Biomedicine & Pharmacotherapy 98 (2018) 701–708

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

The protective effect of shikonin on renal tubular epithelial cell injury induced by high glucose

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ARTICLE INFO

Keywords:
Shikonin
Hyperglycemia
NRK-52E cell
Diabetic nephropathy
Oxidative stress

ABSTRACT

Hyperglycemia-induced oxidative stress is thought to play a critical role in the pathogenesis of diabetic nephropathy (DN). Treating high-glucose (HG)-induced proximal tubule injury has become a patential therapeutic option to attenuate the onset and progression of DN. The present study aimed to investigate the renoprotective effect of shikonin, the chief active compound extracted from the roots of the traditional Chinese herb Lithospermum erythrorhizon, on HG-induced cytotoxicity in NRK-52E cells. Treating cells with HG significantly reduce cell viability while also significantly increasing content of reactive oxygen species (ROS). Treating the cells with shikonin improved these changes induced by HG. Shikonin strongly stabilized mitochondrial membrane potential in HG-induced NRK-52E cells. In addition, treatment with shikonin upregulated antioxidant system in response to ROS by increasing levels of SOD and CAT. Furthermore, shikonin also strongly decreased the levels of activated caspase-3, Bax and p-GSK-3β while increased the p-AKT level. These findings provide that the renoprotective effects of shikonin against HG-induced cytotoxicity in NRK-52E cells may be mediated in inhibiting oxidative stress through activating of the AKT signalling pathway.

1. Introduction

Diabetic nephropathy (DN) is a complication of diabetes mellitus (DM) characterized by glomerular and tubulointerstitial injury in developed countries. Due to a lack of effective treatments, DN has become one of the most devastating diseases in the world [1–3]. The major pathological characteristics of DN are glomerular hypertrophy, tubulointerstitial disease, glomerulosclerosis and basement membrane thickening and accumulation of extracellular matrix [4,5]. Although mesangial hypertrophy and glomerular hyperfiltration of glomerulus have been investigated diabetes extensively, proximal tubule (PT) injury is thought to be an important role in the early stage of DN [6–9]. In addition, PT injury is closely linked to the loss of renal ability and can be used to predict the progression of DN. It is reported that hyperglycemia is a potential trigger in tubular cells damage [8].

How high-glucose (HG) induces PT injury is an area of active investigation, and several studies suggest that hyperglycemia-induced oxidative stress plays an important effect in the PT injury, leading to the development and progression of DN [7,10,11]. Furthermore, increasing evidences indicate that high contents of glucose significantly increase oxidative stress in renal tubular epithelial cell. Therefore, numerous studies have investigated the ability of antioxidant drugs to ameliorate DN [3,12,13]. Currently, several antioxidants have been used in the clinic to delay PT injury in DN patients, including taurine, melatonin, vitamin C and vitamin E [4,14,15]. However, the clinical usefulness of these therapies is severely restricted by one or more factors such as cost, stability and safety. Thus, searching for a safer and more effective drug for the treatment of DN remains an important challenge in drug discovery.

Shikonin, a naphthoquinone pigment, is the chief active component extracted from the root of Lithospermum erythrorhizon, which has long been used in traditional oriental medicine for wound healing, urticaria and other allergic diseases [16]. It is reported that shikonin possesses several pharmacological properties, including antioxidant, anti-platelet activation, antiatherosclerosis, antithrombotic, anti-inflammatory, antitumor and antimicrobial [17–22]. These activities of shikonin are considered to be mediated through its scavenging function on oxygen radicals [23,24]. According to reports, shikonin has ability to against

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https://doi.org/10.1016/j.biopha.2017.12.112
Received 15 September 2017; Received in revised form 7 December 2017; Accepted 28 December 2017

several types of reactive oxygen species (ROS), indicating that shikonin could protect PT injury from oxidative damage. Furthermore, it is suggested that shikonin plays a significant protective role in hepatic ischemia/reperfusion injury via reducing reactive oxygen species [25]. Functions of tubular epithelial cells can be affected by many external factors. It is reported that the AKT signalling network plays an important role in the development and progression of DN patient. Additionally, phosphorylated AKT has ability to reduce glycogen synthase kinase-3β (GSK-3β) and cleaved caspase-3 protect renal tubular cell apoptosis under high glucose conditions [26–29]. According to these insights and our previous work, we examined the renoprotective activity of shikonin against high-glucose induced cytotoxicity in renal tubular epithelial cells (NRK-52E) and performed initial experiments to explore the potential mechanism of that activity.

2. Material and methods

Shikonin and D-(+)-glucose powder were obtained from Sigma–Aldrich Inc. (St Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM), trypsin EDTA (Calcium disodium) and fetal bovine serum (FBS) were purchased from Hyclone laboratories Inc. (South Logan, UT, USA). The kits for malonyldialdehyde (MDA), Superoxide Dismutase (SOD), Catalase (CAT) and lactate dehydrogenase (LDH) were provided from Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Reactive oxygen species (ROS) assay kit and mitochondrial membrance potential assay kit with JC-1 were purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Polyclonal antibodies against Bax (#2772), cleaved caspase-3 (#9661), p-AKT (#4060) and p-GSK-3β (#5558) were purchased from Cell Signaling Technology (Danvers, MA, USA). β-actin specific antibody (bs-0061R) was purchased from Bioss (Beijing, China).

2.1. Cell culture

Rat renal tubular epithelial cells (NRK-52E cells) were obtained from the American Type Culture Collection (Rockville, MD, USA). NRK-52E cells were cultured in high glucose DMEM supplemented with 10% HS, 2 mM lglutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. NRK-52E cells were incubated and the medium was changed every two days.

2.2. Cell viability and LDH assay

Cell viability was assessed by quantitative colorimetric assay via MTT method as described previously. Briefly, the NRK-52E cells were plated in 96-well plates at a density of 8×10^3 cells/well and incubated for 24 h for viability assay. NRK-52E cells were treated with normal glucose (NG, 5.6 mM glucose), high glucose (HG, 30 mM glucose) and high glucose with shikonin at different concentration (1, 3, 10 μg/ml) for 48 h. MTT solution (5 mg/ml) was added to each well and incubated for 4 h at 37°C. The supernatants were then aspirated, 150 μl of DMSO was added to solubilize the formazan crystals with shaking for 5 min. Cell viability was measured by reading absorbance at 570 nm in a plate reader (Thermo, Varioskan Flash).

The release of LDH activity as an in vitro marker for cellular toxicity was measured by using a commercial assay kit. In brief, 6-well plates were seeded at 8×10^4 cells/well for LDH release determination, NRK-52E cells were treated with normal glucose (NG, 5.6 mM glucose), high glucose (HG, 30 mM glucose) and high glucose with shikonin at different concentration (1, 3, 10 μg/ml) for 48 h. The culture medium was collected for the extracellular LDH activity assay. Then adherent cells were washed with PBS buffer, scraped into 500 μl of PBS, and homogenized. The homogenate was centrifuged and supernatants were used to measure intracellular LDH activity. The absorbance of each sample was measured at 450 nm via microplate reader. LDH release rate was calculated as follows:

$$\text{LDH release rate}(\%) = \frac{\text{LDH activity in the culture medium}}{\text{LDH activity in the culture medium} + \text{LDH activity in cells}} \times 100\%$$

2.3. TUNEL staining

The degrees of DNA nick formation and genomic DNA fragmentation was detected by the terminal deoxynucleotidyl transferase nickend labeling (TUNEL) assay. Briefly, NRK-52E cells were seeded onto coverslips and treated with 10 μg/ml shikonin and HG as described for the cell viability assays, media was discarded from plates and washed with PBS three times. Then the cells were fixed in 4% paraformaldehyde for 10 min. The cells were further incubated in a blocking and permeabilizd with 0.1% TritonX-100 in 0.1% sodium acetate for 5 min at 4°C. Thereafter, the cells were labeled by incubation with the TUNEL reaction and fluorescent dUTP mixture for 60 min at 37°C. Nuclei were counterstained 4′,6′-diamidino-2-phenylindole (DAPI). Subsequently, the cells were observed by fluorescent microscope (Carl Zeiss Shanghai Co., Ltd).

2.4. Lipid peroxidation assay

The content of Malondialdehyde (MDA), an index of lipid peroxidation, was assessed using a commercial assay kit according to the manufacture's instruction. In brief, NRK-52E cells were seeded into 6-well plates at a density of 8×10^4 cells/well and treated with shikonin and HG as described for the cell viability assays. At the end of the treatment, NRK-52E cells were washed with PBS three times and homogenized in 0.5 ml buffer solution. The homogenate was centrifuged at 4000 g for 10 min and supernatants were used to measure the content of MDA.

2.5. Dichlorofluorescein assay for ROS

ROS was measured using the DCFH-DA method. Concentration of intracellular ROS can be detected with DCFH-DA, which crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF), which is readily measured by fluorescent microscope and flow cyctometry. Briefly, NRK-52E cells were seeded in 6-well plates at a density of 8×10^4 cells/well and incubated with shikonin and HG as described for the cell viability assays. At the end of treatment, the cells were washed with PBS solution and incubated with DCFH-DA at a final concentration of 10 μM for 30 min at 37°C. After the cells were washed three times with PBS solution to remove the extracellular DCFH-DA, the cells were observed by fluorescent microscope and the fluorescence intensity of DCF was measured by flow cytometry (Beckman Coulter).

2.6. SOD and CAT assay

The activities of antioxidant enzymes, including SOD and CAT, were measured using commercial assay kits according to the manufacturer's instructions. In brief, NRK-52E cells were seeded in 6-well plates at a density of 8×10^4 cells/well and incubated with shikonin and HG as described for the cell viability assays. At the end of the treatment, NRK-52E cells were washed with PBS three times and homogenized in 0.5ml buffer solution. The homogenates were centrifuged and the supernatants were used to measure the activities of antioxidant system. The level of SOD was detected with xanthine oxidase method at 550 nm wavelength with a spectrophotometer. The level of CAT was measured using a spectrophotometric assay at 405 nm wavelength.