacking gel. After the gel was soaked for 30 minutes in transfer buffer (25 mmol Tris, 190 mmol glycine, 20% methanol), the proteins were transferred to 0.45 mm nitrocellulose for 1 hour at 0.08 mA using the Bio-Rad mini trans blot cell model. The blots were incubated overnight at 4℃ with rabbit polyclonal antibodies against c-Fos (1:1000), c-Jun (1:1000) and HSP27 (1:1000), goat polyclonal antibodies against μ-calpain (1:1000), and mouse monoclonal antibodies against HSP70 (1:1000). All antibodies were purchased from Santa Cruz (Beijing Zhongshan Biotechnology, China). The antigen-antibody complex was visualized on an X-ray film using secondary antibodies linked to horseradish peroxidase (1:2000) and an ECL Western blotting chemiluminescent analysis system. Control experiments without primary antibody were negative. The optical density of the film was quantified by the free software (NIH2.0, Scion Image), and calculated as the value of integrated density (ID).[13]

Statistical analysis

The data were expressed as mean ± SD. Statistical analysis was performed using nonparametric tests (Wilcoxon's testing). The relationships between the ratio of 76/80 kD of μ-calpain and ID of other proteins were analyzed by multiple linear regression analysis (stepwise method). *P* values ≤0.05 were considered statistically significant.

Results

Assessment of μ-calpain activations in cortical and hippocampal samples from neonatal rats after HI

The cleavage of cytosolic μ-calpain as a hallmark for its activation after HI was observed in both the cerebral

cortex and hippocampus of neonatal rats (Fig. 1). The ratio of 76/80 kD of μ-calpain was increased significantly after HI and reached a maximum level at 24 hours after HI particularly in the hippocampal samples.

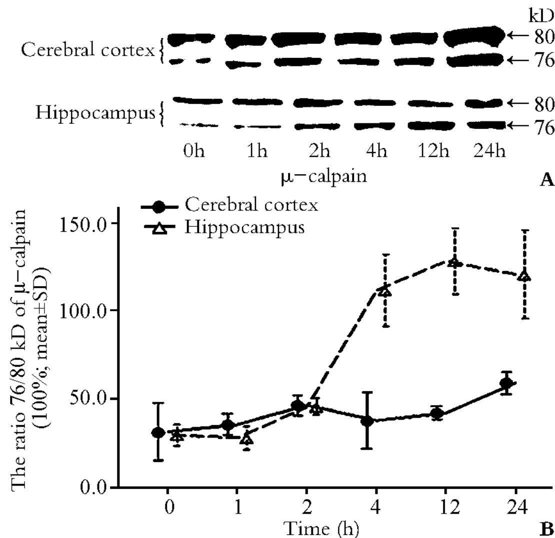

Fig. 1. Cleavage of μ-calpain in the cerebral cortex and the hippocampus in neonatal rats at different time points after hypoxic-ischemia (HI). **A**: Representative Western blots from the cerebral cortex (top) and hippocampus (bottom) protein extracts probed with antibodies against μ-calpain (upper rows = 80 kD; lower rows = 76 kD). **B**: The ratio of 76/80 kD semiquantitative densitometry of μ-calpain from cortical and hippocampal samples at different time interval after HI insults (data shown as mean ± SD, $P \leq 0.05$; $n = 6$ per group; ID = integrated density).

Assessment of c-Fos and c-Jun expressions in cortical and hippocampal samples from neonatal rats after HI

In the second series of experiments, the up-expressions of nuclear c-Fos and c-Jun were observed from both cortical and hippocampal samples of the ischemic sides of the brain after HI. c-Fos expression in the cerebral cortex of neonatal rat peaked at 2 hours and remained elevated at 4, 12 and 24 hours after HI. In the hippocampus, c-Fos was markedly elevated at 1 hour, peaked at 2 hours, decreased at 4 and 12 hours and approached the levels seen at 0 at 24 hours after the appearance of the lesion. Compared with the control group, the expression of c-Fos increased significantly at 1, 2, 4, 12 and 24 hours after HI in both cortical and hippocampal samples ($P < 0.05$). c-Jun expression increased at 0 and 1 hour, peaked at 2 hours and remained at a high level until 24 hours after HI in the cerebral cortex and the hippocampus, demonstrating a significant difference at 0, 1, 2, 4, 12 and 24 hours after HI ($P < 0.05$), as compared with that observed in the normal control group. The significant differences in c-Fos expression between the cerebral cortex and the hippocampus were observed at 2 and 4 hours after HI ($P < 0.05$) (Fig. 2).

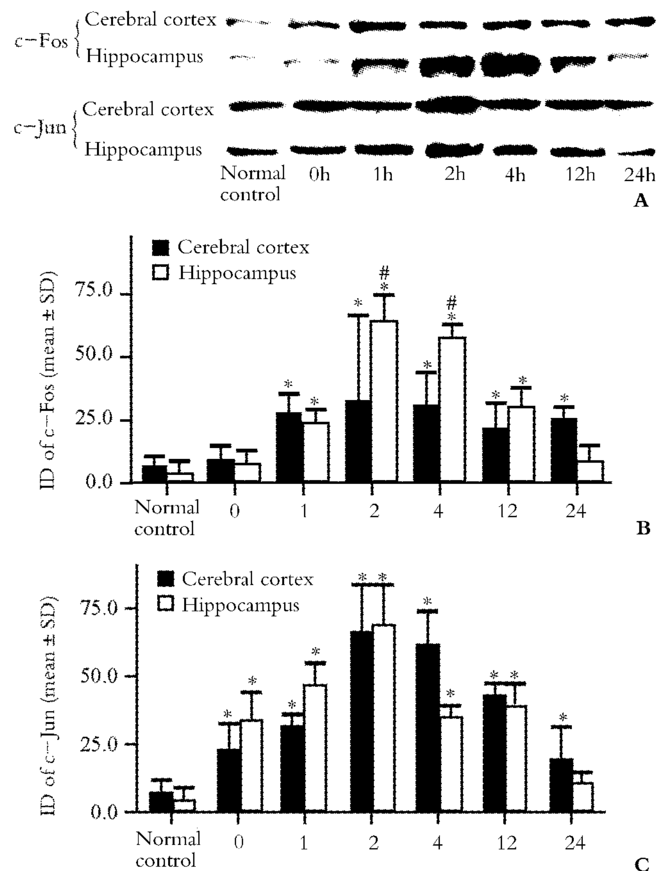

Fig. 2. Changes of c-Fos and c-Jun expressions from cortical and hippocampal samples in neonatal rats after hypoxic-ischemia (HI). **A**: Representative Western blots from the cerebral cortex (upper rows) and the hippocampus (lower rows) protein extracts probed with antibodies against c-Fos (top) and against c-Jun (bottom) at different time interval post HI insults. **B**: Semiquantitative densitometry data of c-Fos expression. c-Fos expression in the neonatal rat cerebral cortex peaked at 2 hours and remained elevated at 4, 12 and 24 hours after HI. In the hippocampus, c-Fos was dramatically elevated at 1 hour, peaked at 2 hours, decreased at 4 and 12 hours and approached the levels seen at 0 and at 24 hours after the lesion. **C**: Semiquantitative densitometry data of c-Jun expression. c-Jun expression increased at 0 and 1 hour, peaked at 2 hours and remained at a high level until 24 hours after HI in both cerebral cortex and hippocampus. *: $P < 0.05$ vs normal control group; #: $P < 0.05$ vs cerebral cortex at the same time-point after HI (data shown as mean ± SD, $P \leq 0.05$; $n = 6$ per group; ID = integrated density).

Assessment of HSP70 and HSP27 expressions in cortical and hippocampal samples from neonatal rats after HI

HSP70 expression in the cerebral cortex of neonatal rat was markedly elevated at 0, 1, 2, 4 and 12 hours, and peaked at 24 hours after the appearance of the lesion. In the hippocampus, HSP70 expression peaked at 1 hour and remained elevated at 2, 4, 12 and 24 hours after HI. Compared with that observed in the control group, a significant difference in its expression was observed at 12 and 24 hours after HI in both cortical and hippocampal samples in neonatal rats ($P < 0.05$). However, a relatively later up-expression of HSP27 was observed.

HSP27 expression peaked at 24 hours after HI in the cerebral cortex and the hippocampus. Compared with that observed in the control group, significant differences in HSP27 expression could only be seen at 24 hours in the cerebral cortex ($P<0.05$), as well as 12 ($P<0.05$) or 24 hours ($P<0.01$) in the hippocampus after HI. The significant differences in HSP70 and HSP27 expressions between the cerebral cortex and the hippocampus were demonstrated ($P<0.05$) at 24 hours after HI (Fig. 3). HSP70 expression was evident in the cerebral cortex in contrast to HSP27 expression in the hippocampus.

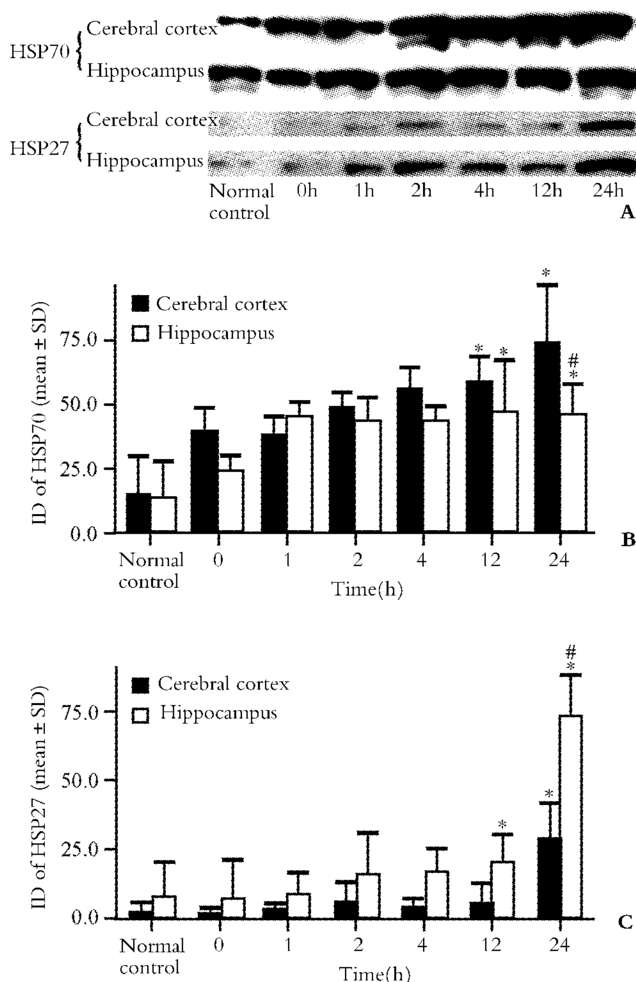

Fig. 3. Changes of HSP70 and HSP27 expressions from cortical and hippocampal samples in neonatal rats after hypoxic-ischemia (HI). **A**: Representative Western blots from the cerebral cortex (upper rows) and the hippocampus (lower rows) protein extracts probed with antibodies against HSP70 (top) and against HSP27 (bottom) at different time interval post HI insults. **B**: Semiquantitative densitometry data of HSP70 expression. HSP70 expression in the neonatal rat cerebral cortex was dramatically elevated at 0, 1, 2, 4 and 12 hours, and peaked at 24 hours after the lesion. In the hippocampus, HSP70 expression peaked at 1 hour and remained elevated at 2, 4, 12 and 24 hours after HI. **C**: Semiquantitative densitometry data of HSP27 expression. A relatively latter up-expression of HSP27 was observed. HSP27 expression peaked at 24 hours after HI in both the cerebral cortex and the hippocampus. *: $P<0.05$ vs normal control group; #: $P<0.05$ vs cerebral cortex at the same time-point after HI (data shown as mean ± SD, $P\leqslant0.05$; $n=6$ per group; ID = integrated density).

Statistical analysis of correlation between μ-calpain activations and altered expressions of c-Fos, c-Jun, HSP70 and HSP27 after HI

With the ratio of 76/80 kD of μ-calpain from cortical samples as the dependent variable Y_1 and the ID values of c-Fos, c-Jun, HSP70 and HSP27 from cortical samples as variables X_1, X_2, X_3 and X_4 respectively, HSP27 and HSP70 were stepped into the multiple linear regression model ($F=26.4$, $P<0.01$). Similarly, taking the ratio of 76/80 kD of μ-calpain from hippocampal samples as the dependent variable Y_1 and the ID values of c-Jun, HSP70 and HSP27 from hippocampal samples as the variables X_1, X_2, X_3 and X_4 respectively, HSP27 was stepped into the multiple linear regression model ($F=16.0$, $P<0.01$).

Discussion

It is commonly accepted that a sustained rise of intracellular Ca^{2+} level is pivotal in cell death. The elevation of the intracellular Ca^{2+} level served as a signal to apoptosis in neurons by activation of Ca^{2+}-dependent enzymes. Several enzymes including lipases and proteases are activated by Ca^{2+}. Among proteases, calpains, a family of the most important Ca^{2+}-dependent enzymes activated by elevated intracellular Ca^{2+} levels, can induce proteolytic modifications in a number of proteins associated with multiple signaling cascades for mediating apoptosis.

The findings of this investigation demonstrated that there was a cleavage of cytosolic μ-calpain after HI from both cortical and hippocampal samples in neonatal rats; and that the ratio of 76/80 kD of μ-calpain was increased significantly after HI and reached a maximum at 24 hours after HI. Our results indicate that calpains may play a key role in the pathogenesis of HIBI in neonatal rats. Furthermore, the distribution of its activated ratio corresponded to the distinct pathologic characteristics of the brain region (cerebral cortex vs hippocampus) as shown in the present study that the ratio of 76/80 kD of μ-calpain persisted at a higher level from hippocampal samples than that from cortical samples, which may contribute to interpreting why the hippocampus is more sensitive and more vulnerable to HI insult than the cerebral cortex. Finally, the time-course in calpain activation that was consistent with the expression of signaling intermediates was observed during HIBI, suggesting that neuronal injury induced by HI insults appears to involve many ongoing and simultaneous mechanisms.

Recent studies have shown that translocation of calpains to the cell nucleus may play a role in apopto-

sis. In this regard, Kubbutat and Vousden[14] observed calpain activity in the nuclear fraction, and Mellgren[15] reported that μ-calpain, not m-calpain, was transported into nuclei in an ATP-dependent fashion. It is supposed that calpains translocated into the nucleus under hypoxic-ischemic conditions result in interaction with and regulation of, for instance, the p53 molecule that plays an essential role in enhancing apoptotic cell death. It is possible that the nuclear translocation of calpains may lead to the activation of unidentified DNAses that consequently could be responsible for DNA degradation. In the present study, cytosolic calpain activations were preceded by up-regulations of nuclear c-Fos and c-Jun. Obviously, further study is needed to clarify the time-course of nuclear μ-calpain activation after HI and whether its translocation into the nucleus under hypoxic-ischemic conditions would result in a latter nuclear c-Fos and c-Jun up-regulation. Although we have focused generally on the role of calpain activation in neural cell demise that occurs after cerebral HI, calpains seem to be play-ing central roles in apoptosis.

HI injury induces elevated expressions of HSPs in the brain. HSPs contribute to the cellular repair by refolding denatured proteins and acting as molecular chaperones in normal processes such as protein translocation and folding. The highly inducible member of the 70-kD family of HSPs, HSP70, has been associated with the self-neuroprotective mechanism from HI injury in the brain. Other HSPs also have been implicated in cellular resistance to injury. The 27-kD HSP (HSP27) has been shown to increase cellular resistance to HI injury.[16]

Generally, the distribution of the elevated neuronal HSP70 immunoreactivity was correlated negatively with that of death-neuron density in neonatal hypoxic-ischemic rat brain. In this study, during the critical period of HIBI (24 hours after HI), an up-regulation of HSP70 was observed from both cortical and hippocampal samples. These findings were consistent with the mRNA expression data.[16] Such HSP70 expression has been used as an indicator of cellular stress; but expression of HSP70 does not indicate whether neurons will die or survive following cerebral HI.[17] Although HI induced the transient expression of HSP70 in neurons, it seemed to produce a relatively later remarkable synthesis of HSP27 as indicated by the present results that were consistent with those of using immunohistochemistry for HSP27. This latter remarkable HSP27 increase may be associated with remodeling and plasticity/recovery of the brain after stroke.[18] In addition to its particular sensitivity to the insulting stimulation, the brain and characteristic distribution patterns within and outside the occluded cerebral artery territory suggest that this protein is regulated differently following HI as compared to HSP70.[19]

The neuronal injury induced by HI insult appears to involve many ongoing and simultaneous mechanisms. In order to identify the most important contributing factor(s) in HIBI, we need to understand more about changes in gene expression after HI. The development of tools such as antisense oligonucleotides, viral transfection vectors, appropriate transgenic animals, and/or selective blocking antibodies, which can interfere with these differentially expressed genes and gene products, will make the suppression of programmed cell death pathways or enhancement of protective mechanisms possible.

In conclusion, μ-calpain is activated over the course of cerebral HI, and the distribution of its activation ratio corresponds to the pathologic state of the brain region. Furthermore, after HI insults the brain induces a significant neuroprotection with an increased HSP70 expression from both cortical and hippocampal samples and a relatively later remarkable HSP27 expression in the hippocampus. The altered expression of transcription factors such as c-Fos and c-Jun that occur immediately after HI may contribute to neuronal apoptosis.

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