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Protective effect of gliclazide on diabetic peripheral neuropathy through Drp-1 mediated-oxidative stress and apoptosis

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HIGHLIGHTS

- DPN was induced by intra-peritoneal injection of streptozotocin.
- Expressions of Drp-1, Bax, caspase-3 and MDA increased in sciatic nerve of DPN rat.
- SOD activity and NCV decreased in sciatic nerve of DPN rat.
- Changes of Drp-1, Bax, Bcl-2, caspase-3, MDA and NCV were blocked by gliclazide.

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ABSTRACT

Objective: To investigate the protective effect of gliclazide and the role of dynamin-related protein l (Drp-1)-mediated oxidative stress and apoptosis in diabetic peripheral neuropathy (DPN). *Methods*: Diabetic rats developed through intra-peritoneal injection of streptozotocin were randomly assigned to treatment group receiving gliclazide or non-treatment group without gliclazide treatment. Rats in control group received intra-peritoneal injection of vehicle and no gliclazide treatment. Eight weeks later, the nerve conduction velocity (NCV) of sciatic nerve was measured and the morphological alterations, the malondialdehyde (MDA) level and superoxide-dismutase (SOD) activity, the expressions of Drp-1, caspase-3, Bax and Bcl-2 in sciatic nerve were evaluated. *Results*: When compared to rats in control group, rats in non-treatment group showed significantly decrease of NCV, obvious demyelinative alteration of sciatic nerve, increased expressions of Drp-1, caspase-3, Bax, Bcl-2 and MDA, and decreased SOD activity. Compared to rats in non-treatment group, rats in treatment group showed significantly increase of NCV, less demyelination of sciatic nerve, decreased expressions of Drp-1, caspase-3, Bax and MDA, and increased activity of SOD. The expression of Bcl-2 was not significantly different between treatment and non-treatment groups. *Conclusion*: Gliclazide showed protective effect on DPN through modulating Drp-1-mediated oxidative stress and apoptosis.

1. Introduction

Diabetic peripheral neuropathy (DPN) is one common chronic complication induced by diabetic hyperglycemia, and severely influences the life quality of patients. Under condition of hyperglycemia, massive free radicals are produced by the mitochondrial respiratory chain and neuronal apoptosis is increased [1]. Recently, oxidative stress, apoptosis and neurotrophic deficit have become the hot-spots for studying in mechanism of DPN [2,3]. As an organelle modulating cellular apoptosis and producing massive free radical, mitochondria in dysfunctional situation is closely correlated with DPN [1,4] and the imbalance between mitochondrial biogenesis and fission is suggested to be involved in the pathogenesis of diabetic neuropathy through the dys-regulation of energy production and the activation of caspase-3 [4].

Dynamin-related protein l (Drp-1) is one member of dynamin super-family and one molecule mediating mitochondrial fission [5]. Previous study indicated that Drp-1 is closely correlated with neurodegenerative diseases [5,6] and regulation of mitochondrial fission [7]. Under endoplasmic reticulum (ER) stress conditions, induction of wild-type DRP-1 enhances caspase-3 activation and generation of reactive oxygen species (ROS) in pancreatic β-cells [8]. These results suggested that Drp-1 may be related with the pathogenesis of DPN through the mediation of oxidative stress and apoptosis.

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As one hyperglycemia-controlling drug for diabetic patients, gliclazide has been shown to be beneficial for DPN [9]; however, whether the mechanism is through scavenging free radical and tumor necrosis factor-alpha (TNFα) is not consistent [9–11]. In addition, it was indicated that gliclazide is able to significantly reduce high glucose-induced apoptosis, mitochondrial alterations [12], and improve the changes of ROS, caspase-3 and Bcl-2 expression [13]. These results suggest that the effect of gliclazide may be related with Drp-1 mediated oxidative stress and apoptosis; however, there was no such report. The present study investigated the changes of mitochondrial Drp-1 expression in sciatic nerve of diabetic rats, the correlation of Drp-1 with oxidative stress and cellular apoptosis, and the effect of gliclazide intervention on DPN.

2. Materials and methods

2.1. Animals and DPN model development

Male Sprague-Dawley rats (8 weeks, 200 ± 20 g) were purchased from the Experimental Animal Center of Nanjing Medical University. All the experiments were approved by the Animal Care and Control Committee of the Nanjing Medical University. The DPN model was developed according to previous study [14]. Briefly, after one-week accommodation and 12-h fasting, the animals were given 1% streptozotocin (Sigma, USA) in 0.1 mol/L citric acid buffer through intra-peritoneal (i.p.) injection (40 mg/kg). After 72 h, blood samples were taken from the tail vein to measure the blood glucose and blood glucose ≥ 16.67 mmol/L was defined as successful development of DPN model. Thirty-five rats successfully developed DPN were randomly assigned to diabetic model group (group D, $n = 17$) or gliclazide group (group G, $n = 18$). Litters, age and weight-matched normal rats were randomly selected as control group (group N, $n = 15$), receiving i.p. injection of same volume of citric acid buffer. After model development, rats in group G received gliclazide (20 mg/kg/day; Servier (Tianjin), China) through intragastric perfusion, while rats in groups N and D received same volume of saline through intragastric perfusion.

The general features of rats and the blood glucose level were monitored. During the whole experiments, the body weight of animals was monitored and the blood glucose was examined every two weeks, and the animals with blood glucose < 16.7 mmol/L in groups D and G were excluded.

2.2. Nerve conduction velocity measurement

At the end of the 8th week after i.p. injection, the animals were anesthetized with 12% urethane (i.p., 10 ml/kg). The animals were fixed at the prone position, the stimulating electrode was inserted intramuscularly into the tuber ischiale, the recording electrode was placed between the malleolus medialis and the second toe, and the referral electrode was located between the recording electrode and the stimulating electrode. Square-waves stimulation (2.0 mV in intensity, 3.0 ms in width) was applied through the electromyograph recording system (Viking IV, Nicolet, USA) to stimulate the sciatic nerve. The action potential latency (L) of sciatic nerve and the distance (D) between the stimulating and recording electrodes were measured to calculate the nerve conduction velocity (NCV). The formula was: NCV (m/s) = D/L. In each experiment, NCV was repeated three times to calculate the mean value. During the recording, the limbic temperature was kept at 37 °C.

2.3. Specimen and homogenate preparation

After fixation of the rat at supine position, the bilateral skin at femoral area of the rat was cut to isolate the muscle and fascia and to expose the sciatic nerve which was cut with sharp scissor and rinsed off the blood with ice-cold saline. The left sciatic nerve was cut into two segments, one segment was fixed in 10% formol for pathological and immunohistochemical staining, another segment was stored at −80 °C for further experiment. The right sciatic nerve was immediately made into 10% homogenate (tissue gram/saline volume in milliliter = 1:9) used for oxidative stress measurement.

2.4. Oxidative stress measurement

Malondialdehyde (MDA) content was measured using thiobarbituric acid reactive substances (TBARS) assay (Jiancheng Bioengineering, Nanjing, China) by measuring the absorbance value at wavelength of 532 nm. Superoxide-dismutase (SOD) activity was measured using xanthine oxidase method to measure the absorbance value at 550 nM with SOD kit (Jiancheng Bioengineering, Nanjing, China). Quantitative bromochloroacetate (BCA) protein assay kit (Boster, Wuhan, China) was used to measure protein contents in samples according to the manual.

2.5. Immunohistological measurement of caspase-3

The samples were successively fixed, dehydrated, embedded with paraffin, sectioned (Leica, German). Then, the sections were successively treated with the following steps: regular dewaxing, repair of hot antigens, blockade of endogenous peroxidase with 3% H_2O_2, blockade with normal goat serum, incubation with primary polyclonal antibody (Boster, Wuhan, China) at 4 °C for overnight, incubation with secondary antibody and SABC complex (Boster, Wuhan, China), DAB coloration (Boster, Wuhan, China). Under light microscope, Schwann cells, axons and surrounding tissues with brownish-yellow or yellowish-brown particles were considered as positive expression. Three sections from each sample and three fields from each section were randomly selected for image analysis. The positive protein expression was calculated through measuring the averaged light density.

2.6. Drp-1, Bax and Bcl-2 expression

The sciatic nerve sample stored at −80 °C was completely ground in phenylmethylsufonyl fluoride (PMSF) and liquid nitrogen. Then, protein-extracting solution (500 μL/100 mg) was added and the mixture was incubated and centrifuged. The supernatant was aspirated and the protein concentration was measured using BCA protein quantitative assay kit. The supernatant was boiled at 100 °C for 5 min and electrophoresis was performed in polyacrylamide gel to isolate the targeting protein, which was transferred to PVDF membrane and blocked with 5% skimmed milk at room temperature for 3 h. The membrane was successively rinsed with TBST, incubated with rabbit against mice polyclonal antibodies (Drp-1, Bax, Bcl-2, β-actin, 1:1000 dilution; Santa Cruz, USA) at 4 °C for overnight, rinsed with TBST, incubated with goat against rabbit IgG (1:1000), labeled by horseradish peroxidase (Zhongshan Goldenbridge Biotechnology Co. Ltd., Beijing, China), diluted by 5% skimmed milk at 37 °C for 2 h, and rinsed with TBST. Finally, the membrane was luminescenced by ECL and exposed to X-film to form image. The gray levels of images were used to analyze the protein expression in different groups.

2.7. Statistical analysis

All data were expressed as mean ± S.D. SPSS13.0 software was used for analysis and the difference between different groups was compared with one-way ANOVA while the difference between different time-points within same group was compared with t-test. $p < 0.05$ was set as significant level.