Ginseng Saponins Diminish Adverse Vascular Effects Associated with Chronic Methionine-Induced Hyperhomocysteinemia

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Recent studies have shown that *Panax* ginseng has a variety of beneficial effects on the cardiovascular systems. Homocysteine (Hcy), which is derived from L-methionine (Met), has been closely associated with the increased risk of cardiovascular diseases. In the present study, we examined whether *in vivo* long-term administration of ginseng saponins (GS), active ingredients of *Panax* ginseng, attenuate adverse vascular effects associated with chronic Met-induced hyperhomocysteinemia (H-Hcy). We found that plasma Hcy level, which was measured after 30 and 60 d, in GS (100 mg/kg)+Met co-administration group was significantly reduced when it was compared with Met alone treatment group. We could also observe the alleviation of endothelial damages of aortic artery vessels in GS (100 mg/kg)+Met co-administration group compared with Met alone treatment group. We compared aortic vasoconstrictile and vasodilatory responses between Met alone and GS (100 mg/kg)+Met co-treatment groups. We found that norepinephrine-induced vasoconstrictile responses were greatly decreased in GS (100 mg/kg)+Met co-treatment group and that carbachol-induced dilatory responses were greatly enhanced in GS (100 mg/kg)+Met co-administration groups as compared with Met alone treatment group. The present results indicate that *in vivo* long-term administration of GS attenuates adverse vascular effects associated with chronic Met-induced H-Hcy in rats.

Key words *Panax* ginseng; Ginseng saponin; methionine; hyperhomocysteinemia; vascular dysfunction

Homocysteine (Hcy) is a thiol-containing amino acid derived from L-methionine (Met), one of an essential amino acids. Hcy is produced entirely from the methylation cycle, since it is totally absent from any dietary source.^{1,2} Three forms of Hcy are usually present in plasma, whereby most of it (80–90%) is bound to proteins; a smaller part is available as free amino acid, and traces are present as disulphide forms. Studies in animal models and humans have demonstrated that hyperhomocysteinaemia (H-Hcy) induces endothelial dysfunctions.^{3–5} Endothelium dysfunctions are known as an initial step of arteriosclerosis and alterations of blood vessel contraction and relaxation, permeability, and changing platelet and leukocyte adhesions.

Although the molecular mechanisms by which H-Hcy impairs the blood vessel wall and promotes arteriosclerosis are still not clearly elucidated, it is believed that a high concentration of plasma Hcy is the main causes of the vascular endothelium injury by inducing the increased oxidative stress and decreased nitric oxide (NO) bioavailability.^{6–9} On the other hand, H-Hcy can also affect left ventricular (LV) hypertrophy and cardiac fibrosis in animal models that is reversed with folate treatment.^{10,11}

Ginseng, a widely recognized herbal drug, has been reported to have a wide range of therapeutic and pharmacological uses. Ginseng has been used extensively in Korean and Chinese medicines and has become increasingly popular in the Western world for its alleged tonic effect and possible preventive and restorative properties. There are increased experimental evidences concerning the beneficial effects of ginseng roots in cardiovascular abnormalities. These studies

have shown that ginseng saponins (GS), the major constituents of ginseng, have protective effects from vascular injuries due to various causes.^{12,13} For example, it has been reported to be effective in treating cardiovascular diseases such as coronary thrombosis and in improving cardiac muscle microcirculation. Administration of GS improved myocardial relaxation function,¹⁴ lowered cerebrovascular resistance, suppressed proliferation of aortic smooth muscle cells induced by hypercholesterolemic serum, ameliorated cardiac function in the early stage postburn,¹⁵ and finally enhanced fibrinolytic activity of bovine aortic endothelial cells.¹⁶ Those effects provide one possibility that GS might be also a valuable approach for the protection to the injured blood vessels by H-Hcy. However, no previous work has examined whether GS attenuates Met-induced H-Hcy and attenuate vascular dysfunctions associated with Met-induced H-Hcy.

Here, we investigated whether the long-term administration of GS could attenuate chronic Met-induced H-Hcy and also examined whether the long-term treatment of GS could ameliorate H-Hcy-induced vascular dysfunctions such as arterial vasoconstrictile and vasodilatory responses by NE¹⁷ and carbachol¹⁸ and H-Hcy induced-endothelial injury in rats.^{19,20} We found that co-administration of GS with Met not only reduced Met-induced H-Hcy but also attenuated endothelial damages of aortic artery vessels. Furthermore, co-treatment of GS with Met maintained agonist-induced vascular responses near to normal level as compared with Met alone. These results indicate that GS might be useful agent in attenuating chronic Met-induced H-Hcy as well as H-Hcy-

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associated vascular abnormalities.

MATERIALS AND METHODS

Animals Eighty male Wistar rats (weighing *ca.* 140–150 g; Charles River, KFT) were housed with free access to commercial food pellets, as well as tap water *ad libitum*. All animals was kept in light–dark conditions L:D=12:12. Light was turned on at 07:00. Animal care and handling was in accordance with the highest standards of institutional guidelines. The animals were divided into four groups with twenty rats. Group 1: Normal control group with only tap water. Group 2: Met and succinylsulfathiazole (SST) administration in the tap water for a period of 30 and 60 d. Group 3: GS alone treatment group for a period of 30 and 60 d. Group 4: GS+Met+SST co-treatment group for a period of 30 and 60 d.

Drug Administrations Figure 1 shows the chemical structures of ginsenosides. Ginseng total saponins (GS), which was isolated according to the method of Tanaka *et al.* (1966),²¹⁾ was kindly provided by the Korea Ginseng Corporation (Daejeon, South Korea). The percentage of GS from ginseng was about 3.4%. GS contained Rb₁ (17.1%), Rb₂ (9.07%), Rc (9.65%), Rd (8.26%), Re (9%), Rf (3%), Rg₁ (6.4%), Rg₂ (4.2%), Rg₃ (3.8%), Ro (3.8%), Ra (2.91%) and other minor ginsenosides. GS dissolved in saline was administered intraperitoneally (i.p.) to rats at various dosages (0, 6.25, 12.5, 25 or 50 mg/kg, twice/d, every 12 h interval for 30 and 60 d). These dosages were based on previous report.²²⁾ GS administration began on day 0, followed by Met on day 3. Met was administered *via* oral (*p.o.*) route to rats. Met-caused H-Hcy was induced by administration of the dissolved Met (1.0 g/kg body weight/d) and SST (0.5 g/kg body weight/d) in the tap water for a period of 30 and 60 d. The dosage of Met and SST administered per animal was based on average daily fluid intake and SST was used to avoid bacterial proliferation and subsequent folate production.^{23–25)} Control vehicle group was administered only with tap water. Animals of each group were housed separately, and fed standard rat chow, and were weighed at 30 and 60 d, respectively.

Determination of Serum Hcy Blood was collected from the femoral artery of fasting rats. It was immediately cooled on ice and centrifuged at 3000×*g* for 20 min at 4 °C to limit the release of Hcy from blood cells. Serum was then stored at –20 °C until assayed. Total Hcy concentrations were measured by a high-performance liquid chromatography (HPLC) technique with fluorometric detection according to Ulbink *et al.*²⁶⁾ Briefly, 240 µl of serum and 60 µl of internal standard (*N*-acetyl-L-cysteine, 50 µM final concentration) was reduced for 30 min at 4 °C with 30 µl of tri-*n*-butyl phosphine (10%). Deproteinization was performed with 300 µl of 10% trichloroacetic acid. After centrifugation, 100 µl of the clear supernatant was mixed with 20 µl of 1.55 M NaOH, 250 µl of 0.125 M borate buffer (pH 9.5), and 50 µl of 1 mg/ml 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate. After derivatization at 60 °C (1 h), the sample was analyzed by HPLC (JASCO International Co., Ltd.), equipped with a fluorescence detector (LC 1255; GBC Scientific Equipment Pty Ltd.). Separation was carried out on a 2003×4.6 mm×5 µM Nucleosil C18 column. The eluant was 0.1 M acetate buffer (pH 4.0) containing 2% methanol. The fluorescence intensi-

Fig. 1. Structures of the Nine Representative Ginsenosides

They differ at three side chains attached the common steroid ring. Abbreviations for carbohydrates are as follows: Glc, glucopyranoside; Ara (pyr), arabinopyranoside; Rha, rhamnopyranoside. Superscripts indicate the carbon in the glucose ring that links the two carbohydrates.

ties were measured with excitation at 386 nm and emission at 516 nm.

Measurement of Blood Pressure Arterial pressure (AP) was continuously recorded in conscious unrestrained rats.²⁷⁾ Briefly, the rats were anesthetized with a mixture of ketamine (100 mg/kg, i.p.) and acepromazine (5 mg/kg, i.p.). A polyethylene catheter (PE-10 connected to PE-50) was chronically placed into the lower abdominal aorta *via* the left femoral artery for measurement of AP. The catheter was tunneled subcutaneously and exteriorized through the interscapular skin. After 7 d of recovery, the aortic catheter was connected to a pressure transducer *via* a rotating swivel that allowed the rat to move freely in the cage. After approximately 3 h of habituation, the AP signal was digitized by a microcomputer for 2 h (12:00–14:00 h). Systolic AP and diastolic AP values were determined on-line. Using off-line analysis, the mean values of these parameters were calculated for a period of 2 h and served as systolic AP and diastolic AP. The standard deviation of the pressure during a 2 h interval was calculated and defined as the quantitative parameter of blood pressure.

Vasomotor Activity At 60 d after experiments, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) in each group. In each group, after blood was drawn from the inferior vena cava, descending thoracic aorta was immediately exposed and isolated from surrounding tissues. The aorta then was dissected out and placed in a silicone-lined Petri dish containing cold (0 to 4 °C) physiological salt solution (PSS) composed of (in mM): NaCl 112.0, KCl 5.0, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1, NaHCO₃ 29.7, glucose 11.5 and EDTA 0.016, and aerated with 95% O₂ and 5% CO₂. Adhering fat and connective tissues of the vessels were removed. Aorta was cut into 2- to 3-mm-wide rings. And, the aortic rings were suspended horizontally between two stainless steel stirrups in organ chambers filled with 3 ml of 37.8 °C, pH 7.4 PSS bubbled with 95% O₂ and 5% CO₂. One of the stirrups was anchored to the organ chamber and the other was connected to a force transducer (Narco bio-system) for the recording of isometric tension. And, the aortic rings were divided into four groups: normal control group, GS alone treatment group, Met alone treatment group and GS+Met

combined group. In each group, endothelium was removed by perfusion of the vessel with air for 1 min at a perfusion pressure of 20 mmHg. The efficacy of endothelial denudation was ascertained by arteriolar responses to acetylcholine (ACh; 0.1 μ M; an endothelium dependent dilator agent), and SNP (0.1 μ M; an endothelium independent agent) before and after the administration of the air bolus. The infusion of air resulted in loss of function of the endothelium, as indicated by the absence of dilation to ACh, whereas dilation to SNP remained intact. Aortic rings in the absence of endothelium were allowed to equilibrate for 90 min at a resting tension of 2.0 g, and then exposed to high potassium (70 mM). When the high-potassium induced contraction reached a plateau, the rings were rinsed with PSS, and allowed to re-equilibrate for 30 min. After a stable plateau of vasoconstriction had been reached by 70 mM KCl, the aortic rings were stabilized by PSS for 30 min. This response was repeated three times. Then, after rings were stabilized by PSS, constrictor responses of arterioles to norepinephrine (NE; 0.1 nM to 10 μ M) were measured. The vascular tone was reached to stable for 30 min. NE was added to the bath in a cumulative manner from 0.1 nM to 10 μ M. The response to each concentration was allowed to reach a plateau before the addition of the next concentration of NE. The amplitude of contraction induced by NE was measured for each concentration. The effects on contraction by NE were expressed as a percentage of the response to a maximal value contracted by 70 mM KCl administered initially in each aorta. In all cases, each experiment was repeated four to five times.

The dilatory effects on carbachol (CCh) were investigated in aortic rings with endothelium. After a stable plateau of vasoconstriction had been reached by 70 mM KCl, the rings were stabilized by PSS for 30 min. This response was repeated three times. And, the rings were again contracted by 70 mM KCl and aortic rings were dilated by CCh with dose-dependent manner (0.1 nM to 10 μ M) to test dilative effects of aorta in the presence of the endothelium. The response to each concentration was allowed to reach a plateau before the addition of the next concentration of CCh. The amplitude of dilation induced by CCh was measured for each concentration. The dilatory effects on contraction by CCh were expressed as a percentage of the response to a maximal value contracted by 70 mM KCl administered initially in each aorta in the presence of endothelium. In all cases, each experiment was repeated four to five times.

Histological Examination In each group, the chest cavities of the rats were opened to remove the thoracic arteries shortly after blood samples were taken. The arteries were

removed and fixed in 4% paraformaldehyde for 2 h. After fixation, the arteries were cut into segments of 3–4 mm and embedded in paraffin. Serial cross-sections were stained with hematoxylin and eosin (HE) and then stained immunohistochemically for endothelial nitric oxide synthase (eNOS) with the avidin–biotin complex immunoperoxidase procedure. Monoclonal antibody against human eNOS (1:1000) was used.²⁸⁾

Data Analysis All data are reported as means $\pm$ S.E.M. Statistical analysis was performed with two-tailed Student's unpaired *t* test, except for the vascular reactivity study, for which two-way ANOVA for repeated measures was used to compare two curves. The threshold for statistical significance was $p < 0.05$.

RESULTS

Body Weight We have measured the body weight, food, and water intake during the long-term administration of normal control, GS alone, Met alone, or GS+Met group. The initial body weight of normal control, GS alone, Met alone, or GS+Met group was 140 ± 13.3 , 142 ± 8.3 , 138 ± 10.1 , 141 ± 6.7 g, respectively. The body weight in normal control, GS, Met, and GS+Met group after 30 and 60 d was not significantly different among them. Thus, the body weight of normal control, GS (100 mg/kg), Met, and GS (100 mg/kg)+Met group was 375.8 ± 24.3 , 369.3 ± 19.1 , 391.7 ± 23.3 and 371.2 ± 23.8 g after 30 d, respectively and 475.7 ± 31.5 , 472.7 ± 28.8 , 498.3 ± 31.8 , and 482.6 ± 32.5 g after 60 d, respectively. We could not also observe significant body weight changes in different dosages of GS (12.5, 25, 50 mg/kg) compared with other treatment groups (data not shown). No significant changes were also observed for initial plasma Hcy level in all four groups before Met administration (data not shown). During whole experimental periods, the amounts of water and food intakes in each group were not significantly different among four groups (data not shown).

Measurements of Blood Pressure and Heart Rate among Various Treatment Group We next measured blood pressure and heart rate changes in each group. Systolic and diastolic arterial pressure (SAP and DAP) among normal control, GS (100 mg/kg), Met, and GS (100 mg/kg)+Met groups were measured. Heart rate (HR) among normal control, GS (100 mg/kg), Met, and GS (100 mg/kg)+Met groups was also measured. There was no significantly difference in SAP, DAP and HR among groups (Table 1). Other hemodynamic parameters such as pulse pressure (PP) and mean AP also did not significantly differ among all four groups (Table 1).

Table 1. Measurement of Blood Pressure and Heart Rate among Various Treatment Groups

	After 30 d				After 60 d			
	Con	GS	Met	GS+Met	Con	GS	Met	GS+Met
SAP	133.3 ± 5.3	128.1 ± 4.8	139.4 ± 5.6	132.9 ± 5.6	133.3 ± 5.3	129.1 ± 6.3	138.8 ± 4.7	134.2 ± 6.4
DAP	115.3 ± 5.1	117.2 ± 1.6	120.6 ± 8.2	114.8 ± 4.3	121.6 ± 5.1	122.5 ± 6.5	125.7 ± 6.8	122.8 ± 4.7
HR	384.2 ± 9.8	391.7 ± 10.1	393.3 ± 12.5	386.8 ± 13.1	391.5 ± 6.7	389.1 ± 11.9	397.5 ± 14.2	386.7 ± 13.1
PP	16.3 ± 6.5	12.3 ± 5.8	18.5 ± 7.2	15.9 ± 4.3	13.5 ± 4.1	17.3 ± 7.2	14.7 ± 8.1	15.8 ± 6.3
MAP	122.2 ± 4.8	119.7 ± 4.2	124.7 ± 6.8	118.4 ± 5.1	125.3 ± 5.1	124.7 ± 5.8	129.6 ± 6.2	124.7 ± 5.3

Met; methionine, SAP; systolic arterial pressure (mmHg), DAP; diastolic arterial pressure (mmHg), HR; heart rate (beats/min), PP; pulse pressure (mmHg), MAP; mean arterial pressure (mmHg). The data were obtained from groups of control vehicle (Con), GS (100 mg/kg) alone, GS (100 mg/kg)+Met, and Met alone. Data are mean $\pm$ S.E.M. ($n=20$, each group).

Effects of GS on Plasma Hcy Concentration after the Long-Term Administration of Met We measured plasma Hcy levels in each administration group. Plasma Hcy levels between normal control and GS alone group after 30 and 60 d were not significantly different from each other. Con: GS = 8.7 ± 3.8 : $9.3 \pm 3.6 \mu\text{M}$ after 30 d and 7.8 ± 3.1 and $7.5 \pm 3.4 \mu\text{M}$ after 60 d, respectively. Plasma Hcy levels were significantly elevated after 30 and 60 d in Met alone administration group compared with normal control and GS alone treatment group. The concentration of Hcy in Met alone treatment group was 62.9 ± 6.3 and $79.0 \pm 6.9 \mu\text{M}$ after 30 and 60 d, respectively. Interestingly, co-administration of GS with Met at dose of 100 mg/kg but not other doses significantly reduced plasma Hcy levels after 30 and 60 d; The concentrations of Hcy were 68.3 ± 7.6 , 65.6 ± 6.9 , 58.3 ± 7.2 , and $31.2 \pm 6.1 \mu\text{M}$ at 12.5, 25, 50 and 100 mg/kg after 30 d ($*p < 0.01$, significantly different from Met alone administration). The concentrations of Hcy were 80.5 ± 6.4 , 79.3 ± 7.1 , 65.6 ± 8.3 , and $41.3 \pm 9.1 \mu\text{M}$ at 12.5, 25, 50 and 100 mg/kg after 60 d ($*p < 0.01$, significantly different from Met alone administration). Thus, co-administration of GS (100 mg/kg) reduced the mean Hcy levels by 50.4% and 47.7% after 30 and 60 d compared with Met alone group, respectively (Fig. 2). However, GS alone administration did not affect plasma Hcy level after 30 and 60 d, indicating that GS itself did not affect basal plasma Hcy level but GS co-administration with Met could inhibit Met alone-caused elevation of plasma Hcy.

Effects of GS on Vasomotor Activity in H-Hcy Rats Since administration of Met enhances the contractile response to NE of rat blood vessel and exhibit stronger contraction in the presence of NE than those of normal rats,²⁹⁾ we next examined whether *in vivo* long-term co-administration of GS with Met affects NE-induced aortic ring contraction in H-Hcy rats. For this, we prepared endothelium-denuded aortic rings from normal control, GS alone, Met, and GS+Met for 60 d and examined NE-induced dose-response contractions in the range of 0.1 nM to 10 μM . % Contractive values compared to 70 mM KCl as 100% were shown in Fig.

Fig. 2. Plasma Hcy Levels

Normal control, GS alone, Met alone or various doses of GS+Met group was treated 30 and 60 d as described in Materials and Methods and plasma total Hcy was measured. Hcy levels were significantly decreased in GS (100 mg/kg)+Met combined group as compared with Met alone group after oral administration of Met. However, this parameter was not significant different between GS alone group and normal control. $*p < 0.01$, vs. Met ($n=20$, each group). Data are means $\pm$ S.E.M.

3A. As shown in Fig. 3A, the contractions induced by NE were not significantly different between normal control and GS alone group. The EC_{50} values were 6.8 ± 0.57 and $8.7 \pm 0.12 \text{ nM}$ for normal control and GS group, respectively. However, we could observe that there were significant increases on contractions induced by NE with dose-dependent manner in Met alone group with reduction of EC_{50} values compared with GS+Met group. Thus, the EC_{50} values were 3.7 ± 0.3 and $6.3 \pm 0.42 \text{ nM}$ for Met and GS+Met group, respectively ($*p < 0.01$, compared with Met alone group) (Fig. 3A). These results are well consistent with previous reports that aortas with H-Hcy are more sensitive to NE than those of normal and indicate that *in vivo* long-term administration of GS attenuates the sensitivity of aorta to NE in H-Hcy rats.

We next measured vasodilatory responses of aorta with endothelium to various concentrations of CCh.¹⁸⁾ % Dilatatory values induced by CCh as compared to 70 mM KCl as 100% were shown in Fig. 3B. As shown in Fig. 3B, the dilatations induced by CCh were not significantly different between normal control, GS alone and GS+Met groups after 60 d except Met alone group. The EC_{50} values were 67.1 ± 7.3 , 61.1 ± 4.4 , and $75.8 \pm 7.3 \text{ nM}$ in normal control, GS alone and GS+Met group, respectively. However, we could also observe that there were significant decreases in dilatory re-

A.

B.

Fig. 3. Effect of GS on Carbachol (CCh)- or Norepinephrine (NE)-Induced Coronary Artery Contraction

Aortic contraction by NE or dilation by CCh was induced by NE or CCh in each group after 60 d. (A) Concentration-dependent contractile curves of NE on coronary artery ring without endothelium. Contractions were significant decreased in GS+Met group (∇) compared with Met alone group ($\blacktriangle$). There was no difference between GS alone group ($\bullet$) and control group ($\blacksquare$). (B) Concentration-dependent dilatory curves of CCh on coronary artery ring with endothelium. Dilatations were significant increased in GS+Met group (∇) compared with Met alone group ($\blacktriangle$). Data are expressed as means $\pm$ S.E.M ($n=20$, each group). $*p < 0.05$, vs. Met.

Fig. 4. Protective Effect of GS on Rat Aortic Artery 60 d after Met Administration

Hematoxylin–eosin (HE)-stained histological sections (5 μ m) of aortic segments from male rats on control (Con) (A), Met (B) and GS+Met group (C). Arrows point the endothelial layer. Scale bar, 50 μ m. Magnification E. 10×0.25 . (D)–(F) Immunohistochemical staining of endothelial nitric oxide synthase (eNOS). Rat aortic arteries were prepared as described in Materials and Methods. eNOS levels were stained with the avidin–biotin complex immunoperoxidase procedure. *In vivo* long-term Met-treated vessels showed less staining of eNOS protein in comparison with control vessels (Fig. 4, arrowhead). In contrast, the GS+Met group showed a staining level of eNOS protein comparable to the control group. Magnification $\times 400$.

sponses induced by CCh with dose-dependent manner in Met alone group. The EC_{50} value was 101.7 ± 10.5 nM (Fig. 3B).

Effects of GS on Endothelial Changes of Artery and eNOS Immunoreactivity After 60 d of administration of normal control, GS, Met, or GS+Met, we prepared arteries and then examined the apparent endothelial morphological changes in each group. As shown in Fig. 4, the morphology of blood vessel after GS alone treatment was not significantly different from normal control group (Fig. 4A), whereas in Met alone group endothelial cells with typical fusiform were lined up in order and we could observe that the intima of vessel was damaged, internal elastic lamina was exposed, endothelial cells was also partially lost, and basal lamina was damaged with swelling (Fig. 4B). Moreover, the surface of endothelial cells remained distorted after 60 d (Fig. 4B, arrow). In contrast, in GS+Met group the endothelial cells were regularly spaced, elongated and normal fusiform with intact borders. We could also not observe the swelling of endothelial cells in this group (Fig. 4C). In eNOS immunoreactivity, Met alone-treated vessels showed significantly less staining of eNOS protein in comparison with control vessels (Figs. 4D, E, arrowhead). In contrast, GS+Met group showed staining levels of eNOS protein comparable to that in the control group, indicating the protective effect of GS on endothelial cells (Fig. 4F).

DISCUSSION

Ginseng becomes popular in the Western world for its alleged tonic effects and possible curative and restorative properties. Accumulating evidences support the potential benefits of ginseng in the cardiovascular systems. For example, administration of ginsenosides, active ingredients extracted from *Panax* ginseng, has been shown to decrease blood pressure in both hypertensive patients and experimental animals.^{30,31} The anti-hypertensive effects of ginsenosides may

be at least partially due to their ability to regulate vascular tones. Indeed, ginsenosides have been shown to concentration-dependently relax the phenylephrine-induced contraction of isolated rabbit aortas.³⁰ However, no previous works have examined whether GS also have beneficial effects on long-term Met-induced H-Hcy and H-Hcy-caused vascular injury in rats.

The present study was performed to know whether *in vivo* long-term administration of GS exhibits beneficial effects on long-term Met-induced H-Hcy and H-Hcy-induced vascular damages. Our results revealed three major findings. First, *in vivo* long-term co-administration of 100 mg/kg GS but not lower doses of GS with Met inhibited the elevation of plasma Hcy induced by long-term administration of Met (Fig. 2). Second, the long-term co-administration of GS (100 mg/kg) with Met ameliorated long-term Met alone-induced arterial vasoconstrictive and vasodilatory dysfunctions (Figs. 3A, B). And third, the long-term co-administration of GS (100 mg/kg) with Met reduced Met alone-induced endothelial injury in arteries (Fig. 4). Thus, the major findings of the present study are that GS has preventive effects against H-Hcy and attenuates the vascular dysfunctions caused by Met-induced H-Hcy.

The previous reports showed that co-administration of folate, vitamin B12, or vitamin B6 prevents the long-term administration of Met-induced H-Hcy.^{3,4,32} The folate and vitamin B12 are involved in the remethylation of Hcy to Met, and vitamin B6 is a necessary cofactor in the degradation of Hcy to cysteine.³³ Thus, the attenuating effect of GS against H-Hcy is unlikely due to that GS is involved in remethylation or degradation of Hcy as those vitamins do. We could not currently explain how the long-term administration of GS inhibits the elevation of plasma H-Hcy induced by long-term administration of Met. There could be several speculations as adaptogenic effects of GS. First, GS might inhibit Met uptake in gastrointestinal systems. Second, GS might facili-

tate Met metabolism and excretions with different ways from those of vitamins. Future studies will be required to assess these possibilities.

In the present study, we have shown that *in vivo* long-term administration of Met induced H-Hcy and the maintenance of H-Hcy was also coupled to endothelial injury (Fig. 4). These results are well consistent with previous reports.^{4,34} It is known that the main mechanisms of endothelial damages by H-Hcy are due to oxidative stress and reduction of NO production caused by eNOS loss. Free radicals have been shown to play a key role in arteriosclerotic plaque formation and to be involved in various vascular injuries.³⁵ Many studies have been conducted on the protective effects of ginseng against free radical damage on the vascular endothelium. For example, Gillis showed the protective effects of ginsenosides on an injured rabbit pulmonary endothelium induced by a variant of reactive oxygen species.¹³ He further showed that ginseng prevented manifestations of oxygen-derived free radical injury by promoting the release of NO.¹³ Thus, the protective effect of GS against H-Hcy-induced endothelial damages might derive from the inhibition of formations of a variant of reactive oxygen free radicals species.

NO produced from eNOS is a known regulator of vascular tone, blood pressure, and antithrombotic activity.^{36–38} Thus, inadequate NO production and decreased eNOS expression due to H-Hcy-induced endothelial damages are seen in arteriosclerosis and other vascular injury.³⁹ As mentioned above, homocysteine also increases potent reactive oxygen free radicals, and this may also explain the decreased availability of NO, because superoxide anion is readily reacted with NO to form peroxynitrite anion.⁴⁰ In our study the long-term Met-induced H-Hcy rat arteries also showed a decrease in eNOS immunoreactivity in comparison with control rings (Fig. 4). The combination of GS and Met led to eNOS staining levels comparable to control rat arteries.

On the other hand, it is known that vascular tone is usually determined by balance between contraction and relaxation. The loss of the basal release of endothelium-derived relaxant factors by H-Hcy results in significant alterations of constriction and relaxation of vascular smooth muscle.²⁸ In further experiments to know whether the long-term co-administration of GS (100 mg/kg) with Met attenuates alterations of constriction and relaxation induced by hormones in blood vessel, we have tested the responses of aortic rings to NE and CCh. We could observe that aortic samples of H-Hcy induced by the long-term administration of Met showed an increased vasoconstriction in the presence of NE and decreased vasodilation in the presence of CCh, whereas aortic samples prepared from the long-term co-administration of GS (100 mg/kg) with Met induced the significant recovery near to normal control level. Thus, the attenuating effects of GS on H-Hcy-caused alterations of constriction and relaxation of aortic vessels might derive from the prevention of endothelial function loss by lowering plasma Hcy.

In the present study, we found that administration of 100 mg/kg GS attenuated Met-induced H-Hcy but the lower doses (12.5, 25, 50 mg/kg) of GS did not prevent Met-induced H-Hcy (Fig. 2). It seems that this effective dosage might be higher than that of human, since Kennedy *et al.* (2004) and Reay *et al.* (2005) showed that single dose of ginsenosides (200 to 400 mg/adult) *via* oral route led to signifi-

cant reductions in blood glucose levels and improve cognitive performance.^{41,42} It is not clear why much higher dosage of GS is required for its pharmacological actions in rat compared with human. The discrepancy between rat and human on the effective dosage of GS might be due to difference of species. In addition, little known about exact metabolic capacity or bioavailability of GS in human and rat, respectively. The elucidation of pharmacokinetics of GS in human and rat might help to determine the effective dosage of GS between human and rat, respectively.

When ginsenosides were administered *via* oral route, ginsenosides metabolites are formed by intestinal microorganisms. Thus, protopanaxadiol ginsenosides are metabolized into compound K (CK) with a glucose at the carbon-20 position, whereas protopanaxatriol ginsenosides are metabolized into M4 leaving only backbone structures of ginsenosides without carbohydrate components. These metabolites are absorbed into the blood in humans and rats (Kanaoka *et al.*, 1994; Karikura *et al.*, 1991).^{43,44} Accumulating evidences have shown that ginsenoside metabolites might exhibit pharmacological and physiological effects and that ginsenosides might play a role as pro-drugs for these metabolites (Wakabayashi, *et al.*, 1997; Wakabayashi, *et al.*, 1998; Hasegawa *et al.*, 2002).^{45–47} In the present study, we administered GS *via* i.p. route rather than oral route in the investigation to know whether GS might affect long-term Met-induced H-Hcy. The present results show a possibility that most of GS after i.p. administration might be directly absorbed into the blood and that ginsenosides rather than ginsenoside metabolites might attenuate Met-induced H-Hcy. The main reason that we examined the effect of GS on Met-induced H-Hcy *via* i.p. rather than oral route was to observe the effect of ginsenosides rather than ginsenoside metabolites. However, further studies will be required to confirm whether ginsenoside metabolites such as CK, M4 and other metabolites might be also involved in attenuation of Met-induced H-Hcy after oral administration of ginsenosides or direct administration of these metabolites.

In summary, the present study showed that the long-term administration of Met induced H-Hcy and caused endothelial damages, and altered vasomotor activity, whereas co-administration of GS with Met diminished Met-induced H-Hcy and attenuated vascular abnormalities caused by H-Hcy. These results show a possibility that GS can be used as one of useful agents attenuating Met-induced H-Hcy and H-Hcy caused-vascular dysfunctions.

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