The effects of ginsenoside Rb1 on endothelial damage and ghrelin expression induced by hyperhomocysteine

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Objective: **Studies have indicated that ginsenoside Rb1 and ghrelin could both prevent homocysteine (Hcy)-induced endothelial dysfunction through the endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) mechanism. This study investigated whether endogenous ghrelin mediates the endothelial protection of ginsenosidee Rb1 through in vitro and in vivo experiments.**

Methods: **Rats were randomized into a control group, a hyperhomocysteine (HHcy) model group with a high methionine diet, a ginsenosides (GS) group, and HHcy plus GS group. Plasma ghrelin was detected by enzyme-linked immunosorbent assay. Aortic rings for control and HHcy groups were treated with ghrelin or not. Endothelium-dependent vasodilatation function was evaluated by the aortic ring assay, and the structural changes were visualized by hematoxylin and eosin staining. Human umbilical vein endothelial cells (HUVECs) were cultured, and the experimental conditions were optimized according to NO production. After treatment, the NO, ghrelin, and von Willebrand factor (vWF) levels in the media were detected and analyzed with linear regression. Ghrelin and eNOS expression were observed by cell immunohistochemical staining. Ghrelin receptor antagonist was used to detect the mechanism of ginsenoside Rb1 on NO production, which was reflected by diacetylated 4,5-diaminofluorescein-2 diacetate fluorescence.**

Results: **In vivo experiments demonstrated that plasma ghrelin levels in the HHcy group were significantly elevated vs controls ($P < .05$) and were significantly increased in the HHcy plus GS group ($P < .01$). Compared with control, endothelium-dependent vasodilatation function was greatly reduced in the HHcy group ($P < .01$), which was significantly increased in HHcy plus ghrelin group compared with HHcy group ($P < .01$). The arterial walls of HHcy group exhibited characteristic pathologic changes, which were repaired in HHcy plus ghrelin group. In vivo, compared with Hcy (200 μM) group, HUVECs pretreated with ginsenoside Rb1 (10 μM) for 30 minutes showed significant increases in NO and ghrelin levels and evident reduction in vWF levels. Linear regression analysis demonstrated that ghrelin levels were significantly positively correlated with NO levels and significantly negatively correlated with vWF levels. The addition of Rb1 to Hcy also greatly reversed Hcy-induced downregulation of ghrelin and eNOS expression. Ghrelin inhibition significantly abolished the upregulation of NO levels induced by Rb1.**

Conclusion: **Ghrelin can prevent Hcy-induced vascular endothelial dysfunction and structural damage. The compensatory elevation of plasma ghrelin levels in an Hcy-induced endothelial injury model may be a protective response. Ginsenoside Rb1 can significantly stimulate the ghrelin endocrine to inhibit endothelial injury. Ginsenoside also upregulates the NO signaling pathway reduced by Hcy through the ghrelin molecular mechanism. (J Vasc Surg 2011;53:156-64.)**

Clinical Relevance: **Homocysteine is an independent risk factor for endothelial injury and dysfunction. Ginsenoside Rb1, a major constituent of ginseng (traditional Chinese herb), can effectively block homocysteine-induced dysfunction of endothelium-dependent vasorelaxation as well as endothelial nitric oxide synthase (eNOS) downregulation. Ghrelin, a novel brain-gut peptide, has multiple cardiovascular protective effects, including vasorelaxation. The objective of this study was to determine the effect and molecular changes of ginsenoside Rb1. Our results showed that ginseng compounds have effects of vasorelaxation and endogenous ghrelin upregulation. Exogenous ghrelin can block homocysteine-induced endothelial injury and dysfunction in the rat thoracic aortic. Ginsenoside Rb1 can improve NO production, ghrelin, and eNOS expression in human umbilical vascular endothelial cells. This study indicated that ginsenoside Rb1 has a prospective clinical future in controlling homocysteine-associated vascular diseases through the ghrelin molecule and NO-signaling mechanism.**

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Hyperhomocysteine (HHcy) is recognized as one independent risk factor for atherosclerosis. About 40% of patients with coronary heart disease or cerebrovascular atherosclerosis have hyperhomocysteinemia. Therefore, prevention and treatment of atherosclerosis, calcification, hypertension, and other chronic vascular diseases may lie in the inhibition of excessive production of HHcy and alleviation of its toxic effects on the vascular endothelium.

Ginseng is one of the most widely used traditional Chinese medicines and is reported to have a wide range of therapeutic and pharmacologic applications. Ginsenosides, the major pharmacologically active ingredients of ginseng, appear to be responsible for most of the activities of ginseng.[1] In addition to influencing the central nervous system, ginsenosides can be used in clinical endocrinology and many cardiovascular and cerebral diseases because they enhance NO release from endothelial cells.[2,3] Ginsenosides are also shown to have potential benefits on the cardiovascular system through diverse mechanisms, including antioxidant, modifying vasomotor function, reducing platelet adhesion, influencing ion channels, altering autonomic neurotransmitters release, improving lipid profiles, and involving glucose metabolism.[4]

Our previous study used a high-methionine diet to induce HHcy and establish an HHcy-induced endothelial injury model in rats.[5] Ginsenosides were administered as a medication during establishment of the rat model, indicating that ginsenosides can counteract the effect of Hcy on endothelial injury and vascular vasodilatation function.[6] The detailed mechanisms are still unclear, however.

Ghrelin is a newly identified vasoactive peptide. It functions as a paracrine/autocrine factor to maintain homeostasis of the cardiovascular system and is important in cardiovascular diseases.[7,8] Ghrelin can prevent endothelial cell death, increase the expression of nitric oxide (NO) synthase in endothelial cells (eNOS), directly induce NO synthesis, and protect endothelial functions, which can also reverse Hcy-induced endothelial dysfunction in the porcine coronary artery through regulation of the NO signaling pathway.[9] Data show that the ginsenoside monomer Rb1 can improve Hcy-induced porcine coronary artery dysfunction through regulation of the NO signaling pathway.[10]

Because the effects and mechanisms of ghrelin and ginsenosides in endothelial protection are so similar, we hypothesized that ginsenosides could protect against homocysteine-induced endothelial injury by mechanisms involved in ghrelin induction and activation and in the upregulated NO signaling pathway. In this study, we investigated the effects and molecular mechanisms of endothelium protection by the ginsenoside monomer Rb1. This study suggests a potential clinical application of ginsenoside Rb1 and ghrelin in controlling Hcy-associated vascular injuries, and provides a thought of seeking both a temporary solution and permanent cure in clinical vascular diseases.

MATERIALS AND METHODS

Chemicals and reagents. Total ginsenosides and the ginsenoside monomer Rb1 (Rb1) were provided by Yiling Pharmaceutical Industry Ltd Co (Shijiazhuang, China). M199 media, trypsin, and fetal bovine serum were purchased from Gibco (Invitrogen, Carlsbad, Calif). Endothelial cell growth supplement was purchased from BD Biosciences (San Jose, Calif). L-type methionine, succinylsulfathiazole (SST) and Hcy were purchased from Sigma (St Louis, Mo). Diacetylated 4,5-diaminofluorescein-2 diacetate (DAF-2 DA) was purchased from Cayman Chemical Co (Ann Arbor, Mich). Ghrelin polypeptide and its receptor antagonist, [D-Lys3]-GHRP-6 was purchased from Phoenix Pharmaceuticals Inc (Burlingame, Calif). The ghrelin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Shanghai Shellgene Biotech Co, Ltd (Shanghai, China). The nitric oxide (NO) testing kit was obtained from the Nanjing Jiancheng Biotechnology Institute (Nanjing, China). The ELISA kit for von Willebrand factor (vWF) was purchased from Shanghai Taiyang Biotech Co, Ltd (Shanghai, China). The primary antibodies for eNOS and ghrelin and the streptavidin-biotin complex (SABC) immunohistochemistry kit were purchased from Wuhan Boster Biological Technology Co, Ltd (Wuhan, China).

Endothelial injury model and grouping. Male Wistar rats (200-250 g) were obtained from the Experimental Animal Center of Sun Yat-sen University (Clean grade, Certificate No SCXK2004-0011) and randomly assigned for the experiments. Animal use was in accordance with the Chinese Council on Animal Care Guidelines. The HHcy-induced endothelial dysfunction model was made using intragastric administration of methionine as described previously.[11] In HHcy group and HHcy plus ginsenosides (GS) group, rats were intragastrically administered 1 g/kg/day L-methionine and supplied with drinking water containing 0.5 g/kg succinylsulfathiazole for 4 weeks. Then, rats in HHcy plus GS group were administered 1.2 g/kg ginsenoside for 7 days, whereas rats in control group were administered equal volumes of double-distilled H_2O and supplied with drinking water without succinylsulfathiazole. After treatment, all rats were fasted overnight, and blood samples were collected the next morning. After anesthesia, blood samples were collected by cardiac puncture into ethylenediaminetetraacetic acid-coated anticoagulant tubes, mixed with aprotinin (0.6 TIU/mL blood sample), and centrifuged at 4°C, 1600*g* for 15 minutes to separate plasma. Samples were preserved at −80°C before detection of ghrelin.

Thoracic arteries from control and HHcy groups were isolated, placed in modified Kerbs solution (118 mmol/L NaCl, 4.7 mmol/L KCl, 216 mmol/L CaCl2, 1.2 mmol/L MgSO4·7H2O, 112 mmol/L KH2 PO4, 25 mmol/L NaHCO3, 5.5 mmol/L glucose, pH 7.4) and aerated with a mixture of 95% O_2 and 5% CO_2. After dissociating the surrounding fat and connective tissues carefully, each artery was truncated into 3-mm-thick rings and incubated in a 37°C water bath. Then the aortic rings from control and

HHcy groups were incubated in Krebs solution with or without ghrelin (final concentration: 10^{-6}M) for 1 hour. Finally, a portion of aortic rings was examined histologically with hematoxylin and eosin (HE) staining as reported,[12] the remaining portion of the aortic ring was kept in Krebs solution for the vascular ring experiment.

Thoracic aortic ring relaxation assay. Endothelium-dependent vasodilation function was detected as previously described.[13] These aortic rings were put into 4 mL Krebs solution. Each aortic ring was transfixed with two L-shape stainless steel hooks, with one fixed at the bottom of the thermostatic tank and the other connected to a tension transducer (JZ101) by a thread. Data were recorded with the PowerLab system. The Krebs solution was aerated with a mixed gas containing 95% O_2 and 5% CO_2, and the solution was replaced every 20 minutes. The temperature in the tank was maintained at about 37°C. The rings were allowed to equilibrate for 1.5 hours after being subjected to a tension of 1.5 g. The contraction signal was transmitted through an 8-channel connector of MP-285 Micromanipulator GSys to a personal computer that was connected to a PowerLab system (ADInstruments Inc, Colorado Springs, Colo). The data were recorded and analyzed with the PowerLab system and its software, Chart.

Before this experiment, vessels were contracted with 0.1 mol/L KCl. If the difference between two consecutive contraction amplitudes was <10%, the vessel reactions were considered repeatable; otherwise, the vessels were discarded. During the following experiment, the vessels were thoroughly washed after each step, and then the next step was performed until the contraction signals curve returned to baseline and stabilized for 25 minutes.

The vessel rings were precontracted in a high-potassium solution, washed, and normalized to the baseline level. Then the rings were soaked in 4 mL Krebs solution supplemented with 40 μL phenylephrine (final concentration: 10^{-6} mol/L). After the contraction reaction reached a plateau, the rings were treated with a range of cumulative concentrations of acetylcholine (10^{-10} to 10^{-6} mol/L). The relaxation percentages of vessel rings under different concentrations of acetylcholine were recorded, and acetylcholine-induced endothelium-dependent vascular relaxation was monitored.

Human umbilical vein endothelial cells culture and grouping. Human umbilical cords were provided by the Maternity Department at the Second Affiliated Hospital of Sun Yat-sen University. This study was approved by the Ethical Committee of Sun Yat-sen University Medical Faculty and by the mothers whose umbilical cords were donated for human umbilical vein endothelial cells (HUVECs). Fresh umbilical cords were washed with phosphate-buffered saline (PBS) in a cell culture hood to remove blood and residual blood clots, and digested with 0.25% trypsin containing ethylenediaminetetraacetic acid. HUVECs were collected by low-speed centrifugation, resuspended, and seeded onto cell culture flasks, plates, or dishes that were precoated with 0.2% gelatin. Cells were maintained in Roswell Park Memorial Institute medium 1640 containing 15% inactivated fetal calf serum, 100 U/mL penicillin, 100 U/mL streptomycin, and 2 mmol/L L-glutamine. Endothelial cell growth supplements and 0.005 U/L heparin were added. The culture media was changed every 2 days. Cells were passaged 2 to 3 days after reaching 80% confluence. HUVECs were harvested, and cell type was confirmed by vWF immunofluorescent positive staining with negative controls.

The third-generation HUVECs were seeded and incubated with endothelial cell growth supplement until reaching 90% confluence. HUVECs were divided into the following groups: control, Hcy-induced endothelial injury groups (stimulated with 50, 100, 200, 300, or 500 μM Hcy), a dose range of ginsenoside monomer Rb1 plus Hcy groups (pretreatment with 0.01, 0.1, 1, 10, or 100 μM Rb1 before Hcy stimulation), and a time-course of ginsenoside Rb1 plus Hcy groups (pretreatment for 5, 15, 30, or 60 minutes Rb1 before Hcy stimulation). The NO levels in the culture media were detected 48 hours after the treatment, and the model optimization for the following experiments were established accordingly. Then HUVECs were incubated for 48 hours with Hcy (200 μM), Rb1 (10 μM), or Hcy plus Rb1. After treatment, cell culture media were collected and preserved at −80°C before the detection of NO, ghrelin, and vWF levels. HUVECs were fixed and examined with immunohistochemical staining for intracellular ghrelin and eNOS expression.

Detection of biochemical indices in blood and culture media. Ghrelin levels in plasma and the culture media and vWF content in the culture media were detected with a double-antibody ELISA. The NO level in the culture media was analyzed with the nitrate reductase assay, which involves nitrate reductase deoxidization of NO_3^- to NO_2^-. The NO level can be calculated by measuring the NO_2^- concentration, as detected by chromometry. All assays were performed according to the manufacturer's instruction of each kit.

Cell immunohistochemistry and detection of intracellular NO content by fluorescent microscopy. After treatment, cells were washed and incubated with anti-eNOS or antighrelin primary antibody and the corresponding secondary antibodies. A group of negative controls was used in each experiment, for which the primary antibody was replaced with PBS. Finally, the coverslips were developed in 3,3′-diaminobenzidine reagent and observed under the light microscope for the subcellular localization of each protein. The protein levels were determined by semiquantitative analysis.

To detect intracellular NO production, HUVECs were added with DAF-2 DA. This dye is hydrolyzed intracellularly by cytosolic esterases releasing DAF-2, which can be converted into a fluorescent product (DAF-2T) by NO. The DAF-2 DA was loaded at a concentration of 1 μmol/L in Krebs solution in the dark at 37°C for 30 minutes as reported.[14] Then cells were washed twice with PBS and images were viewed and scanned by a Fluoview fluorescent microscope (Olympus, Tokyo, Japan) at 485-nm excitation wavelength and 530-nm emission wavelength for DAF-2 DA fluorescence. The green fluorescent densities were calculated lastly.

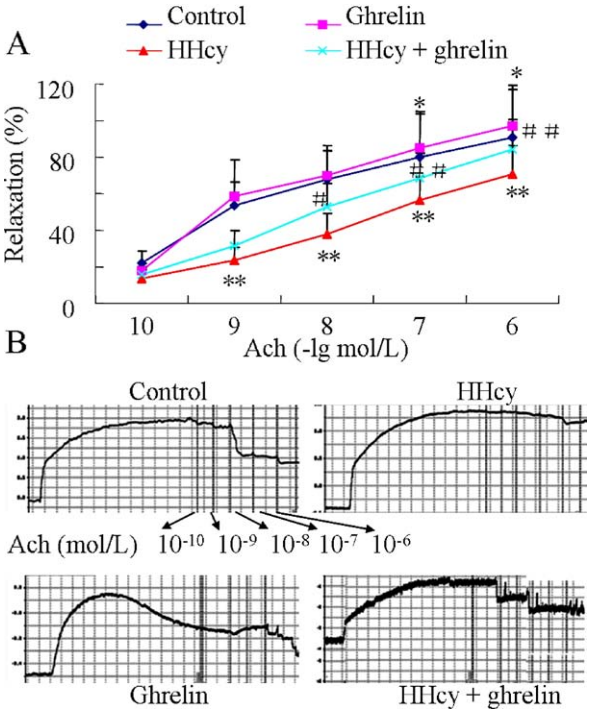

Fig 1. Effects of hyperhomocysteine (*HHcy*) and ghrelin on the rat aortic vasodilatation. **A,** The control and HHcy-induced model aortic rings were truncated and incubated in Krebs solution with or without ghrelin (10^{-6} M). There was significant difference in maximum vessel contraction in response to a range of acetylcholine (*Ach*, 10^{-10} to 10^{-6} mol/L) among all groups (n = 8). Mean data are shown with the standard deviation. **B,** The contraction signals curve relaxations of aortic rings were monitored, and the percentage of vasodilatation was recorded and analyzed. $^{*}P < .05$, $^{**}P < .01$ vs control group; $^{\#}P < .05$, $^{\#\#}P < .01$ vs HHcy group.

Statistical analysis. All of the data are presented as the mean ± standard deviation and analyzed with SPSS 11.0 software (SPSS Inc, Chicago, Ill). Two-sided *t* tests were performed for the data between treatment and control groups. One-way analysis of variance (ANOVA) was used in a multigroup comparison of the measured data. The correlations among NO, ghrelin, and vWF levels in the culture media of HUVECs were determined by linear regression. A value of $P \leq .05$ was considered statistically significant.

RESULTS

Changes in aortic ring relaxation function. Compared with the control, the endothelium-dependent relaxation function was significantly reduced in the HHcy group ($P < .01$; Fig 1). Compared with the HHcy group, the percentage of aortic ring relaxation was significantly increased in Hcy plus ghrelin group ($P < .01$), suggesting that ghrelin can prevent the aorta from undergoing endothelial dysfunction induced by HHcy. The endothelium-dependent relaxation function of the aortic rings with a high dose of acetylcholine (10^{-6} mol/L) was also significantly increased in the ghrelin group compared with the control group ($P < .05$).

Pathologic changes in aortic structure. After treatment, the aortic rings from rats in each group were fixed and analyzed with pathologic examinations. The arterial walls from the rats in the control group (Fig 2, *a-c*) and the ghrelin group (Fig 2, *j-l*) did not show any abnormal changes. However, rat vessels in the HHcy group (Fig 2, *d-f*) were easily disrupted, and endothelium was extruded and disconnected by lipid plaque. The elastic membrane was severely distorted, and some vascular smooth muscle cells underwent necrosis as demonstrated by deeply stained cytoplasm and condensed nuclei. In addition, surrounding connective tissues were infiltrated with granulocytes and monocytes, whereas ghrelin treatment reduced the HHcy-induced vascular injury and improved the vessel wall restoration in the HHcy plus ghrelin group (Fig 2, *g-i*).

Changes in plasma ghrelin levels. After treatment, the endogenous plasma ghrelin levels were detected by ELISA in the four groups. Compared with the control group, the endogenous plasma ghrelin level was upregulated in the HHcy group ($P < .05$), and compared with the control and HHcy groups, the plasma ghrelin level was significantly increased in the HHcy plus GS group ($P < .01$, $P < .05$; Fig 3). Ginsenoside alone did not affect the endocrine level of ghrelin in the GS group.

Primary culture and identification of HUVECs. Using an inverted microscope or phase-contrast microscopy, endothelial cells were visualized as flat, short spindle or polygon shapes with uniform cell size and clear nuclei. HUVECs were identified by indirect immunofluorescent staining with a vWF antibody. Primary and passaged cultures of endothelial cells were stained with yellowish-green fluorescent staining in the cytoplasm and dark green in the nuclei, showing a clear outline. Green spots without any cell outline were occasionally seen in the negative control (data not shown).

Determination of optimal conditions for Hcy-induced HUVECs injury. HUVECs were treated with a range of Hcy concentrations, and the NO content in the culture media was measured by the nitrate reductase assay. Compared with the control group, NO levels in the 200 μM Hcy group and 300 μM Hcy group for 48 hours were significantly reduced ($P < .05$), with 200 μM Hcy group showing the most significant reduction (Fig 4, *A*). Then, cells were pretreated with a range of ginsenoside Rb1 doses for 30 minutes before Hcy stimulation. The NO content was significantly increased in the 10 μM ginsenoside Rb1 pretreatment group compared with the Hcy group ($P < .05$, Fig 4, *B*). Cells were also pretreated with ginsenoside Rb1 before Hcy stimulation over a time-course. The results demonstrated that NO levels were increased in the Hcy plus ginsenoside Rb1 (pretreatment for 30 or 60 minutes) groups significantly than in the Hcy group ($P < .05$), and the Hcy plus Rb1 (pretreatment for 30 minutes) group illustrating the most significant increase. Therefore, 30 minutes was set as the optimal pretreatment time for Rb1 in the following experiments (Fig 4, *C*).

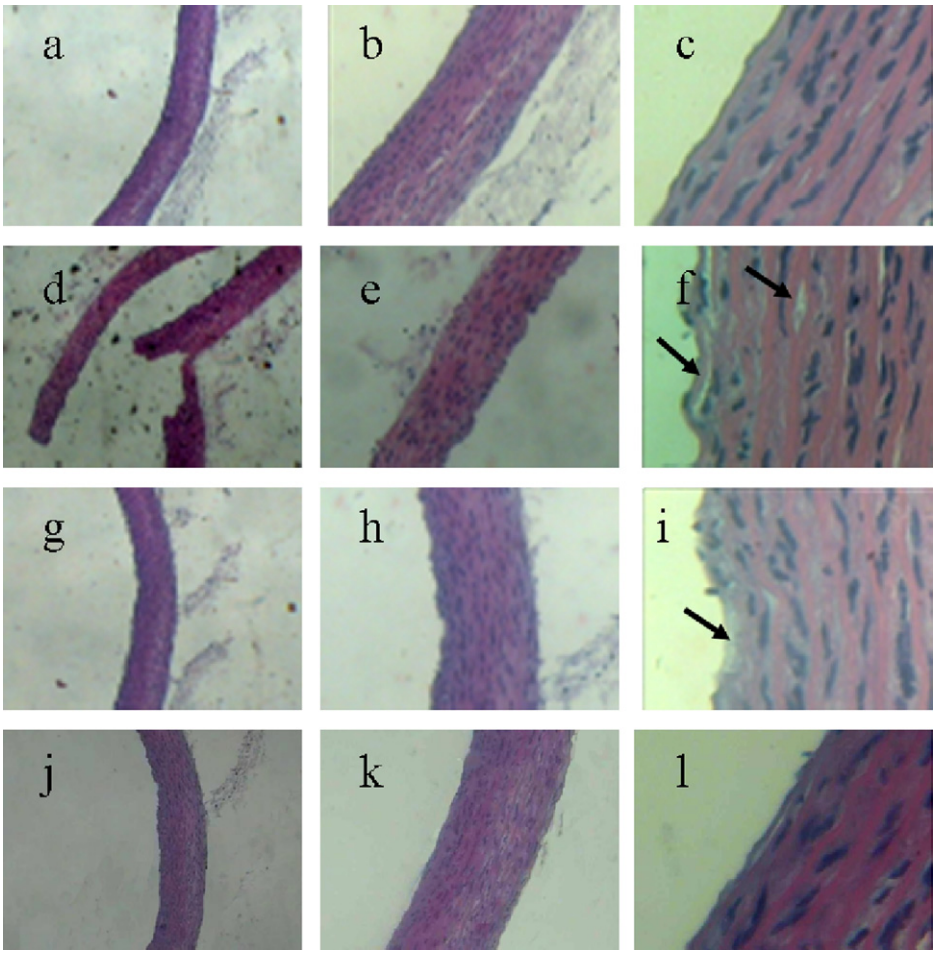

Fig 2. Effects of hyperhomocysteine (*HHcy*) and ghrelin on the aortic endothelial injury and restoration. Aortic walls of the four groups were examined for evidence of pathologic changes using routine hematoxylin and eosin staining. **Panels a, b,** and **c** show the control group; **d, e** and **f,** the HHcy group; **g, h,** and **i,** the HHcy + ghrelin group; and **j, k,** and **l**, the ghrelin group. **Panels a, d, g,** and **j** are at original magnification ×40; **b, e, h,** and **k** are at original magnification ×100; and **c, f, i,** and **l** are at original magnification ×400. **c,** Aortic wall structure is normal. **f,** The *arrows* show the foam cells of aorta lipid plaque and fractured endothelium in the HHcy group. **i,** The *arrows* show improved endothelium in HHcy + ghrelin group. **l,** No obvious pathologic changes were observed in ghrelin group.

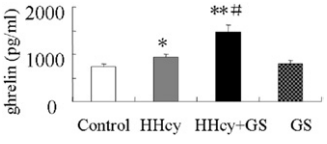

Fig 3. Effects of hyperhomocysteine (*HHcy*) and total ginsenosides (*GS*) on the plasma ghrelin levels in rats. After ginsenoside treatment, the plasma ghrelin levels of the four groups were detected by enzyme-linked immunosorbent assay. $^{*}P < .05$, $^{**}P < .01$ vs control group; $^{\#}P < .05$ vs HHcy group. Mean data are shown with the standard deviation.

The concentrations of NO, ghrelin, and vWF in the culture media of HUVECs and correlation analyses of different factors. As reported in the Table, compared with the control group, NO and ghrelin levels in the culture media of HUVECs were significantly decreased ($P < .01$), and the vWF level was significantly elevated in the Hcy group ($P < .01$). Compared with the Hcy group, NO and ghrelin levels in the culture media of HUVECs were significantly increased ($P < .01$, $P < .05$), and the vWF level was significantly reduced in the Hcy plus Rb1 group ($P < .05$). Linear regression indicated that the ghrelin level was significantly and positively correlated with the NO level ($r = 0.71$, $P < .01$, Fig 5, *A*), whereas it was significantly and negatively correlated to the vWF level ($r = -0.55$, $P < .05$, Fig 5, *B*).

Cell immunohistochemical analysis of ghrelin and eNOS expressions in HUVECs. The protein expression levels of ghrelin and eNOS are shown in Fig 6, *A* and *B*, respectively. The positive signal was defined as brownish-yellow particles in the cytoplasm and/or nuclei. No positive signal was detected in the negative control cells. As shown

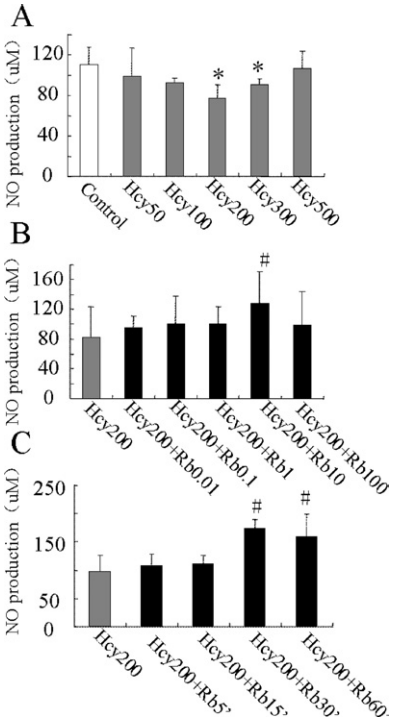

Fig 4. Effects of homocysteine (*Hcy*) and ginsenoside Rb1 *(Rb)* on the nitric oxide (*NO*) levels in the culture media of human umbilical vein endothelial cells (HUVECs). **A,** The NO levels in the culture media of HUVECs treated with a range of Hcy doses for 48 hours (Hcy, 50-500 μM). **B,** The doses effects on NO release into the culture media by ginsenoside Rb1 pretreatments (0.01-100 μM, 30 minutes) plus Hcy (200 μM) stimulation for 48 hours. **C,** The time-courses effects on NO release into the culture media by ginsenoside Rb1 pretreatment (10 μM, 5-60 minutes) plus Hcy (200 μM) for 48 hours. $^{*}P < .05$ vs control group; $^{\#}P < .05$ vs Hcy 200 μM group. Mean data are shown with the standard deviation.

Table. The impacts of homocysteine (*Hcy*) and Rb1 on nitric oxide (*NO*), ghrelin, and von Willebrand factor (*vWF*) levels in the culture media (n = 6)

Groups	*NO (uM/L) Mean ± SD*	*Ghrelin (ng/mL) Mean ± SD*	*vWF (%) Mean ± SD*
Control	121.7 ± 10.37	14.18 ± 0.9949	103.1 ± 10.02
Hcy	100.0 ± 9.844[a]	11.65 ± 1.525[b]	159.2 ± 13.32[b]
Hcy + Rb1	137.5 ± 12.55[c]	13.69 ± 1.254[c]	137.9 ± 12.57[c]

[a]$P < .01$ vs control group; [b]$P < .05$; [c]$P < .01$ vs Hcy group.

in Fig 6, *A-b*, the cells in the control group were clearly stained brown-yellow in the cytoplasm, implying that endothelial cells had a high level of ghrelin expression. As shown in Fig 6, *B-b*, cells in the control group were stained with brownish-yellow particles in the cytoplasm and nuclei, indicating that endothelial cells had a high level of eNOS expression. Compared with the control group, ghrelin and eNOS signals were weakened in the Hcy group ($P < .01$, Fig 6, *A-c* and *B-c*), whereas ghrelin and eNOS signals were both significantly increased in the Hcy plus Rb1 group compared with the Hcy group ($P < .01$, Fig 6, *A-d* and 6, *B-d*). The expression levels of ghrelin and eNOS were further analyzed with a cell image analysis system. Semiquantitative analysis indicated that Rb1 significantly upregulated ghrelin and eNOS expression levels that were down regulated by Hcy (Fig 6, *C*).

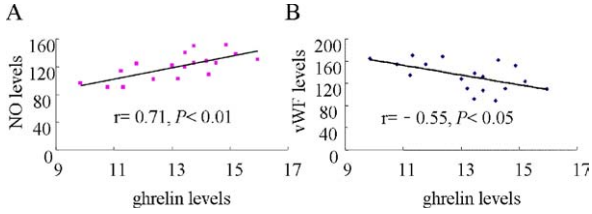

Fig 5. The correlations are shown among nitric oxide (*NO*), ghrelin, and von Willebrand factor (*vWF*) levels. Human umbilical vein endothelial cells were incubated for 48 hours with homocysteine, or homocysteine plus Rb1, and then the culture media were collected for detection. **A,** The correlation between NO and ghrelin levels in the culture media was analyzed by linear regression ($r = 0.71$, $P < .01$). **B,** The correlation between vWF and ghrelin levels in the culture media was analyzed by linear regression ($r = -0.55$, $P < .05$).

The effects of ghrelin inhibition on NO production. Fluorescence images are shown in Fig 7, *A*. The intracellular fluorescence intensity in the Hcy group (Fig 7, *A-b*) was significantly reduced compared with the control (Fig 7, *A-a*). Analysis of mean fluorescence intensity of Hcy group and Hcy plus Rb1 group (Fig 7, *A-d*) confirmed a significant increase of NO content in response to Rb1. One hundred micromoles of [D-Lys3]-GHRP-6,[15] ghrelin receptor antagonist plus Rb1 was added 30 minutes before Hcy stimulation, then intracellular NO content was also detected. Rb1 group (Fig 7, *A-c*) and ghrelin receptor antagonist group (Fig 7, *A-f*) had no effect on NO production, but the ghrelin receptor antagonist significantly abolished the increase of NO production induced by ginsenoside Rb1 in Hcy plus Rb1 plus ghrelin receptor antagonist group (Fig 7, *A-e*).

DISCUSSION

Our study showed that exogenous ghrelin can prevent Hcy-induced vascular endothelial dysfunction and structural damage. The compensatory elevation of plasma endogenous ghrelin level in an HHcy-induced endothelial injury model may be a protective response against injury in rats. We also showed that ginsenoside Rb1 prevented Hcy-induced impairment of endothelium-dependent vasodilatation and improved Hcy-induced reduction of NO production. To our knowledge, this is the first study that shows ghrelin molecule and NO mechanism mediated the protective effects of ginsenosides on endothelium and ginsenoside Rb1 on HUVECs. Our results suggest the potential clinical application of ginsenosides in prevention of HHcy-associated cardiovascular disease.

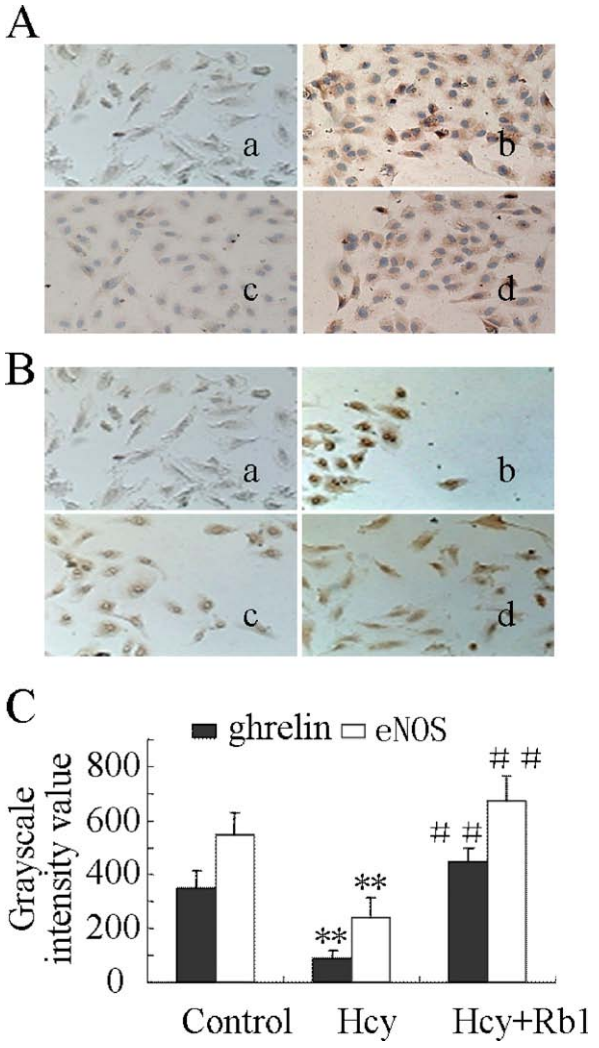

Fig 6. Effects of homocysteine (*Hcy*) and ginsenoside Rb1 is shown on ghrelin and endothelial nitric oxide synthase (*eNOS*) immunoreactivity in human umbilical vein endothelial cells (HUVECs). **A** and **B,** HUVECs were incubated for 48 hours with Hcy, or Hcy plus Rb1, and then intracellular ghrelin and eNOS protein levels (*brown-yellow* staining signal) were determined with immunohistochemical staining. **Panel a,** negative; **b,** control group; **c,** Hcy group; **d,** Hcy + Rb1 group; original magnification ×200. **C,** The staining signal was analyzed by grayscale scanning use the image analysis system. $^{**}P < .01$ vs control group; $^{\#\#}P < .01$ vs Hcy group. Mean data are shown with the standard deviation.

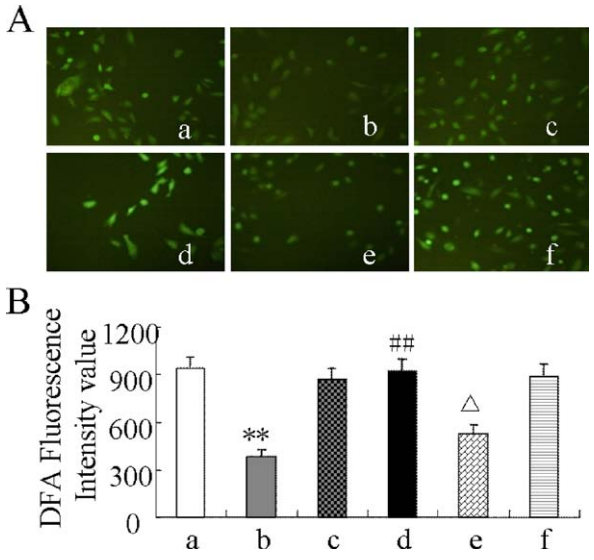

Fig 7. Effects of ghrelin receptor antagonist and ginsenoside Rb1 on intracellular nitric oxide (*NO*) in human umbilical vein endothelial cells (HUVECs) loaded with the NO-sensitive dye 4,5-diaminofluorescein-2 diacetate (DAF-2 DA). **A,** HUVECs were cultured for 48 hours in the following groups: control, Hcy, Rb1, Hcy plus Rb1, Hcy plus Rb1 plus ghrelin receptor antagonist, ghrelin receptor antagonist, then the intracellular NO contents reflected by DAF-2DA fluorescence signal were observed. **(a)** Control; **(b)** Hcy; **(c)** Rb1; **(d)** Hcy + Rb1; **(e)** Hcy + Rb1 + ghrelin receptor antagonist; and **(f)** ghrelin receptor antagonist. The images were obtained by confocal laser microscopy, original magnification ×200. **B,** The bar chart shows the quantification of mean DAF-2DA fluorescence intensity for intracellular NO. $^{**}P < .01$ vs control group; $^{\#\#}P < .01$ vs Hcy group; $^{\Delta}P < .05$ vs Hcy + Rb1 group. Mean data are shown with the standard deviation.

HHcy is regarded as an independent risk factor for myocardial infarction, stroke, and vascular diseases. It was found that prolonged exposure of endothelial cells to Hcy impaired the production of endothelium-derived relaxing factor NO.[16] Hcy can also significantly impair endothelium-dependent vasodilatation function and inhibit the activity of eNOS by reducing Arg transport[17] and activating protein kinase C.[18] This study established an endothelial injury model with a wide dose range of Hcy (50–500 μM Hcy) as reported,[19] and these doses crossed and represented the different severity degrees of clinical HHcy. Our results demonstrated that Hcy can suppress endothelium-dependent vasodilatation function, inhibit eNOS expression and NO production, and induce vWF release, a key parameter and biomarker of endothelial injury.[20]

There is increased clinical evidence concerning the potential benefits of ginseng in cardiovascular diseases. Studies show that ginsenosides may prevent vascular injury and dysfunction through inducing NO release.[21,22] Ginsenosides can enhance the activity of calcium-activated potassium ion channels in vascular smooth muscles and increase the opening rate of the $Ca2^{+}$-activated K^{+} channels (KCa) on the endothelial cell membranes.[23] Our previous study also showed that the Chinese herbal medicine ginseng and its ginsenoside extracts can both prevent Hcy-induced endothelial injury.[6] The ginsenoside Rb1 possesses potent pharmacologic activity toward the cardiovascular system, which has been proven to increase NO production in the vascular endothelium[3,24] and ameliorate the abnormal expression profiles of inflammatory and oxidative stress genes during endothelial injury in rats with a deficiency of vital energy, thereby protecting the vascular

endothelium from injury.[25] Ginsenoside Rb1 has also been shown to enhance eNOS activity, promote NO release, reduce reactive oxygen species production, and inhibit endothelial proliferation and dysfunction induced by Hcy.[26,27] This study also found that eNOS protein expression was downregulated, NO production was reduced, and vWF release, as a marker reflecting endothelial injury and activation, was increased in HUVECs treated with Hcy. However, these effects were all reversed by pretreatment with 10 μM ginsenoside Rb1, a dose that is consistent with the dose referred to in a previous study.[10]

Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor (GHSR), which has an important function in regulating secretion of growth hormone, and circulating ghrelin is mainly synthesized and secreted by the gastric mucosa.[15] However, other tissues, such as cardiac muscle, aorta, coronary artery, and pulmonary artery and vein, can also express ghrelin and its receptor.[28] Ghrelin causes multiple cardiovascular protective effects, including inhibition of inflammation, increase of cardiac contractility and vascular relaxation, reduction of reactive oxygen species production, and prevention of myocardial cell apoptosis during cardiac ischemia.[29]

Shimizu et al[30] found that ghrelin can exert its cardiovascular effects, including inhibition of endothelial death, increase of eNOS expression in endothelial cells, direct induction of NO synthesis, and improvement of endothelial functions, in a growth hormone-independent manner.

Furthermore, ghrelin is the most potent endothelin-1 antagonist in vivo. In endothelium-deprived human internal mammary artery, ghrelin counteracted vasoconstriction induced by the long-lasting vasoconstrictor endothelin-1, suggesting that ghrelin can cause endothelium-independent vasodilatation effects that may be mediated by multiple factors.[31] Studies have shown that ghrelin could improve endothelial function, elevate the biologic activity of NO, directly relax vascular smooth muscle, and suppress sympathetic activity to reduce blood pressure through affecting the central nervous system.[32] Animal experiments have indicated that plasma ghrelin content and ghrelin messenger RNA in the aortic vascular tissue were decreased in rats with angiosteosis, whereas after ghrelin treatment, calcifications of blood vessels and vascular cells were significantly alleviated.[33]

It was reported that the plasma ghrelin level was significantly increased in heart failure patients with cachexia.[34] We previously found that the ghrelin levels in the plasma and myocardial tissues were compensatorily increased during the pathologic course of Adriamycin-induced rat heart failure, which protected the heart by protection of mitochondria, improvement of cardiac energy metabolism, enhancement of antioxidative capacity, and inhibition of myocardial cell apoptosis.[35,36] In this study, we found for the first time that the plasma ghrelin level was significantly elevated in the HHcy-induced endothelial injury rat model compared with control, and that exogenous ghrelin can evidently improve HHcy-induced endothelium-dependent vasodilation dysfunction. This finding suggested that elevation of plasma ghrelin levels in the HHcy group might be a compensatory and protective reaction to injury. A recent study showed that ghrelin might prevent Hcy-induced endothelial dysfunction in porcine coronary artery through regulation of the eNOS/NO signaling pathway.[9] This study first found that ginsenosides may trigger the integral ghrelin endocrine to protect the local vascular endothelium, whether ginsenosides can upregulate the local expression of ghrelin and enhance its interaction with ghrelin receptor (GHSR) in blood vessels has yet to be determined.

Ghrelin is primarily secreted into the blood stream by gastric X/A-like cells in the submucosal region. Approximately 60% to 70% of the ghrelin in circulation is derived from the gastric mucosa, with a secondary contribution from the small intestine. Other tissues of the body may also produce a small amount of ghrelin. In this study, the Hcy-induced compensatory elevation of the plasma ghrelin level may be associated with the "relative scarceness" of local ghrelin autocrine in the endothelial microenvironment. Ginsenosides may regulate energy metabolism and enhance the gastrointestinal ability to produce and secrete more ghrelin into circulation. So, ginsenoside treatment could significantly stimulate ghrelin endocrine. Normal vascular endothelium can synthesize, activate, and release a variety of active substances through metabolism. As the largest endocrine store, it has an important function in regulating vascular tone, hemostasis, antithrombosis, and maintaining normal vascular functions by secretion of multiple active substances. It has been reported that rat and human myocardial cells can synthesize and secrete ghrelin to inhibit cell apoptosis in a paracrine/autocrine manner.[37] In the present study, we found that normal endothelial cells can function like the myocardial cells to express and secrete ghrelin. Furthermore, when endothelial cells were injured, the autocrine ghrelin level was significantly reduced, and the ghrelin level was negatively correlated with the vWF level. All these suggested that the autocrine ghrelin level was associated with the degree of endothelial injury. In addition, this study also showed that ghrelin levels secreted by endothelial cells were positively correlated with NO production.

CONCLUSIONS

Ginsenoside Rb1 protected endothelial cells through upregulation of ghrelin secretion, NO production, and eNOS protein expression, which was reduced by Hcy, implying that ghrelin peptide mediated endothelial protection by ginsenoside Rb1 through regulating the NO signaling pathway. This study provides a thought of seeking both a temporary solution, such as exogenous ghrelin treatment or surgery, and permanent cure with Chinese herbs in clinical vascular diseases.

AUTHOR CONTRIBUTIONS

Conception and design: ZX, TL, WW, YW
Analysis and interpretation: ZX, TL, WW
Data collection: ZX, TL

Writing the article: ZX, TL
Critical revision of the article: ZX, TL, WW, YW
Final approval of the article: ZX, TL, WW, YW
Statistical analysis: ZX, TL
Obtained funding: ZX, WW, YW
Overall responsibility: ZX

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