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Astragalosides IV protected the renal tubular epithelial cells from free fatty acids-induced injury by reducing oxidative stress and apoptosis

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ABSTRACT

Renal tubular injury is associated with the development of diabetic nephropathy (DN) and the end-stage renal disease (ESRD). Free fatty acids (FFAs)-associated lipotoxicity contributes to injury of proximal renal tubular epithelial (HK-2) cells in diabetes. Palmitic acid (PA) which is the most abundant saturated fatty acid in FFAs is closely associated with the gradual decline of renal function. Astragalosides IV (AS-IV) has a variety of pharmacological effects such as anti-inflammation and anti-oxidation. In the current study, we investigated the effects of AS-IV on PA-induced apoptosis of HK-2 cells and the underlying mechanisms. The results showed that AS-IV (10, 20, 40 μmol/L) could alleviate PA-induced apoptosis of HK-2 cells. We found that AS-IV reduced the expression of Bax and cleaved-caspase3, but increased the expression of Bcl-2 and phosphorylated Nrf2 in HK-2 cells. Moreover, AS-IV reduced the level of reactive oxygen species (ROS) in the cells. Our study suggests that AS-IV could protect against PA-induced apoptosis in HK-2 cells by inhibiting ROS generation and apoptotic protein expression. This study may provide a new theoretical option for the patients with type 2 diabetes.

1. Introduction

Diabetic nephropathy (DN) accounts for 30–47% of the end-stage renal disease (ESRD) and is a major cause of death in diabetic patients [1]. Although mesangial cells and podocytes are main targets of pathological stimuli associated with diabetes in kidney, a large amount of evidence showed that renal tubular injury also contributes to DN and ESRD [2,3]. Accumulation of lipid deposits and excessive free fatty acids (FFAs) in kidneys lead to injury of HK-2 cells and consequently decline of renal function [4–6]. Both excessive FFAs and renal tubular injury were found in rats with diabetes mellitus (DM) [7,8]. Excessive FFAs levels were also detected in obese patients and type 2 diabetic patients. However, the specific mechanisms for the free fatty acids-induced renal tubular damage has not yet been identified. Oxidative stress is one of the most important mechanisms leading to diabetic complications. The increase of ROS can promote the apoptosis of renal tubular cells and aggravate renal injury [9]. However, whether oxidative stress is associated with FFAs-induced HK-2 cell damage remains unclear.

At present, there is no effective medical treatment for DN. AS-IV is a traditional Chinese medicine extracts with various pharmacological effects [10]. It is reported that AS-IV has protective effect on the kidneys of diabetic rats and alleviated renal damage caused by DN [11]. AS-IV also inhibited the apoptosis of HK-2 cells induced by high glucose [12]. However, whether AS-IV has protective effects on HK-2 cells from FFAs-induced injury remains unclear. The present study was carried out to explore the potential protective effects of AS-IV on HK-2 cells against PA-induced injury and the mechanisms involved. Our results showed that AS-IV decreased ROS production and abundance of apoptosis-related proteins, such as Bax, Bcl-2, cleaved-caspase3, and increased the level of Nrf2 in HK-2 cells treated with PA, suggesting that AS-IV has protective effects on the renal tubular epithelial cells in DN.

2. Materials and methods

2.1. Preparation of palmitic acid (PA)

Briefly, Sodium palmitate acid (PA, sigam-aldrich, USA) was dissolved in distilled water and then gently mixed at 70℃ until completely dissolved. The PA solution was then mixed with fatty acid-free bovine serum albumin (BSA, Shanghai Yeasen Biotechnoligy Co., Ltd, China) at a ratio of 6:1 (PA : BSA) at 55 ℃ for 2 h to produce a stock solution. The complex fatty acid solution was added to the serum-free medium to obtain the final PA concentration of 200 μmol/L.

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2.2. Cell culture and treatment

HK-2 cell line was gifted from college of pharmacy, Anhui Medical University. HK-2 cells were cultured (at 37℃, in a 5% CO_2 atmosphere) in DMEM/F12 containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin until the cell density reaches 70–80%. The cells were transferred to serum-free medium for 24 h before used for experiments. The cells were divided into the following groups: 1) control group: the HK-2 cells were treated with BSA (0.2%); 2) model group: the HK-2 cells were stimulated with PA (200 μmol/L) for 24 h; 3) AS-IV-treated groups: the HK-2 cells were stimulated with PA (200 μmol/L) and different concentrations of AS-IV (10, 20, 40 μmol/L) for 24 h. All treatments were triplicated.

2.3. Cell viability assay

Cell proliferation was assessed by 3-(4, 5-Dimethylthiazol-2-yl) -2, 5- diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) colorimetric assay to determine the role of PA and AS-IV (Astragaloside IV, purity > 98%, Nanjing Zelang Medical Technology Co., Ltd, China). The cells were seeded as 5×10^3 per well in a 96-well plate. Then the different concentrations of AS-IV (10, 20, 40 μmol/L) or PA (200 μmol/L) and AS-IV (10, 20, 40 μmol/L) were given simultaneously for 24 h, and the same concentration of vehicle was added to the control group. After incubation, MTT solution (5 mg/ml) was added to the media and incubated for 4 h, then dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) was used to dissolve the formazan formed in living cells, and absorbance of the solution was measured at 490 nm using a microplate reader (Thermo Fisher company, USA). The experiment was conducted in triplicate. Cell viability was calculated as (administration group OD - blank group OD) / (control group OD - blank group OD) × 100%.

2.4. Oil red O staining

The cells were rinsed with phosphate buffered saline (PBS) and then fixed with 4% formaldehyde at room temperature for 15 min or longer. Then cells were washed with PBS and immersed in an Oil red O working solution (Oil red O dye to distilled water at a volume ratio of 3:2) at room temperature for 20 min. The cells were washed with water to remove any unbound dyes and then visualized under a microscope (BX-51, Olympus Co. Ltd, Japan). The contents of lipid accumulation in HK-2 cells were quantified by Image-Pro Plus 6.0.

2.5. Apoptosis analysis

For analysis of cell apoptosis, HK-2 cells were stained with Hoechst-33258 (Beyotime Biotechnology, Shanghai, China) and Annexin V-FITC/PI (Beibo Biology Co. Ltd, Shanghai, China) assay kits. For Hoechst 33258 staining, the cells were incubated with Hoechst 33258 at room temperature for 5 min and were then washed by PBS. The percentage of apoptotic cells was counted using fluorescent microscopy. The nucleus of normal cells was shown blue, while the nucleus of apoptotic cells was somewhat white, densely stained or fragmented. For Annexin V-FITC/PI staining, according to the manufacturer's instructions, Annexin V-FITC-stained green fluorescence represents the early apoptosis and PI-stained red fluorescence represents the late apoptosis. Cells were stained with Annexin V-FITC/PI (annexin V-FITC:PI, 1:2) and incubated at room temperature for 15 min in dark. Apoptotic cells were visualized under a fluorescence microscope (BX-51, Olympus Co., Ltd. Japan).

2.6. ROS assay

HK-2 cells were treated as before and incubated with DCFH-DA (Sigma, USA, 10 μmol/L) at 37 ℃ for 30 min in dark. DCFH-DA was a cell-permeable, nonfluorescent precursor of DCF. This non-fluorescent product accumulates in the cells and is oxidized and yields the highly fluorescent DCF product which can be visualized under a fluorescence microscope (BX-51, Olympus Co., Ltd. Japan). The production of ROS was quantified by densitometry with Image-Pro Plus 6.0 software.

2.7. Western blotting analysis

Cells were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer containing 1 mM PMSF and protease inhibitors to extract proteins. The protein concentration of each sample was determined by BCA protein concentration test kit (Beyotime Biotechnology, Shanghai, China). The same amount of proteins were loaded to and were separated by SDS-PAGE, and were transferred to PVDF membrane (Millipore Co., Ltd. USA). The PVDF membranes were blocked with 5% skim milk in TBST buffer, and incubated overnight at 4℃ with corresponding primary antibodies, anti cleaved-caspase3 (1:500), anti-Bax (1:500), anti-Bcl-2 (1:500), anti-Nrf2-phosphos 40 (1:1000), anti-H3 (1:500) and βactin (1:2000). Then the membranes were washed with TBST and incubated with HRP-conjugated secondary antibody (1:10,000). Antibody-antigen complexes were visualized with ECL and analyzed quantitatively by densitometry with Image J software. The relative density of immunoreactive bands was normalized to the density of the corresponding β-actin bands.

2.8. Statistical analysis

Data were collected from the repeated experiments at least three times. Results were expressed as mean ± SD. Statistical significance was analyzed via one-way analysis of variance (ANOVA) using SPSS 17.0 software. The $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of PA and AS-IV on cell viability

To assess the cytotoxicity of AS-IV, the HK-2 cells were treated with different concentrations of AS-IV ranging from 6.25 to 200 μmol/L. The MTT results showed that the AS-IV at concentrations from 6.25 to 100 μmol/L had no significant effect on normal HK-2 cell activity, while AS-IV 200 μmol/L could decrease the cell viability (Fig.1A). Therefore, we selected AS-IV at concentrations of 10, 20 and 40 μmol/L for all subsequent experiments. To investigate whether AS-IV protects against HK-2 cell injury induced by PA, we examined the effect of AS-IV (10, 20, or 40 μmol/L) on HK-2 cells for 24 h, and the results showed that the AS-IV treatment at all the concentrations significantly increased the cell survival rate(Fig.1B).

3.2. Effect of AS-IV on lipid accumulation in HK-2 cells

To evaluate the effect of AS-IV on intracellular lipid deposition in HK-2 cells, we treated the cells with PA plus AS-IV (10, 20, or 40 μmol/L) for 24 h. Oil Red Staining was used to determine the effects of AS-IV on lipid deposition. As shown in Fig. 2, compared with control group, intracellular lipid deposition was significantly increased after PA stimulation. However, AS-IV treatment significantly decreased the PA-induced lipid deposition, compared to the PA model group. These results suggest that AS-IV can reduce lipid deposition in HK-2 Cells.

3.3. Effect of AS-IV on apoptosis in HK-2 cells

To investigate whether AS-IV protected against PA-induced apoptosis, HK-2 cells were subjected to apoptosis assay by Hoechst 33258 (Fig. 3) and Annexin V-FITC/PI Kits (Fig. 4). We found that the apoptosis rate significantly increased in PA group compared with the control group. Compared with the model group, simultaneous treatment with