Effect of Ginsenoside Rh1 on Proliferation, Apoptosis, and Oxidative Stress in Vascular Endothelial Cells by Regulation of the Nuclear Erythroid 2-related Factor-2/Heme Oxygenase-1 Signaling Pathway

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Abstract: This study aimed to investigate the role of ginsenoside Rh1 in regulating the proliferation, apoptosis, and oxidative stress in oxidized low-density lipoprotein (ox-LDL)-treated human vascular endothelial cells (VECs) and the underlying mechanisms. VECs were treated with ox-LDL to generate an in vitro atherosclerosis model. The effect of ginsenoside Rh1 on cell viability and proliferation was examined by MTT and colony formation assays, respectively, and cell apoptosis was determined by flow cytometry and transferase dUTP nick end-labeling assay. The levels of reactive oxygen species, malondialdehyde, and superoxide dismutase activity were detected using biological assays. Finally, the effect of ginsenoside Rh1 on the levels of BAX and BCL-2 and the nuclear erythroid 2-related factor-2/heme oxygenase (HO)-1 signaling pathway was determined by quantitative real-time polymerase chain reaction and western blot assays. Treatment with ginsenoside Rh1 significantly increased the proliferation and decreased the apoptosis of ox-LDL–treated VECs in a dose-dependent manner. Moreover, ginsenoside Rh1 also relieved oxidative stress in ox-LDL–treated VECs by activating the Nrf2/HO-1 signaling pathway. Thus, ginsenoside Rh1 affects the proliferation, apoptosis, and oxidative stress in ox-LDL–treated VECs by activating the Nrf2/HO-1 signaling pathway.

Key Words: ginsenoside Rh1, atherosclerosis, vascular endothelial cells, oxidative stress

(*J Cardiovasc Pharmacol*™ 2022;79:335–341)

INTRODUCTION

Atherosclerosis (AS) is a leading cause of coronary heart disease.[1–4] Vascular endothelial cells (VECs) are a major cell type that reside in the arterial walls, and aberrant behaviors of VECs may contribute to the development of AS.[5] A previous study found that excessive reactive oxygen species (ROS) produced as a result of external stimulation leads to oxidative stress injury, which is the main cause of endothelial dysfunction.[6] In addition, excessive ROS production leads to an antioxidant/prooxidant imbalance that triggers the activation of inflammatory signals and mitochondria-mediated apoptosis, which aggravates the occurrence and development of AS.[7] Furthermore, increased levels of oxidized low-density lipoprotein (ox-LDL) have also been identified as risk factors for AS, and ox-LDL contributes to the pathogenesis of AS by affecting the growth of VECs.[8] Therefore, suppressing ox-LDL–induced apoptosis and oxidative stress in VECs may be a novel and effective therapeutic strategy for the long-term management of AS.

In recent years, the role of traditional Chinese medicine in the treatment of heart disease has been discussed in many previous studies. Ginsenoside, an active component of ginseng, has significant therapeutic effects on a variety of cardiovascular diseases, including antioxidant effect, improvement of hemodynamics, regulation of vascular function, antithrombosis, protection of myocardial ischemia–reperfusion, and so on.[9–11] Ginsenosides are divided into protopanaxadiol type (Rb1 and Rb2) and protopanaxatriol type (Rg1 and Rh1) according to whether there are hydroxyl groups on the 6-carbon.[12–14] The effects of the active components of ginseng, such as Rb1 or Rg1, on VECs have been discussed previously[9,15]; however, the effect of ginsenoside Rh1 on ox-LDL–induced apoptosis and oxidative stress in VECs remains unclear.

Therefore, in the current study, we investigated the effect of ginsenoside Rh1 on oxidative stress injury in ox-LDL–treated VECs and its potential mechanism to determine whether Rh1 can ameliorate endothelial injury associated with AS and thus be potentially applied in the treatment of coronary heart disease. We hypothesized that ginsenoside Rh1 regulates the proliferation and apoptosis of ox-LDL–treated VECs and relieves oxidative stress by regulating the nuclear erythroid 2-related factor 2 (Nrf2)/heme oxygenase (HO)-1 signaling pathway.

METHODS

Cell Culture and Treatment

Human VECs were purchased from the Cell Bank (Shanghai Institutes for Biological Sciences, Shanghai,

Received for publication April 22, 2021; accepted July 15, 2021.

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The authors report no conflicts of interest.

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China). The VECs were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco, NY), 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO_2 and 95% humidity. Cells were passaged once every 2 days, until 3–6 passages. Then the cells were divided into 4 groups: control group, ox-LDL group, ox-LDL + 25 μg/mL ginsenoside Rh1 group, and ox-LDL + 50 μg/mL ginsenoside Rh1 group. The cells in the control group were cultured in RPMI-1640 medium. The cells in the ox-LDL group were cultured with 50 μg/mL ox-LDL. The cells in the ginsenoside Rh1 group were cultured with 50 μg/mL ox-LDL + 25 μg/mL ginsenoside Rh1 or 50 μg/mL ox-LDL+50 μg/mL ginsenoside Rh1. The cells in each group were cultured for 24 hours and then collected for further experiments.

Cell Viability Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to examine the viability of VECs in the different groups. In brief, cells were seeded in 96-well plates (5×10^3 cells/well) and incubated with 100 μL of MTT solution (0.5 mg/mL) for 4 hours in an incubator, and the precipitate was dissolved in 150 μL of dimethylsulfoxide. The optical density of each well was measured at 570 nm after shaking for 10 minutes. Three duplicates were performed for each sample.

Colony Formation Assay

VECs were plated in 24-well plates (2×10^3 cells/well) and cultured for 14 days. Crystal violet was added to each well, and the cells were stained at room temperature for 15 minutes. Colonies were visualized using an inverted light microscope. Three duplicates were performed for each sample.

Terminal Deoxynucleotidyl Transferase deoxyuridine triphosphate Nick End-Labeling Assay

VECs were fixed in 4% neutral formalin. The cells were then washed with phosphate-buffered saline, and hydrogen peroxide was added. Next, the cells were washed with phosphate-buffered saline thrice and stained with transferase deoxyuridine triphosphate nick end-labeling (TUNEL) mixture in the dark. The TUNEL-positive cells were imaged with a microscope, and the number of cells was calculated using the Image-Pro Plus 5.0 software. Three duplicates were performed for each sample.

Flow Cytometry

Forty-eight hours after transfection, the cells from different groups were collected and 5 μL of Annexin-V-fluorescein isothiocyanate (FITC) and 2.5 μL of propidium iodide (PI) were added to the cells. Cell apoptosis was determined using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). A scatter diagram of the apoptotic cells in the various stages was distributed as follows: Q3: early stage apoptotic cells (FITC+/PI−) and Q2: advanced stage apoptotic cells (FITC+/PI+). The apoptotic rate was calculated as the ratio of cells in Q3 + Q2 to the total number of cells. Three duplicates were performed for each sample.

Determination of ROS, Malondialdehyde, and Superoxide Dismutase Levels

The levels of ROS, malondialdehyde (MDA), and superoxide dismutase (SOD) activity were detected using the respective kits (Nanjing Jiancheng Biological Reagent Research Institute). All steps were performed according to the manufacturer's instructions. Three duplicates were performed for each sample.

Quantitative Real-time Polymerase Chain Reaction

Total RNA was collected from the cells to detect the mRNA expression of Nrf2, HO-1, SOD1, BAX, and B-cell lymphoma-2 (BCL-2). RNA was reverse transcribed into cDNA using the TaqMan MicroRNA Array kit (Applied Biosystems, CA). Polymerase chain reaction (PCR) was performed using the SYBR Green RT-PCR Master Mix (Applied Biosystems). The thermocycling conditions used were as follows: 95°C for 5 seconds; 45 cycles of 95°C for 5 seconds, 60°C for 1 minute, 95°C for 5 seconds, and 60°C for 1 minute. The mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method. glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. The primer sequences used are listed in Table 1. Three duplicates were performed for each sample.

Western Blotting

All antibodies were purchased from Abcam (Cambridge, MA). The protein samples were electrophoresed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane using an iBlot Gel Transfer Device (Thermo Fisher). The membranes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) reagent and then incubated with anti-Nrf2 (1:600; Abcam, MA; ab62352), anti-HO-1 (1:1000; Abcam; ab52947), anti-BAX (1:1000; Abcam; ab32503), and anti-BCL-2 (1:1000; Abcam; ab32124) at 4°C overnight. On day 2, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (1:3000, Cell Signaling Technology, MA, 7074S) followed by incubation with enhanced chemiluminescence Prime western blotting detection reagents (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). Finally, the ImageQuant TL GE Healthcare Life Sciences system was used to digitize the strength of the protein bands. Three duplicates were performed for each sample.

Statistical Analysis

Data are expressed as the means (n = 3) ± SD. Comparisons between 2 groups or multiple groups were performed using one-way analysis of variance followed by the Tukey test. Statistical significance was set at $P < 0.05$. Statistical analysis was performed using SPSS (version 22.0; Chicago, IL).

TABLE 1. Sequences for Real-Time Polymerase Chain Reaction Primers

Gene	Accession Number	Sequences (5′→3′)	Product Length (bp)
Nrf2	NM_198461	F: GGCGTTAGAAAGCATCCTTCC	79
		R: GCAGAGGGCACACTCAAAGT	
HO-1	NM_001161357	F: CTGTGCCACCTGGAACTGAC	88
		R: TCTTGTGGGTCTTGAGCTGTT	
SOD1	NM_000454	F: GGTGGGCCAAAGGATGAAGAG	227
		R: CCACAAGCCAAACGACTTCC	
BAX	NM_003217	F: CATATAACCCCGTCAACGCAG	77
		R: GCAGCCGCCACAAACATAC	
BCL-2	AF089746	F: CCAGCGTATATCGGAATGTGG	116
		R: CCATGTGATACCTGCTGAGAAG	
GAPDH	NM_014364	F: TGTGGGCATCAATGGATTTGG	116
		R: ACACCATGTATTCCGGGTCAAT	

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

RESULTS

Ginsenoside Rh1 Promotes the Viability and Proliferation of Ox-LDL–Treated VECs In Vitro

First, the effect of ginsenoside Rh1 on the viability and proliferation of ox-LDL–treated VECs was examined by MTT and colony formation assays. As shown in Figure 1, ox-LDL markedly decreased the viability and proliferation of VECs (Figs. 1B, C, $P < 0.01$), whereas ginsenoside Rh1 improved the viability and proliferation of ox-LDL–treated VECs in a dose-dependent manner (Figs. 1B, C).

Ginsenoside Rh1 Inhibits Apoptosis of Ox-LDL–Treated VECs In Vitro

The effect of ginsenoside Rh1 on apoptosis of ox-LDL–treated VECs was examined by flow cytometry and TUNEL assay. We found that ox-LDL significantly promoted

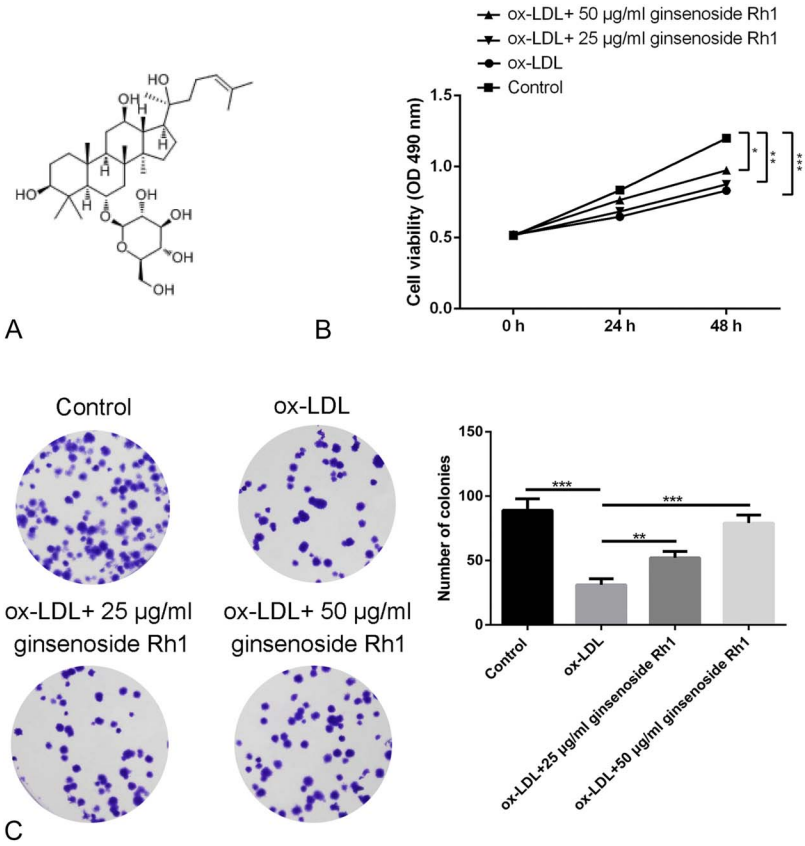

FIGURE 1. Effect of ginsenoside Rh1 on the viability and proliferation of ox-LDL–treated VECs in vitro. A, Chemical structure of ginsenoside Rh1. B, Results of MTT assay. C, Results of colony formation assay. Values were means and SD (n = 3). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

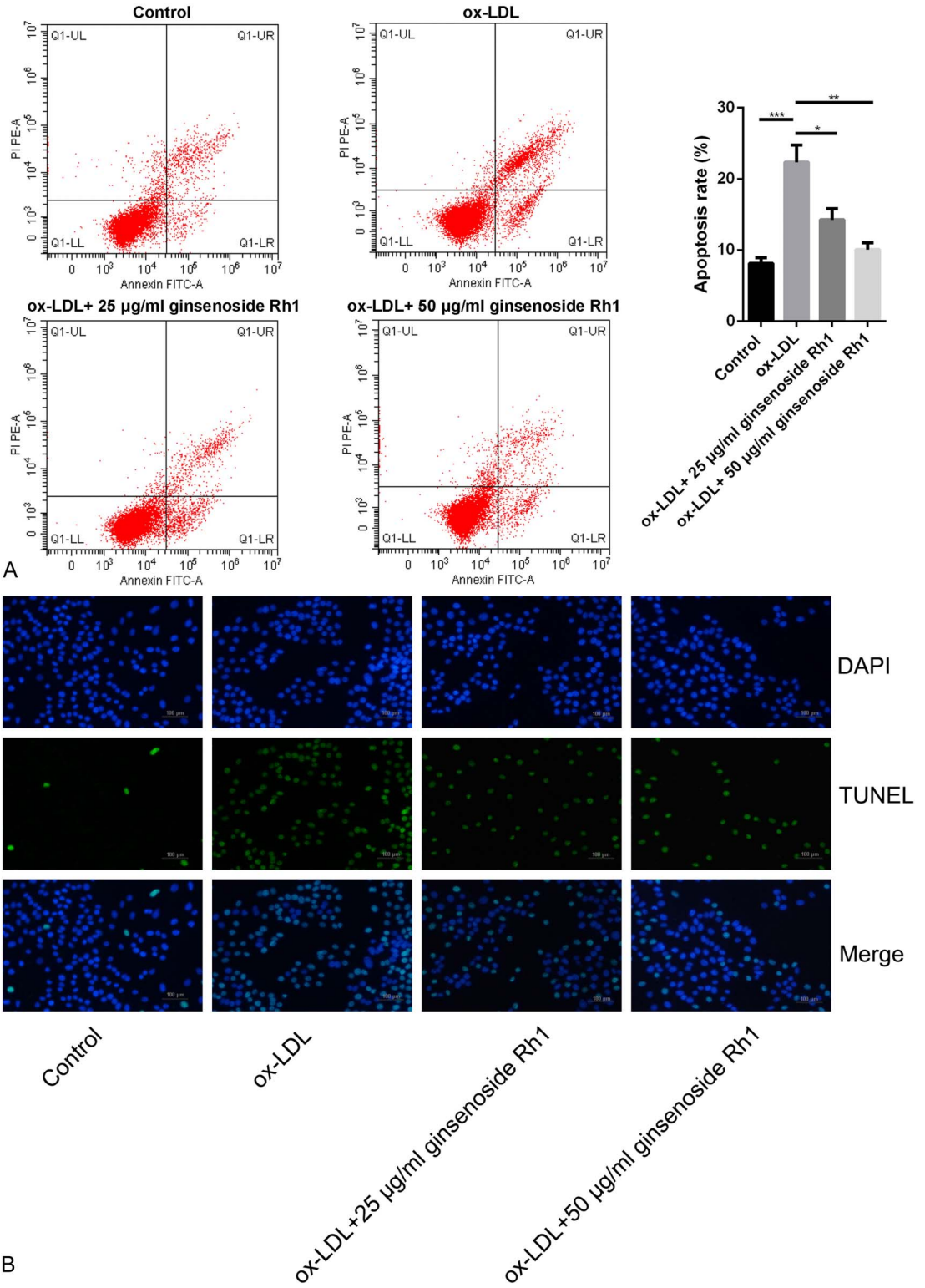

FIGURE 2. Effect of ginsenoside Rh1 on apoptosis of ox-LDL–treated VECs in vitro. Apoptosis of cells was determined by flow cytometry (A) and TUNEL (B) assays. Scale bar: 100 μm. Values were means and SD (n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

apoptosis of VECs, whereas ginsenoside Rh1 inhibited the apoptosis of ox-LDL–treated VECs in a dose-dependent manner (Fig. 2).

Ginsenoside Rh1 Decreases ROS and MDA Levels and Increases SOD Activity in Ox-LDL–Treated VECs In Vitro

Next, the effect of ginsenoside Rh1 on ROS, MDA levels, and SOD activity was examined in ox-LDL–treated VECs. As shown in Figure 3, ox-LDL markedly increased the levels of ROS and MDA and decreased SOD activity in the VECs (Fig. 3, $P < 0.01$), whereas treatment with ginsenoside Rh1 decreased the levels of ROS and MDA and increased the SOD activity in the ox-LDL–treated VECs in a dose-dependent manner (Fig. 3).

Ginsenoside Rh1 Activates the Nrf2/HO-1 Signaling Pathway in Ox-LDL–Treated VECs In Vitro

Finally, the effect of ginsenoside Rh1 on the expression of BAX, BCL-2, and Nrf2/HO-1 signaling pathways was examined in ox-LDL–treated VECs by quantitative real-time PCR and western blot assays. We found that the mRNA expression and protein levels of BAX were significantly upregulated, whereas those of Nrf2, HO-1, SOD1, and BCL-2 were significantly downregulated. After treatment with ginsenoside Rh1, the expression of BAX was significantly downregulated and that of Nrf2, HO-1, SOD1, and BCL-2 was significantly upregulated (Fig. 4).

DISCUSSION

In this study, we investigated the effects of ginsenoside Rh1 in ox-LDL–treated VECs and the underlying mechanisms. We found that ginsenoside Rh1 promotes proliferation, inhibits apoptosis, and relieves oxidative stress in ox-LDL–treated VECs by regulating the Nrf2/HO-1 signaling pathway.

Previous studies have indicated that the development of AS is strongly associated with vascular injury, which is induced by aberrant proliferation and apoptosis of VECs.[16–18] Ox-LDL was reported to be overexpressed in AS, and the upregulation of ox-LDL is considered to be an important cause for the abnormal behaviors of VECs.[19] Consistent with the results of previous studies, in this study, we found that ox-LDL inhibits the proliferation and promotes the apoptosis of VECs in vitro, suggesting that ox-LDL may function as an important inducer of vascular injury in AS. Simultaneously, we found that the mRNA expression and protein levels of BAX were upregulated and those of BCL-2 were downregulated in ox-LDL–treated VECs. However, after ginsenoside Rh1 treatment, VEC apoptosis was inhibited. Apoptosis is a form of programmed cell death mediated mainly by the caspases. The BCL-2 protein family is the central regulator of caspase activation and includes the antiapoptotic protein BCL-2 and the proapoptotic protein BAX.[20] Guo et al[21] demonstrated that the upregulation of BCL-2 and the downregulation of BAX inhibit macrophage apoptosis induced by ox-LDL. Therefore, we hypothesized that ginsenoside Rh1 may play an antiapoptotic role by attenuating ox-LDL–induced oxidative stress injury in VECs. However, some reports have shown that active components of ginseng alone treatment can induce angiogenesis. Zhang et al[22] confirmed that ginsenoside F1 induces angiogenesis by regulating the insulin-like growth factor 1 (IGF-1)/IGF1R pathway. Therefore, whether the effect of ginsenoside Rh1 is in an ox-LDL–specific manner remains to be further explored.

Recently, it has been suggested that ox-LDL stimulates cells to produce excessive ROS and upregulates the expression of adhesion molecules, which recruit monocytes to the endothelium. This impairs the secretory function of endothelial cells, reduces their antioxidant capacity, and induces apoptosis of endothelial cells.[23] Therefore, eliminating

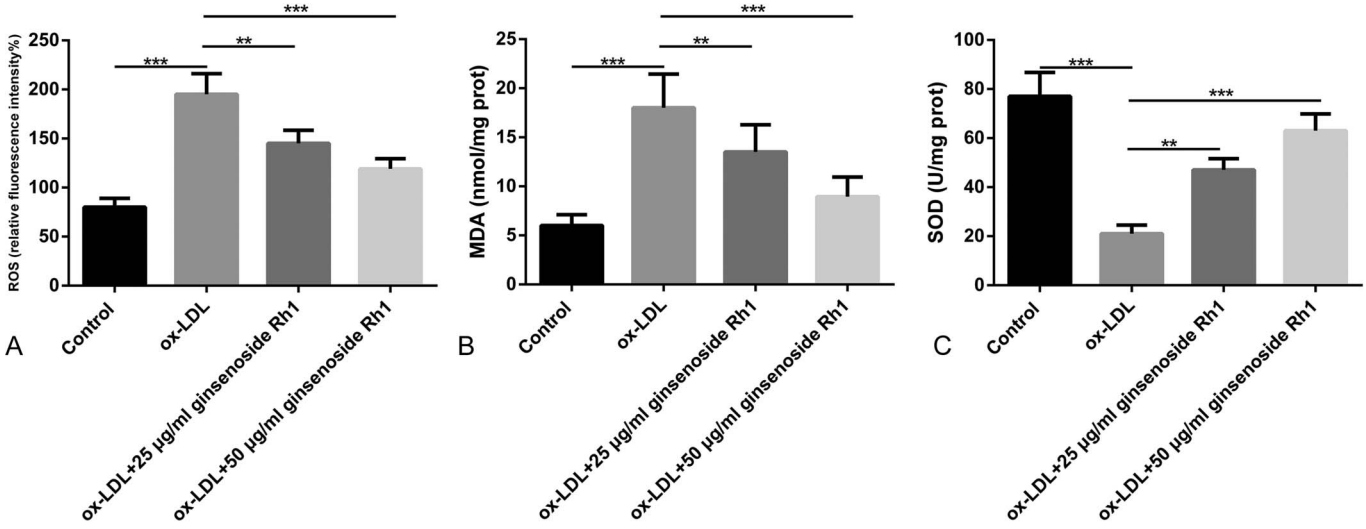

FIGURE 3. Effect of ginsenoside Rh1 on the levels of ROS (A) and MDA (B), and activity of SOD (C) in ox-LDL–treated VECs in vitro. Values were means and SD (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$.

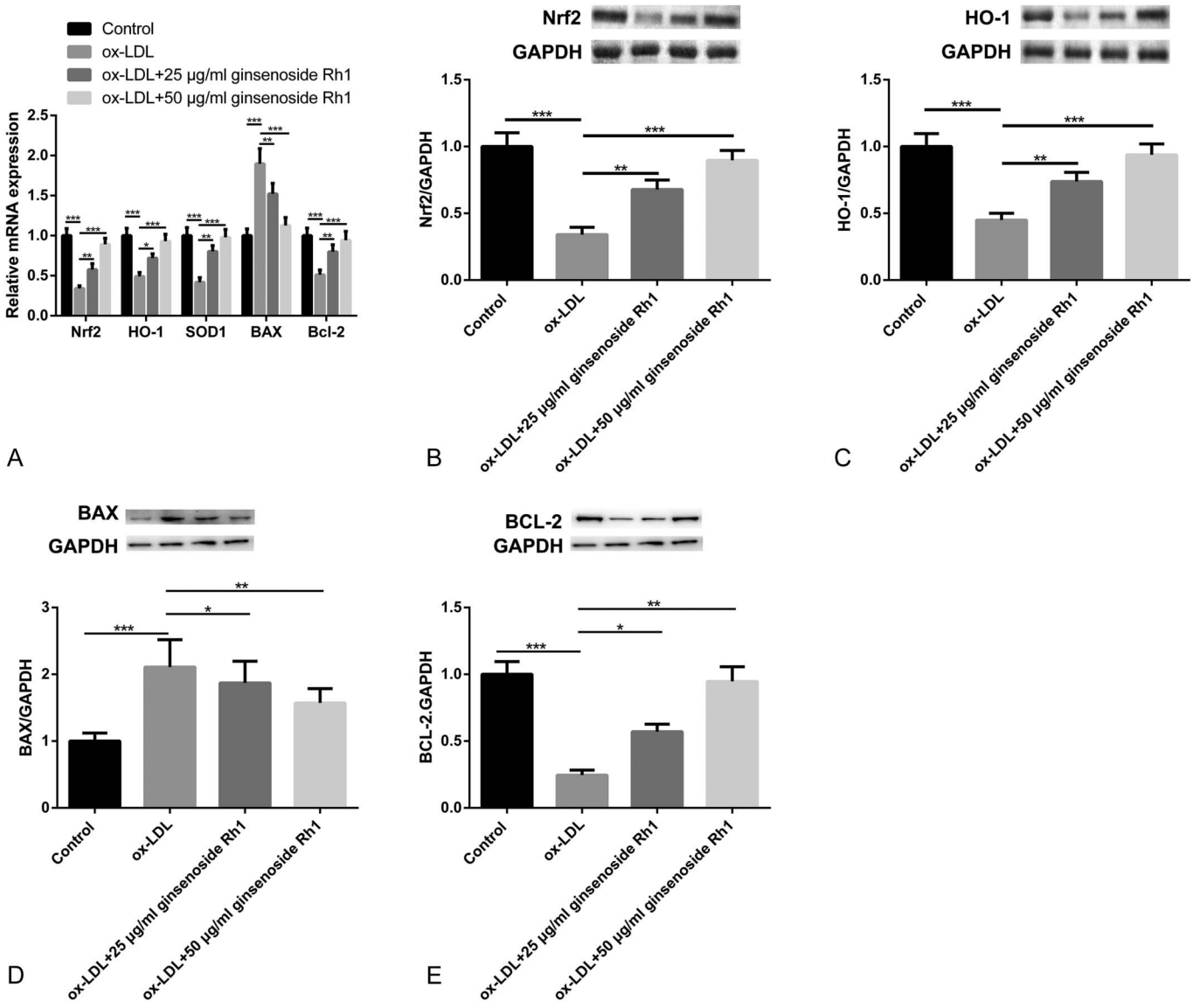

FIGURE 4. Effect of ginsenoside Rh1 on the Nrf2/HO-1 signaling pathway in ox-LDL–treated VECs in vitro. A, mRNA expression of Nrf2, HO-1, BAX, BCL-2, and SOD1 was determined by quantitative real-time polymerase chain reaction. B–E, Protein levels of Nrf2, HO-1, BAX, and BCL-2 were determined by western blot analysis. Values were means and SD (n = 3). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

excessive ROS and improving the antioxidant capacity through antioxidants may be the key strategy to inhibit ox-LDL–induced endothelial injury. Our study showed that ROS was produced in ox-LDL–treated VECs and caused an imbalance in the redox homeostasis in VECs by a decrease in SOD activity and an increase in MDA content. However, ginsenoside Rh1 treatment significantly reduced the production of ROS and MDA and enhanced SOD activity. These results suggest that ginsenoside Rh1 may inhibit ox-LDL–induced endothelial injury by increasing the intracellular antioxidant concentration. Gai et al[24] found that ginsenoside Rh1 inhibits isoproterenol-mediated cardiotoxicity by reducing the amount of MDA and increasing the activity of SOD and other antioxidant enzymes. In addition, although there are limited reports on the antioxidant functions of ginsenoside Rh1, many studies have found that ginsenosides Rg1 and Rb1 have immense potential in the treatment of oxidative stress–mediated diseases.[9,25–27] In general, our results confirmed that ginsenoside Rh1 also has a significant antioxidant effect.

To explore the specific mechanism underlying Rh1-mediated antioxidant functions, we analyzed the effect of ginsenoside Rh1 on the Nrf2/HO-1 signaling pathway. The Nrf2/HO-1 signaling pathway is a key mechanism of cellular antioxidant defense. An increasing number of studies have shown that natural active products protect cardiovascular functions by activating the Nrf2/HO-1 signaling pathway.[9,25–28] Nrf2 is a key regulator in maintaining redox homeostasis and cellular antioxidant defense[29] and can regulate the

expression of various antioxidants and phase II detoxification enzymes (HO-1 and SOD) through an antioxidant response element.[30] Among these antioxidants and phase II detoxification enzymes, HO-1 plays a key protective role in AS diseases.[31] Previous studies have confirmed that the upregulation of HO-1 enables cells to resist external stimuli and respond to oxidative stress. In this study, we found that ginsenoside Rh1 upregulated the mRNA expression of SOD1 and the mRNA and protein expression of Nrf2 and HO-1 in VECs. These results indicate that Rh1 may mediate antioxidative stress and antiapoptosis by activating the Nrf2/HO-1 signaling pathway.

However, there are still some limitations in this study. The in vivo experiment needs to be performed to further explore the role of ginsenoside Rh1 in AS treatment. We will conduct animal experiments in the future.

CONCLUSION

In conclusion, this study is the first to report that ginsenoside Rh1 may regulate the proliferation and apoptosis of VECs and relieve oxidative stress by activation of the Nrf2/HO-1 signaling pathway. Our study provides a theoretical basis for the application of ginsenoside Rh1 as a potential alternative medicine for the treatment of AS.

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