Ginsenoside Rg1 Delays Tert-Butyl Hydroperoxide-Induced Premature Senescence in Human WI-38 Diploid Fibroblast Cells

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Tert-butyl hydroperoxide (t-BHP), an analog of hydroperoxide, induced characteristic changes of senescence in human diploid fibroblasts WI-38 cells. It was reported that ginsenoside Rg1, an active ingredient of ginseng, ameliorated learning deficits in aged rats. The present study was aimed to investigate whether ginsenoside Rg1 can delay the premature senescence of WI-38 cells induced by t-BHP and to explore the underlying molecular mechanisms. First, Rg1 pretreatment markedly reversed senescent morphological changes in WI-38 cells induced by t-BHP. Second, t-BHP treatment alone resulted in an increase in the protein levels of P16 and P21, and a decline in intracellular adenosine 5'-triphosphate (ATP) level and mitochondrial complex IV activity. Ginsenoside Rg1 pretreatment had significant effects of attenuating these changes. These data indicate that ginsenoside Rg1 has an anti-aging effect on t-BHP-induced premature senescence in WI-38 cells. This effect may be mediated by regulating cell cycle proteins and enhancing mitochondrial functioning.

Key Words: Tert-butyl hydroperoxide—Senescence—Ginsenoside Rg1—p16—p21—Mitochondrial respiratory chain complex—ATP.

Ginseng, the root of Panax ginseng C. A. Meyer (Araliaceae), has been used as a remedy in Chinese traditional medicine for over 2000 years mostly for general health improvement, including fighting against aging. Over the past several decades, ginseng has been drawing great attention all over the world, especially in China, Japan, Korea, and the United States. Ginseng, a complex system consisting of multiple compounds, has a wide range of pharmacological activities, including immunomodulatory effects, anti-inflammatory activity, improvement of physical stamina, and stimulation of appetite. It is also thought to have beneficial effects on learning, memory, and behavioral control (1).

Ginsenoside Rg1 is one of the major active components of ginseng. The chemical structure of Rg1 is shown in Figure 1. The two sugar side-chains lie at C-6 and C-20. As ginseng, Rg1 is shown to have several physiological effects, such as stimulation of the central nervous system, facilitation of memory, anti-fatigue activity, and promotion of acetylcholine, protein, and lipid synthesis (2). Ginsenoside Rg1 is believed to be the principle compound of ginseng for anti-aging. It is reported that ginsenoside Rg1 ameliorated learning deficits in aged rats (3,4). Zhao and colleagues (5) have reported that Rg1 can delay the premature senescence in WI-38 cells induced by tert-butyl hydroperoxide (t-BHP). However, it is still not clear by which mechanisms ginsenoside Rg1 delays the premature senescence induced by t-BHP. Progress has been made in past decades in the study of the mechanisms of senescence. Multiple causes including cell cycle regulation, free radicals, mitochondrial DNA damage, telomere shortening, as well as protein carbonylation are found to be involved in the process of senescence (6–9).

The WI-38 cell is a human diploid fibroblast derived from embryonic lung tissue, and is considered to be young at population doubling (PD) ≤30 and to be fully senescent at PD ≥55. WI-38 cells in replicative senescence usually have features such as changes in their shape and size, irreversible cell cycle arrest mostly in the G1 phase, increase in the percentage of cells with positive senescence-associated beta-galactosidase (SA-β-gal) staining, as well as reduction in telomere length (10–12). Tert-butyl hydroperoxide is an analog of hydroperoxide. Studies have shown that the cells exposed to t-BHP display many biomarkers of senescence resembling replicative–senescent cells; this has been referred to as stress-induced premature senescence (13). Our previous study also demonstrated that treatment four times with t-BHP resulted in WI-38 cell senescence, indicating that t-BHP is a potent inducer in modeling premature senescence (5).

The aims of the current study were to confirm whether ginsenoside Rg1 can delay the premature senescence of WI-38 cells induced by t-BHP and to explore the possible mechanisms in terms of cell cycle regulation, mitochondrial functioning, and telomerase activity. The results of present studies have revealed a more complete view of the anti-aging effects of ginsenoside Rg1. Our findings also have highlighted the importance of cycle regulatory proteins P16 and P21 and mitochondrial functioning in the process of senescence.
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MATERIALS AND METHODS

Cell Cultures and Treatments

Ginsenoside Rg1 (molecular weight 800, purity 98%) was obtained from the Department of Biochemistry of Jilin University, China. The stock of Rg1 at 1 M was prepared with minimal essential medium (MEM; Gibco, now part of Invitrogen, San Diego, CA). The chemical structure of ginsenoside Rg1 is shown in Figure 1.

WI-38 cells were derived from American Type Culture Collection (ATCC) and cultured with MEM, which contained 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acid, penicillin at 100 U/mL, and streptomycin at 100 μg/mL. The cultures were grown in an incubator at 37°C with 5% CO₂. The cells were subcultured at a dilution of 1:2 or 1:4 when the confluence of cells was about 80%. In slowly growing cultures, the culture medium was changed every 3–4 days.

WI-38 cells at 24 PDs were grouped randomly into the following groups: young group (at 30 PDs without any pretreatment), control group (at 38 PDs without any pretreatment), t-BHP alone group (premature senescence model group), and ginsenoside Rg1 pretreatment groups (5, 10, and 20 μM). For the cell premature senescence modeling, the WI-38 cells from 30 PDs were treated with 100 μM t-BHP four times for 1 hour at the interval of every two PDs. Rg1 pretreatment groups were made by adding different concentrations of ginsenoside Rg1 in MEM culture starting from 24 PDs, and Rg1 was no longer administered during the period of 1 hour t-BHP induction.

Figure 1. Chemical structure of ginsenoside Rg1.

Methyl Thiazolyl Tetrazolium Assay

Methyl thiazolyl tetrazolium is converted in living cells to formazan, which has a specific absorption maximum. The WI-38 cells before 30 PDs were treated with ginsenoside Rg1 (0–80 μM) for indicated times, then the culture medium was changed to the fetal bovine serum–free medium containing 5% CO₂/95% air overnight. The absorbance of the supernatant was measured at 570 nm on an automated microtiter plate reader. Data were expressed as the mean percentage of viable cells versus controls.

Lactate Dehydrogenase Cytotoxicity Assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells; it rapidly releases into the cell culture supernatant upon damage of the plasma membrane. LDH oxidizes lactate to pyruvate, which then reacts with tetrazolium salt INT to form formazan. The amount of formazan produced in culture supernatant directly correlates to the number of lysed cells. The WI-38 cells before 30 PDs were treated with ginsenoside Rg1 (0–80 μM) for indicated times, then the culture supernatant was collected and centrifuged at 250 g for 5 minutes, and the supernatant was transferred into the reaction mixture solution. The absorbance of all samples was measured at 490 nm on an automated microtiter plate reader. The formula to calculate the percentage of cytotoxicity is $\text{Cytotoxicity (%) = \left( \frac{\text{Test sample} - \text{Low control}}{\text{High control} - \text{Low control}} \right) \times 100\%}$

Electronic Microscopy

The investigated cells were trypsinized, centrifuged, collected, then fixed in a prechilled solution consisting of 3% glutaraldehyde and 1% paraformaldehyde for at least 4 hours. They were then washed three times with phosphate-buffered saline (PBS) and postfixed at 1% perosmic acid for 2 hours at 4°C. After being dehydrated, soaked, embedded, sectioned, and stained, the samples were examined with an HU-12A electron microscope (Hitachi, Tokyo, Japan). This study was repeated three times.

SA-β-gal Staining

SA-β-gal was stained according to a modified method (12). The percentage of SA-β-gal-positive cells of the total number of cells was calculated. An average percentage was obtained from five independent experiments.

Cell Cycle Analysis

Cell cycle analysis was performed as described (14). The cells of the t-BHP alone group and the Rg1 pretreatment groups were plated at approximately 70% confluence, and the cells of young group and control group were plated about 20% and 50%, respectively. All cells were left to grow for an additional 4 days. When the experiments were performed, the cell confluence in the t-BHP alone and Rg1 pretreatment groups had changed little, and the cell confluence in the young and control groups had grown to approximately 70%–80%. The investigated cells were trypsinized, washed, and then exposed to propidium iodide (PI) solution (PI at 500 mg/L and RNase A at 50 mg/L) for 30 minutes at 37°C. After being washed twice with PBS, the cells were measured by fluorescence-activated cell sorting on a Becton-Dickinson FACScan Flow Cytometry System (BD, Franklin Lakes, NJ). The data were analyzed using CellFIT software.

Determination of Telomere Length

Genomic DNA in WI-38 cells was extracted with a genomic DNA purification kit (Promega, Madison, WI).
DNA was digested, subjected to electrophoresis, and transferred to a nylon membrane, then was prehybridized and hybridized with a digoxigenin-labeled probe specific for telomeric repeats (TeloTAGGG Telomere Length Assay Kit, Roche, Mannheim, Germany). After scanning the chemiluminescence signals with a Lumi-Imager (Roche) and analyzing by using computer software, we calculated the mean terminal restriction fragments (TRF) using the formula: 

\[
L = \frac{\sum(OD_i)}{\sum(OD_i/L_i)}
\]

where \(OD_i\) and \(L_i\) are, respectively, the signal intensity and TRF length at position \(i\) on the gel image.

**Determination of Telomerase Activity and Expression of hTERT Gene**

The telomeric repeat amplification protocol (TRAP) assay was performed using a TRAP EZE enzyme-linked immunosorbent assay (ELISA) Telomerase Detection Kit (Chemicon, now Millipore, Billerica, MA) according to the manufacturer's instructions. Briefly, frozen cell pellets (10^3–10^6 cells) were homogenized in 200 µL of CHAPs lysis buffer and incubated on ice for 30 minutes. The lysates were centrifuged at 12,000 g for 20 minutes at 4°C. Protein concentration was determined with a protein assay kit (Bio-Rad, Hercules, CA), and 1 µg of protein was used for each TRAP assay. Polymerase chain reaction (PCR) was performed at 94°C for 30 seconds, 55°C for 30 seconds for 30 cycles. The PCR product was detected by ELISA and native-polyacrylamide gel electrophoresis (PAGE) analysis.

HepG2 cell and SGC-7901 cells were used as positive controls, and total RNA was extracted by Trizol reagent (Invitrogen). Reverse transcriptase–PCR (RT–PCR) was performed from 1 µg of total RNA to detect expression of the hTERT gene by the Access RT-PCR System (Promega). Primers for sense of hTERT were 5'-CGGAAGAGGTAGAGGGAGGAGA-3' and for antisense 5'-CGGAAGAGTGTTGGTCACAGG-3', respectively. Primers for sense of GAPDH were 5'-GAAGGTTAAGGTCCAGATGTC-3' and for antisense 5'-CCAGTGTCATGGATGACC-3', respectively. Amplification conditions were as follows: 45°C for 45 minutes, 95°C for 2 minutes, then (95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds for 30 cycles), and 72°C for 5 minutes. Products of RT–PCR were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

**Western Blot Analysis of P16 and P21**

The cells were washed twice in prechilled PBS, lysed in buffer (1% Nonidet-P40, Tris at 50 mmol/L pH 7.5, NaCl at 150 mmol/L, phenylmethylsulfonyl fluoride [PMSF] at 1 mmol/L, and aprotinin, leupeptin, and pepstatin at 0.02 mg/mL) on ice for 5 minutes, and centrifuged at 10,000 g for 20 minutes at 4°C. The lysates were centrifuged at 12,000 g for 20 minutes at 4°C. The pellets were dispersed immediately in 100 µL of CHAPs lysis buffer solution consisting of 1.5 M aminocaproic acid, 50 mM Tris (pH of 7.0) prior to being added to 20 µL of 10% dodecyl maltoside, chilled on ice for 5 minutes and centrifuged at 20,000 g for 30 minutes at 4°C. Protein concentration of supernatant was determined using the method previously described and was adjusted to 200 mg/mL and pH 7.4. Mitochondrial activities were determined according to Van Remmen and Richardson (16). Mean values were obtained from five independent experiments.

**Statistical Assay**

All data were expressed as means ± standard deviation (SD). Differences among groups were analyzed using a one-way analysis of variance (ANOVA), followed by Tukey’s test. A probability value of \( p < .05 \) was considered statistically significant.

**RESULTS**

**Effects of Ginsenoside Rg1 on Cell Viability**

The cell viability was assessed by MTT reduction assay. As illustrated in Figure 2, ginsenoside Rg1 (1.25–80 µM) had no significant effects on cell survival in WI-38 cells <30 PDs. These data indicated that ginsenoside Rg1 at the range of 1.25–80 µM was safe to WI-38 cell viability.
Effects of Ginsenoside Rg1 on Cytotoxicity Measured by Release of LDH

Cell death or cytotoxicity is evaluated classically by the quantification of plasma membrane damage. As illustrated in Figure 3, the amount of LDH in the culture supernatant released from the cells treated by 80 μM Rg1 was lower than that in the supernatant of the control group (p < .05). At 4 or 5 days, the amount of LDH in the supernatant from the cells treated with 5, 10, or 40 μM Rg1 was lower than that in the supernatant of the control group (p < .05). These data indicated that 5, 10, or 40 μM ginsenoside Rg1 has protective effects on WI-38 cells.

Effect of Ginsenoside Rg1 on Cell Morphology

Cell morphology was evaluated by light microscopy. As illustrated in Figure 4, the WI-38 cells in 30 PDs without any treatment were mostly fusiform or in irregular triangle shapes and stretched out two to three protrusions in the cytoplasm. The nucleolus was clear in the orbicular-ovate nucleus (Figure 4A). There were no obvious differences in cell morphology between the control group (38 PDs) and the young group except for the 38 PD cells having a bigger volume and being a little more granular (Figure 4B). The cells treated with t-BHP alone displayed morphological senescent characteristics such as irregular shape, big cell volume, flat body, and granular abundance, as well as a decrease in the ratio of nucleus to cytoplasm (Figure 4C). Compared with the t-BHP alone group, the cells pretreated with ginsenoside Rg1 had more regular shapes, smaller volumes, and less granular cytoplasm (Figure 4D and F), especially at a dose of 10 μM (Figure 4E). This finding suggested that ginsenoside Rg1 has a strong effect in maintaining the nonsenescent phenotype.

Effects of Ginsenoside Rg1 on Cell Ultramicrostructure

The cell ultramicrostructure was evaluated by electron microscopy. As illustrated in Figure 5, the cells in the young group appeared fusiform or had irregular nuclei, clear nucleoli, abundant rough endoplasmic reticulum, rarely secondary lysosomes (including lipofuscin), and normal mitochondria (Figure 5A). Compared with those in the young group, cells in control group had no obvious differences except for the presence of a small amount of swelling mitochondria and clustering secondary lysosomes in some cells (Figure 5B). On the contrary, the cells in the...
t-BHP alone group showed abundant secondary lysosomes, mitochondrial swelling, and the disappearance of mitochondrial cristae (Figure 5C). The cells in the Rg1 pretreatment group at a dose of 5 μM had no obvious differences when compared with those in the t-BHP alone group (Figure 5D). However, the cells in the Rg1 pretreatment group at 10 μM appeared to have fewer secondary lysosomes in cytoplasm, and no more swelling mitochondria (Figure 5E). The integrity of mitochondria in the 10 μM Rg1 pretreatment group was similar to that in the control and young groups. In addition, when compared with the t-BHP alone group, the cells pretreated with Rg1 at 20 μM had fewer secondary lysosomes, but no change in swelling mitochondria and lipid droplet formation (Figure 5F).

**Effects of Ginsenoside Rg1 on SA-β-gal Staining**

As illustrated in Figure 6 and summarized in Table 1, the cells with positive SA-β-gal staining appeared light green in cytoplasm and red in nucleus. The percentages of SA-β-gal-positive cells in all experimental groups were higher than those in the young group (p < .01). However, Rg1 pretreatment significantly reduced the number of SA-β-gal-positive cells when compared with the t-BHP alone group (p < .01), and with the most-pronounced effect when the dose of Rg1 was 10 μM.

**Effects of Ginsenoside Rg1 on Cell Cycling**

The cell cycle analysis in this study was performed by quantification of the percentage of cells in the G1 phase. As...
summarized in Table 1, the percentage of cells in the t-BHP alone group in G1 phase was significantly higher than that in the young and the control groups ($p < .05$). However, the cells in G1 phase in all groups of Rg1 pretreatment were significantly reduced when compared with the t-BHP alone group ($p < .05$), and had the most pronounced effect at a dose of 10 μM. A higher percentage of S phase cells was observed in the Rg1 pretreatment group than in the t-BHP alone group (data not shown).

Effects of Ginsenoside Rg1 on Telomere Length

The telomere length of WI-38 cells shortens with culture PDs. The telomere length in the cells of the t-BHP alone group was 3789 ± 659 bp, which was significantly shorter than that of cells in the young group (5929 ± 1253 bp) and control group (4953 ± 807 bp). However, Rg1 pretreatment significantly attenuated telomere length shortening when compared to the t-BHP alone group ($p < .05$). In addition, telomere length was significantly longer in the Rg1-pretreated...
group (5261 ± 1298) at a dose of 10 μM than at other doses (p < .05). Results are shown in Figure 7A and B.

**Effects of Ginsenoside Rg1 on Telomerase Activity and hTERT Gene Expression**

To investigate whether ginsenoside Rg1 was involved in the activation of telomerase in WI-38 cells, activity of telomerase in WI-38 cells was determined by the TRAP ELISA method. The results indicated that telomerase activity in WI-38 cells was negative (data not shown). We also determined (with RT–PCR) the levels of hTERT messenger RNA (mRNA) in all WI-38 cells pretreated with Rg1. The hTERT mRNA was readily detectable in HepG2 cells and SGC-7901 cells, but hardly detectable in all WI-38 cells (Figure 8).

**Effects of Ginsenoside Rg1 on P16 and P21 Protein Levels**

The levels of P16 and P21 protein were increased along with senescence of WI-38 cells when compared with the young and control groups (p < .01). However, Rg1 pretreatment significantly decreased P16 and P21 protein levels compared with the t-BHP alone group (p < .01) (Figure 9A and B).

**Effects of Ginsenoside Rg1 on ATP Level**

ATP was determined by an ATP Bioluminescence Assay Kit and was expressed as mean fluorescence intensity (MFI). As illustrated in Figure 10, the MFI of WI-38 cells was decreasing along with cell senescence. The MFI in the t-BHP alone group was 3550.80 ± 525.29, which was significantly lower than that in the young group (9555.56 ± 286.00) and control group (6160.20 ± 667.11). However, Rg1 pretreatment significantly increased intracellular ATP levels, with a pronounced effect at a dose of 10 μM (6585.6 ± 771.42) when compared with the t-BHP alone group (p < .05).

**Effects of Ginsenoside Rg1 on Mitochondrial Respiratory Chain Complex I–IV Activity**

As summarized in Table 2, there were no significant differences in mitochondrial respiratory chain complex I, II, and III activities in cells among all groups pretreated with ginsenoside Rg1. Respiratory chain complex IV activity in the control group was higher than that in the young group,
but it decreased significantly in the t-BHP alone group. However, Rg1 pretreatment significantly increased complex IV activity in comparison with the t-BHP alone group with a most pronounced effect at a dose of 10 μM (p < .01).

DISCUSSION

The present study has confirmed that t-BHP, an analog of hydroperoxide, can induce premature senescence in human diploid fibroblast WI-38 cells, consistent with another report (13). More importantly, for the first time our experiments demonstrated the anti-aging effects of ginsenoside Rg1 and revealed the potential underlying molecular mechanisms in terms of cell cycle regulating and mitochondrial functioning.

MTT assay confirmed that ginsenoside Rg1 (1.25–80 μM) has no effects on viability of WI-38 cells before 30 PDs. Meanwhile, the LDH cytotoxicity assay indicated that ginsenoside Rg1 (5, 10, 40, and 80 μM) has protective effects on WI-38 cells. Therefore, in subsequent experiments, we chose ginsenoside Rg1 (5, 10, and 20 μM) in treating WI-38 cells.

One of hallmarks of senescent cells is that they sustain their metabolic activities for a long time, but lose the reactivity to mitogen and the ability of synthesizing DNA, and stop cell cycling in G1 phase without entering S phase (10). It is proposed that cyclin, cyclin dependant kinase (CDK), and cyclin dependant kinase inhibitor (CDI) play important roles in regulating cell cycle progression in senescent fibroblast cells (17–19). P16, located at 9p21, prevents cells from entering the G1 phase through binding CDK4 especially and inhibiting the activity of the cyclinD–CDK4 complex. P16 is a key gene concerned with cell senescence that regulates cell cycle and affects telomere length and cell life span (20). Expression of P16 in senescent cells was obviously higher than that in young cells (21). Consistent with those reports, our results also showed that P16 increased in t-BHP alone treated cells, and moreover that P16 decreased in ginsenoside Rg1-pretreated cells in a dose-dependant manner.

P21, located at 6p21.2, inhibits phosphorylation of cyclin D–CDK4 and cyclin E–CDK2 complexes, and dephosphorylates Rb protein and inhibits E2F release and DNA synthesis, resulting in cell cycle arrest in the G1 phase and induces cell senescence (11,22,23). PCNA is a cofactor of DNA polymerase δ/ε. P21 binds to PCNA to form the p21–PCNA complex. This complex prevents polymerase from extending, causing polymerase to deviate from templates, and thus directly inhibits DNA replication (24). In the present study, the expression of P21 increased along with the ongoing senescence in WI-38 cells, and it decreased in a dose-dependant manner in WI-38 cells pretreated with ginsenoside Rg1. These results indicate that ginsenoside Rg1 may regulate the levels of P16 and P21 to

Table 1. Effects of Ginsenoside Rg1 on SA-β-gal Staining and Cell Cycle

<table>
<thead>
<tr>
<th>Group</th>
<th>Cells in the G1 Phase (%)</th>
<th>SA-β-gal-Positive Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young group</td>
<td>67.61 ± 1.77</td>
<td>3.00 ± 1.00</td>
</tr>
<tr>
<td>Control group</td>
<td>68.25 ± 2.86</td>
<td>37.33 ± 2.08*</td>
</tr>
<tr>
<td>t-BHP alone group</td>
<td>86.32 ± 3.23</td>
<td>90.00 ± 2.65*</td>
</tr>
<tr>
<td>Rg1 pretreatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μM</td>
<td>79.91 ± 1.84</td>
<td>45.33 ± 1.15*</td>
</tr>
<tr>
<td>10 μM</td>
<td>69.56 ± 2.38</td>
<td>35.33 ± 1.53*</td>
</tr>
<tr>
<td>20 μM</td>
<td>74.61 ± 1.65</td>
<td>53.00 ± 4.00*</td>
</tr>
</tbody>
</table>

Notes: *p < .01, †p < .05 vs young group, n = 5.
†p < .05, ‡p < .01 vs t-BHP alone group, n = 5.
SA-β-gal = senescence-associated beta-galactosidase; t-BHP = tert-butyl hydroperoxide.

Figure 7. Effects of ginsenoside Rg1 on telomere length. Telomere length was measured with Southern blotting of terminal restriction fragments derived from digestion of genomic DNA using frequent-cut restriction enzymes. Lane 1: Young group; lane 2: Control group; lane 3: Tert-butyl hydroperoxide (t-BHP) alone group; lane 4: Rg1 pretreatment group (5 μM); lane 5: Rg1 pretreatment group (10 μM); lane 6: Rg1 pretreatment group (20 μM); lane M: Marker. Values are mean ± standard deviation from three independent experiments. *p < .05 compared with young group; †p < .05 compared with t-BHP alone group.
promote cells in the G1 phase into the S phase, resulting in the delay of WI-38 cell premature senescence. We also noticed the phenomenon of the ability of Rg1 at a high concentration (20 \( \mu \)M) to reduce the levels of p21 and p16 expression below the levels in young and control groups (Figure 9), which can not be explained by the present study. We are unclear about the mechanisms. Further work will be pursued to discover how and why ginsenoside Rg1 reduces the level of p21 and p16.

A telomere is a specialized structure at the ends of eukaryotic chromosome consisting of two 20-Kb short repeat sequences. It plays an important role in chromosome positioning, stabilizing, replicating, and regulating cell growth and life span (25–27). It is also correlated with cell apoptosis, transformation, and immortalization (28). von Zglinicki and colleagues (29) have reported that WI-38 cells may stop splitting when the length of telomere shortens to close to 4 Kb. In the present study, the length of telomere in WI-38 cells treated with t-BHP four times was 3.8 ± 0.7 Kb, and it was reduced by 2100 bp from those of cells without treatment, consistent with the report by Dumont and colleagues (30). However, the shortening of telomere was significantly diminished when WI-38 cells were pretreated with ginsenoside Rg1. We hypothesize that ginsenoside Rg1

Figure 8. Effects of ginsenoside Rg1 on hTERT messenger RNA (mRNA) expression. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis was performed for detecting mRNA expression for hTERT complementary DNA in all groups. HepG2 cells and SGC-7901 cells were used as positive controls. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Lane A: Young group; lane B: Control group; lane C: Tert-butyl hydroperoxide (t-BHP) alone group; lane D: Rg1 pretreatment group (5 \( \mu \)M); lane E: Rg1 pretreatment group (10 \( \mu \)M); lane F: Rg1 pretreatment group (20 \( \mu \)M); lane G: SGC-7901 cells; lane H: HepG2 cells.

Figure 9. Effects of ginsenoside Rg1 on P21 and P16 expression. Expression of P21 and P16 protein was assayed by Western blotting. \( \beta \)-actin was used as a control of protein loading. Lane 1: Young group; lane 2: Control group; lane 3: Tert-butyl hydroperoxide (t-BHP) alone group; lane 4: Rg1 pretreatment group (5 \( \mu \)M); lane 5: Rg1 pretreatment group (10 \( \mu \)M); lane 6: Rg1 pretreatment group (20 \( \mu \)M). The bands of Western blot were scanned, and the ratios of optical density of specific bands and \( \beta \)-actin were illustrated. Values represent the mean ± standard deviation with five independent experiments. * \( p < .01 \), * \( p < .001 \), compared with t-BHP alone group.
may activate telomerase in WI-38 cells to compensate for t-HBP-induced shortening of telomere in WI-38. hTERT is the human telomerase reverse transcription gene, which plays an important role in activation of telomerase (31,32). hTERT mRNA expression temporarily parallels telomerase activity during cellular differentiation and neoplastic transformation (33,34). However, the present data demonstrate that hTERT mRNA in WI-38 cells is undetectable. Additionally, pretreatment with ginsenoside Rg1 cannot increase hTERT mRNA level in WI-38 cells. In the present study, we did not detect the telomerase activity and hTERT expression in WI-38 cells. We inferred that the antisenescent effect of Rg1 is not due to telomerase activation, but may be due to other pathways.

Mitochondria play an important role in the process of senescence. It has been reported that the mitochondrial function is decaying continuously and the energy homeostasis is disturbed in the process of aging (35–37). In the present study, the data from ultramicroscopy demonstrated that the mitochondria were swollen and the cristae had disappeared in the WI-38 cells treated with t-BHP alone. The amount of swelling mitochondria was significantly decreased in WI-38 cells pretreated with Rg1. We also evaluated the mitochondrial function in terms of ATP production and mitochondrial complex I–IV activities in WI-38 cells. As mentioned above, ginsenoside Rg1 pretreatment significantly increased ATP production in WI-38 cells when compared with t-BHP treatment alone. Furthermore,

<table>
<thead>
<tr>
<th>Group</th>
<th>Respiratory Chain Complex I</th>
<th>Respiratory Chain Complex II</th>
<th>Respiratory Chain Complex III</th>
<th>Respiratory Chain Complex IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young group</td>
<td>5.623 ± 0.9685</td>
<td>0.4415 ± 0.0901</td>
<td>0.2296 ± 0.0786</td>
<td>0.3832 ± 0.0938</td>
</tr>
<tr>
<td>Control group</td>
<td>5.2217 ± 1.3218</td>
<td>0.4813 ± 0.0816</td>
<td>0.1847 ± 0.0883</td>
<td>0.5032 ± 0.1218*</td>
</tr>
<tr>
<td>t-BHP alone group</td>
<td>3.5325 ± 1.4090</td>
<td>0.4544 ± 0.0287</td>
<td>0.1650 ± 0.0873</td>
<td>0.2653 ± 0.0210</td>
</tr>
<tr>
<td>Rg1 pretreatment groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μM</td>
<td>3.7798 ± 1.7274</td>
<td>0.4689 ± 0.0878</td>
<td>0.1764 ± 0.0752</td>
<td>0.4079 ± 0.0581*</td>
</tr>
<tr>
<td>10 μM</td>
<td>4.7021 ± 1.5232</td>
<td>0.5050 ± 0.0906</td>
<td>0.2158 ± 0.0874</td>
<td>0.4816 ± 0.0300*</td>
</tr>
<tr>
<td>20 μM</td>
<td>4.3837 ± 1.1329</td>
<td>0.4511 ± 0.0551</td>
<td>0.1759 ± 0.0675</td>
<td>0.4416 ± 0.0464*</td>
</tr>
</tbody>
</table>

Notes: Data are shown as μmol/mg/min.
*< .05 vs Young group, n = 5.
1*< .05 vs t-BHP alone group, n = 5.
2*p < .01 vs Young group and Control group, n = 5.
3t-BHP = tert-butyl hydroperoxide.
t-BHP treatment alone resulted in a decrease in the activity of complex IV, which was consistent with the report by Benzi and colleagues (38). Rg1 pretreatment markedly suppressed the decrease of the activity of complex IV. The level of respiratory chain complex IV activity in controls being higher than that in young cells was likely caused by an mechanism of compensation (p < .05), similar to another report (37). These results further confirm that oxidants may damage the mitochondrial function and render cell senescence. In short, the present data indicate that Rg1 may stabilize the membrane system of mitochondria, increase mitochondrial complex IV activity and ATP level, and protect mitochondria from oxidative damage.

The present data indicated that pretreatment with ginsenoside Rg1 at a dose of 10 μM has a more pronounced effect than that at doses of 5 and 20 μM with regard to ATP production, complex activity, morphological changes, SA-β-gal staining, and cell cycle analysis in WI-38 cells. Meanwhile, the data also showed that ginsenoside Rg1 treatment at 20 μM had no protective effects on LDH cytotoxicity and mitochondrial ultrastructure morphology changes in WI-38 cells induced by t-BHP. Although the exact mechanism is not clear, it appears that Rg1 treatment at 20 μM starts to lose the protection to mitochondrial functioning, which is likely to play a pivotal role in Rg1’s protective functions. Therefore, it needs to be noted that the optimal dose for anti-aging effects of ginsenoside Rg1 in vitro should be lower than 20 μM.

Summary

We demonstrated in the present study that ginsenoside Rg1 delays cell premature senescence induced by chronic oxidative stress probably through reducing the expression of P16 and P21, pushing the entry of WI-38 cells from the G1 phase into the S phase and also attenuating telomere shortening. Furthermore, the present study indicates that ginsenoside Rg1 effectively protects mitochondria from oxidation damage by increasing complex IV activity and ATP production. Delaying fatigue of the mitochondrial function is an effective antisenescence pathway, and ginsenoside seems to be a potential natural product with such a capability.

Ginsenoside Rg1 has previously been found to act as a scavenger of free radicals. Our previous study also indicated that Rg1 has protective effects on dopamine or 1-methyl-4-phenylpyridinium/1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)–induced apoptosis in dopaminergic neurons probably due to a decrease in the level of reactive oxygen species, increase of glutathione, and decreasing the vitality of superoxide dismutase (39,40). Further work will be pursued to discover how ROS production and antioxidant activity are regulated to account for the anti-aging property of Rg1.

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