Introduction

Aging is considered to be a major risk factor for developing atherosclerosis and is also associated with reducing the regenerative capacity of the endothelium and causing endothelial senescence[1].

Atherosclerosis is a very common condition associated with increased cardiovascular risk, and endothelial dysfunction is thought to promote its development[2]. However, the underlying mechanisms remain to be determined. Moreover, atherosclerosis is also associated with an increase in endothelial cell turnover, and endothelial cell apoptosis plays a pivotal role in developing atherosclerotic plaques[3].

The silent information regulator 2 (Sir2) is an NAD-dependent deacetylase. It is well known that an overexpression of Sir2, or its homologue, can extend the lifespan of a wide range of lower eukaryotes, including yeasts, worms and flies[4]. In mammals, Sir2 is represented by seven homologues (Sirt1~7), of which Sirt1 is the most closely related to the yeast Sir2 and has been studied extensively.

Recent studies have demonstrated that Sirt1 plays an important role in regulating cell survival by inhibiting apoptosis induced by stress[5-7]. Researchers speculate that Sirt1 could also regulate cell aging because it has been reported that apoptosis and senescence in vascular endothelial cells are closely related to atherosclerosis progression.

In this study, we investigated the ability of Sirt1 to interfere with apoptosis and cellular senescence in human umbilical vascular endothelial cells (HUVECs) and explored the effect of Rhein lysinate (RHL) on Sirt1’s function.

Effects of Rhein lysinate on H2O2-induced cellular senescence of human umbilical vascular endothelial cells

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Aim: To observe the effect of Rhein lysinate (RHL) on cellular senescence of human umbilical vascular endothelial cells (HUVECs) and elucidate its action mechanism.

Methods: Cell viability was determined using MTT assay. The expression levels of Sirt1 mRNA and protein were measured by RT-PCR and Western blot, respectively. Senescence associated (SA)-β-galactosidase activity was detected to evaluate cell senescence. Apoptosis and cell cycle progression were determined using flow cytometry.

Results: Treatment with RHL (10 μmol/L) for 48 h significantly increased the proliferation of HUVECs. In contrast, treatment with H2O2 (25, 50 and 100 μmol/L) for 6 d dose-dependently increased β-galactosidase positive cells. Spontaneous cell senescence appeared as the cell passage increased. Pre-treatment with RHL (10 μmol/L) reversed H2O2 or increased cell passage-induced cell senescence. H2O2 (100 μmol/L) significantly arrested HUVECs at G1 phase (73.8% vs 64.6% in the vehicle group), which was blocked by RHL (10 μmol/L). RHL (5 and 10 μmol/L) enhanced both mRNA transcription and protein expression of Sirt1. H2O2 (100 μmol/L) significantly decreased Sirt1 expression, and induced up-regulation of p53 acetylation and p16ink4a, which were blocked by pre-treatment with RHL (10 μmol/L). Interference with siRNA for Sirt1 abolished the effect of RHL. H2O2 (100 μmol/L) did not induce HUVEC apoptosis. The expression of apoptosis-associated proteins, such as p53, p21, Bcl-2, and Bax, did not significantly change in the presence of H2O2 (100 μmol/L) or RHL (10 μmol/L).

Conclusion: RHL protected HUVECs against cellular senescence induced by H2O2, via up-regulation of Sirt1 expression and down-regulation of the expression of acetyl-p53 and p16ink4a.

Keywords: Rhein lysinate; H2O2; cell cycle; senescence; Sirt1; human umbilical vascular endothelial cells (HUVECs); RNA interference

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one of the major bioactive constituents of the rhubarb rhizome (R. palmatum L or R. tanguticum Maxim)\(^8\). In previous studies, Rhein was found to have a variety of bioactivities, such as inhibiting IL-1 induced chondrocyte activation\(^9\), decreasing hypertrophy in mesangial cells\(^10\), inhibiting tumor cell proliferation, and inducing tumor cell apoptosis\(^11\). In our previous study, we also found that a dose of more than 20 µmol/L RHL could inhibit tumor cell proliferation, and it acted synergistically with Taxol when combined, both in vitro and in vivo\(^12\). However, less than 20 µmol/L RHL can also improve cell viability. Therefore, we investigated the effect of RHL on cell viability in HUVECs and explored its mechanisms.

Materials and methods

Reagents

RHL, the salt of Rhein and lysine, was made in our department with 95% purity. Its structural formula was shown in our previous article. Gelatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), collagenase I, and heparin were obtained from Sigma Aldrich (Shanghai, China); endothelial cell growth factor (ECGF) was purchased from Roche (Shanghai, China); and trypsin was purchased from Gibco (Grand Island, NY, USA). Sirt1 and p53, p21, p16INK4a, Bcl-2, and Bax, and β-Actin were purchased from Santa Cruz Technology (Danvers, MA, USA). Pre-stained Protein Marker p7708V was purchased from New England Biolabs Ltd (Pickering, Ontario, Canada). Western Blotting Luminol Reagent and PVDF membrane were purchased from Millipore (Billerica, MA, USA).

Cell culture and determining cell viability

HUVECs isolated from newborn umbilical cord were grown in M199 supplemented with 20% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) at 37°C under 5% CO\(_2\) in a humidified atmosphere. Cells were incubated with different concentrations of RHL (0, 5, 10, 15, and 20 µmol/L) at 37°C for 48 h. Moreover cells were incubated with 10 µmol/L RHL for 0, 6, 12, 24, and 48 h. Cell viability was assessed by MTT method.

Reverse transcription-PCR

The mRNA level of Sirt1 in HUVECs was measured by RT-PCR. Total RNA in HUVECs was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA). After treatment with RNase-free Dnase for 30 min, the total RNA (50 ng/µL) was reverse transcribed with oligo (dT) primers. The Sirt1 transcription level relative to GAPDH was determined by means of RT-PCR. The following primers were used: Sirt1 forward (F) 5'-CCTGACTTCAGATCAAGAGACGGT-3', reverse (R) 5'-TCCACCAC-CCTTGGTCTGTA-3'\(^13\). Amplification was performed on an Eppendorf thermocycler for 30 cycles with denaturing at 94°C for 1.5 min.

Immunoblot analysis

Cells were lysed on ice for 30 min in lysis buffer [50 mmol/L HCl, pH 7.6, 150 mmol/L NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L dithiothreitol, 1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 10 mmol/L sodium fluoride]. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose filters. First, the membrane was inoculated in a blocking buffer containing BSA (1%) and Tween 20 (0.1%, v/v) in PBS (PBS/Tween 20) at room temperature for 1 h. Then, it was incubated overnight at 4°C with the proper primary antibodies. Finally, it was inoculated with the proper secondary antibodies at room temperature for 2 h. Each membrane was developed using an enhanced ChemiImager5500 chemiluminescence system (Alpha Innotech Corporation, Miami, FL, USA).

RNA interference

Synthetic Sirt1 small interfering RNA (siRNA) was purchased from GenePharma Co, Ltd (Shanghai, China). The 21-nt siRNA sequence targeting Sirt1 corresponded to the coding region 5'-GCAAACACGCAUCUGCAGUUGUA-3' and 5'-UACAAAAUCGCAAGAUCCUGUUGC-3'. The scrambled control siRNA sequences were 5'-UUCUCGGACGUUGCACGUTT-3' and 5'-ACGUGACACGUUGCAATT-3'. After 10 µmol/L RHL treatment for 24 h, these siRNA were transfected into HUVECs using the HiPerFect Transfection Reagent (QiAGEN, Shanghai, China). After siRNA transfection for 24 h, the cells were incubated with 100 µmol/L H\(_2\)O\(_2\) for 6 d. Then, the cells were stained using galactosidase (β-gal).

Galactosidase (β-gal) staining

HUVECs were treated with or without different concentrations of H\(_2\)O\(_2\), RHL or H\(_2\)O\(_2\) plus RHL. After that, the cells were washed twice with phosphate-buffered saline (PBS) and then fixed for 5 min with PBS containing 2% formaldehyde and 0.2% glutaraldehyde. The cells were then incubated at 37°C for 10 h with a staining solution (40 mmol/L citric acid, sodium phosphate, pH 6.0, 1 mg/mL 5-bromo-4-chloro-3-isothiocyanate (X-gal, sigma), 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 150 mmol/L NaCl, and 2 mmol/L MgCl\(_2\)). Senescence-associated (SA)-β-gal-positive cells were observed by microscopy, and over 400 cells were counted in three independent fields\(^14\).

Annexin V FITC/PI assay

Annexin V-FITC/PI double staining was achieved using an Annexin V-FITC/PI apoptosis detection kit. HUVECs were treated with or without 100 µmol/L H\(_2\)O\(_2\), 10 µmol/L RHL, or both for 48 h. The cells were washed with PBS and collected by trypsinization. The cells were then treated according to the instructions in the Annexin V-FITC/PI apoptosis detection protocol.
(Bao Sai, Beijing), which indicate the following:

- The Annexin V-FITC+/PI− population reflects normal healthy cells.
- The Annexin V-FITC+/PI+ cells show early apoptosis.
- The Annexin V-FITC+/PI+ cells are in late apoptosis or necrosis.
- The Annexin V-FITC−/PI+ cells are necrotic.
- The percentage of normal, early apoptotic, late apoptotic, and necrotic cells were calculated using FACS Calibur and Cell Quest software (Becton-Dickinson, Franklin Lakes, NJ, USA) [13].

Cell cycle assay
To determine the effect of H2O2 and RHL on cell cycle progression, HUVECs were grown for 48 h (one-cell cycle) with or without 100 μmol/L H2O2, 10 μmol/L RHL, or both. The cells were washed with PBS and collected by trypsinization. The cells were fixed with 70% ethanol and treated with 5 mg/mL (Rnase) for 30 min. After staining with 50 mmol/L propidium iodide, the cells were subjected to flow cytometric analysis with FACS Calibur and Cell Quest software (Becton-Dickinson).

Statistical analysis
Statistical analysis was performed between the control group and the different treatment groups. Comparisons of the means were conducted by one-way ANOVA. All values were expressed as the mean±SD, and P<0.05 was considered to be statistically significant.

Results
Low levels of RHL improve HUVEC proliferation and enhance Sirt1 transcription and expression
Our previous study indicated that low levels of RHL (less than 20 μmol/L) could improve cell proliferation in MCF-7 breast cancer cells. The same effect of RHL on HUVECs was also observed in a time-dependent manner; however, only 10 μmol/L RHL could significantly promote HUVEC proliferation (Figure 1A, 1B). In the following study, RHL (5 and 10 μmol/L) enhanced Sirt1 transcription and expression in a dose-dependent manner compared to the vehicle (Figure 2).

Establishing senescence model in HUVECs and Sirt1 can delay HUVEC cellular senescence progress
To investigate the effect of Sirt1 on senescence in HUVECs, we first established the senescence model in HUVECs. Four main groups were respectively treated with 0, 25, 50, or 100 μmol/L H2O2, and senescent cells were found to increase with
increasing dosages of H₂O₂ (Figure 3A); however, the expression of Sirt1 decreased (Figure 4A). In the meantime, we also observed that the number of senescent cells increased with increasing cell passage (Figure 3B), and the expression of Sirt1 decreased (Figure 4B). Furthermore, 10 µmol/L RHL could antagonize cell senescence induced by H₂O₂ and the increased passage of HUVECs (Figure 3C, 3D); moreover, siRNA (Sirt1) antagonized the effect of RHL (Figure 3E, 3F).

RHL has no effect on the apoptosis signaling pathway

H₂O₂ inhibited HUVEC survival with IC₅₀ of about 100 µmol/L. To determine whether or not H₂O₂ inhibits HUVEC survival by inducing apoptosis, we investigated the effect of H₂O₂ on apoptosis. H₂O₂ 100 µmol/L induced HUVECs senescence but could not induce HUVEC apoptosis (Table 1). Furthermore, we examined the associated protein expression in the apoptosis signaling pathway. It indicated that the protein expression associated with apoptosis, such as p53, p21, Bcl-2, and Bax, did not change in the H₂O₂ 100 µmol/L or RHL 10 µmol/L treatment groups (P>0.05, Figure 5A).

Table 1. The effect of RHL on necrosis HUVECs induced by H₂O₂. n=3. Mean±SD. aP<0.05, bP<0.01 vs control. cP<0.05, dP<0.01 vs H₂O₂ 100 µmol/L.

| Concentration (µmol/L) | Normal (%) | Necrotic (%) | Early apoptotic (%) | Late apoptotic (%) |
|------------------------|------------|--------------|---------------------|-------------------|
| Control                | 80.9±6.7   | 7.4±0.7      | 6.6±0.6             | 5.1±0.8           |
| RHL 10                 | 83.2±6.5   | 6.2±0.8      | 4.0±0.5             | 6.6±0.5           |
| H₂O₂ 100               | 60.4±4.8   | 21.4±1.2     | 8.8±0.7             | 9.4±1.0           |
| RHL+H₂O₂               | 71.0±4.1   | 15.6±1.4     | 6.8±0.9             | 6.6±0.6           |

H₂O₂ induced G₁ arrest in HUVECs, and RHL prevented against H₂O₂-induced G₁ arrest

Cell cycle plays an important role in cellular senescence. Senescence occurs during the G₁ period. Treatment with 100 µmol/L H₂O₂ arrested HUVECs at G₁ phase. The proportion of cells in the G₁ phase was approximately 73.8%. However, it was about 64.6% in the vehicle group. RHL 10 µmol/L reversed the G₁ arrest effects of H₂O₂. The proportion of cells in G₁ phase decreased to 63.1% (Table 2). H₂O₂ 100 µmol/L induced the p53 acetylation and p16INK4a up-regulation and treatment with 10 µmol/L RHL prevented this process (Figure 5B).

Table 2. The effect of RHL on cell cycle arrest of HUVECs induced by H₂O₂. n=3. Mean±SD. aP<0.05, bP<0.01 vs control. cP<0.05, dP<0.01 H₂O₂ 100 µmol/L.

| Concentration (µmol/L) | G₀/G₁ (%) | S (%) | G₀/M (%) |
|------------------------|-----------|-------|----------|
| Control                | 64.6±4.6  | 22.4±2.3 | 13.1±1.7 |
| RHL 10                 | 62.0±4.5  | 25.2±2.8 | 12.8±1.5 |
| H₂O₂ 100               | 73.8±5.5b | 13.3±1.2c | 12.9±1.3 |
| RHL+H₂O₂               | 63.1±5.1a | 24.5±2.6b | 12.4±2.2 |

Discussion

In our previous study, we found that high concentrations of RHL (more than 20 µmol/L) could inhibit cell proliferation in a dose-dependent manner[^12]. In the meantime, we also found that low concentrations of RHL (less than 20 µmol/L) could improve cell proliferation and alleviate the cytotoxicity induced by Taxol (data was not shown). However, how RHL improves cell proliferation is unclear. In this study, we also observed that RHL improved HUVEC proliferation at low concentrations (less than 20 µmol/L), especially at 10 µmol/L (Figure 1). As is well known, increased cellular senescence is associated with decreased cell proliferation in vivo because senescent cells cannot divide[^36]. Therefore, it can be concluded that by delaying cell senescence, low concentrations of RHL can improve HUVEC proliferation.

Cellular senescence is a process by which cells irreversibly exit the cell cycle and cease to divide in response to a variety of stresses, including oxidative stress[^57]. It was reported that oxidative stress damages DNA, leading to activation of the tumor suppressor p53, a key regulator of the cell cycle and cellular senescence. It has also been reported that p53 acetylation promotes expression of growth-suppressive genes and induces cellular senescence[^18, 19]. In this study, we established the cell senescence model using H₂O₂-treated HUVECs. We found that H₂O₂ could induce HUVEC senescence in a dose-dependent manner (Figure 3A). We also found cell spontaneous senescence appeared with an increase in cell passage (Figure 3B), and that HUVECs could only survive to the fifth generation without epidermal growth factor (EGF) supplementation. The characteristics of HUVECs made it easier to establish this model of cell senescence. As we investigated the HUVEC senescence mechanism, we observed that the expression of Sirt1 decreased in a dose-dependent manner following treatment with various concentrations of H₂O₂, that the expression of Sirt1 decreased with the increase in cell passage (Figure 4), and that 10 µmol/L RHL could antagonize cell senescence induced by H₂O₂ and the increase in the passage of HUVECs (Figure 3C, 3D); moreover, siRNA (Sirt1) could antagonize the effect of RHL (Figure 3E, 3F). Therefore, it can be concluded that RHL prevented cellular senescence induced by H₂O₂ treatment, as well as the increase of cell passage, through enhancing Sirt1 expression (Figure 3C-3F).

Endothelial senescence causes endothelial dysfunction, promotes atherogenesis and contributes to age-related vascular disorders. Sirt1 is a key sensor system for regulating endothelial cell survival, proliferation, and senescence. The protective activities of Sirt1 may be achieved at least in part by fine tuning the acetylation/deacetylation of key proteins[^59]. In this study, we also observed that the p53 acetylation level was inversely related to the expression of Sirt1 in H₂O₂-induced
Figure 3. SA-β-gal activity of different passages of HUVECs treated with various concentrations of H$_2$O$_2$.  (A) HUVECs were treated with 0, 25, 50, and 100 µmol/L H$_2$O$_2$ at 37 °C for 3 d and then placed in a medium with the same concentration of H$_2$O$_2$ for further 3 d.  cP<0.01 vs vehicle.  (B) P1, P2, P3, and P4 represent the different passages of HUVECs.  HUVECs were cultured for 3 d and then propagated.  cP<0.01 vs P1.  (C) HUVECs were pre-treated with 10 µmol/L RHL at 37°C for 24 h, followed by 100 µmol/L H$_2$O$_2$ treatment.  cP<0.01 vs H$_2$O$_2$.  (D) P3 generation HUVECs were treated with 10 µmol/L RHL at 37°C for 6 d.  cP<0.01 vs vehicle.  (E) HUVECs were treated with 10 µmol/L RHL at 37°C for 24 h, followed by siRNA (Sirt1) for 24 h and incubation with 100 µmol/L H$_2$O$_2$ for 6 d.  cP<0.01 vs H$_2$O$_2$.  fP<0.01 vs RHL+H$_2$O$_2$.  (F) The expression of Sirt1 in the various groups. n=3. Mean±SD.
cellular senescence. However, the expression of p53 did not change (Figure 5).

The p53 pathway is known to respond to a wide variety of stress signals, including telomere shortening, hypoxia, mitotic spindle damage, heat or cold shock, unfolded proteins, improper ribosomal biogenesis, nutritional deprivation, and mutational activation of some oncoproteins. It is a single core module that governs the three potent tumor suppression mechanisms: growth arrest, senescence, and apoptosis[20].

In this study, we explored whether apoptosis inhibited cell proliferation. Although 500 µmol/L H2O2 has been shown to induce cell apoptosis in PC12 cells (rat pheochromocytoma cell line)[21], HUVEC apoptosis was not affected by H2O2 (less than 100 µmol/L) treatment as measured by flow cytometry (Table 1) and Western blot (Figure 5). However, in the cell cycle analysis, we found that 100 µmol/L H2O2 induced G1 period block, 10 µmol/L RHL prevented G1 period block (Table 2), and that G1 period block is the feature of cell senescence[22]. Therefore, we can conclude that the senescence induced by H2O2 was not due to apoptosis but to G1 period block, and that RHL can interrupt the G1 period block induced by H2O2.

Most normal mammalian cells have a finite lifespan, which is thought to constitute a protective mechanism against unlimited proliferation. This phenomenon, called senescence, is driven by telomere attrition, which triggers tumor suppressors to induce factors, including p16 INK4a[23]. In this study, we also found that 10 µmol/L RHL could antagonize p16INK4a upregulation induced by 100 µmol/L H2O2. It can be inferred that H2O2 could partially induce HUVEC G1 period block by increasing p16INK4a expression and that RHL could antagonize this phenomenon by the same signaling pathway. However, the effect of H2O2 and RHL on cyclin needs further investigation.

In conclusion, the decrease in Sirt1 expression inhibited cell proliferation induced by H2O2, as well as the spontaneous cell senescence with increased cell passage. Subsequently p53 was acetylated, and p16INK4a was upregulated, which caused G1 period block and aging. However, RHL resisted this process. In addition, during HUVEC senescence, it was G1 period block, not apoptosis, that played an important role, and RHL interrupted the G1 period block induced by H2O2.

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**Author contribution**

Gang HU designed research; Ya-jun LIN, Bo LIU, and Zong-yuan YU performed research; Jie WEI and Yong-zhan ZHEN contributed new analytical tools and reagents; Ya-jun LIN and Yong-zhan ZHEN analyzed data; Ya-jun LIN and Gang HU wrote the paper.

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