Ginsenoside Rb1 reduces H_2O_2-induced HUVEC dysfunction by stimulating the sirtuin-1/AMP-activated protein kinase pathway

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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 (H_2O_2) 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 H_2O_2-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 H_2O_2-stimulated HUVEC aging. It was identified that Rb1 restored the H_2O_2-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 H_2O_2-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 H_2O_2-induced endothelial aging. Taken together, these results provide new insights into the possible molecular mechanisms by which Rb1 protects against H_2O_2-induced HUVEC senescence via the SIRT1/AMPK pathway.

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Introduction

Endothelial cell aging is a major risk factor for cardiovascular disease (CVD) development (1-3). Numerous studies have shown that hydrogen peroxide (H_2O_2)-induced vascular endothelial dysfunction is partially responsible for the development of aging (4-6). H_2O_2-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 H_2O_2-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% CO_2 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 H_2O_2 (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 H_2O_2 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 H_2O_2 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 Greiss reagent I and 50 μl Greiss 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 ($K_4[Fe(CN)_6]3H_2O$), 5 mmol/l potassium ferricyanide ($K_3[Fe(CN)_6]$), 150 mmol/l sodium chloride, 2 mmol/l magnesium chloride and 1 mg/ml X-gal] overnight at 37˚C without CO_2. 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 ($1x10^6$/well) were seeded in six-well plates and exposed to the experimental conditions. To measure the $NADP^+$/NADPH ratio, the cells were harvested, lysed with 200 μl NAD^+/NADH 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^+/NADH 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

buffer [1X Tris-buffered saline (TBS) and 0.1% Tween-20 with 5% non-fat dry milk] for 1 h at room temperature. The primary antibodies were rabbit polyclonal anti-eNOS (1:500; Cell Signaling Technology, Inc.), Rabbit polyclonal anti-PAI-1 (1:500; Cell Signaling Technology, Inc.), Rabbit polyclonal anti-AMPK (1:2,000; Cell Signaling Technology, Inc.), rabbit polyclonal anti-phosphorylated AMPK (Thr172; 1:1,000; Cell Signaling Technology, Inc.), rabbit polyclonal anti-SIRT1 (1:1,000; Cell Signaling Technology, Inc.), and rabbit polyclonal anti-GAPDH (control, 1:5,000; Cell Signaling Technology, Inc.). The membrane was incubated with the primary antibody in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4˚C. After washing three times for 10 min each with 15 ml of 10X TBS/0.1% TBST, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.) in 10 ml blocking buffer with gentle agitation for 1 h at room temperature, followed by 3 washes for 10 min each. 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.).

Statistical analysis. Results were obtained from at least three independent experiments (n≥3) if not otherwise stated. Data are expressed as the mean ± standard deviation (n≥3). Statistical significance was calculated by one-way or two-way analysis of variance with Bonferroni's post-hoc test using GraphPad Prism software (version 5.0; GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of H_2O_2 and Rb1 treatment in HUVECs. A number of studies have reported that H_2O_2 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. H_2O_2 was used at a concentration of 60 μM in the present study based on our previous studies (16,36,37) in which 60 μM H_2O_2 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 H_2O_2 increased the SA-β-gal$^+$ cell number (blue staining). In addition, the H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 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 Rb1 on SIRT1 and phosphorylation of the catalytic subunit of AMPK (Thr172) in the presence or absence of H_2O_2 was examined. The results demonstrated that 60 μM H_2O_2 inhibited SIRT1 expression (Fig. 2A and B) as well as phosphorylation of AMPK (Fig. 2A and C) and that treatment with Rb1 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 Rb1 was further examined and it was identified that treatment with 60 μM H_2O_2 reduced the NAD$^+$/NADH ratio, which was restored with Rb1 pretreatment (Fig. 2D). The data confirmed that H_2O_2-induced decrease in the NAD$^+$/NADH ratio were associated with reduced SIRT1 expression and it was demonstrated for the first time, to the best of the authors' knowledge, that treatment with Rb1 at 10 and 20 μM Rb1 prevented the reductions in SIRT1 and the NAD$^+$/NADH ratio, and inhibited phosphorylated AMPK, in HUVECs exposed to H_2O_2.

Involvement of AMPK in Rb1-mediated inhibition of the H_2O_2-induced oxidative response. Next, it was determined whether AMPK was involved in the inhibitory effects of Rb1 on the H_2O_2-induced oxidative response. HUVECs were treated with 10 or 20 μM Rb1 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 Rb1 on H_2O_2-induced PAI-1 expression (Fig. 3C). Rb1 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 Rb1 on H_2O_2-induced senescence and the oxidative response in HUVECs.

Involvement of SIRT1 in Rb1-mediated inhibition of the H_2O_2-induced oxidative response. To further understand the role of SIRT1 in the AMPK pathway, it was investigated whether inactivation of SIRT1 by nicotinamide affected H_2O_2-induced cell injury. As Guo *et al* (27) reported that 20 mM NAM decreases SIRT1 gene expression significantly, HUVECs were treated with H_2O_2 in the presence or absence of 20 mM NAM, followed by observation of the effects on H_2O_2-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 Rb1 on the ATP level were next investigated. Rb1 protected HUVECs from H_2O_2-induced PAI-1 expression (Fig. 4C) and rescued the downregulation of

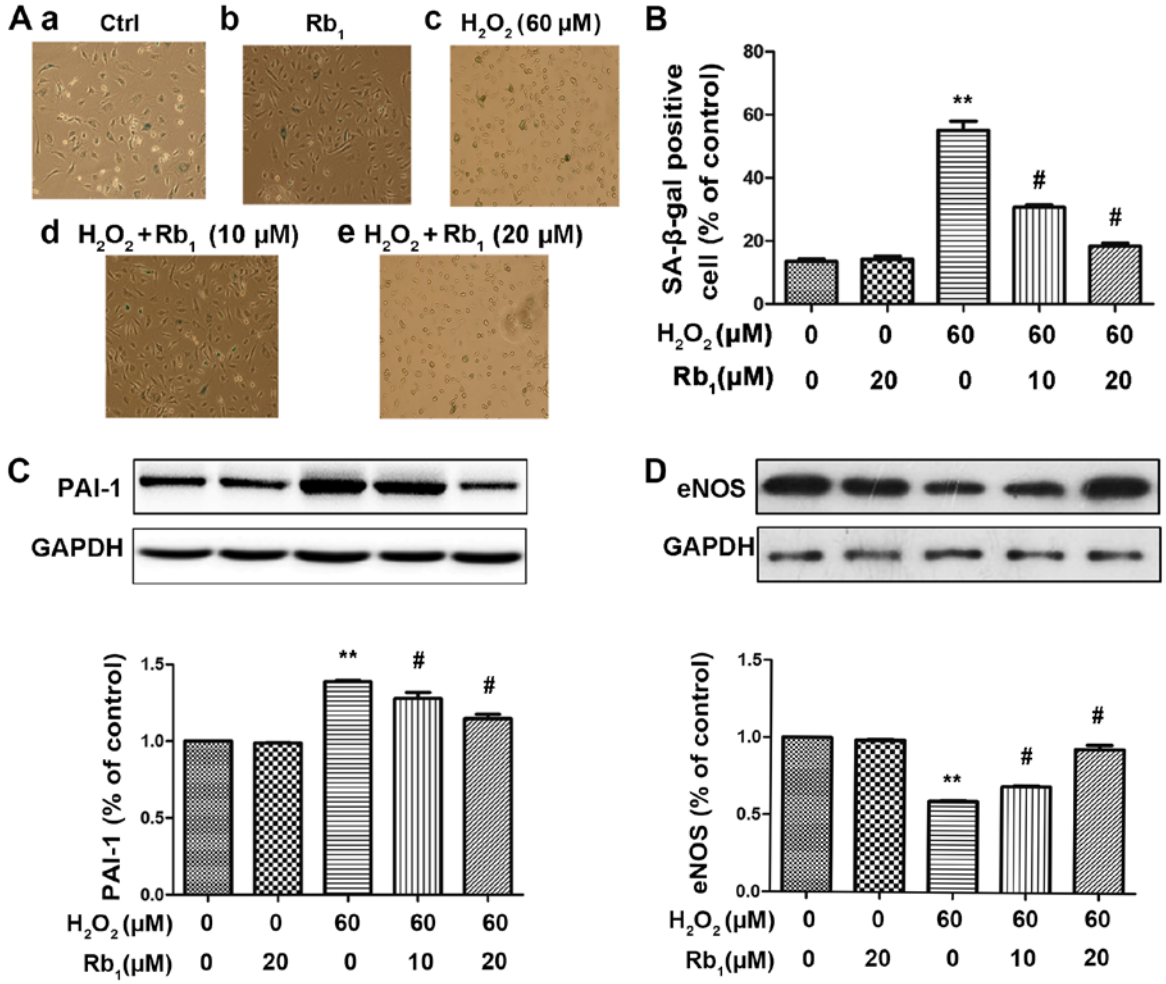

Figure 1. Rb1 inhibits H_2O_2-induced senescence and represses PAI-1 as well as eNOS expression in HUVECs. The HUVECs were pretreated with 10-20 μM Rb1, treated with or without 60 μM H_2O_2 for 1 h and then cultured for 24 h. (A) Representative phase contrast images of SA-β-gal$^+$ cells (blue staining; magnification, x100). (B) Ratio of SA-β-gal-positive cells calculated from 400 cells per group. The protein levels of PAI-1 (C) and eNOS (D) were detected by western blot analysis and semi-quantified by densitometry. Representative blots of three independent experiments are shown. **P<0.01 vs. Ctrl group; $^{\#}$P<0.05 vs. 60 μM H_2O_2 group. Rb1, ginsenoside Rb1; H_2O_2, hydrogen peroxide; PAI-1, plasminogen activator inhibitor-1; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cell; SA-β-gal, senescence-associated β-galactosidase; Ctrl, control.

eNOS expression (Fig. 4D) and NO production (Fig. 4E). As shown in Fig. 4F, treatment with Rb1 increased the ATP level under H_2O_2-induced oxidative stress. However, the promotional effect on the ATP level by Rb1 was not observed in the NAM-treated group (Fig. 4F). The beneficial effects of Rb1 on H_2O_2-induced injuries were reversed by treatment with NAM (Fig. 4). These *in vitro* data confirmed that inhibition of SIRT1 by NAM blocks Rb1 to increase phosphorylation of AMPK, ATP production, eNOS expression and NO production, which collectively indicate that Rb1 plays a protective role against H_2O_2-induced injury of HUVEC via the SIRT1/AMPK pathway.

Discussion

Our previous studies (16,36,37) demonstrated that endothelial senescence and dysfunction in HUVEC were characterized by enhanced H_2O_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_2O_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_2O_2-induced senescence and dysfunction and decreased the protective effect of Rb1 on HUVEC. Collectively, the results provide novel mechanisms for Rb1 protected H_2O_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

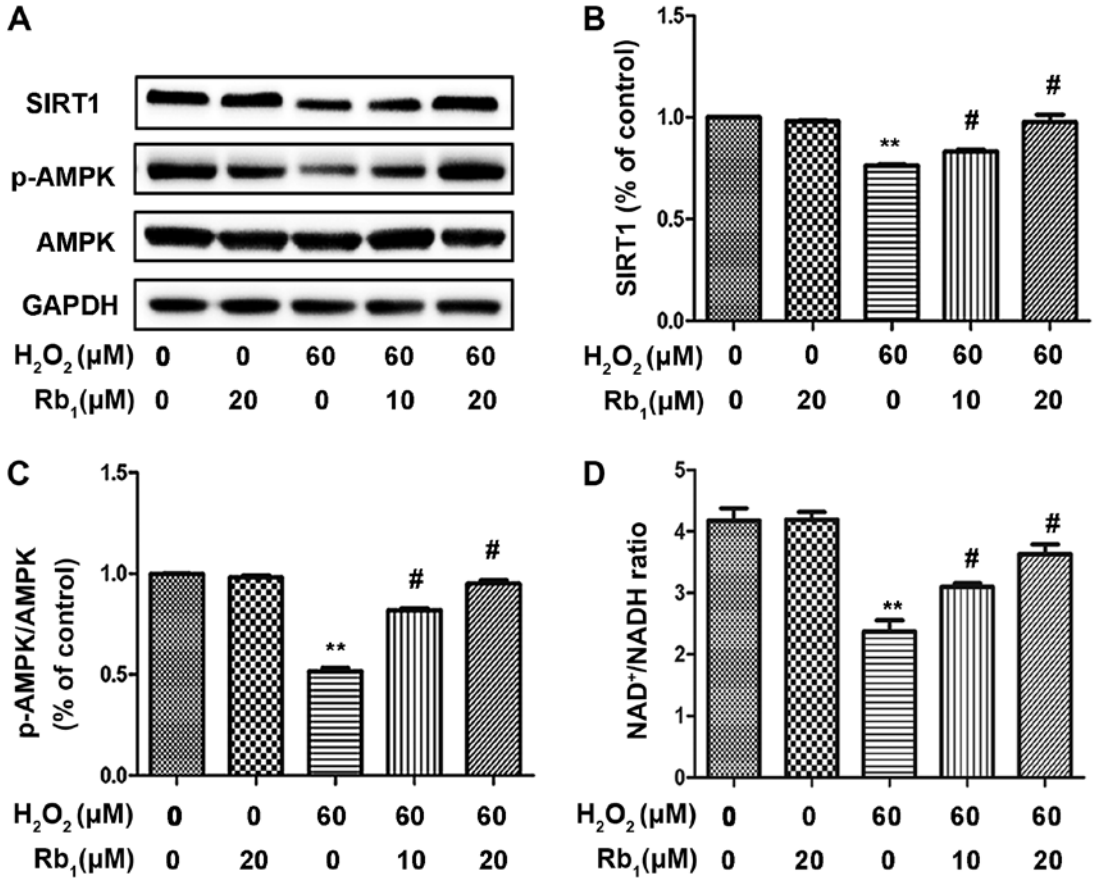

Figure 2. Rb1 restores the H_2O_2-induced reduction of SIRT1, promotes phosphorylation of AMPK and increases the NAD^+/NADH ratio. The HUVECs were pretreated with 10-20 μM Rb1, treated with or without 60 μM H_2O_2 for 1 h and then cultured for 24 h. (A) The levels of SIRT1, phosphorylated AMPK, AMPK 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 H_2O_2 group. Rb1, ginsenoside Rb1; H_2O_2, hydrogen peroxide; SIRT1, Sirtuin-1; AMPK, AMP-activated protein kinase; HUVEC, human umbilical vein endothelial cell; p, phosphorylated.

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 H_2O_2-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 H_2O_2-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 H_2O_2-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 H_2O_2 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 H_2O_2-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

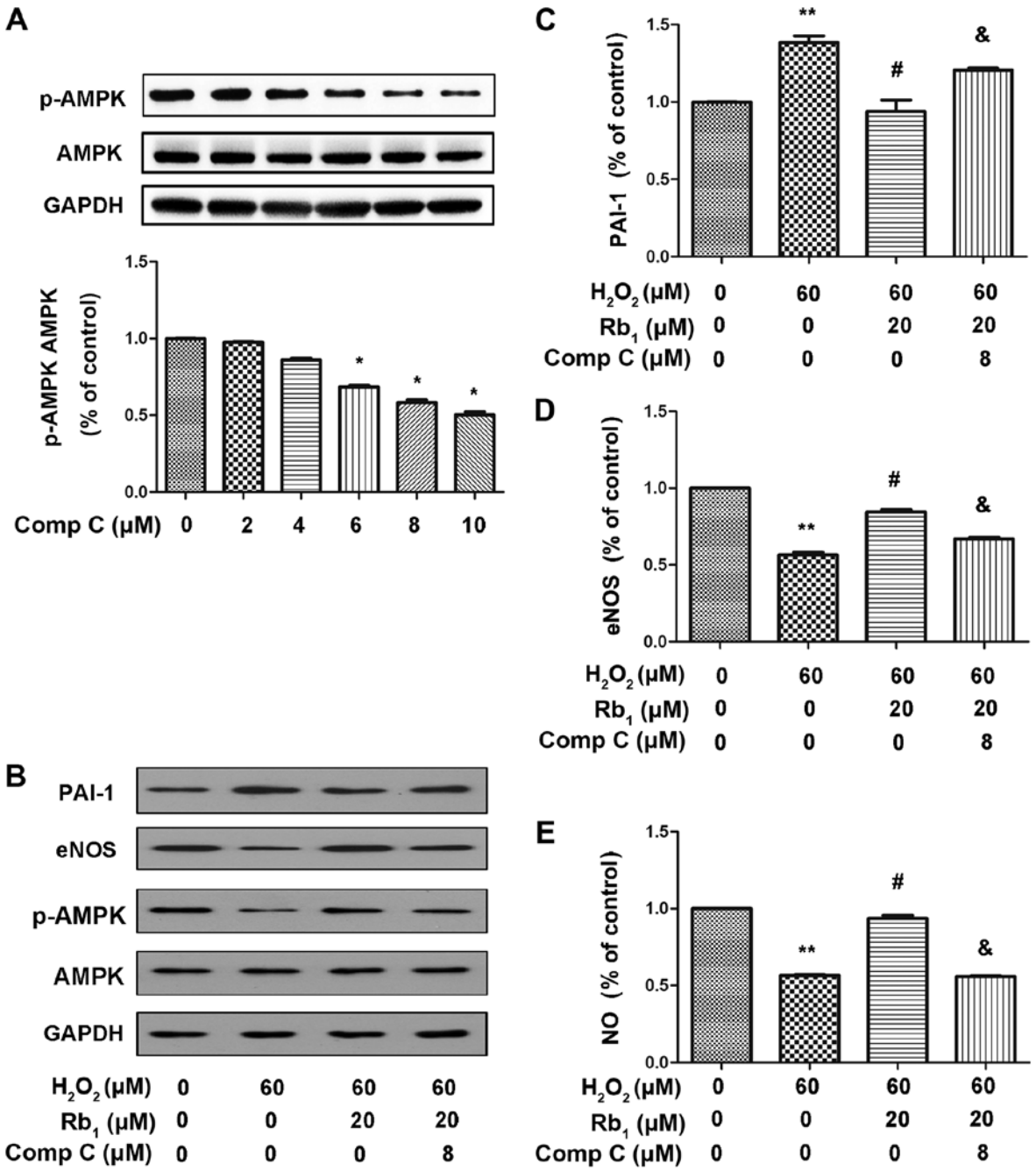

Figure 3. Compound C suppresses the beneficial effects of Rb1 on H_2O_2-induced antioxidant and anti-inflammatory functions. The HUVECs were pretreated with the indicated concentrations of compound C for 30 min and then pretreated with 20 μM Rb1 for 30 min, followed by treatment with or without 60 μM H_2O_2 for 1 h and then culture for 24 h. (A) Phosphorylation of AMPK in HUVECs pretreated with compound C (0, 2, 4, 6, 8 and 10 μM) for 30 min detected by western blot analysis and semi-quantified by densitometry. Total AMPK and GAPDH were also determined. (B) The protein levels of PAI-1, eNOS, phosphorylated AMPK, AMPK and GAPDH were determined by western blot. (C) Quantitative analysis of PAI-1 expression ratio and PAI-1 expression levels. (D) Quantitative analysis of eNOS expression ratio and eNOS expression levels. (E) NO production measured by the Griess reaction and expressed as the percentage of the control. Results represent three independent experiments. *P<0.05 and **P<0.01 vs. control group; $^{\#}$P<0.05 vs. 60 μM H_2O_2 group; $^{\&}$P<0.05 vs. 60 μM H_2O_2+Rb1 group. Rb1, ginsenoside Rb1; H_2O_2, hydrogen peroxide; HUVEC, human umbilical vein endothelial cell; AMPK, AMP-activated protein kinase; PAI-1, plasminogen activator inhibitor-1; eNOS, endothelial nitric oxide synthase; p, phosphorylated; NO, nitric oxide; Comp C, compound C.

SIRT1 in the endothelium reverses H_2O_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 Rb1 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

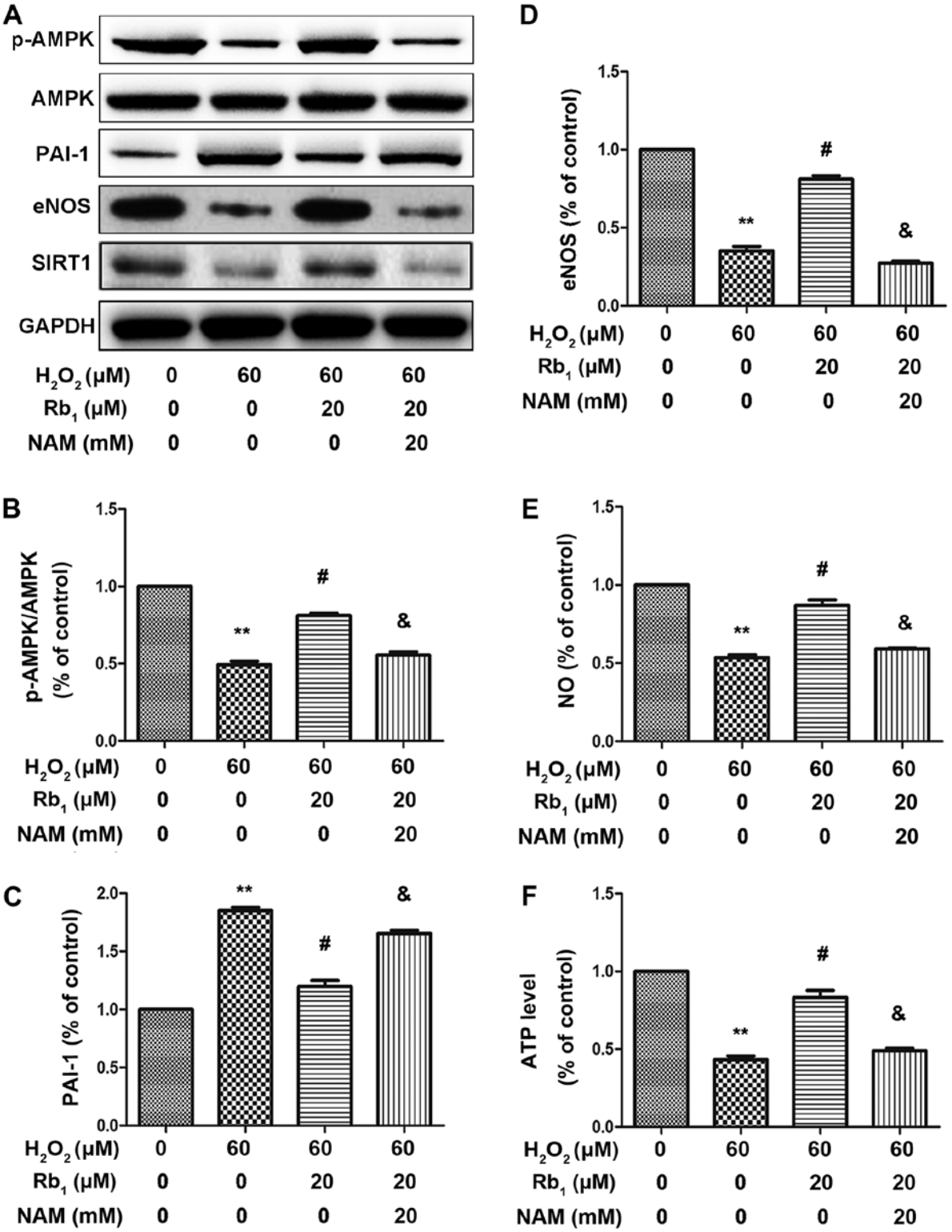

Figure 4. NAM blocks the protective effects of Rb1 against H_2O_2-induced senescence in HUVECs. (A) The protein levels of phosphorylated AMPK, AMPK, PAI-1, eNOS, SIRT1 and GAPDH were determined by western blot. (B) Quantitative analysis of phosphorylated AMPK expression ratio and phosphorylated AMPK levels. Total AMPK and GAPDH were also determined. (C) Quantitative analysis of PAI-1 expression ratio and PAI-1 expression levels. (D) Quantitative analysis of eNOS expression ratio and eNOS expression levels. (E) NO production measured by the Griess reaction and expressed as the percentage of the control. (F) The amount of ATP in the cells was measured using an ATP determination kit. Results represent three independent experiments. **P<0.01, vs. control group. $^{\#}$P<0.05 vs. 60 μM H_2O_2 group. $^{\&}$P<0.05, vs. 60 μM H_2O_2+Rb1 group. NAM, nicotinamide; Rb1, ginsenoside Rb1; H_2O_2, hydrogen peroxide; HUVEC, human umbilical vein endothelial cell; AMPK, AMP-activated protein kinase; PAI-1, plasminogen activator inhibitor-1; eNOS, endothelial nitric oxide synthase; SIRT1, Sirtuin-1; p, phosphorylated; NO, nitric oxide.

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 H_2O_2-induced endothelial dysfunction. However, *in vivo* 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 H_2O_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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