Rb1 Protects Endothelial Cells from Hydrogen Peroxide-Induced Cell Senescence by Modulating Redox Status

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Senescence of endothelial cells has been proposed to play an important role in endothelial dysfunction and atherogenesis. In the present study we aimed to investigate whether ginsenoside Rb1, a major constituent of ginseng, protects endothelial cells from H₂O₂-induced endothelial senescence. While H₂O₂ induced premature senescence-like phenotype of human umbilical vein endothelial cells (HUVECs), as judged by increased senescence-associated β -galactosidase (SA- β -gal) activity, enlarged, flattened cell morphology and sustained growth arrest, our results demonstrated that Rb1 protected endothelial cells from oxidative stress induced senescence. Mechanistically, we found that Rb1 could markedly increase intracellular superoxide dismutase (Cu/Zn SOD/SOD1) activity and decrease the malondialdehyde (MDA) level in H₂O₂-treated HUVECs, and suppress the generation of intracellular reactive oxygen species (ROS). Consistent with these findings, Rb1 could effectively restore the protein expression of Cu/Zn SOD, which was down-regulated in H₂O₂ treated cells. Taken together, our data demonstrate that Rb1 exhibits antioxidant effects and antagonizes H₂O₂-induced cellular senescence.

Key words senescence; human umbilical vein endothelial cell; reactive oxygen species; antioxidant

Aging is considered as a high risk factor for cardiovascular diseases, especially for atherosclerosis.^{1,2)} Vascular aging is believed to be associated with pro-oxidant and pro-inflammatory phenotypic and functional changes, which promote the progression of cardiovascular diseases.³⁾

At the cellular level, senescence is a state of permanent growth arrest in which cells are alive and metabolically active for months, but are unresponsive to mitogenic stimuli.⁴⁾ Free radical-dependent cellular damage theory was developed since 1956 and remains the theory basis of aging.⁵⁾ Accumulating evidence supports the link between oxidative stress and aging by showing that oxidative stress induces cellular senescence.^{6–9)} Hydrogen peroxide is widely used to achieve oxidative stress-induced premature senescence within a short time period.

Ginsenoside Rb1 is one of 40 bioactive extracts from ginseng which has been used as tonic remedy in traditional Chinese Medicine for two thousand years.^{10,11)} Previous studies have demonstrated that increased generation of reactive oxygen species (ROS) in the injured vascular can be reversed by Rb1 treatment which provides a protective effect on the vascular injury.¹²⁾ Therefore, we postulate that ginsenoside Rb1 may play a role in protecting vascular cells from senescence through modulating the balance of the antioxidant related enzyme and pro-oxidant related enzyme. To evaluate the protective effects of Rb1, we employed human umbilical vascular endothelial cells (HUVECs) as a model and determined the superoxide dismutase (SOD) and malondialdehyde (MDA) levels in the cells treated by Rb1.

SOD, along with other enzymatic and non-enzymatic antioxidants, plays a pivotal role in preventing cellular damage caused by ROS. It exerts its role through reducing the superoxide radical to form hydrogen peroxide and oxygen. There are three forms of SODs which are designated as Cu/Zn-SOD (SOD1), mitochondrial Mn-SOD (SOD2), and extracellular SOD (SOD3).¹³⁾ SOD1, the most extensively studied

antioxidant enzyme, is constitutively expressed in all mammalian cells and is localized in the cell cytosol and the nucleus.¹⁴⁾ MDA is a major and stable end product formed during the peroxidation of lipids and is regarded as marker of lipid peroxidation in tissues.¹⁵⁾

Here, we reported that Rb1 antagonizes H₂O₂-induced HUVECs senescence through upregulating endogenous antioxidants SOD and decreasing vascular lipid peroxidation.

MATERIALS AND METHODS

Chemicals and Reagents Rb1 was purchased from the Division of Chinese Materia Medica and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Public Health (Beijing, China). Hydrogen peroxide (H₂O₂) and dimethylsulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, U.S.A.). The reagent kits for the measurement of the levels of MDA and SOD were purchased from Nanjing Institute of Jiancheng Bioengineering (Nanjing, China). 2',7'-Dichlorofluorescein diacetate (H₂DCFDA) and TRIzol reagent were obtained from Invitrogen (Carlsbad, CA, U.S.A.). PrimeScriptTM RT reagent Kit and SYBR Premix Ex TaqTM Kit were obtained from TaKaRa (TaKaRa, Dalian, China). All other reagents used were of analytical grade.

Cell Culture Primary HUVECs were prepared from newborn human umbilical cords within 6 h by 0.1% collagenase (Gibco) perfusion as described previously.¹⁶⁾ HUVECs were plated into a 0.2% gelatin-coated cell culture flask and grown in Medium 199 (M199) (Gibco) supplemented with 20% Fetal Bovine Serum (FBS) (Gibco), 20% Serum-Free Medium (SFM) (Gibco), 2 mmol/l glutamine, 60 μ g/ml Endothelial Cell Growth Supplement (ECGS) (BD) and 5 U/ml heparin, kept in a humid incubator at 37 °C in 5% CO₂. Subcultures were obtained by treating cells with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Gibco).

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Experiments were conducted on cells with a passage number between passages 2 and 3. The cell type was determined by “cobblestone” cell morphology and by flow cytometric analysis with a monoclonal anti-human CD31 antibody (BD, U.S.A.). More than 99% of the cells were positive for CD31. The protocol was approved by the Research Committee of the Third Affiliated Hospital of Sun Yat-sen University and conformed to the principles outlined in the Declaration of Helsinki. The parturients enrolled in the current protocol were negative for Human Immunodeficiency Viruses (HIV), Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) and gave written informed consent to providing their umbilical cord.

Cell Treatment The exponentially growing HUVECs were plated at a cell density of 1×10^5 /well in 6-well plate and cultured overnight at 37 °C. The next day, the medium was changed with M199 supplemented with 2% FBS for at least 8 h to starve the cells. Then the cells were exposed to various concentrations of H_2O_2 to induce premature senescence. After 1 h, the medium was replaced with normal medium. Different concentration of Rb1 (10, 20, 40 μ mol/l) was added in the media 30 min before the induction of senescence by addition of H_2O_2 . Finally the cells were harvested in indicated time for Western blot analysis and real-time polymerase chain reaction (PCR).

Senescence-Associated β -Galactosidase (SA- β -Gal) Assay To determine the number of senescent cells, senescence-associated β -galactosidase staining was performed as described previously.¹⁷⁾ Briefly, cells were washed twice in phosphate buffered saline (PBS) and fixated in 2% formaldehyde and 0.2% glutaraldehyde for 5 min at room temperature, washed twice and incubated for 18 h at 37 °C (no CO_2) with fresh SA- β -gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-D-galactoside, 40 mmol/l citric acid/sodium phosphate dibasic at pH 6.0, 150 mmol/l NaCl, 2 mmol/l $MgCl_2$, 5 mmol/l $K_3[Fe(CN)_6]$ and 5 mmol/l $K_4[Fe(CN)_6] \cdot 3H_2O$). Light microscopic pictures were taken on an inverted microscope with a $\times 100$ magnification. Senescent cells were identified as blue-stained cells, and a total of 400 contiguous cells were counted to determine the percentage of SA- β -gal positive cells.

Measurement of Intracellular SOD Activity and MDA Level The MDA level and SOD activity were determined using commercially available kits following the manufacturer's instructions. The results were expressed as units per milligram of protein. The assay for SOD activity was based on its ability to inhibit the oxidation of hydroxylamine by O_2^- produced from the xanthine-xanthine oxidase system. One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50%. MDA was measured at a wavelength of 532 nm by reacting with thiobarbituric acid (TBA) to form a stable chromophoric product. The result was expressed by thiobarbituric acid reactive substances (TBARS).

Real-Time Polymerase Chain Reaction (PCR) Total RNA in HUVECs was isolated using Trizol (Invitrogen, U.S.A.) following the manufacturer's instructions. cDNA was generated by reverse transcript from mRNA using the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The SYBR Premix Ex Taq™ Kit (TaKaRa, Dalian, China) was then used for real-

time PCR reaction. β -Actin, a housekeeping gene, was used as internal control to account for variations in mRNA loading. The β -actin (GenBank No. NM_001101.3) primer sequences were: forward 5'-AGC GGG AAA TCG TGC GTG AC-3' and reverse 5'-TCC ATG CCC AGG AAG GAA GG-3'. The SOD1 (GenBank No. NM_000454.4) primer sequences were: forward 5'-CAA AGA TGG TGT GGC CGA TG-3' and reverse 5'-TTT CCA CCT TTG CCC AAG TCA-3'. Real-time PCR was performed in an ABI 7000 PCR detection system. The thermal cycle condition used for reverse transcription was as follows: 15 min at 37 °C and 5 s at 85 °C. The condition used for real-time PCR was as follows: 30 s at 95 °C, 40 repeats of 5 s at 95 °C and 31 s at 60 °C, and 1 repeat of 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C.

Western Blotting The cells were lysed with whole cell lysis buffer (KeyGen BioTechnology, Nanjing, China) according to the manufacturer's instructions. Cells lysates were quantified by BCA methods according to the manufacturer's instructions (Sangon BioTechnology, Shanghai, China), then resuspended in the sample buffer and boiled for 5 min at 100 °C. The same amount of protein was electrophoresed on 4–12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride membranes (Millipore, U.S.A.). Membranes were blocked using 5% nonfat dried milk in TBST for 1 h at room temperature with gentle shaking, then probed with a rabbit antihuman SOD1 monoclonal antibody (EPITOMICS, U.S.A.) at the dilution of 1 : 100000 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G secondary antibody at the dilution of 1 : 10000. Membranes were developed using the enhanced chemiluminescence detection method (Millipore, U.S.A.), and the signals were quantified using Quantity One Software (Bio-Rad, U.S.A.).

Flow Cytometric Evaluation of ROS Intracellular ROS generation was monitored by flow cytometry using peroxide-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (H_2DCFDA , Invitrogen). H_2DCFDA is converted by intracellular esterases to DCFH, which is oxidized into the highly fluorescent dichlorofluorescein (DCF) in the presence of a proper oxidant. The cells were loaded with 1 μ mol/l H_2DCFDA in serum-free medium at 37 °C for 30 min and then washed twice with PBS. Next, the cells were monitored with a flow cytometer (BD, U.S.A.) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. ROS was determined by comparing the changes in fluorescence intensity to those of the control.

Statistical Analysis Data were expressed as mean $\pm$ the standard error (S.E.) of the mean (S.E.M.) and analyzed using a LSD analysis. A p value < 0.05 was considered statistically significant.

RESULTS

Rb1 Antagonizes H_2O_2 -Induced HUVECs Senescence 40–100 μ mol/l H_2O_2 could effectively induce HUVECs senescence (data not shown), and we chose the concentration of 60 μ mol/l H_2O_2 to set up the senescent model. As shown in Fig. 1, based on the SA- β -gal staining in which positive senescent cells were stained blue, 60 μ mol/l H_2O_2 induced

Fig. 1. Rb1 Antagonizes H₂O₂-Induced Senescence in HUVECs

HUVECs were pretreated with Rb1 for 30 min before the administration of 60 μM H₂O₂. The medium was replaced with normal medium 1 h later and cultured for another 24 h. Then the cells were stained for assessment of senescence cells. The cells stained blue were positive senescent cells. Shown were representative pictures from three independent experiments on 2–3 passaged cells after separate primary cultures with similar results.

Fig. 2. Rb1 Reduces TBARS (MDA) Level in HUVECs

HUVECs were pretreated with different doses of Rb1 for 30 min before the administration of 60 μM H₂O₂. The medium was replaced with normal medium 1 h later and cultured for another 24 h. TBARS level was measured and expressed as units per milligram of protein. Values were means ± S.E.M. from three independent experiments on 2–3 passaged cells after separate primary cultures. **p* < 0.05, ***p* < 0.01 versus 60 μM H₂O₂ alone.

the senescence of HUVECs and 20 μmol/l Rb1 antagonized the senescence induced by H₂O₂ effectively.

Effects of Rb1 on MDA Content and SOD Activity

The effects of Rb1 on lipid peroxidation and endogenous antioxidant preservation were measured. Treatment with H₂O₂ caused significant increase in intracellular TBARS level (*p* < 0.01 vs. untreated control), while pre-incubation with Rb1 (20, 40 μmol/l) markedly attenuated the increase (*p* < 0.05) (Fig. 2). As the control, Rb1 alone did not significantly change the TBARS level. Similarly, the SOD activity was comparable between the non-treated control and Rb1 alone treated group. Compared to control group, treatment with H₂O₂ decreased the activity of SOD to 50.4 ± 6.9%. However, pre-incubation with Rb1 (10, 20, 40 μmol/l) significantly increased SOD activity compared with H₂O₂ alone treated group (*p* < 0.01) (Fig. 3).

Rb1 Promotes the Expression of SOD1 in HUVECs

To evaluate whether Rb1 plays a role in the reversal of senescence through modulating the redox status, we examined the expression of SOD1 at mRNA level in HUVECs treated by Rb1. Real-time PCR analysis showed that at the time point of 24 h, SOD1 mRNA in the 60 μmol/l H₂O₂ treated group decreased by 1.99 fold, compared with untreated group (*p* < 0.01). However, SOD1 mRNA increased by 1.54, 1.77, 1.75 fold in 10, 20, 40 μmol/l Rb1 pretreated group respectively, compared with H₂O₂ alone treated group (*p* < 0.01)

Fig. 3. Rb1 Upregulates SOD Activity in HUVECs

HUVECs were pretreated with different doses of Rb1 for 30 min before the administration of 60 μM H₂O₂. The medium was replaced with normal medium 1 h later and cultured for another 24 h. SOD activity was measured and expressed as units per milligram of protein. Values were means ± S.E.M. from three independent experiments on 2–3 passaged cells after separate primary cultures. ***p* < 0.01 versus 60 μM H₂O₂ alone.

Fig. 4. Rb1 Promotes SOD1 mRNA Expression in HUVECs

HUVECs were pretreated with 10, 20, 40 μM Rb1 for 30 min before the administration of 60 μM H₂O₂. The medium was replaced with normal medium 1 h later and cultured for another 24 h. The mRNA level of SOD1 was measured by real-time PCR and normalized against β-actin, shown as 2^{−[Ct(Cu/ZnSOD)−Ct(β-actin)]}. Values were means ± S.E.M. from three independent experiments on 2–3 passaged cells after separate primary cultures. ***p* < 0.01 versus 60 μM H₂O₂ alone.

(Fig. 4). These data suggest that Rb1 upregulates SOD1 mRNA expression in HUVECs. To provide further evidence for this, we examined SOD1 protein level in HUVECs. Western blotting results demonstrated that the level of SOD1 protein reduced in H₂O₂ treated group compared to untreated group after 24, 48 and 72 h incubation (Fig. 5). However, Rb1 reversed the decreased expression of SOD1 protein in HUVECs treated with H₂O₂ in a dose-dependent manner (Fig. 5). As the control, Rb1 alone did not significantly change the expression of SOD1 at either mRNA or protein level. Taken together, these data demonstrate that Rb1 upregulates the expression of SOD1 in H₂O₂ treated HUVECs.

Intracellular Radical Scavenging Ability of Rb1 To confirm whether ROS production is affected as results of the changes of SOD1 activity and expression in the non-treated, H₂O₂-treated, and Rb1-pretreated groups, we measured intracellular ROS level using the ROS-sensitive probe H₂DCF-DA by a flow cytometry. In accordance with the previous results of SOD activity and expression, Rb1 alone did not change the ROS generation. However, compared to the non-treated control group, 60 μmol/l H₂O₂ significantly increased

Fig. 5. Rb1 Promotes SOD1 Protein Expression in HUVECs. The Reversal Effect of Rb1 on Cu/Zn SOD Protein Expression in H₂O₂-Induced Senescent HUVECs in Indicated Time Points (A, 24 h; B, 48 h; C, 72 h) after Treatment

HUVECs were pretreated with indicated doses of Rb1 for 30 min before the administration of 60 μM H₂O₂. The medium was replaced with normal medium 1 h later and cultured for 24 h (A), 48 h (B) or 72 h (C). Then the cells were collected and lysated. The protein level of SOD1 was evaluated by Western blot with SOD1 antibody. GAPDH served as loading control. Shown were representative blots of three independent experiments on 2–3 passaged cells after separate primary cultures with similar results and expressed as means ± S.E.M. **p* < 0.05, ***p* < 0.01 versus 60 μM H₂O₂ alone.

DCF fluorescence (Fig. 6D) whereas pre-treatment with 20 μmol/l Rb1 markedly inhibited the production of ROS induced by H₂O₂ (Fig. 6E).

DISCUSSION

Consistent with previous studies showing the protective effects of Rb1 on vascular cells against injury *in vitro*,^{12,18)} we report here that Rb1 reversed HUVECs senescence induced by H₂O₂ through modulating endogenous antioxidants and pro-oxidants products. Although intensive research has been focused on Rb1 regarding to its protective effect on different cell type damage including endothelial^{12,18)} and neural cell,¹⁹⁾ few studies have investigated the potential anti-senescence or anti-aging role of Rb1,²⁰⁾ especially for endothelial cells. Our findings were based on oxidative stress induced HUVECs senescence by using H₂O₂. In this system, after sublethal dose of H₂O₂ treatment, HUVECs exhibited typical senescent phenotypes which were confirmed by the morphology marker of SA-β-gal. Our data show that Rb1 reversed the senescent phenotypic changes. This is the first report to show a role of Rb1 in protecting cellular senescence in HUVECs.

It is known that some of ROS products are extremely unstable and metabolized rapidly *via* a delicate oxidative-antioxidative system.²¹⁾ Thus it is not accurate to illustrate the extent of cell senescence or injury by a transient indicator. Since the free-radical theory of aging proposes that degenerative senescence is largely dependent on the cumulative effect of ROS,²¹⁾ we examined ROS products within senescent cells by using a stable and metabolic product or long lived metabolic enzyme. Consistence with increased ROS production in the senescent HUVECs, the intracellular peroxidized end product MDA elevated in HUVECs, while the antioxidants SOD1 was defective in the senescent cells. These results support the free-radical theory of aging,^{5,21)} and prove that the model of H₂O₂-induced HUVECs senescence used in our experiments is suitable to characterize the anti-senescence function of Rb1. It is well known that SODs act as enzymes that catalyze the superoxide anion radical into hydrogen peroxide.¹³⁾ SOD is recognized as antioxidant defense enzyme and its abnormal expression is implicated in life span.²²⁾ Decreased level and activity of SOD after the administration of exogenous H₂O₂ is also reported in other H₂O₂-injury models.^{23,24)} It is supposed to be a negative feedback that endogenous SOD is reduced by an elevated level of exogenous H₂O₂. In addition, the decreased level and activity of SOD could result in the accumulation of superoxide anion radical in the cells which may be partly responsible for H₂O₂-induced injury and the increased level of lipid peroxidation showed in our study.

Interestingly, Rb1 was found to improve endothelial proliferation and decrease the production of superoxide anion in Hcy induced SVEC4–10 cells injury.¹²⁾ Here we described a specific type of cell injury, senescence, which is induced by H₂O₂ treatment. Furthermore, our data demonstrate that Rb1 exhibited antagonistic effects against H₂O₂-induced senescence. It is important to note that the change of SOD activity and MDA level was not perfectly coincident in HUVECs pretreated with different concentrations of Rb1, suggesting that the reduction of lipid peroxidation induced by Rb1 may also depend on other ROS scavenging system, not limited to

Fig. 6. Rb1 Antagonizes H₂O₂-Induced ROS Production in HUVECs

The fluorescence intensity was measured with a flow cytometer. (A) negative control (without H₂DCF-DA); (B) blank control (without H₂O₂); (C) 20 μ mol/l Rb1 alone; (D) 60 μ mol/l H₂O₂ treatment for 60 min; (E) 60 μ mol/l H₂O₂ treatment for 60 min plus pre-incubation with Rb1 for 30 min; (F) Effects of Rb1 on the production of intracellular ROS. "Marker" indicated a line separating regions on single parameter histograms that are treated statistically separate. "All" indicated all the area within (and on) the boundary. "M1" indicated positive area which is set according to the blank control. The values were expressed as percentages of the fluorescence intensity. ** $p < 0.01$ versus 60 μ M H₂O₂ alone. The shown graph was representative of three independent experiments on 2–3 passaged cells after separate primary cultures with similar results.

SOD1. Indeed, Rb1 has been shown to upregulate the expression of catalase, an enzyme that catalyzes the conversion of hydrogen peroxide to water and oxygen²⁵⁾ and glutathione peroxidase (GSH-Px), an enzyme that catalyzes the breakdown of hydrogen peroxide and organic hydroperoxides,²⁶⁾ affect heme oxygenase (HO-1) expression and suppress p47phox expression.^{27–29)} Furthermore, a recent study showed that Rb1 protected liver from ischemia-reperfusion injury through upregulating NO concentration and SOD1 activity and reducing MDA level.³⁰⁾

Although Rb1 works as a direct scavenger or increases the level of antioxidants to indirectly scavenge the ROS,^{27–30)} our results in this study provide evidence that Rb1 is more likely to increase the antioxidant and act as an indirect ROS scavenger: (1) Rb1 mitigates the oxidative stress *via* reducing

the stable metabolic molecular MDA level and decreasing the intracellular ROS level in H₂O₂-induced senescent HUVECs. (2) Enhanced SOD activity and expression are observed after Rb1 pre-treatment in H₂O₂-induced senescent HUVECs. The present study is performed on the well characterized model of H₂O₂-induced senescence which is based on arrested cell growth caused by imbalanced ROS scavenging. In our study, the correlation of the activity and expression of SOD and the production of ROS is well coincident in the senescent model. By analyzing the changes of SOD, ROS (or stable metabolic substance, MDA), and senescent phenotype, it is clearly indicated that Rb1 protects HUVECs from H₂O₂-induced senescence through regulating the redox status.

In conclusion, our data demonstrate that ginsenoside Rb1

modulates redox status such as upregulating SOD1 expression, scavenging ROS, and decreasing the peroxidation to prevent the cellular senescence in HUVECs.

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