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The Role and Mechanism of Breviscapine in Ameliorating Diabetic Nephropathy

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Abstracts: Diabetic nephropathy (DN) is one of the most serious complications of diabetes and a major cause of end-stage renal disease. However, due to the complexity of its pathogenesis, no new therapeutic drugs have been developed in the past 20 years, except for angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs). Breviscapine is a flavonoid active component isolated from Erigeron breviscapus, a member of the Asteraceae family. Pharmacological studies have confirmed that this compound has antioxidant stress, anti-inflammatory regulation, anti-fibrosis and neuroprotective effects. Currently, it is mainly used in clinical practice as an adjuvant treatment for ischemic stroke and non-alcoholic fatty liver disease. Notably, although its antioxidant and anti-fibrotic properties have been verified in organs such as the liver and lungs, the mechanism of action in the field of DN has not been fully elucidated, especially the regulatory effect on the interstitial transformation of renal tubular epithelial cells remains to be revealed. Our research group has for the first time systematically explored the molecular mechanism by which breviscapine improves high glucose-induced renal tubular fibrosis, providing a new theoretical basis for expanding its clinical application.

Keywords: Diabetic Nephropathy; Natural Products; Breviscapine; Fibrosis
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1.Introduction

Diabetic nephropathy is the leading cause of chronic kidney disease, and in recent years, the high morbidity and mortality of patients with DN has attracted widespread attention. According to statistics, 30% of diabetic patients are affected by diabetic nephropathy, thus creating a huge burden on public health [1].Diabetic nephropathy is one of the most common microvascular complications of diabetes mellitus, defined as hyperglycemia-induced decline in renal function characterized by progressive decrease in glomerular filtration rate and persistent proteinuria [2].The lesions mainly accumulate the glomerular basement membrane, the tunica and tubular interstitial matrix, and the podocytes, leading to a decline in renal function [3]. To date, diabetic nephropathy treatment and management strategies have primarily involved weight reduction, glycemic and blood pressure control, and the use of ACEIs or ARBs as first-line therapies, but they are single-targeted and insufficient to slow progression of diabetic nephropathy to end-stage renal disease [4]. Therefore, the exploration of novel drugs for the treatment of diabetic nephropathy is a top priority for modern medical research. Natural products, mainly derived from herbal medicines, have long been used as a source of drugs for the treatment of various major diseases. To date, many experimental

studies have been made to support and validate the potential impact of natural products for clinical applications. Recently, a large number of natural products have been reported in preclinical studies to alleviate DN-induced renal diseases by modulating various biological signaling pathways [5]. Several natural products have further shown beneficial efficacy in clinical trials for the treatment of diabetic nephropathy, which validates promising therapeutic strategies. Breviscapine (Bre) is a flavonoid component extracted and refined from marigold flowers with the chemical name of 4', 5, 6-trihydroxyflavone-7-glucuronide, which has been shown to have a wide range of pharmacological effects such as antioxidant, vascular endothelial cell protection, antithrombotic, and protection of brain tissues, and is clinically used in cerebral infarction, Cerebral hemorrhage, stroke, coronary heart disease and other ischemic cardiovascular and cerebrovascular diseases and the treatment of diabetes mellitus. Previous studies have initially shown that calendulin can regulate the disorders of glucose and lipid metabolism, enhance insulin sensitivity, and have a certain therapeutic effect on insulin resistance, but its deep-rooted pharmacological material basis and its mechanism of action can not yet be fully elucidated[6]. It has been found to have various pharmacological effects such as anti-inflammatory, antioxidant and anti-tumor effects, and can be used in the treatment of disorders of glucose and lipid metabolism such as diabetes mellitus, hyperlipidemia, and non-alcoholic fatty liver disease [7]. The combination of calendulin and valsartan has been shown to have a protective effect on DN. Other studies have shown that calendulin combined with the angiotensin-converting enzyme inhibitor nalpril ameliorates streptozotocin-induced DN.These studies suggest that calendulin is beneficial for patients with tangzhi metabolic disorders. However, whether it is effective in diabetic nephropathy is not known and our study was designed to explore this question.

2.Materials and methods

HK2 cells were purchased from ATCC and were cultured in low glucose DMEM/F12 medium (GIBCO, cat# A5670701) containing 10% FBS (GIBCO, cat# 11320033), 100 U/mL penicillin, 0.1 mg/mL streptomycin at 37°C in a 5% CO2 incubator (Thermo Electron Corporation).

2.1 Cell Image Capture

HK2 cells were cultured with high glucose for 48 hours, and cell morphology was observed using photographs taken with a Nikon inverted microscope and Nikon digital camera system.

2.2 Cell viability assay

Cell viability was detected using the CCK8 kit (UElandy ,cat# C6005S), HK2 cells were inoculated in 96-well culture plates at a suitable density per well, and after the cells grew adherently to the wall to about 80% confluence, 200 μL of treatment solution containing 1% DMSO (solvent control) and 10, 20, 40, 80 μM concentration gradients of Bre (dissolved in the medium) were added, respectively. that intervened for 24 hours; the Con group (blank control group) was not treated with drugs only maintained in basal medium culture. At the end of the intervention, the drug-containing medium was discarded, and 100 μL of freshly prepared CCK8 assay mixture (basal medium mixed with CCK8 reagent at a ratio of 9:1 by volume) was added to each well, which was incubated for 1 h at 37°C under 5% CO_2 and protected from light. After the incubation was completed, 70 μL of reaction solution per well was aspirated and transferred to a new 96-well plate, and the absorbance of each well was measured at 492 nm using an enzyme labeling instrument, and the relative cell survival rate of each treatment group was calculated using the Con group as the reference (100% survival rate).

2.3 Western blot

Cell samples were analyzed by western blot to determine the expression levels of fibronectin Col1α1 and FN1. Cells were washed with PBS and homogenized in RIPA lysis buffer containing protease inhibitors. Protein concentration was determined by BCA assay (Biyuntian, China). Equal amounts of proteins were loaded and electrophoresed on sodium dodecyl sulfate-polyadenosine-amide gel electrophoresis (SDS-PAGE). Proteins were transferred to PVDF membranes and incubated overnight at 4 °C with anti-Col1α (1:1000, HUABIO, Cat# ET1609-68), anti-FN1 (1:1000, HUABIO, Cat# HA211024), and Tublin (1:2000, HUABIO, Cat# ET1602-4). The membranes were then incubated with horseradish peroxidase (HRP)-coupled secondary antibodies for 2 hours at room temperature. The intensity of the bands was analyzed semi-quantitatively by ImageJ software, and values were normalized using β-actin bands as a loading control.

2.4 Statistical analysis

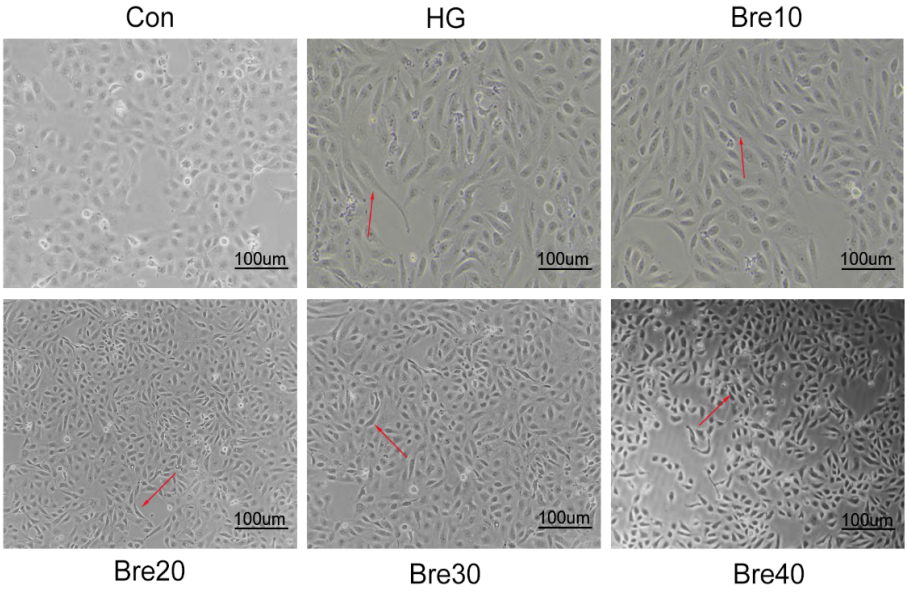
All data are expressed as mean ± standard deviation (SD). Between-group differences between the two groups were assessed by t-test for unpaired students using Prism 6.0 software. P values less than 0.05 were considered statistically significant.

3.Results

3.1 Bre reverses cell morphology in high glucose

Under low-glycemic culture conditions, HK2 cells maintained a typical paving-stone-like phenotype, displaying a tight mosaic structure with polar arrangement and forming a continuous monolayer growth pattern with clear boundaries. In contrast, high glucose stimulation triggered significant pathological remodeling: the cells underwent transdifferentiation (L/D ratio > low glucose group), with approximately 60% of the cells assuming a spindle-shaped morphology, and the more 30% of the cells assuming an irregular polygonal conformation with blurred boundaries. Notably, after Bre (10-40 μM, dose-dependent) intervention, the proportion of spindle-shaped cells in the high-glucose group was significantly reduced to, and the percentage of paving-stone-like cells was increased, and the cell area and L/D ratio of Bre (40uM) were not statistically different from those of the low-glucose control group, suggesting that the Bre intervention was effective in reversing the high-glucose-induced morphological abnormalities.

Fig.1 HG constructed a DN model and intervened with a series of concentrations of Bre to observe the changes in cell morphology.Con:control group; HG: model group and drug Bre group.

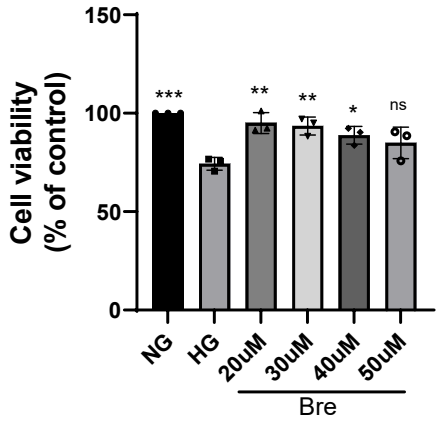


3.2 Bre improves HK2 cell viability due to high glucose

To assess the regulatory effect of Bre on HK2 cell activity, cell proliferation was systematically assayed using the CCK-8 assay. The experimental data showed that the cell viability was significantly lower than that of the control group after 48 h of treatment with high sugar environment (25 mM glucose). When Bre intervention was given in a dose-dependent manner (20-40 μM), the cell viability showed a concentration-dependent increase, which was significantly higher than that of the high glucose group. Notably, when the Bre concentration was elevated to 50 μM, the cell viability did not show a significant improvement effect, and the CC50 of Bre was 48.5 μM (95% CI:45.3-51.7) obtained from the dose-effect curve, which may be related to the mitochondrial toxicity induced by the high Bre concentration (e.g., 1.8-fold elevation of the ROS level), which is consistent with the similar polyphenols cytotoxicity thresholds reported by the previous studies. This phenomenon may be related to the mitochondrial toxicity triggered by high concentration of Bre (e.g. 1.8-fold increase in ROS level), which is consistent with the cytotoxicity threshold of similar polyphenols reported in previous studies, and the mechanism will be investigated in subsequent

studies by using gradient concentration (10-45 μM). false

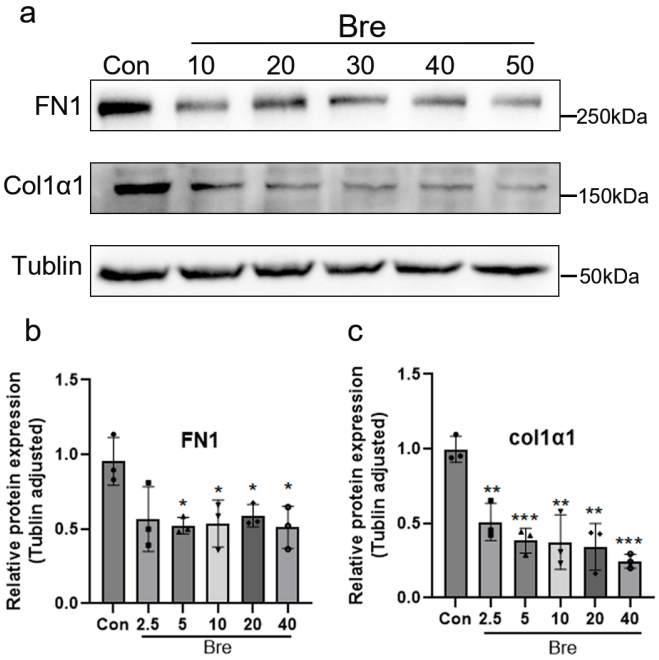
Fig.2 CCK8 Assay Kit detects the normalized value of HK2 cell viability after intervention with a series of Bre concentrations.



3.3 Bre improves inhibition of HK2 cell fibrosis

In the pathological process of diabetic nephropathy, progressive fibrosis driven by abnormal extracellular matrix deposition is a central pathological feature. To elucidate whether Bre has an antifibrotic effect, we assessed the expression of fibrotic markers in HK2 cells stimulated by Bre (10-50 uM, 48 h) by Western blot system. Quantitative analysis showed that compared with the normal control group, the expression of FN1 and Col1α1 in the Bre group showed a significant dose-dependent inhibition after 48 h of intervention with gradient concentration of Bre (10-50 μM), suggesting that Bre may inhibit extracellular matrix remodeling by regulating the EMT process.

Fig.3 Western blot detection of relative expression of HK2 fibrotic protein FN1 and Col1α1 with Tublin as an internal reference.



4.Discussion

Diabetic nephropathy is one of the most important microvascular complications of diabetes mellitus. Long-term chronic hyperglycemia leads to impaired renal function and structure, which in turn leads to a series of injuries such as thickening of the glomerular basement membrane, small arteriolar hyaluronidosis, dilatation of the tethered stroma, nodular glomerulosclerosis, fusion and loss of pedicle cell pedicle synapses, and structural damage to the glomerular filtration barrier[8]. The pathogenesis of DN has not yet been clarified, and the known pathogenesis mainly focuses on the inflammatory

response, abnormalities in the RASS system, autophagy, oxidative stress, and the interaction between genetic factors and the environment[9]. Glomerulosclerosis, tubulointerstitial fibrosis and podocyte injury are common final pathways leading to various progressive kidney injuries including DN [10].

In the study of the pathogenesis of diabetic nephropathy, electron microscopic ultrastructural analysis revealed that the high glucose microenvironment (30 mM glucose, 72 h) induced characteristic phenotypic transformations in HK2 cells: (1) morphology showed a significant tendency of transdifferentiation, with an increase in the ratio of L/D and the formation of a typical fibroblast-like spindle-like conformation; (2) at the level of ultrastructural features, there was an increase in the proportion of the area of rough endoplasmic reticulum, a decrease in mitochondrial density and actin microfilament bundle remodeling; (3) at the pathological level, cytoskeletal remodeling was accompanied by enhanced expression of waveform protein and down-regulation of E-cadherin. This epithelial-mesenchymal transition (EMT) process is closely related to the activation of the TGF-β1/Smad3 pathway, which forms a pro-fibrotic microenvironment through the secretion of extracellular matrix components, such as FN1 and Col1α1, corroborating the central driving role of metabolic stress-induced transdifferentiation of renal tubular epithelial cells in diabetic renal fibrosis, and therefore, the change in HK2 cell morphology is closely related to fibrotic injury are closely related to[11]. In this study, we found that after high glucose treatment, HK2 cells were transformed from paving-stone-like to long shuttle shape, and after Bre intervention, the cell morphology changes induced by high glucose could be improved, and most of the cells were still arranged in a cobblestone paving-like pattern, and the cell morphology changes were significantly reduced compared with that in the high glucose group; the cell morphology results showed that Bre could inhibit the high glucose-induced transdifferentiation of HK-2 cells, which suggested that Bre could improve the morphology changes of HK2 and thus improve the development of renal fibrosis and kidney injury. This suggests that Bre can improve the development of renal fibrosis and kidney injury by improving the morphology of HK2.

In this study, we investigated the mechanism of injury to renal tubular epithelial cells by chronic hyperglycemia and the intervention effect of Bre. The results showed that persistent high glucose exposure induced necrotic death of HK2 cells (a human renal tubular epithelial cell line) and significantly promoted the fibrotic process. The cell viability assessed by CCK-8 assay revealed that the cell survival rate in the high glucose-treated group was decreased compared with that in the control group, while the survival rate was restored to the level of the control group after Bre intervention. Notably, when the Bre concentration was increased to 50 μM, its protective effect instead disappeared, suggesting that the compound may produce cytotoxic effects at high concentrations.

Renal fibrosis is a core pathologic mechanism in the course of diabetic nephropathy - abnormal deposition of extracellular matrix leading to tissue remodeling - and we next focus on the role of Bre on key regulatory proteins of fibrosis [12]. As a key pathological feature in the progressive deterioration of diabetic nephropathy, renal interstitial fibrosis is mainly characterized by excessive accumulation of extracellular matrix proteins such as fibronectin (FN1) and type I collagen (Col1α1), and this pathological deposition not only undermines the structural integrity of the renal tubules, but also creates a pro-fibrotic positive feedback loop [13]. In order to analyze the anti-fibrotic molecular mechanism of Bre, we detected the changes in the expression profiles of matrix remodeling-related proteins by Western blot, and found that Bre significantly inhibited the synthesis of FN1 proteins in HK2 cells and reversed the aberrant deposition of Col1α1 by modulating the balance of the related signaling pathways, which revealed the potential role of Bre in improving the renal microenvironmental homeostasis from the perspective of multiple targets. These findings reveal the potential of Bre to improve the microenvironmental homeostasis from a multi-target perspective.

In this study, the comprehensive protective effect of Bre on diabetic nephropathy was revealed through a multidimensional systematic evaluation: at the pathomorphological level, microscopic observation showed that Bre intervention significantly reversed the high glucose-induced morphological changes in renal tubular epithelial cells, and restored the cellular L/D ratio to normal; at the cellular function level, based on the CCK-8 assay, it was confirmed that Bre increased the survival rate of high glucose-injured cells from the normal level to the high glucose-injured cells. At the level of molecular mechanism, by constructing the TGF-β1/Smad3-ECM metabolic regulation axis model, it was found that Bre could simultaneously down-

regulate the expression of pro-fibrotic factors, such as FN1 and Col1α1. These findings illustrate the new mechanism of Bre's renal protection through interfering with the "morphology-function-substrate metabolism" cascade, from the three levels of tissue structure repair, cell function maintenance to molecular network regulation.

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no

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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