

Original Article

Inhibitory effect of interleukin-1 β antibody for NLRP3 inflammasome on epilepsy rat model

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Abstract: Background: Although the important role of interleukin-1 β (IL-1 β) in the pathogenesis of epilepsy had been evidenced by clinical survey, its molecular mechanisms details were still unclear; Objective: This study was to explore the inhibitory effects of NLRP3 inflammasome by IL-1 β maturation; Methods: The experiments were performed in rat model with temporal lobe epilepsy. Pilocarpine was used to establish an epilepsy rat model. Intervention followed intraperitoneally injection with IL-1 β -Ab, which could neutralize IL-1 β and block NLRP3 inflammasome. We detected the NLRP3, IL-1 β and NF-kB gene expression by RT-PCR and protein S100B expression by ELISA; Results: The IL-1 β antibody could cross the brain blood barrier (BBB), and reach hippocampus by high concentrations more than 3.00 μ g, for the treatment of epileptic rat model. It found that the expression of NLRP3, IL-1 β and NF-kB genes had decreased significantly after intervention by IL-1 β -Ab ($P < 0.05$). Simultaneously, the target protein S100B was decreased significantly too after intervention 72 hours in their serum; Conclusion: The IL-1 β antibody could relieve epileptic symptoms through inhibiting the expression of IL-1 β and NLRP3 gene, crossing through the BBB.

Keywords: IL-1 β , NLRP3, epilepsy, antibody intervention

Introduction

Epilepsy is one of common serious neurological malfunction, characterized by recurrent unprovoked seizures. It always accompanied with multitude of complications as cognitive, behavioral, and psychiatric disorders [1, 2]. There are almost 30% people suffered from intractable epilepsy disease, who had to take long-term medication with anti-convulsant drugs. The worse situations were much different epileptic pathological structures induced long-term clinical multi-drug resistant. On previous studies, it found more sophisticated pathophysiological mechanism changes and more participation of inflammation at subsequent stage of epilepsy. The involving inflammation was a complex process, including cytokine and chemokine, which could activate the gliosis [3, 4].

But, recent studies have showed that the inflammatory mediators could aggravate the apoptotic rate of nerve cells, and activated gliosis by inflammatory cytokine for double times [5, 6]. After analysis of surgical resection results, interleukin-1 β (IL-1 β) and interleukin-1

receptor 1 (IL-1R1) were at the higher level than normal, which supported that high expression levels of IL-1 β and IL-1R1 might play an important role in epileptic patients. Activated NALP3 in turn triggers an immune response. NLRP3 inflammasome is an essential factor for IL-1 β expression. It was consisted of two independent steps. The NF-kB as a transcription factor could reduce the signal transmission intensity of immune cells, when TLRs and IL-1R were activated by a nonnative precursor IL-1 β (pro-IL-1 β) [7, 8]. Then, NLRP3 released from the cytoplasm. And some kinds of cytokines and chemokine produced through paracrine and autocrine, related to high level of NF-kB gene expression [9-11].

Considering the cell signaling pathway of inflammatory, it might be effective that IL-1 β antibodies inhibited epilepsy-related autoimmune responses.

In this study, it used IL-1 β antibody as a main method of intervention. NLRP3 inflammasome activation was detected in an epilepsy rat model. It could find the difference of gene

IL-1 β inhibited NLRP3 on epilepsy rat model

Table 1. The primer sequences for RT-PCR

Gene	Primer sequence	Size (bp)
IL-1 β	F: 5'-GCCAACAAGTGGTATTCTCCA-3' R: 5'-TGCCGTCTTTCATCACACAG-3'	800
NF κ B	F: 5'-TGGGACGACACCTCTACACA-3' R: 5'-GGCTCAAAGTTCTCCACCAG-3'	300
NLRP3	F: 5'-GCAAGAGGTGCTGATGAAGA-3' R: 5'-CCTTCTCGGGTGGGTAATC-3'	166
ASC	F: 5'-TTGCTGGATGCTCTGTATGG-3' R: 5'-CCAAGTAGGGCTGTGTTTGC-3'	207
Caspase-1	F: 5'-AGATGCCAACCACTGAAAGG-3' R: 5'-GCATGATTCCCAACACAGGT-3'	885

expression and protein expression during treatment. All that finding would be helpful for the epilepsy treatment in the future.

Materials and methods

Experimental animal source

30 male Sprague-Dawley (SD) rats with 8 weeks old, whose weights from 180 to 220 g, were purchased from the LuKang Pharmaceutical Co. (Shandong, China). All rats were feeding under a strict sterile laminar flow sterile, by independent rearing room, with room temperature of 25°C and air humidity of 50%.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Jining Medical University (Permit Number: JNMULA201409-37279). All of the surgeries were performed under sodium pentobarbital with 10 mg/kg (i.m.) anesthesia, and all efforts were to minimize their suffering.

Constructing mode animals

All rats were injected (i.p.) lithium chloride with dose of 127 mg/kg and methylscopolamine with dose of 1 mg/kg. These drugs were purchased from Sigma-Aldrich, St. Louis, (MO, USA). Immunization program was implemented once every 12 hours.

After 24 hours later, they were administered injection (i.p.) with pilocarpine, according to the speed of 30 mg/kg every 30 minutes until their onset of generalized convulsive seizure activity.

The total number of pilocarpine injections was limited to three times injections per rat. Diazepam, which was purchased from Xupu Pharma (China), was taken to suppress seizure activity, after more 1 hour later, which was according to speed of 10 mg/kg (i.p.).

As for the control group, it was treated with normal saline instead of pilocarpine. All 30 rats were managed by using normal saline (4 ml i.p., b.i.d.) and 5% glucose (5 ml i.p., b.i.d.) for a few days until they resumed normal feeding behavior.

Drug intervention

It used IL-1 β antibody with concentration of 20 ug per 1 ml to treat the epilepsy animal, purchased from R&D Systems Inc. (Minneapolis, USA). The schedule of drug intervention was following as 1st, 7th and 14th day after administration of pilocarpine. The using dose and dosing interval for IL-1 β antibody was according to Professor Clausen's publication in 2011 [5].

Detection of important indicators

Antibody concentration detection: 12 rats were euthanized at 1st day after drug intervention by IL-1 β antibody (IL-1 β -Ab) for these epilepsy model animals. Then collected their hippocampus and weighted it freshly. Each hippocampus sample was homogenized in lysis buffer and PMSF (Beyotime Institute of Biotechnology No.p0013, No.ST506; Jiangsu, China), using a glass grinder for 30 min at room temperature. The supernatant was separated by centrifugation. Then IL-1 β -Ab concentration was detected by Western blot method. The supernatant was collected and analyzed using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then it transferred the proteins onto a polyvinylidene di-fluoride (PVDF) membrane (Millipore; California). The membrane was blocked with 5% BSA Tris-HCl buffered saline and 0.05% Tween-20 for 2 h at 25°C. It incubated with primary antibodies of mouse anti IL-1 β monoclonal antibody and secondary antibodies of rabbit anti mouse monoclonal antibodies. It calculated the WB Protein bands with GAPDH as internal standard using an enhanced chemiluminescence system (ECL, Bestbio, China). The PBS was used as a negative control.

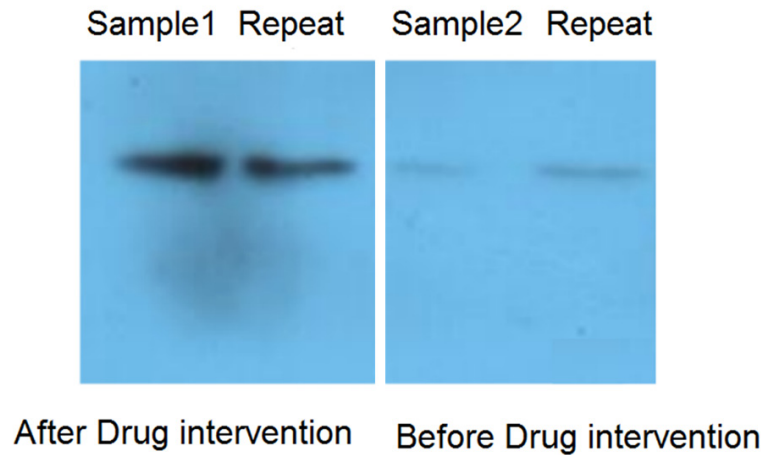


Figure 1. The results of Western blot before and after drug intervention.

Detection of mRNA expression level

Another 12 rats were euthanized at 14th day after drug intervention by IL-1 β -Ab. It collected their hippocampus tissues according to previously describe and extracted its mRNA according to the manual. It was used UNIQ-10 Column Trizol Total RNA Isolation Kit Trial (Sangon Biotech, Shanghai, China) to extract the nucleic acid. Tested and calculated the concentration of mRNA by UV spectrophotometer, and adjusted to 100 ng of total RNA for subsequent research with RT-PCR. All the used primers sequences were listed in **Table 1**. And the Fast Quant RT Kit with SYBR Green (TIANGEN biotech co. LTD, Beijing, China) was used for fluorescent labeling. The average threshold cycle (Ct) values were calculated.

Serological detection

It collected 2 ml arterial blood from the central abdominal aorta at 14th the day after drug intervention. Then separated the serum and detected the changes of S100B protein concentration using ELISA method. The S100B ELISA kits were purchased from CUSABIO (Wuhan, China). It implemented testing according to the manufacturer's instructions.

Histopathological examination

We detected the hippocampus tissue by histological and immuno-histochemical methods, to evaluate tissue loss and gliosis at 21st day after drug intervention by IL-1 β -Ab. Firstly, fixed the tissues in formalin for 5 min at 30°C, and sliced

it as 5 μ m thickness. After staining with 1% toluidine blue at 55°C for 2 minutes and blocking with ice-cold acetone at 25°C for 1 minute, it was examined by specific antibodies for chromogenic reaction. The examination was following that: blocked nonspecific binding by 10% normal goat serum (NGS) for 2 h, with Concentration of 1:400; incubated with mouse anti-rat glial fibrillary acidic protein (GFAP) as primary antibodies for 12 hour at 4°C; combined with rabbit anti-rat antibodies of anti-Iba1 (Abcam, ab5076) as secondary antibodies. Finally, it used the donkey anti-rabbit antibody (Alexa Fluor 488) as chromogenic antibody with concentration of 1:800. Under Olympus Fluor view laser scanning confocal microscope (LEICA, DM4000B, German), it collected the fluorescence signal and calculated the target protein expression.

Statistical analysis

Statistical analysis was performed using the SPSS software version 19.0 (Chicago, USA). All data are represented as means \pm SD ($\bar{x} \pm s$) of three or more independent experiments. The data are changed into normal distribution with logarithm if the original data are positive Skewness distribution. If the Data are homogenous, Analysis of variance, Student-Newman-Keulsa and Pearson's correlation will be used. If the data are not homogenous, Kruskal-Wallis, Games-Howell test, as well as Spearman's correlation analysis will be used. *P* Values less than 0.05 were considered to be statistically significant.

Results

Animal conditions

After construction of epilepsy model animals by lithium chloride and pilocarpine, their symptoms were consistent with the clinical manifestations of epilepsy, including syncope, hyperventilation, narcolepsy, panic attacks and psychogenic non-epileptic seizures (PNES). All these results suggested the construction of epilepsy animal model was successful.

IL-1 β inhibited NLRP3 on epilepsy rat model

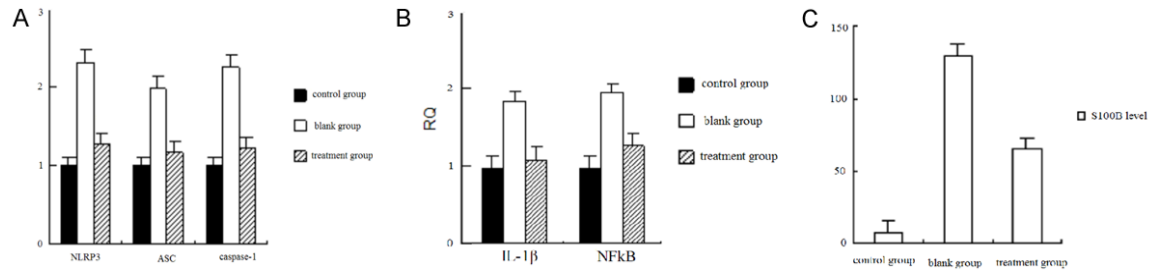


Figure 2. Differential expression of mRNA by RT-PCR testing. A: Higher level expression of NLRP3, ASC and caspase-1 mRNA, in epilepsy group than healthy control group ($P < 0.05$); B: Treatment with IL-1 β antibody was with statistically significant ($P < 0.05$); C: Serum level about S100B was higher in epilepsy group ($P < 0.001$).

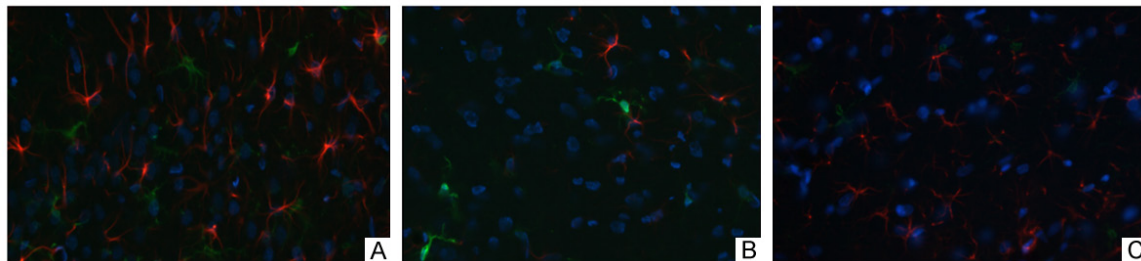


Figure 3. Immunohistochemical analysis of hippocampus. A: Compared with control group, only 48% of the neurons remained at the same area of hippocampus of rats in blank group; B: The treatment of IL-1 β antibody in the epileptic rats retained 77.8% of the neurons compared with the control group; C: Compared with control group, there were less activated microglia and astrocytes in the IL-1 β antibody treated group.

By drug intervention with IL-1 β antibody, their clinical symptoms were relieved, with normal eating, drinking, no severe fainting or twitch. This observation suggested that drug was effective in some extent.

Antibodies distributed in the hippocampus

Using IL-1 β -Ab to treat these epilepsy model animals, it detected the antibody concentration in hippocampus at the 1st day. There was a significantly increased about IL-1 β -Ab concentration in treatment group, compared with control group (**Figure 1**).

On the other hand, it means that antibody could cross the blood-brain barrier to achieve the purpose of treatment of certain disease. However, they did not produce sufficient amounts of antibody in the control group.

Differential expression of mRNA

Through the fluorescence real-time quantitative RT-PCR testing, it calculated the expression levels of NLRP3, ASC and caspase-1 genes between epilepsy model animal group and

healthy animal group; and calculated the expression levels of IL-1 β and NF- κ B genes in the drug intervention group, according to value of average Ct (**Figure 2A**). There was higher levels of NLRP3, ASC and caspase-1 mRNA expression, in epilepsy group than healthy animal group ($P < 0.05$), which indicated that NLRP3 inflammasome genes expressing had an important relationship with occurrence of epilepsy diseases.

By treating with IL-1 β -Ab, the expression of both IL-1 β and NF- κ B mRNA were at low levels, with 49.34% and 35.32% decreasing respectively. It meant the treatment with IL-1 β -Ab were statistically significant ($P < 0.05$) (**Figure 2B**).

Changes of S100B in serum

Since S100B was an important indicator of neurochemical, the changes of S100B in serum could reflect the degree of inflammatory response and the effect of IL-1 β antibody treatment. It found that S100B was higher in epilepsy group ($P < 0.001$) than the control group (**Figure 2C**). By comparing before and after drug intervention, it found that IL-1 β antibody could decrease its level by 50% ($P < 0.001$).

IL-1 β inhibited NLRP3 on epilepsy rat model

Immunohistochemistry analysis

Using the nissl-staining method, we could observe and calculate the different expression in hippocampal tissue. Compared with control group, there was only 48% neurons in situ retention in hippocampus of epilepsy model animal (45 ± 5 versus 22 ± 3 , $P < 0.001$, **Figure 3A**). However, after treatment with IL-1 β antibody, it retained 77.8% neurons, compared with the control group (45 ± 5 versus 35 ± 4 , $P < 0.01$, **Figure 3B**).

Furthermore, in order to analyze the effect of neutralization of IL-1 β antibody on glial activation for epilepsy disease, it detected the expression of the GFAP and Iba1, which were indicators for glial in the hippocampus. The expression of GFAP and Iba1 increased significantly in model animals compared with healthy animals (respectively 8 ± 3 and 32 ± 5 versus 2 ± 2 and 5 ± 3 , $P < 0.001$). On the other hand, there were less activated microglia and astrocytes in the IL-1 β antibody intervention group, compared with control group (respectively 3 ± 2 and 14 ± 4 versus 8 ± 3 and 32 ± 5 , $P < 0.001$, **Figure 3C**).

Discussion

Epilepsy is a group of neurological diseases characterized by epileptic seizures [11, 12]. Epileptic seizures are episodes that can vary from brief and nearly undetectable to long periods of vigorous shaking [13]. These episodes can cause physical injuries including occasionally broken bones [13]. In epilepsy, seizures tend to recur, and have no immediate underlying cause [14]. Isolated seizures that were provoked by a specific cause such as poisoning are not deemed to represent epilepsy [14]. People with epilepsy in some areas of the world experience stigma due to the condition [13]. The cause of most cases of epilepsy was unknown, although some people develop epilepsy as the result of brain injury, stroke, brain tumors, infections of the brain, and birth defects [15, 16]. Known genetic mutations were directly linked to a small proportion of cases [17-19]. Epileptic seizures are the result of excessive and abnormal nerve cell activity in the cortex of the brain [20]. The diagnosis involves ruling out other conditions that might cause similar symptoms such as fainting and determining if another cause of seizures is present.

IL-1 β is a member of the interleukin 1 family of cytokines. This cytokine is produced by activated macrophages as a pro-protein, which is processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. The induction of cyclooxygenase-2 (PTGS2/COX2) by this cytokine in the central nervous system (CNS) is found to contribute to inflammatory pain hypersensitivity. This gene and eight other interleukin 1 family genes form a cytokine gene cluster on chromosome 2 [21-23]. Increased production of IL-1B causes a number of different autoinflammatory syndromes, most notably the monogenic hybrid conditions referred to as CAPS, due to mutations in the inflammasome receptor NLRP3 which triggers processing of IL-1B [23, 24].

It was the first time to reveal the relationship between the IL-1 β antibody and epilepsy in this study. We constructed an epilepsy animal model by pilocarpine, and analyzed the inhibitory effect of interleukin-1 β antibody for NLRP3 inflammasome to treat the epilepsy rat. That IL-1 β antibody was in high penetration into the hippocampus, which could reduce the loss of glial activation in brain tissue, and S100B levels in serum. The cell signaling pathway might be affected by NLRP3 inflammasome to achieve. The IL-1 β and NF- κ B genes expression were at lower level in the drug intervention group.

As previously studies, intravenous, subcutaneous or intraperitoneal pathway for administration with β -amyloid, insulin-like growth factor-1 or interferon- γ could pass an intact BBB [12-15]. In our study, it found that antibody could cross the BBB to achieve the purpose of treatment.

Additionally, the NF- κ B mRNA was also increased in the hippocampal tissue from TLE patients and animal brains following epilepsy [17], similar to what was observed in our study. We observed that NLRP3, ASC and caspase-1 were significantly up-regulated in the experimental models, and neutralization of IL-1 β suppressed the NLRP3 inflammasome genes expression levels. Furthermore, some scientist found that IL-1 β had negative effects on astrocytes of brain tissue and reduced microglia

activation. These were consistent with the results of our study.

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Disclosure of conflict of interest

None.

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