Baicalin serves a protective role in diabetic nephropathy through preventing high glucose-induced podocyte apoptosis

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Abstract. Diabetic nephropathy (DN) is one of the late complications of diabetes, which seriously affects the lives of patients. Baicalin (BA) is a flavone glycoside that has been identified to improve renal function in patients with DN. The present study aimed to investigate the roles and mechanisms of BA in DN. For that purpose, podocytes were cultured for 48 h under conditions of high glucose (HG; 30 mM D-glucose) or normal glucose (NG; 5 mM D-glucose). Then, the cells were treated with different concentrations of BA (6.25, 12.5 and 25 µM) for 24 h. Cell viability and apoptosis were determined using an MTT assay and flow cytometry, respectively. Protein and mRNA expression levels were analyzed using western blotting and reverse transcription-quantitative PCR, respectively. BA treatment was identified to promote the viability of podocytes and suppress cell apoptosis in a dose-dependent manner. Compared with the results in the NG group, HG stimulation significantly decreased the viability of podocytes and increased the apoptotic rate, whereas BA treatment following HG stimulation increased the viability of podocytes and decreased the apoptotic rate. Moreover, the effect of BA was revealed to be associated with the sirtuin 1/NF-κB signaling pathway in DN. In conclusion, the results of the present study suggested that BA treatment may significantly decrease HG-induced podocyte apoptosis, which indicated that BA might be a promising agent for DN treatment.

Introduction

Diabetic nephropathy (DN) is a serious and harmful chronic complication caused by diabetes and it is a common cause of end-stage renal disease (1). Diabetes can cause glomerular damage, accompanied by proteinuria and subsequent tubulo-interstitial lesions, which ultimately culminates in end-stage renal disease (2-4). At present, DN occurs in 20-40% of patients with diabetes (5). The development of DN takes years and involves numerous characteristic pathological changes, such as the excessive accumulation of the extracellular matrix, glomerular sclerosis, tubular dilatation and atrophy, and interstitial fibrosis (6). A major pathological feature of DN is the abnormal apoptosis of podocytes. In a previous study, it was identified that the apoptosis of podocytes was associated with the decreased expression levels of multiple proteins, including podocin, nephrin and Slit-associated proteins (7), which resulted in considerable proteinuria in DN (8). Therefore, it is important to prevent podocyte apoptosis and cure DN at an early stage.

Baicalin (BA; 5,6,7-trihydroxyflavone), a herbal medicine found in the Chinese Pharmacopoeia, is one of the main flavonoids purified from the roots of Astragalus membranaceus (9). It has been reported that BA exhibits significant anticancer effects in a variety of malignant tumors, including colorectal (10), breast (11) and lung cancer (12), where it has been identified to serve a crucial role in the vascular system through both anti-inflammatory and antioxidation mechanisms (13). However, to the best of our knowledge, there are currently no existing studies on the effect of BA on high glucose (HG)-induced podocyte apoptosis.

Sirtuin 1 (SIRT1) is an important nicotinamide adenine dinucleotide-dependent nuclear histone deacetylase that has been identified to serve a crucial role in the vascular system through regulating cell proliferation and the cell cycle (14). It was previously demonstrated that SIRT1 served an important role in the pathogenesis of kidney disease (15). In fact, the activation of SIRT1 has been suggested to be a novel target for the treatment of patients with chronic kidney disease, including DN (16).

NF-κB, one of the most important transcription factors in macrophages, extensively regulates cell proliferation, apoptosis and inflammatory cytokine expression (17), and it is also identified to be activated in diabetic conditions (18).
The activated and phosphorylated form of NF-kB subunits (p65 and p50) translocate to the nucleus, which indicates the activation of the NF-kB signaling pathway (19). Numerous reports have revealed that hyperglycemia-induced DN and the associated inflammation is induced by NF-kB; the majority of proinflammatory cytokines are regulated by NF-kB transcription (20-22). Notably, NF-kB was identified to be involved in regulating apoptosis in podocytes (21,22).

The present study aimed to investigate the effects of BA treatment on podocytes in vitro and to determine whether BA protected against DN through inhibiting HG-induced podocyte apoptosis. Furthermore, the underlying molecular mechanisms were analyzed to reveal the potential role and value of BA in DN. The results of the present study provided a theoretical basis, as well as treatment strategies for the treatment of DN.

Materials and methods

Cell culture and HG-induced podocyte injury model. The conditionally immortalized mouse podocyte MPC-5 cell line (cat no. C1389) were obtained from Shanghai Guandao Biological Engineering Co., Ltd. Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% (v/v) penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

For HG-induction, following 12 h of serum starvation, podocytes were cultured at 37°C for 48 h under conditions of HG (30 mM D-glucose; Sigma-Aldrich; Merck KGaA) or normal glucose (NG; 5 mM D-glucose; Sigma-Aldrich; Merck KGaA) (23).

MTT assay. Cell viability was analyzed using an MTT assay (Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's protocol. For MTT detection, after 12 h of serum starvation, podocytes were cultured for 48 h under conditions of HG (30 mM D-glucose; Sigma-Aldrich; Merck KGaA) or normal glucose (NG; 5 mM D-glucose; Sigma-Aldrich; Merck KGaA) (23).

Flow cytometric analysis of apoptosis. Following the treatment of podocytes with 6.25, 12.5 or 25 µM BA, apoptosis was analyzed using flow cytometry. Briefly, the podocytes were digested from 6-well plates using trypsin, resuspended in fresh medium and centrifuged at 500 x g for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in 1X binding buffer. The cells were subsequently stained with Annexin V-FITC solution and PI at 4°C for 15 min using the Annexin V-FITC Apoptosis Detection kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Apoptotic cells were analyzed using a flow cytometer and the flow cytometry data were analyzed by FlowJo software (version 7.6.1; FlowJo LLC) to determine the percentage of apoptotic cells.

Western blotting. Following the treatment of podocytes with 6.25, 12.5 or 25 µM BA, total protein was extracted from the conditionally immortalized mouse podocytes using RIPA lysis buffer (Beyotime Institute of Biotechnology). Total protein was quantified using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) and 30 µg protein/lane was separated by 10% SDS-PAGE. The separated proteins were subsequently transferred onto a PVDF membrane (EMD Millipore) and blocked at room temperature with 5% fat-free powdered milk dissolved in tris-buffered saline (TBS) containing 0.1% Tween for 1.5 h. The membrane was incubated with the following primary antibodies at 4°C overnight: Anti-SIRT1 (1:1,000; Cell Signaling Technology, Inc.), anti-cleaved caspase-3 (1:1,000; Santa Cruz Biotechnology, Inc.), anti-pro-caspase-3 (1:1,000; Santa Cruz Biotechnology, Inc.), anti-GAPDH (1:5,000; Cell Signaling Technology, Inc.), anti-p65 (1:1,000; Abbcam) and anti-phosphorylated (p)-p65 (1:1,000; Abbcam). Following the primary antibody incubation, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (cat no. ab7090; 1:2,000; Abbcam) at room temperature for 2 h. The protein bands were visualized using an enhanced chemiluminescence reagent (EMD Millipore) and GAPDH served as a loading control for normalization.

Reverse transcription-quantitative PCR (RT-qPCR). Following the treatment of podocytes with 6.25, 12.5 or 25 µM BA, total RNA was extracted from podocytes using TRIzol® reagent on ice (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following the RNA extraction, the concentration of each sample was measured using an ultraviolet spectrophotometer. Total RNA was reverse transcribed into cDNA using a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd.), according to the manufacturer's protocol. The following RT temperature protocol was used: 70°C for 5 min, 37°C for 5 min and 42°C for 60 min. qPCR was subsequently performed using a HiScript II One Step qRT-PCR SYBR Green kit (Vazyme Biotech Co., Ltd.) according to the manufacturer's protocol. The following thermocycling conditions were used for the qPCR: 95°C for 3 min; followed by 40 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s; GAPDH served as the internal loading control for normalization. Primer sequences for PCR were listed as following: SIRT1 forward, 5'-AAT CCA GTC ATT AAC AAG GTT CTACAA-3' and reverse, 5'-TAGGACCAT TAC TGCC CAGAGG-3'; p65 forward, 5'-CATGCGT CCTGGCA AT CG-3' and reverse, 5'-GGTCCC GTTCTT TACACAC-3' and GAPDH forward, 5'-CCATGGGAAGG TGAAGGTC-3' and reverse, 5'-GAAGGGGT CATTTAGGGCAAC-3'. Data were quantified using the 2^{-ΔΔCt} method (24).

Statistical analysis. Experiments were repeated three times. Statistical analysis was performed using SPSS 22.0 software (IBM Corp.) and data are presented as the mean ± SD. Statistical differences between groups were determined using one-way ANOVA with a Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference.
Figure 1. BA promotes podocyte viability and decreases apoptosis. Podocytes were treated with 6.25, 12.5 or 25 µM BA for 24 h. (A) MTT assay was used to determine the cell viability. (B) Apoptotic rate of podocytes was analyzed using flow cytometry.
Results

Effects of BA on podocyte viability and apoptosis. To investigate the effects of BA, an MTT assay and flow cytometry were performed to determine the cell viability and rate of apoptosis, respectively. Compared with the untreated group, BA significantly increased the viability of podocytes in a dose-dependent manner (Fig. 1A). In addition, compared with the untreated group, BA significantly inhibited cell apoptosis in a dose-dependent manner (Fig. 1B and C). The results from the western blotting also revealed that BA treatment reduced the cleaved-caspase-3/pro-caspase-3 ratio compared with that in the untreated cells (Fig. 1D and E). Taken together, these results suggested that BA treatment may increase cell viability and decrease the rate of apoptosis.

Effects of BA on the HG-induced podocyte injury model. To investigate the effects of BA on the HG-induced podocyte injury model, podocytes were cultured for 48 h under conditions of HG (30 mM D-glucose) or NG (5 mM D-glucose) following serum starvation for 12 h. Then, the cells were treated with different concentrations of BA for 24 h. The MTT assay demonstrated that HG stimulation significantly decreased the viability of podocyte cells, whereas the treatment of HG-induced podocytes with BA significantly weakened the effects of HG in a dose-dependent manner; a significantly increased cell viability was observed in the HG + BA-treated groups compared with the HG group (Fig. 2).

Flow cytometry and western blotting were subsequently performed to determine the rate of apoptosis and
Figure 3. BA improves HG-induced podocyte apoptosis. Following serum starvation for 12 h, podocytes were cultured with HG (30 mM D-glucose) or normal glucose (5 mM D-glucose) for 48 h and then treated with 6.25, 12.5 or 25 µM BA for 24 h. (A and B) Flow cytometry was used to determine the cell apoptotic rate. (C) Western blotting was performed to determine the protein expression levels of the apoptosis-associated proteins pro-caspase-3 and cleaved caspase-3. (D) Semi-quantification of the ratio between cleaved-caspase-3/pro-caspase-3. All data are presented as the mean ± SD. *P<0.01 vs. NG group. #P<0.05 and ##P<0.01 vs. HG treatment group. BA, baicalin; HG, high glucose; PI, propidium iodide; NG, normal glucose.
the expression levels of apoptosis-associated proteins, respectively. These results indicated that compared with the NG group, HG stimulation significantly increased the apoptotic rate of podocytes (Fig. 3A and B) and enhanced the cleaved-caspase-3/pro-caspase-3 ratio (Fig. 3C and D). Moreover, compared with that in the HG group, BA
Effects of BA on the expression levels of SIRT1 and p-p65 in HG-induced podocytes. RT-qPCR and western blotting assays were performed to detect the expression of SIRT1 and p-p65 mRNA and protein, respectively. Compared with the NG group, HG-induced podocytes exhibited decreased SIRT1 protein expression levels (Fig. 4A) and significantly decreased SIRT1 mRNA expression levels (Fig. 4B). Moreover, compared with the results in the control group, HG stimulation increased p-p65 protein expression levels (Fig. 4C) and the p-p65/p65 ratio (Fig. 4D). Notably, BA treatment increased the expression levels of SIRT1 protein and mRNA in a dose-dependent manner (Fig. 4A and B), whilst decreasing p-p65 protein expression levels (Fig. 4C) and the ratio between p-p65/p65 compared with the HG group (Fig. 4D). There were no significant differences observed in the expression levels of p65 at the protein or mRNA level between the groups (Fig. 4C and E).

Discussion

At present, the main risk of diabetes is the occurrence of chronic complications, for example, high levels of blood glucose can lead to damage in the retina, nerves and kidneys (25). Therefore, it is important to prevent the occurrence of such complications. DN is the most important chronic complication of patients with diabetes and it demonstrates a complex pathogenesis, including the involvement of genetic factors and the activation of polyol signaling pathways, the inflammatory response and the oxidative stress response (1).

BA, a natural molecule, is a major bioactive flavonoid (26). Multiple studies have demonstrated that BA serves a variety of biological functions, including antibacterial (27), antiviral (28-30), antitumor (31) and anti-inflammatory effects (32,33). For example, Lu et al (34) identified that BA promoted osteoclast maturation and function through the MAPK/microphthalmia-associated transcription factor signaling pathway; Zheng et al (35) suggested that BA may serve as a potential therapeutic against leukemia; and Ju et al (36) revealed that BA protected against thrombin-induced cell damage by inhibiting the expression of proteinase-activated receptor 1 and NF-κB activation. However, the role of BA in DN remains relatively unclear. Therefore, the present study investigated the effects of BA on HG-induced podocytes. The results demonstrated that BA significantly improved podocyte viability and decreased the rate of cell apoptosis. Following the establishment of a HG-induced podocyte injury model, it was also revealed that BA significantly increased the viability of HG-induced podocyte cells and decreased the rate of cell apoptosis.

Previous studies have reported that SIRTs were highly expressed and activated in the kidney, liver, spleen, lung, heart and muscle (37,38). In addition, SIRT was identified to be heterogeneous, serving different physiological and pathological roles in different cells and tissues under certain stress conditions (39). SIRT1 is the most studied member of the SIRT family, which is most likely due to its ability to regulate the cell cycle, mitochondrial metabolism, energy homeostasis, inflammation, oxidative stress and apoptosis (38). SIRT1 was found to serve a crucial role in systemic metabolic homeostasis; the downregulation of SIRT1 expression in visceral adipose tissue led to metabolic abnormalities associated with visceral obesity in diabetic and obese women (40). In the present study, BA treatment increased SIRT1 expression levels in a dose-dependent manner in HG-induced podocytes. In addition, BA treatment decreased the p-p65/p65 ratio, whilst there were no significant differences observed in the expression levels of total p65 at both the protein and mRNA level between BA-treated cells and the control cells. Thus, these data indicated that BA may inhibit the HG-induced activation of the NF-κB signaling pathway. Previous studies have demonstrated that the high expression levels of SIRT1 decreased the acetylation of NF-κB and decreased the side effects of cisplatin on renal tubular toxicity (41). Taken together, it was suggested that BA may suppress HG-induced podocyte apoptosis in DN. However, it is worth noting that in the present study, the apoptotic rate of the NG group was >10%, which may just represent the normal rate of programmed cell death in podocytes or it may be due to the cell culture environment; thus, further confirmation is required. Another interesting result was that in contrast with our study, a previous study reported that BA induced apoptosis in cancer cells (10-12).

In conclusion, the results of the present study suggested that BA treatment may inhibit podocyte apoptosis in a HG environment and serve a protective role in DN. In addition, the mechanism of action of BA may be associated with its regulation over SIRT1/NF-κB signaling pathway activation. However, this study is only a preliminary in vitro study of the role of BA in DN. To further validate the role of BA in DN, more in-depth studies are required; for example, determining how BA affects the SIRT1/NF-κB signaling pathway. In addition, the effect of BA in DN needs to be verified in vivo, which is our aim in future studies.

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Availability of data and materials

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

Authors' contributions

JL designed the study, collected the data, performed the statistical analysis, interpreted the data and prepared the manuscript. YL, SYi, SYa and MK contributed to the data collection and helped perform the statistical analysis. ZL contributed to the data collection, statistical analysis and helped prepare the manuscript. All authors read and approved the final manuscript.
Ethics approval and consent to participate
Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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