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Salidroside Protects Against Advanced Glycation End Products-Induced Vascular Endothelial Dysfunction

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Background: Salidroside, the major active compound in *Rhodiola*, has been reported to provide beneficial effects on cardiovascular diseases, but its effects on diabetes-induced vascular endothelial dysfunction are less known. Here, we examined the protective effects of salidroside on endothelial function in diabetes and explored the potential underlying mechanism.

Material/Methods: First, we assessed the endothelium-dependent relaxation response to acetylcholine, with or without salidroside treatment, in aortas isolated from Sprague-Dawley rats. Then, cell viability, oxidative biomarkers, and protein expression were tested to determine the effect of salidroside treatment on human umbilical vein endothelial cells (HUVECs) *in vitro*.

Results: Advanced glycation end product (AGE)-induced endothelial dysfunction was significantly improved by salidroside treatment ($P < 0.05$), as shown by a reduced relaxation response to the vasodilator acetylcholine. Further, incubation with salidroside restored NO levels and reduced reactive oxygen species formation in AGE-stimulated HUVECs in a concentration-dependent manner ($P < 0.05$). We also showed that nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/heme oxygenase 1 (HO-1) and nuclear factor kappa B (NF- κ B) signaling was critical for the salidroside-mediated beneficial regulation.

Conclusions: Our results demonstrate that salidroside protects against AGE-induced endothelial dysfunction, and its effects may be in part attributed to the induction of HO-1 and attenuation of phosphorylated NF- κ B p65.

MeSH Keywords: **Diabetes Mellitus, Type 2 • Endothelial Cells • Glycosylation End Products, Advanced**

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Background

The endothelium is regarded as the key regulator of vascular homeostasis, which not only forms a barrier between the vascular lumen and wall but is also involved in circulation of active signals for sensation and transportation [1]. When endothelial function is lost, the vasculature is predisposed to vasoconstriction, leukocyte adherence, platelet activation, thrombosis, and atherosclerosis [2].

Diabetes mellitus (DM) has become one of the main contributors to overall morbidity and mortality in the past decades [3]. Vascular endothelial dysfunction is a prominent feature of DM. The causes of endothelial dysfunction in diabetes include inflammation, dyslipidemia, hyperglycemia, and oxidative stress [4]. These risk factors can initiate the endothelial dysfunction and, in turn, vascular dysfunction, and contribute to the development of atherosclerotic diseases, including coronary artery disease, acute coronary syndrome, stroke, and thrombosis.

To establish a therapeutic strategy for DM, pharmacological effects of medicinal plants have been widely studied. Plant-derived antioxidant molecules have been shown to provide beneficial effects on endothelial function [5]. Salidroside (*p*-hydroxyphenethyl- β -D-glucoside), a major active compound in *Rhodiola*, has been reported to inhibit cardiomyocyte apoptosis by decreasing excessive oxidative stress [6]. Additionally, increasing evidence shows that salidroside suppresses cell injury through the activation of antioxidant signaling pathways [7–9]. In this study, we hypothesized that salidroside exerts a protective effect on endothelial function in diabetes and investigated the potential underlying mechanism of action.

Material and Methods

Materials

Unless otherwise stated, all chemical reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Tissue preparation and measurement of tension

All experiments were performed according to the Guidelines on Animal Experiments from the Committee on Medical Ethics at the National Health Department of China and were approved by the Laboratory Center of the Shanghai Tenth People's Hospital. Fifty male Sprague–Dawley (SD) rats (250–300 g) were purchased from Shanghai Slac Laboratory Animal Co., Ltd., and housed in plastic cages with well-ventilated stainless-steel grid tops at room temperature under a 12-h light/dark cycle.

Aortic rings were prepared as reported previously [10]. The descending thoracic aortic rings were dissected and incubated in Krebs solution (NaCl, 118 mM; KCl, 4.75 mM; NaHCO₃, 25 mM; MgSO₄, 1.2 mM; CaCl₂, 2 mM; KH₂PO₄, 1.2 mM; and glucose, 11 mM) at 37°C and gassed with 95% O₂ and 5% CO₂ for 5 h in a cell culture incubator. The rings were set at 1-g passive tension and allowed to equilibrate for 60 min. After the equilibration, rings with the endothelium were stimulated with a single concentration of acetylcholine (ACh) in the absence or presence of bovine serum albumin (BSA), BSA-advanced glycation end products (AGEs), and N^G-nitro-L-arginine methyl ester (L-NAME), an endothelial nitric oxide synthase inhibitor, and treated with increasing concentrations of salidroside (0.1–10 μ M).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). HUVECs were routinely maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂ in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). For all experiments, HUVECs were used between passages 3 and 10.

Cell viability assay

The viability of HUVECs was determined by using a cell counting kit-8 (CCK-8) according to the manufacturer's directions (Beyotime Institute of Biotechnology, Jiangsu, China). CCK-8 utilizes nonradioactive, sensitive colorimetric assays to determine the number of viable cells, cell proliferation, and cytotoxicity. HUVECs (100 μ L) were seeded in 96-well plates at a density of 1–2 \times 10⁴ cells/mL, incubated for 12–72 h, and treated with various concentration of salidroside for 24 h. Then, 10 μ L of the CCK-8 reagent was added to each well for 2 h, and the absorbance was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA).

Nitric oxide assay

Cells were pretreated with AGEs either at 200 μ g/mL for 6–72 h or at various concentrations for 24 h. Then, HUVECs were incubated with salidroside (0.1–10 μ M) for 24 h, followed by stimulation with 200 μ g/mL AGEs for 24 h. The cells were collected and incubated on ice in 500 μ L of cell lysis buffer [1 mM ethylenediaminetetraacetic acid (EDTA), 10 mg/mL aprotinin, 0.5 mg/mL leupeptin, 0.7 mg/mL pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.0]. After centrifugation at 10 000 \times g for 5 min, the supernatant was collected to determine the nitric oxide (NO) levels using a commercial kit (Jian Cheng Biological Engineering Institute, Nanjing, China).

Intracellular reactive oxygen species measurement

The level of intracellular reactive oxygen species (ROS) was measured with the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology). HUVECs were seeded onto a 24-well plate at a density of $1-2 \times 10^5$ cells per well. After synchronization for 24 h, the cells were incubated with or without various concentrations of salidroside for 24 h, followed by the addition of AGEs at 200 $\mu\text{g}/\text{mL}$ and incubation for an additional 2 h. After the treatment, the cells were incubated with a serum-free medium containing DCFH-DA (10 μM) for 30 min at 37°C in the dark, washed twice with phosphate-buffered saline, trypsinized, then resuspended, and immediately subjected to a flow cytometric analysis (EPICS XL, Beckman Coulter, Fullerton, CA, USA). Fluorescence signals were acquired by using a 530-nm band-pass filter for DCF. Each determination was based on a mean fluorescence intensity of 10 000 cells. In blank controls, the cells were incubated without DCFH-DA.

Protein extraction and western blot analysis

Total proteins from HUVECs were analyzed by Western blot. For the analysis, cells were removed from plates after treatments using a lysis solution containing 4% sodium dodecyl sulfate (SDS), 2 mM EDTA, and 50 mM Tris-HCl, pH 6.8. The homogenates were centrifuged at $15\ 000 \times g$ for 15 min at 4°C, and total protein was quantified in the supernatants by the Bradford method. Equal amounts of protein (50 μg) were separated by 8% or 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The blots were then washed with Tris-buffered saline, blocked with 5% non-fat dry milk (except phosphorylated myosin phosphatase-targeting protein, which was blocked with 5% BSA) in Tris-buffered saline with Tween 20 for 1 h, and incubated with the appropriate primary antibody at dilutions recommended by the supplier. The membrane was then washed and incubated with a secondary antibody, conjugated to horseradish peroxidase, for 1 h at room temperature. The blots were developed with a SuperSignal-enhanced chemiluminescent substrate solution (Pierce Chemical Company, Rockford, IL, USA). Anti- β -actin, anti-nuclear factor (erythroid-derived 2)-like 2 (Nrf2), anti-kech-like ECH-associated protein 1 (Keap1), anti-heme oxygenase 1 (HO-1), and anti-histone H3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-nuclear factor kappa B (NF- κB) p65 and anti-NF- κB p65 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical analysis

All data are expressed as the mean \pm standard deviation and were analyzed with the GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Statistical analyses

were performed using one-way analysis of variance, followed by Turkey's post-test. Differences with $P < 0.05$ were considered statistically significant.

Results

Salidroside increased endothelium-dependent aortic ring relaxation

We first examined the contractile activity of the aorta rings from SD rats in the presence of AGEs at 200 $\mu\text{g}/\text{mL}$ and L-NAME at 10 μM . As shown in Figure 1, the vascular relaxation function was significantly inhibited by L-NAME, and the aortic rings showed impaired vasodilator sensitivity to the ACh application compared to that of the control, with or without AGEs (200 $\mu\text{g}/\text{mL}$) (Figure 1A). The aortic rings from SD rats exhibited increased vasodilator sensitivity to salidroside in a concentration-dependent manner compared to that in the control and in the presence of AGEs (Figures 1B–1D).

Effect of salidroside on cell viability

In the experiments, performed with a medium containing 10% FBS, no significant differences were observed in the cell viability of HUVECs treated with 0.1 to 10 μM salidroside compared with that in the control (Figure 2A). However, HUVEC viability was markedly inhibited in the presence of 100 and 1000 μM salidroside compared with that of control cells or cells treated with 10 μM salidroside. Then, HUVECs were treated with various concentrations of salidroside for 12 to 72 h. The cell viability was significantly inhibited in the presence of 1 and 10 μM salidroside after 48 to 72 h compared with that of the cells treated with salidroside for 24 h (Figure 2B). Hence, for all subsequent experiments, primary cultures of HUVECs were treated with 0.1 to 10 μM salidroside for 24 h.

Changes in NO levels

The levels of NO in HUVECs treated with AGEs and in those treated with salidroside were significantly different (all $P < 0.05$). Figure 3 shows the NO levels in the supernatants of both AGE- and salidroside-treated HUVECs. The levels of NO were significantly lower in HUVECs incubated with AGEs at 200 $\mu\text{g}/\text{mL}$ for 12 h and were significantly decreased in HUVECs treated with AGEs at 100, 200, 300, and 400 $\mu\text{g}/\text{mL}$. However, there were no marked differences in the NO levels among the treatments with 200 $\mu\text{g}/\text{mL}$ AGEs for 24, 48, and 72 h ($P > 0.05$, Figure 3A). Similarly, there were no marked differences in the NO levels among the treatments with AGEs at 200, 300, and 400 $\mu\text{g}/\text{mL}$ for 24 h ($P > 0.05$, Figure 3B). As shown in Figure 3C, incubation with salidroside restored NO levels in AGE-treated cells in a concentration-dependent manner ($P < 0.05$).

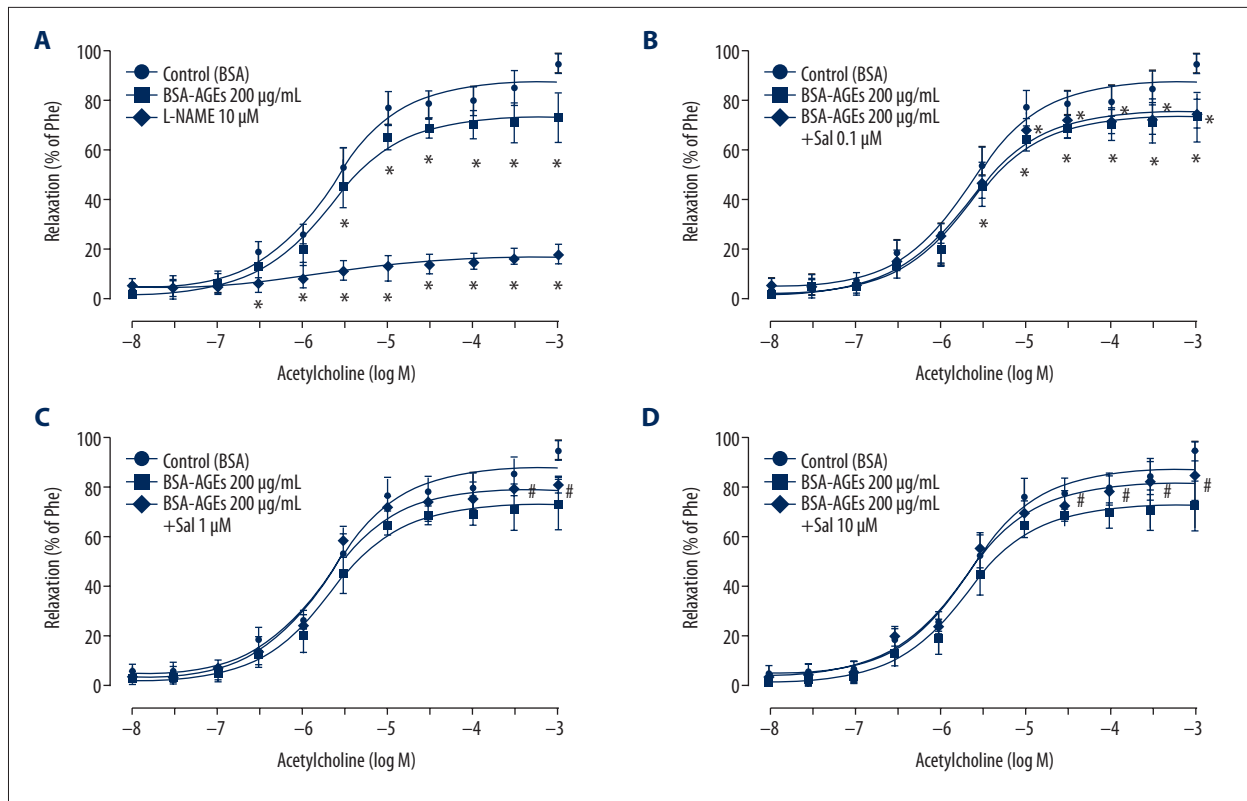


Figure 1. Endothelium-dependent relaxation response to AGEs and salidroside treatments in thoracic aortic rings from SD rats, precontracted with acetylcholine: **(A)** Vascular relaxation function was significantly inhibited by L-NAME compared to that of the control, with or without AGEs (200 µg/mL). **(B–D)** Aortic rings from SD rats exhibited increased vasodilator sensitivity to salidroside in a concentration-dependent manner compared to that in the controls and in the presence of AGEs. Rings in each group were 10 (n=10). * $P < 0.05$ versus the control group. # $P < 0.05$ versus the BSA-AGEs (200 µg/mL) treated group. L-NAME – N^G-nitro-L-arginine methyl ester; BSA – bovine serum albumin; AGEs – advanced glycation end products; Sal – salidroside.

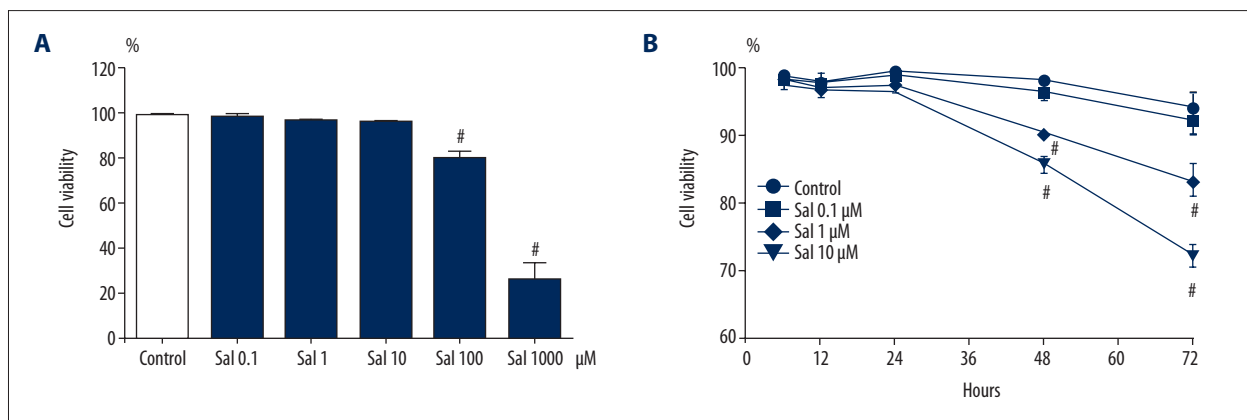


Figure 2. Cell viability assay: **(A)** Cytotoxic effects of salidroside at varying concentrations on HUVECs treated for 24 h. **(B)** Cells were incubated with salidroside for 6 to 72 h in the incubation medium as described in the Materials and Methods section, and viability was assessed by the CCK-8 assay. Values are expressed as the means ± standard deviation of triplicate experiments. * $P < 0.05$, # $P < 0.01$ versus the control group. Sal – salidroside.

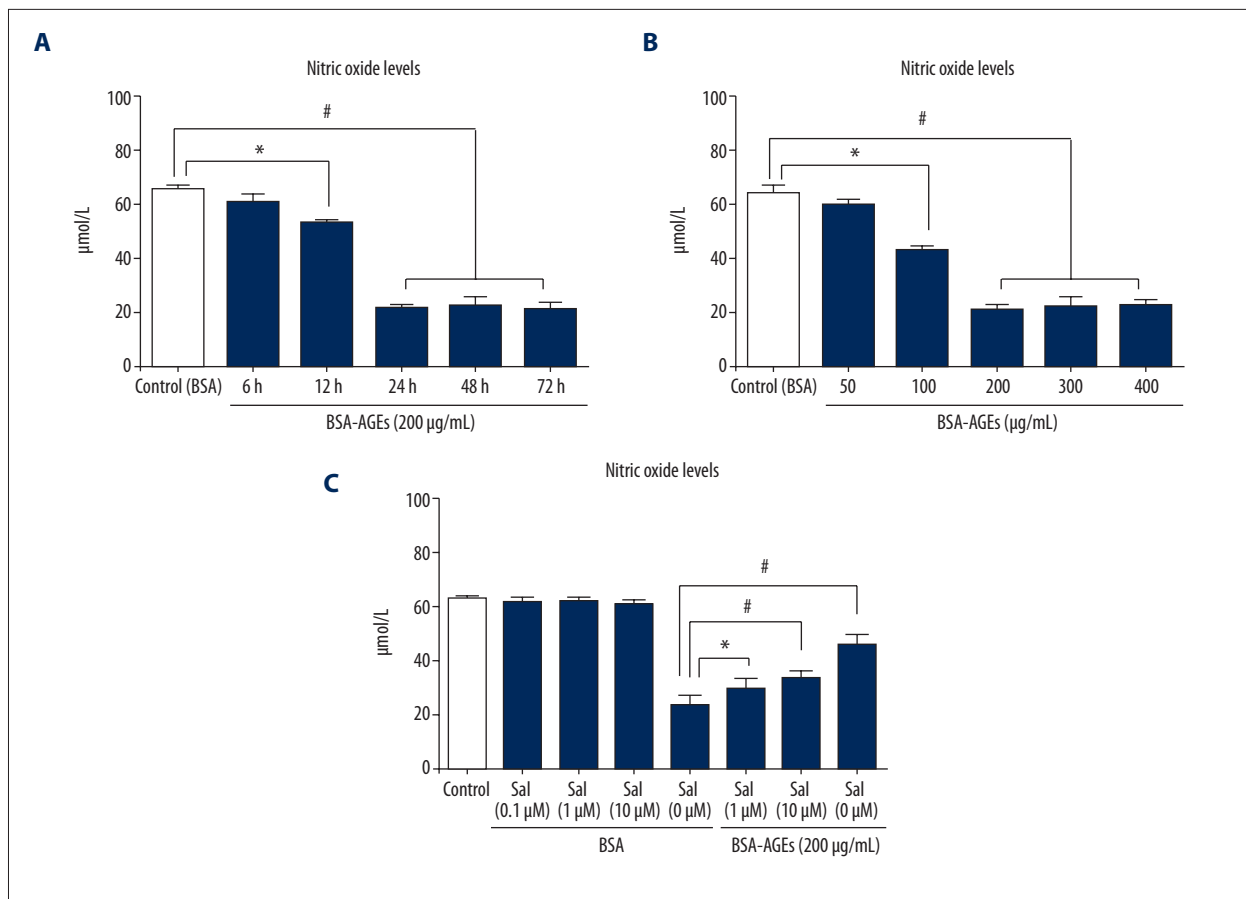


Figure 3. Assay of NO levels: (A) NO levels were measured at different time points in HUVECs treated with AGEs (200 µg/mL). (B) NO levels were measured in HUVECs treated with varying concentrations of salidroside for 24 h. (C) Salidroside treatment restored the NO levels after AGE treatment in a concentration-dependent manner. * $P < 0.05$, # $P < 0.01$ versus the control group. BSA – bovine serum albumin; AGEs – advanced glycation end products.

Salidroside reduced AGE-induced ROS formation in cultured HUVECs

The intracellular ROS production was remarkably increased in HUVECs treated with AGEs for 30 min (Figure 4A). However, salidroside pretreatment resulted in a significant decrease in ROS generation (Figures 4A and 4B). In HUVECs treated with different concentrations of salidroside (0.1 to 10 µM), the fluorescence intensity was significantly reduced (Figure 4B, all $P < 0.05$).

Effect of salidroside on Nrf2/HO-1 and NF-κB signaling

To investigate the effect of salidroside on the Nrf2/HO-1 signaling pathway, protein expression of Nrf2, Keap1, and HO-1 was measured by immunoblotting (Figures 5A–5C). The protein expression of Nrf2 was markedly decreased upon incubation of cells with 200 µg/mL AGEs for 24 h. This effect was attenuated by the pretreatment with 0.1 and 1 µM salidroside.

In HUVECs, the protein expression of Keap1 increased after incubation with AGEs (200 µg/mL) for 24 h, and salidroside at 0.1 and 1 µM reduced the Keap1 expression (Figure 5B). On the contrary, the protein expression of HO-1 in HUVECs incubated with AGEs (200 µg/mL) decreased after 24 h, and the levels of HO-1 expression were restored following a treatment with 0.1 and 1 µM salidroside for 24 h (Figure 5C, $P < 0.05$).

The NF-κB pathway, a typical inflammatory signaling pathway, was also assessed in our study, and the protein expression of phospho-NF-κB p65 and total NF-κB was measured by immunoblotting (Figure 5D). The protein expression of phospho-NF-κB p65 was markedly increased upon incubation of cells with AGEs (200 µg/mL) for 24 h. However, this effect was attenuated by salidroside pretreatment (Figure 5D).

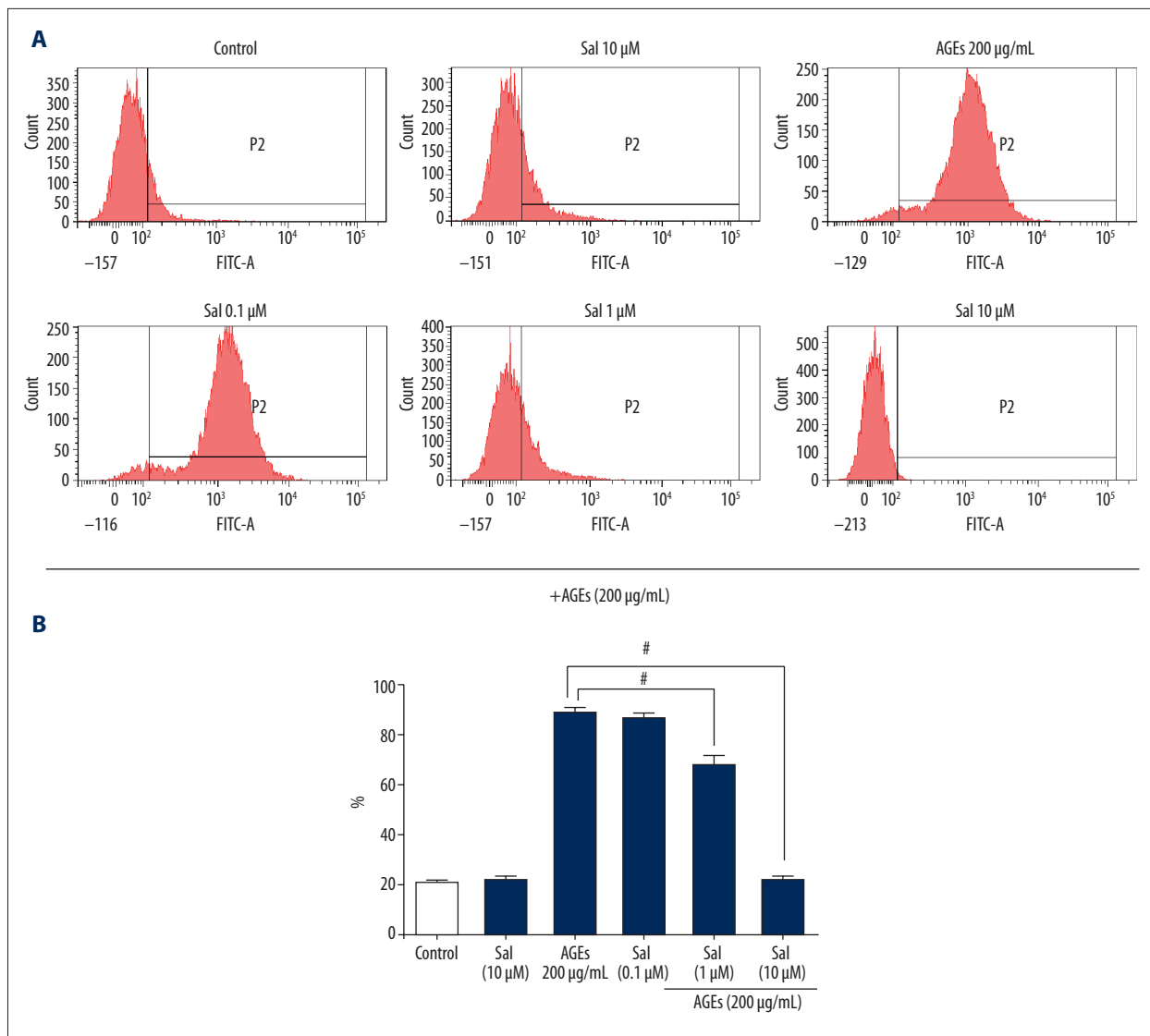


Figure 4. Flow cytometry results of intracellular ROS production after AGE treatment: **(A)** Representative flow cytometric images of HUVECs incubated with or without different concentrations of salidroside (0.1, 1, or 10 µM) prior to AGE exposure. **(B)** Quantitative analysis of the percentage of DCFH-DA probe-positive cells. # $P < 0.05$ versus the control group. Sal – salidroside; BSA-AGEs – bovine serum albumin-advanced glycation end products.

Discussion

The main aims of this study were to investigate whether salidroside could protect HUVECs against the AGE-induced vascular endothelial dysfunction and to explore the potential mechanisms. The data complemented the findings from our previous study [11], which suggested that salidroside decreased the atherosclerotic plaque formation by lowering lipids and inflammation in *Ldlr*^{-/-} mice.

The endothelium not only acts as a semi-selective barrier but also serves physiological and metabolic functions. Endothelial dysfunction represents an imbalance in the production of

vasodilator factors, and it has been proposed that the disturbance of this balance is an initial event in the pathogenesis of atherosclerosis [12]. Diabetes or insulin resistance promotes endothelial dysfunction and leads to the development of metabolic syndrome, which precedes cardiovascular disease [13]. AGEs are diverse compounds generated via a non-enzymatic reaction between reducing sugars and amine residues. It has been established that AGEs play specific roles in the intra- and extracellular deposition of insoluble complexes, interfere with the activity of endothelial nitric oxide synthase, NO bioavailability, and endothelial-dependent vasodilation, and also activate AGE receptor-dependent molecular pathways [14]. Therefore, numerous studies have identified

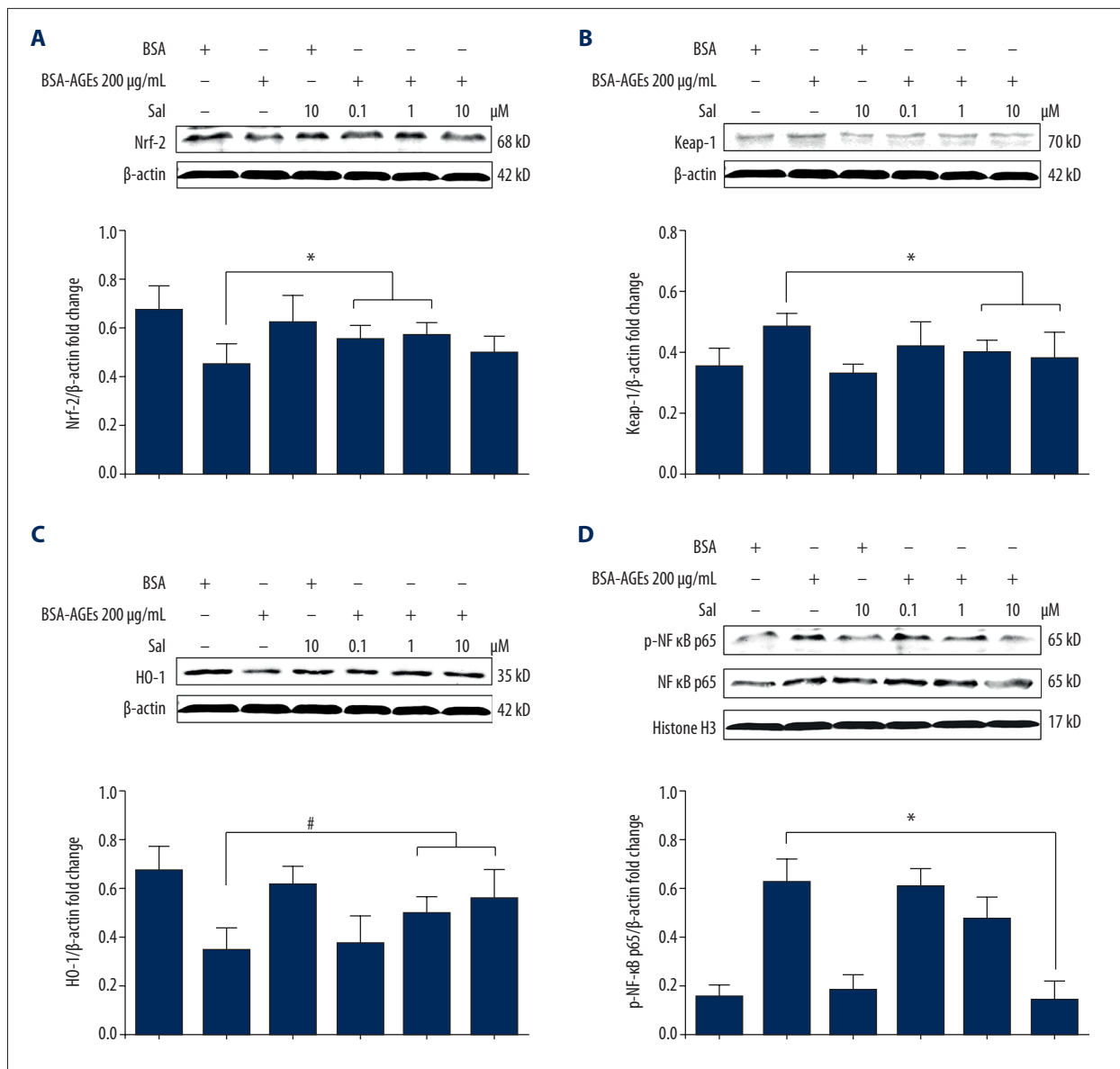


Figure 5. Western blot data showing the effects of salidroside on Nrf2 (A), Keap1 (B), HO-1 (C), and phospho-NF-κB p65 (D) levels in AGE (200 µg/mL)-exposed HUVECs. Total cell proteins were probed with primary antibodies against Nrf2, Keap1, HO-1, and phospho-NF-κB p65. β-Actin and histone H3 were used as internal controls. Values are expressed as the means ± standard deviation from 3 independent experiments. * $P < 0.05$, # $P < 0.01$ versus the control group.

AGEs as a therapeutic target for the prevention of cardiovascular complications in diabetes [15–17].

Development of antidiabetic medications from natural products or phytochemicals has been receiving more attention recently [18]. Many studies have highlighted not only the benefits of phytochemicals with hypoglycemic effects but also their importance in the management of diabetic complications. *Rhodiola* (Crassulaceae), an arctic–alpine plant, has been extensively used in traditional folk medicine in Asian and European countries. Several studies have demonstrated that salidroside,

a major active compound in *Rhodiola*, exhibits adaptogenic, neuroprotective, antitumor, anti-apoptosis, cardioprotective, and antidepressant effects [19–22]. It has been reported that salidroside showed a cardioprotective effect in diabetes and its complications in *in vivo* and *in vitro* studies [23–25]. Thus, our investigation focused on the role of salidroside in the diabetes-related vascular endothelial dysfunction induced by AGEs.

Tan et al. [26] have reported that salidroside inhibits the activation of caspase-3, decreases the expression of B-cell lymphoma 2-associated X protein (Bax), and restores the balance

of pro- and anti-apoptotic proteins in endothelial cells. In addition, some studies have shown that salidroside improves the endothelial dysfunction by inhibiting oxidative stress or by regulating metabolism [27–29]. However, the exact molecular mechanism of salidroside-related beneficial effects on the cardiovascular system under diabetic conditions is still unclear. A better understanding of the salidroside molecule and its cardioprotective effects may lead to more advanced strategies for treating cardiovascular complications associated with diabetes.

Nrf2 and NF- κ B are 2 key transcription factors that regulate the cellular responses to oxidative stress and inflammation, respectively [30,31]. Pharmacological and genetic studies have suggested that there is a functional crosstalk between these 2 important pathways [32]. Zhu et al. [7] have also reported that salidroside might suppress HUVEC cell injury induced by oxidative stress through activation of the Nrf2 signaling pathway. Lu et al. suggested that salidroside reduces HG-induced ROS generation and apoptosis and improves podocytes viability by upregulating HO-1 expression [33]. In this study, we focused on the role of salidroside in inflammation and redox signaling and found that the compound attenuated NF- κ B signaling and increased Nrf2/HO-1 protein expression. Therefore, these results suggest that salidroside exerts its protective effect against AGE-induced endothelial dysfunction via antioxidant mechanisms that involve the activation of the Nrf2 pathway.

Although we observed protective and antioxidant effects of salidroside in the present study, the effects of this compound are believed to be more profound than previously reported. In particular, little is known about the interactions of salidroside with other signaling networks in response to AGE-induced endothelial injuries. Further investigation of salidroside under diabetes conditions will be required to gain more comprehensive understanding of the beneficial effects of salidroside on the endothelium.

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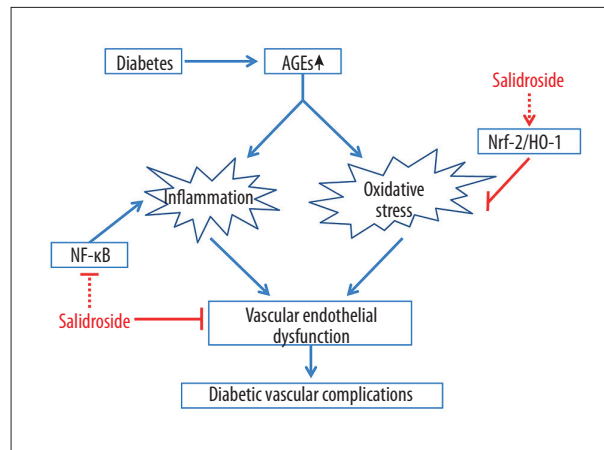


Figure 6. Schematic representation of the role of salidroside in AGE-induced vascular endothelial dysfunction.

Conclusions

In conclusion, this study demonstrates that salidroside protects against AGE-induced vascular endothelial dysfunction through inhibiting NF- κ B and activating the Nrf2 signaling pathway (Figure 6). Salidroside may be considered a candidate therapeutic drug for diabetic cardiovascular diseases; however, further *in vivo* experiments and clinical studies are needed to confirm its therapeutic effects.

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