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Original Article

Effects of Rhein lysinate on H_2O_2-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 H_2O_2 (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 H_2O_2 or increased cell passage-induced cell senescence. H_2O_2 (100 μmol/L) significantly arrested HUVECs at G_1 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. H_2O_2 (100 μmol/L) significantly decreased Sirt1 expression, and induced up-regulation of p53 acetylation and $p16^{INK4a}$, which were blocked by pre-treatment with RHL (10 μmol/L). Interference with siRNA for Sirt1 abolished the effect of RHL. H_2O_2 (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 H_2O_2 (100 μmol/L) or RHL (10 μmol/L).
Conclusion: RHL protected HUVECs against cellular senescence induced by H_2O_2, via up-regulation of Sirt1 expression and down-regulation of the expression of acetyl-p53 and $p16^{INK4a}$.

Keywords: Rhein lysinate; H_2O_2; cell cycle; senescence; Sirt1; human umbilical vascular endothelial cells (HUVECs); RNA interference

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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.

Rhein (4,5-dihydroxy-anthraquinone-2-carboxylic acid) is

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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 acetyl-p53 (lys382) antibody were purchased from Upstate (New York, USA); antibodies against p53, p21, $p16^{INK4a}$, Bcl-2, Bax, and β-Actin were purchased from Santa Cruz Technology (Santa Cruz, CA, USA); and secondary antibodies against rabbit or mouse were purchased from Cell Signaling 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 d(T) 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′-CTGATTAAAAATGTCTCCACGAAC AG-3′; GAPDH F 5′-ACCACAGTCCATGCCATCAC-3′, R 5′-TCCACCAC-CCTGTTGCTGTA-3′[13]. Amplification was performed on an Eppendorf thermocycler for 30 cycles with denaturing at 94 °C for 30 s, annealing at 58 °C for 40 s, and extension at 72 °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 inoculated 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′-GCAACAGCAUCUUGCCUGAUUUGUA-3′ and 5′-UACAAAUCAGGCAAGAUGCUGUUGC-3′. The scrambled control siRNA sequences were 5′-UUCUCCGAACGUGU-CACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-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_2O_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_2O_2, RHL or H_2O_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-isolyl-β-*D*-galactoside (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_2O_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 kit

(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)[15].

Cell cycle assay

To determine the effect of H_2O_2 and RHL on cell cycle progression, HUVECs were grown for 48 h (one-cell cycle) with or without 100 μmol/L H_2O_2, 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).

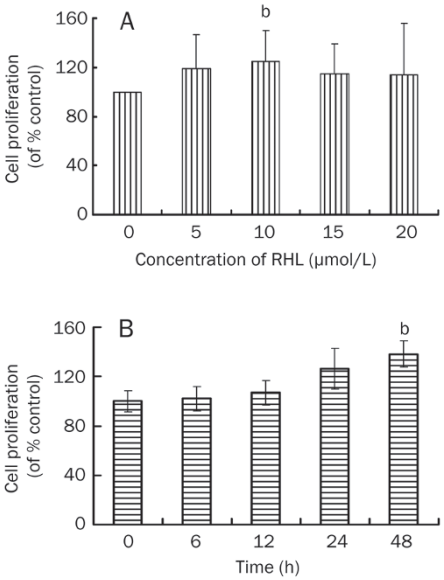

Figure 1. Rhein lysinate at low concentrations promoted the proliferation of HUVECs. HUVECs were treated with various concentrations of RHL at 37 °C for 48 h (A), and then cells were incubated with 10 μmol/L RHL for 0, 6, 12, 24, and 48 h (B). The effects on cell proliferation were examined by MTT assay, and cell proliferation was calculated as the percentage of control. n=3. Mean±SD. $^bP<0.05$ *vs* control.

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 H_2O_2, and senescent cells were found to increase with

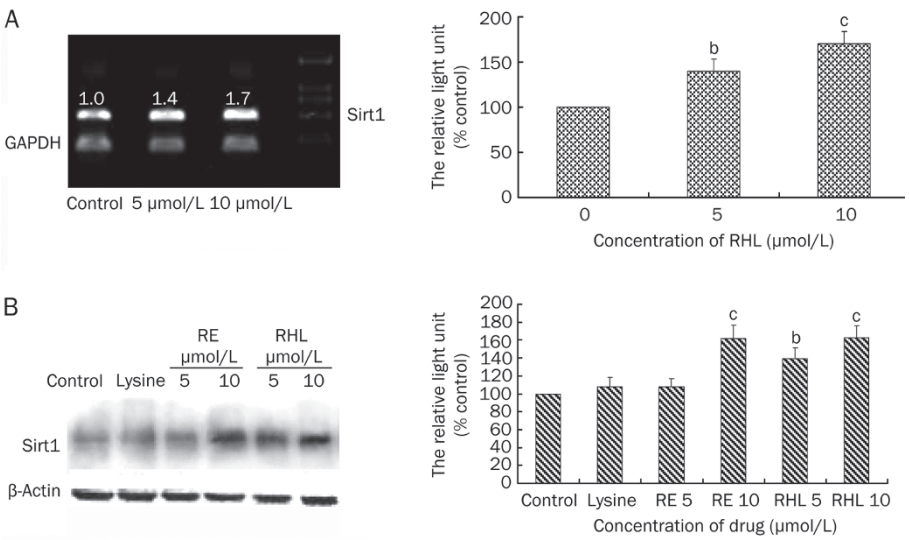

Figure 2. Rhein lysinate at low concentrations increased Sirt1 transcription and expression. HUVECs were treated with 5 or 10 μmol/L RHL at 37 °C for 48 h. Sirt1 transcription was examined by RT-PCR (A), and Sirt1 expression was examined by Western blot (B). RE=resveratrol (positive control); RHL=Rhein lysinate. n=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control.

increasing dosages of H_2O_2 (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 H_2O_2 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

H_2O_2 inhibited HUVEC survival with IC_{50} of about 100 μmol/L. To determine whether or not H_2O_2 inhibits HUVEC survival by inducing apoptosis, we investigated the effect of H_2O_2 on apoptosis. H_2O_2 100 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 H_2O_2 100 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 H_2O_2. n=3. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* control. ^eP<0.05, ^fP<0.01 *vs* H_2O_2 100 μmol/L.

Concentration (μmol/L)	Normal cells (%)	Necrotic cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)
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
H_2O_2 100	60.4±4.8^c	21.4±1.2^c	8.8±0.7	9.4±1.0
RHL+H_2O_2	71.0±4.1^{be}	15.6±1.4^{bf}	6.8±0.9	6.6±0.6

H_2O_2 induced G_1 arrest in HUVECs, and RHL prevented against H_2O_2-induced G_1 arrest

Cell cycle plays an important role in cellular senescence. Senescence occurs during the G_1 period. Treatment with 100 μmol/L H_2O_2 arrested HUVECs at G_1 phase. The proportion of cells in the G_1 phase was approximately 73.8%. However, it was about 64.6% in the vehicle group. RHL 10 μmol/L reversed the G_1 arrest effects of H_2O_2. The proportion of cells in G_1 phase decreased to 63.1% (Table 2). H_2O_2 100 μmol/L induced the p53 acetylation and $p16^{INK4a}$ up-regulation and treatment with 10 μmol/L RHL prevented this process (Figure 5B).

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[16]. Therefore, it can be concluded that by delaying cell senescence, low concentrations of RHL can improve HUVEC proliferation.

Table 2. The effect of RHL on cell cycle arrest of HUVECs induced by H_2O_2. n=3. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* control. ^eP<0.05, ^fP<0.01 H_2O_2 100 μmol/L.

Concentration (μmol/L)	G_0/G_1 (%)	S (%)	G_2/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
H_2O_2 100	73.8±5.5^b	13.3±1.2^c	12.9±1.3
RHL+H_2O_2	63.1±5.1^e	24.5±2.6^f	12.4±2.2

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[17]. 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 H_2O_2-treated HUVECs. We found that H_2O_2 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 H_2O_2, 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 H_2O_2 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 H_2O_2 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[20]. In this study, we also observed that the p53 acetylation level was inversely related to the expression of Sirt1 in H_2O_2-induced

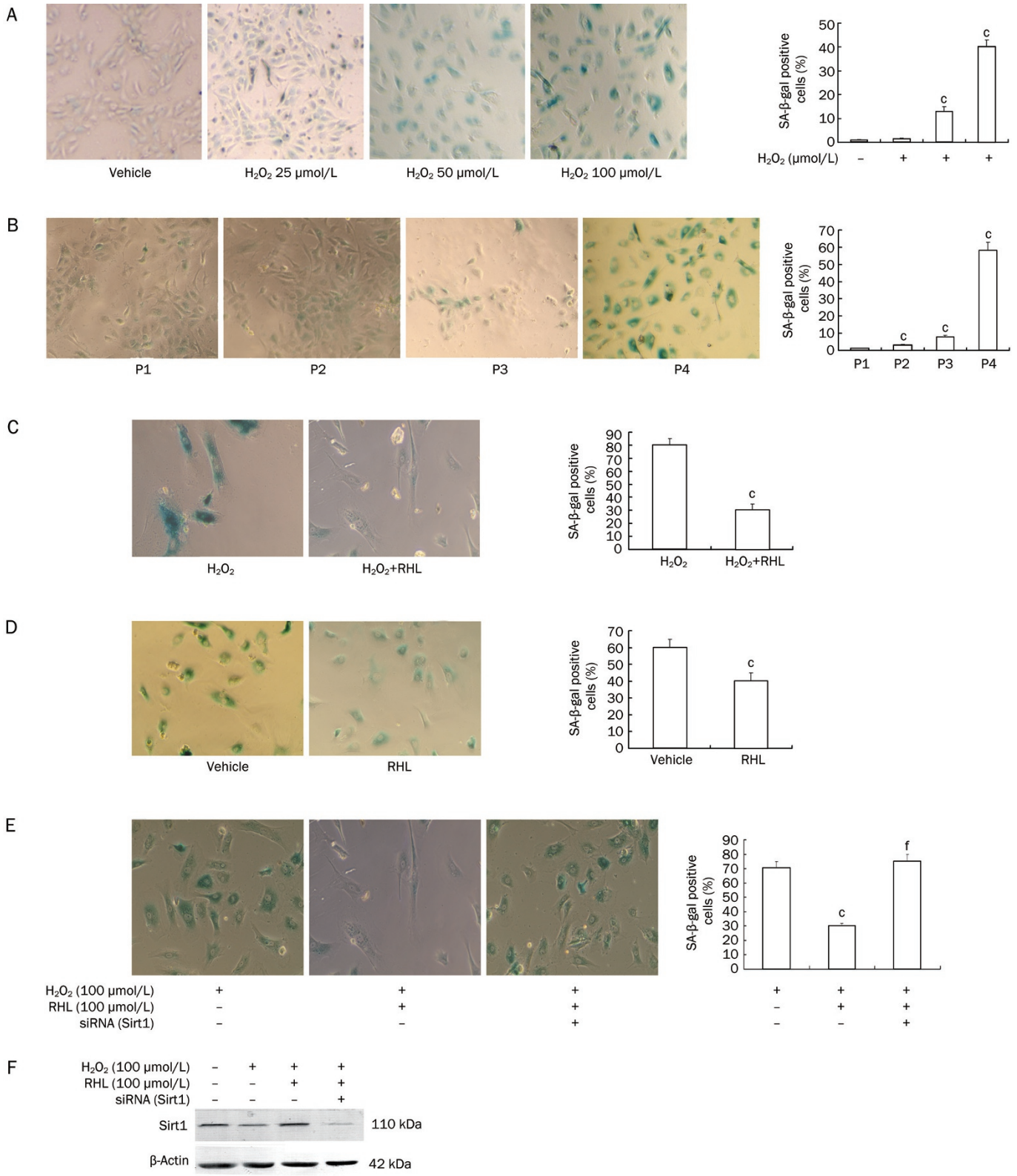

Figure 3. SA-β-gal activity of different passages of HUVECs treated with various concentrations of H_2O_2. (A) HUVECs were treated with 0, 25, 50, and 100 μmol/L H_2O_2 at 37 °C for 3 d and then placed in a medium with the same concentration of H_2O_2 for further 3 d. $^cP<0.01$ *vs* vehicle. (B) P1, 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_2O_2 treatment. $^cP<0.01$ *vs* H_2O_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_2O_2 for 6 d. $^cP<0.01$ *vs* H_2O_2. $^fP<0.01$ *vs* RHL+H_2O_2. (F) The expression of Sirt1 in the various groups. n=3. Mean±SD.

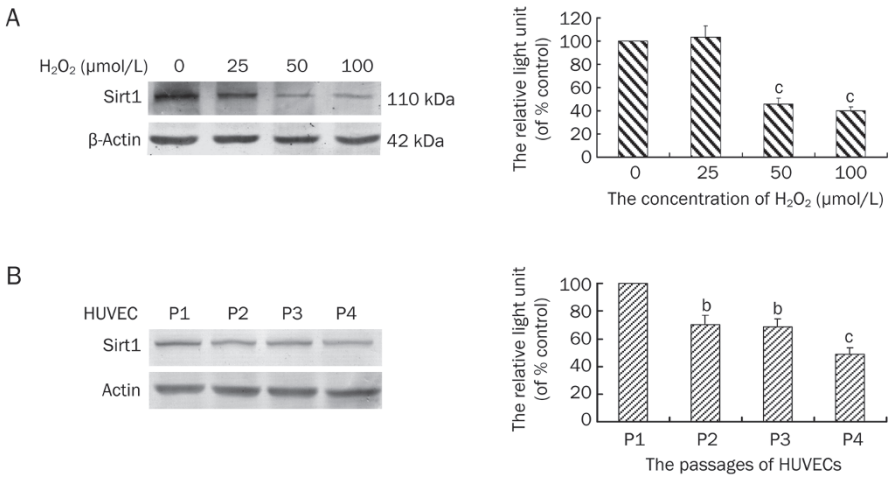

Figure 4. Effects of various concentrations of H_2O_2 on Sirt1 expression, and Sirt1 expression at different cell passages. (A) HUVECs were treated with 0, 25, 50, and 100 μmol/L H_2O_2 at 37 °C for 3 d and then placed in a medium with the same concentration of H_2O_2 for 3 more days. The Sirt1 expression was examined by Western blot. (B) P1, P2, P3, and P4 represented different passages of HUVECs. HUVECs were cultured for 3 days and then propagated. After incubation, Sirt1 expression was also determined, as in (A). n=3. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* control.

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 oncogenes. It is a single core module that governs the three potent tumor suppression mechanisms: growth arrest, senescence, and apoptosis[20].

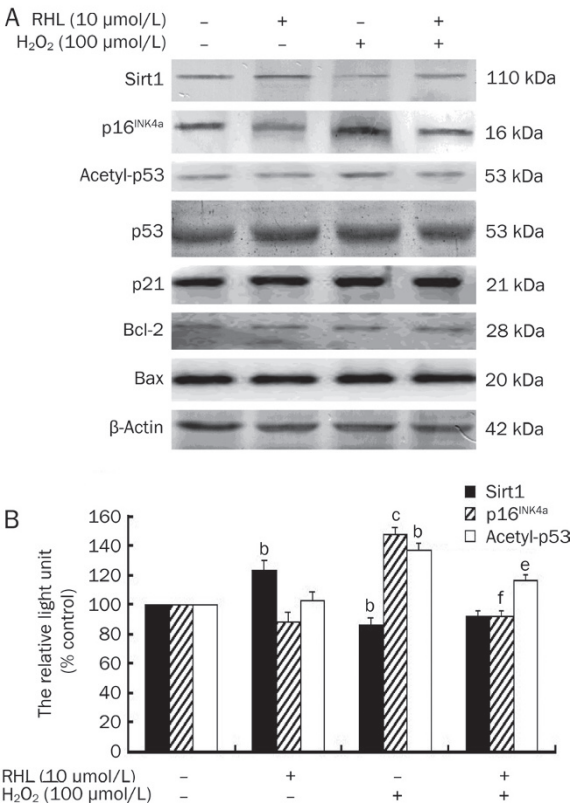

Figure 5. (A) Effect of RHL on the expression of Sirt1 and its downstream proteins. (B) Relative density of the protein levels. HUVECs were treated with 100 μmol/L H_2O_2 and/or RHL 10 μmol/L at 37 °C for 3 d and then placed in a medium with the same concentration of H_2O_2 for 3 more days. The expression of Sirt1, p53, p21, $p16^{INK4a}$, Bcl-2, Bax, and β-Actin was determined by Western blot. n=3. Mean±SD. ^bP<0.05, ^cP<0.01 *vs* control. ^eP<0.05, ^fP<0.01 *vs* H_2O_2 100 μmol/L.

In this study, we explored whether apoptosis inhibited cell proliferation. Although 500 μmol/L H_2O_2 has been shown to induce cell apoptosis in PC12 cells (rat pheochromocytoma cell line)[21], HUVEC apoptosis was not affected by H_2O_2 (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 H_2O_2 induced G_1 period block, 10 μmol/L RHL prevented G_1 period block (Table 2), and that G_1 period block is the feature of cell senescence[22]. Therefore, we can conclude that the senescence induced by H_2O_2 was not due to apoptosis but to G_1 period block, and that RHL can interrupt the G_1 period block induced by H_2O_2.

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 $p16^{INK4a}$ upregulation induced by 100 μmol/L H_2O_2. It can be inferred that H_2O_2 could partially induce HUVEC G_1 period block by increasing $p16^{INK4a}$ expression and that RHL could antagonize this phenomenon by the same signaling pathway. However, the effect of H_2O_2 and RHL on cyclin needs further investigation.

In conclusion, the decrease in Sirt1 expression inhibited cell proliferation induced by H_2O_2, as well as the spontaneous cell senescence with increased cell passage. Subsequently p53 was acetylated, and $p16^{INK4a}$ was upregulated, which caused G_1 period block and aging. However, RHL resisted this process. In addition, during HUVEC senescence, it was G_1 period block, not apoptosis, that played an important role, and RHL interrupted the G_1 period block induced by H_2O_2.

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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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