Tropical Journal of Pharmaceutical Research February 2023; 22 (2): 239-244
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v22i2.3

Original Research Article

Protective effect of epalrestat on peripheral nerves in rats with diabetic peripheral neuropathy via NF-κB pathway

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Sent for review: 10 November 2022 *Revised accepted: 29 January 2023*

Abstract

Purpose: *To study the protective effect of epalrestat on peripheral nerves in rats with diabetic peripheral neuropathy (DPN).*
Methods: *A total of 36 Sprague-Dawley rats were randomly divided into normal, model, and epalrestat groups, each containing 12 rats. The morphology of the neurons was assessed using hematoxylin-eosin (H&E) staining. The expressions of B-cell lymphoma-2 (Bcl-2), and Bcl-2 associated X protein (Bax) were determined via immunohistochemistry. The relative protein expressions of NF-κB and Caspase3 were determined via Western blotting, and apoptosis was determined using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.*
Results: *The morphology of the neurons was clear and intact in the normal group, disordered and destroyed in the model group, and was improved in the epalrestat group. The other two groups had a significantly higher positive expression of Bax and a significantly lower positive expression of Bcl-2 than the normal group ($p < 0.05$), while the epalrestat group had a significantly lower positive expression of Bax, and a significantly higher positive expression of Bcl-2 than the model group ($p < 0.05$). Furthermore, the protein expressions of NF-κB and Caspase3 were increased in other groups compared with normal group ($p < 0.05$), while they declined in epalrestat group compared with model group ($p < 0.05$). The apoptosis rate was significantly lower in epalrestat group than in model group ($p < 0.05$).*
Conclusion: *Epalrestat inhibits neuronal apoptosis by suppressing the NF-κB signaling pathway, thereby exerting a neuroprotective effect in DPN rats. Further studies would be required to validate the molecular mechanism.*

Keywords: *Diabetic peripheral neuropathy, Epalrestat, NF-κB, Apoptosis*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Diabetes mellitus is the most common endocrine and metabolic disease in clinical practice, which often causes lesions in other systems, such as diabetic peripheral neuropathy (DPN) and diabetic retinopathy [1]. With the changes in people's lifestyle and improvement of living standards, the incidence rate of diabetes also becomes increasingly higher, accompanied by increasing incidence of related complications. Diabetic peripheral neuropathy is one of the most important complications of diabetes, also with a rising incidence, which often leads to limb

paresthesia, hyperesthesia and even long-term pain, as well as absence of neural reflex in severe cases. Therefore, DPN has gradually attracted the attention of researchers.

Currently, studies have shown that one of the main pathological mechanisms of DPN is closely related to massive neuronal apoptosis, thus causing damage and necrosis [2]. As an important apoptotic pathway, the nuclear factor-κB (NF-κB) signaling pathway may regulate the expressions of important apoptosis-related molecules, such as B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax) and Caspase3, thereby playing an important regulatory role in apoptosis. Therefore, the NF-κB signaling pathway is considered to be involved in the pathological mechanism and pathogenesis of DPN due to its regulatory effect on apoptosis.

Epalrestat is one of the main drugs used for the clinical treatment of DPN, which possesses a good neuroprotective effect in clinical application. However, the mechanism of its neuroprotective effect in DPN remains unclear. Therefore, this present study aimed to investigate the protective effect of epalrestat on peripheral nerves in DPN rats through the NF-κB pathway.

EXPERIMENTAL

Animals

A total of 36 specific pathogen-free Sprague-Dawley (SD) rats aged one month, were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (license no. SCXK, 2014-0003, Shanghai, China). The rats were fed with normal feed and sterile filtered water every day in the Laboratory Animal Center under 12/12 h light-dark cycle, room temperature and regular humidity. This study was approved by the Animal Ethics Committee of Shaoxing Stomatological Hospital Animal Center. All procedures were conducted in accordance with the 'Animal Research: Reporting *In vivo* Experiments guidelines 2.0' [3].

Animal grouping and treatment

The 36 SD rats were divided into a normal group (n = 12), model group (n = 12) and epalrestat group (n = 12) using a random number table. The rats were adaptively fed in the Laboratory Animal Center for 7 days before experiments.

The rats in the normal group were fed normally without any treatment. In the model group, the diabetic rat models were established, and then normal saline was intraperitoneally injected every day. In the epalrestat group, the diabetes model was also established, and then epalrestat (Yangtze River Pharmaceutical Group, Taizhou, China) was intraperitoneally injected every day at a dose of 0.5 mg/kg/day. The samples were taken after 4 weeks of intervention.

Modeling

Streptozotocin solution (Sigma, St. Louis, MO, USA) at a concentration of 1 % was prepared and intraperitoneally injected into rats (60 mg/kg). After 3 days, blood glucose was measured after blood was drawn from the tail vein. The blood glucose > 16.7 mmol/L indicated the successful establishment of diabetes model.

Sampling

The rats were anesthetized using an inhaled anesthetic. After the successful application of anesthesia, the blood was drawn from the abdominal aorta, and sciatic nerve tissues were directly taken from 6 rats in each group, washed with normal saline and stored in the EP tube at -80 °C for subsequent Western blotting. The remaining 6 rats in each group were perfused and fixed to get the specimens: the chest was cut to expose the heart, and the left auricle was perfused with 400 mL 4 % paraformaldehyde. Their sciatic nerve tissues were harvested and fixed in 4 % paraformaldehyde for immunohistochemistry and TUNEL (Vazyme, Nanjing, China).

H & E staining

The paraffin-embedded sciatic nerve tissue was sectioned into 5 μm thick sections using a paraffin microtome, scoped flat in 42 °C warm water, dried and prepared into paraffin sections. Then the sections were soaked and routinely deparaffinized in xylene solution and gradient alcohol, stained with hematoxylin dye for 5 min, placed in pure water for 10 min, color-separated with 95 % ethanol for 5 s, transparentized for 10 s, and sealed with neutral balsam.

Immunohistochemistry

Using paraffin tissue sections, sections were soaked and routinely deparaffinized in xylene solution and gradient alcohol, placed in citric acid buffer, and heated in a microwave for 3 times (3 min/time, braised for 5 min each time) for complete antigen retrieval. After washing the sections, endogenous peroxidase blocking agent was added dropingly to the reaction for 10 min. The sections were rinsed again and sealed with goat serum for 20 min. After the goat serum was

discarded, the anti-Bax (1 : 200) and anti-Bcl-2 (1 : 200) primary antibodies were added for incubation in a refrigerator at 4 °C overnight. On the next day, the sections were washed, reacted with secondary antibodies for 10 min, After thorough washing, the sections were reacted with streptavidin-peroxidase solution for 10 min and stained with diaminobenzidine (DAB). Finally, the nuclei were counterstained with hematoxylin, and the sections were sealed and observed under a microscope.

Western blotting

The cryopreserved heart tissues were added with lysis buffer, subjected to ice bath for 1 h and centrifuged in a centrifuge at 14,000 g for 10 min. The protein was quantified using bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The absorbance of protein was determined using a microplate reader and the standard curve was plotted, based on which the protein concentration was calculated. After protein denaturation, the extracted proteins were separated using a 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride membrane (Roche, Basel, Switzerland), sealed with the sealing buffer for 1.5 h and incubated with the anti-NF-κB (1 : 1000) and anti-Caspase3 (1 : 1000) primary antibodies (10 h) and secondary antibodies (1 h) (1 : 1000). After the membrane was washed, the image was fully developed using the chemiluminescent reagent for 1 min.

Statistical analysis

SPSS statistical analysis software (version 26.0) (IBM, Armonk, NY, USA) was used for statistical analysis. Enumeration data were expressed as mean ± standard deviation. *t*-test was used for the data in line with normal distribution, and non-parametric test for the data not in line with normal distribution and homogeneity of variance.

RESULTS

Neuronal morphology

As shown in Figure 1, the morphology of neurons was clear and intact, and there were abundant Nissl's bodies in normal group. In model group, the morphology of neurons was disordered, and there were fewer Nissl's bodies with partial lysis. In epalrestat group, the morphology of neurons was improved compared with that in model group, and the number of Nissl's bodies was larger with improved morphology.

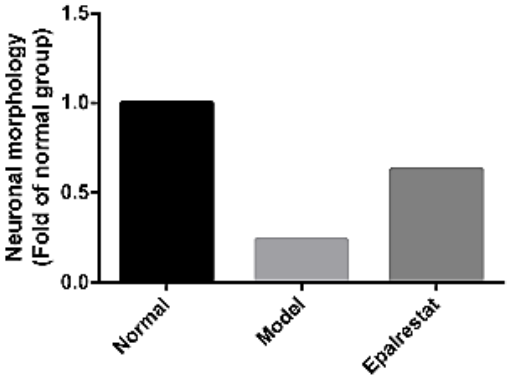

Figure 1: Epalrestat improved the morphology of neurons

Immunohistochemistry results

According to the statistical results (Figure 2), compared with those in normal group, the mean optical density of Bax positive expression was significantly increased, while that of Bcl-2 significantly declined in the other two groups, showing statistically significant differences ($p < 0.05$). Besides, compared with those in model group, the mean optical density of Bax positive expression significantly declined, while that of Bcl-2 was significantly increased in epalrestat group, showing statistically significant differences ($p < 0.05$).

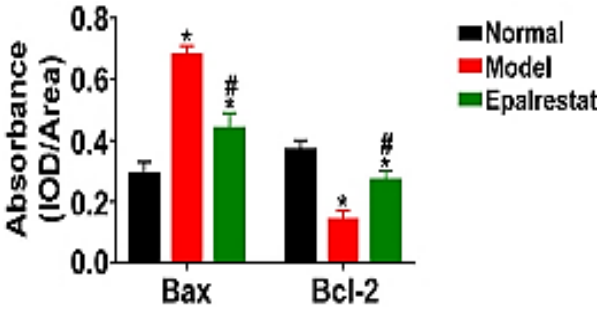

Figure 2: Mean absorbance of Bax and Bcl-2 in each group. Note: *$P < 0.05$ *vs.* normal group; #$p < 0.05$ *vs.* model group

Relative protein expressions

According to the statistical results of protein expression (Figure 3), the relative protein expressions of NF-κB and Caspase3 were obviously increased in the other two groups compared with those in normal group, and the differences were statistically significant ($p < 0.05$). Besides, compared with those in model group, the relative protein expressions of NF-κB and Caspase3 obviously declined in epalrestat group, and the differences were statistically significant ($p < 0.05$).

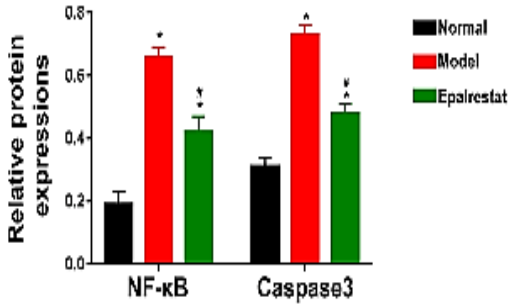

Figure 3: Relative protein expressions of neurons in each group. Note: *P < 0.05 *vs.* normal group, #p < 0.05 *vs.* model group

TUNEL assay results

The apoptosis rate remarkably rose in the other two groups compared with that in normal group, and the differences were statistically significant (p < 0.05), while it remarkably declined in epalrestat group compared with that in model group, and the difference was statistically significant (p < 0.05) (Figure 4).

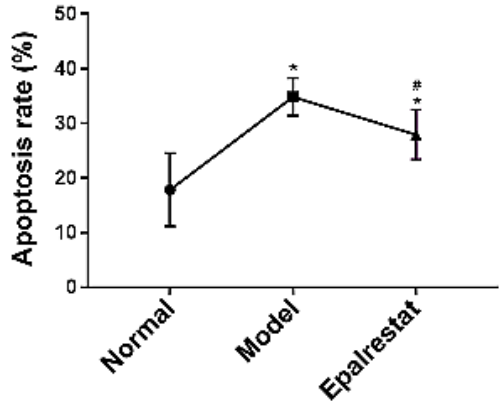

Figure 4: Apoptosis rate of neurons in each group. *P < 0.05 *vs.* normal group; #p < 0.05 *vs.* model group

DISCUSSION

Diabetic peripheral neuropathy is one of the important complications of diabetes, and its incidence is also on the increase, with rising morbidity of diabetes. According to epidemiological statistics, about 55 % of diabetic patients suffer from DPN, which causes limb sensory disorder, limb hyperesthesia, heat perception dysfunction, pain (stabbing pain and swelling pain), and even loss of limb sensory function and neurodegeneration. Therefore, it is of utmost importance to further study the pathogenesis, and methods of clinical prevention and treatment of DPN. With the deepening of research on DPN, it has been recognized that apoptosis, especially neuronal apoptosis, is one of the main pathological responses and mechanisms of DPN. Hyperglycemia induced by diabetes can further cause damage to the microvessels and nervous system, and lead to neuronal injury in the nervous system under the action of damage factors, further causing neuronal apoptosis, affecting the normal structure and normal physiological function of the nervous system, and producing a series of typical clinical manifestations. Further research has revealed that the NF-κB signaling pathway is closely related to apoptosis, which participates in and regulates the pathophysiological mechanism and related processes of apoptosis [4-7].

Studies have demonstrated that NF-κB, as an important member of the nuclear transcription factor protein family, may enter the nucleus and bind to various gene transcriptional promoters, thereby regulating the transcription of gene transcriptional promoters and the translation of multiple downstream proteins [8,9]. Under normal conditions, NF-κB binds to its inhibitory protein IκB, thus losing its physiological effect. Under the action of damage factors and various inflammatory factors, IκB kinase is activated to degrade IκB protein, so that NF-κB is released into the nucleus and participates in the regulation of the transcription of various downstream proteins, thus activating the NF-κB signaling pathway [10,11].

The NF-κB signaling pathway is an important apoptotic pathway. After the activation of NF-κB signaling pathway, it enters the nucleus and binds to transcription factors, causing the expression changes in apoptosis-related factors Bax and Bcl-2, and then regulating the expression of downstream apoptotic effector Caspase3 [12-15]. In this study, it was found that in the sciatic nerves of DPN rats, the NF-κB signaling pathway was activated, along with changes in expressions of Bax and Bcl-2, abnormally high expression of Caspase3, and massive neuronal apoptosis. After epalrestat intervention, the activation of NF-κB signaling pathway was inhibited, the expression of Bax declined, the expression of Bcl-2 was enhanced, the expression of Caspase3 was decreased, the neuronal apoptosis was weakened, the morphology of neurons was improved, and the nervous system was protected.

CONCLUSION

Epalrestat inhibits neuronal apoptosis by suppressing NF-κB signaling pathway, thereby exerting a neuroprotective effect in DPN rats. Thus, it is a potential agent for development for the management of DNP.

DECLARATIONS

Acknowledgements

None provided.

Funding

None provided.

Ethical approval

None provided.

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

The authors 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 them.

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