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Full paper

Salidroside protects renal tubular epithelial cells from hypoxia/reoxygenation injury *in vitro*

Yan Sun [a], Liru Xun [a], Gang Jin [a], Lei Shi [b, *]

[a] Department of Nephrology, Shaanxi Provincial People's Hospital, Xi'an, 710068, China
[b] Department of Infectious Disease, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, 710061, China

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ABSTRACT

Oxidative stress, inflammation and cell apoptosis are important mechanisms of renal ischemia/reperfusion (I/R) injury. Salidroside, a natural phenylpropanoid glycoside, possesses anti-inflammatory, anti-oxidative, and anti-apoptotic effects. However, the effect of salidroside on renal I/R injury has not been fully elucidated. The present study aimed to investigate the effect of salidroside on renal I/R injury *in vitro*. Our results showed that salidroside improved the viability of human renal tubular epithelial cells (HK-2) in response to hypoxia/reoxygenation (H/R). Salidroside caused apparent decrease in the levels of reactive oxygen species (ROS) and malondiaidehyde (MDA), and significant increase in superoxide dismutase (SOD) activity in HK-2 cells. Pretreatment with salidroside markedly inhibited the production levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 in a dose-dependent manner. Salidroside treatment exhibited significant increase in Bcl-2 expressions, and decrease in Bax expressions and caspase-3 activity when compared with the H/R group. Salidroside decreased the levels of toll-like receptor 4 (TLR4) and p-p65 in HK-2 cells. Overexpression of TLR4 significantly attenuated the effects of salidroside on cell viability, oxidative stress, cytokine production and cell apoptosis in HK-2 cells. These findings indicated that salidroside protected HK-2 cells from H/R stimulation, which was mediated by the TLR4/NF-κB pathway.

Introduction

Acute kidney injury (AKI) is a clinical condition which is characterized as a rapid loss of kidney function.[1] Patients with AKI present an abrupt increase in serum creatinine that result from the kidney injury.[1] AKI may cause various complications, such as changes in fluid balance, metabolic acidosis, uremia, and influences on other organs, which may lead to death.[2] Therefore, better understanding of the cause and mechanism is helpful for the management and treatment of AKI.

It is known that renal ischemia/reperfusion (I/R) is a primary cause of AKI.[3] Renal I/R injury is an unavoidable incident that commonly occurs in blockage of renal blood flow and renal transplantation.[3,4] Therefore, it is important to develop novel therapies for controlling renal I/R injury. It has been demonstrated that several biological processes including oxidative stress, inflammation, cell apoptosis, and necrosis play essential roles during renal I/R injury.[5–7] Consequently, many researchers focus on the anti-inflammatory and anti-oxidant agents in order to explore a new medicine for effective management of renal I/R injury.[6,7] However, the available therapy for curing renal I/R injury remains very limited.

Salidroside, a phenylpropanoid glycoside, is a bioactive constituent extracted from *Rhodiola rosea L*[8]. Pharmacological studies have proved that salidroside possesses a wide range of activities such as anti-inflammatory,[9] anti-oxidative,[10] anti-apoptotic effects.[11] In addition, salidroside has been reported to attenuate I/R injury.[12,13] However, the effects of salidroside on renal I/R injury in human renal tubular epithelial cells remain to be elucidated.

In the current study, we investigated the effect of salidroside on renal I/R injury *in vitro* and explored the possible molecular mechanism. The results indicated that salidroside protected HK-2 cells from hypoxia/reoxygenation (H/R) stimulation. The protective effect of salidroside was mediated by the toll-like receptor 4 (TLR4)/NF-κB signaling pathway.

* Corresponding author.
E-mail address: shilei_xa@163.com (L. Shi).
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Materials and methods

Cell culture

Human renal tubular epithelial cells (HK-2) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in DMEM/F12 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin (Sigma, St. Louis, MO, USA) and 0.1 mg/mL streptomycin (Sigma). The HK-2 cells were cultured in a normoxic atmosphere (5% CO_2) at 37 °C.

H/R model establishment

H/R model was established to simulate I/R injury *in vitro*. HK-2 cells were incubated in serum-free DMEM/F12 medium under hypoxia atmosphere (containing 5% CO_2, 1% O_2, and 94% N_2) at 37 °C for 24 h, and then subjected to a 12 h exposure to reoxygenation atmosphere (containing 5% CO_2, 21% O_2, and 74% N_2) in DMEM/F12 medium supplemented with 10% FBS. To explore the effect of salidroside on H/R injury, the different concentrations of salidroside (6.25, 12.5, 25 μM) were added to the medium 2 h before the H/R induction. Salidroside was purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO; Sigma).

Cell transfection

HK-2 cells were transiently transfected with TLR4 over-expressing plasmid (Santa Cruz Biotechnology, CA, USA). After 48 h, the transfected cells were subjected to H/R induction with or without the presence of salidroside.

Cell viability assay

HK-2 cells (1×10^4 cells/well) were plated in 96-well plates and incubated for 24 h. Then the cells were exposed to a series concentration of salidroside (0, 6.25, 12.5, 25, 50 μM) for 24 h; or exposed to different concentrations of salidroside (6.25, 12.5, 25 μM) for 2 h and then subjected to H/R induction. MTT assay was performed to evaluate the cell viability of HK-2 cells. In brief, 50 μl MTT solution (5 mg/ml, Sigma) was added to each well and incubated for 4 h. Subsequently, 150 μl dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Finally, the absorbance at the wavelength of 490 nm was determined using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of reactive oxygen species (ROS) level

The ROS levels were measured using a ROS Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The 2′,7′-dichlorofluorescein diacetate (DCFH-DA) solution was added to the medium of HK-2 cells and incubated for 30 min at 37 °C. Then the cells were trypsinized, washed, and analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA).

Measurement of malondialdehyde (MDA) level and superoxide dismutase (SOD) activity

The cell lysates of HK-2 cells were prepared using RIPA buffer as described previously.[14] Then the MDA level and SOD activity in cell lysates were respectively detected using MDA assay kit (Nanjing Jiancheng Bioengineering Institute) and SOD assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Western blotting

The total proteins were extracted from HK-2 cells using a Total Cell Protein Extraction Kit (Millipore, Billerica, MA, USA). After determination of protein concentration with a BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China), proteins (30 μg per lane) from each sample were electrophoresed through 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Then the proteins were transferred to nitrocellulose membranes (Millipore). The membranes were blocked in 5% skim milk at room temperature for 1 h, and then incubated with primary antibodies against Bcl-2 (dilution 1:3000, Santa Cruz Biotechnology, CA, USA), Bax (dilution 1: 2000, Santa Cruz Biotechnology), TLR4 (dilution 1: 500, Abcam, Cambridge, MA, USA), p-p65 (dilution 1: 1500, Abcam), p65 (dilution 1: 500, Abcam) and GAPDH (dilution 1: 2000, Santa Cruz Biotechnology) at 4 °C overnight. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000, Santa Cruz Biotechnology) for 1 h at 37 °C. Finally, the immunoreactive bands on the membranes were visualized using an enhanced chemiluminescence (ECL) system (Thermo Scientific, Waltham, MA, USA) and the density was quantified using Quantity One software (version 4.6.2, Bio-Rad Laboratories).

Measurement of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 cytokine levels

The supernatant of HK-2 cells was collected for the measurement of cytokines secretion. The levels of TNF-α, IL-1β and IL-6 were determined by ELISA method using commercial kits (R&D Systems, Minneapolis, MN, USA).

Caspase-3 activity assay

The caspase-3 activity in cell lysates of HK-2 cells was measured using a caspase-3 activation kit (R&D Systems). The cell lysates were incubated with the caspase-3 substrate and reaction buffer in the dark at 37 °C for 2 h. The absorbance at 405 nm was read by a BioTek microplate reader.

Statistical analysis

The statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, IL, USA). All data were shown as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to analyze the differences among groups. A $p < 0.05$ was thought to be statistically significant.

Results

Salidroside pretreatment significantly improved HK-2 cell viability in response to H/R

We first assessed the cytotoxicity effect of salidroside on HK-2 cells. As shown in Fig. 1A, treatment with low doses of salidroside (6.25, 12.5, and 25 μM) did not affect the cell viability of HK-2 cells; however, the high dose of salidroside (50 μM) caused significant reduction in cell viability. Therefore, the low doses of salidroside, 6.25, 12.5, and 25 μM, were selected for the following experiments. In order to explore the effect of salidroside on I/R injury, HK-2 cells were subjected to H/R induction to simulate I/R injury *in vitro*. The different concentrations of salidroside (6.25, 12.5, 25 μM) were added to the medium 2 h before the H/R induction. The results of MTT assay indicated that H/R induction markedly suppressed the cell viability, whereas salidroside pretreatment reversed the inhibition effect of H/R induction on cell viability in a dose-dependent manner (Fig. 1B).

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