

Original Article

Role of GRP78, CHOP and TRB3 in cerebral ischemia-reperfusion injury in rats

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Abstract: Objective: This study aims to observe the expression level changes of endoplasmic reticulum stress-related molecules GRP78, CHOP and TRB3 in rat brains after cerebral ischemia-reperfusion injury (CIRI), and investigate its roles in CIRI. Methods: Male SD rats were randomly divided into three groups: normal group, sham operation group and CIRI group. The CIRI group was further divided into four groups: IR-2h, IR-6h, IR-24h and IR-48h groups. GRP78, CHOP and TRB3 protein and mRNA expressions in brain tissues were detected by western blot and real-time PCR, respectively; and brain cells apoptosis was detected by the TUNEL method. Results: At the 6th, 24th and 48th hour of ischemia reperfusion, the protein and mRNA expression of GRP78, CHOP and TRB3 in brain tissues significantly increased compared with in the expression in the normal and sham operation groups ($P<0.01$). Conclusion: Endoplasmic reticulum stress may promote brain cell apoptosis by regulating the protein expression of GRP78 downstream signaling molecules CHOP and TRB3, and participate in the occurrence and development of CIRI.

Keywords: Cerebral ischemia-reperfusion injury, GRP78, CHOP TRB3, apoptosis

Introduction

With the improvement of the standard of living of people, changes in life style and the increase of population aging, the incidence of cerebrovascular diseases has significantly increased; among which, ischemic cerebrovascular disease accounts for 60-80% of these diseases [1]. After cerebral ischemia, some of the blood vessels spontaneously recanalize, and some are recanalized after thrombolysis, cell damage further aggravates, and cerebral ischemia reperfusion injury (CIRI) occurs [2]. In recent years, due to the findings in some studies, the role of endoplasmic reticulum stress (ERS) in CIRI has received more and more attention [3-7]. In this study, the role of ERS in CIRI was investigated in the animal model of rats.

Materials and methods

Main reagents

GRP78 polyclonal antibody and the TUNEL apoptosis detection kit (Wuhan Boster Biological Technology, Ltd.); CHOP first antibody and β -actin first antibody (Cell Signaling, USA); TRB3 first antibody (Beijing Bioss); First strand cDNA

synthesis kit and total RNA extraction kit (Fermentas, USA). Primers were designed and synthesized by Dalian Takara Biotech. The sequences were as follows: GRP78, F: 5'-TGG AAT CTT CAC CTC AGA GTG-3'; R: 5'-ATA TCC AAG GTG AAC ACA CAC-3'; CHOP, F: 5'-CCT CGC TCT CCA GAT TCC A-3'; R: 5'-CTC ATT CTC CTG CTC CTT CTC C-3'; TRB3, F: 5'-TCA AGT TGC GTC GAT TTG TCT TC-3'; R: 5'-CAG TCA TCA CAC AGG CAT CCTC-3'; β -actin, F: 5'-GGA AGC TTG TCA TCA ATG G-3'; R: 5'-CTG TGG TCA TGA GTC CTT C-3'.

Main instruments

Real-time PCR machine (Bio-Rad, USA); Olympus optical microscope and image acquisition system (Olympus, Japan).

Establishment of animal models and specimen acquisition

Forty healthy male Sprague-Dawley (SD) rats (provided by the experimental animal center of Guizhou Medical University) were used in this study. These rats had a body weight of 200 ± 25 g. Rats were randomly assigned into the following groups: normal group (n=5), sham operation group (n=5), and CIRI group (n=30). The CIRI

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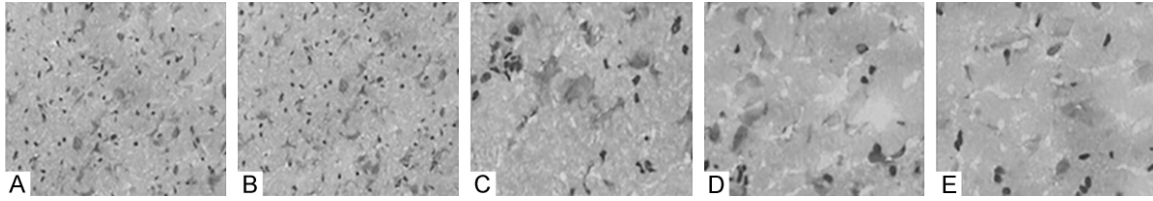


Figure 1. HE staining results of rats brain (A, B: $\times 100$; C-E: $\times 200$). A: Control group; B: Sham operation groups; C: IR 6 h group; D: IR 24 h group; E: IR 48 h group.

Table 1. Cell apoptosis rate in different groups ($\bar{x} \pm s$)

Group	Number	Apoptosis Rate (%)
Control	5	2.96 \pm 0.61
Sham-operation	5	3.04 \pm 0.87
I/R 6 h	9	21.63 \pm 8.95*
I/R 24 h	9	40.85 \pm 16.83* [#]
I/R 48 h	8	28.51 \pm 10.30*

Note: compared with control and sham-operation group, * $P < 0.01$; compared with IR 48 h, [#] $P < 0.01$.

group was further divided into three groups: IR-6h, IR-24h and IR-48h groups ($n=10$, each group). The CIRI model of rats was established by referring to the modified Longa suture method [8]. During the establishment process of the model, one rat died in the IR-6h group, one rat died in the IR-24h group, and two rats died in the IR-8h group. Brain tissues between the optic decussation and pituitary stalk on the left cerebral hemisphere were obtained from each of the rats, and were quickly placed in liquid nitrogen for cryopreservation.

Staining observation

Brain tissues were fixed in 10% formalin, embedded with paraffin, sliced, stained with hematoxylin-eosin (H&E), and observed under light microscopy.

Cell apoptosis detection

Operation procedures were based on the instructions of the reagents.

GRP78 CHOP and TRB3 protein expression levels were analyzed by western blot.

GRP78 CHOP and TRB3 mRNA expressions were detected by real-time PCR.

Statistical analysis

Data were analyzed using SPSS 17.0 software. Measurement data were expressed as mean \pm

standard deviation ($\bar{x} \pm SD$). Inter-group comparisons were conducted using univariate analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Staining of rat brain tissues

As shown in **Figure 1**, in the normal and sham operation groups, rats had complete brain tissue structures, complete nuclear membranes and clear nucleolus. With the extension of ischemia reperfusion time, a large number of foci with liquefaction necrosis and cystic lesions appeared.

Cell apoptosis rate

As shown in **Table 1**, compared with the normal group and sham operation group, the apoptosis rate in each IR group significantly increased ($P < 0.01$), and this reached its peak at the 24th hour of ischemia reperfusion.

Western blot results

As shown in **Figure 2**, there was a low expression of GRP78 protein in brain tissues in rats in the normal group and sham operation group; while in each IR group, GRP78 protein expression was significantly increased compared with the normal and sham operation groups ($P < 0.01$), which reached a peak at the 6th hour of ischemia reperfusion.

As shown in **Figure 3**, there was a low expression of CHOP protein in brain tissues of rats in the normal group and sham operation group; while in each IR group, CHOP protein expression significantly increased compared with the normal group and sham operation group ($P < 0.01$), which reached a peak at the 24th hour of ischemia reperfusion.

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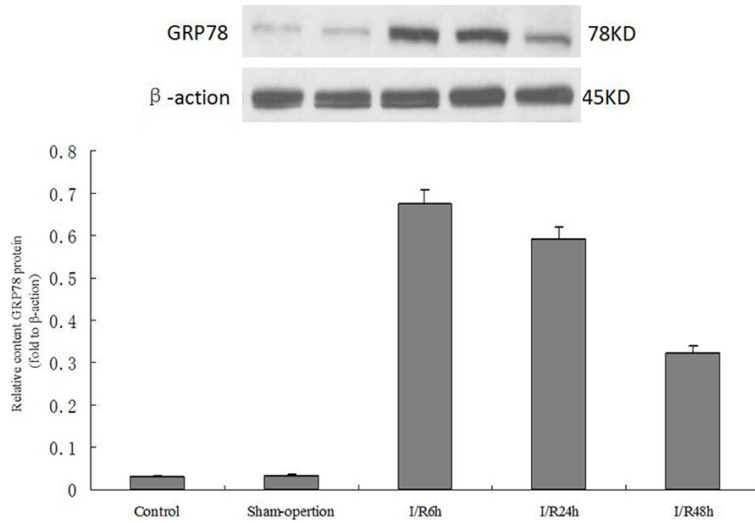


Figure 2. Expression of GRP78 in different groups.

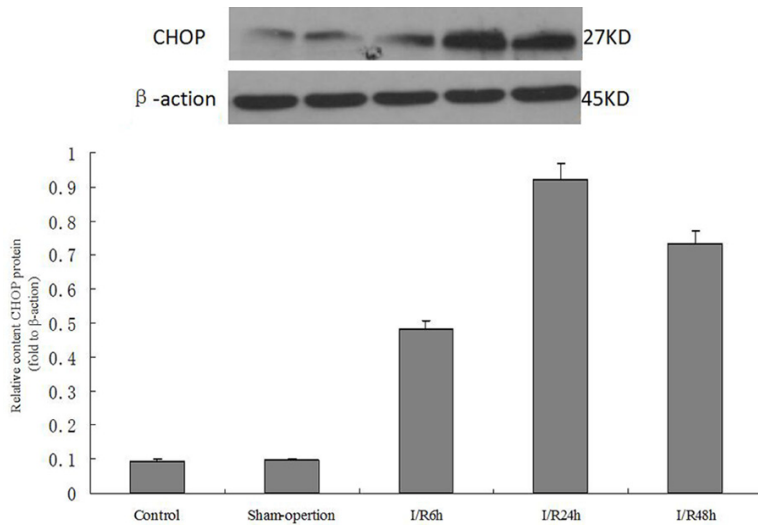


Figure 3. Expression of CHOPD in different group.

As shown in **Figure 4**, there was a low expression of TRB3 protein in brain tissues of rats in normal group and sham operation group; while in each IR group, TRB3 protein expression was significantly increased compared with the normal group and sham operation group ($P < 0.01$), which reached a peak at the 24th hour of ischemia reperfusion.

Results of real-time fluorescent PCR detection

As shown in **Table 2**, the mRNA expression of GRP78, CHOP and TRB3 in brain tissues of rats significantly increased in each IR group, compared with that in the normal group and sham operation group ($P < 0.01$). GRP78 mRNA expres-

sion reached a peak at the 6th hour of ischemia-reperfusion ($P < 0.01$), CHOP mRNA expression reached a peak at the 24th hour of ischemia-reperfusion ($P < 0.01$), and TRB3 mRNA expression reached a peak at the 24th hour of ischemia-reperfusion ($P < 0.01$).

Discussion

Recent studies have revealed that ERS was a new way to mediate apoptosis [9]. GRP78 was a key regulator protein that promotes protein maturation in normal cells, and was considered as a marker protein in ERS [10]. This study revealed that the gene and protein expression level of GRP78 reached a peak at the 6th hour of ischemia reperfusion, which was significantly higher in rats in the CIRI group than that in rats in the normal and sham operation groups ($P < 0.01$). The expression level of GRP78 in the IR-24h and IR-48h groups gradually decreased with time. This suggested that ERS was involved in CIRI.

CHOP is a specific transcription factor in ERS [11]. Under normal conditions, CHOP expression levels in cells are very low. This expression level significantly increased when ERS occurred [12, 13]. The results of this study revealed that CHOP was activated and CHOP expression level increased during CIRI, while continuous CHOP overexpression caused by ERS promoted cell apoptosis. Through silencing the CHOP gene, Zheng *et al.* [14] found that TUNEL positive cells in the damaged areas of rat brains significantly decreased. This revealed that CHOP plays a role in mediating cell apoptosis and aggravating brain injury in CIRI [15-17]. These were consistent with the results of this study.

TRB3 protein is a kinase-like protein [18]. It can directly bind with serine/threonine protein

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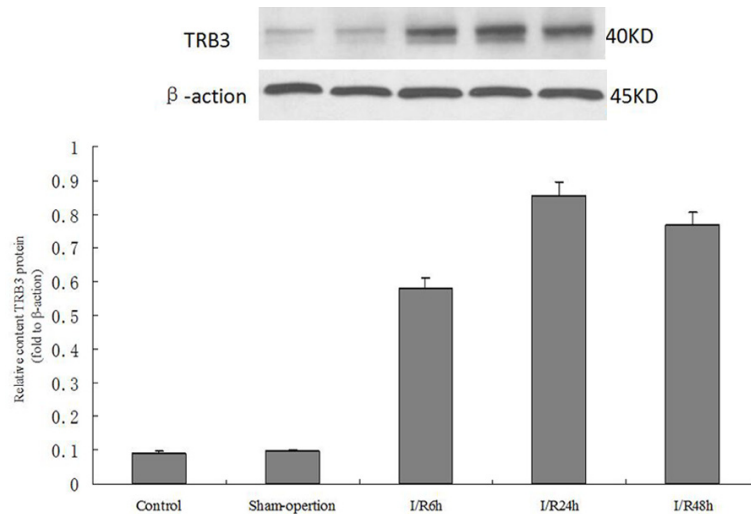


Figure 4. Expression of TRB3 in different group.

Table 2. The mRNA expression of GRP78, CHOP and TRB3 in brain tissues of rats in different groups ($\bar{x} \pm s$)

Groups	GRP78	CHOP	TRB3
Control	18.69±6.77	37.33±12.70	33.34±10.18
Sham-operation	19.35±7.43	38.73±11.56	34.11±8.75
IR 6 h	334.54±98.33*.*	215.95±86.33*.*	190.46±76.44**.*
IR 24 h	223.98±98.68*	353.53±122.21*	327.35±114.48*
IR 48 h	194.17±79.81*	258.67±107.01*	281.7±92.32*

Note: compared with control and sham-operation groups, * $P < 0.01$; compared with IR 24 h, * $P < 0.05$; compared with IR 24 h, ** $P < 0.01$; compared with IR 48 h, * $P < 0.05$; compared with IR 48 h, ** $P < 0.01$.

kinase AKT to inhibit AKT phosphorylation; thus, inhibiting its activity. In addition, AKT is an important anti-apoptosis signal molecule [19, 20]. The result of this study revealed that TRB3 mRNA expression levels in brain tissues of CIRI rats significantly increased, compared with that in rats in the normal group and sham operation group ($P < 0.01$).

Based on our TUNEL results, it was revealed that compared with the normal and sham operated group, the apoptosis rate of cerebral cells in rats in the CIRI group significantly increased ($P < 0.01$). This suggested that the apoptosis rate of cerebral cells was consistent with CHOP and TRB3 protein and mRNA expression levels in CIRI rats. The reason may be that continuous ERS occurred due to ischemia, hypoxia and a large number of free radicals in brain cells after cerebral ischemia; which promoted CHOP expression. Furthermore, CHOP further bound

with the promoter region of the TRB3 gene and induced TRB3 expression. On the one hand, TRB3 inhibited CHOP expression via negative feedback mechanism, which made CHOP and TRB3 protein expression levels lower in brain tissues from CIRI rats at the 48th hour of cerebral ischemia, compared to that at the 24th hour of cerebral ischemia. On the other hand, TRB3 may cause apoptosis in brain cells by inhibiting the phosphorylation of AKT [21]. However, this study revealed that the apoptosis rate of brain cells did not gradually increase with the increase in CIRI. Furthermore, the apoptosis rate was lower in CIRI rats at the 48th hour of cerebral ischemia than that at the 24th hour of cerebral ischemia. This result was similar to those of Song *et al.* [22]. Therefore the author speculates that ERS aggravated with time, which initiated different signaling pathways, causing significantly increased CHOP and TRB3 expression in this period. During the late

stage, CHOP and TRB3 expression levels decreased with the increase in the negative feedback effect of TRB3 [23]; and brain cell apoptosis mediated by this also significantly decreased. Under this condition, the way that the damaged brain cells removed was mainly necrosis, which was converted from apoptosis in the early stage. This change cause a vigorous release of various proinflammatory factors in CIRI brain tissues. CIRI becomes severe with time.

In summary, ERS-related GRP78, CHOP and TRB3 protein and gene expression levels significantly increase during CIRI. Changes in CHOP and TRB3 levels were consistent with the apoptosis rate of brain cells in rats. This suggests that ERS may promote brain cell apoptosis; and thus, it is involved in the occurrence and development of CIRI through regulating the protein expression of GRP78 downstream signaling molecules CHOP and TRB3.

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