Biochemical Pharmacology 82 (2011) 148–155

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Ginsenoside Rb1 prevents homocysteine-induced endothelial dysfunction via PI3K/Akt activation and PKC inhibition

Tao-Hua Lan [a,b], Zhi-Wei Xu [a], Zhi Wang [c], Yi-Ling Wu [d], Wei-Kang Wu [a,b,*], Hong-Mei Tan [a,**]

[a] Department of Pathophysiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, PR China
[b] Institute of Integrated Traditional Chinese and Western Medicine, Sun Yat-sen University, Guangzhou 510080, PR China
[c] Department of Anesthesiology, The Second Affiliated Hospital of Sun Yat-sen University, Guangzhou 510000, PR China
[d] Yiling Medicinal Academy, Shijiazhuang 050035, PR China

ARTICLE INFO

Article history:
Received 31 January 2011
Accepted 6 April 2011
Available online 14 April 2011

Keywords:
Ginsenoside Rb1
Homocysteine
NO
PI3K/Akt
PKC

ABSTRACT

Hyperhomocysteinemia (HHcy), a risk factor for cardiovascular disease, is associated with endothelial dysfunction. Ginsenoside Rb1, the major active constituent of ginseng, potently attenuates homocysteine (Hcy)-induced endothelial damage. However, the underlying mechanism remains unknown. In this study, we have investigated the effect of Ginsenoside Rb1 on Hcy-induced endothelial dysfunction and its underlying signal pathway in vivo and in vitro. Ginsenosides prevented Hcy-induced impairment of endothelium-dependent relaxation and Rb1 reversed Hcy-induced reduction of NO production in a dose-dependent manner as detected by nitrate reductase method. Rb1 activated serine-1177 phosphorylation of endothelial nitric oxide synthase (eNOS) and serine-473 phosphorylation of Akt, while inhibited threonine-495 phosphorylation of eNOS as detected by western blotting. Rb1-induced phosphorylation of serine-1177 was significantly inhibited by wortmannin, PI3K inhibitor or SH-5, an Akt inhibitor, and partially reversed by Phorbol 12-myristate 13-acetate (PMA), a PKC activator. PMA also stimulated phosphorylation of threonine-495 which was inhibited by Rb1. Here we show for the first time that Rb1 prevents Hcy-induced endothelial dysfunction via PI3K/Akt activation and PKC inhibition. These findings demonstrate a novel mechanism of the action of Rb1 that may have value in prevention of HHcy associated cardiovascular disease.

1. Introduction

Hyperhomocysteinemia has been recognized as an independent risk factor for atherosclerosis since McCulley first made the association between homocysteine and atherosclerosis in 1969 [1]. Even a slightly high plasma homocysteine level is associated with cardiovascular disease, miocardial infarction, peripheral artery disease, cerebrovascular disease and shock. It is widely accepted that endothelial dysfunction plays a crital role in pathogenesis and development of cornary atherosclerotic disease [2]. A growing body of evidence showed that oxidative stress, vascular smooth muscle cell proliferation and impairment of endothelial function play important roles in HHcy-induced pathophysiological process of cardiovascular disease [3–5].

Ginseng is one of the most popular and well-known herbal drugs in traditional Chinese medicine. It is used worldwide for its various beneficial effects on the cardiovascular, central nervous and endocrine systems. Studies have showed that ginsenosides, the active ingredient of ginseng, have protective roles in many cardiovascular and cerebral diseases via preventing free-radical injury, reducing platelet adhesion, improving lipid profiles, and modifying vasomotor function [6]. Among more than 40 ginsenosides, ginsenoside Rb1 appears to be responsible for most of the pharmaceutical actions of ginseng. Although it has been reported that ginsenoside Rb1 could block homocysteine-induced endothelial dysfunction in porcine coronary arteries [7], the underlying signaling pathways of the ginsenoside Rb1 action have not been fully elucidated.

Abbreviations: Akt, protein kinase B; ECGS, endothelial cell growth supplement; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; GS, ginsenosides; Hcy, homocysteine; HHcy, Hyperhomocysteinemia; HUVECs, human umbilical vein endothelial cells; L-NAME, N-nitro-L-arginine methyl ester hydrochloride; M199, medium 199; NO, nitric oxide; PBS, phosphate-buffered saline; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PMA, Phorbol 12-myristate 13-acetate; SNP, sodium nitroprusside; SST, Succinylsulfathiazole; vWF, von Willebrand factor.

* Corresponding author at: Institute of Integrated Traditional Chinese and Western Medicine, Sun Yat-sen University, Guangzhou 510080, PR China.
Tel.: +86 20 87332028; fax: +86 20 87332028.
** Corresponding author. Department of Pathophysiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, PR China.
Tel.: +86 20 87330028; fax: +86 20 87330028.
E-mail addresses: w2004wk@yahoo.com.cn (W.-K. Wu), thmei40@yahoo.com.cn (H.-M. Tan).

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.04.001

In the current study, we investigated the effect of ginsenoside Rb1 on Hcy-induced endothelial dysfunction and the underlying signaling pathways in vivo and in vitro.

2. Materials and methods

2.1. Materials

Ginsenoside Rb1 (Rb1) is a reference compound (purity >98%) purchased from the Division of Chinese Materia Medica and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), Ministry of Public Health, China. Ginsenosides were provided by Yiling Pharmaceutical Industry Ltd Co (Shijiazhuang, China). M199, trypsin, penicillin, streptomycin and FBS were purchased from Gibco (Invitrogen, Carlsbad, CA, USA). Endothelial cell growth supplement was purchased from BD Biosciences (San Jose, CA, USA). L-Methionine, SST, Hcy, acetylcholine, L-NAME, wortmannin, PMA, heparin and β-actin antibody were purchased from Sigma (St Louis, MO, USA). A23187 and SH-5 were purchased from Merck (Darmstadt, Germany). The NO Assay Kit was obtained from the Nanjing Jiancheng Biotechnology Institute (Nanjing, China). The ELISA kit for vWF was purchased from Shanghai Taiyang Biotech Co, Ltd (Shanghai, China). Antibodies against eNOS, eNOS phosphothreonine 495, eNOS phosphoserine 1177, Akt, Akt phosphoserine 473 were purchased from Cell Signaling Technology (Boston, MA, USA).

2.2. Experiments in rats

2.2.1. HHcy-induced endothelial dysfunction animal model establishment

Male Sprague-Dawley rats (specific-pathogen-free, weighed 150–180 g) were provided by Experimental Animal Center of Sun Yat-sen University. All rats were kept in a room maintained at 24 °C with a 12-h light/dark cycle and fed standard rat chow. The animal experiment was approved by the Animal Care and Use Committee of Sun Yat-sen University. All animal care and procedures conformed to the Council for International Organizations of Medical Sciences (CIOMS) guidelines. Rats were randomized into control group, HHcy model group and HHcy + GS group. Rats in HHcy group and HHcy + GS group were intragastricly administrated L-methionine by 1 g kg^{-1} day^{-1} for 4 weeks [8]. Succinylsulfathiazole (SST) (0.5 g kg^{-1} day^{-1}) was added to the drink of rats in order to avoid bacterial proliferation and subsequent folate production. Rats in HHcy + GS group had been administered ginsenosides (1.2 g kg^{-1} day^{-1}) since 1 week before. Control rats were administered the same volume of purified water.

2.2.2. Plasma NO and vWF analysis

Rats were fasted overnight and anesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.). Blood was collected the next morning, cooled immediately on ice and then centrifuged at 3000 rpm for 20 min at 4 °C. Plasma was separated and stored at −80 °C for assay. The plasma NO levels were assayed by using a NO Assay Kit and the plasma vWF levels were measured by ELISA.

2.2.3. Thoracic aortic ring relaxation assay

The thoracic aortas were isolated immediately after the blood collection and carefully excised of fat and connective tissue, then cut into rings about 3 mm long. The aortic rings were hung on stainless steel hooklets in improved Krebs solution (mmol L^{-1}: NaCl 118, KCl 4.7, $CaCl_2$ 2.6, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, Glucose 5.5, PH 7.4) bubbled with 95% O_2 and 5% CO_2 at 37 °C. Rings were connected to transducers and then the Powerlab system (AD instruments, USA). After that, the rings were equilibrated for 1.5 h under a resting tension of 1.5 g and the Krebs solution was changed every 20 min. The viability of the ring preparation was assessed by contracting vessels with 100 mmol L^{-1} KCl before each experiment. The contraction signals were recorded by the Powerlab system and analyzed with the Chart 5 for Windows software (AD instruments, USA). 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 min. The rings were soaked in 4 ml Krebs solution supplemented with norepinephrine (10^{-6} mol L^{-1}). When the vasoconstriction curves of rings reached the plateau phase, acetylcholine (10^{-10}–10^{-6} mol L^{-1}), A23187 (10^{-5} mol/L, a calcium ionophore that activates eNOS) and SNP (10^{-9}–10^{-5} mol L^{-1}) were added to observe the endothelium-dependent and independent relaxation of aortic rings respectively. In some experiments, vessels were preincubated with eNOS inhibitor, N-nitro-L-arginine methyl ester hydrochloride (L-NAME) (3×10^{-5} mol L^{-1}) 20 min before the addition of acetylcholine. The contraction tension was recorded and relaxation in response to acetylcholine, A23187 and SNP was calculated as the percent reduction from norepinephrine-induced tension.

2.3. Experiments in human umbilical vein endothelial cells

2.3.1. Human umbilical vein endothelial cells (HUVECs) culture

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 HUVECs. Fresh umbilical cords were washed with phosphate-buffered saline (PBS) in a cell culture hood to remove blood and residual blood clots. HUVECs were isolated from human umbilical veins digestion and maintained on gelatin coated T25 flasks in medium 199 (M199) supplemented with 20% fetal bovine serum (FBS), 50 μg ml^{-1} endothelial cell growth supplement (ECGS), 100 U ml^{-1} penicillin, 100 U ml^{-1} streptomycin and 50 μg ml^{-1} heparin [9]. Cultures were maintained at 37 °C in humidified 5% CO_2 incubator. HUVECs were identified by their typical cobblestone appearance and the presence of von Willebrand factor (factor VIII). HUVECs from 2 to 4 passages were used for experiment. HUVECs were divided into the following groups: control, Hcy groups (stimulated with 100, 200, or 500 μmol L^{-1} Hcy), a dose range of ginsenoside Rb1 plus Hcy groups (pretreated with 10^{-2}, 10^{-1}, 1, or 10 μmol L^{-1} Rb1 for 30 min before Hcy stimulation), and a time-course of ginsenoside Rb1 plus Hcy groups (pretreated with 10 μmol L^{-1} Rb1 for 5, 15, 30, or 60 min before Hcy stimulation).

2.3.2. Measurement of NO concentration in culture medium

The concentrations of NO in culture medium were determined through detecting the concentration of nitrite (NO^{2-}), the stable product of NO. HUVECs were seeded in 24-well plate at a density of 4×10^4/well. After treated with indicated concentrations of Hcy and ginsenoside Rb1 for 48 h, culture medium was collected. NO^{2-} content was measured using a microplate method based on the Griess reaction by a NO Assay Kit. The optical densities at 560 nm wavelength were obtained using a Microplate Reader (Thermo, USA) and concentrations of NO were calculated according to the calibration curve.

2.3.3. Western blot analysis

HUVECs were treated with indicated concentrations of Hcy and ginsenoside Rb1. Akt phosphorylation at Ser-473, eNOS phosphorylation at Ser-1177 and Thr-495 were tested by Western blotting.

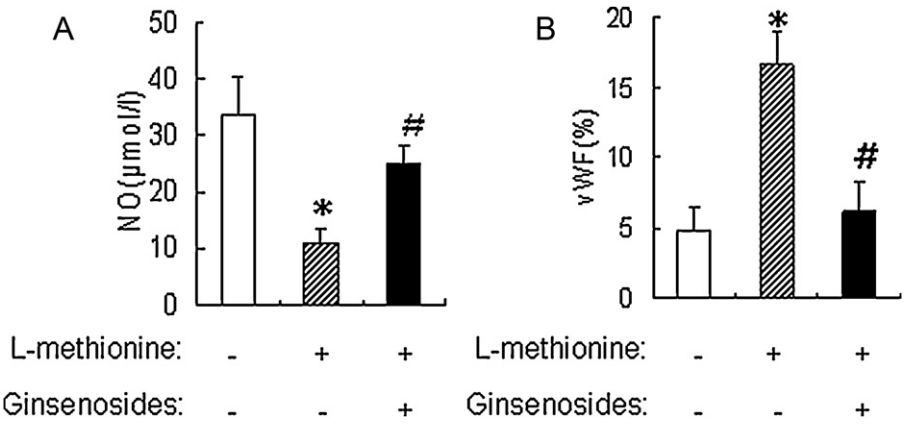

Fig. 1. The impacts of L-methionine administration and ginsenosides on plasma NO (A) and vWF (B) in rats. The HHcy-induced endothelial dysfunction animal model was established by long-term intragastric administration with L-methionine in rats. Ginsenosides were added in the treatment group. Plasma NO and vWF concentration were measured by nitrate reductase method or ELISA respectively. The data are shown as mean ± SD ($n = 8$). $^{*}p < 0.05$ versus control rats, $^{\#}p < 0.05$ versus L-methionine-administrated rats.

To determine the roles of PI3K, Akt and PKC in Rb1-induced changes of eNOS phosphorylation, PI3 K inhibitor wortmanin (5 μmol L^{-1}), Akt inhibitor SH-5 (10 μmol L^{-1}) and PKC activator PMA (100 nmol L^{-1}) were added into the culture medium 60 min or 10 min before cells lysis. Whole cell extract (40 μg) from each sample was analyzed with specific antibodies against eNOS (1:1000 dilution), eNOS phosphoserine 1177 (1:1000 dilution), Akt (1:1000 dilution), Akt phosphoserine 473 (1:1000 dilution) and eNOS phosphothrine 495 (1:1000 dilution). Incubation with monoclonal β-actin antibody (1:10000 dilution) was performed as the loading control. The blots were detected on Kodak X-Omat film by the enhanced chemiluminescence. Quantification of band intensity was performed using the Image J software (NIH, USA).

2.4. Statistical analysis

All in vitro data were from at least three independent experiments. Results were expressed as the mean ± SD. Statistical

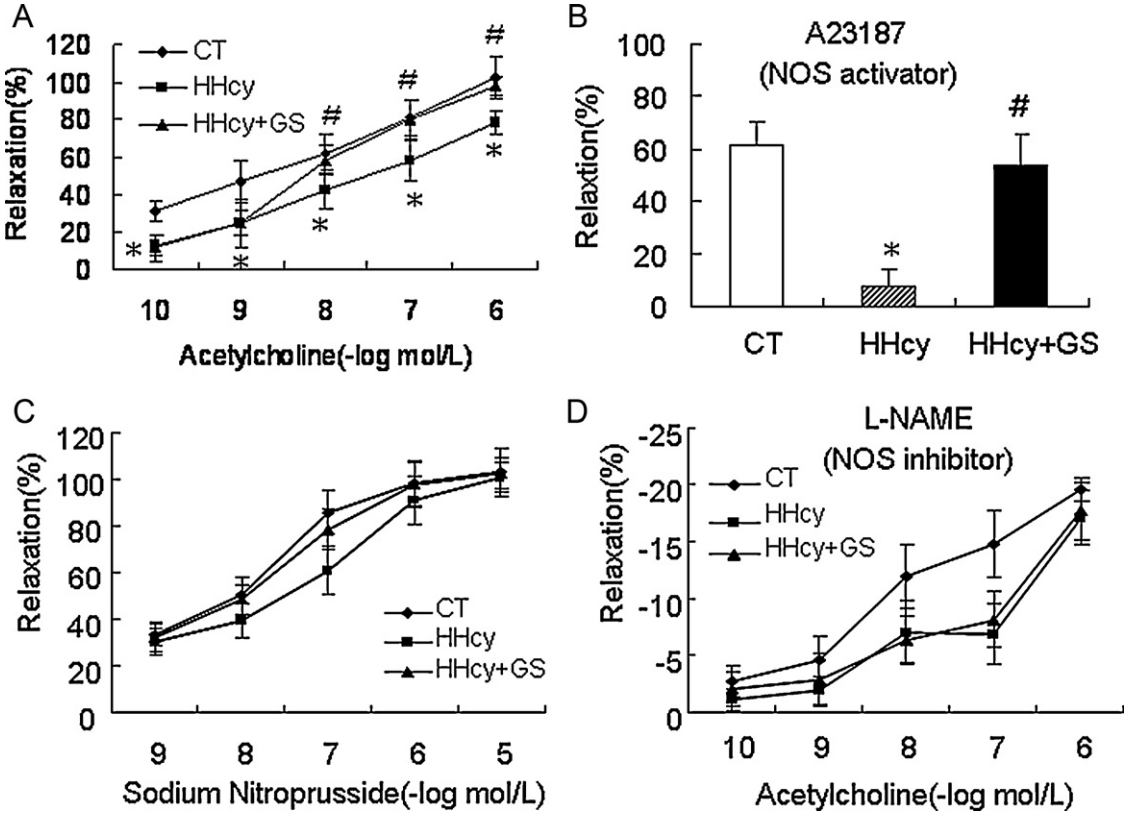

Fig. 2. Effects of L-methionine administration and ginsenosides on the rat aortic relaxation. Thoracic aortic rings were precontracted with KCl and contracted with phenylephrine. Dose-response relaxation was measured for cumulative increments of 10^{-10}–10^{-6} mol L^{-1} acetylcholine (A). Relaxation was also measured in response to the addition of 10^{-5} mol L^{-1} A23187 (B) or 10^{-9}–10^{-5} mol L^{-1} SNP(C). Then, aortic rings were precontracted with KCl, treated with 3×10^{-5} mol L^{-1} L-NAME for 20 min, and contracted with phenylephrine. Dose-response relaxation was measured for cumulative increments of acetylcholine (D). The data are shown as mean ± SD ($n = 8$). $^{*}p < 0.05$ versus control group, $^{\#}p < 0.05$ versus HHcy group. CT, untreated control; HHcy, with only L-methionine administration; HHcy + GS, with both L-methionine and ginsenosides administration.

comparison between two groups was performed using Student's *t* test. Statistical comparison among multiple groups was performed by one-way ANOVA followed by LSD test using the SPSS 11.0 software (SPSS Inc, Chicago). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Ginsenosides induced the increase of plasma NO and the decrease of plasma vWF in rats with HHcy-induced endothelial dysfunction

Homocysteine is a thiol-containing amino acid derived from L-methionine, one of the essential amino aids. The HHcy-induced endothelial dysfunction animal model was established by long-term intragastric administration with L-methionine in rats. Ginsenosides were added in the treatment group. Plasma NO and vWF concentration were measured by nitrate reductase method or ELISA respectively. As shown in Fig. 1, HHcy significantly reduced the level of plasma NO but increased the level of plasma vWF, which were significantly reversed by ginsenosides administration.

3.2. Ginsenosides reversed HHcy-induced impairment of endothelium-dependent relaxation

Endothelial-dependent vasomotion has been the most widely used clinical end point for assessment of endothelial function [10]. To assess whether ginsenosides prevent HHcy-induced endothelial dysfunction, relaxation in response to acetylcholine and sodium nitroprusside (SNP) were performed on aortic rings. Acetylcholine produced a dose-dependent relaxation in precontracted aortic rings from all three groups. However, as showed in Fig. 2A, HHcy significantly impaired relaxation to acetylcholine, while ginsenosides attenuated HHcy-induced impairment of relaxation to acetylcholine with a maximal induction at 10^{-6} mol L^{-1} acetylcholine. After incubation with 10^{-6} mol L^{-1} acetylcholine, control group aortic segment relaxed by $102 \pm 11\%$ and that of HHcy + GS group relaxed by $98 \pm 4\%$, whereas HHcy group aortic segment relaxed by only $78 \pm 6\%$. Similar changes were observed in the relaxation to A23187 (Fig. 2B). Endothelium-independent relaxation produced by NO donor SNP was similar between these three groups (Fig. 2C). To define the contribution of NO in the ginsenosides-induced enhancement of endothelium-dependent relaxation, L-NAME, a specific NOS inhibitor, was added before the addition of acetylcholine. As showed in Fig. 2D, pretreatment of vessels with L-NAME abolished the relaxation response to acetylcholine in all three groups. These results confirmed that HHcy impairs endothelium-dependent relaxation and ginsenosides reverse Hcy-induced impairment of relaxation via eNOS activation.

3.3. Rb1 reversed Hcy-induced reduction of NO production in HUVECs

HUVECs were treated with various concentrations of Hcy for 48 h, and NO content in the culture medium was measured by the NO Assay Kit. NO levels in the presence of Hcy showed a dose-dependent reduction at concentrations 100–500 μmol L^{-1} with a maximal induction at 500 μmol L^{-1} Hcy (Fig. 3A). Then, cells were pretreated with various concentrations of Rb1 for 30 min before 200 μmol L^{-1} Hcy stimulation. As showed in Fig. 3B, Rb1 induced a dose-dependent increase of NO production at concentrations 10^{-2} to 10 μmol L^{-1} with a maximal induction at 10 μmol L^{-1} Rb1. Cells were also pretreated with 10 μmol L^{-1} Rb1 for indicated times before 200 μmol L^{-1} Hcy stimulation. NO levels in the presence of Rb1 showed significant increases for 30 and 60 min pretreatment (Fig. 3C).

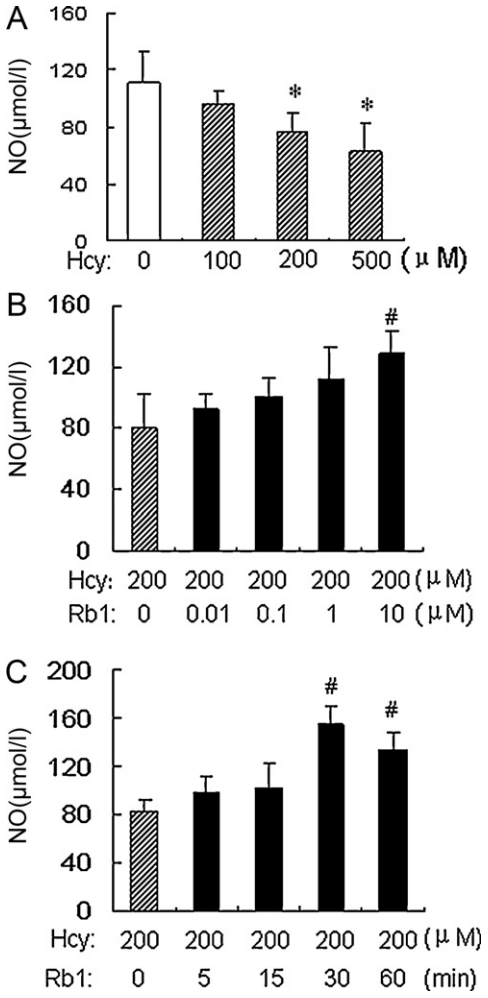

Fig. 3. The effects of Hcy and ginsenoside Rb1 on NO production in HUVECs. HUVECs were treated with various concentrations of Hcy for 48 h (A) and were pretreated with various concentrations of Rb1 for 30 min (B) or with 10 μmol L^{-1} Rb1 for indicated times(C) before Hcy stimulation. NO concentrations in culture medium were measured by using a NO Assay Kit. Experiments were repeated three times and data are shown as mean ± SD. $^{*}p < 0.05$ versus control cells, $^{\#}p < 0.05$ versus 200 μmol L^{-1} Hcy-treated cells.

3.4. Rb1 activated Ser-1177 and inhibited Thr-495 phosphorylation of eNOS in HUVECs

To examine involvement of eNOS in the NO production, the effects of Hcy and Rb1 on eNOS phosphorylation at Ser-1177 and Thr-495 were tested by Western blotting. As showed in Fig. 4, Hcy resulted in a significant inhibition of eNOS phosphorylation at Ser-1177 but a significant activation of eNOS phosphorylation at Thr-495. However, total levels of eNOS protein were not affected by Hcy. Rb1 reversed Hcy-induced dephosphorylation of eNOS at Ser-1177 and phosphorylation of eNOS at Thr-495 with a maximal induction at 10 μmol L^{-1} Rb1 for 30 min pretreatment, without altering the protein expression of total eNOS (Fig. 5a and 6). These results indicate that phosphorylation of eNOS at Ser-1177 and dephosphorylation of eNOS at Thr-495 were involved in the Rb1-induced increase of NO production.

3.5. Rb1 activated Ser-473 phosphorylation of Akt in HUVECs

Since the active kinase that phosphorylates eNOS at Ser-1177 is most probably Akt, we investigated the effects of Hcy and Rb1 on phosphorylation status of Akt. As showed in Fig. 4, Hcy inhibited

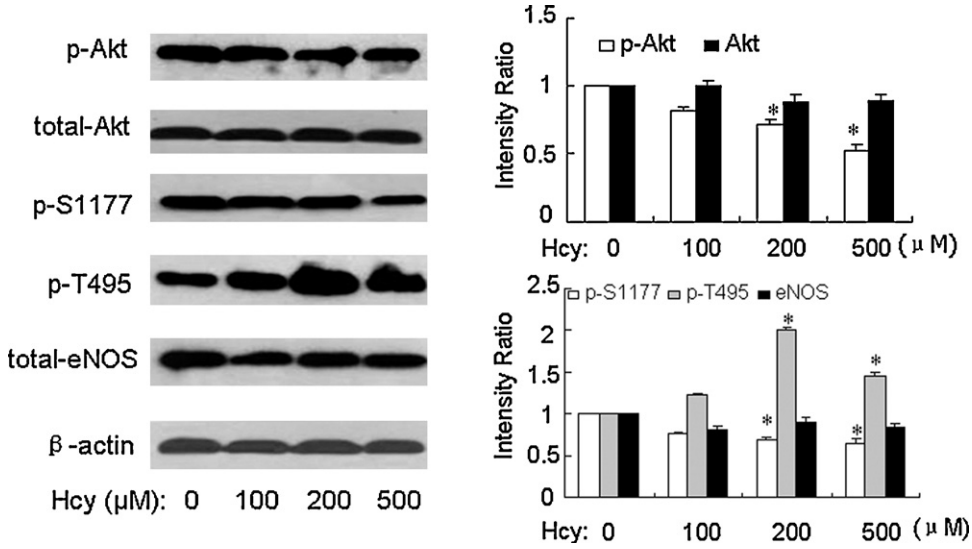

Fig. 4. Effect of Hcy on Akt and eNOS phosphorylation in HUVECs. HUVECs were treated with indicated concentrations of Hcy for 48 h, and phosphorylated Akt at Ser-473, phosphorylated eNOS at Ser-1177, phosphorylated eNOS at Thr-495, total Akt and total eNOS were determined by Western blot analysis. Incubation with monoclonal β-actin antibody was performed as the loading sample control. Experiments were repeated three times and data are shown as mean ± SD. $^{*}p < 0.05$ versus control cells.

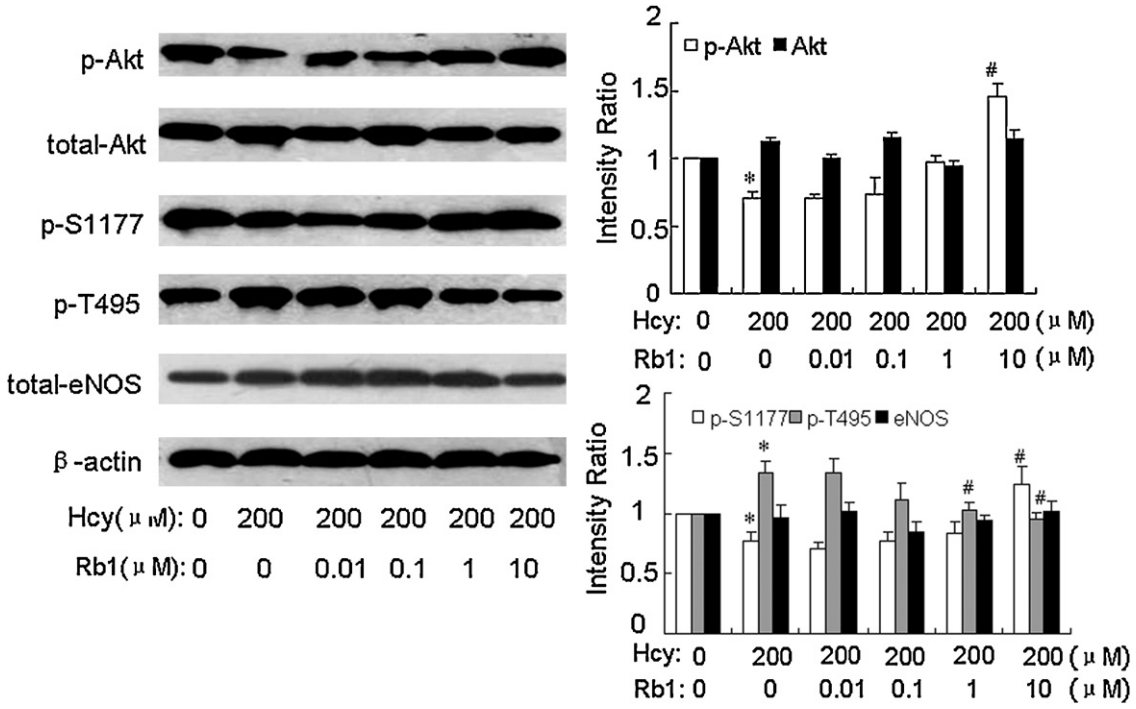

Fig. 5. Effect of indicated concentrations of Rb1 on Akt and eNOS phosphorylation in HUVECs. HUVECs were pretreated with indicated concentrations of Rb1 for 30 min before 200 μmol L^{-1} Hcy stimulation, and phosphorylated Akt at Ser-473, phosphorylated eNOS at Ser-1177, phosphorylated eNOS at Thr-495, total Akt and total eNOS were determined by Western blot analysis. Incubation with monoclonal β-actin antibody was performed as the loading sample control. Experiments were repeated three times and data are shown as mean ± SD. $^{*}p < 0.05$ versus control cells. $^{\#}p < 0.05$ versus 200 μmol L^{-1} Hcy-treated cells.

Akt phosphorylation at Ser-473 in a dose-dependent manner with a maximal induction at 500 μmol L^{-1} Hcy. However, Hcy had no effect on total Akt. Rb1 reversed Hcy-induced dephosphorylation of Akt with a maximal induction at 10 μmol L^{-1} Rb1 for 30 min pretreatment, without altering the protein expression of total Akt (Fig. 5 and 6). These results indicate that Akt phosphorylation at Ser-473 may be involved in the Rb1-induced phosphorylation of eNOS.

3.6. Rb1 induced eNOS phosphorylation at Ser-1177 via PI3K/Akt activation in HUVECs

To determine whether PI3K/Akt plays a role in Rb1-induced eNOS phosphorylation at Ser-1177, PI3K inhibitor wortmannin and Akt inhibitor SH-5 were used before cells lysis. As showed in Fig. 7, Rb1-induced eNOS phosphorylation at Ser-1177 was significantly inhibited by wortmannin and SH-5. These results indicate that Rb1-induced eNOS phosphorylation at Ser-1177 is dependent on the activation of PI3K/Akt signaling pathway.

3.7. Rb1 induced eNOS phosphorylation at Ser-1177 and inhibited eNOS phosphorylation at Thr-495 via PKC inhibition in HUVECs

To determine whether PKC plays a role in Rb1-induced eNOS phosphorylation at Ser-1177 and eNOS dephosphorylation at Thr-495, PKC activator PMA was used before cells lysis. As showed in Fig. 8, Rb1-induced eNOS phosphorylation at Ser-1177 was

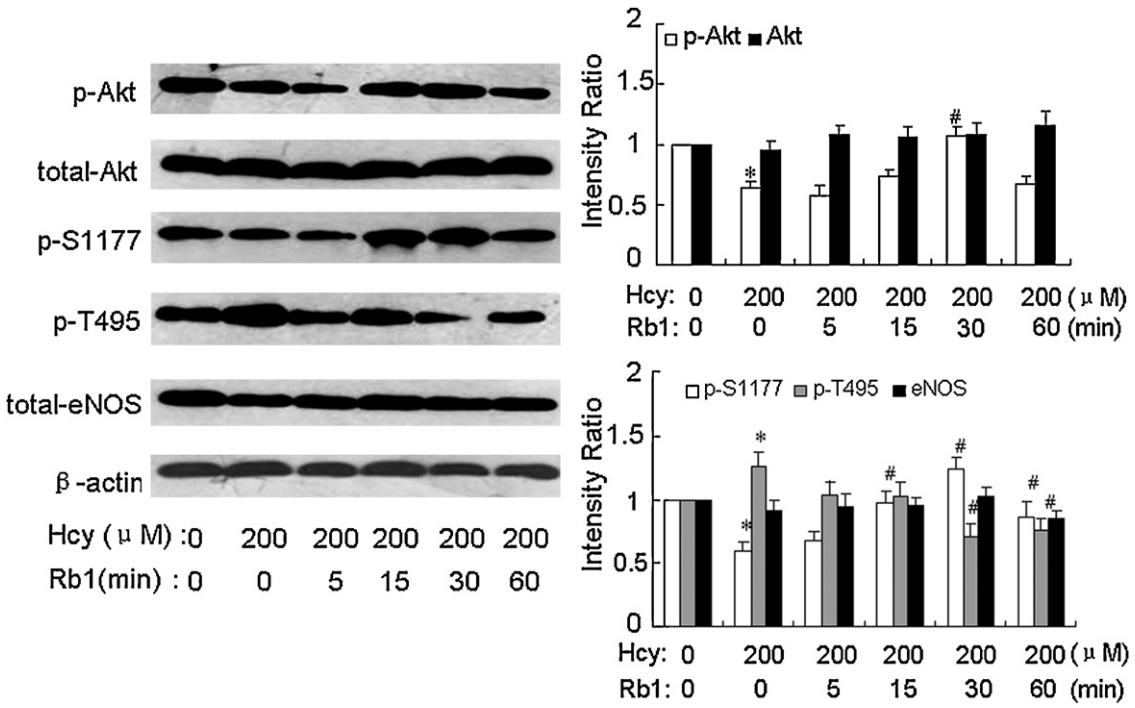

Fig. 6. Effect of Rb1 pretreated for indicated times on Akt and eNOS phosphorylation in HUVECs. HUVECs were pretreated 10 μmol L^{-1} Rb1 for indicated times before 200 μmol L^{-1} Hcy stimulation, and phosphorylated Akt at Ser-473, phosphorylated eNOS at Ser-1177, phosphorylated eNOS at Thr-495, total Akt and total eNOS were determined by Western blot analysis. Incubation with monoclonal β-actin antibody was performed as the loading sample control. Experiments were repeated three times and data are shown as mean ± SD. $^{*}p < 0.05$ versus control cells. $^{\#}p < 0.05$ versus 200 μmol L^{-1} Hcy-treated cells.

partially inhibited by PMA, while Rb1-induced dephosphorylation of eNOS at Thr-495 was significantly reversed by PMA. These results indicate that Rb1-induced eNOS phosphorylation at Ser-1177 and dephosphorylation of eNOS at Thr-495 are partially dependent on the inhibition of PKC signaling pathway.

4. Discussion

HHcy is now a common risk factor for cardiovascular disease, similar to hyperlipidemia, hypertension, and smoking [11,12]. It is believed that a high concentration of plasma Hcy, which is derived from L-methionine, is the main causes of the vascular endothelium injury by inducing the increased oxidative stress and decreased NO bioavailability [13–15]. Endothelium dysfunction is known as an initial step of arteriosclerosis and alterations of blood vessel contraction and relaxation, permeability, and changing platelet and leukocyte adhesions. It has been showed that HHcy impaired endothelial function and eNOS activity via PKC activation [16]. Our results confirmed that L-methionine-induced HHcy could impair endothelium-dependent relaxation, reduce the production of endothelium-derived relaxing factor NO and induce the release of vWF, a key parameter and biomarkers of endothelial injury [17]. In addition, we demonstrated that Hcy blocked eNOS activity via inhibiting eNOS phosphorylation at Ser-1177 and Akt phosphorylation at Ser-473, while activating eNOS phosphorylation at Thr-495.

Ginseng becomes popular in the Western world for its alleged tonic effects and possible curative and restorative properties. Ginsenosides, the active ingredient of ginseng, have attracted much attention for its multiple pharmacological effects on

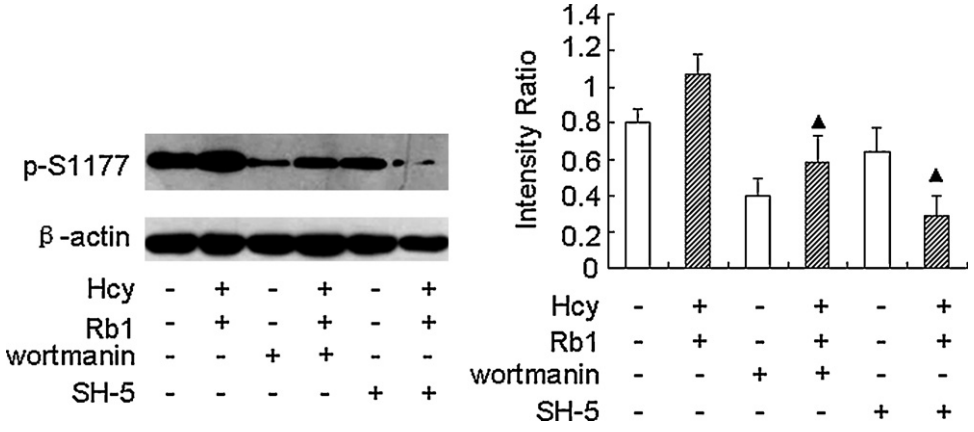

Fig. 7. PI3K/Akt activation was involved in Rb1-induced eNOS phosphorylation in HUVECs. HUVECs were pretreated with 10 μmol L^{-1} Rb1 for 30 min before 200 μmol L^{-1} Hcy stimulation. 5 μmol L^{-1} wortmannin (PI3K inhibitor) and 10 μmol L^{-1} SH-5 (Akt inhibitor) were added into the culture medium 60 min before cells lysis. The change of phosphorylation status of Ser-1177 was detected by Western blot analysis. Incubation with monoclonal β-actin antibody was performed as the loading sample control. Experiments were repeated three times and data are shown as mean ± SD. $^{\blacktriangle}p < 0.05$ versus Rb1-treated cells.

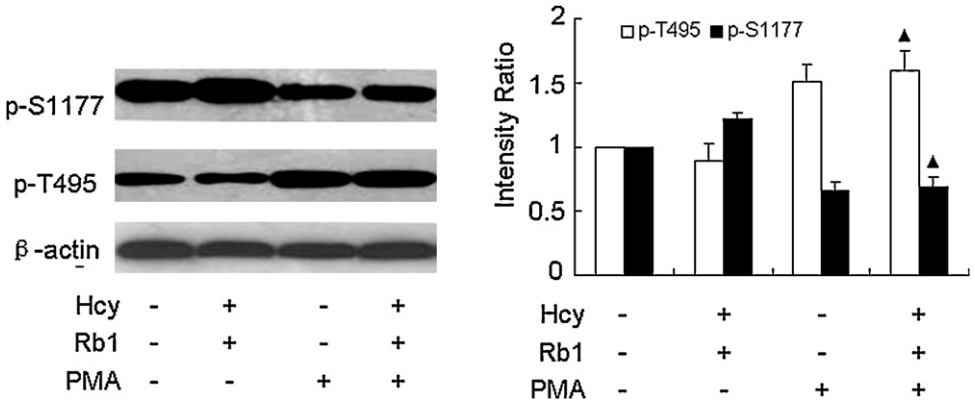

Fig. 8. PKC inhibition was involved in Rb1-induced eNOS phosphorylation at Ser-1177 and eNOS dephosphorylation at Thr-495 in HUVECs. HUVECs were pretreated with 10 μmol L^{-1} Rb1 for 30 min before 200 μmol L^{-1} Hcy stimulation. 100 nmol L^{-1} PMA (PKC activator) was added into the culture medium 10 min before cells lysis. The change of phosphorylation status of and Thr-495 were detected by Western blot analysis. Incubation with monoclonal β-actin antibody was performed as the loading sample control. Experiments were repeated three times and data are shown as mean ± SD. ▲$p < 0.05$ versus Rb1-treated cells.

cardiovascular system, central nervous system, endocrine system and immune system [18]. There is increased clinical evidence concerning the potential benefits of ginseng in cardiovascular diseases. It has been reported that co-administration of ginseng saponins with methionine not only reduced methionine-induced HHcy but also attenuated endothelial damages of aortic artery vessels [19]. Studies showed that ginsenosides may prevent vascular dysfunction through inducing NO release [20,21]. The present study established a methionine-induced HHcy rat model, and showed that ginsenosides significantly attenuated HHcy-induced impairment of relaxation to acetylcholine and A23187, the eNOS activator, which was inhibited by L-NAME, a NOS specific inhibitor, while relaxation to SNP, the NO donor, was preserved. Our results suggested that eNOS-NO signaling, but not altered vascular smooth muscle cell responsiveness, was responsible for the effects of ginsenosides on HHcy-induced endothelial dysfunction.

The components of ginsenosides belong either to the protopanaxadiol or protopanaxatriol groups [22]. Ginsenosides Rb1, the major active constituent of ginseng, represents protopanaxadiols. Rb1 possesses potent pharmacologic activity toward the cardiovascular system, which has been proven to increase NO production in the vascular endothelium [23]. It was found that PI3K/Akt and MEK/ERK pathways and androgen receptor are involved in the regulation of acute eNOS activation by Rb1 in human aortic endothelial cells [24]. Ginsenoside Rb1 prevents Hcy-induced inhibition of endothelial proliferation and suppresses ROS production [25]. It was also reported that Rb1 effectively blocked Hcy-induced endothelial damage through improving Hcy-induced reduction of eNOS expression, reducing Hcy-induced oxidative stress in porcine coronary arteries [7]. However, the underlying signaling pathways of the effects of ginsenoside Rb1 on Hcy-induced endothelial dysfunction have not been fully elucidated.

Nitric oxide is one of the most important substances released from vascular endothelial cell [26]. Endothelial NO has antiatherosclerotic properties, such as inhibition of platelet aggregation, leukocyte adhesion, smooth muscle cell proliferation, and expression of genes involved in atherosclerosis [27]. Our study found that Rb1 reversed the Hcy-induced reduction of NO production in cultured HUVECs. Furthermore, endothelial nitric oxide synthase is the direct enzyme responsible for NO generation in endothelial cells and the post-translational regulation of eNOS activity is mainly influenced by the coordinated phosphorylation and dephosphorylation of amino acid [28,29]. Ser-1177, a serine residue in the reductase domain, and Thr-495, a threonine residue, are two important phosphorylation sites in regulating eNOS activity [30]. Phosphorylation of Ser-1177 is critical for eNOS activation while phosphorylation of Thr-495 leads to inhibition of eNOS activity in endothelial cells. In the present study, we showed for the first time that Rb1 not only reversed Hcy-induced inhibition of Akt phosphorylation at Ser-473 and eNOS phosphorylation at Ser-1177, but also inhibited Hcy-induced phosphorylation of eNOS at Thr-495.

Evidence suggested that PI3K/Akt contributes to enhanced eNOS phosphorylation at Ser1177 and production of NO [31,32]. PKC signaling inhibits eNOS activity not only by phosphorylating Thr-495 but also by dephosphorylating Ser-1177 in endothelial cells [33,34]. Our study further found that Rb1-induced eNOS phosphorylation at Ser-1177 was inhibited by PI3K inhibitor wortmannin and Akt inhibitor SH-5, and PKC activator PMA partially reversed Rb1-induced eNOS phosphorylation at Ser-1177 and dephosphorylation of eNOS at Thr-495. These data lead us to conclude that PI3K/Akt activation and PKC inhibition mediated Rb1-induced phosphorylation of eNOS at Ser-1177 and dephosphorylation of eNOS at Thr-495, leading to increase in NO production in HUVECs.

However, it has been reported that PKC isoforms play the intricate roles in regulating eNOS activity. AlphaPKC is upstream of Akt activation and eNOS phosphorylation on Ser1177 [35]. BetaPKC and zetaPKC are responsible for the phosphorylation of eNOS on Thr495 [36,37]. Monti et al. recently demonstrated that reactive oxygen species formation in endothelial dysfunction results from uncontrolled endothelial nitric oxide synthase (eNOS) activity mediated by activation of deltaPKC or inhibition of varepsilonPKC [38]. Inhibition of deltaPKC or activation of varepsilonPKC corrects the perturbed phosphorylation state of eNOS, thus increased endothelial cell survival. Therefore, to find out the roles of PKC isoforms in Rb1-induced eNOS phosphorylation, a further study is needed.

Taken together, our present study indicated that ginsenoside Rb1 prevents homocystine-induced endothelial dysfunction via PI3K/Akt activation and PKC inhibition. Although more detailed signaling pathways need to be determined, our results demonstrate a novel mechanism of the effects of Rb1 on HHcy-induced endothelial dysfunction and provide new evidence for the potential clinical application of ginseng in prevention of HHcy associated cardiovascular disease.

Acknowledgement

The present work was supported by the National Key Basic Research and Development Project of China (973 Project, No. 2005CB523305).

References

[1] McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. Am J Pathol 1969;56:111–28.
[2] Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362:801–9.
[3] Lentz SR, Sobey CG, Piegors DJ, Bhopatkar MY, Faraci FM, Malinow MR, et al. Vascular dysfunction in monkeys with diet-induced hyperhomocyst(e)inemia. J Clin Invest 1996;98:24–9.
[4] Liu X, Shen J, Zhan R, Wang Z, Wang Z, Zhang Z, et al. Proteomic analysis of homocysteine induced proliferation of cultured neonatal rat vascular smooth muscle cells. Biochim Biophys Acta 2009;1794:177–84.
[5] Ventura E, Durant R, Jaussent A, Picot MC, Morena M, Badiou S, et al. Homocysteine and inflammation as main determinants of oxidative stress in the elderly. Free Radic Biol Med 2009;46:737–44.
[6] Zhou W, Chai H, Lin PH, Lumsden AB, Yao Q, Chen CJ. Molecular mechanisms and clinical applications of ginseng root for cardiovascular disease. Med Sci Monit 2004;10:187–92.
[7] Zhou W, Chai H, Lin PH, Lumsden AB, Yao Q, Chen C. Ginsenoside Rb1 blocks homocysteine-induced endothelial dysfunction in porcine coronary arteries. J Vasc Surg 2005;41:861–8.
[8] Ungvari Z, Pacher P, Rischák K. Dysfunction of nitric oxide mediation in isolated rat arterioles with methionine diet-induced hyperhomocysteinemia. Arterioscler Thromb Vasc Biol 1999;19:1899–904.
[9] Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973;52:2745–56.
[10] Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. Circulation 2007;115(10):1285–95.
[11] Wang H, Tan H, Yang F. Mechanisms in homocysteine-induced vascular disease. Drug Discov Today (Dis Mech) 2005;2:25–31.
[12] Yang F, Tan HM, Wang H. Hyperhomocysteinemia and atherosclerosis. Acta Physiol Sin 2005;57:103–14.
[13] Harker LA, Ross R, Slichter S, Scott RC. Homocystine-induced arteriosclerosis. The role of endothelial cell injury and platelet response in its genesis. J Clin Invest 1976;58:731–41.
[14] Rodgers GM, Conn MT. Homocysteine, an atherogenic stimulus, reduces protein C activation by arterial and venous endothelial cells. Blood 1990;75:895–901.
[15] Welch GN, Loscalzo J. Homocysteine and atherothrombosis. N Engl J Med 1998;338:1042–50.
[16] Jiang X, Yang F, Tan H, Liao D, Bryan Jr RM, Randhawa JK, et al. Hyperhomocystinemia impairs endothelial function and eNOS activity via PKC activation. Arterioscler Thromb Vasc Biol 2005;25:2515–21.
[17] Weyer C, Yudkin JS, Stehouwer CD, Schalkwijk CG, Pratley RE, Tataranni PA. Humoral markers of inflammation and endothelial dysfunction in relation to adiposity and in vivo insulin action in Pima Indians. Atherosclerosis 2002;161:233–42.
[18] Glenn MB, Lexell J, Ginseng J. Head Trauma Rehabil 2003;18:196.
[19] Kim JH, Cho SY, Kang CW, Yoon IS, Lee JH, Jeong SM, et al. Ginseng saponins diminish adverse vascular effects associated with chronic methronine-induced hyperhomocysteinemia. Biol Pharm Bull 2006;29(12):2425–31.
[20] Li Z, Niwa Y, Sakamoto S, Shono M, Chen X, Nakaya Y. Induction of inducible nitric oxide synthase by ginsenosides in cultured porcine endothelial cells. Life Sci 2000;67:2983–9.
[21] Chen X. Cardiovascular protection by ginsenosides and their nitric oxide releasing action. Clin Exp Pharmacol Physiol 1996;23:728–32.
[22] Shibata S, Tanaka O, Ando T, Sado M, Tsushima S, Ohsawa T. Chemical studies on oriental plant drugs XIV. Protopanaxadiol, a genuine sapogenin of ginseng saponins. Chem Pharm Bull (Tokyo) 1966;14:595.
[23] Xie H, Hu G, Suo Z. Effect of ginsenoside Rb1 and astragalus polysaccharide on the excretion of NO IL-6 and TNF-α in micro-vascular endothelial cells. Acta Vet Zool Sin 2006;37:903–7.
[24] Yu J, Eto M, Akishita M, Kaneko A, Ouchi Y, Okabe T. Signaling pathway of nitric oxide production induced by ginsenoside Rb1 in human aortic endothelial cells: a possible involvement of androgen receptor. Biochem Biophys Res Commun 2007;353:767–9.
[25] Ohashi R, Yan S, Mu H, Chai H, Yao Q, Lin PH, et al. Effects of homocysteine and ginsenoside Rb1 on endothelial proliferation and superoxide anion production. J Surg Res 2006;133:89–94.
[26] Albrecht EW, Stegeman CA, Heeringa P, Henning RH, van Goor H. Protective role of endothelial nitric oxide synthase. J Pathol 2003;199:8–17.
[27] Lowenstein CJ, Dinerman JL, Snydel SH. Nitric oxide: a physiologic messenger. Ann Intern Med 1994;120:227–37.
[28] Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J 1994;298:249–58.
[29] Fleming I, Busse R. Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. Am J Physiol Regul Integr Comp Physiol 2003;284:R1–2.
[30] Mount PF, Kemp BE, Power DA. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. J Mol Cell Cardiol 2007;42:271–9.
[31] Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 1999;399:601–5.
[32] Peng X, Haldar S, Deshpande S, Irani K, Kass DA. Wall stiffness suppresses Akt/eNOS and cytoprotection in pulse-perfused endothelium. Hypertension 2003;41:378–81.
[33] Fleming I, Fisslthaler B, Dimmeler S, Kemp BE, Busse R. Phosphorylation of Thr495 regulates Ca^{2+}/calmodulin-dependent endothelial nitric oxide synthase activity. Circ Res 2001;88:e68–75.
[34] Michell BJ, Chen ZP, Tiganis T, Stapleto D, Katsis F, Power DA, et al. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. J Biol Chem 2001;276:17625–8.
[35] Partovian C, Zhuang Z, Moodie K, Lin M, Ouchi N, Sessa W, et al. AlphaPKC activates eNOS and increases arterial blood flow in vivo. Circ Res 2005;97:482–7.
[36] Payne GA, Glenn Bohlen H, Deniz Dincer U, Borbouse L, Tune JD. Periadventitial adipose tissue impairs coronary endothelial function via PKC-beta-dependent phosphorylation of nitric oxide synthase. Am J Physiol Heart Circ Physiol 2009;297:H460–5.
[37] Oubaha M, Gratton J-P. Phosphorylation of endothelial nitric oxide synthase by atypicalPKC{zeta} contributes to angiopoietin-1-dependent inhibition of VEGF-induced endothelial permeability in vitro. Blood 2009;114:3345–51.
[38] Monti M, Donnini S, Giachetti A, Mochly-Rosen D, Ziche M. δPKC inhibition or εPKC activation repairs endothelial vascular dysfunction by regulating eNOS post-translational modification. J Mol Cell Cardiol 2010;48:746–56.