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Original Research Article

Eriocalyxin B relieves kidney damage in a rat model of membranous nephropathy by inhibiting JAK/STAT3 pathway

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Abstract

Purpose: *To investigate the function of eriocalyxin B (EriB) in membranous nephropathy (MN) in a rat model and the related mechanisms.*
Methods: *Cationic C-BSA was injected into Sprague-Dawley (SD) rats to generate a rat model of MN. The MN rats were treated with different concentrations of EriB (2.5, 5, and 10 mg/kg). After treatment, their kidneys were excised, sectioned, and hematoxylin and eosin (H&E) stained to determine renal injury, while Masson's trichrome staining was performed to determine renal tissue fibrosis. Bradford assay was used to determine rat proteinuria and ELISA employed to determine blood urea nitrogen (BUN) and serum creatinine (SCr) levels. ELISA was also used to measure IL-1β, IL-6, and TGF-α levels, while immunoblot analysis was used to assess protein expressions of related indicators in JAK/STAT3 pathway.*
Results: *Compared with sham rats, MN rats showed increased the contents of proteinuria, BUN, and SCr ($p < 0.01$), induced serious renal injury, and increased expression of collagen I and III ($p < 0.01$), and fibrosis. It also elevated the levels of IL-1β, IL-6, and TGF-α ($p < 0.01$), causing inflammation and activation of the JAK/STAT3 pathway. EriB treatment alleviated renal injury, fibrosis, and inflammation in MN rats. With increase in EriB concentration, renal injury in MN rats was gradually alleviated.*
Conclusion: *EriB alleviates renal injury in MN rats by inhibiting JAK/STAT3 pathway. Thus, EriB is a potential drug for the management of MN.*

Keywords: *Membranous nephropathy, Eriocalyxin B, JAK/STAT3 pathway, Fibrosis, Inflammatory response*

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INTRODUCTION

Membranous nephropathy (MN), also known as membranous glomerulopathy, is a common cause of nephrotic syndrome in adults worldwide [1]. The MN is characterized by the presence of immune complex deposits between the glomerular basement membrane and podocytes [2]. A new therapeutic strategy is needed to reduce the risk of chronic nephrotoxicity.

Eriocalyxin B (EriB) is a biologically active

substance extracted from *Isodon eriocalyx* var. I. Eriocalyx has long been used in traditional Chinese medicine as an anti-inflammatory agent, and EriB has been used to treat sore throat and inflammation [3]. EriB also has anti-cancer effects and can inhibit the proliferation, migration, invasion, and other malignant phenotypes of malignant tumor cells, such as pancreatic cancer cells [4].

In terms of the anti-inflammatory effect, it has been illustrated that EriB treatment has an alleviating effect on experimental autoimmune encephalomyelitis and can reduce the production of reactive oxygen species [5]. The EriB reduced the degree of inflammation and the severity of pelvic pain in a mouse model of prostatitis [6]. In Parkinson's disease treatment, EriB selectively regulated microglial activation, thereby exerting an effective anti-inflammatory effect, which eliminated neuronal damage and relieved symptoms [7]. However, few studies have reported the effect of EriB on chronic kidney diseases, such as uremia, and the related mechanism remains unclear.

It has been shown that EriB inhibits the JAK/STAT3 pathway [8,9] and that activation of the STAT3 pathway contributes to chronic kidney disease progression and promotes the onset of renal fibrosis [10]. Thus, blocking the STAT3 pathway may inhibit the inflammatory response and subsequent kidney damage [11]. This study aimed to investigate the function of EriB in MN using a rat model and to discuss the related mechanisms.

EXPERIMENTAL

Establishment of a rat MN model

Rat treatments were approved by the Ethics Committee of Changzhou Second People's Hospital Affiliated with Nanjing Medical University (approval no. 2021001) and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [12]. Adult male Sprague-Dawley (SD) rats (Chengdu Dossy Experimental Animal Co. Ltd., China) that weighed an average of 225 ± 20 g, were divided randomly into sham and MN groups. Rats in the MN group were injected intravenously with cationic C-BSA daily for 28 days. The MN was induced by gradually increasing the C-BSA dose from days 1 – 7 (1, 1, 1, 1.5, 1.5, 2, and 2 mg) followed by a constant dose of 2.5 mg over the next 3 weeks. The MN status of the rats was confirmed by measuring 24-h proteinuria using a Bradford assay kit (Sigma-Aldrich, MO, USA) [13].

EriB treatment

The MN rats were injected intravenously with EriB (QCHENG Bio, Shanghai, China, 2.5, 5, or 10 mg/kg) daily from days 14 – 28 [6]. The SD rats were divided into sham, MN, MN + 2.5 mg/kg EriB, MN + 5 mg/kg EriB, and MN + 10 mg/kg EriB groups with 6 rats in each group. On day 27, the rats were placed in metabolic cages and urine was collected. After interference treatment, the rats were euthanized and blood was collected via cardiac puncture. Kidneys were excised for further studies.

Kidney function and inflammation analysis

The amount of protein in urine was determined using the Bradford assay (Biolab Technology Co. Ltd., Beijing, China). A Beckman DU-650 spectrophotometer was used to measure absorbances of samples that were diluted at 1:100. A standard curve was prepared with bovine serum albumin. Blood urea nitrogen (BUN) and serum creatinine (SCr) in serum were determined using the ELISA kits (Shanghai Jun Yu Biotechnology Co. Ltd., Shanghai, China). The IL-1β, IL-6, and TGF-α in kidney homogenates were determined using ELISA kits (SPBio, Wuhan, China).

Histopathological analysis

Kidneys were fixed with 4 % paraformaldehyde and sectioned into 5 μm paraffin slices. For Hematoxylin and Eosin (HE) staining, slices were stained with hematoxylin staining solution for 5 min, immersed in 0.2 % ammonia or saturated lithium carbonate for 30 sec, and counterstained with eosin solution. For Masson's trichrome staining, slices were stained with hematoxylin solution for 8 min, stained with ponceau acid fuchsin solution for 5 min, differentiated with phosphomolybdenum-phosphotungstic acid for 5 min, stained with aniline blue solution for 5 min, and then treated with 0.2 % acetic acid for 2 min. Finally, the sections were observed under a microscope.

Immunoblot analysis

Tissues were lysed with RIPA lysis buffers and protein concentrations were quantified using the BCA method. Proteins were separated with 12 % SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with the following primary antibodies: anti-collagen I (1:1000, ab34710, Abcam), anti-collagen II (1:1000, ab188570), anti-p-p65 (1:1000, ab76302), anti-p65 (0.5 μg/mL, ab16502), anti-p-IκBα (1:10000, ab133462), anti-

IκBα (1:1000, ab32518), p-JAK2 (1:1000, ab32101), JAK2 (1:5000, ab108596), p-STAT3 (1:1000, ab267373), STAT3 (1:1000, ab68153), and anti-β-actin (1:1000, ab8227) overnight. After TBST washing (three times), a secondary antibody (1:1000, Cell Signaling, MA, USA) was added for incubation at room temperature for 3 h. After TBST washing, enhanced chemiluminescence was used to develop protein blots. The images captured by a gel imager were analyzed using Image J software. β-actin was an internal reference to determine the relative expression levels of each target protein.

Statistical analysis

The SPSS 22.0 software was used for data analysis. Data are expressed as mean ± standard deviation (SD). One-Way ANOVA and Tukey's multiple comparisons test were used to compare multiple sets of data. *P*-value < 0.05 indicated statistical significance.

RESULTS

EriB treatment reduced renal injury in a rat model of MN

The H&E staining showed inflammatory infiltration and tubular dilation in MN rats, and these pathological conditions were alleviated after EriB treatment (Figure 1 a). Protein in urine was measured by the Bradford method, and an increase in proteinuria was found in MN rats; however, the concentration of protein in the urine reduced in MN rats after treatment with EriB (Figure 1 b). Interestingly, as the concentration of EriB increased, BUN and SCr levels decreased gradually (Figure 1 c). In general, renal injury in MN rats was severe. However, EriB treatment has an alleviating effect on MN rats to reduce renal injury.

EriB treatment reduced renal fibrosis

Masson's trichrome staining was performed on rat kidney tissue to observe renal fibrosis. EriB treatment reduced fibrotic lesions in the kidneys of MN rats in a concentration-dependent manner (Figure 2 a). Levels of the fibrosis-related indicators collagen I and collagen III were analyzed using the immunoblot analysis method. Protein expressions of collagen I and collagen III were increased in MN rats. Collagen I and collagen III protein expressions were decreased in a concentration-dependent manner after EriB treatment (Figure 2 b). Overall, EriB treatment ameliorated fibrotic lesions in the renal tissues of MN rats.

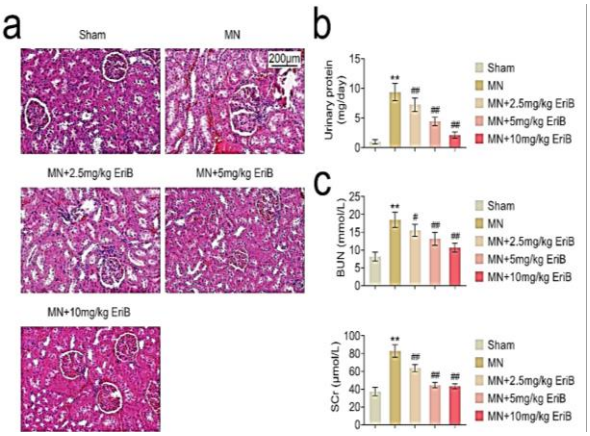

Figure 1: EriB treatment reduces renal tissue damage in a rat model of MN. a: Renal tissues stained with H&E solution to observe renal injury; b: Urinary protein measured by the Bradford method; c: BUN and SCr. n = 6, $^{**}p < 0.01$ vs. sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. MN. Data was expressed as mean ± SD

EriB treatment alleviated renal tissue inflammatory response in a rat model of MN

The ELISA was used to monitor levels of inflammatory indicators IL-1β, IL-6, and TGF-α in MN rats and immunoblot analysis was used to evaluate the protein expression of p-p65, p65, p-IκBα, and IκBα. All of these indicators were elevated with the onset of MN in rats, but EriB treatment reduced their levels in a concentration-dependent manner (Figure 3 a and b). EriB treatment alleviated the inflammatory response in the renal tissues of MN rats.

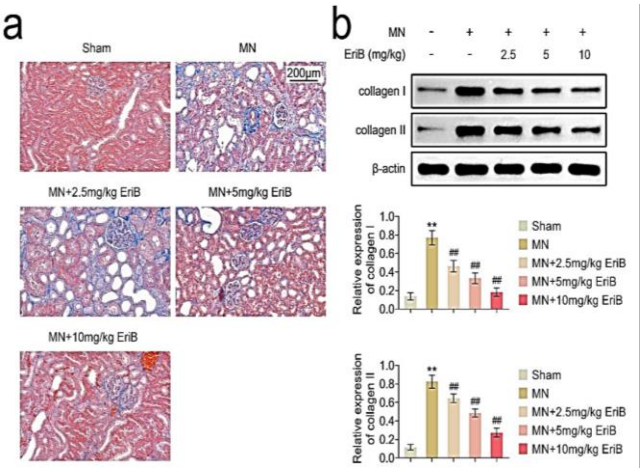

Figure 2: EriB treatment reduces renal fibrosis in a rat model of MN. a: Masson's trichrome staining of kidney tissue; b: immunoblot analysis of protein expressions of collagen I and collagen III. n = 6, $^{**}p < 0.01$ vs. sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. MN. Data are mean ± SD

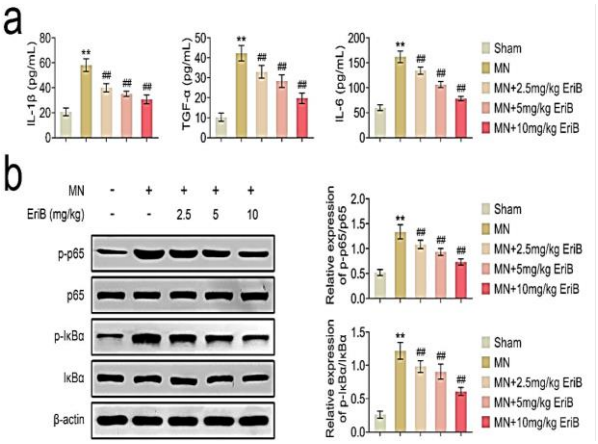

Figure 3: EriB treatment alleviated the inflammatory response in renal tissue in a rat model of MN. a: IL-1β, IL-6, and TGF-α in kidney homogenates measured by the ELISA kits; b: immunoblot analysis of protein expressions of p-p65, p65, p-IκBα, and IκBα. n = 6, ***p* < 0.01 vs. sham; #*p* < 0.05, ##*p* < 0.01 vs. MN. Data are expressed as mean ± SD

EriB inhibited the JAK/STAT3 pathway

Activation of the STAT3 pathway is associated with chronic kidney disease progression and can promote renal fibrosis. In this study, protein expressions of p-JAK2, JAK2, p-STAT3, and STAT3 were measured in the kidney tissues of MN rats. The outcomes demonstrated that the JAK/STAT3 pathway was overactivated in MN rats. With the increase of EriB concentration, JAK/STAT3 pathway was gradually inhibited in MN rats (Figure 4). In brief, EriB can inhibit the JAK/STAT3 pathway and therefore exert an treatment effect on MN.

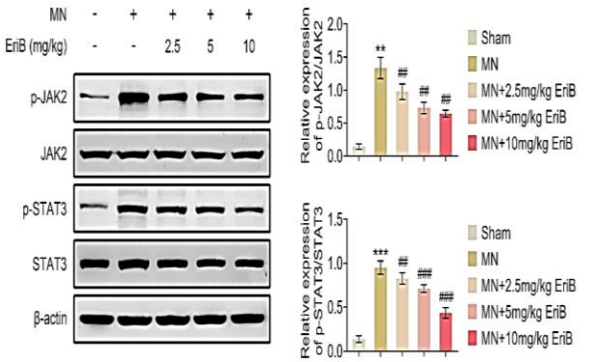

Figure 4: EriB inhibited the activation of JAK/STAT3 pathway. Immunoblot analysis of protein expressions of p-JAK2, JAK2, p-STAT3, and STAT3. n = 6, ***p* < 0.01 vs. sham; #*p* < 0.05, ##*p* < 0.01 vs. MN. Data was expressed as mean ± SD

DISCUSSION

The MN is the primary glomerular disease that recurs after kidney transplantation. At present, effective treatments for MN are lacking and disease activity cannot be predicted and diagnosed. This study focused on the efficacy of EriB and ultimately discussed how EriB attenuates MN. The EriB inhibits the JAK/STAT3 pathway, thereby alleviating renal injury in a rat model of MN.

The BUN is a metabolite of the human body excreted through the kidneys, and high BUN levels associate closely with damage to renal function. The SCr is derived from the metabolism of exogenous and endogenous proteins and is excreted mainly through the kidneys. The SCr levels are normally maintained at a relatively stable level. Typically, BUN and SCr are the indicators used to assess renal function [14,15]. Thus, this study investigated the effects of EriB on MN by measuring BUN and SCr levels and found that EriB treatment decreased BUN and SCr levels and alleviated kidney pathologies in MN rats in a concentration-dependent manner. Until now, the effects of EriB on renal function and pathological injury have not been reported. However, EriB has an alleviating effect on inflammation and pelvic pain in autoimmune prostatitis [6] and dopaminergic neuron injury [7].

Renal fibrosis is a pathophysiological change of kidney function injury and loss. Due to the stimulation of various pathogenic factors, the inherent cells of the kidney are damaged and a large amount of collagen is deposited and accumulates resulting in the gradual hardening of the renal parenchyma and scar formation. The MN rats developed renal fibrosis and protein expression of collagen I/III increased. However, EriB treatment attenuated fibrotic lesions in the kidney in a concentration-dependent manner. To the best of our knowledge, until now, the anti-fibrotic effects of EriB had not been studied, thus our results are novel.

IL-1β and TNF-α are cytokines produced by cells in response to infection, and IL-6 is a lymphokine produced by activated T cells and fibroblasts. The three are all pro-inflammatory cytokines and are inflammatory markers in membranous glomerulonephritis. IL-6 and TNF-α levels were found to be higher in patients with MN [16], which is consistent with our results. Proteins p-p65, p65, p-IκBα, and IκBα are involved in nuclear factor κB (NF-κB) signaling, and inhibition of the NF-κB pathway may be a possible mechanism for the treatment of MN [17]. In this study, increased levels of IL-1β, IL-6, and TGF-α and increased ratios of p-p65/p65 and p-IκBα/IκBα indicated that inflammatory responses were activated in MN rats. After EriB treatment, the increases in these inflammatory

indices were reversed suggesting that EriB exerted anti-inflammatory effects in MN.

Targeted control of the JAK/STAT pathway has been suggested as therapeutically feasible for diabetes-induced kidney damage. Inactivation of the JAK/STAT3 pathway could reduce renal fibrosis in rat models of diabetic nephropathy. A report on septic acute kidney injury indicated that fish oil-based therapy reduces renal injury and inflammatory response by inhibiting the JAK/STAT3 pathway [18]. This study revealed that EriB protected MN rats from renal injury by inhibiting the JAK/STAT3 pathway.

CONCLUSION

The findings of this study show that EriB protects against renal injury, fibrosis, and inflammation in MN by inactivating JAK/STAT3 pathway. However, EriB does not act alone. Numerous genes interact with one another in MN and, therefore, the complete mechanism of MN remains to be elucidated.

DECLARATIONS

Acknowledgements

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None provided.

Ethical approval

The animals for this study were approved by the Ethics Committee of Changzhou Second People's Hospital Affiliated with Nanjing Medical University (approval no. 2021001) and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [12]..

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Shanmai Guo and Xi Guan designed and carried out the study, supervised the data collection, analyzed and interpreted the data, prepared the manuSCript for publication, and reviewed the draft of the manuSCript. Both authors have read and approved the manuSCript.

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