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Effect of the JAK2/STAT3 signaling pathway on nerve cell apoptosis in rats with white matter injury

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Abstract. – OBJECTIVE: The Janus activated kinase 2 (JAK2)/signal transducer and the activator of transcription 3 (STAT3) pathway are involved in many physiological processes, such as cell survival, inflammation, development, proliferation and differentiation. Increasing evidence has shown that this pathway also has neuron-specific functions in the central nervous system. In this study, the functional significance of the JAK2/STAT3 signaling pathway in nerve cell apoptosis in rats with white matter injury was evaluated.

MATERIALS AND METHODS: The rat model of white matter injury was established by ligating bilateral common carotid arteries, and the changes of the JAK2 and STAT3 phosphorylation in hippocampal neurons were evaluated using the immunohistochemistry. In addition, the effects of JAK2 inhibitor AG490 and STAT3 small interfering ribonucleic acids (siRNAs) on the expression of phosphorylated-JAK2 (pJAK2), STAT3 messenger RNAs (mRNAs) and pSTAT3 in hippocampal neurons of white matter injury rats were studied. The effects of both on cerebral infarction volume and neuron apoptosis in white matter injury rats were also investigated.

RESULTS: The expression of pJAK2 and pSTAT3 were significantly increased after white matter injury in rats ($p<0.05$). JAK2 inhibitor AG490 markedly decreased the phosphorylation of JAK2 and STAT3 in hippocampal neurons in the model group ($p<0.05$). STAT3 siRNAs remarkably reduced the expression levels of STAT3 mRNA and protein in hippocampus neurons in the model group ($p<0.05$), while having no effect on the expression level of pJAK2 protein. AG490 and STAT3 siRNAs notably attenuated the volume of cerebral infarction in the model group, as well as reduced neuron apoptosis after white matter injury.

CONCLUSIONS: The inhibition of the JAK2/STAT3 signaling pathway contributed to reducing the volume of cerebral infarction and neuron apoptosis in rats with white matter injury.

Key Words:
JAK2/STAT3 signaling pathway, White matter injury, Nerve cells, Apoptosis.

Introduction

White matter injury is the main cause of vascular dementia and disability in the elderly. Age and hypertension are considered the two most important risk factors to this[1]. The pathogenesis of white matter injury is mainly related to vascular edema, microbleeds or/and chronic cerebral hypoperfusion, and also accompanied by endothelial dysfunction. Collagen disease, retinal and intraparenchymal venules dilatation in the periventricular and subcortical regions are associated with the severity of white matter injury. Cerebral venous hypertension caused by hemorrhage and injury in the downstream veins may play a major role in the pathogenesis of white matter injury. Pro-inflammatory cytokines are important promoters of inflammatory neuronal injury after ischemia[2], which can diffuse through the Janus activated kinase (JAK)/signal transducer and the activator of the transcription (STAT) signaling pathway[3]. Cytokines binding to receptors can induce the phosphorylation of related JAK and its downstream STAT[4]. JAK exists in three isoforms (JAK1, JAK2 and JAK3) and STAT exists in seven forms (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6). Among them, JAK2 and STAT3 are considered to be the most conservative isoforms. Previous studies[5-7] have shown that STAT1 and STAT3 phosphorylation was up-regulated after focal cerebral ischemia in rodents. Takagi et al[8] revealed that increased phosphorylation of STAT1 in neurons resulted in white matter injury, and the mice that knock out

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STAT1 displayed smaller infarctions. However, the significance of STAT3 activation in white matter injury has not been evaluated before. Therefore, the effect of the JAK2/STAT3 signaling pathway on neuronal apoptosis in white matter injury rats was investigated in this study.

Materials and Methods

Main Test Reagents

JAK2 phosphorylation inhibitor AG490 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Polyclonal antibody of phosphorylated-STAT3 (pSTAT3) and polyclonal antibody pJAK2 from Cell Signaling Technologies (Danvers, MA, USA), β-actin antibody from Sigma-Aldrich, Ltd. (St. Louis, MO, USA), in-situ cell death detection kit from Roche Molecular Biochemicals (Basel, Switzerland), reverse transcriptases from Life Technologies (Gaithersburg, MD, USA), and TaqMan 5700 sequence detection system from Applied Biosystems (Foster City, CA, USA).

Test Animals

In this study, 120 adult male Sprague-Dawley (SD) rats (280-300 g) were used; 90 rats underwent bilateral common carotid artery ligation to establish the white matter injury model and 30 rats underwent the sham operation. Among all rats in the model group, 30 rats of the model group were treated with AG490, 30 rats with STAT3 small interfering ribonucleic acids (siRNAs) and 30 rats with control siRNAs, respectively. Of the 30 rats receiving sham operation, 10 rats were treated with AG490, 10 with STAT3 siRNAs and 10 with control siRNAs. This study was approved by the Animal Ethics Committee of the Nanjing Medical University Animal Center.

Animal Model Preparation

The rats received solid and liquid fasting; they were abdominally anesthetized with 10% chloral hydrate followed by fixation and disinfection in the supine position. The incision along the midline of the neck was then made and the bilateral common carotid arteries were bluntly separated from the sympathetic nerve chains. After that, the sympathetic nerves were stripped and bilateral common carotid arteries were ligated, the muscle and skin tissues were sutured and wounds were sterilized. In the sham operation group, the common artery was not ligated.

Intracerebral Injection of AG490

AG490 was continuously injected into the lateral ventricles in the rats. The drug was filled into a permeable micro-pump, which was pumped at a rate of 1 μL/h. Each pump was connected to a stainless-steel tube for brain infusion through a peristalsis tube and was triggered overnight at 37°C. The cannula was implanted into the lateral ventricle in a three-dimensional position and fixed to the skull with dental cement, and the pump was placed in the skin folds of the rat neck. The cannula and pump were implanted under halothane anesthesia.

Injection of siRNAs Into the Brain

In order to selectively inhibit the STAT3 phosphorylation, RNA interference (RNAi) technique was applied. STAT3 messenger RNAs (mRNAs) were degraded by STAT3 siRNAs, which limited the phosphorylation of available STAT3 proteins after white matter injury. The STAT3 siRNA sequence is as follows: 5'-CCA ACG ACC UGC AAU AUU-3'. In addition, the non-targeted siRNA was designed as the negative control, which contained equivalent guanine-cytosine to the functional siRNA while lacked recognizable targets. The mismatch was confirmed by the Basic Local Alignment Search Tool analysis. The sequence of the control siRNA is as follows: 5'-AUG AAC GUG AAU UGC UCA A-3'. The STAT3 siRNA or control siRNA was suspended in siRNA universal buffer and the concentration was adjusted to 50 μM. Then, 17 μL siRNAs were incubated with 3 μL oligofetamines at room temperature for 15 min. The STAT3 siRNA or control siRNA was slowly injected into the cerebral cortex using the Hamilton syringe. One hour later, white matter injury or sham operation was performed in the rat group.

Immunohistochemistry

The positive cells of pJAK2 and pSTAT3 were evaluated in brain slices of rats that died after white matter injury, and rats receiving sham operation were set as controls. The rats were perfused with 4% buffered paraformaldehyde through the heart, and the brain was cut open. The slices were rinsed with 0.1 Tris-Buffered Saline (TBS) containing 0.1% Triton-X100, incubated in 1% hydrogen peroxide (H_2O_2) for 30 min and washed in TBS with Tween 20 (TBST) (Beyotime, Shanghai, China). Subsequently, the slices were blocked in 5% normal goat serum for 1 h followed by incubation with polyclonal pSTAT3 antibody

and polyclonal pJAK2 antibody overnight. After washing in TBS, the slices were incubated with biotinylated goat anti-rabbit antibody for 1 h. Combined with an avidin-biotinylase marker, the slices were then subjected to color development with 3,3'-diaminobenzidine + H_2O_2. After dehydration and cleaning, the slices were installed in Permount.

Western Blotting

The effects of AG490 and STAT3 siRNAs on the expression of pJAK2 and pSTAT3 proteins were tested by Western blotting. The tissue was lysed in the lysate, and the protein content in the supernatant was determined by centrifugation. The samples were electrophoresed on the polyacrylamide gel prepared by Bio-Rad (Hercules, CA, USA), transferred onto the polyvinylidene difluoride membrane (PVDF) and bound to polyclonal antibodies of pJAK2 (1:400) and pSTAT3 (1:500) as well as horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (1:1000). Finally, the protein band recognized by the antibody was detected by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA).

Detection of Cell Apoptosis via Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)

In-situ cell death detection kit was used to detect apoptotic cells. Procedures are as follows: the slices were treated with 1% protease K for 15 min, washed with Phosphate-Buffered Saline (PBS) and incubated in the TUNEL reaction mixture (Solarbio, Beijing, China) at 37°C for 1 h. Then, the samples were rinsed in PBS, placed in anti-fading solution and analyzed using a fluorescence microscope to count the proportion of apoptotic cells.

Real Time-Polymerase Chain Reaction (PCR) Detection

The total RNA was extracted from the cortex of each rat using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Each sample was reversely transcribed using Reverse Transcription (RT) kit (TaKaRa, Otsu, Shiga, Japan), and Real Time-PCR was performed as described by Vemuganti et al[9]. Complementary deoxyribonucleic acids (cDNAs) and specific primers were added to SYBR Green PCR Master Mix, and PCR amplification was carried out in TaqMan 7500 sequence detection system. With 18S ribosomal RNA (rRNA) and β-actin as internal controls, the Ct values were compared to quantitatively amplify transcripts. The primer sequences of STAT3 are as follows: F-GCGAGTTCCCCATGGA, R-CACGAGTGTGACT.

Statistical Analysis

Data were expressed as mean ± standard deviation and analyzed by GraphPad Prism 5.0 (La Jolla, CA, USA). The intergroup comparison was performed *via* the one-way analysis of variance, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Effects of White Matter Injury on the Phosphorylation of JAK2 and STAT3 in Rat Hippocampal Neurons

First of all, the levels of pJAK2 and pSTAT3 in hippocampus neurons in the model group and the sham operation group were detected after the establishment of the white matter injury rat model. The results manifested that the expression of pJAK2 and pSTAT3 in the model group were markedly higher than those in the sham operation group ($p<0.05$) (Figure 1A-1B).

Effects of JAK2 Inhibitor AG490 on pJAK2 and pSTAT3

In order to study the effects of JAK2 in the JAK2/STAT3 signaling pathway on hippocampal neurons in white matter injury rats, we investigated the effects of JAK2 inhibitor AG490 on phosphorylation of JAK2 and STAT3 in hippocampal neurons in white matter injury rats. Immunohistochemistry and Western blotting results confirmed that AG490 significantly reduced the levels of pJAK2 and pSTAT3 in hippocampal neurons of white matter injury rats ($p<0.05$) (Figure 2A-2C).

Effects of STAT3 siRNAs on the STAT3 mRNA Expression and pSTAT3 in Hippocampal Neurons of White Matter Injury Rats

In order to verify the effects of STAT3 in the JAK2/STAT3 signaling pathway on hippocampal neurons of white matter injury rats, RNAi technique was adopted to interfere with the translation of STAT3 mRNAs. By minimizing the synthesis of new STAT3 proteins, the role of pSTAT3 in hippocampal neurons of white matter injury rats was clarified. The results demonstrated that STAT3 siRNA treatment evidently decreased the

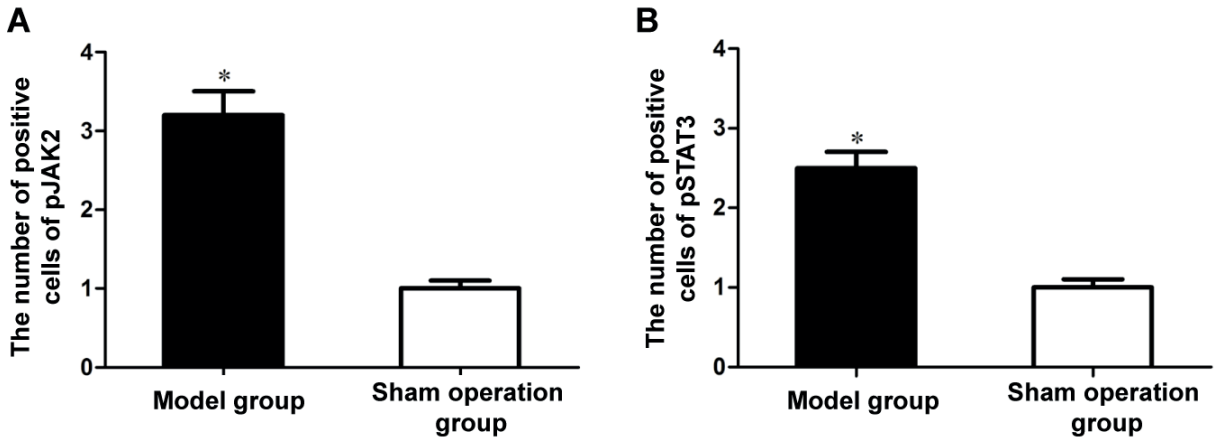

Figure 1. Detection of the expressions of the JAK2 and STAT3 phosphorylation *via* immunohistochemistry. ***A,*** The number of positive cells of pJAK2 in hippocampus neurons of rats in the white matter injury model group (multiple compared with that in the sham operation group) (**p*<0.05). ***B,*** Number of positive cells of pSTAT3 in hippocampus neurons of rats in the white matter injury model group (multiple compared with that in the sham operation group) (**p*<0.05).

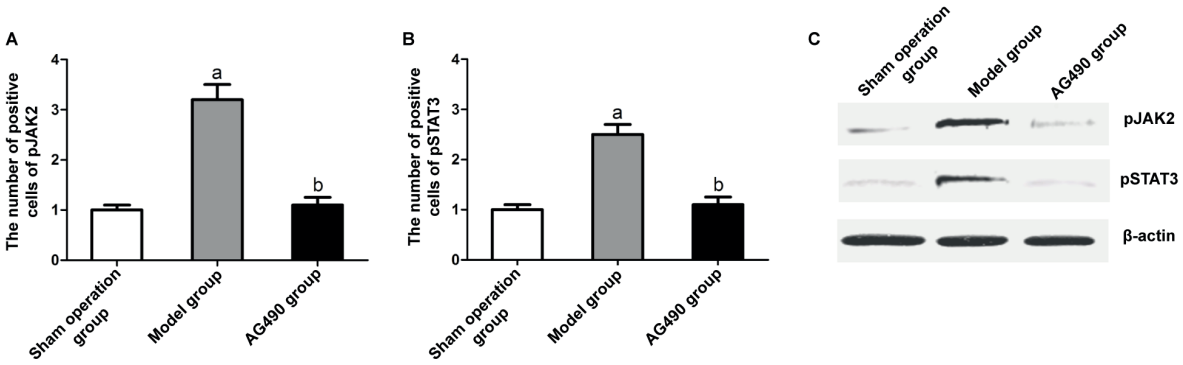

Figure 2. Examination of the effects of AG490 on the JAK2 and STAT3 phosphorylation in hippocampal neurons of white matter injury rats by immunohistochemistry and Western blotting. ***A-B,*** Detection of the down-regulation effect of AG490 on the phosphorylation of JAK2 and STAT3 *via* immunohistochemistry. a indicates that pJAK2 or pSTAT3 in the model group is remarkably increased compared with that in the sham operation group ($p<0.05$), and b indicates that pJAK2 or pSTAT3 in the AG490 group is significantly reduced compared with that in the model group. ***C,*** Detection of the effects of AG490 on the expression of pJAK2 or pSTAT3 using Western blotting.

expression level of STAT3 mRNAs instead of pJAK2 proteins in hippocampus neurons of white matter injury rats ($p<0.05$) (Figure 3A).

Effects of AG490 and STAT3 siRNAs on the Infarction Volume

To further explore the effect of the JAK2/STAT3 signaling pathway on white matter injury rats, the rats were treated with AG490 and STAT3 siRNAs, respectively, and their effects on cerebral infarction volume were investigated. As shown in Figure 4, there was no significant difference in the infarction volume between the siRNA control group and the model control group [control group: (221.3 ± 18.1) mm^3 and siRNA control group: (228.3±19.3) mm^3]. Compared with the control group, AG490 and STAT3 siRNAs significantly reduced the infarction volume in the siRNA control group [AG490: (138.5 ± 14.4) mm^3 and STAT3 siRNAs: (140.3 ± 14.0) mm^3] ($p<0.05$).

Effects of AG490 and STAT3 siRNAs on Neuron Apoptosis After White Matter Injury

Finally, the effects of AG490 and STAT3 siRNAs on neuron apoptosis after white matter injury were determined *via* TUNEL. According to the results, AG490 markedly reduced the proportion

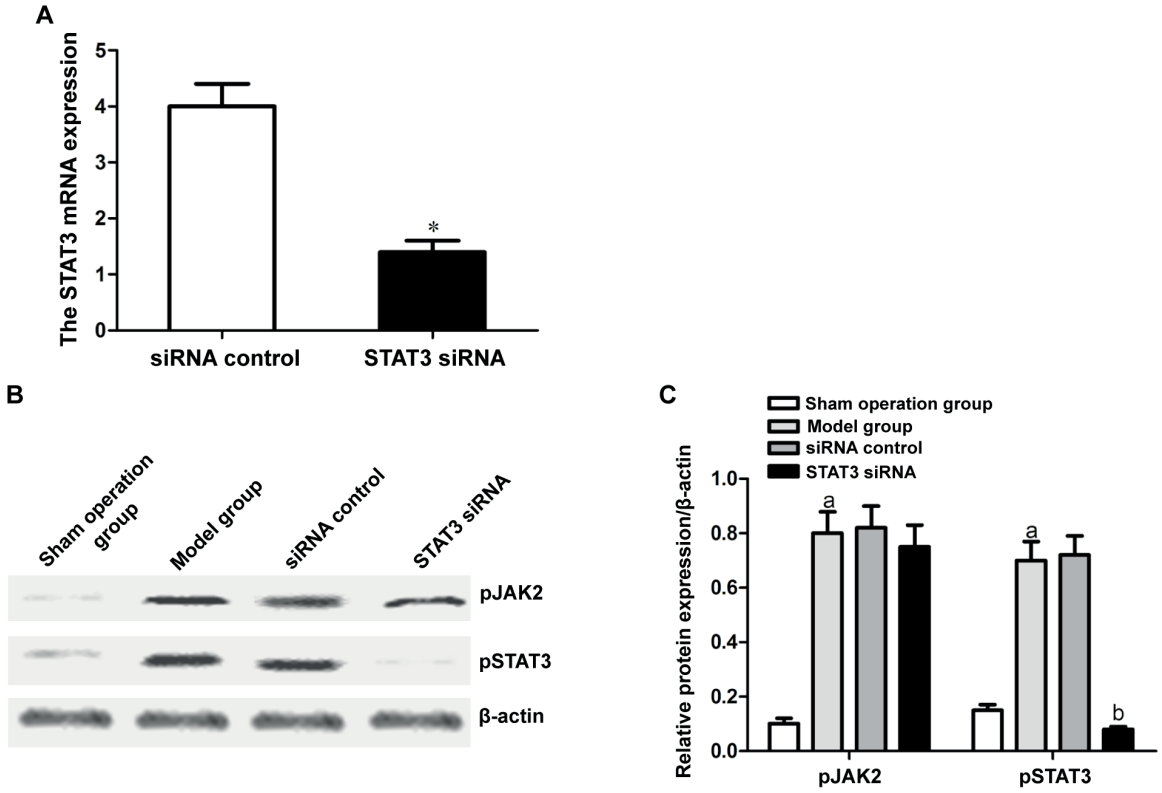

Figure 3. Effects of STAT3 siRNAs on the STAT3 mRNA expression and pSTAT3 in hippocampus neurons of white matter injury rats. ***A,*** RT-PCR detection results reveal that STAT3 siRNA treatment in rats can reduce the expression of STAT3 mRNAs induced by white matter injury (*p<0.05). ***B-C,*** Western blotting detection results verified that the pSTAT3 expression in hippocampus neurons of rats with white matter injury treated with STAT3 siRNAs is markedly decreased (*p<0.05).

of neuron apoptosis in the AG490 group compared with the model control group [control group: (38.5 ± 4.2)%, AG490 group: (20.4 ± 2.2)%] (p<0.05). Compared with the siRNA control group, STAT3 siRNAs also significantly down-regulated the proportion of neuron apoptosis [siRNA control group: (35.6 ± 4.5)%, STAT3 siRNA group: (18.2 ± 2.5)%] (p<0.05). The above results suggested that AG490 and STAT3 siRNAs decreased neuron apoptosis after white matter injury (Figure 5).

Discussion

This study revealed that the JAK2/STAT3 activation was closely related to neuron apoptosis in white matter injury rats. JAK2, as an upstream signal molecular of STAT3, is assumed to regulate the phosphorylation of STAT3, thus mediating the downstream biological responses to STAT3 activation. In order to verify this hypothesis, the rats were injected with JAK phosphorylation inhibitor AG490 at the first day of this study, and the results showed that it eliminated JAK2 and STAT3 phosphorylation in the ischemic brain. AG490 infusion also resulted in a smaller infarction volume and a decreased proportion of apoptotic cells compared to the control rats injected with carriers. The above results indicated that the prevention of the phosphorylation of JAK2 and STAT3 has the effect of reducing neuronal apoptosis. It has also been reported that pJAK2 phosphorylated STAT1 except for STAT3. Previous studies[10] have shown that AG490 inhibited the phosphorylation of STAT1 and STAT3 in pre-treated hearts. Besides, Takagi et al[8] revealed that focal ischemia in STAT1 knockout mice induced less brain injury. Therefore, it could not be ruled out that AG490 being neuroprotected by blocking phosphorylation of STAT1 and STAT3 Therefore, the effect of siRNA specifically preventing STAT3 phosphorylation on neuron apoptosis in white matter injury rats was further studied. RNAi technique was adopted for specifically post-transcriptional gene silencing and degradation of targeted homologous gene transcripts, thus achieving the efficient and high-throughput gene

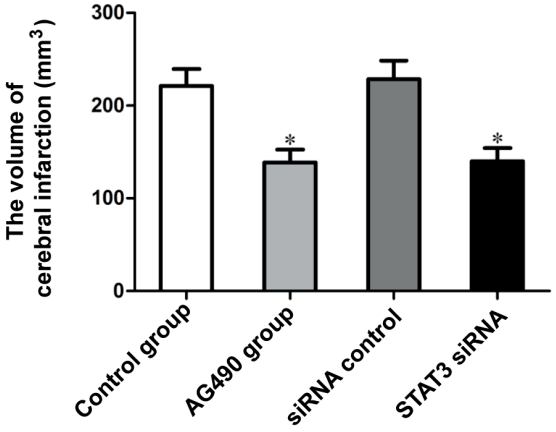

Figure 4. Effects of AG490 and STAT3 siRNAs on the infarction volume. AG490 and STAT3 siRNAs significantly decrease the volume of cerebral infarction (*p<0.05).

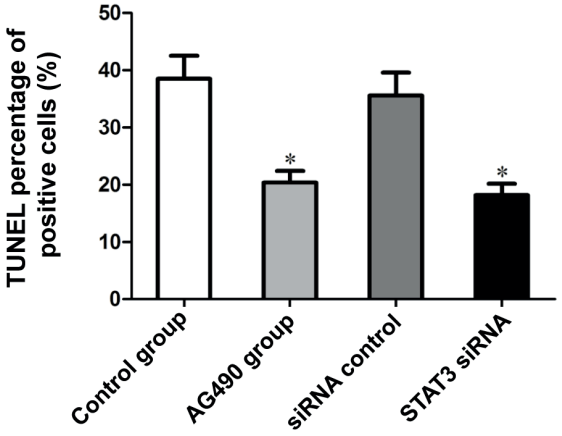

Figure 5. Detection of the effects of AG490 and STAT3 siRNAs on neuron apoptosis after white matter injury *via* TUNEL. G490 and STAT3 siRNAs decrease neuron apoptosis after white matter injury (*p<0.05).

function analysis[11]. Studies have shown that the siRNA-induced knockdown of tumor suppressor gene[12] and the knockout of the brain-derived neurotrophic factor[13] after spinal cord hypoxia could induce neuroprotection in adult rats during global cerebral ischemia. In this work, compared with the control siRNA, STAT3 mRNA and STAT3 phosphorylation in the brain of white matter injury rats were significantly reduced using siRNAs. Although normal levels of STAT3 activation are crucial to cell function[14], this paper showed that excessive STAT3 activation after white matter injury was harmful to the brain. This is understandable because interleukin-6 (IL-6) is known to form excessively after white matter injury and its pro-inflammatory effect is mediated by rapid activation of the JAK/STAT signal transduction[15]. In fact, STAT3 was first identified as a transcription factor which participates in the acute inflammatory reaction downstream of IL-6 activation[16,17]. PSTAT3 also stimulates the expression of the cytokine signaling inhibitor protein family, which acts as intracellular negative feedback for the JAK and STAT phosphorylation to turn off or inhibit the cytokine signaling[18]. Currently, the functional significance of JAK2/STAT3 activation induced by white matter injury is unclear. Sun et al[19] have reported that the JAK/STAT pathway played an important role in normal astrocyte differentiation during the development and the abnormal astrocyte response after central nervous system injury[20]. Increased phosphorylation of STAT3 in glial fibrillary acidic protein + astrocytes was observed in the ischemic brain. In addition, previous studies have shown that the up-regulation of STAT3 phosphorylation in neurons was involved in neuroprotection after conditioned spinal cord injury[21]. Hence, the increased pSTAT3 might promote the survival of neurons by inducing the expression of neuroprotective cell signal transduction inhibitor 3. However, the results of this work manifested that the JAK2/STAT3 activation in nerve cells might have neurotoxicity, since JAK2 inhibitor AG490 and STAT3 siRNAs induced significant neuroprotection in nerve cells after white matter injury.

Conclusions

We showed that the JAK2/STAT3 pathway was closely associated with the apoptosis of neurons in white matter injury rats, and the inhibition of its activity helps to reduce the cerebral infarction volume and neuron apoptosis in white matter injury rats.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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References

1) Pantoni L, Garcia JH. Pathogenesis of leukoaraiosis: a review. Stroke 1997; 28: 652-659.

2) Popko K, Gorska E, Stelmaszczyk-Emmel A, Plywaczewski R, Stoklosa A, Gorecka D, Pyrzak B, Demkow U. Proinflammatory cytokines Il-6 and TNF-alpha and the development of inflammation in obese subjects. Eur J Med Res 2010; 15 Suppl 2: 120-122.

3) O'Shea JJ, Pesu M, Borie DC, Changelian PS. A new modality for immunosuppression: targeting the JAK/STAT pathway. Nat Rev Drug Discov 2004; 3: 555-564.

4) Darnell JJ, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994; 264: 1415-1421.

5) Avalle L, Pensa S, Regis G, Novelli F, Poli V. STAT1 and STAT3 in tumorigenesis: a matter of balance. JAKSTAT 2012; 1: 65-72.

6) Scarabelli TM, Townsend PA, Chen Scarabelli C, Yuan Z, McCauley RB, Di Rezze J, Patel D, Putt J, Allebban Z, Abboud J, Chilukuri K, Gardin J, Saravolatz L, Knight RA, Latchman DS, Stephanou A. Amino acid supplementation differentially modulates STAT1 and STAT3 activation in the myocardium exposed to ischemia/reperfusion injury. Am J Cardiol 2008; 101: 63E-68E.

7) Hong F, Jaruga B, Kim WH, Radaeva S, El-Assal ON, Tian Z, Nguyen VA, Gao B. Opposing roles of STAT1 and STAT3 in T cell-mediated hepatitis: regulation by SOCS. J Clin Invest 2002; 110: 1503-1513.

8) Takagi Y, Harada J, Chiarugi A, Moskowitz MA. STAT1 is activated in neurons after ischemia and contributes to ischemic brain injury. J Cereb Blood Flow Metab 2002; 22: 1311-1318.

9) Vemuganti R, Dempsey RJ. Carotid atherosclerotic plaques from symptomatic stroke patients share the molecular fingerprints to develop in a neoplastic fashion: a microarray analysis study. Neuroscience 2005; 131: 359-374.

10) Bolli R, Dawn B, Xuan YT. Role of the JAK-STAT pathway in protection against myocardial ischemia/reperfusion injury. Trends Cardiovasc Med 2003; 13: 72-79.

11) Ichim TE, Li M, Qian H, Popov IA, Rycerz K, Zheng X, White D, Zhong R, Min WP. RNA interference: a potent tool for gene-specific therapeutics. Am J Transplant 2004; 4: 1227-1236.

12) Ning K, Pei L, Liao M, Liu B, Zhang Y, Jiang W, Mielke JG, Li L, Chen Y, El-Hayek YH, Fehlings MG, Zhang X, Liu F, Eubanks J, Wan Q. Dual neuroprotective signaling mediated by downregulating two distinct phosphatase activities of PTEN. J Neurosci 2004; 24: 4052-4060.

13) Baker-Herman TL, Fuller DD, Bavis RW, Zabka AG, Golder FJ, Doperalski NJ, Johnson RA, Watters JJ, Mitchell GS. BDNF is necessary and sufficient for spinal respiratory plasticity following intermittent hypoxia. Nat Neurosci 2004; 7: 48-55.

14) Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000; 19: 2474-2488.

15) Campbell IL. Cytokine-mediated inflammation, tumorigenesis, and disease-associated JAK/STAT/SOCS signaling circuits in the CNS. Brain Res Brain Res Rev 2005; 48: 166-177.

16) Akira S, Nishio Y, Inoue M, Wang XJ, Wei S, Matsusaka T, Yoshida K, Sudo T, Naruto M, Kishimoto T. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. Cell 1994; 77: 63-71.

17) Xiong L, Yu KH, Zhen SQ. MiR-93 blocks STAT3 to alleviate hepatic injury after ischemia-reperfusion. Eur Rev Med Pharmacol Sci 2018; 22: 5295-5304.

18) Rakesh K, Agrawal DK. Controlling cytokine signaling by constitutive inhibitors. Biochem Pharmacol 2005; 70: 649-657.

19) Sun Y, Nadal-Vicens M, Misono S, Lin MZ, Zubiaga A, Hua X, Fan G, Greenberg ME. Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell 2001; 104: 365-376.

20) Xia H, Mao Q, Paulson HL, Davidson BL. siRNA-mediated gene silencing in vitro and in vivo. Nat Biotechnol 2002; 20: 1006-1010.

21) Qiu J, Cafferty WB, McMahon SB, Thompson SW. Conditioning injury-induced spinal axon regeneration requires signal transducer and activator of transcription 3 activation. J Neurosci 2005; 25: 1645-1653.