Resveratrol Protects against High-Fat Diet Induced Renal Pathological Damage and Cell Senescence by Activating SIRT1

Nannan Zhang, Zhongchi Li, Kang Xu, Yanying Wang, and Zhao Wang*

MOE Key Laboratory of Protein Sciences, Department of Pharmacology, School of Medicine, Tsinghua University; Beijing 100084, P. R. China; and Peking Union Medical College; Beijing 100050, P. R. China.

Received January 26, 2016; accepted June 12, 2016

Obesity-related renal diseases have been a worldwide issue. Effective strategy that prevents high fat-diet induced renal damage is of great significance. Resveratrol, a natural plant polyphenol, is famous for its antioxidant activity, cardioprotective effects and anticancer properties. However whether resveratrol can play a role in the treatment of renal diseases is unknown. In this study, we added resveratrol in normal glucose or high glucose medium and provide evidences that resveratrol protects against high-glucose triggered oxidative stress and cell senescence. Moreover, mice were fed with standard diet, standard diet plus resveratrol, high-fat diet or high-fat diet plus resveratrol for 3 months, and results show that resveratrol treatment prevents high-fat diet induced renal pathological damage by activating SIRT1, a key member in the mammalian sirtuin family that responds to calorie restriction life-extension method. This research confirms the potential role of resveratrol in the treatment of renal diseases and may provide an effective and convenient method to mimic the beneficial effects of calorie restriction.

Key words resveratrol; kidney; SIRT1; pathology; senescence

For years, the ever increasing number of overweight individuals has led to severe health problems, including diabetes, metabolic syndrome and organ degeneration. Among these obesity-related complications, renal disease has been reported to be a significant issue. High-fat diet induces renal lipid metabolism disorder, renal lipotoxicity and subsequent renal injuries. Moreover, renal structural and functional changes have been observed in the mice on a high-fat diet (HFD).

Effective strategy that prevents obesity and diabetic-induced renal damage is desperately needed.

SIRT1, an oxidized form of nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases and mono-ADP-ribosyltransferases, is a member of the mammalian sirtuin family. SIRT1 has been reported to play a key role in stress response, metabolism and lifespan regulation. Activation of SIRT1 protects mice from diet-induced obesity, metabolic disorders and oxidative injury, while SIRT1 inactivation is associated with lipid accumulation, cell injury and diabetic nephropathy.

Resveratrol (3,5,4'-trihydroxystilbene), a natural plant polyphenol first isolated from the roots of white hellebore, is reported as an activator of SIRT1. Several evidences indicated the therapeutic potential of resveratrol, such as its antioxidant activity, cardioprotective effects and anticancer properties. However, it is unclear whether resveratrol can play a beneficial role in preventing diet-induced renal diseases. In this study, we provide evidences that resveratrol prevent high-fat diet induced renal pathological damage and cell senescence by activating SIRT1, further confirming the potential of resveratrol in the treatment of renal diseases.

MATERIALS AND METHODS

Animals and Treatment Male C57BL/6 mice were obtained from the experimental animal facility of Tsinghua University, Beijing, China and resveratrol (J&K Scientific, Beijing, China) was administered orally. Three-month-old mice were housed under pathogen free conditions, 12/12 light/dark cycle and fed with a standard diet (SD), standard diet plus resveratrol (SDR; 0.04% resveratrol mixed with standard diet), high-fat diet (HFD; 60% energy from fat), or high-fat diet plus resveratrol (HFD-R; 0.04% resveratrol mixed with high-fat diet) for 3 months. Experiments were performed in accordance with the approved guidelines and with the approval of the Institutional Ethical Committee of China.

Pathological Staining Samples were fixed in 4% buffered paraformaldehyde then embedded in paraffin. Hematoxylin–eosin (H&E) staining (Disinbio, Beijing, China) and Masson’s trichrome staining (Sigma, St. Louis, MO, U.S.A.) were conducted according to standard protocols.

Cell Culture WI38 cells The American Type Culture Collection (ATCC) were propagated in Dulbecco’s modified Eagle’s medium (DMEM) (Corning, Manassas, VA, U.S.A.) medium plus 10% fetal bovine serum, 37°C and 5% CO₂. Cells were cultured under different conditions: Normal glucose (NG; 5.5 mmol/L), normal glucose medium supplemented with 1.4 µg/mL resveratrol (NGR; normal glucose medium supplemented with 1.4 µg/mL resveratrol), high glucose (HG; 25 mmol/L), high glucose medium supplemented with 1.4 µg/mL resveratrol (HGR; high glucose medium supplemented with 1.4 µg/mL resveratrol). Resveratrol (Sigma) suspended in dimethyl sulfoxide (DMSO) was used in the in vitro study. Initial population doubling level (PDL): 20. Indexes were inspected after four-week propagation.

Cell Counting Kit-8 (CCK-8) Assay Cell viability was measured using the CCK-8 assay (Beyotime Biotechnology, Shanghai, China). Ten microliters reagents were added into 100 µL medium then incubated for 1 h. Optical density was measured with spectrophotometer (Biorad, Hercules, CA, U.S.A.) at 450 nm.

Cell Senescence Associate β-Gal (SA-β-Gal) Staining SA-β-Gal assay was performed with senescence cells histo-
chemical staining kit (Sigma) according to the manual. Five random views in each group were selected to calculate the percentage of β-Gal positive cells.

**Western Blot Analysis** Proteins were extracted by RIPA lysis buffer (Beyotime Biotechnology) then calculated the concentration with bicinchoninic acid (BCA) method. Antibodies were purchased from the indicated sources: p16 (Santa Cruz Biotechnology, CA, U.S.A.), MnSOD (Abcam, Cambridge, U.K.), SIRT1 (Abcam), β-actin (Cell Signaling Technology, Danvers, MA, U.S.A.), Goat anti-rabbit immunoglobulin G (IgG) (Cell Signaling Technology). ImageJ software was used for gray intensity analysis.

**Immunohistochemical Analysis** Immunohistochemical analysis was performed according to the protocol of IHC Kit (Maxim, Fujian, China). SIRT1 antibody (Abcam, Cambridge, MA, U.S.A.) was used as a primary antibody, and other reagents were provided by the kit.

**Statistical Analyses** Data are expressed as the mean±standard error of the mean (S.E.M.). p Values were determined by 2-way ANOVA with Bonferroni’s multiple comparison test.

![Fig. 1](image.png)

Resveratrol Protects against High-Fat Diet Induced Renal Pathological Damage

(A) Body weight profile of mice on standard diet (SD), standard diet plus resveratrol (SDR), high-fat diet (HFD) or high-fat diet plus resveratrol (HFDR). (B) HE staining was used to detect renal histomorphological changes. Arrow indicated necrosis area, original magnification: ×200. Renal failure index was measured according to Paller’s morphologic study method. (C) Masson’s trichrome staining was conducted to detect renal fibrosis. Arrows indicated glomerulosclerosis and tubulointerstitial fibrosis, original magnification: ×400. Statistical comparison was made by calculating the percentage of fibrosis area. SD, standard diet, n=7; SDR, standard diet plus resveratrol (SDR), n=8; HFD, high-fat diet, n=6; HFDR, high-fat diet plus resveratrol, n=7. *p<0.05, ***p<0.001.
RESULTS

Resveratrol Prevents High-Fat Diet Induced Renal Pathological Damage  To determine the effect of resveratrol on renal pathologies, 3-month-old mice were fed with SD, SDR, HFD, or HFDR for 3 months. Body weight profile was demonstrated to indicate the amount of calorie intake in different group (Fig. 1A). The profile show that mice in HFD or HFDR group gained more weight than SD or SDR mice. Besides, mice in HFDR group were slightly lighter than HFD mice, but there was no significant difference. Furthermore, we applied histological analysis to evaluate the effects of resveratrol on diet-induced renal injuries. H&E staining was conducted to determine renal histomorphological changes (Fig. 1B) and renal failure index was measured according to Paller’s morphologic study method.19

![Cell Viability](image)

![H&E Staining](image)

Fig. 2. Resveratrol Protects against Cell Senescence
(A) Cell viability was measured at different resveratrol concentration to explore the optimal dose for cell growth. (B) Senescent-associated β-gal (SA-β-gal) assay was performed to evaluate the effects of resveratrol on cell senescence and (C) the rate of β-gal positive cells was calculated. *p<0.05, **p<0.01 (n=3).

![Western Blot](image)

Fig. 3. Resveratrol Plays Cytoprotective Effect by Enhancing SIRT1 Expression
(A) Western blot analysis was performed to measure the level of p16INK4A, MnSOD, and SIRT1. Normalized to GAPDH. (B) The results of gray intensity analysis showed relative expression level of indicated proteins. *p<0.05, **p<0.01 (n=3).
Significantly renal tubule epithelium swelling was observed in the mice on a HFD, accompanied with several necrosis areas (indicated by arrow), which are hallmarks of renal degeneration. While the administration of resveratrol exhibited renoprotective activity against the diet-induced renal damage as almost no necrosis area was detected in HFDR group. On the other hand, there was no significant difference between SD and SDR mice, suggested that resveratrol treatment improved HFD-induced renal insufficiency, but had few effect in regulating normal renal function.

Masson’s trichrome staining was conducted to detect renal fibrosis (Fig. 1C), which is associated with chronic kidney disease and renal aging. In addition, we made statistical comparison by calculating the percentage of fibrosis area. Consistent with histomorphological changes, severe glomerulosclerosis and tubulointerstitial fibrosis were detected under HFD condition, showing the diet-induced renal damages distinctly. Besides, there was still no significant difference of renal fibrosis between SD and SDR mice. Results indicated that treatment with resveratrol may not have distinct role in normal condition, but is particularly resistant to the diet-induced renal pathological damages.

**Resveratrol Protects Cells against High Glucose (HG) Triggered Senescence Acceleration**

High glucose culture condition is reported to induce oxidative stress and accelerate cell senescence. In order to clearly investigate the effect of resveratrol, we cultured human diploid fibroblast WI38 in normal glucose (NG), normal glucose plus resveratrol (NGR), HG, or high glucose plus resveratrol (HGR) conditions to mimic the effect of high-energy intake in vitro. Firstly, we measured cell viability by CCK-8 assay at different resveratrol concentration to explore the optimal dose for cell growth (Fig. 2A). Results show that cell viability elevated first then declined along with the increasing of resveratrol concentration,
and the optional concentration for cell growth is 1.4 μg/mL.

Furthermore, we performed senescent-associated β-gal (SA-β-gal) assay to evaluate the effect of resveratrol on cell senescence (Fig. 2B). The accumulation of β-galactosidase is a solid biomarker to identify senescent cells.23) Five random views in each group were selected to calculate the rate of β-gal positive cells (Fig. 2C). Condensed coloration and higher number of β-gal positive cells were observed in HG group, indicated an accelerated aging process. Supplementation with resveratrol rescued the high-glucose triggered cell senescence. Moreover, the percentage of β-gal positive cells under NGR condition was also decreased compared with NG control group, although there was no significant difference. Results confirm the vital function of resveratrol on senescent resistance and cell protection.

**Resveratrol Plays Antiaging and Antioxidant Effect by Targeting SIRT1**
To explore the mechanism by which resveratrol play cell protection and antiaging effect, we measured the level of some key proteins that regulate cell senescence and oxidative stress by Western blot analysis (Fig. 3). Results showed that HG condition markedly increased the level of βNFKαA, which is a solid biomarker of aging.24) Treatment with resveratrol effectively rescued the high-glucose triggered βNFK4A accumulation, thus resisted cell senescence.

As resveratrol is famous for its antioxidant activity, we measured the level of manganese superoxide dismutase (MnSOD), an important antioxidant,25) to investigate the oxidative status among different culture conditions. Results displayed that HG culture condition strongly down-regulated MnSOD expression, showing the insufficiency of antioxidant function in HG condition. In contrast, resveratrol treatment restored the level of MnSOD, although without significant difference, it can still provide evidence towards the antioxidant effect and cell protection capacity of resveratrol.

In addition, we measured the level of SIRT1, which is responsible for aging and oxidative stress resistance.26) Results showed a significant decrease of SIRT1 expression under HG condition, which may contribute to cell senescence, inflammatory response and metabolic disorders. But, resveratrol treatment elevated the level of SIRT1 and rescued HG-induced SIRT1 deficiency. These results provide evidence that resveratrol play antiaging and antioxidant effect by activation of SIRT1.

**Resveratrol Plays Renoprotective Effect by Activating SIRT1**
In vitro results confirmed the role of SIRT1 in the process of resveratrol mediated cell protection. We wondered whether the renoprotective effect of resveratrol is also SIRT1-dependent. We stained SIRT1 by immunohistochemistry (Fig. 4A) and compared its level by Western blotting analysis (Fig. 4B) in mice kidneys among different diet groups. Consist with histomorphological results, resveratrol effectively prevents HFD-induced renal pathological damage (such as necrosis areas, indicated by arrow). Moreover, enhanced SIRT1 expression was observed with resveratrol treatment, based on standard or high-fat diet. These results provide the evidence that resveratrol protects against high-fat diet induced renal injuries by the activation of SIRT1.

In summary, this study links resveratrol to aging and oxidative stress, therefore preventing high-fat diet induced renal damage by activating SIRT1 (Fig. 4C). These results confirm the potential role of resveratrol in the treatment of renal diseases.

**DISCUSSION**

Nowadays, obesity-related renal diseases attract widespread attention. Calorie restriction (CR), a dietary regimen that reduces 30–40% in intake, is reported to be the most robust way to prevent organ insufficiency,27,28) however it is difficult to keep strictly within the limit of calorie restriction method in daily life. Therefore, development of calorie restriction mimetics is desperately needed.

SIRT1, a number of the mammalian sirtuin family, is linked to the CR-related lifespan regulation.29) The levels of SIRT1 are induced under CR condition,30) and SIRT1 transgenic mice display calorie restriction-resembled phenotypes.31) Moreover, HFD is also associated with increased production of mitochondrial reactive oxygen species (ROS),32) which are generated endogenously in the process of mitochondrial oxidative phosphorylation and responsible for oxidative damage.33,34) The MnSOD, an important antioxidative enzyme, is reported to regulates ROS metabolism and detoxify mitochondrial ROS.35) As a consequence, methods that enhance SIRT1 and MnSOD activity are probably have the potential to prevent HFD-induced renal injuries, where resveratrol are demonstrated to have a role in this study.

**HFD and HG Culture Comparison**
In this study, we applied HG culture condition to mimic the effect of high-energy intake in vitro. Although there should be different biochemical changes, but these two situations have something in common. First of all, both of the two methods increased energy intake, and then regulate energy consumption and metabolism. Secondly, HFD induces antioxidant imbalance and oxidative injury,36) and HG culture also induces oxidative stress.37) In addition, the alteration of some key proteins or signaling pathways is similar under HFD or HG culture. For example, they both have impaired insulin-like growth factor 1 (IGF-1) signaling,38,39) and they both target p53 protein,40,41) which plays significant roles in aging and apoptosis. Therefore, even they are different biochemical changes, the HG culture condition may be acceptable to mimic the effect of HFD in the *in vitro* study.

**Fibroblast Senescence and Tubulointerstitial Fibrosis**
In the *in vivo* test, we measured the severity of tubulointerstitial fibrosis and demonstrated that resveratrol protect against HFD-induced renal fibrosis. In the *in vitro* study, we measured fibroblast senescence and showed that resveratrol prevent HG-induced cell senescence. It is not difficult to understand the distinct roles of resveratrol in two situations. In the *in vitro* situation, resveratrol activates several anti-aging genes and play anti-oxidative effect to delay cell replicative senescence.11,13) But in the *in vivo* study, we administrated resveratrol on 3-month old mice, which is far from aging. Therefore, resveratrol rescued HDF-induced inflammatory response and oxidative stress which will strongly induce renal fibrosis.42–44) These results demonstrated that resveratrol can play beneficial roles both *in vivo* and *in vitro*.

**ROS and Cell Senescence Induce Renal Damage**
Previous study indicated that oxidative stress play a critical role in the initiation and progression of renal disease, and agents targeting ROS can prevent the kidney from oxidative damage.45) Moreover, it has been reported that all forms of acute and
chronic renal injuries, regardless of species, is associated with enhanced generation of ROS, which linked ROS to renal damage. Besides, cell senescence also plays a pivotal role in renal damage. Previous study showed that cell senescence induces renal tubular epithelial cell dysfunction and loss in the model of renal disease. It is also reported that cell senescence could contribute to organ aging and the expression of senescence-associated genes are enhanced in aging kidney. These results provide evidence towards the effect of ROS and cell senescence in renal damage, as we show in Fig. 4C. In this study, we confirm that resveratrol prevents high-fat diet induced oxidative stress, cell senescence, and renal damage by elevating the level of SIRT1. This work suggests resveratrol as a novel therapeutic agent for renal diseases and may provide an effective and simple method to mimic the effect of CR.

Acknowledgments This work was funded by the National Basic Research Program of China (973 Program, No. 2013CB530802) and the National Natural Science Foundation of China (No. 81270425).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES


