Ginsenoside Rb1 blocks homocysteine-induced endothelial dysfunction in porcine coronary arteries

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Objective: Homocysteine (Hcy) is an independent risk factor for atherosclerosis. This study investigates the effects of ginsenoside Rb1, a major constituent of ginseng, on Hcy-induced endothelial dysfunction and molecular changes in porcine coronary arteries.

Methods: The coronary arteries were harvested from pig hearts and cut into 5-mm ring segments, which were then divided into six groups, including control, Hcy alone (50 μM), low-dose (1 μM) or high-dose (10 μM) Rb1 alone, and Hcy plus low-dose or high-dose Rb1. After 24-hour incubation, the rings were analyzed for vasomotor function in response to thromboxane A2 analog U46619, bradykinin, and sodium nitroprusside (SNP), respectively. In addition, superoxide anion was assessed by lucigenin-enhanced chemiluminescence analysis. Endothelial nitric oxide synthase (eNOS) was studied using real-time polymerase chain reaction and Western blot.

Results: Endothelium-dependent relaxation (bradykinin) was significantly reduced in rings treated with Hcy alone as compared with the control (49.80% vs 71.77%, n = 8, P < .05), whereas neither high-dose nor low-dose Rb1 alone affected the endothelium-dependent relaxation. The low-dose Rb1-Hcy combined group had a partially improved endothelium-dependent relaxation (54.44%), whereas the high-dose Rb1-Hcy combined group showed a complete recovery of endothelium-dependent relaxation (72.89%). There was no substantial difference in maximal contraction induced by U46619 or endothelium-independent relaxation by SNP among all groups (P > .05). Furthermore, superoxide anion was markedly increased by 137% in the Hcy-treated group as compared with the control, but there were no statistically significant changes from the control in all other groups (P > .05). Lastly, eNOS mRNA and protein levels were substantially reduced in the Hcy-treated group, but not in the Rb1-Hcy combined groups.

Conclusions: This is the first study to show that ginsenoside Rb1 can effectively block Hcy-induced endothelial dysfunction and superoxide anion production as well as eNOS downregulation in porcine coronary arteries. This study suggests that ginseng and its active constituents may have potential clinical applications in controlling Hcy-associated vascular injuries. (J Vasc Surg 2005;41:861-8.)

Clinical Relevance: Homocysteine (Hcy) is an independent risk factor for atherosclerosis and other vascular lesions. It causes endothelial dysfunction and oxidative stress. Ginseng compounds have effects of vasorelaxation and antioxidation. The purpose of this study was to determine the effect of ginsenoside Rb1, a major constituent of ginseng, on Hcy-induced endothelial dysfunction and molecular changes in porcine coronary arteries. Our results showed that ginsenoside Rb1 can effectively block Hcy-induced dysfunction of endothelium-dependent vasorelaxation as well as superoxide anion production and eNOS downregulation. This study suggests that ginseng compounds may have potential clinical applications in controlling Hcy-associated vascular diseases and other vascular lesions.

Since McCulley first made the association between homocysteine and atherosclerosis in 1969, multiple studies have shown that Hcy is an independent risk factor for atherosclerotic diseases.1 Plasma homocysteine (Hcy) levels correlated with cardiovascular mortality and with peripheral and coronary artery disease.2-4 A growing body of evidence suggests that oxidative stress plays an important role in the effects of Hcy on the cardiovascular system. In earlier studies, we showed that Hcy significantly impaired endothelium-dependent vasorelaxation and decreased endothelial nitric oxide synthase (eNOS) immunoreactivity in porcine coronary arteries.5,6 It is widely accepted that endothelial dysfunction is an initial step of atherosclerosis by virtue of impairing endothelium-dependent factors, altering blood vessel permeability, and changing platelet and leukocyte adhesions. Additionally, a strong correlation between plasma Hcy levels and the oxidative stress marked by plasma malondialdehyde was identified in patients with cardiovascular diseases.7 Ginseng, a widely recognized herbal drug, has been reported to have a wide range of therapeutic and pharmacologic uses. Ginseng’s genus name Panax is derived from the Greek words pan (all) and akos (cure), meaning cure-all. Ginseng root has been used extensively in Chinese medi-
cinine and has become increasingly popular in the Western world for its alleged tonic effect and possible curative and restorative properties. There is increased clinical evidence concerning the potential benefits of ginseng roots in cardiovascular diseases. Studies have shown that the ginseng root and ginsenosides, the major constituents of ginseng, have protective roles in atherosclerotic plaque formation and various vascular injuries in part because of the prevention of free-radical injury to the vascular endothelium.\(^8\,^9\) We hypothesized that ginseng and its active constituents could protect the vessels from Hcy damage. In this study, we investigated the molecular mechanisms of and the functional effects of ginsenoside Rb1 (Rb1) on Hcy-induced endothelial dysfunction in porcine coronary arteries. The data from our study suggest a potential clinical application of ginseng in controlling Hcy-associated vascular injuries.

**MATERIALS AND METHODS**

**Chemical and reagents.** Hcy (DL-homocysteine), dimethyl sulfoxide (DMSO), thromboxane A2 analog (9, 11-dideoxy-11a, 9 a-epoxymethanoprostaglandin F2a) (U46619), bradykinin, Rb1 (from Panax quinquefolium root, dissolved in dimethyl sulfoxide), Tri-Reagent, tris-buffered saline (TBS) solution, phosphate-buffered saline (PBS) solution, antihuman β-actin monoclonal antibody, and Tween 20 were obtained from Sigma (St. Louis, MO). Dulbecco modified Eagle medium (DMEM) was obtained from Life Technologies, Inc (Grand Island, NY), and antibiotic-antimycotic was obtained from Mediatech Inc (Herndon, VA). The iScript cDNA Synthesis Kit and iQ SYBR Green SuperMix Kit were obtained from Bio-Rad Laboratories (Hercules, CA). The protein assay kit and polyacrylamide gels were obtained from Bio-Rad Laboratories. Antibody against human eNOS was obtained from BD Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated goat antimouse secondary antibodies and the enhanced chemiluminescence kit were obtained from Amersham life Sciences (Buckinghamshire, England). The biotinylated horse antimouse immunoglobulin G and avidin-biotin complex kit were obtained from Vector Labs (Burlingame, CA).

**Isometric tension of porcine coronary arteries.** The myograph system used in our laboratory has been previously described.\(^5\,^10\,^12\) Pig hearts (n = 8) were harvested from 6- to 7-month-old farm pigs within 10 minutes of killing at a local slaughterhouse. The right coronary artery was harvested and cut into multiple 5-mm rings, which were then incubated in DMEM and divided into six groups (eight rings per group): DMSO control, Hcy alone (50 μM), low-dose (1 μM) or high-dose (10 μM) Rb1 alone, and Hcy plus low-dose or high-dose Rb1. After 24-hour incubation in a 37° C and 5% CO2 cell culture incubator, the artery rings were analyzed using myograph analysis (Danish Myo Technology Organ Bath 700 MO, Aarhus, Denmark). Each ring was treated with thromboxane A2 analogue U46619 (10−7 M) to generate maximal contraction. The dose of 10−6 M of U46619 was chosen because our previous experiments showed that this was an optimal dose for the contraction of porcine coronary rings (data not shown). A relaxation dose-response curve was generated by adding 60 μL of five cumulative doses of the endothelium-dependent vasodilator bradykinin (10−9, 10−8, 10−7, 10−6, and 10−5 M) at 3-minute intervals. Bradykinin is a potent vasodilator that acts through endothelial B2 kinin receptors to stimulate the release of NO through eNOS activation.\(^13\) Subsequently, endothelium-independent relaxation was induced by adding sodium nitroprusside (SNP, 10−8 M). Contractility and percentage of relaxation were calculated based on the tension changes. The data of the coronary artery rings from eight different pig hearts were averaged and represented as one data point for statistical analysis.

**Real-time polymerase chain reaction (PCR).** The porcine coronary artery endothelial cells were collected from cultured artery rings by scraping the luminal surface with surgical blades. Total RNA from cultured porcine coronary artery endothelial cells was isolated by using TRI-Reagent following the manufacturer’s instructions. cDNA was generated by reverse transcript from mRNA using the iScript cDNA Synthesis Kit according to the manufacturer’s instructions. The iQ SYBR Green SuperMix Kit was then used for real-time PCR reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as internal control for eNOS expression to account for variations in mRNA loading. The same total RNA, 1 μg, was loaded for all samples. Porcine eNOS and GAPDH primers were designed using Beacon Designer. The eNOS (GenBank no. AF266137) primer sequences are: forward primer 5′-CCCTACAACCGGCTCCCCCTC-3′ and reverse primer 5′-GCTGTCGTTGTTACTGGAATTCCTTT-3′. The GAPDH (GenBank no. AF017079) primer sequences are: forward primer 5′-TGTACCAACAACTGTTGCCC-3′ and reverse primer 5′-GGCATGGACTGTGGTCAATGAGA-3′. Real-time PCR was performed in an iCycler iQ real-time PCR detection system. The thermal cycle condition used for reverse transcription was as follows: 5 minutes at 25° C, 30 minutes at 42° C, and 5 minutes at 85° C. The condition used for real-time PCR was as follows: 3 minutes at 95° C, 40 repeats of 20 seconds at 95° C and 1 minute at 60° C. Controls were performed with no reverse transcription (mRNA sample) or water control for both eNOS and GAPDH to show the specificity of the primers and the lack of DNA contamination in samples. Sample cycle threshold (Ct) values were determined from plots of relative fluorescence units (RFU) vs PCR cycle numbers during exponential amplification so that sample measurement comparisons were possible. The eNOS gene expression level for each sample was calculated as 2^[Ct_ (GAPDH)−Ct_ (eNOS)]

**Western blotting.** The protein was isolated from endothelial cells using TRI-Reagent according to the manufacturer’s instructions. Cells were then resuspended in 20 μL of 10 M urea and heated for 3 minutes at 100° C. The protein concentration was determined using the Bradford method.
protein assay with bovine serum albumin as a standard. The same amount of endothelial protein was resolved electrophoretically by one-dimensional SDS-PAGE (10% polyacrylamide) for approximately 1 hour at 100 V. Subsequently, the gels were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.03% SDS, and 20% methanol), and the proteins were electrophoretically transferred to nitrocellulose filters at 100 V for 1 hour on ice. Filters were blocked using 5% nonfat dried milk in PBS with 0.05% Tween 20 (PBS-T) for 1 hour at room temperature. eNOS was detected using a mouse antihuman monoclonal antibody diluted 1:1000, and β-actin was detected using a mouse antihuman monoclonal antibody diluted 1:10,000. The eNOS and β-actin primary antibodies were detected with horseradish peroxidase (HRP)–conjugated goat anti-mouse immunoglobulin G secondary antibodies diluted 1:5000. Blots were developed using ECL™ plus and analyzed with an AlphalImager gel documentation system (Alpha Innotech Co, San Leandro, CA).

Immunohistochemistry. Rings of treated porcine coronary arteries were fixed overnight in 10% neutral buffered formalin and subsequently were stored in 70% alcohol until processing. Samples were later embedded in paraffin, cut into 5-μm-thick cross-sections, and mounted onto slides. Sections were treated with 3% H2O2 in PBS (pH, 7.4) for 10 minutes to quench the endogenous peroxidase activity. After three 10-minute rinses in 0.1 M PBS, sections were incubated in monoclonal antibody against human eNOS (1:1000) diluted in 0.1 M PBS containing 5% normal horse serum, 0.1% Triton-X 100 overnight at 4° C. After another three 10-minute rinses in 0.1 M PBS, sections were incubated with biotinylated antimouse immunoglobulin G (1.250) at room temperature for 40 minutes. For diaminobenzidine visualization, sections were incubated in avidin–biotin–peroxidase solution at room temperature for 1 hour, followed by 0.1% diaminobenzidine and 0.003% H2O2 in TBS for 5 to 10 minutes. Sections were counterstained with hematoxylin-eosin, cover-slipped, and viewed on an Olympus BX41 microscope (Olympus USA Inc, Melville, NY). Images were captured with an attached SPOT-RT digital camera and software (Diagnostic Instruments Inc, Sterling Heights, MI).

Lucigenin-enhanced chemiluminescence analysis. Levels of superoxide anion produced by endothelial cells were detected using the lucigenin-enhanced chemiluminescence method with Sirius Luminometer and FB12 software from Berthold Detection System GmbH (Pforzheim, Germany). The rings were cut open longitudinally and trimmed into approximately 5 × 5-mm pieces. They were then rinsed briefly in a modified Krebs HEPES buffer solution (KHBS, 120 mM NaCl, 4.7 mM KCl, 1.18 mM KH2PO4, 20 mM HEPES, 2.5 mM CaCl2, 1.17 mM MgSO4, and 25 mM NaHCO3). An assay tube (12 × 75 mm) was filled with 500 μL of Krebs HEPES buffer solution and 25 μL of lucigenin. After gently vortexing, the vessel segments were placed endothelium-side-down in the tubes. A time-based reading of the luminometer was recorded by FB12 software. The data in relative light units per second (RLU/sec) for each sample were averaged between 5 and 10 minutes. Values of blank tubes containing the same reagents as the vessel ring samples were subtracted from their corresponding vessel samples. The area of each vessel segment was measured using a caliper and was used to normalize the data for each sample. Final data were represented as RLU/sec/mm².

Statistical analysis. Data from the different groups were analyzed using an unpaired Student’s t test (two-tail, Minitab software, Sigma Breakthrough Technologies Inc, San Marcos, TX). In addition, an analysis of variance (ANOVA) test was used to analyze data of endothelium-dependent relaxation in response to bradykinin. A P value < 0.05 was considered statistically significant. Statistics are reported as mean ± the standard error (SE) of the mean (SEM).

RESULTS

Rb1 blocks Hcy-induced endothelial vasomotor dysfunction. The effects of Hcy and Rb1 on the contractility of pig coronary artery rings are shown in Fig 1. There was no substantial difference in maximum vessel contraction in response to U46619 (10⁻⁷ M) among all groups, including DMSO control, Hcy (50 μM) alone, low-dose Rb1 (1 μM) or high-dose Rb1 (10 μM) alone, and Hcy combined with low-dose or high-dose Rb1 (P > .05). Endothelium-dependent vasorelaxation induced by bradykinin was significantly different between the Hcy-treated group and the controls. As shown in Fig 2, A, precontracted arterial rings in the Hcy-alone group showed a marked reduction in endothelium-dependent vasorelaxation (4.16 ± 3.01%, 14.7 ± 6.94%, 26.8 ± 5.14%, 39.7 ± 3.95%, 49.8 ± 2.49%, respectively) in response to each cumulative dose of bradykinin (10⁻⁷, 10⁻⁶, 10⁻⁵, respectively) as compared with the DMSO controls (8.84 ± 2.27%, 24.6 ± 4.01%, 46.7 ± 3.58%, 62.7 ± 4.68%, 71.8 ± 2.32%, respectively). Additionally, both low-dose and high-dose Rb1-alone groups showed a similar vasorelaxation pattern as the control (9.13 ± 5.17%, 22.4 ± 7.67%, 45.1 ± 6.4%, 62.3 ± 38%, 71.3 ± 1.84%; and 8.8 ± 2.68%, 38 ± 5.69%, 55.3 ± 4.29%, 64.9 ± 3.7%, 72 ± 2.55%, respectively) Furthermore, low-dose Rb1-Hcy did not show a significant improvement in endothelium-dependent relaxation as compared with Hcy alone, whereas high-dose Rb1-Hcy completely reversed the effect of Hcy and showed a similar vasorelaxation pattern as the control group. Lastly, Fig 2, B shows the vasorelaxation pattern of each group in response to the high concentration of bradykinin (10⁻⁵). Endothelium-dependent relaxation was significantly impaired in rings treated with Hcy alone as compared with the controls (49.80% vs 71.77%, n = 8, P < .05), whereas neither high-dose nor low-dose Rb1-alone affects endothelium-dependent relaxation (71.34 ± 1.84% and 72 ± 2.55%, respectively). The low-dose Rb1-Hcy combined group had partially improved endothelium-dependent relaxation (54.44 ± 2.63%), whereas the high-dose Rb1-Hcy combined group showed complete recovery of endothelium-dependent relaxation (72.89 ± 2.2%). Our
data also showed that there was no significant difference in endothelium-independent relaxation induced by SNP among all groups ($P > .05$) (Fig 3), thus indicating that smooth muscle NO–dependent cyclic guanosine monophosphate (cGMP)–mediated signaling function remained intact.

**Rb1 improves Hcy-induced impairment of eNOS mRNA expression.** The levels of eNOS mRNA expression in porcine coronary arteries were measured using real-time PCR analysis to determine the influences of Hcy and Rb1 on mRNA expression in the treated arterial rings (Fig 4). All of the values were normalized with GAPDH, which served as the internal control. The Hcy-alone group showed a 46% reduction of eNOS mRNA level (0.013 ± 0.003) as compared with the DMSO control (0.024 ± 0.004, $P < .05$), whereas both the low-dose and the high-dose Rb1 group had a comparable level of eNOS mRNA as the controls (0.027 ± 0.001 and 0.024 ± 0.001, respectively; $P > .05$). In addition, the high-dose Rb1–Hcy combined group showed a significant improvement in eNOS mRNA level (0.018 ± 0.002) as compared with the Hcy-alone group (0.013 ± 0.003, $P < .05$).

**Rb1 counteracts Hcy-induced reduction of eNOS protein levels.** The level of eNOS protein production was determined by Western blot analysis, and the data were normalized using the internal loading controls of $\beta$-actin (Fig 5). Based on the results from the mRNA data, only the high-dose Rb1–Hcy combined group had statistically significant improvement in the eNOS mRNA level, thus only high-dose Rb1 was chosen for eNOS protein analysis. Consistent with the eNOS mRNA data, Hcy-treated vessels showed substantially less density of eNOS protein bands and a 65% reduction in the eNOS protein–$\beta$-actin ratio (0.272 ± 0.06) as compared with the control group (0.787 ± 0.11), and the high-dose Rb1–Hcy combined group showed a significantly improved eNOS protein level (eNOS protein–$\beta$-actin ratio, 0.555 ± 0.06, $P < .05$). The eNOS protein level in the high-dose Rb1-alone group had no significant differences as compared with that in the DMSO control group ($P > .05$).

eNOS protein expression in porcine coronary arterial endothelium was further depicted using immunohistochemical staining (Fig 6). The DMSO control group showed a strong positive staining pattern of eNOS on the endothelial layer of the arterial walls in contrast to the poor staining pattern of eNOS in the Hcy-alone group. Again, only high-dose Rb1 was used for comparison and showed similar staining patterns as the DMSO controls. Importantly, the high-dose Rb1–Hcy combined group showed a strong positive staining pattern similar to that of the control group.
group, which indicated the recovery of eNOS protein in the endothelial layer of the high-dose Rb1-Hcy group.

Rb1 attenuates Hcy-induced superoxide anion production. Levels of superoxide anion produced by endothelial cells were determined using lucigenin-enhanced chemiluminescence (Fig 7). Superoxide anion was markedly increased by 137% in Hcy treated groups as shown by significantly higher levels of peak chemiluminescence (17.5 ± 5.4 RLU/s/mm², n = 8) than the DMSO controls (8.75 ± 1.72 RLU/s/mm², n = 8). High-dose or low-dose Rb1 alone showed no effects on the level of peak chemiluminescence as compared with the controls (7.09 ± 0.84, 8.93 ± 1.4, 8.75 ± 1.72 RLU/s/mm², respectively). Again, both high-dose and low-dose Rb1-Hcy combined groups showed a substantial reduction in superoxide anion level as shown by a dramatically decreased level of peak chemiluminescence (9.22 ± 1.4 and 9.34 ± 0.28 RLU/s/mm², respectively).

**DISCUSSION**

Although ginseng and its active constituents, ginsenosides, have gained increased popularity worldwide for a myriad of beneficial effects, the underlying mechanisms are still unclear. The information regarding the effects of ginseng on cardiovascular diseases is even more scattered. Herein we investigated the cellular mechanism as well as the functional influence of ginsenoside Rb1 on protecting against Hcy-induced endothelial damage. This study showed that Rb1 prevented Hcy-induced impairment of endothelium-dependent vasorelaxation and improved Hcy-induced reduction of eNOS production. To our knowledge, this is the first study that shows the beneficial role of ginsenoside in preventing Hcy-induced vascular injury in porcine coronary arteries and shows the potential underlying mechanisms of interaction between ginsenoside and Hcy on endothelium. Our results suggest the potential clinical application of ginseng and its major constituents in protecting the endothelium against Hcy and superoxide-associated injuries, thus having a potential role in cardiovascular disease prevention.

Hcy is a known risk factor for cardiovascular disease. Severe hyperhomocysteinemia (>100 μM) has been observed in patients with homozygous deficiency of cystathionine-β-synthase or methylene tetrahydrofolate reductase and is usually associated with premature atherosclerosis or thrombosis. Even a mild to moderate elevation of Hcy (10 to 100μM) is associated with increased risks for cardiovascular and cerebrovascular diseases. A study from our labo-
Latory showed that Hcy significantly decreased endothelium-dependent vasorelaxation and eNOS immunoreactivity as well as induced marked endothelial injury in both porcine coronary and carotid arteries. Additionally, Eberhardt and colleagues showed that mild hyperhomocysteinemia impaired endothelium-dependent vasodilatation by increasing reactive oxygen species, and a subsequent losing of NO bioavailability in an animal model. Furthermore, Lang et al confirmed that the inhibitory effect of Hcy on endothelium-dependent relaxation is caused by an increase in the endothelial cell intracellular levels of superoxide.

Lastly, Tawakol et al studied human brachial artery dilatation during reactive hyperemia in subjects with hyperhomocysteinemia and proved that endothelium-dependent vasodilatation was significantly impaired in the hyperhomocysteinemic subjects as compared with control subjects, whereas endothelium-independent vasodilatation was no different between the two groups. However, Tasatargil et al documented that short-term exposure to Hcy significantly decreased vascular responsiveness in human internal mammary arteries through other mechanisms, including possible Ca++ channel involvement and other undefined direct effects. Based on our previous study, a Hcy concentration of 50 μM (mild to moderate hyperhomocysteinemia) was chosen to induce impairment on endothelium-dependent relaxation without affecting endothelium-independent relaxation or smooth muscle contraction.

Studies have shown that ginseng and ginsenosides induce NO release, a primary vasodilator. Chen et al showed the relaxation effects of ginsenoside Rb1 and Rg1 on pulmonary vessels. However, Kang et al studied the effects of two different groups of ginsenosides, protopanaxatriol (Rg1 and Re) and protopanaxadiol (Rb1 and Re), on rat aortic rings. They indicated that ginsenosides from the protopanaxatriol group, but not the protopanaxadiol group, enhanced the release of NO from endothelial cells. Other endothelium-derived vasomotor factors have also been shown to be involved in vasorelaxation. Fang et al studied myocardial infarction and reperfusion on a dog model and discovered that ginsenoside decreased thromboxane A2 and increased 6-keto PGF1 levels after both coronary occlusion and reperfusion, which confirmed the protective effects of ginsenosides through vasomotor factors. Yuan et al also studied the effects of ginseng extracts on human umbilical vein endothelial cells and showed that ginseng extracts inhibited thrombin-induced endothelin release, a potent vasoconstrictor. Kim et al showed that ginsenoside Rg3 mediated endothelium-dependent relaxation through eNOS activation in rat arteries. Kwan et al showed that ginsenoside Rb1 on Hcy-induced endothelium dysfunction. Our data suggest that Rb1 effectively blocks the effect of Hcy through maintaining eNOS functions and reducing oxidative stress. Based on our knowledge, this is the first report that ginsenoside Rb1 counteracts the effects of Hcy.

Results on homocysteine-induced vasomotor dysfunction from this study are consistent with previous studies from our laboratory in showing that Hcy significantly impairs the endothelium-dependent vasodilatation that was induced by cumulative doses of bradykinin, and exerted no effect on maximal contraction or endothelium-independent relaxation induced by thromboxin analog and SNP, respectively. Furthermore, our study investigated the effects of ginsenoside Rb1 on Hcy-associated vasomotor dysfunction, showing that Rb1 by itself had no effect on vasomotor function of porcine coronary arterial rings.
However, when combined with Hcy, a high-dose Rb1-Hcy combined group had a complete recovery of endothelium-dependent vasorelaxation and a low-dose Rb1-Hcy group had only a partial improvement in vasorelaxation. Although the low-dose Rb1-Hcy combined group apparently had decreased endothelium-dependent relaxation beyond the reduction seen with Hcy alone at low concentrations of bradykinin, the reduction was not statistically significant. We believe that fluctuations in the values may be caused by the variability of tissue samples and native conditions of the porcine coronary arteries. In addition, our results of Rb1 alone having no effect on vasomotor function is consistent with the study by Kang et al, who showed that ginsenosides in a protopanaxadiol group, including Rb1, do not enhance NO release from endothelial cells.18 Although a study from Chen et al concluded otherwise.18 From the vasomotor study, we postulate that Rb1 dose-dependently blocks Hcy-induced impairment of endothelium-dependent vasorelaxation, which indicates a potential clinical application of Rb1 in preventing Hcy-induced vascular dysfunction.

Molecular mechanisms of Hcy-induced endothelium dysfunction have been extensively studied. Oxidative stress and eNOS reduction have proven to be the major mechanisms through which Hcy exerts its effects on vascular endothelium. Eberhardt et al showed that an increased superoxide production is associated with Hcy-induced endothelial damage in a mouse model.14 Additionally, our previous study showed that Hcy suppressed eNOS immunoactivity, and thus induced endothelium dysfunction.5 Moreover, Li et al used a complementary DNA microarray to disclose a link between Hcy and cholesterol dysregulation, and showed the possible role of Hcy-induced oxidative stress in this response.24 Lastly, Pruefer et al examined the acute effect of homocysteine on endothelial dysfunction in isolated rat arteries and on microcirculatory leukocyte-endothelium interaction in vivo.25 They concluded that acute hyperhomocysteinemia induces endothelial dysfunction, characterized by a loss of endothelial-derived NO, leading to an inflammatory state. We investigated the molecular mechanisms of Hcy-induced endothelial dysfunction by studying eNOS mRNA and protein production using real-time PCR and Western blot. Because Hcy may cause injury and loss of endothelial cells, we used internal controls to ensure an equal amount of tissue samples for the study among all groups. Total RNA or proteins were measured before real-time PCR or Western blot was performed. The housekeeper gene GAPDH was used as the control in real-time PCR analysis, and β-actin was used as the internal control for Western blot. All of the values were normalized before final analysis. The Hcy-treated group showed a marked reduction of eNOS mRNA and protein levels as compared with the control group. Additionally, decreased eNOS expression in the porcine coronary arterial endothelium was depicted by immunohistochemical staining, which was consistent with Western blot results. Furthermore, the fact that high-dose Rb1 completely reversed the adverse effects of Hcy on endothelial cells with no significant effects on the eNOS level when incubated with porcine coronary rings alone implies that Rb1 was protecting the endothelium from Hcy-induced damage with little effect on the healthy endothelium. However, the changes in NO levels of Hcy- or Rb1-treated vessels were not studied. It could be interesting to measure eNOS activity by the conversion of arginine to citulline and NO production.

Free radicals have been shown to play a key role in atherosclerotic plaque formation and to be involved in various vascular injuries. Extensive studies have been conducted on the protective effects of ginseng against free-radical damage on the vascular endothelium. Zhong et al examined cellular structures of free radical damage on myocardial cells induced by xanthine. They measured free radicals with an electron spin resonance technique and discovered certain ginsenosides (Rb1, Rb2, Rb3, Re, Rg1, Rg2, Re, and Rh1) counteracting the action of free radicals induced by xanthine.26 In an animal model, Chen et al showed that ginsenoside protected against myocardial reperfusion injury with a concomitant increase in 6-keto-PGF1α and a decrease in lipid peroxidation, and also protected the rabbit pulmonary and aortic endothelium against electrolysis-induced free radical damage.18 Additionally, Gillis showed the protective effects of ginsenoside on an injured rabbit pulmonary endothelium induced by a variant of reactive oxygen species.9 He further reviewed other studies and confirmed that ginseng prevented manifestations of oxygen-derived free radical injury by promoting the release of NO. In our study, Hcy-induced free radical production and the effect of Rb1 were investigated using lucigenin-enhanced chemiluminescence. We showed that the Hcy-treated group had a significant increase in free radical production, whereas Rb1 blocked the effect of Hcy, as indicated by dramatically decreasing levels of peak chemiluminescence in Rb1-Hcy combined groups. Our study proved that either high-dose or low-dose Rb1 fully blocked free radical production.

Admittedly, mechanisms of Hcy-induced endothelial damages have not yet been fully elucidated. Our results indicated that other cellular pathways may be involved in the functional effects of Hcy that are not influenced by Rb1. As shown by our data, both low-dose and high-dose Rb1 completely reversed superoxide anion production induced by Hcy in Hcy-Rb1 combined groups, whereas low-dose Rb1 only partially recovered the eNOS mRNA level and endothelial-dependent relaxation. Furthermore, our data suggested that ginsenoside Rb1 functions in a dose-dependent manner, thus low-dose Rb1 may not be enough to overcome the adverse effects induced by Hcy. Further studies are warranted to explore the detailed molecular and cellular mechanisms of Rb1. It is interesting to compare the antioxidative effect of Rb1 with a well-known antioxidant enzyme superoxide dismutase. Tissue-model-based analysis is limited because of the rapid deterioration of the endothelial cells and the variable conditions of each porcine coronary artery. Therefore, to overcome the limited viability and great variability of tissue samples, a cell culture model has been created in our laboratory to further
investigate the cellular pathways of Rb1. Emerging data show that different constituents of ginseng may have antagonistic actions.\textsuperscript{27} This indicates a more complex interaction with more detailed studies required in the future.

In summary, our study showed that ginsenoside Rb1 effectively blocks Hcy-induced impairment of endothelium vasomotor function in porcine coronary arteries. In addition, Rb1 successfully improved Hcy-induced reduction of eNOS expression, and reduced Hcy-induced oxidative stress. This is the first study that has shown the functional relationship and molecular mechanisms of the effects of ginsenoside Rb1 on Hcy-induced endothelial dysfunction. However, the observations made during this investigation do not directly translate into human arteries, and there is extremely limited study in the literature regarding serum levels of ginsenosides after oral ginseng doses in human. Therefore, clinical studies are warranted to determine the effects of Rb1 on human endothelium and to determine the optimal oral dose of ginsenoside Rb1 and ginseng. Consistent with our previous studies,\textsuperscript{11,12} ginsenoside Rb1, similar to red wine and estrogen, effectively reduces Hcy-induced endothelial dysfunction and free radical production. These studies imply an important future therapeutic strategy of using antioxidant-based pharmacotherapy in counteracting the detrimental effects of hyperhomocysteinemia. Moreover, the antioxidant-based pharmacotherapy perhaps also has a protective role in other free-radical-induced vascular injuries.

REFERENCES


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