Eriocalyxin B inhibits inflammation induced by CCI-induced microglia activation to relieve neuropathic pain through inhibition of JAK2/STAT3 and NF-κB pathways

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Abstract

Neuropathic pain is a very troublesome disease that seriously affects human life. Eriocalyxin B (ErIB) has been revealed to attenuate various diseases through its anti-inflammatory effects, but its regulatory effects on neuropathic pain remains unclear. The paw withdrawal threshold and paw withdrawal thermal latency were detected through mechanical allodynia and thermal hyperalgesia tests. The spinal injury was assessed through hematoxylin and eosin staining. The cell apoptosis was measured through terminal deoxynucleotide transferase-mediated dUTP nick end-labeling assay. The protein expressions were examined through Western blot analysis. The mRNA expression was examined through reverse transcription-quantitative polymerase chain reaction. The ionized calcium-binding adaptor molecule 1 level in the spinal cord was evaluated through immunofluorescence assay. The levels of tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 were measured through enzyme-linked-immunosorbent serologic assay. The chronic constriction injury (CCI) rat model was constructed for the study. Our results demonstrated that ErIB relieved CCI-stimulated neuropathic pain and nerve damage. In addition, the enhanced neural apoptosis mediated by CCI induction was reduced after ErIB treatment. In addition, ErIB inhibited CCI-induced microglia activity and inflammation. At last, the Janus kinase 2–signal transducer and activator of transcription 3 (JAK2/STAT3) and nuclear factor κB (NF-κB) pathways were activated in CCI rat model, which were attenuated following ErIB treatment. Importantly, ErIB (10 mg/kg) had a strong effect that was similar to the positive control (1-μg/kg dexmedetomidine), suggesting that ErIB may be an effective drug for neuropathic pain. This study demonstrated that ErIB inhibited inflammation caused by CCI-induced microglia activation to relieve neuropathic pain through inhibition of JAK2/STAT3 and NF-κB pathways. This study may highlight the regulatory functions of ErIB in the treatment of neuropathic pain.

Keywords: ErIB; inflammation; microglia activation; neuropathic pain; JAK2/STAT3 and NF-κB pathways

Introduction

Neuropathic pain is a common and persistent disease (Baron et al., 2010; Devor, 1991). The International Association for the Study of Pain (IASP) has defined neuropathic pain as a disorder of the somatosensory nervous system that disrupts the central nervous system (CNS) and/or the peripheral nervous system (Bouhassira, 2019). The pathogenesis of neuropathic pain is complex, and some studies have proved that tissue damage is the direct consequence of neuropathic pain that influences the nervous system and stimulate the ectopic discharge phenomenon of bypass conduction (Cohen and Mao, 2014; Guo et al., 2022). Nevertheless, the mechanism of neuropathic pain has not been fully understood; hence, most of the current treatments are based on narcotic drugs, such as dexmedetomidine, to attenuate discomfort (Huang et al., 2017; Mücke et al., 2018; Xu. and
Eriocalyxin B relieves neuropathic pain

Xu., 2021). Inflammation is a very remarkable feature of neuropathic pain; thus, reducing inflammation is the first step to relieve this disease. Therefore, seeking drugs that are more effective has become crucial.

In recent years, traditional Chinese medicine has played an increasingly important role in the treatment of neuropathic pain (Feng et al., 2014; Li et al., 2020). Eriocalyxin B (EriB) extracted from Isodon eriocalyx var is a biologically active ingredient. In traditional Chinese medicine, Isodon eriocalyx var has been used as a drug for anti-inflammatory treatment, and its extract EriB has also been developed as a drug for treating sore throats and inflammation (Leung et al., 2006; Niu et al., 2002). Studies have illustrated that EriB has anticaner effect, which can suppress cell proliferation, migration, invasion, and other malignant phenotypes in colon, pancreatic, and breast cancers (Duan et al., 2021; Li et al., 2012; Riaz et al., 2019). EriB exerts anti-inflammatory effects through inhibiting the differentiation of T helper 1 (Th1) and Th17 cells and the enhancement of reactive oxygen species (ROC), thereby improving autoimmune encephalomyelitis (Lu et al., 2013). Moreover, EriB exerts anti-inflammatory effects through selectively regulating the conversion of microglia to M2 phenotype (resolution of inflammation and tissue repair) by targeting the nuclear factor kappa B (NF-kB) signaling pathway, thereby alleviating Parkinson’s symptoms (Dou et al., 2018). However, the effects of EriB in neuropathic pain and its regulatory mechanism remain unclear.

Both Janus kinase 2–signal transducer and activator of transcription 3 (JAK2/STAT3) and NF-kB pathways have been revealed to participate in the progression of neuropathic pain (Fei et al., 2017; Popiolek-Barczyk and Mika, 2016; Song et al., 2021). However, whether EriB affects JAK2/STAT3 and NF-kB pathways to relieve neuropathic pain is unclear.

This study aimed to explore the regulatory role of EriB in the progression of neuropathic pain. Our study showed that EriB inhibited inflammation caused by chronic constriction injury (CCI)-induced microglia activation to relieve neuropathic pain through inhibition of JAK2/STAT3 and NF-kB pathways. These findings suggested that EriB could be a useful novel drug to treat neuropathic pain.

Materials and methods

CCI rat model

Male Sprague-Dawley (SD) mice (aged 6–8 weeks, n = 30) were acquired for experiments (Vital River, Beijing, China). Free food and water were supplied to animals, and they were kept at 25°C into a 12-h light/dark cycle. All animal experiments were performed in line with the guidelines of the Animal Experiments Ethics Committee of Changzhou Traditional Chinese Medicine Hospital.

Mice (n = 6 in each group) were randomly separated into the following five groups: Sham, CCI, CCI+5-mg/kg EriB, CCI+10-mg/kg EriB, and CCI+1-μg/kg dexamethasone. The CCI-induced rat model for neuropathic pain was created as described by Zhang et al. (2019). After anesthetizing with pentobarbital sodium (40 mg/kg), mice in the CCI group were subjected to expose sciatic nerves, and ligated with 4-0 chromic gut suture (Ethicon Inc., Cincinnati, USA), to induce CCI. EriB (5 or 10 mg/kg) or dexamethasone (1 μg/kg) was injected intraperitoneally to CCI mice for 7 days post-operation. Mice in the sham group were subjected to the same surgery but no sciatic nerve ligation was made. Finally, mice were euthanized with pentobarbital sodium (160 mg/kg). The L4–L6 spinal cord was removed from euthanized animals for additional experiments. EriB (B30248, purity >98%) was bought from Shanghai YuanYe Biotechnology Co (Shanghai, China).

Mechanical allodynia and thermal hyperalgesia tests

The paw withdrawal threshold (PWT) was measured through von Frey hair stimulation (Stoelting Co.) to assess mechanical allodynia (Inoue et al., 2009). von Frey filaments were put into the hind paw’s dorsal surface with sufficient force. When mice withdrew their paws, PWT was recorded in terms of pressure (g). Each trial was done for six times at 3-min interval.

The paw withdrawal thermal latency (PWL) was examined to evaluate thermal hyperalgesia (Zheng et al., 2019). Briefly, the infrared light beam from a modified Hargreaves device (Ugo Basile SRL) was irradiated to the hind paw’s plantar surface. Subsequently, when mice withdrew their paws, the PWL was recorded. All experimental mice underwent thermal and mechanical pain tests at 0, 3, 7, 14, and 21 days post-operation.

Hematoxylin and eosin (H&E) staining

The collected spinal cords were fixed in 4% paraformaldehyde. Next, the spinal cords embedded in paraffin and dehydrated, and cut into 5-μm sections. All sections were stained in H&E solution. Finally, histopathologic changes were observed under a light microscope.

The scoring criteria of spinal cord injury were as described by Shan et al. (2021) as follows:

0 = no lesion;
1 = gray matter containing 1–5 eosinophilic neurons;
2 = gray matter containing 6–10 eosinophilic neurons; 
3 = gray matter containing >10 eosinophilic neurons; 
4 = infarction of <1/3 of the gray matter area; 
5 = infarction of 1/3–1/2 of the gray matter area; and 
6 = infarction of >1/2 of the gray matter area.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

The in situ cell death detection kit (Cat. No. 11684817910; Roche, Basel, Switzerland) was applied for measuring cell apoptosis. Sections of the spinal cord were permeabilized through Triton X-100 (0.1%) and sodium citrate. The sections were then incubated with TUNEL staining solution at 37°C in darkness for 1 h. After washing, incubation with converter-peroxidase (POD) and diaminobenzidine (DAB) was performed for spinal cord sections. 4',6-Diamidino-2-phenylindole (DAPI) was used for staining of nucleus. Eventually, the fluorescent images were developed under fluorescence microscope (Olympus, Tokyo, Japan).

Western blot analysis

Proteins were isolated from spinal cords through radioimmunoprecipitation (RIPA) lysis assay buffer (Thermo Fisher Scientific, MA). These proteins were separated through 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Beyotime, Shanghai, China). After blocking, the membranes were incubated at 4°C for 12 h with the following primary antibodies: B-cell lymphoma-2 (BCL-2, 1:1,000; ab196495; Abcam, Shanghai, China), Bcl-2-associated X (BAX 1:2,000; ab182733), cleaved caspase-3 (1:5,000; ab214430), cluster of differentiation (CD) 86 cells (1 µg/mL; ab112490), CD206 (1 µg/ml; ab64693), phospho Janus kinase 2 (p-JAK2, 1:1,000; ab32101), (JAK2, 1:5,000; ab108596), p-signal transducer and activator of transcription 3 (STAT3, 1:1,000; ab32143), STAT3 (1:1,000; ab68153), and β-actin (1 µg/mL; ab8226). Next, the appropriate secondary antibodies (1:2,000; ab7090; Abcam) were added to membranes. Ultimately, the chemiluminescence detection kit (Thermo Fisher Scientific Inc.) was used for evaluating the blots (Rahmati and Taherabadi, 2021).

Immunofluorescence (IF) staining

After washing, sections of the spinal cords were blocked in bovine serum albumin phosphate–buffered saline solution (BSA/PBS, 3%) and fetal bovine serum (FBS)/PBS (10%). Next, the sections were incubated with primary antibody of ionized calcium-binding adaptor molecule 1 (IBA1; 0.5 µg/mL, ab178846; Abcam) for 12 h at 4°C. The secondary antibodies (1:1,000, ab150080) were mixed with sections for 2 h post-washing. Fluorescence microscope (Olympus) was utilized for acquiring fluorescent images (Bostani et al., 2020; Rahmati and Rashno, 2021).

Enzyme Linked Immunosorbent Serologic Assay (ELISA)

The levels of tumor necrosis factor-α (TNF-α; ab236712), interleukin (IL)-1β (ab255730), and IL-6 (ab234570) in serum were examined by using commercial ELISA kits (Abcam) in line with the manufacturer’s instructions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The isolation of RNAs from the spinal cords of mice was conducted using the Trizol reagent (Termo Fisher, Waltham, MA). The synthesis from RNAs to complementary DNA (cDNA) was performed through the PrimeScript™ RT reagent kit (Takara, Dalian, China), and RT-qPCR was executed using the SYBR Green PCR kit (Toyobo, Japan). The calculation of mRNA expressions was done through the 2ΔΔCt method, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) acting as an internal control. The primers are listed in Table 1.

Statistical analysis

GraphPad Prism 9.0 (GraphPad Software Inc.) was employed for statistical analysis. The data were presented as mean ± standard deviation (SD). Comparisons in the groups (two or multiple) were evaluated through the Student’s t-test or one-way ANOVA with Tukey’s post hoc test. Normality and variance homogeneity was checked for all cases. P < 0.05 was considered as statistically significant.

Table 1. The sequences of primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<tr>
<td>TNF-α</td>
<td>F: 5’-GAA ACA CAC GAG ACG CTG AA-3’</td>
<td>R: 5’-AGG GAG GCC TGA GAC ATC TT-3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>F: 5’-TAC AGG GTC CCA GAT GAA CAA C-3’</td>
<td>R: 5’-TTT GAG GCC CAA GGC CAC AG-3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>F: 5’-CCA GAA ACC GCT ATG AAG TTC C-3’</td>
<td>R: 5’-GTGGGAGTGGATCTCTCTCTGA-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5’-ACC ACA GTC CAT GCC ATC AC-3’</td>
<td>R: 5’TCC ACC CTG TTG CTG TA-3’</td>
</tr>
</tbody>
</table>
Results

EriB relieved CCI-stimulated neuropathic pain and nerve damage

At first, the CCI rat model for neuropathic pain was constructed. In Figure 1A, the PWT decreased in CCI rat model, but this change reversed after treatment with EriB (5 mg/kg or 10 mg/kg) or dexmedetomidine (1 μg/kg). Moreover, the PWL demonstrated similar results (Figure 1B). H&E staining showed that nerve fiber swelling and fiber structure disorder were observed in the CCI model group, but they were restored after EriB or dexmedetomidine treatment (Figure 1C). In addition, histopathology score was strengthened in the CCI model group, but it was reduced following EriB or dexmedetomidine treatment (Figure 1D). Importantly, EriB (10 mg/kg) had a strong effect, which was similar to the positive control (1-μg/kg dexmedetomidine). These findings indicated that EriB relieved CCI-stimulated neuropathic pain and nerve damage.

EriB suppressed CCI-mediated neural apoptosis

Cell apoptosis was enhanced in CCI rat model, but it was weakened after EriB or dexmedetomidine treatment (Figure 2A). In addition, the BCL-2 protein expression was decreased, while the BAX and cleaved caspase-3 protein expressions were increased in CCI rat model, but these changes were attenuated by EriB or dexmedetomidine treatment (Figure 2B). The effect of EriB (10 mg/kg) was similar to the positive control (1-μg/kg dexmedetomidine). Taken together, EriB suppressed CCI-mediated neural apoptosis.

EriB inhibited CCI-induced microglia activity

As displayed in Figure 3A, the IBA1 level was enhanced in CCI rat model, but this effect was reduced following EriB or dexmedetomidine treatment. In addition, the CD86 protein expression was increased in CCI rat model, but this effect was also reduced following EriB or dexmedetomidine treatment. These findings indicated that EriB inhibited CCI-induced microglia activity.

Figure 1. EriB relieved CCI-stimulated neuropathic pain and nerve damage. Mice were divided into sham, CCI, CCI+5-mg/kg EriB, CCI+10-mg/kg EriB, CCI+1-μg/kg dexmedetomidine groups. (A) PWT was observed through mechanical allodynia test. (B) PWL was examined through thermal hyperalgesia test. (C) Spinal injury was assessed through H&E staining. (D) Histopathology score was also verified. ***P < 0.001 vs the sham group; #P < 0.05, ###P < 0.001 vs the CCI group.

Figure 2. EriB suppressed CCI-mediated neural apoptosis. Mice were divided into sham, CCI, CCI+5-mg/kg EriB, CCI+10-mg/kg EriB, and CCI+1-μg/kg dexmedetomidine groups. (A) Cell apoptosis in the spinal cord was measured by TUNEL assay. (B) The protein expressions of BCL-2, BAX, and Cleaved caspase-3 were assessed by Western blot analysis. ***P < 0.001 vs the sham group; #P < 0.05, ###P < 0.001 vs the CCI group.
this change was weakened after EriB or dexmedetomidine treatment. The CD206 protein expression did not change after CCI induction and was increased after EriB or dexmedetomidine treatment (Figure 3B). Compared to the positive control (1-μg/kg dexmedetomidine), EriB (10 mg/kg) had the similar attenuated effects. These data confirmed that EriB inhibited CCI-induced microglia activity.

**EriB reduced CCI-triggered inflammation**

Results from ELISA indicated that the levels of TNF-α, IL-1β, and IL-6 were increased in CCI rat model, but these changes were attenuated following EriB or dexmedetomidine treatment (Figure 4A). In addition, the mRNA expressions of TNF-α, IL-1β, and IL-6 also had similar changes (Figure 4B). The remission effects of EriB (10 mg/kg) were similar to the positive control (1-μg/kg dexmedetomidine). In short, EriB reduced CCI-triggered inflammation.

**EriB retarded JAK2/STAT3 and NF-κB pathways**

Finally, the effects of EriB were investigated on JAK2/STAT3 and NF-κB pathways. Figure 5A illustrated that p-JAK2/JAK2 and p-STAT3/STAT3 levels were increased in CCI rat model, but these effects were relieved after EriB or dexmedetomidine treatment. Similarly, the p-P65/P65 and p-IκBα/IκBα levels were enhanced in CCI model. The CD206 protein expression did not change after CCI induction and was increased after EriB or dexmedetomidine treatment (Figure 3B). Compared to the positive control (1-μg/kg dexmedetomidine), EriB (10 mg/kg) had the similar attenuated effects. These data confirmed that EriB inhibited CCI-induced microglia activity.
Eriocalyxin B relieves neuropathic pain

Figure 5. EriB retarded JAK2/STAT3 and NF-κB pathways. Mice were divided into sham, CCI, CCI+5-mg/kg EriB, CCI+10-mg/kg EriB, and CCI+1-μg/kg dexmedetomidine groups. (A) The protein expressions of p-JAK2, JAK2, p-STAT3, and STAT3 were assessed by Western blot analysis. (B) The protein expressions of p-P65, P65, p-IκBα, and IκBα were evaluated by Western blot analysis. ***P < 0.001 vs the sham group; ###P < 0.001 vs the CCI group.

Discussion

Neuropathic pain is caused by nerve inflammation, nerve damage, virus infection, and other factors (Gierthmühlen and Baron, 2016). More and more Chinese herb extracts have been investigated to ameliorate neuropathic pain (Luo et al., 2020; Xu et al., 2016). EriB was discovered to have regulatory effects on some diseases (Dou et al., 2018; Duan et al., 2021; Leung et al., 2006; Li et al., 2012; Lu et al., 2013; Riaz et al., 2019), but its roles in neuropathic pain remained unclear. In this study, a CCI rat model was constructed. Our results demonstrated that EriB relieved CCI-stimulated neuropathic pain and nerve damage. Moreover, the enhanced neural apoptosis mediated by CCI induction was reduced after EriB treatment.

Microglia are vital glial cells in the spinal cord that contribute to sensitize and maintain chronic pain (Prinz and
Priller, 2014). Cortical and spinal microglial cells are key immune components with important functions in regulating inflammatory responses in the CNS (Fujita and Yamashita, 2019; Streit, 1993). When immune homeostasis is disrupted, microglia change into two extremely polarization states: M1 phenotype (pro-inflammatory) and M2 phenotype (Orihuela et al., 2016). Studies have revealed that activated microglia stimulates pro-inflammatory response and produces pro-inflammatory factors, such as TNF-α, IL-1β, and ROS, which additionally contribute to neuronal damage, thus inducing neuropathic pain (Wu et al., 2020; Zheng et al., 2019). In addition, a previous study has demonstrated that blocking of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways could suppress the activity of microglia BV2, and decreased neuroinflammatory factors to relieve neuropathic pain (Choi et al., 2012). In this study, our experiments manifested that EriB inhibited CCI-induced microglia activity and inflammation.

The JAK2/STAT3 and NF-κb pathways also have been shown to take part into the regulation of neuropathic pain progression. For instance, red nucleus IL-6 modulates JAK/STAT3 and ERK signaling pathways to affect the maintenance of neuropathic pain (Ding et al., 2018). In addition, ligustrazine retards JAK/STAT3 pathway to relieve neuropathic pain (Wang et al., 2016). Astaxanthin suppresses MAPK and NF-κb pathways to decrease neuropathic pain (Zhao et al., 2021). Moreover, gallic acid represses the P2X7 receptor-mediated NF-κb/STAT3 pathways to mitigate neuropathic pain behaviors (Yang et al., 2021). Moreover, valproic acid affects STAT1/NF-κb and JAK2/STAT3 signaling pathways to regulate microglial function and reduce neuroinflammatory response, thereby relieving spinal nerve ligation-triggered neuropathic pain (Guo et al., 2021). In our study, the results showed that JAK2/STAT3 and NF-κb pathways were activated in CCI rat model, but these were attenuated following EriB treatment. Importantly, EriB (10 mg/kg) had the similar strong effects with the positive control (1-μg/kg dexmedetomidine), suggesting that EriB may be a useful drug to treat neuropathic pain.

Conclusion

This study demonstrated that EriB inhibited inflammation caused by CCI-induced microglia activation to relieve neuropathic pain through inhibition of JAK2/STAT3 and NF-κb pathways. However, this study also had some limitations for the regulatory functions of EriB on neuropathic pain progression, such as lacking other phenotypes and human samples. More experiments are required in the future to foster probe the other roles of EriB in neuropathic pain.

Availability of Data and Materials

All data generated or analyzed in this study are included in this published article. The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Competing Interests

The authors stated that they had no conflict of interest to declare.

Author Contributions

Zhifeng Sheng designed the study, completed the experiment, and supervised data collection. Xiaoyan Pan analyzed and interpreted the data. Both authors prepared the manuscript for publication and reviewed its draft. Both authors read and approved the final manuscript.

References


206 Quality Assurance and Safety of Crops & Foods 15 (2)