Abstract. Endothelial dysfunction and senescence are closely associated with cardiovascular diseases including atherosclerosis and hypertension. Ginsenoside Rb1 (Rb1), the major active constituent of ginseng, has been investigated intensively because of its anti-obesity and anti-inflammatory effects. In a previous study, hydrogen peroxide (H2O2) was applied to induce human umbilical vein endothelial cell (HUVEC) aging. It was demonstrated that Sirtuin-1 (SIRT1) was activated by Rb1 to protect HUVECs from H2O2-induced senescence. However, the mechanisms are not fully understood. The present study examined the role of AMP-activated protein kinase (AMPK), an energy sensor of cellular metabolism, in the signaling pathway of SIRT1 during H2O2-stimulated HUVEC aging. It was identified that Rb1 restored the H2O2-induced reduction of SIRT1 expression, which was consistent with our previous study, together with the activation of AMPK phosphorylation. Using compound C, an AMPK inhibitor, the role of AMPK in the protective effect of Rb1 against H2O2-induced HUVEC senescence was examined. It was identified that the induction of phosphorylated AMPK by Rb1 markedly increased endothelial nitric oxide synthase expression and nitric oxide production, and suppressed PAI-1 expression, which were abrogated in HUVECs pretreated with compound C. Further experiments demonstrated that nicotinamide, a SIRT1 inhibitor, downregulated the phosphorylation of AMPK and reduced the protective effects of Rb1 against H2O2-induced endothelial aging. Taken together, these results provide new insights into the possible molecular mechanisms by which Rb1 protects against H2O2-induced HUVEC senescence via the SIRT1/AMPK pathway.

Introduction

Endothelial cell aging is a major risk factor for cardiovascular disease (CVD) development (1-3). Numerous studies have shown that hydrogen peroxide (H2O2)-induced vascular endothelial dysfunction is partially responsible for the development of aging (4-6). H2O2-induced endothelial dysfunction reflects a loss of the balance between pro- and anti-oxidant, pro- and anti-inflammatory, and pro- and anti-thrombotic signals, all of which contribute to increased release of proinflammatory cytokines, including plasminogen activator inhibitor-1 (PAI-1), and a decline in antioxidants including endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) (7-11). Thus, the prevention of endothelial dysfunction is essential to treat endothelial aging and CVD.

Ginseng, the root of Panax ginseng CA Meyer, is one of the most popular herbs in traditional Asian medicine. A growing body of evidence suggests that ginsenoside Rb1 (Rb1), a major component of ginsenosides extracted from ginseng, has various biological activities including antioxidative stress relief, anti-obesity and anti-inflammation (12-14). One of our previous studies also suggested that Rb1 at the concentration of 10-40 µM inhibits free fatty acid-induced inflammation partially through the blockade of nuclear factor (NF)-κB phosphorylation in 3T3-L1 adipocytes (15). Additionally, another study by our group demonstrated that Rb1 at the concentration of 20 µM attenuates human umbilical vein endothelial cell (HUVEC) senescence by improving the redox status (16). However, the range of effective concentrations and further modulated mechanisms of Rb1 in the endothelium are not fully elucidated.

AMP-activated protein kinase (AMPK) is a heterotrimeric member of an evolutionarily conserved protein kinase family that is sensitive to changes in oxygen tension and ATP consumption (17). Accumulating evidence has revealed that AMPK participates in the regulation of lipid metabolism, inflammation and angiogenesis in various animal models and cell types (18-22). Ido et al (23) reported that AMPK protects endothelial cells from the adverse effects of sustained
hyperglycemia. Nagata et al (22) demonstrated that endothelial AMPK signaling plays a critical role in blood vessel recruitment to tissues responding to ischemic stress. In addition, studies have shown that AMPK exerts its beneficial role through multiple signaling pathways, including activation of eNOS and production of NO (24,25). However, it is still unclear whether endothelial senescence, eNOS activation and NO synthesis in HUVECs in response to Rb1 are related to the activation of AMPK.

Complementing our previous studies, the present study was undertaken to investigate the protective effects of Rb1 against H₂O₂-induced HUVEC dysfunction mediated by AMPK and the underlying mechanisms.

Materials and methods

Cell culture and treatments. Primary HUVECs were isolated from different six neonatal umbilical cords as previously described (26). Briefly, HUVECs at passages 2-4 were maintained in M199 medium (Invitrogen, Thermo Fisher Scientific, Inc.) supplemented with 20% fetal bovine serum (HyClone, GE Healthcare Life Sciences) and 60 µg/ml endothelial cell growth supplement (BD Biosciences) at 37°C in a 5% CO₂ incubator and then exposed to the desired treatment in triplicate. The isolation procedure for HUVECs was approved by the Research Committee at the Third Affiliated Hospital of Sun Yat-sen University (approval nos. 2010-2-48 and 2018-02-057-01). The donors were negative for human immunodeficiency virus and hepatitis B virus and provided written informed consent to donate the umbilical cords.

To induce senescence, isolated HUVECs were treated with 60 µM H₂O₂ (Sigma-Aldrich, Merck KGaA) for 1 h and then cultured for another 24 h at 37°C. Rb1 (16071307, Chengdu Pufei De Biotech Co., Ltd.) used in the present study was extracted from Panax ginseng by HPLC according to the manufacturer’s instructions and the purity of Rb1 used in the present study was 98.85%. To evaluate the effect of Rb1 on senescence, the cells were pretreated with 10 or 20 µM Rb1 for 30 min prior to H₂O₂ treatment. To measure the effect of the Sirt1 inhibitor nicotinamide (NAM; Sigma-Aldrich, Merck KGaA) and AMPK inhibitor compound C (Sigma-Aldrich, Merck KGaA), the cells were incubated with 20 mM NAM (27) or 8 µM compound C for 30 min as reported previously (18,28,29) and then treated with or without Rb1 at concentrations of 10 or 20 µM for 30 min before H₂O₂ treatment. At the end of each experiment, the cultured supernatants and monolayered cells were harvested for analyses.

Measurement of NO production. NO production was evaluated by measuring the accumulation of nitrites. The Griess method (30) was used to detect NO using a NO assay kit (Beyotime Institute of Biotechnology), following the manufacturer’s instructions. Briefly, after the cells were cultured and treated as described above, 50 µl culture supernatant was incubated with 50 µl Griess reagent I and 50 µl Griess reagent II in a 96-well microplate at room temperature for 30 min. The optical density was measured with a Victor microplate reader (PerkinElmer, Inc.) at 540 nm. Nitrite concentrations in the medium were calculated according to a standard curve.

Senescence-associated β-galactosidase activity assay. Senescence was detected using a senescence-associated β-galactosidase (SA-β-gal)-positive approach according to a published protocol (31). After HUVECs were washed twice with prechilled PBS, the cells were fixed with 2% formaldehyde plus 1% glutaraldehyde for 5-10 min at room temperature. The cells were then washed twice with prechilled PBS for 3 min and stained with a staining solution [40 mmol/l citric acid/sodium phosphate buffer, 5 mmol/l potassium ferrocyanide (K₃[Fe(CN)₆]·3H₂O), 5 mmol/l potassium ferricyanide (K₃[Fe(CN)₅]·2H₂O), 150 mmol/l sodium chloride, 2 mmol/l magnesium chloride and 1 mg/ml X-gal] overnight at 37°C without CO₂. Senescent cells were identified as blue-stained cells under a TS100 inverted microscope (Nikon Corporation) at x100 magnification. At least 400 cells were examined to determine the percentage of SA-β-gal-positive cells in each group.

NAD⁺/NADH assay. The cellular NADP⁺/NADPH ratio was determined using a NAD⁺/NADH Quantification Kit (Beyotime Institute of Biotechnology), according to the manufacturer’s instructions. In brief, HUVECs (1x10⁵/well) were seeded in six-well plates and exposed to the experimental conditions. To measure the NAD⁺/NADPH ratio, the cells were harvested, lysed with 200 µl NAD⁺/NADPH buffer and gently pipetted to promote cell lysis. Then, 50-100 µl of the samples were collected and incubated for 30 min at 60°C. Then, 20 µl of the reacted samples were added to a 96-well plate and analyzed at 450 nm as the reference wavelength in the Victor microplate reader. The NAD⁺/NADPH ratio was calculated according to a standard curve and normalized to the cell number, following the manufacturer’s protocol.

Measurement of intracellular ATP. Determination of intracellular ATP in the indicated groups of HUVECs was performed by a bioluminescence assay (ATP Assay kit; Beyotime Institute of Biotechnology), according to the manufacturer’s instructions. Briefly, the cells were washed twice with prechilled PBS and then lysed in lysis buffer on ice. Then, the samples were harvested and centrifuged at 12,000 x g for 5 min at 4°C and the supernatants were collected for subsequent analysis. After the reaction solutions containing luciferase and luciferin were added and background luminescence was measured, the ATP standard solution and samples were added and luminescence was measured. Then, the background luminescence was subtracted and the standard curve was constructed. The ATP concentrations were calculated from the standard curve and normalized to total protein content.

Western blot analysis. HUVECs were grown in six-well plates. After the indicated treatments, the cells were washed twice with prechilled PBS and then lysed in radioimmunoprecipitation buffer with a protease inhibitor cocktail, phenylmethylsulfonyl fluoride and sodium orthovanadate (Santa Cruz Biotechnology, Inc.). The protein concentration was measured by the Bradford method. Proteins (30 µg) in 30 µl reducing sample buffer were boiled for 5 min at 100°C and then resolved by SDS-PAGE (8 or 12% gels) for 2 h at 100 V. The proteins were transferred onto a polyvinylidene difluoride membrane for 90 min at 100 V. After transfer, the membrane was incubated in 25 ml blocking
The results demonstrated that 60 µM H2O2 increased Pai-1 expression were then measured after the same treatment of HuVecs. In line with our previous studies (16,36,37), it was reported to induce cell senescence and oxidative stress in HuVecs and other different cell lines (32-35), which plays a central role in cell dysfunction. H2O2 was used at a concentration of 60 µM in the present study based on our previous studies (16,36,37) in which 60 µM H2O2 was reported to induce cell senescence and oxidative stress sufficiently. Senescence-associated β-galactosidase (SA-β-gal) staining was used to determine the degree of cell senescence. Aging cells stained blue indicated the senescent phenotype staining was used to determine the degree of cell senescence. Membranes were developed using the enhanced chemiluminescence detection method (EMD Millipore). The signals were quantified using Quantity One software (version 4.6.9; Bio-Rad Laboratories, Inc.).

**Results**

**Effects of H2O2 and Rb1 treatment in HUVECs.** A number of studies have reported that H2O2 is a major inducer of cell senescence and oxidative stress in HUVECs and other different cell lines (32-35), which plays a central role in cell dysfunction. H2O2 was used at a concentration of 60 µM in the present study based on our previous studies (16,36,37) in which 60 µM H2O2 was reported to induce cell senescence and oxidative stress sufficiently. Senescence-associated β-galactosidase (SA-β-gal) staining was used to determine the degree of cell senescence. Aging cells stained blue indicated the senescent phenotype of HUVECs. In line with our previous studies (16,36,37), it was demonstrated that 60 µM H2O2 increased the SA-β-gal* cell number (blue staining). In addition, the H2O2 treated group had more cells with a clear outline, enlarged cell body and difficulty in adhering to the bottom of the culture plate, all characteristics of stressed senescent cells (38,39). By contrast, pretreatment with Rb1 decreased the SA-β-gal* cell number and attenuated cell senescence induced by H2O2 in a dose-dependent manner (Fig. 1A and B). Pai-1 and eNOS expression were then measured after the same treatment. The results demonstrated that 60 µM H2O2 increased Pai-1 expression (Fig. 1C) and decreased eNOS expression (Fig. 1D) in HUVECs, which were restored by Rb1 in a dose-dependent manner.

*Effects of H2O2 and Rb1 on SIRT1 expression, AMPK phosphorylation and the NAD+/NADH ratio.* A number of studies have shown that SIRT1 exhibits anti-inflammatory (40-42) and antioxidant effects (43-45) in the endothelium. The effect of Rbl on SIRT1 and phosphorylation of the catalytic subunit of AMPK (Thr172) in the presence or absence of H2O2, was examined. The results demonstrated that 60 µM H2O2 inhibited SIRT1 expression (Fig. 2A and B) as well as phosphorylation of AMPK (Fig. 2A and C) and that treatment with Rbl at 10 and 20 µM restored Sirt-1 expression (Fig. 2A and B) and AMPK phosphorylation (Fig. 2A and C) in a dose-dependent manner.

The intracellular NAD+/NADH ratio in the presence or absence of Rbl was further examined and it was identified that treatment with 60 µM H2O2 reduced the NAD+/NADH ratio, which was restored with Rbl pretreatment (Fig. 2D). The data confirmed that H2O2-induced decrease in the NAD+/NADH ratio was associated with reduced SIRT1 expression and it was demonstrated for the first time, to the best of the authors' knowledge, that treatment with Rbl at 10 and 20 µM Rbl prevented the reductions in SIRT1 and the NAD+/NADH ratio, and inhibited phosphorylated AMPK, in HUVECs exposed to H2O2.

*Involvement of AMPK in Rbl-mediated inhibition of the H2O2-induced oxidative response.* Next, it was determined whether AMPK was involved in the inhibitory effects of Rbl on the H2O2-induced oxidative response. HUVECs were treated with 10 or 20 µM Rbl for 24 h in the presence or absence of compound. C, a specific inhibitor of AMPK. As shown in Fig. 3A, compound C clearly downregulated the phosphorylation of AMPK at 6, 8 and 10 µM in a dose-dependent manner. According to the results, 8 µM compound C was chosen to inhibit the phosphorylation of AMPK in the following experiment. Pretreatment of HUVECs with compound C significantly abolished the inhibitory effects of Rbl on H2O2-induced Pai-1 expression (Fig. 3C). Rbl also significantly restored eNOS expression (Fig. 3D) and NO production (Fig. 3E) and this beneficial effect was markedly reversed by pretreatment of HUVECs with compound C (Fig. 3). The results suggest that AMPK activation is essential for the inhibitory effect of Rbl on H2O2-induced senescence and the oxidative response in HUVECs.

*Involvement of SIRT1 in Rbl-mediated inhibition of the H2O2-induced oxidative response.* To further understand the role of SIRT1 in the AMPK pathway, it was investigated whether inactivation of SIRT1 by nicotinamide affected H2O2-induced cell injury. As Guo et al (27) reported that 20 mM NAM decreases SIRT1 gene expression significantly, HUVECs were treated with H2O2 in the presence or absence of 20 mM NAM, followed by observation of the effects on H2O2-induced senescence and the expression of antioxidant genes. As shown in Fig. 4, NAM reduced the phosphorylation of AMPK (Fig. 4A and B), indicating that AMPK is the downstream protein modulated by SIRT1.

As AMPK is one of the most important proteins in modulating the cellular energy metabolism and ATP is a major downstream product of mitochondrial energy coordinators, the effects of NAM with or without Rbl on the ATP level were next investigated. Rbl protected HUVECs from H2O2-induced Pai-1 expression (Fig. 4C) and rescued the downregulation of
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Discussion

Our previous studies (16,36,37) demonstrated that endothelial senescence and dysfunction in HUVEC were characterized by enhanced H$_2$O$_2$-induced β-galactosidase activity and impaired anti-oxidant capacity. This was associated with an increased protein expression of PAI-1, decreased NO production and eNOS expression in HUVEC. The present study identified that Ginsenoside Rb1 protects against cell senescence and dysfunction through activation of Sirt1. However, the involved molecular mechanisms have yet to be elucidated. In line with our previous studies (16,36,37), the present study demonstrated that Rb1 significantly increased NO content, eNOS expression and Sirt1 expression in H$_2$O$_2$-induced HuVec senescence. In addition, it was identified for the first time, to the best of the authors’ knowledge, that these changes were associated with upregulated phosphorylation of AMPK. Compound C, an inhibitor of AMPK, was used to clarify the relation of AMPK and Sirt1. It was identified that treatment with compound C markedly attenuated the protective effects of Rb1 in HuVecs. Furthermore, the role of Sirt1/AMPK pathway was further confirmed by administration of nicotinamide, which enhanced H$_2$O$_2$-induced HuVEC senescence. The present study also provided novel mechanisms for Rb1 protected H$_2$O$_2$-induced HUVEC senescence involving SIRT1/AMPK pathway.

A number of studies have reported that Rb1, a main constituent of the root of *P. ginseng*, has various pharmacological...
effects that include mitigating endothelial inflammation and obesity in vitro and in vivo. Zhou et al (46) reported that Rb1 protects HUVECs from TNF-α-induced oxidative stress and inflammation by inhibiting NF-κB, JNK and p38 signaling pathways. Yuan et al (47) reported that Rb1 protects pulmonary microvascular endothelial cells from lipopolysaccharide-induced expression of inflammatory cytokines. Our previous studies demonstrated that treatment with Rb1 at the concentration of 20 µM significantly reduces H₂O₂-induced cell senescence and inhibits PAI-1 expression (37) partly through stimulation of SirT1 (36). The present study demonstrated that Rb1 at the concentration of 10-20 µM played a beneficial role on H₂O₂-induced endothelial dysfunction, which complements our previous research. However, the molecular mechanisms need further elucidation.

Previous studies have suggested a critical role of AMPK in stabilizing endothelial functions through regulation of eNOS signaling to suppress inflammation and oxidative stress (48-51). In addition, there are some studies on the effects of AMPK activators on the expression of eNOS, suggesting that AMPK acts as an eNOS activator (52-54). Thus far, there has been a lack of studies from the present authors concerning the role of AMPK in the regulation of H₂O₂-induced oxidative stress and inflammatory responses by Rb1 in HUVECs. However, the present study explored the use of compound C to inhibit AMPK and determined whether Rb1 upregulated eNOS expression and NO production through AMPK. As a result, it was identified that AMPK activity was enhanced by Rb1. The present study not only confirmed that the expression and activity of eNOS suppressed by H₂O₂ stimulation was enhanced by Rb1 treatment, which is in accordance with previous findings, but also identified that the beneficial effects of Rb1 were abolished by compound C, indicating a novel role of AMPK in regulating Rb1-dependent eNOS activity and NO synthesis. However, the mechanism of Rb1 in AMPK-dependent eNOS activation and NO production in HUVECs requires further investigation.

SIRT1 is highly sensitive to cellular redox states and considered to have a cardioprotective effect (55,56) that maintains endothelial functions by counteracting the effects of reactive oxygen species as a NAD⁺-dependent class III histone deacetylase (57). SIRT1 has been reported to regulate the functions of several important transcription factors with anti-inflammatory effects (58-60). It antagonizes H₂O₂-induced premature senescence via negative modulation of p53 by deacetylation of Lys-373, Lys-382 and Lys-320 in the human endothelium (61). Ota et al (62,63) demonstrated that the overexpression of

Figure 2. Rb1 restores the H₂O₂-induced reduction of SIRT1, promotes phosphorylation of AMPK and increases the NAD⁺/NADH ratio. The HUVECs were pretreated with 10-20 µM Rb1, treated with or without 60 µM H₂O₂ for 1 h and then cultured for 24 h. (A) The levels of SIRT1, phosphorylated AMPK, AMPK and GAPDH were determined by western blot analysis. (B) Quantitative analysis of SIRT1 expression ratio and SIRT1 expression levels. (C) Quantitative analysis of phosphorylated AMPK ratio and phosphorylated AMPK expression levels. (D) NAD⁺/NADH ratio measured using a colorimetric assay. *P<0.01 vs. control group; **P<0.05 vs. 60 µM H₂O₂ group. Rb1, ginsenoside Rb1; H₂O₂, hydrogen peroxide; SIRT1, Siruin-1; AMPK, AMP-activated protein kinase; HUVEC, human umbilical vein endothelial cell; p, phosphorylated.
SIRT1 in the endothelium reverses H$_2$O$_2$-induced premature cellular senescence through an eNOS-dependent signaling pathway. The present study also confirmed that inhibition of SIRT1 by nicotinamide caused a stress-induced increase in PAI-1 with a concomitant decrease in eNOS expression and NO production, which are consistent with Cacicedo et al (64). Furthermore, the present study demonstrated that Rbl induced AMPK phosphorylation, which was abolished by nicotinamide. Previous studies have reported the functional connections between the two master regulators SIRT1 and AMPK: Gao et al (65) reported that activated SIRT1 functionally interacts with AMPKα and upregulates its phosphorylation in aortic endothelial and smooth muscle cells of Klotho knockout mice and other studies have shown that AMPK can also function as a SIRT1 activator by increasing the NAD$^+/$/NADH ratio (66,67). These studies
suggest that AMPK may crosstalk with SIRT1 to modulate downstream targets. The present study demonstrated that inhibition of SIRT1 by nicotinamide functionally suppressed the phosphorylation of AMPK, which in turn abolished the
protective effect against Rb1 on \( \text{H}_2\text{O}_2 \)-induced endothelial dysfunction. However, in vitro experiments are required to confirm the anti-aging and antioxidant effects of Rb1 demonstrated in vitro. Further research is also required to elucidate the complex molecular mechanisms underlying the involvement of AMPK and SIRT1 in anti-aging and antioxidant processes.

In conclusion, Rb1 improved \( \text{H}_2\text{O}_2 \)-induced HUVEC senescence and dysfunction through the SIRT1/AMPK signaling pathway. The results of the present study suggested that the associated mechanisms may be related to decreased PAI-1 expression and upregulated eNOS expression and NO production. The present study provides evidence to support the novel role of AMPK in the beneficial effects of Rb1 on HUVEC senescence, which can be explored further in animal models and clinical studies.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

ZZ, JZ and XQ designed the study, ZZ, MW, CC, DL and LW conducted the research. ZZ and MW analyzed the data and wrote the manuscript. ZZ and MW revised the discussion section of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The isolation procedure for HUVECs was approved by the Research Committee at the Third Affiliated Hospital of Sun Yat-sen University. The donors provided written informed consent to donate the umbilical cords.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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