CORRELATION BETWEEN NF-K B SIGNALING PATHWAY AND ACTIVATION OF NLRP3 INFLAMMASOME IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS IN MICE

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ABSTRACT

Objective: *To analyze the correlation between the NF-κB signaling pathway and NLRP3 inflammasome activation in an experimental autoimmune encephalomyelitis (EAE) model in mice.*

Methods: *Thirty C57BL/6 mice were selected and divided into the control group, the EAE model group, the EAE onset group (EAE mice score ≤1 points), and the peak group (EAE mice score ≤4 points), with 10 rats in each group. The EAE model group mice were injected with MOG to establish the mouse EAE model, and the control group mice were injected with CFA emulsion under the skin. RT-PCR was used to detect the expression of the NLRP3 inflammatory corpuscle mRNA and NF-κb-p65 mRNA in spleen mononuclear cells and spinal cords of EAE mice. Western blot was used to detect the protein expression of NLRP3 inflammatory bodies. The ELISA method was used to detect the levels of IL-1 β and IL-18 in mice blood.*

Results: *There was no significant difference in the mRNA and protein expression of NLRP3 inflammatory corpuscles in the spleens of EAE mice in each group (P>0.05). The expression of NLRP3 inflammasome mRNA and protein in the spinal cords of EAE mice was significantly higher than that of the control group (P < 0.05), and the expression of NLRP3 inflammasome mRNA and protein in the spinal cords of EAE mice during the peak period was significantly higher than that in the early stage of EAE (P<0.05). The ratio of p-nf -κb-p65/NF- κb-p65 in spinal cords of EAE mice was significantly higher than that of the control group (P<0.05), and the ratio of p-nf-κb-p65/NF-κb-p65 in spinal cords of EAE mice was significantly higher than in the early stage of EAE. The expression of NLRP3 inflammatory bodies was positively correlated with the ratio of p-nf-κb-p65/NF-κb-p65 (r=0.784, 0.614, both P<0.05). The serum levels of IL-1 β and IL-18 in EAE mice were significantly higher than those in the control group (P<0.05). The levels of IL-1 β and IL-18 in EAE mice during the peak period were significantly higher than those in the early stage (P<0.05).*

Conclusion: *The content of NLRP3 inflammasome in the spinal cords of EAE model mice was significantly high, and the activation of NF-κB signaling pathway in the spinal cord was significantly increased, which is significantly positively correlated with the level of NLRP3 inflammatory bodies, which are involved in the occurrence and development of EAE.*

Keywords: *Experimental autoimmune encephalomyelitis, NF-κB signaling pathway, NLRP3 inflammatory bodies, correlation.*

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Introduction

Multiple sclerosis is a demyelinating disease of the central nervous system that affects normal nerve transmission by destroying the white matter around the ventricle, the spinal cord, and the protective layer of the optic nerve. The disease's clinical symptoms and signs are diversiform; about 50% of patients' starting symptoms include one or more body, motor, and sensory disorder, frequent illness, a high recurrence rate, and morbidity. Multiple sclerosis is one of the most common and impactful diseases of the nervous system, and can cause serious damage to population productivity and quality of life in young adults[1-2]. The etiology and pathogenesis of multiple sclerosis have not yet been fully defined, and a variety of studies have confirmed that its occurrence is the result of the combined action of multiple factors, and that inflammation plays a crucial role in the pathogenesis of this disease[3]. Experimental

autoimmune encephalomyelitis (EAE) is a classic animal model of multiple sclerosis, which mainly targets the immune response mediated by myelin antigen-specific T cells, and inflammatory cell infiltration and myelin demyelination are the main pathological changes[4]. Recent studies have found that NLRP3 inflammasome mediated inflammation is closely related to many infectious diseases and autoimmune diseases[5].

NLRP3 inflammasome is a multi-protein complex, which can activate caspase-1 by identifying pathogen-associated molecular patterns or host-derived danger signal molecular patterns, thereby promoting the secretion of inflammatory cytokines IL-1 and IL-18, and causing a series of inflammatory responses[6].

In this study, an EAE animal model was established to analyze the role of NLRP3 inflammasome in multiple sclerosis and its correlation with the NF-κB signaling pathway.

Materials and methods

Reagents and instruments

This research used 10% chloral hydrate (Shanghai Lianshuo Biotechnology Co., Ltd.), inactivated mycobacterium tuberculosis (BD DIFCO, USA), 1640 medium (Emmett Technology Co., Ltd.), erythrocyte lysate (Shanghai Zhenyu Biotechnology Co., Ltd.), myelin solid blue staining kit (Shanghai Zhuocai Biotechnology Co., Ltd.), xylene (Shanghai Baoman Biotechnology Co., Ltd.), neutral gum (Beijing Solaibao Technology Co., Ltd.), trizol extract reagent (Shanghai Jinganti Biological Engineering Co., Ltd.), DEPC (Shanghai Shunna Biotechnology Co., Ltd.), adsorption column method RNA extraction kit (Beijing Biolaibo Technology Co., Ltd.), and ELISA kit (Beijing Jiayuan Xingye Technology Co., Ltd.).

The tools used in this research are inverted microscope (Beijing Jinda Sunshine Technology Co., Ltd.), paraffin embedding machine (Dongguan Pubiao Experimental Equipment Technology Co., Ltd.), water bath (Jiangsu Jirui Biotechnology Co., Ltd.), real-time fluorescence quantitative PCR instrument (Shanghai Aiyan Biotechnology Co., Ltd.), RNA concentration tester (Guangzhou Beimat Instrument Equipment Co., Ltd.), low temperature high speed centrifuge (Shanghai Tusen Vision Technology Co., Ltd.), piper (Shanghai Co., Ltd.), and an electric thermostatic drying oven (Beijing Ganming Gene Technology Co., Ltd.).

Preparation of EAE animal models

Thirty C57BL/6 mice, aged 6 to 8 weeks and weighing 18 to 20g, were selected and purchased from Beijing Vitong Lihua Experimental Animal Technology Co., Ltd. They were divided into the control group, the EAE initial onset group, and the EAE peak group, with 10 mice in each group. Mice were anesthetized by intraperitoneal injection of 2% chloral hydrate, and subcutaneously injected with 0.05 mL of Myelin oligodendrocyte glycoprotein (MOG) emulsion in 4 parts on the back of mice in the EAE model group, respectively. Mice in the control group were subcutaneously injected with Complete Freund's Adjuvant (CFA) emulsion, and were intraperitoneally injected with 300ng percough toxin/mouse on the day of MOG or CFA injection, and then injected again 48 hours later. The score standard was: 0 was normal; 0.5 was drooping tail tip of mice; 1 was tail tension disappeared or the waddling gait of the mice; 1.5 was disappearance of tail tension accompanied by staggering gait; 2 divided into incomplete paralysis of both hind limbs or paralysis of single hind limbs; 2.5 divided into paralysis of one hind limb with incomplete paralysis of the other hind limb; 3 divided into two hind limbs paralysis; 3.5 divided into paralysis of two hind limbs with weakness of one forelimb; 4 was quadriplegic; 5 was dying or dead.

In chloral hydrate anesthetized mice, blood was collected from eyeballs, and supernatant was taken after centrifugation and stored in a refrigerator at -80°C. The mice were sacrificed by cervical vertebra dislocation, the connective tissues around the spleen were collected, and the mononuclear cells were separated by immunomagnetic bead separation technique. The dorsal skin of mice was cut, and the thoracolumbar section of the spine was cut off. The spinal cord was flushed out by PBS and stored in liquid nitrogen.

Detection method

mRNA expression of NLRP3 inflammasome in spleen mononuclear cells and the spinal cord of EAE mice and mRNA expression of NF-κB-p65 in spinal cords were detected by RT-PCR. Total RNA of EAE mouse visceral mononuclear cells and spinal cords were extracted according to the instructions of trizol reagent, and the RNA concentration was measured. The extracted total RNA was synthesized into cRNA according to the instructions of the reverse transcription kit, and the reaction system was prepared for reverse transcription on the PCR

instrument. The reaction conditions were as follows: At 95°C for 30 sec, a total of 40 cycles, 95°C for 5 sec, and 60°C for 30 sec. The expression levels of NLRP3 mRNA and NF-κB-p65 were analyzed using the $2^{-\triangle\triangle Ct}$ formula.

Detection of NLRP3 inflammasome protein expression was calculated using Western blot. According to the protein extraction kit, RIPA protein lysate was added to extract and measure the total protein of EAE mouse visceral mononuclear cells and spinal cords, and the protein concentration was determined using the BCA method. 20μg protein was taken for SDS-PAGE electrophoresis, and the separation gel and concentrated gel were prepared. After electrophoresis, the membrane was transferred. The corresponding secondary antibody was added, DAB color rendering and exposure determined.

ELISA was used to detect the levels of IL-1β and IL-18 in the blood of mice: the experimental procedures were performed in strict accordance with the instructions of the kit, and the absorbance value was measured at 450nm wavelength by enzyme plate instrument.

Statistical methods

All measurement data in this study were expressed as ($\bar{x}\pm s$). The mean between the two groups was compared by independent sample t test, and the mean between multiple groups was compared by analysis of variance. The correlation between the NF-κB signaling pathway and the activation of NLRP3 inflammasome was analyzed by Pearson, and P<0.05 was considered statistically significant. All data in this study were analyzed using the SPSS20.0 software package.

Results

mRNA and protein expression of NLRP3 inflammasome in spleen mononuclear cells of EAE mice at different stages of EAE

There was no significant difference in the mRNA and protein expression of NLRP3 inflammasome in individual and cell spleens of EAE mice (P>0.05). See Table 1.

mRNA and protein expression of NLRP3 inflammasome in spinal cord of EAE mice at different stages of disease

The expression of NLRP3 inflammatory small body mRNA protein was significantly higher in the early and peak onset in EAE mice than in the control group (P<0.05), the mRNA and protein expressions of NLRP3 inflammasome in the spinal cord of EAE mice during the peak of onset were significantly higher than those in the early onset, and the difference was statistically significant (P<0.05). See Table 2.

Group	Sample (n)	NLRP3 mRNA	NLRP3
Control group	10	0.59±0.16	0.38±0.15
Early onset	10	0.63±0.11	0.41±0.18
Peak of onset	10	0.57±0.22	0.40±0.16
P	0.406	< 0.001	< 0.001

Table 1: mRNA and protein expression of NLRP3 inflammasome in spleen mononuclear cells of EAE mice at different stages of EAE ($\bar{x}\pm s$).

Group	Sample (n)	NLRP3 mRNA	NLRP3
Control group	10	0.03±0.01	0.30±0.11
Early onset	10	0.19±0.08*	0.48±0.17*
Peak of onset	10	0.35±0.11*#	0.60±0.16*#

Table 2: mRNA and protein expression of NLRP3 inflammasome in spinal cord of EAE mice at different stages of EAE ($\bar{x}\pm s$).
*Note: Compared with the control group, *P<0.05; compared with the beginning of the disease, #P<0.05.*

The level of NF-κB in the spinal cord of EAE mice at different stages of disease

The ratio of p-NF-κB-p65/NF-κB-p65 in the spinal cord of EAE mice was significantly higher than that in the control group (P<0.05), the ratio of p-NF-κB-p65/ NF-κB-p65 in the spinal cord of EAE mice in the peak of onset was significantly higher than that in the early onset, and the difference was statistically significant (P<0.05), as shown in Table 3. Pearson analysis showed that the expression of NLRP3 inflammasome was significantly positively correlated with the ratio of p-NF-κB-p65/ NF-κB-p65 in EAE mice (r=0.784, 0.614, P<0.05).

Group	Sample (n)	NLRP3 mRNA
Control group	10	0.03±0.01
Early onset	10	0.19±0.08*
Peak of onset	10	0.35±0.11*#

Table 3: The level of NF-κB in the spinal cord of EAE mice at different stages of disease ($\bar{x}\pm s$).
*Note: Compared with the control group, *P<0.05; compared with the beginning of the disease, #P<0.05.*

Levels of IL-1β and IL-18 in the blood of EAE mice at different stages of disease

The levels of IL-1β and IL-18 in EAE mice were significantly higher than those in control group (P<0.05), the levels of IL-1β and IL-18 in the blood of EAE mice during the peak of onset were significantly higher than those in the early onset, and the difference was statistically significant (P<0.05). See Table 4.

Group	Sample(n)	IL-1β (pg/ml)	IL-18 (pg/ml)
Control group	10	1.02±0.07	0.85±0.06
Early onset	10	4.35±1.47*	3.13±1.26*
Peak of onset	10	5.74±1.05*#	3.89±1.43*#

Table 4: Levels of IL-1β and IL-18 in blood of EAE mice at different stages of disease ($\bar{x}\pm s$).
*Note: Compared with the control group, *P<0.05; compared with the beginning of the disease, #P<0.05.*

Discussion

Multiple sclerosis is an autoimmune disease of the central nervous system, involving the brain nucleus and spinal cord. It is characterized by focal inflammatory response, white matter demyelination, loss of axonal nucleus neurons, paralysis of limbs, visual impairment, and paresthesia. Multiple sclerosis has a chronic course and tends to affect young people. In the late stages of the disease, it often results in severe neurological dysfunction, loss of work ability, and blindness, and many patients can only rely on ethical action[7]. At present, millions of people around the world suffer from this disease, with 50% of patients suffering from depression and a high suicide rate. It is one of the major neurological diseases leading to disability among young and middle-aged people, and at the same time it places a great burden on countries and governments and affects social and economic development. EAE, an animal model of multiple sclerosis, has a similar pathological basis and plays an important role in clinical neuroimmunology studies. It mainly uses MOG to attack the central nervous system and induce its own immune response, resulting in primary demyelination of axons and impaired axonal conduction of the central nervous system. Demyelinating lesions were observed in the white matter and spinal cord of the infected mice, accompanied by inflammatory infiltration and neurodegeneration, which were similar to the typical pathological changes of multiple sclerosis[8-9].

At present, cyclohexanone dihydrazone has been used clinically to establish an animal demyelination model, but due to the lesions, white matter, and the long modeling time, the clinical application is not widespread[10].

NLRP3 inflammatory corpuscle is a protein complex that plays a key role in the process of various diseases due to its natural inherent immunity as an important component, its induction as an immune response to perception and microbial attack damage signals, as well as other types of pathogens or danger signals, and its existence in the cytoplasm of peripheral immune cells, glial cells, nerve cells, and other cells[11]. In recent years, many studies have reported that inflammasomes (mainly NLRP3 inflammasomes) may play an important role in the process of central nervous system autoimmune demyelination, but the direct role is not fully understood[12]. Studies by foreign scholars have found that the levels of NLRP3 inflammasomes in the brain tissue and spinal cords of EAE rats are significantly higher than those in normal rats, suggesting that the inflammatory response mediated by NLRP3 inflammasomes may be involved in the pathogenesis of EAE[13]. Other scholars found that the levels of activated caspase-1 in spleen cells and central nervous systems and inflammatory factors in blood were significantly increased in EAE models, suggesting that NLRP3 inflammasomes can promote the maturation of caspase-1 and inflammatory factors[14]. The NF-κB signaling pathway is closely related to inflammatory response, and p65 plays an important role in its members. Relevant data have shown that inflammasomes can secrete exosomes to activate the NF-κB signaling pathway in macrophages and promote the immune response to autoimmune diseases[15].

In this study, EAE mice were established as the model of multiple sclerosis, and the expression of NLRP3 inflammasomes in spleen mononuclear cells and spinal cords of mice at different stages of disease was analyzed. It was found that mRNA and protein expression of NLRP3 inflammasomes in the spinal cords of EAE mice were significantly higher than those in the control group (P<0.05), which confirmed that the NLRP3 inflammasome was involved in the destruction of central nervous system lesions of EAE, but there was no difference in the expression of NLRP3 in splenic mononuclear cells. Further analysis of the levels of NF-κB in the spinal cord of EAE mice at different stages of disease showed that the ratio of P-NF-κB-p65/NF-κB-p65

in the spinal cord of EAE mice was significantly higher than that in the control group ($P<0.05$), and was positively correlated with the expression of the NLRP3 inflammasome, suggesting that NF-κB/NLRP3 was involved in the progression of EAE. The results of this study also showed that the levels of IL-1β and IL-18 in EAE mice were significantly higher than those in the control group ($P<0.05$), supporting the role of the NLRP3 inflammasome in the pathogenesis of EAE.

In conclusion, the level of NLRP3 inflammasomes in the spinal cords of EAE model mice was significantly high, and the blood activity of the NF-κB signaling pathway was significantly increased in the spinal cord, which is significantly positively correlated with the level of NLRP3 inflammasomes, and is synergically involved in the occurrence and development of EAE.

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