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Panax ginseng Extract Rich in Ginsenoside Protopanaxatriol Attenuates Blood Pressure Elevation in Spontaneously Hypertensive Rats by Affecting the Akt-Dependent Phosphorylation of Endothelial Nitric Oxide Synthase

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ABSTRACT: Nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS) is a fundamental regulator of systemic blood pressure. Ginsenosides from *Panax ginseng* have been investigated in vitro for the molecular and biochemical mechanisms by which they stimulate NO release in vascular endothelial cells; however, little research has been done to confirm the physiological relevance of these in vitro studies. To address this research gap, the effects of a *P. ginseng* extract rich in ginsenosides from protopanaxatriol on spontaneously hypertensive rats (SHRs) was examined. Ginseng extract administration stimulated nongenomic Akt-mediated eNOS activation, enhanced NO production, improved vessel wall thickening, and alleviated hypertension in SHRs, confirming the physiological relevance of previous in vitro studies with ginsenosides.

KEYWORDS: *ginsenoside, nitric oxide, endothelial nitric oxide synthase, spontaneously hypertensive rats*

INTRODUCTION

Blood pressure (BP) is regulated through the integration of cardiac, neuronal, humoral, and vascular mechanisms. Among the interacting homeostatic regulators identified in the pathogenesis of hypertension, those of the renin−angiotensin system and autonomic nervous system primarily serve to protect the body from hypotension. Indeed, attenuation of the activities of these regulators has little impact on hypertension.[1] On the other hand, endothelium-derived relaxing factor (EDRF), an unstable humoral mediator released from arteries and veins that was later identified as nitric oxide (NO), plays a pivotal role in establishing the baroreceptor set point.[1−4] Studies have provided clear evidence of the involvement of NO in BP regulation. For example, rats treated with compounds that diminish NO bioavailability, such as pharmacologic inhibitors of endothelial nitric oxide synthase (eNOS) including L-nitroarginine or L-*N*-arginine methyl ester, display reduced vascular responsiveness to normal vasodilatory stimuli.[5] The results of studies using eNOS gene knockout animals also support the roles of NO in BP regulation.[1]

Dietary interventions are very important to restore NO bioavailability through improving eNOS function.[6] *Panax ginseng* has drawn wide attention due to an array of potential health benefits, including well-documented cardiovascular-protective effects evidenced by anecdotal descriptive clinical observations and detailed molecular biological studies.[7,8] Since the first isolation of ginsenoside in 1963, over 30 ginsenosides have been identified. These compounds appear in two major variants, ginsenosides from 20(*S*)-protopanaxadiol (g-PPDs) (Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, and Rs1) and ginsenosides from 20(*S*)-protopanaxatriol (g-PPTs) (Re, Rf, Rg1, Rg2, and Rh1), on the basis of the presence of a carboxyl group at the C-6 position.[8]

Much effort has been focused on elucidating the mechanism by which individual ginsenosides mediate NO release from endothelial cells (ECs).[7] One of the major ginsenosides, Rg1, is known to increase phosphorylation of phosphatidylinositol 3-kinase (PI3K), Akt, and eNOS, thereby increasing NO production in ECs.[9−12] Comparison of g-PPTs and g-PPDs has revealed differences in their NO-releasing effects. For example, Rg1 and Re (g-PPTs), but not Rb1 or Rc (g-PPDs), induce endothelium-dependent relaxation in rat aortas.[13] Rg1 and Re (g-PPTs) are functional ligands of glucocorticoid receptor (GR),[11,14] whereas Rb1 (g-PPD) is a functional ligand of estrogen receptor.[15]

Given the poor oral bioavailability of intact ginsenosides in rats[16,17] and the fact that the mechanisms of ginsenosides have largely not been verified in vivo, a more profound understanding of the biological relevance of ginsenosides is needed. Therefore, the goal of the present study was to examine the vasodilatory effects of a *P. ginseng* extract rich in PPT on a common model for human hypertension, the spontaneous hypertensive rat (SHR).

MATERIALS AND METHODS

Preparation of Protopanaxatriol-Rich Ginseng Extract. PPT-rich ginseng extract was kindly provided by CJ Cheiljedang Corp. (Seoul, Korea). Briefly, dried Korean ginseng (*Panax ginseng* C.A. Meyer) roots were cut into small pieces and extracted with ethanol, followed by elution on a Diaion HP-20 ion-exchange resin (Mitsubishi Chemical Co., Tokyo, Japan). An analytical procedure that used an Agilent

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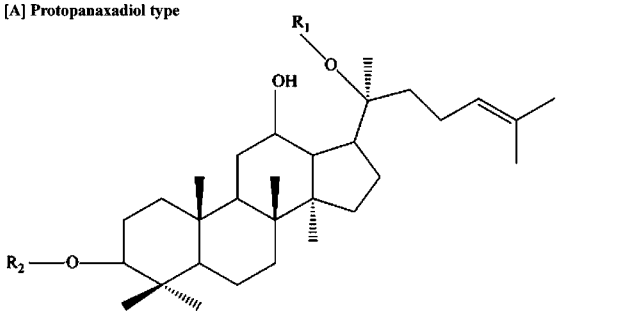
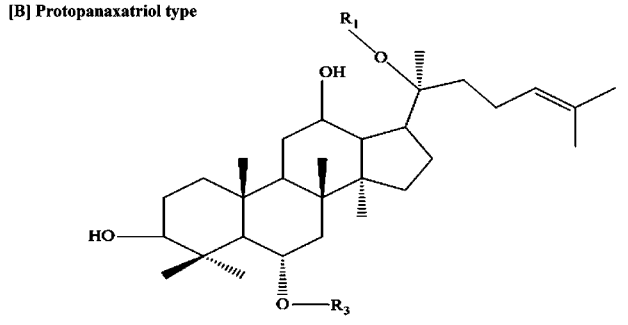
1100 HPLC system (Palo Alto, CA) equipped with a binary gradient pump and a reversed-phase column (Venusil XBP C18, 250 × 4.6 mm, i.d. 5 μm, Agela Technology, Newark, DE) was employed for quantitative determination of major ginsenosides, including Rg1 (137 mg/g), Re (326 mg/g), Rb1 (63.1 mg/g), Rb2 (29.2 mg/g), Rc (73,2 mg/g), and Rd (11.5 mg/g) (Figure 1). The mobile phase consisted of acetonitrile (A) and water (B) with flow rate at 1.6 mL/min, and the column was kept constant at 30 °C. The eluate was monitored at 203 nm and the ginsenosides were identified by comparison with standards. Single ginsenosides (Rg1, Re, Rb1, Rb2, Rc, and Rd, purity >95%) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and BTGin Co., Ltd. (Chungnam, Korea).



Ginsenosides	R_2	R_1
Rb1	Glc^{1}-^{2}Glc-	Glc^{1}-^{6}Glc-
Rb2	Glc^{1}-^{2}Glc-	Ara(p)^{1}-^{6}Glc-
Rc	Glc^{1}-^{2}Glc-	Ara(f)^{1}-^{6}Glc-
Rd	Glc^{1}-^{2}Glc-	Glc-



Ginsenosides	R_3	R_1
Rg1	Glc-	Glc-
Re	Rha^{1}-^{2}Glc-	Glc-

Figure 1. Molecular structures of ginsenosides. (A) Protopanaxadiol (PPD) type: Rb1, Rb2, Rc, and Rd. (B) Protopanaxatriol (PPT) type: Rg1 and Re.

Measurement of Systolic Blood Pressure in Spontaneously Hypertensive Rats. All experimental procedures were conducted in compliance with the guidelines and protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Ewha Womans University. Forty 10-week-old male SHRs were purchased from the Jung-Ang Lab Animal Inc. (Seoul, Korea). During the experimental period, the rats were housed individually with 12 h light/dark cycle under a temperature of 23 ± 1 °C and allowed free access to water and a semipurified high-fat diet, modifying a base AIN-93G formulation. The diet approximately consisted of 40 energy percent (En%) carbohydrates (cornstarch, dextrinized cornstarch, and sucrose), 20 En% protein (casein), and 40 En% fats (lard and soybean oil). After a 1-week acclimation period, rats were randomly divided into four groups (n = 10 in each group): 0 (vehicle, control), 50 (low-dose group, LG), 100 (medium-dose group, MG), or 500 mg/kg body weight (high-dose group, HG). PPT-rich ginseng extract or vehicle (saline) was administered to rats by oral gavage once daily for 4 weeks. These doses were estimated on the basis of our preliminary in vitro studies (data not published) and bioavailability of ginsenosides.[17,18] Systolic blood pressure (SBP) of unanesthetized, prewarmed, restrained rats was measured by a noninvasive tail-cuff method (BP-2000 blood pressure analysis system, Visitech Systems, Apex, NC) at t = 0, 2, 4, 6, and 8 h postadministration on the first day and once a week thereafter. The SBP was presented as the mean of five measurements for each rat.

Histological Examination of Aortic Wall. The rats were killed and segments of thoracic aorta were fixed in 10% neutral buffered formalin solution for 24 h. The fixed aortas were dehydrated through a graded series of ethanol and embedded in paraffin according to standard procedures. Paraffin sections (5 μm thick) were stained with hematoxylin and eosin and examined microscopically.[19,20]

Western Blot Analysis of Phosphorylated eNOS and Phosphorylated Akt. The aorta vessels frozen in liquid nitrogen were homogenized in an ice-cold protein extraction solution with a polytron homogenizer and then centrifuged at 13 000 rpm for 10 min. The supernatants were collected, and protein concentrations were analyzed via bicinchoninic acid (BCA) protein assay. Equal amounts of proteins were subjected to immunoblot. Membranes were probed with antibodies against phosphorylated p-eNOS (Ser1177) and p-Akt (Ser473) and ß-actin from Cell Signaling Technology, Inc. (Danvers, MA). The immunoreactive proteins were detected by a chemiluminescence detection system. Equal protein loading was verified by use of a housekeeping ß-actin antibody. The protein bands were digitally imaged on a LAS-3000 imaging system and Multi Gauge software (Fujifilm, Cypress, CA).

Citrulline Assay. The eNOS activity was determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]-citrulline[21] with a NOS activity assay kit (Calbiochem–Novabiochem International Corp., La Jolla, CA). In brief, aorta vessels were homogenized in lysis buffer. The reaction was initiated by adding cofactors and L-[^{3}H]arginine and terminated by adding stop buffer, followed by elution on resin. [^{3}H]Arginine did not bind to the column resin. Thus, the activity of eNOS was calculated from the formation of L-[^{3}H]citrulline by use of a LS6500 liquid scintillation counter (Beckman Coulter Inc., Brea, CA). Data was calculated as counts per minute (cpm) per microgram of protein and expressed as percent of control.

RNA Isolation and Microarray Analysis of Gene Expression. For the microarray analysis, total RNA was extracted from each aorta of three rats selected from the HG and control using the TRI Reagent (Ambion, Austin, TX). Double-stranded cDNA was synthesized from each total RNA via the MAUI hybridization system (BioMicro Systems, Inc. Salt Lake City, UT). After analysis of its quality, biotinylated cRNA was transcribed from the cDNA and was analyzed by the Roche NimbleGen rat genome oligo 12-plex chip (Madison, WI), covering over 26 419 genes. The arrays were analyzed on

an Axon GenePix 4000B scanner with associated software (Molecular Devices, Sunnyvale, CA). All scanning data were analyzed with GeneSpring GX 7.3.1 (Agilent Technologies, Santa Clara, CA).

Statistical Analysis. Results were expressed as mean ± standard error (SE). The data were analyzed by one-way analysis of variance (ANOVA) with post hoc Dunnet's multiple comparison test. In microarray analysis, the average values from three samples are presented as the mean ± standard deviation (SD). Significant difference between the treatment group and the control group was evaluated by using Student's t test. Statistical significance was indicated by $P < 0.05$. Statistical analyses were performed by using the Statistical Analysis Systems package, version 9.2 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Hypotensive Effects of Protopanaxatriol-Rich Ginseng Extract in Spontaneously Hypertensive Rats. The baseline SBP for all groups was 184.67 ± 2.13 mmHg. After a single administration of PPT-rich ginseng extract, a general line model for repeated measures showed dose-dependent variations of SBP over 8 h postadministration ($P < 0.001$). The maximum effect was seen in the HG group at 6 h postadministration ($P = 0.0269$ versus control) and recovered to the initial value thereafter (Figure 2a). Miguel et al.[22] also reported that single administration of milk-casein-derived peptides showed a maximum decrease of SBP 4−6 h postadministration in SHRs and then a progressive increase of SBP to reach values similar to those of the control rats 24 h postadministration. Repeated administrations of PPT-rich ginseng extract for 4 weeks also led to significant variations over time ($P = 0.0263$), with the HG group showing a remarkable decrease in SBP from week 2 ($P = 0.0081$) (Figure 2b). PPT-rich ginseng extract intake had no effect on body weight or food intake (data not provided), which suggests that the beneficial effect on BP is not due to alterations of these parameters.

Effect of Protopanaxatriol-Rich Ginseng Extract on Endothelium-Dependent Vasodilation via Phosphatidylinositol 3-Kinase/Akt Signal Pathway. To link the BP-lowering effect of PPT-rich ginseng extract with its underlying molecular mechanisms, we determined its direct effect on the protein expression and phosphorylation of eNOS and Akt in the SHR aorta by Western blotting. Active Akt directly phosphorylated eNOS at Ser 1177/1179.[23,24] Although the total eNOS and Akt protein expression displayed no changes in quantity (data not shown), phosphorylations of eNOS and Akt in the HG group were 3.5-fold ($P = 0.061$) and 4-fold higher ($P = 0.0051$), respectively, than those of the control (Figure 3a). To confirm this result, eNOS activity was measured from the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in aortic homogenates from SHRs. Approximately 2-fold higher L-[^{3}H]citrulline levels were found in the HG group compared to the control ($P = 0.001$) (Figure 3b).

Both increased eNOS expression and increased eNOS enzymatic activity can reduce BP. Of the three main isoforms of NOS enzymes, neuronal NOS and eNOS are generally constitutively expressed. Thus, the main switch for eNOS activity is not at the level of transcription but enzyme activation.[1] Consistent with our findings, a previous in vitro study showed no changes in the total quantity of eNOS with ginsenoside treatment.[11] Taken together, these results suggest that the BP-lowering effect of PPT-rich ginseng extract may depend, at least partially, on the nontranscriptional PI3K/Akt signal pathway.

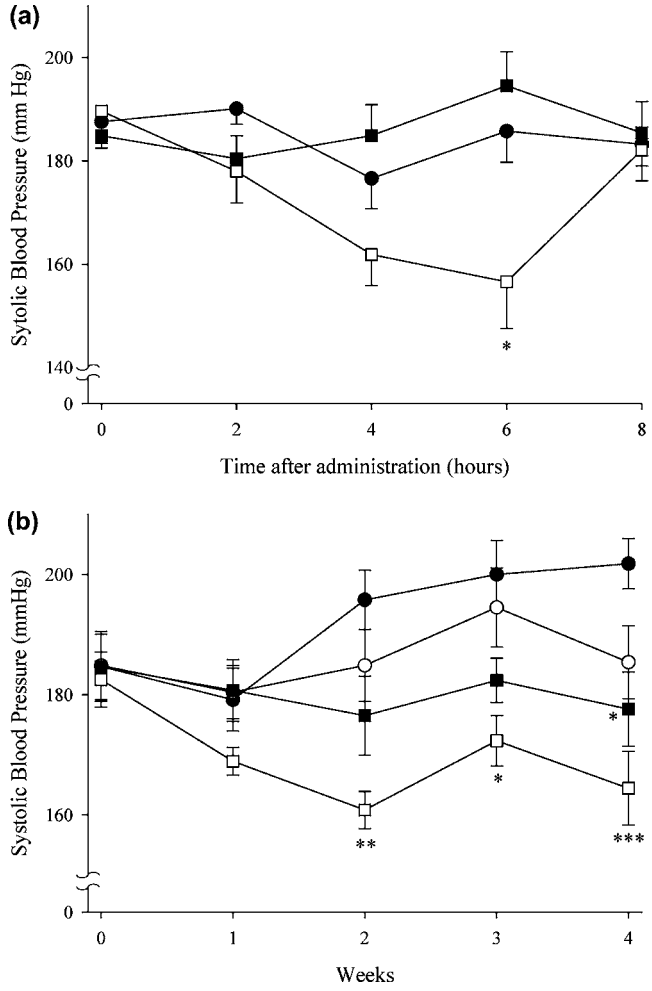


Figure 2. Changes of SBP in SHRs after (a) single or (b) repeated administration of PPT-rich ginseng extract for 4 weeks at the following doses: (●) vehicle, (○) 50 mg/kg, (■) 100 mg/kg, or (□) 500 mg/kg. For single administration, SBP was monitored in the groups vehicle, 100 mg/kg, and 500 mg/kg only. Data are mean value ± SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs vehicle-treated control, based on ANOVA with post hoc Dunnet's multiple range test.

PPT-rich ginseng extract administration reduced aortic wall thickness in SHRs (Figure 4), confirming the results of a previous study showing that higher BP is associated with increased aortic wall thickness.[25] However, it remains unknown whether PPT-rich ginseng extract inhibits the proliferation of vascular smooth muscle cells, although previously it was shown that eNOS-derived NO inhibits vascular smooth muscle cell growth.[26] This question requires further investigation.

Effect of Protopanaxatriol-Rich Ginseng Extract on Gene Expression in Aortic Vessel. Total RNA was successfully prepared from the aorta of rats from the HG and control and gene profiling was performed with 23,146 genes. The signal intensities of 448 genes were differently expressed between the 2 groups at levels of more than 1.5-fold or below 0.66-fold (data not shown). Genes associated with vascular and endothelial pathways were analyzed in detail to obtain extensive information about the in vivo metabolic pathway associated with the antihypertensive effect of PPT-rich ginseng extract. The changes in gene expression after the administration of

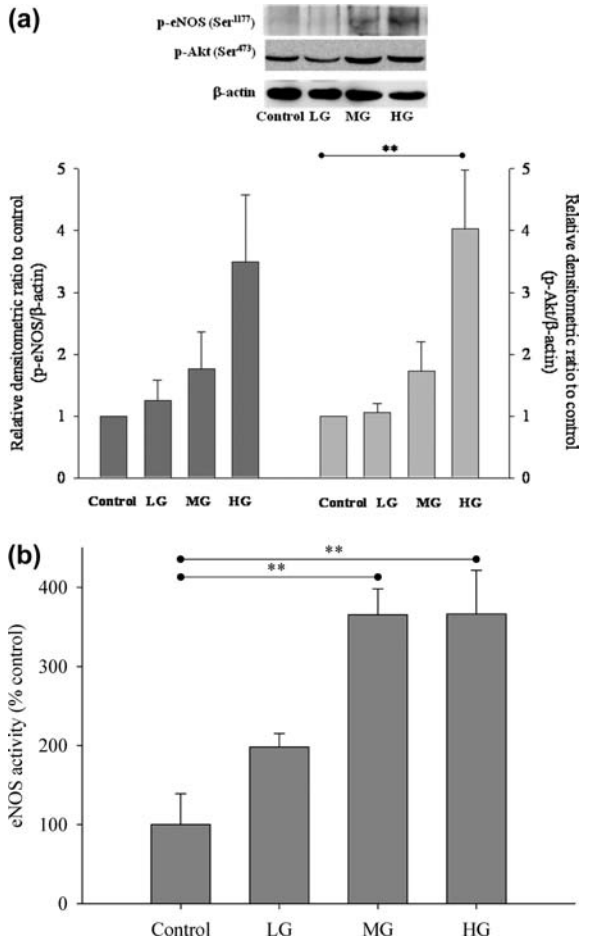


Figure 3. Direct effects of PPT-rich ginseng extract on eNOS activation in SHR aorta, after repeated administration of PPT-rich ginseng extract at different doses (vehicle, 50, 100, or 500 mg/kg) for 4 weeks. (a) Phosphorylated eNOS (Ser 1177) and Akt (Ser 473) increased in a dose-dependent manner. (b) eNOS activity measured by conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline also increased dose-dependently. Protein loading was normalized by β-actin. eNOS activity is expressed as percent of control. Data are mean value ± SE (n = 10). **P < 0.01 vs vehicle-treated control, based on ANOVA with post hoc Dunnet's multiple range test.

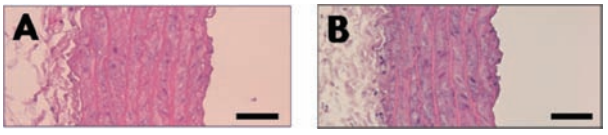


Figure 4. Thoracic aorta from SHR rats treated for 4 weeks with (A) vehicle and (B) PPT-rich ginseng extract, 500 mg/kg. Note marked decrease in the thickness of individual smooth muscle cell layers of aorta in the treated rat (B) when compared those of control rat (A). H&E stain, bar = 50 μm.

PPT-rich ginseng extract were not large, but there were some significant differences between the HG and the control group (Table 1). The most significant difference was a decrease in intercellular adhesion molecule 1 (ICAM-1) gene with about 0.65-fold (P = 0.01). ICAM-1 is a cell surface glycoprotein expressed on various cell types, including vascular smooth muscle cells,[27,28] and has been known to increase the progression of aortic atherosclerosis.[29]

Table 1. Changes in Gene Expression Associated with Vascular and Endothelial Pathways[a]

description	control (n = 3), mean ± SD	HG (n = 3), mean ± SD	x-fold change	P-value
Vascular Function				
intercellular adhesion molecule 1 (ICAM-1)	642 ± 335	418 ± 31**	0.65	0.010
nitric oxide synthase 2 (iNOS)	2223 ± 183	2215 ± 108	1.00	0.884
endothelial nitric oxide synthase (eNOS)	1914 ± 132	1812 ± 101	0.95	0.225
Akt	2329 ± 173	2135 ± 283	0.92	0.361
phosphatidylinositol 3-kinase (PI3K)	785 ± 56	808 ± 136	1.03	0.877
caveolin 1	4699 ± 646	5091 ± 491	1.08	0.297
C-terminal Hsp70-interacting protein (CHIP)	1129 ± 348	897 ± 177	0.79	0.156
nitric oxide synthase-interacting protein (NOSIP)	196 ± 42	163 ± 13	0.83	0.051
Blood Coagulation				
plasminogen	506 ± 100	633 ± 61	1.25	0.056
urokinase-type plasminogen activator (U-TPA)	1825 ± 330	1705 ± 122	0.93	0.237
Renin–Angiotensin System				
angiotensin-converting enzyme	975 ± 130	932 ± 67	0.96	0.370
angiotensinogen	652 ± 36	667 ± 97	1.02	0.855
renin	1665 ± 141	1732 ± 143	1.04	0.509

[a]Significant differences between the control and HG group were evaluated by Student's t-test (** P < 0.001).

For genes associated with eNOS synthesis, there were no significant changes in the expressions of eNOS (0.95-fold, P = 0.225), Akt (0.92-fold, P = 0.361), and PI3K (1.03-fold, P = 0.877), confirming the importance of posttranslational modifications of eNOS at multiple sites in endothelial NO production. In addition, translocation of eNOS from the plasma membrane to intracellular compartments is also important in regulating eNOS activity.[30] In resting cells, caveolin-1 binds to membrane-bound eNOS and inhibits its activity.[31] The C-terminal Hsp70-interacting protein (CHIP) negatively regulates eNOS trafficking into the Golgi complex,[32] and the nitric oxide synthase-interacting protein (NOSIP) inhibits NO production by negatively regulating eNOS localization in the plasma membrane.[33] The caveolin-1 gene showed no change (1.08-fold, P = 0.297), whereas genes for CHIP (0.79-fold, P = 0.156) and NOSIP (0.83-fold, P = 0.051) showed a tendency toward slightly lower expression after PPT-rich ginseng extract administration.

Next, genes associated with blood coagulation and renin–angiotensin system that are known to have effects on blood pressure were analyzed. The HG showed an increasing tendency in plasminogen gene with about 1.25-fold (P = 0.056). However, no other significant differences were detected for the other genes. Taken together, thess microarray data suggest that PPT-rich ginseng extract might have additional preventive potential in cardiovascular function other than acting as NO stimulator in the aorta. Additional microarray and reverse transcription–polymerase chain reaction (RT-PCR) analysis for other organs such as lung, kidney, and liver would be helpful for furthering understanding this potential effect of PPT-rich ginseng extract and individual ginsenosides.

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This study clearly demonstrates that administration of the PPT-rich ginseng extract stimulated nongenomic eNOS activation, enhanced NO production, improved vessel wall thickening, and alleviated hypertension in SHRs, confirming the physiological relevance of previous in vitro studies. These findings also imply some other potential molecular mechanisms (ICAM-1 and plasminogen) underlying the physiological effects of PPT-rich ginseng extract in the vasculature. Our choice of PPT-rich ginseng extract was based on our previous observation that PPT-rich ginseng extract has a higher potency of releasing NO from ECs than individual ginsenosides (data not shown), possibly due to the presence of multiple active components. Whether combinational interactions exist between ginsenosides remains to be studied.

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Notes

The authors declare no competing financial interest.
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ABBREVIATIONS:

BP, blood pressure; EDRF, endothelium-derived relaxing factor; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; PPD, protopanaxadiol; PPT, protopanaxatriol; EC, endothelial cells; PI3K, phosphatidylinositol 3-kinase; SHR, spontaneously hypertensive rat; ICAM-1, intercellular adhesion molecule 1

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