Original Article

Upregulated expression of S100A8 in mice brain after focal cerebral ischemia reperfusion

Peng Sun[1], Qian Li[2], Qing Zhang[3], Li Xu[1], Ji-yuan Han[1]

[1] *Department of Emergency Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*

[2] *Department of Emergency Medicine, Liyuan Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430077, China*

[3] *Department of Anesthesiology,Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China*

Corresponding Author: *Qing Zhang, Email: zqann_126@126.com*

BACKGROUND: Recent studies have showed that S100A8 has been implicated in the pathobiology of inflammatory disorders, and that cerebral ischemia reperfusion (I/R) rapidly activates inflammation responses via Toll-like receptor 4 (TLR4). This study aimed to explore the expression of S100A8 and the relationship between S100A8 and TLR4 in focal cerebral ischemia reperfusion injury.

METHODS: C3H/HeJ mice (*n*=30) and C3H/HeN mice (*n*=30) were divided randomly into a C3H/HeJ model group (*n*=18), a C3H/HeJ control group (*n*=12), a C3H/HeN model group (*n*=18), and a C3H/HeN control group (*n*=12). Middle cerebral artery I/R model in mice was produced using a thread embolism method. The brains of the mice were collected after ischemia for 1 hour and reperfusion for 12 hours. Stroke outcome was evaluated by determination of infarct volume and assessment of neurological impairment scores. Brain injury after cerebral I/R was observed by an optical microscope after TTC and HE dyeing. The immunofluorescence technique and real time PCR were used to test the expression level of S100A8 in brain damage.

RESULTS: Compared with C3H/HeN mice, TLR4-deficient mice (C3H/HeJ) had lower infarct volumes and better outcomes in neurological tests. The levels of S100A8 increased sharply in the brains of mice after I/R injury. In addition, mice that lacked TLR4 (C3H/HeJ) had lower expression of I/R-induced S100A8 than C3H/HeN mice in the model group, indicating that a close relationship might exist between the levels of S100A8 and TLR4.

CONCLUSION: S100A8 interaction with TLR4 might be involved in brain damage and in inflammation triggered by I/R injury.

KEY WORDS: S100A8; Toll-like receptor 4; Cerebral ischemia reperfusion; Inflammation

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INTRODUCTION

S100A8 (calgranulin A or migration inhibitory factor related protein 8; MRP-8) belongs to the S100 protein family of calcium-modulated proteins that are highly abundant in neutrophil granulocytes and in some monocyte subsets.[1] Recent studies have showed that S100A8 has been implicated in the pathobiology of inflammatory disorders, such as asthma, rheumatoid arthritis, and inflammatory bowel disease.[2] It is well known that the inflammatory and immune reactions are involved in cerebral ischemia reperfusion (I/R),[3,4] but whether S100A8 mediates the injury in the process of cerebral I/R is still unknown. We here report our findings concerning the expression changes and the role of S100A8 in cerebral I/R.

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METHODS

Animals' preparation

TLR4 wild-type (C3H/HeN) and mutant mice (C3H/HeJ) of 6–8 weeks of age were purchased from Beijing Vitalriver Experimental Animal Center and Shanghai SLAC Animal Center, respectively. All of the mice were divided into 4 groups at random: C3H/HeN control group (*n*=12), C3H/HeJ control group (*n*=12), C3H/HeN model group (*n*=18) and C3H/HeJ model group (*n*=18).

Establishment of mice cerebral ischemia-reperfusion model

The model of middle cerebral artery (MCA) reperfusion was produced in mice according to the Caso method with a small revision.[5] After injection of 1% pentobarbitol sodium (4 mL/kg) through the peritonea, the mice were placed under a microscope and magnification was set to ten times. We incised at cervical median line, and the left external carotid artery (ECA) and left internal carotid artery (ICA) were exposed. After that, we inserted prepared nylon silk into ICA (about 10–12 mm length deep inside) until it blocked the blood supply of the middle cerebral artery. The criteria of a successful model included: 1) left Horner symptom; and 2) right-side hemiparalysis mainly for the forward limbs. We draw back nylon silk after 1 hour and mice brains were removed 12 hours after reperfusion.

Scores of neurological impairment

According to the standard of the Longa 5 grading method,[6] we graded neurologic impairment: 0, no neurologic impairment; 1, endoduction of the right anterior limbs and no wholly stretch while left the tail; 2, circling towards the right side when it walked spontaneously; 3, right-side lateriversion when walking; 4, without spontaneous walk and some consciousness lost. We screened out the mice with 0 or 4 score.

Triphenyltetrazolium chloride (TTC) staining

Six mice were taken respectively from the C3H/HeN model group and C3H/HeJ model group for TTC staining. The brains of the mice were taken and cut into 5 coronal brain slices 2 mm thickness from the frontal pole to the occipital pole. The slices were immediately put into 2% TTC (Sigma Company) away from light, followed by 37 °C incubation for 30 minutes, then fixed with 4% paraformaldehyde. Normal brain tissue was dyed to be bright red, while infarction focus was pale white. We arranged the fixed brain slices in orders, and then measured the whole area of slice and cerebral infarction area with Adobe Photoshop CS software. After that, we calculated cerebral infarction volume and whole brain volume, as well as the volume ratio of cerebral infarction (cerebral infarction volume/ whole brain volume).

Hematoxylin-eosin (HE) staining

Six mice were taken respectively from each group for HE staining of brain tissue. The mice were anesthetized with 10% chloral hydrate and perfused through the left ventricle with 4% paraformaldehyde. The brains of these mice were removed, and brain tissues were used to perform regular dehydration, transparence, paraffin imbedding, slicing, and HE staining. The injury of neurons was observed with a light microscope.

Immunofluorescence

The sections of brain tissue were dewaxed, hydrated and rinsed by distilled water. They were subsequently incubated in the primary antibody over night at 4 °C (10 μg/mL Goat Anti Mouse S100A8 Antibody, Catalog #AF2059, R&D Systems Biotechnology, Inc). The sections were washed with TBS-T, and then incubated in secondary antibody for 30 minutes at 37 °C. QDs-SA was added after the sections were washed again and they were incubated for another 30 minutes at 37 °C. All specimens were examined under a fluorescence microscope.

Real-time PCR

Six mice were taken from each group and their brains were removed rapidly from the skull. Total RNA derived from the ipsilateral (ischemic) hemisphere. Reverse transcription was performed by using reverse transcription kit (TAKARA Biotechnology Co., LTD). PCR reaction mixture was amplified at 94 °C for 3 minutes, which was followed by 40 cycles at 94 °C for 30 seconds, at 58 °C for 30 seconds, at 72 °C for 30 seconds, with a final extension at 72 °C for 10 minutes. PCR primers were as follows: S100A8 5'-CAA GGA AAT CAC CAT GCC CTC T-3' (forward) and 5'-TTT GTG AGA TGC CAC ACC CAC T-3' (reverse); β-actin 5'-TGA GAC CTT CAA CAC CCC AG-3' (forward), 5'-GCC ATC TCT TGC TCG AAG TC-3' (reverse).

Statistical analysis

All the data were expressed as mean±standard deviation. Statistical analysis was performed using SPSS 11.0 for Windows. Student's *t* test or one-way ANOVA was employed for analysis of the results. A probability value less than 0.05 was considered to be statistically significant.

RESULTS

Comparison of neurological impairment scores and brain infarct volume between the model groups

As shown in Table 1, the score of neurological impairment in the C3H/HeN group was higher than that of the C3H/HeJ group and the brain infarct volume of the C3H/HeN group was larger than that of the C3H/HeJ group.

Pathological changes of brain tissue in each group

Mice in the C3H/HeN and C3H/HeJ control groups (as shown in A, C) had normal shape of nerve cells. But in the model groups (as shown in B, D), nerve cells had different levels of swelling, and vacuolization appeared, and the shape of cell nucleus varied. Moreover, the above changes were more obvious in C3H/HeN mice than those in C3H/HeJ mice (Figure 1).

Table 1. Comparison of neurological impairment scores and brain infarct volume between the model groups

Groups	*n*	Neurological score	Brain infarct volume
C3H/HeN	6	2.50±0.55	40.78±3.35
C3H/HeJ	6	1.50±0.55*	26.09±2.90*

Compared with C3H/HeN group, *P<0.01.

Expression of S100A8 protein in each group

Mouse brain tissue was stained with goat anti-S100A8 antibody. Red fluorescence showed the localization of S100A8 (arrows)(magnification×400). Few S100A8-positive cells were observed in the hemisphere in the control group (A, C) and they were almost exclusively observed in the ischemic hemisphere (B, D) in the model group. Additionally, more S100A8-positive cells were found in the ischemic brain of C3H/HeN mice (B) compared with those of C3H/HeJ mice (D) (Figures 2, 3).

Expression of S100A8 mRNA in each group

The relative expression quantity of S100A8 was calculated as $2^{-\Delta\Delta Ct}$. The result indicated that the S100A8 mRNA expression was increased significantly after I/R injury. However, the expression of S100A8 mRNA was lower in the C3H/HeJ model group than in the C3H/HeN model group (Figure 4).

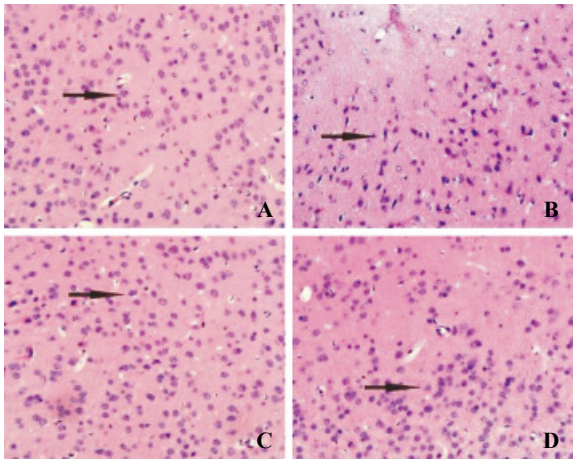

Figure 1. Pathological changes of brain tissues in each group (HE×400). A: C3H/HeN control group; B: C3H/HeN model group; C: C3H/HeJ control group; D: C3H/HeJ model group.

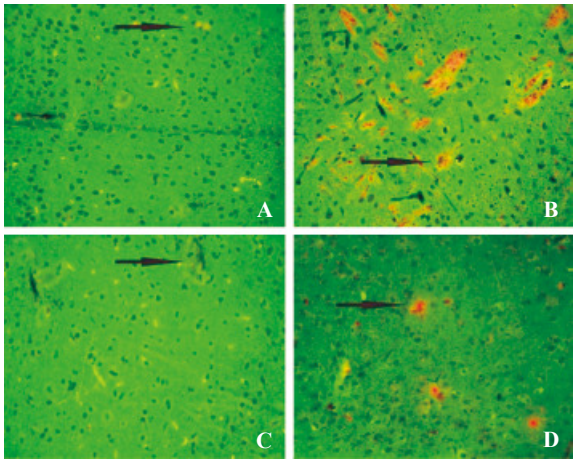

Figure 2. Expression of S100A8 protein in the ischemic mouse brain (magnification×400). A: C3H/HeN control group; B: C3H/HeN model group; C: C3H/HeJ control group; D: C3H/HeJ model group.

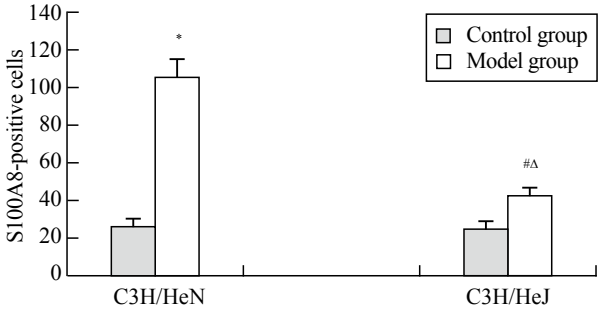

Figure 3. S100A8-positive cells in each group. Compared with C3H/HeN control group, *P<0.01; compared with C3H/HeJ control group, #P<0.01; compared with C3H/HeN model group, ΔP<0.01.

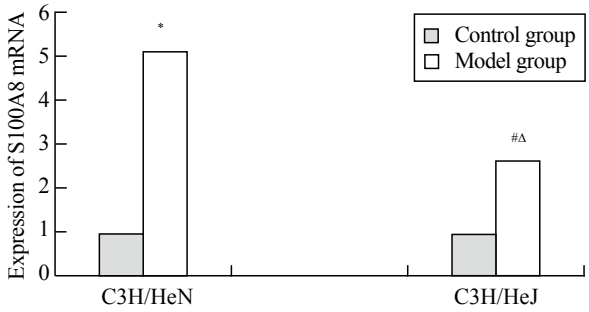

Figure 4. Expression of S100A8 mRNA in each group. Compared with C3H/HeN control group, *P<0.01; compared with C3H/HeJ control group, #P<0.01; compared with C3H/HeN model group, ΔP<0.05.

DISCUSSION

S100 proteins are a family of small Ca^{2+}-binding proteins which share a broad spectrum of functions.[7-9] Marked expression of serum S100 proteins was found in patients with serious brain injury and showed the predictive value for minor brain injury to some extent.[10] Belonging to this family, S100A8 has been implicated in the pathobiology of inflammatory and tumor disorders in recent studies.[11-13]

Under physiologic conditions, S100A8 is mostly expressed as a homodimer or a heterodimer together with S100A9, another member of the S100 protein family. S100A8 and S100A9 play many important roles including regulation of enzyme activity, Ca^{2+}-homeostasis, and interaction with components of the cytoskeleton.[14-16] But experimental data indicate a positive feedback mechanism according to which S100A8 and S100A9, released by primed myeloid cells under inflammatory conditions, promotes further leukocyte recruitment. The heterodimeric complex is involved in innate immunity, leukocyte adhesion, and endothelial transmigration.[17,18]

It has been proved that the inflammatory and immune reactions are involved in cerebral I/R. And our study showed that the expression of S100A8 mRNA and protein were increased sharply after I/R injury in both C3H/HeN and C3H/HeJ model groups, indicating that S100A8 contribute to neuroinflammation and the progression of I/R damage during early stage. The results were approximately in consistent with those reported by Ziegler.[19]

The high expression of S100A8 in ischemic brain provided a foundation for its mediation of cerebral I/R injury. But it is still unclear which signal pathway interacted with S100A8 was involved in brain damage? Recently it has been shown that TLR4 can evoke the inflammatory chain reaction and play a crucial role in innate immune response.[20-23] The expression of TLR4 has been detected in heart, lung and brain I/R injury and their expression has been shown to be modulated by a variety of internal or external stimuli, including TLR agonists, hypoxia, and proinflammatory cytokines.[5,24,25] In our study, the score of neurological impairment in the C3H/HeN group was higher than that of the C3H/HeJ group and the brain infarct volume of the C3H/HeN group was larger than that of the C3H/HeJ group, suggesting that TLR4 might mediate the inflammatory injury in the cerebral I/R process.[26,27] In addition, we demonstrated that mice in the C3H/HeJ model group had a lower expression of I/R-induced S100A8 than C3H/HeN mice in the model group, indicating that a close relationship might exist between the levels of S100A8 and TLR4. The expression of S100A8 has been detected in microglia as well as infiltrating neutrophils during cerebral I/R injury.[19] And TLR4 in microglia serve as one of the important inflammatory receptors.[28,29] Furthermore, the latest study has shown that that S100A8 is an endogenous ligand of TLR4 that is responsible for the amplification of LPS effect triggered by the Mrp8-Mrp14 complex, and TLR4-mutant cells showed a similar lack of response to LPS and S100A8.[30] Therefore, it is supposed that the S100A8-activated TLR4 signal pathway leads to the chained amplification of inflammation reaction in the early stage of cerebral I/R injury. However, it is still unclear that whether S100A8 takes part in cerebral I/R injury via TLR4 or via both TLR4 and other receptor. And when TLR4 is activated, it is still unclear which molecules are involved. Further study is needed.

Recent clinical studies[16] have been focused on elevated serum levels of S100A8 with inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease, and chronic lung disease. Moreover, increased S100A8/A9 expression has become a novel, early, and sensitive marker of acute coronary syndromes.[31] Hence the study on the mechanism of S100A8 mediating cerebral I/R injury is of practical value in early diagnosis and prevention of cerebrovascular events.

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Ethical approval: The present study was approved by Animal Care and Use Committee of the Tongji Medical College, Wuhan, China.

Conflicts of interest: The authors have no competing interests relevant to the present study.

Contributors: Sun P, Zhang Q and Han JY designed research; Sun P, LI Q, Zhang Q and Xu L performed the research; Sun P and Zhang Q wrote the paper.

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