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Original Article

SIRT6/NF-κB signaling axis in ginsenoside Rg1-delayed hematopoietic stem/progenitor cell senescence

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Abstract: Objective: To investigate the role of SIRT6/NF-κB signaling axis in ginsenoside Rg1-delayed hematopoietic stem/progenitor cell senescence and to provide theoretical and experimental evidence for delaying HSC/HPC senescence pathway. Methods: After the separation and purification by immunomagnetic sorting, Sca-1$^+$HSC/HPC was divided into: normal control group; aging group; positive control group; Rg1 delaying group and Rg1 treatment group. Senescence-associated β-galactosidase (SA-β-Gal) staining, flow cytometry analysis of cell cycle and hematopoietic progenitor cells mixed colony (CFU-Mix) culture were performed to determine the delaying or curing roles of Rg1 in Sca-1$^+$HSC/HPC senescence. Quantitative PCR and Western blotting were used to detect the mRNA and protein expression of senescence regulatory molecules, such as SIRT6 and NF-κB. Results: Compared with the aging group, the positive rate of SA-β-gal staining cells and the proportion of cells in G1 phase decreased; the number of CFU-Mix increased; mRNA and protein expression of SIRT6 increased; mRNA and protein expression of NF-κB was down-regulated in Rg1 delaying and treatment groups; the changes of the indicators in Rg1 delaying group were more significant than those in Rg1 treatment group. Conclusion: Rg1 may fight against Sca-1$^+$HSC/HPC senescence induced by t-BHP through regulating SIRT6-NF-κB signaling pathway.

Keywords: Ginsenoside Rg1, SIRT6, NF-Kb, Sca-1$^+$HSC/HPC, senescence

Introduction

Stem cell theory of aging is so far the latest theory to explain body's senescence mechanisms [1-3]. Hematopoietic stem cells (HSC) aging and body aging are closely related with a variety of age-related diseases, which is one of the most noteworthy topics in the field of stem cell senescence.

Ginseng is an important drug of supplementing qi in traditional medicine, with the effects of benefiting qi and nourishing blood, lengthening life and enhancing immunity; our previous study found that: Ginsenoside Rg1 can delay HSC/HPC senescence both in vivo and in vitro, but its targeting regulatory molecules remains unclear. The latest research shows that deacetylase SIRT6 is a key regulatory gene of cell lifespan; SIRT6 regulates telomere-independent cell aging by inhibiting NF-κB [4]. In this study, the tert-butylhydroperoxide (t-BHP)-induced Sca-1$^+$HSC/HPC senescence was used as the model and treated with ginsenoside Rg1to explore the regulatory roles of SIRT6 and NF-κB in the delay of HSC/HPC senescence induced by Rg1, providing theoretical and experimental basis for exploring ways to delay HSC aging and body aging.

Materials and methods

Animals

C57BL/6 mice of clean grade, 6-8 weeks old, male or female, weighing 20-25 g, provided by the Experimental Animal Center of Chongqing Medical University; Animal Certificate of Conformity: SCXK (Chongqing) 2007-0001.

Regents

Ginsenoside Rg1 (Jilin Hongjiu Biotechnology Co., Ltd., purity > 95%); IMDM, fetal bovine

Table 1. Primers used in FQ-PCR

Primer	Forward (5'-3')	Reverse (5'-3')	Product size (bp)
SIRT6	GCTGAGGGACACCATCCTAGA	GTAGCCAGCGGCAGGTTC	147
NF-κB	GTGGGGACTACGACCTGAATG	CTGCACCTTGTCACACAGTAGG	215
β-actin	TGACGTGGACATCCGCAAAG	CTGGAAGGTGGACAGCGAGG	205

serum, horse serum (Gibco Company); Anti-Sca-1 + Micro Bead Kit (Miltenyi company); SAβ-gal Staining Kit (Cell Signaling company); Trizol and RT-PCR kit (TaKaRa company); PCR primers (Invitrogen Corporation); t-BHP, Ficoll separating medium, L-glutamine, methyl cellulose (Sigma); rhGM-CSF and rhEPO (Kirin Kunpeng bio-Pharmaceutical Co, Ltd.); SIRT6, NF-κB primary antibody, HRP secondary antibody (Santa Cruz company); protein lysates (Applygen company); BCA protein Assay kit (Beyotime Biotechnology Co., Ltd.); ECL luminescence kit (Pierce Co).

Grouping

According to the method of our laboratory [5], mouse Sca-1$^+$HSC/HPC were isolated and purified by magnetic activated cell sorting (MACS); the obtained Sca-1$^+$HSC/HPCs were seeded in 6 plates at a density of 1 × 105 cells/well. Normal control group was incubated with IMDM culture medium containing 10% fetal bovine serum (37°C, 5% CO_2) for 6 h; senescence group: on the basis of control group, t-BHP with a final concentration of 100 μmol/L was added; positive control group: on the basis of the control group, Rg1 with a final concentration of 10 μmol/L was added to co-culture for 6 h; Rg1 aging-treatment group: based on the aging group, 10 μmol L Rg1 was added to co-culture for 6 h; Rg1 aging-delay group: based on the positive control group, t-BHP with a final concentration of 100 μmol/L was added. After the cells were treated in each group, the medium was washed for related detection.

SA-β-galactosidase staining

The cells were collected in each group; staining was performed according to the instructions of SA-β-Gal Staining Kit; after dyeing, centrifugation, cytospin slides and sheet-sealing by 70% glycerol were performed. 400 cells were randomly counted in each slide and the percentage of positive cells was recorded.

Cell cycle analysis by flow cytometry

Cells were collected in each group, and washed once by PBS, fixed by 70% ice ethanol overnight; after washed twice by PBS, 100 μl bovine pancreatic ribonuclease (1 mg/ml) was added and cells were incubated in 37°C water bath for 30 min; Cells were stained with propidium iodide dye (50 μg/ml) in dark for 30 min before flow cytometry detection; Multicycle software (Japan PHENIX company) was used for the analysis of cell cycle phases in each group.

CFU-mix culture

According to the literature [6] to improve the method, 2-mercaptoethanol (1 × 10-4 mol/L), 3% L-glutamine, horse serum, rhEPO, IL-3, rhGM-CSF, 1 × 10^4 cells of each group and 2.7% methylcellulose were added in order; the total volume was 2 ml; after mixing well, cells were seeded in 96-well plates (0.2 ml/well) and incubated in an incubator (37°C, 5% CO_2, saturated humidity) for seven days; based on the numbers of Sca-1+HSC/HPC and CFU-Mix, the formation capacity of CFU-Mix and its multiple differentiation potential in each group were evaluated.

Quantitative PCR to detect mRNA expression of SIRT6 and NF-κB

Cells were collected in each group; After Trizol lysis, total RNA was extracted and reverse transcribed into corresponding cDNA; reverse transcription reaction conditions: 42°C, 30 min, 99°C, 5 min, 5°C, 5 min. The cDNA was preserved at -20°C for RT-PCR reactions. The cDNA reverse transcribed from RNA was used as the template to amplify SIRT6, with GAPDH as an internal reference. The primer sequences were shown in **Table 1**. PCR amplification conditions: 94°C 4 min; 94°C 20 S, 60°C 30 S, 72°C 30 S, totally 35 cycles; detecting signals at 72°C. Quantity One software (Bio Rad) was used for data processing and result analysis.

Table 2. Percentage of SA-β-gal staining positive cells ($\bar{x} \pm s$, n=8)

Group	SA-β-Gal positive rate (%)
Normal control	9.08 ± 2.41
Aging	64.50 ± 4.95[1)]
Positive control	7.12 ± 2.53
Rg1 aging-treatment	36.14 ± 2.44[2)]
Rg1 aging-delay	24.50 ± 2.34[2),3)]

Note: Compared with normal control group [1)]P<0.05; compared with aging group [2)]P<0.01; compared with Rg1 aging-treatment group [3)]P<0.05.

Western blotting for the protein expression of SIRT6 and NF-κB

Cells were collected and total protein was extracted; the concentration of total protein was determined according to the instructions of BCA concentration assay kit. An equal amount of total protein was separated by SDS-PAGE and transferred to PVDF membranes; after sealing by 5% skim milk for 2 h, rabbit anti-mouse SIRT6 and NF-κB primary antibody (1:200) were added, allowed to stand at 4°C overnight; and then the protein was rinsed thoroughly by TBST buffer; horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:5000) was added, reacting at room temperature for 2 h; After sufficiently washing the membrane by TBST for three times and ECL enhanced luminescent reagent staining, it was exposed to the gel imaging system for image developing-fixing before observing the results.

Statistical analysis

Experimental data was processed by SPSS statistical software, using the methods of factorial design and single-factor analysis of variance; the data were expressed as $\bar{x} \pm s$; P<0.05 was considered statistically significant.

Results

Percentage of SA-β-gal staining positive cells

After t-BHP induction, the percentage of SA-β-Gal staining positive cells in aging group was significantly higher than that in normal control group; no significant difference was found between the normal control group and positive control group; the percentage of positive cells in Rg1 aging-treatment and aging-delay groups were significantly lower than that in aging group, and the positive rate in Rg1 aging-delay group was lower compared with Rg1 aging-treatment group (**Table 2**).

Distribution of cell cycles of Sca-1$^+$HSC/HPC

Compared with normal control group, cells in aging group were arrested in G1 phase; the percentage of cells in G0/G1 phase was significantly increased, and the proportion of cells in S phase was reduced; the proliferation index PI (S + G2/M) was significantly reduced; the positive control group had a lower percentage of G1 phase cells and a higher PI than the control group; compared with the aging group, the percentages of G1 phase cells in Rg1 aging-treatment and aging-delay groups were significantly reduced, and the PI was significantly higher than the aging group; changes were the most significant in Rg1 aging-delay group (**Table 3**).

Detection of CFU-Mix ability

The colonies in positive control group were more than those in the normal control group; the colonies in aging group were significantly reduced compared with the normal control group; the number of colonies in Rg1 aging-treatment and aging-delay groups was higher than that in aging group, and that in Rg1 aging-delay group was higher compared with Rg1 aging-treatment group (**Table 4**).

Changes in SIRT6 and NF-κB mRNA levels

Compared with the normal control group, increased SIRT6 mRNA expression and decreased NF-κB mRNA expression were found in the positive control group; SIRT6 mRNA expression in aging group was significantly lower than that in the normal control group, while the NF-κB mRNA expression was higher than that in the control group; compared with the aging group, up-regulation of SIRT6 mRNA expression and down-regulation of NF-κB mRNA expression were detected in Rg1 aging-treatment and aging-delay groups. Changes of index in Rg1 aging-delay group were more significant compared with Rg1 aging-treatment group (**Figure 1**).

Table 3. Distribution of cell cycles of Sca-1$^+$HSC/HPC ($\bar{x}$ ± s, *n*=8)

Group	G_0/G_1 (%)	G_2/M (%)	S (%)	PI (%)
Normal control	72.3±3.4	7.3±1.9	19.7±3.1	27.7±3.3
Aging	87.5±3.9[1)]	4.8±1. 5[1)]	7.9±2.1[1)]	12.8±1.6[1)]
Positive control	70.9±1.8[2)]	8.8±0.7[2)]	20.5±2.1[2)]	29.4±2.2[2)]
Rg1 aging-treatment	81.4±1.2[3)]	6.9±1.0[3)]	10.7±1.5[3)]	17.6±2.0[3)]
Rg1 aging-delay	78.2±1.4[3),4)]	7.2±1.0[3),4)]	15.2±1.4[3),4)]	22.4±1.8[3),4)]

Note: Compared with normal control group [1)]*P*<0.05, [2)]*P*<0.01; compared with aging group [3)]*P*<0.01; compared with Rg1 aging-treatment group [4)]*P*<0.05.

Table 4. Number of CFU-mix ($\bar{x}$ ± s, n=8)

Group	Number of CFU-Mix (/10^4)
Normal control	17.04±1.48
Aging	3.04±2.58[1)]
Positive control	20.65±2.17[2)]
Rg1 aging-treatment	8.04±2.42[3)]
Rg1 aging-delay	10.18±1.23[3),4)]

Note: Compared with normal control group [1)]*P*<0.05, [2)]*P*<0.01; compared with aging group [3)]*P*<0.01; compared with Rg1 aging-treatment group [4)]*P*<0.05.

Changes in SIRT6 and NF-κB protein levels

Compared with the normal control group, increased SIRT6 protein expression and decreased NF-κB protein expression were found in the positive control group; and the contrary changes were detected in aging group; compared with the aging group, up-regulation of SIRT6 protein expression and down-regulation of NF-κB protein expression were detected in Rg1 aging-treatment and aging-delay groups; Changes in Rg1 aging-delay group were the most significant (**Figure 2**).

Discussion

Ginseng is an important drug of supplementing *qi* in traditional medicine, with the effects of benefiting qi and nourishing blood, tranquilizing the mind and lengthening life; Ginsenosides Monomer Rg1 is the main active ingredient of ginseng anti-aging with the effect of anti-aging, antioxidant, enhance immunity and so on. The study found that Rg1 can significantly prolong the life of the body and cell, prolong the survival time of old rats, significantly improve the recessive behavioral activity function of aged rats[7, 8]. In this study, we used t-BHP-induced Sca-1$^+$HSC/HPC aging model to study vitro anti-aging effects of Rg1. The results showed that: compared with the control group, the aging Sca-1$^+$HSC/HPC enhanced self-renewal and multi-differentiation capacity after Rg1 treatment and anti-aging treatment, indicating that Rg1 had an effect on anti-t-BHP-induced Sca-1$^+$HSC/HPC senescence.

Deacetylase SIRT6 is a nuclear protein which is widely expressed in mammal. By affecting the DNA damage-repair process to maintenance genomic stability, they reduced aging and extended the life of the organism. Dysfunction of SIRT6 led to senescence. Kawahara et al's [9-12] study confirmed that SIRT6 regulated organism cell aging by inhibiting NF-κB. SIRT6 and NF-κB RELA subunit combined together, promoting NF-κB target gene promoter H3K9 deacetylation, playing its role and enhancing NF-κB signaling pathway which can promote the occurrence of premature and normal aging. The study found that: compared with the control group, in the aging group, the expression of Sca-1$^+$HSC/HPC SIRT6 was decreased, and the expression of NF-κB was increased, which was the same as the SIRT6 and NF-κB expression in the process of cell senescence. After Rg1 acted on aging Sca-1$^+$HSC/HPC, the expression of SIRT6 was up-regulated and NF-κB was down-regulated, indicating that Rg1 en-hanced the intracellular expression of SIRT6 and SIRT6 slowed cell senescence by inhibiting expression of NF-κB, indicating that Rg1 may play its role on t-BHP-induced anti-Sca-1$^+$HSC/HPC senescence by regulating SIRT6-NF-κB signaling pathway. Compare with the Rg1 treatment group, expression changes of SIRT6 and NF-κB in Rg1 aging group was significantly higher, which further suggested that anti-aging effects of Rg1 was superior to treatment of aging.

Cell senescence is affected by many external factors, and environmental factors must play its role through internal gene regulation. Cell cycle arrest is one of the mechanisms of cell senescence; $p16^{INK4a}$, $p19^{Arf}$, p53 and $p21^{Cip1/Waf1}$ are regulators of cell cycle; the activation of

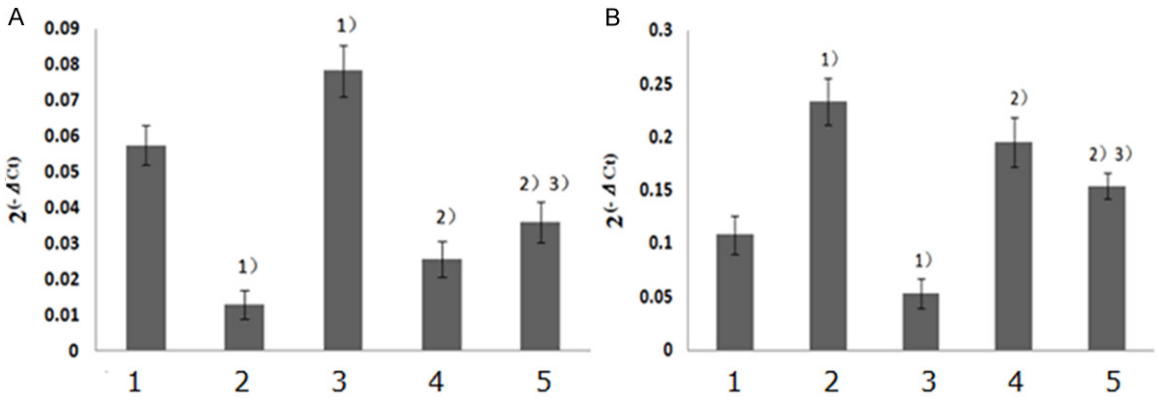

Figure 1. mRNA Expressions of SIRT6 and NF-Kb, A: SIRT6 mRNA expression; B: NF-κB mRNA expression. 1: Normal control group; 2: aging group; 3: positive control group; 4: Rg1 treatment group; 5: Rg1 delay group. Note: Compared with normal control group [1)] $P<0.05$; Compared with aging group [2)] $P<0.01$; compared with Rg1 aging-treatment group [3)] $P<0.05$.

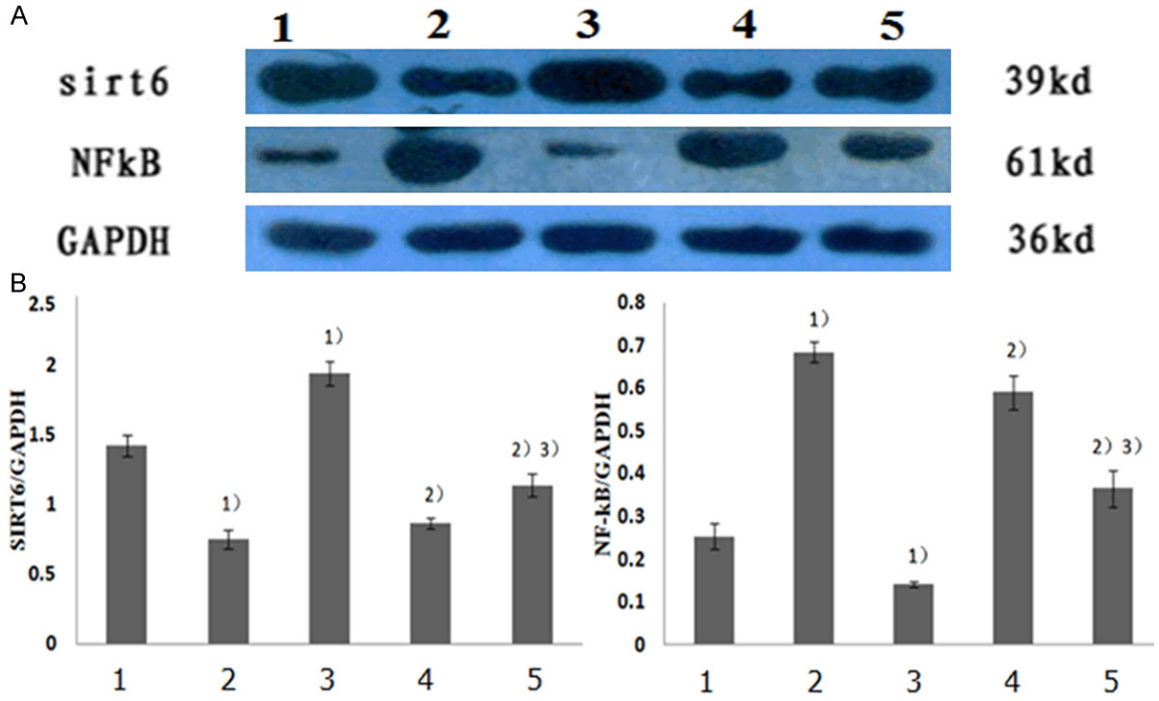

Figure 2. Effect of Rg1 on Protein expressions of SIRT6 and NF-κB in Sca-1^{+}HSC/HPC. A: western blotting of SIRT6 and NF-κB protein expression; B: Changes in SIRT6 and NF-κB protein expressions. 1: Normal control group; 2: aging group; 3: positive control group; 4: Rg1 treatment group; 5: Rg1 delay group.

any signal pathway in $p16^{INK4a}$-Rb and $p19^{Arf}$-Mdm2-p53-$p21^{Cip1/Waf1}$ can induce telomere-dependent organism cell aging. Deacetylase is another regulatory mechanism of cell senescence; SIRT6 regulates telomere-independent organism cell aging by inhibiting NF-κB; our study [13] found that Rg1 played its aging-delay and aging-treatment roles in HSC/HPC through regulating signaling pathways of p16-INK4a-Rb, $p19^{Arf}$-Mdm2-p53-$p21^{Cip1/Waf1}$ and SIRT6-NF-κB; Whether there are extensive multi-level calls among these pathways, and whichever of telomere-dependent and telmere-independent signaling pathways plays a more important role, are still pending further study.

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Disclosure of conflict of interest

None.

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