Original Research Article

Rehmannioside A protects against high glucose-induced apoptosis and oxidative stress of renal tubular epithelial cells by inhibiting the MAPK pathway

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Abstract

Purpose: To investigate the effects and mechanism of rehmannioside A (ReA) on diabetic nephropathy (DN) progression.
Methods: Various concentrations of glucose and ReA were added to HK2 cells, and cell viability was analyzed using the 3-(4,5)-dimethylthiahiazo(-2)-3,5-diphenytetrazoliumromide (MTT) assay. Cell apoptosis, caspase 3 activity, and expression levels of BAX, Bcl-2, and cleaved poly (ADP-ribose) polymerase were evaluated to assess the effect of ReA on cell apoptosis. The effect of ReA on oxidative stress was also evaluated by assessing superoxide dismutase, catalase, and malondialdehyde levels. Lactate dehydrogenase release and reactive oxygen species levels were also measured. Finally, activation of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 1/2 was determined by immunoblot technique.
Results: ReA significantly enhanced the survival of HK2 cells induced with high glucose (HG). In addition, ReA suppressed apoptosis and inhibited oxidative stress of HK2 cells induced with HG ($p < 0.05$). ReA protected against HG-induced apoptosis and oxidative stress of renal tubular epithelial cells by inhibiting the MAPK pathway ($p < 0.05$).
Conclusion: ReA is a potential as a therapeutic agent for DN; however, in vivo and clinical investigations are required to validate this assertion.

Keywords: Diabetic nephropathy, Rehmannioside, Apoptosis, Oxidative stress, MAPK pathway, HK2 cells

INTRODUCTION

Diabetic nephropathy (DN) is a microvascular complication of diabetes characterized by persistent clinical proteinuria and decreased glomerular filtration rate [1]. DN increases the long-term incidence rate and patient mortality and is the main cause of end-stage renal disease [2]. The pathological mechanism of DN has not been fully described, and currently, hyperglycemia is considered to be the driving factor. Hyperglycemia can activate various signal transduction pathways in different types of renal cells promoting glomerular ultrafiltration [3]. The

development of DN is related to renal tubule injury and deterioration of renal function [4]; thus, renal tubule injury is an appropriate therapeutic target of DN.

Apoptosis and oxidative stress are important factors in the pathogenesis of DN [5]. Oxidative stress is thought to deteriorate hemodynamics and renal function by changing the cellular signaling pathways involved in renal metabolism [6]. Hyperglycemia increases the production of reactive oxygen species (ROS) through a variety of mechanisms that aggravate oxidative stress [7]. Excessive oxidative stress induces apoptosis of renal tubular epithelial cells through an oxidant-dependent mechanism [8]. Therefore, inhibition of oxidative stress and apoptosis are two ways to treat DN.

Rehmannioside A (ReA) is a compound extracted from the plant *Rehmannia glutinosa* [9] that displays anti-inflammatory, anti-oxidant, and neuroprotective effects. It has been shown that ReA regulates expression of nuclear factor erythroid 2-related factor 2, nuclear factor kappa B (NF-kB), and caspase-3; improves anti-oxidant, anti-inflammatory, and anti-apoptotic capabilities; and improves cognition in rats with vascular dementia [9]. ReA inhibits the release of inflammatory mediators by inhibiting NF-kB and mitogen-activated protein kinase (MAPK) signaling pathways, promotes macrophage M2 polarization *in vitro*, protects co-cultured neurons from apoptosis, and alleviates spinal cord injury in rats. Therefore, it has promise for the treatment of spinal cord injury [9].

In this study, the effects of ReA on DN progression were investigated. ReA suppressed high glucose (HG)-induced apoptosis and oxidative stress of renal tubular epithelial cells; therefore, ReA has promise for the treatment of DN.

EXPERIMENTAL

Material

Rehmannioside A (purity > 98 %) was purchased from Chengdu Herbpurify Co., Ltd (Chengdu, China).

Cell culture and treatments

HK2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 medium containing 5 % fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM l-glutamine, and 100 U/ml penicillin/streptomycin. Cells were treated with 0, 20, 40, 60 or 80 mM glucose for 24 h. For ReA treatment, cells were incubated with 20, 40, 80, or 100 µM ReA for 24 h.

Determination of cell viability

HK2 cells were plated into 96-well plates at a density of $5×10^3$ cells/well. Cell viability was assessed via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, the plates were washed with phosphate-buffered saline (PBS), 10 % MTT was added to each well, the plates were incubated for 4 h, and then absorbance at 490 nm was measured using a microplate reader.

Assessment of cell apoptosis

Apoptosis was measured using the Annexin V/PI apoptosis kit according to the manufacturer's protocol (Sigma Aldrich, St. Louis, MO, USA). Briefly, cells were digested and resuspended in binding buffer containing Annexin V and propidium iodide (PI) for 5 min at room temperature in the dark. The proportion of intact cells (Annexin V^-/PI^-), early apoptotic cells (Annexin V^+/PI^-), and late apoptotic cells (Annexin V^+/PI^+) were determined using a flow cytometer (BD Biosciences, San Jose, CA, USA).

Determination of caspase-3 activity

For the determination of caspase-3 activity, the Caspase-3 activity kit (ab252897, Abcam, Cambridge, UK) was used according to the manufacturer's instructions.

Western blotting

Cells were harvested and lysed with RIPA buffer (Abcam), protein concentrations were determined by the BSA method, samples were subjected to SDS-PAGE, and then transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5 % BSA in PBST. Then, membranes were probed with primary antibodies targeting BAX (1:1000 dilution, ab32503, Abcam), p-ERK (1:1000 dilution, ab201015, Abcam), ERK (1:1000 dilution, ab184699, Abcam), p-p38 (1:1000 dilution, ab178867, Abcam), p38 (1:1000 dilution, ab170099, Abcam), and β-actin (1:3000 dilution, ab8226, Abcam). Subsequently, membranes were treated with goat anti-rabbit or goat anti-mouse horseradish peroxidase conjugated secondary antibodies, and bands were detected by enhanced chemiluminescence. Relative protein levels were quantified using LabWorks 4.5

software (UVP, LLC, Phoenix, AZ, USA) with β-actin as the internal control.

Evaluation of ROS

The Reactive Oxygen Species Assay Kit (Biyuntian, Shanghai, China) was used to measure ROS production. Cells were treated as indicated and then ROS detection reagent was added for 1 h following the manufacturer's instructions. Immunofluorescence images were obtained with an inverted fluorescence microscope (Olympus IX71, Tokyo, Japan).

Lactate dehydrogenase assay

The lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect cellular LDH release. The medium from each well was collected and transferred to a 96-well flat-bottom plate, reaction mixture was added to each sample, the plate was incubated for 30 min in the dark, and then stop solution was added to each well and the absorbance at 490 nm was measured using a SpectraMax M Series Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA).

Superoxide dismutase, malondialdehyde, and catalase assays

Malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) levels were measured using the appropriate commercial kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

Data are shown as mean ± standard deviation (SD). For statistical analysis, the Student's t-test was used, and $p < 0.05$ was considered statistically significant.

RESULTS

ReA protects HK2 cells from HG-induced cell injury

HK2 cell viability upon exposure to increasing glucose concentrations was determined. As expected, exposure to HG (40, 60, and 80 mM) decreased HK2 cell viability (Figure 1 A). Because significant damage was caused upon exposure to 40 mM glucose, this concentration was used in the further experiments. The toxicity of ReA on HK2 cells was also measured, and no significant effect was found on HK2 cell viability

upon exposure to 20 µM ReA for 24 h (Figure 1 B); thus 20 µM ReA was used in the further experiments. To measure the effect of ReA on HG-induced cell injury, cells were exposed to 40 mM glucose and treated with or without various concentrations of ReA. Cell viability improved in a dose-dependent manner upon treatment with ReA (Figure 1 C). Thus, ReA treatment improved the viability of HK2 cells exposed to HG.
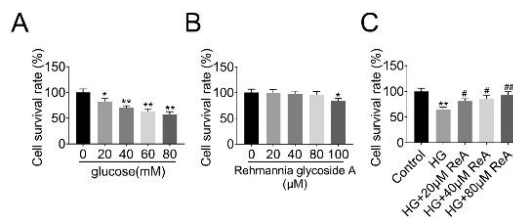

Figure 1: ReA protects HK2 cells from HG-induced cell injury**.** A, Cell viability of HK2 cells exposed to the indicated glucose concentrations assayed by MTT. B, Cell viability of HK2 cells exposed to various ReA concentrations assayed by MTT. C, Cell viability of HK2 cells exposed to HG with or without ReA treatment assayed by MTT. *$P < 0.05$, **$p < 0.01$ vs control, #$p < 0.05$, ##$p < 0.01$ vs HG

ReA alleviated HK2 cell apoptosis induced by HG

Previous studies showed that apoptosis is the main cause of diabetes cardiomyopathy; thus, apoptosis of HK2 cells with or without ReA treatment was evaluated upon HG-induction. A sharp induction of apoptosis was observed in cells exposed to HG. In the ReA treatment groups, the number of apoptotic cells was reduced significantly in a dose-dependent manner (Figure 2 A). Caspase 3 activity was also reduced upon ReA treatment in a dose-dependent manner (Figure 2 B). Further, BAX and cleaved poly (ADP-ribose) polymerase (PARP) protein levels were higher and Bcl-2 levels were lower in the HG group (Figure 3). ReA treatment of HG-induced cells significantly inhibited the accumulation of BAX and cleaved PARP proteins and increased Bcl-2 levels, indicating reduced cell apoptosis following ReA treatment (Figure 3). In conclusion, ReA treatment alleviates HK2 cell apoptosis induced by HG.

ReA represses oxidative stress induced by HG

SOD, MDA, CAT, LDH, and ROS levels were measured. Induction of MDA and reduction of SOD and CAT levels were detected in the HG group. Upon treatment with ReA, the MDA level decreased and the SOD and CAT levels

increased in a dose-dependent manner (Figure 4 A). The LDH level increased upon HG exposure, whereas LDH release was blocked in the ReA treated group (Figure 4 B). HG exposure significantly increased ROS levels in HK2 cells, and ReA treatment inhibited HG-induced ROS production (Figure 4 C). These results suggests that ReA treatment reduces oxidative stress in HK2 cells.

ReA inhibits HG-induced apoptosis via suppression of p38 MAPK and ERK1/2 phosphorylation

Although the p38 MAPK pathway affects HG-induced cell apoptosis in renal tubular epithelial cells, it is unknown whether ReA exerts its effect via the MAPK pathway. As shown in Figure 5, p-p38 MAPK and p-ERK1/2 (extracellular signal-regulated kinase 1/2) levels were significantly induced in HK2 cells exposed to HG. In ReA treated HG-induced HK2 cells, this increase in p-p38 MAPK and p-ERK1/2 levels was suppressed. Thus, the anti-apoptotic function of ReA involves inhibition of p38 MAPK and ERK1/2 phosphorylation in renal tubular epithelial cells.
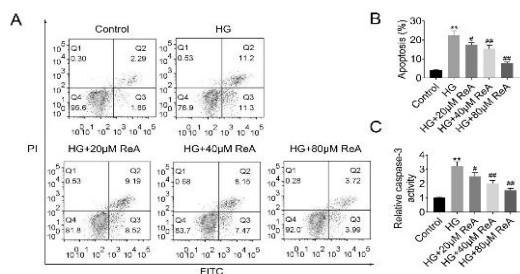

Figure 2: ReA treatment alleviated HK2 cell apoptosis induced by HG. A and B, Cell apoptosis as detected by flow cytometry. C, Caspase 3 activity. **$P < 0.01$ vs control, #$p < 0.05$, ##$p < 0.01$ vs HG
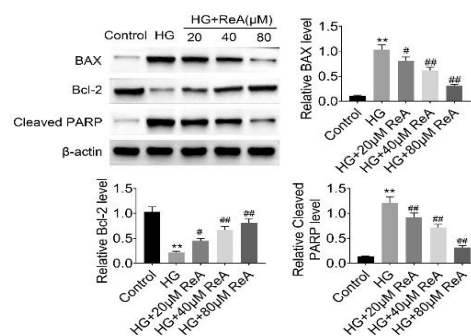

Figure 3: Expression of apoptosis-related proteins Bcl-2, BAX, and cleaved PARP induced by HG upon ReA treatment. **$P < 0.01$ vs control, #$p < 0.05$, ##$p < 0.01$ vs HG
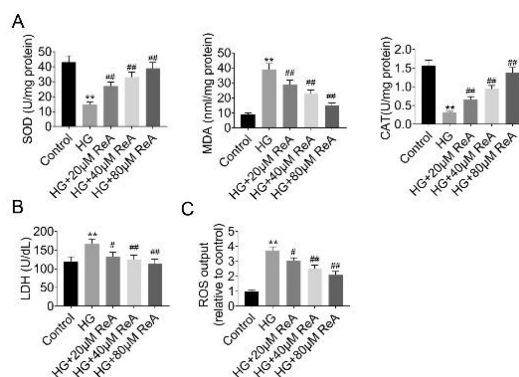

Figure 4: ReA represses oxidative stress induced by HG. A, SOD, MDA, and CAT levels in the various treatment groups. B, LDH release in the various treatment groups. C, Cellular ROS levels in the various treatment groups. **$P < 0.01$ vs control, #$p < 0.05$, ##$p < 0.01$ vs HG
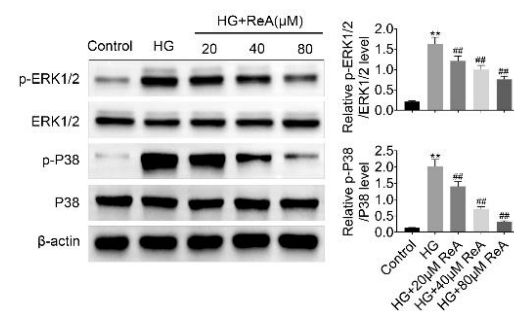

Figure 5: ReA treatment inhibits HG-induced apoptosis via suppression of p38 MAPK and ERK1/2 phosphorylation. Expression of p-p38 MAPK, p38 MAPK, p-ERK1/2, and ERK1/2 in the various treatment groups. **$P < 0.01$ vs control, #$p < 0.05$, ##$p < 0.01$ vs HG

DISCUSSION

In China, the incidence of DN has been increasing annually [10]. Early DN presents as asymptomatic with normal or high blood pressure; however, the incidence of DN increases as diabetes progresses [11]. The development of DN associates with tubular injury and with deterioration of renal function [12]. In this study, HK2 renal tubular epithelial cells were treated with HG to induce DN and the effects of ReA on the HG-induced cells were evaluated. Our results showed that ReA treatment reversed the negative effects of HG on survival, apoptosis, and oxidative stress of renal tubular epithelial cells, indicating that ReA can treat DN.

Multiple biological activities of ReA have been demonstrated. ReA has been shown to mediate microglial polarization and to attenuate neuronal apoptosis by suppressing the NF-κB and MAPK pathways involved in spinal cord injury repair. In

addition, ReA attenuated cognitive deficits in rats with vascular dementia by inhibiting oxidative stress, inflammation, and apoptosis. Thus, ReA has been shown to have anti-inflammatory, anti-oxidative stress, and neuroprotective effects. In this study, the pro-survival, anti-apoptosis, and anti-oxidative stress activities of ReA were observed in the *in vitro* DN cell model. Thus, ReA has potential as a therapeutic drug for multiple diseases. Hyperglycemia exacerbates oxidative stress by increasing ROS production through a variety of mechanisms [13]. Further, excessive oxidative stress induces apoptosis of renal tubular epithelial cells [14]. Therefore, suppression of oxidative stress and apoptosis of renal tubular epithelial cells could be an effective treatment for DN, and numerous drugs have been used for this purpose [15]. ReA suppressed both apoptosis and oxidative stress of HG-induced HK2 cells, so it could be an effective treatment for DN.

In this study, ReA promoted survival, suppressed apoptosis, and suppressed oxidative stress in HG-induced HK2 cells via the MAPK pathway, and the MAPK pathway has been shown to be involved in DN progression [15]. The MAPK pathway regulates proliferation, survival, and apoptosis of multiple cell types, as well as DNA damage and cancer progression [5,16]. The MAPK pathway also mediates cellular oxidative stress [17]. Thus, multiple drugs that target this pathway could be used as therapeutics [18].

CONCLUSION

A HG-induced cell model has been established successfully. The findings of this study show that ReA promotes survival and suppresses apoptosis of HG-induced HK2 cells. In addition, ReA treatment suppresses oxidative stress in HK2 cells upon exposure to HG. Mechanistically, ReA promotes survival but suppresses apoptosis and oxidative stress in HG-induced HK2 cells via MAPK pathway. Therefore, ReA is a promising therapeutic agent for the management of DN.

DECLARATIONS

Funding

Competing interests

The authors state that there are no conflicts of interest to disclose.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lili Yang and Lei Huai designed the study and supervised the data collection. Qunhong Xu analyzed and interpreted the data. Benyong Wang prepared and reviewed the manuscript for publication. All authors have read and approved the manuscript.

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