Ginseng Inhibits Cardiomyocyte Hypertrophy and Heart Failure via NHE-1 Inhibition and Attenuation of Calcineurin Activation

Running Title: Guo et al: Antihypertrophic Effects of Ginseng

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Abstract

Background—Ginseng is a medicinal plant used widely in Asia which has gained popularity in the West during the past decade. Increasing evidence suggests a therapeutic role for ginseng in the cardiovascular system. The pharmacological properties of ginseng are mainly attributed to ginsenosides, the principal bioactive constituents in ginseng. The present study was carried out to determine whether ginseng exerts a direct antihypertrophic effect in cultured cardiomyocytes and whether it modifies the heart failure process in vivo. Moreover, we determined the potential underlying mechanisms for these actions.

Methods and Results—Experiments were performed on cultured neonatal rat ventricular myocytes as well as adult rats subjected to coronary artery ligation (CAL). Treatment of cardiomyocytes with the $\alpha_1$ adrenoceptor agonist phenylephrine for 24 h produced a marked hypertrophic effect as evidenced by significantly increased cell surface area and ANP gene expression. These effects were attenuated by ginseng in a concentration-dependent manner with a complete inhibition of hypertrophy at a concentration of 10 $\mu$g/ml. Phenylephrine-induced hypertrophy was associated with increased gene and protein expression of the Na$^+$-H$^+$ exchanger 1 (NHE-1), increased NHE-1 activity, increased intracellular concentrations of Na$^+$ and Ca$^{2+}$, enhanced calcineurin activity, increased translocation of NFAT3 into nuclei and GATA-4 activation, all of which were significantly inhibited by ginseng. Upregulation of these systems was also evident in rats subjected to 4 weeks of CAL. However, animals treated with ginseng demonstrated markedly reduced hemodynamic and hypertrophic responses which were accompanied by attenuation of upregulation of NHE-1 and calcineurin activity.

Conclusions—Taken together, our results demonstrate a robust antihypertrophic and antiremodeling effect of ginseng which is mediated by inhibition of NHE-1 dependent calcineurin activation.

Key Words: ginsenosides, phenylephrine; NHE-1, calcineurin, heart failure
Ginseng is a popular herbal medicine used in Asia for centuries although in recent years its potential therapeutic effects have become more widely recognized. Ginsenosides are the principal bioactive constituents of ginseng and more than 40 different ginsenosides have been isolated to date.\(^1\) Ginseng exerts numerous pharmacological properties in multiple species including humans.\(^2\) The cardiovascular beneficial effect of ginseng has also been demonstrated including for the treatment of angina pectoris\(^3\) and reducing adriamycin-induced heart failure in rats.\(^4\) Moreover, ginseng has been demonstrated to attenuate right and left ventricular hypertrophy in a number of experimental models.\(^5-7\) Since the underlying basis for the antihypertrophic effect of ginseng is poorly understood, we studied the mechanisms for the antihypertrophic effect of ginseng using cultured ventricular myocytes as well as an in vivo model of heart failure secondary to chronic ischemia. The study centered primarily on the Na\(^+\)-H\(^+\) exchanger-1 isoform (NHE-1) which has been extensively shown to contribute to hypertrophy and heart failure.\(^8-9\) Accordingly, we determined the effect of ginseng on NHE-1 activity and expression and subsequent effects on key signaling mechanisms underlying the hypertrophic program. For example, NHE-1 has been shown to contribute to intracellular Ca\(^{2+}\) overloading resulting in the activation of Ca\(^{2+}\)-dependent pro-hypertrophic processes mediated by the protein phosphatase calcineurin and subsequent activation of pro-hypertrophic transcriptional factors.\(^10,11\) Here we determined the role of this pathway in mediating the antihypertrophic effect of ginseng in cultured ventricular myocytes subjected to hypertrophic stimuli and applied these findings to an in vivo model of heart failure in rats.
Methods

Neonatal Cardiac Myocytes Culture and Treatment Protocol

The studies have been approved by the Animal Use Subcommittee of the University of Western Ontario and procedures conform to the guidelines of the Canadian Council on Animal Care (Ottawa, ON, Canada). Myocytes were prepared from hearts of 1 to 4-day-old Sprague-Dawley rats as described previously and cultured for 24 h in serum containing medium followed by 24 h in serum free medium. To initiate hypertrophy, myocytes were then treated with 10 μM of the α1 adrenoceptor agonist phenylephrine (PE) for 24 h in the absence or presence of ginsenosides (0.1, 1 or 10 μg/ml). For some experiments (see Results) cells were subjected to PE treatment for shorter durations.

Ginsenoside Extraction Procedure

Four year old North American ginseng (Panax quinquefolius) roots were collected from 5 different farms in Ontario, Canada and shipped to Naturex (South Hackensack, NJ) for ginsenoside extraction using a hydroalcoholic process. Briefly, ground ginseng roots were soaked three times over five hours in an ethanol/water (75/25, v/v) solution at 40°C. The extract was filtered and excess solvent removed under vacuum at 45°C. The extract was concentrated again until the total solids on a dry basis were approximately 60%. These concentrates were then lyophilized at the Ontario Ginseng Innovation and Research Consortium central laboratory (University of Western Ontario) to produce a powdered alcoholic ginseng extract which was then subjected to analysis by high pressure liquid chromatography (HPLC) to determine presence of major (Rb1 and Re) and minor (Rg1, Rb2, Rd, and Rc) ginsenosides.
Measurement of Cell Surface Area

Myocytes were visualized using a Leica DMIL inverted microscope (Leica, Wetzlar, Germany) equipped with an Infinity 1 camera. At least 10 random photographs were taken from each dish and the cell surface area of a minimum of 30 cells from each treatment was measured using SigmaScan Software (Systat, Richmond, CA).

Measurement of Intracellular Na\(^+\) and Ca\(^{2+}\) Concentrations

Myocytes were incubated with CoraNa Red (excitation: 554 nm and emission: 578 nm) or Furo-2 (excitation: 338 nm and emission: 510 nm) for 30 min at 37 °C, to measure Na\(^+\) or Ca\(^{2+}\) concentrations, respectively. Myocytes were washed twice with phosphate-buffered saline (PBS) and fluorescence intensity was measured using a spectra Max M5 plate reader. Fluorescence intensity was normalized against control (CoroNa-Red-loaded cells or Furo-2-loaded cells) after subtraction of baseline (CoroNa-Red or Furo-2 without cells).

Measurement of Intracellular pH (pHi)

pHi was measured using the pH-sensitive dye 2', 7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxyethyl ester (Invitrogen, Carlsbad, CA). Briefly, myocytes were loaded with the dye at 37°C for 30 min and placed on the stage of an inverted Zeiss Axiovert 35 microscope. Myocytes were continuously perfused at 1 ml/min with HCO\(_3\) free HEPES buffer solution. The pHi in individual cardiomyocytes was recorded by photometry at 502.5 nm and 440 nm for excitation and 528 nm for emission using a monochromatic Deltascan-4000 system (Photon Technology International, Birmingham, NJ). The NH\(_4\)Cl prepulse technique was used to
determine activity of NHE-1 and the effect of treatments on NHE-1 activity in cardiomyocytes.\textsuperscript{13}

**Calcineurin Phosphatase Activity Assay**

Calcineurin activity was determined using commercially-available kits according to the manufacturer’s instructions (Enzo Life Sciences, Plymouth Meeting, PA).

**Determination of NFAT3 Translocation**

Myocytes were fixed in an acetone and methanol (20:80) mixture as described previously.\textsuperscript{14} After permeabilization and blocking, cells were incubated with NFAT3 antibody (1:100 dilutions) overnight at 4°C followed by incubation with Alexa Fluor 594 goat anti-rabbit IgG (1:250 dilutions) for one hour at room temperature in dark. The cells were mounted on the glass slide using DakoCytomation fluorescent mounting medium and visualized using a Zeiss Axio Observer D1 fluorescence microscope (Zeiss, Gottingen, Germany).

**Electrophoretic-Mobility Shift Assay (EMSA)**

EMSAs were performed using the Panomics EMSA Gel-Shift Kit (Panomics, Inc., Fremont, CA) according to the manufacturer’s protocol.

**RNA isolation, Reverse transcription and Real-Time PCR Analysis**

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. RNA (2 μg) was used to synthesize first strand of cDNA using M-MLV reverse transcriptase according to the manufacturer’s protocol and was used as a template in the following PCR reactions. The expression of ANP, NHE-1 and 18S rRNA (loading control) genes
was determined in 10 μl reaction volumes using SYBR green Jumpstart Tag ReadyMix DNA polymerase and fluorescence was measured and quantified using DNA Engine Opticon 2 System. The following primer sequences were used: 5’- CTGCTAGACCACCTGAGGA-3’ (forward) and 5’-AAGCTGTTGCAGCC TAGTCC-3’ (backward) for ANP; 5’ ATGTGGGCTGG GAAACAAGAC-3’ (forward) and 5’-GACAGTCCCTCCCGTGAAA-3’ (backward) for NHE-1 and 5’-GCCCAATCCAGACAAACAGT-3’ (forward) and 5’-TGATTTTT GGCTTGGGTCTC-3’ (backward) for MCIP1 and 5’-GTAACCC TTGAACCCCATT-3’ (forward) and 5’-CCATCCAAT CCGTA GTAGCG-3’ (backward) for 18S rRNA. PCR conditions and cell cycle number were optimized for each set of primers. Melting curve analysis showed a single PCR product for each gene amplification. PCR conditions to amplify all three genes were 30 s at 94°C followed by annealing at 60°C for 25 s for ANP and NHE1 and 54°C for 20 s for 18S rRNA followed by elongation at 72°C for 30 s. All genes were amplified for 40 cycles except 18S rRNA which was amplified for 35 cycles.

**Western Blotting for GATA-4 and NHE-1**

After appropriate treatments, myocytes were washed with PBS and lysed with 150 μl of lysis buffer. Cell lysates were transferred to 1.5 ml Eppendorf tubes, homogenized, and centrifuged at 10,000 x g for 5 min at 4°C. The supernatant was transferred to a fresh tube and the protein concentration determined by the Bradford protein assay method (Bio-Rad, Hercules, CA). Thirty micrograms of protein were resolved on a 10 % SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked in 5 % milk for 1 h and incubated with primary antibody for GATA-4 or NHE-1 for 1 h followed by secondary antibody for 1 h and then detected by enhanced chemiluminescence reagent (Amersham Biosciences Inc., Piscataway, NJ).
The blots were stripped and reprobed with actin antibodies.

**Coronary Artery Ligation**

Male Sprague-Dawley rats (275-300g) were randomly assigned to the following four treatment groups: sham and coronary artery ligation (CAL) with or without ginsenosides (100 mg/kg) treatment started 24 hours after surgery. Surgery was performed as previously described. All animals received 0.03 mg/kg buprenorphine immediately after completion of surgery for pain management.

**Echocardiography**

Four weeks after CAL, rats were anaesthetized with 2% isoflurane and placed in a supine position on a heated platform. The chest and abdomen were shaved and the extremities were fixed to electrodes on the platform surface using tape and a highly conductive electrode gel. Echocardiography evaluations were performed using a Vevo 770 high-resolution in vivo micro-imaging system equipped with a real-time micro visualization scan head of 17.5 MHz (VisualSonics, Toronto, ON, Canada). M-mode two-dimensional echocardiography images were obtained from the parasternal short-axis. Images were analyzed using the Vevo 770 Protocol-Based Measurements software and calculations for the dimensions of the left ventricle (LV) diameter. Doppler measurements were taken to determine peak early diastolic filling velocity (E wave), peak late diastolic filling velocity (A wave) and to determine E/A ratios.

**Hemodynamic Measurements**

The rats were anesthetized with pentobarbital sodium (50 mg/kg). An anterior thoracotomy was
performed, and the LV was catheterized retrogradely via the right carotid artery using a 2.0F P-V Mikro-Tip catheter (Millar Instruments, Houston, TX) as previously described.\textsuperscript{15} Data were recorded and analyzed by hemodynamic data analysis software (Notocord, Croissy-sur-Seine, France), digitized with a sampling rate of 1000 Hz and recorded on a personal computer using Notocord-hem 4.2 software.

**Statistical Analysis**

Results are presented as means ± SEM. The data were analyzed with one way ANOVA and group differences were detected using a Student-Newman-Keuls post-hoc test when initial ANOVA analysis revealed statistically significant differences. P values of \( P < 0.05 \) were considered significant.

**Results**

**Effect of Ginseng on PE-Induced Cardiomyocyte Hypertrophy**

Myocytes treated with PE for 24 h demonstrated a significant increase in cell surface area from 850 ± 14 \( \mu \text{m}^2 \) to 1090 ± 20 \( \mu \text{m}^2 \) (Figure 1A &B, \( P < 0.05 \)) whereas in the presence of 10 \( \mu \text{g/ml} \) ginsenosides cell surface area in the presence of PE was reduced to 942 ± 44 \( \mu \text{m}^2 \) (Figure 1B, \( P < 0.05 \) PE alone). PE increased gene expression of ANP by greater than two-fold (Figure 1C; \( P < 0.05 \)) compared to controls whereas this was almost completely prevented by 10 \( \mu \text{g/ml} \) ginsenosides. Ginsenosides had no direct effect on either parameter in the absence of PE. It should be added that ginsenosides exerted similar effects against other prohypertrophic stimuli including either 100 nM angiotensin II or 10 nM endothelin-1 (Figure 2).
Effect of Ginseng on PE-Induced Changes in NHE-1 Protein, Gene Expression and Activity

Figure 3 shows that PE-induced a 1.56 ± 0.16 fold upregulation of NHE-1 gene (Figure 3A) and 1.38 ± 0.09 fold increase in protein abundance (Figure 3B) after 24 h treatment which was associated with increased NHE-1 activity (Figure 3C). The upregulation of NHE-1 gene and protein expression was abrogated by the two highest concentrations of ginsenosides with increases for gene and protein levels in the presence of 10 μg/ml ginsenosides reduced to 1.18 ± 0.04 fold and 1.07 ± 0.10 fold, respectively (panels A and B). Moreover, stimulation of NHE-1 activity was reduced to values not significantly different from control by 10 μg/ml ginsenosides (Figure 3C).

Effect of Ginseng on PE-Induced Changes in Intracellular Na\(^+\) and Ca\(^{2+}\) Concentrations

PE-induced a rapid elevation in intracellular concentrations of both Na\(^+\) and Ca\(^{2+}\) which was evident 15 min after PE addition (Figure 4). No effect of ginseng (10 μg/ml ginsenosides) was observed up to 6 h after its addition although a significant reduction in the intracellular concentrations of both Na\(^+\) and Ca\(^{2+}\) was evident 12 and 24 h after administering ginsenosides with values not significantly different from control after 24 h.

Effect of Ginseng on PE-Induced Changes in Calcineurin Activity

As shown in Figure 5A calcineurin activity was rapidly (within 15 min) increased after PE administration with activity steadily declining in the presence of ginsenosides after 6 h, although still significantly greater from control. Ginsenosides alone had no effect on calcineurin activity (Figure 5A).
Effect of Ginseng on PE-Induced Changes in NFAT3 Nuclear Import and GATA-4 Activation

Localization of NFAT3 in control myocytes was primarily restricted to the cytosol (Figure 5B) although substantial translocation to nuclei was evident after 24 h PE treatment. Ginsenosides clearly reduced PE-induced translocation resulting in substantial cytosolic localization of NFAT3 similarly to that seen under control conditions (Figure 5B).

PE significantly increased GATA-4 phosphorylation by 1.3 fold (Figure 5C; \( P<0.05 \)) and increased GATA-4-DNA binding activity as determined by EMSA (Figure 5D). Both responses were prevented by ginsenosides (Figure 5C&D).

Effect of Ginseng on CAL-Induced Left Ventricular Dysfunction

We next determined whether direct antihypertrophic effect of ginsenosides seen in cultured myocytes can be translated to protection \textit{in vivo} in rats subjected to 4 weeks of sustained CAL.

As shown in the Table, CAL produced marked systolic and diastolic abnormalities which was attenuated by ginsenoside treatment. Moreover, ginsenosides treatment significantly reduced the increased in left ventricular inner diameters in rats subjected to sustained CAL (Figure 6A & B). In addition CAL increased the E/A ratio obtained from Doppler echocardiographic analysis which was normalized in animals treated with ginseng indicative of improved diastolic function (Figure 6C &D).
Effect of Ginseng on CAL-Induced Cardiac Hypertrophy

Animals subjected to CAL had significantly reduced body weights at the end of the 4 week ligation period although this was unaffected by ginsenosides (Figure 7A). Animals subjected to CAL exhibited significantly increased left ventricle weights as well as ANP expression (Figure 7D), indicating development of left ventricular hypertrophy (Figure 7B-6D). These responses were completely prevented by ginsenosides.

Effect of Ginseng on CAL-Induced NHE-1 and Expression and Calcineurin Activation

As shown in Figure 8A, rats subjected to CAL had significantly increased NHE-1 expression (1.98 ± 0.16 fold) although this was partially but significantly reduced by ginsenoside treatment (1.41 ± 0.11 fold) (Figure 8A). Two indicators of calcineurin activity, namely modulatory calcineurin interacting protein 1 (MCIP-1) expression (Figure 8B) and calcineurin phosphatase activity (Figure 8C) were significantly increased in hearts subjected to CAL although these responses were completely abrogated by ginsenosides (Figure 8B & C).

Discussion

Although ginseng has been used as a pharmacotherapeutic agent in Asian society for centuries its potential cardiac therapeutic properties have not been extensively studied and are poorly understood. Ginseng is also among the most common of the alternate medicines used by the American population, although not necessarily for cardiovascular disorders.16 Whether the use of natural compounds such as ginseng holds promise for the treatment of cardiovascular disorders is not known, possibly due to a paucity of data demonstrating their effects in well-established experimental models of cardiovascular disease as well as the lack of information on the
mechanism of action of these compounds. Here we determined the potential antihypertrophic effect of the biologically active components of ginseng, the ginsenosides, extracted from North American ginseng, on the hypertrophic response of myocytes exposed to the $\alpha_1$ adrenoceptor agonist PE. We show that ginsenosides provide a robust antihypertrophic influence in this model of hypertrophy although our study also suggests that the antihypertrophic effect of ginseng is likely not restricted to PE particularly as it would be unlikely that the robust salutary effects seen in vivo was mediated solely by an effect restricted to inhibition of $\alpha_1$ adrenoceptor-mediated hypertrophy. Moreover, our study shows that ginsenosides also markedly attenuate the direct hypertrophic of both angiotensin II and endothelin-1 on myocytes.

Overall, our study strongly suggests that the ability of ginsenosides to attenuate hypertrophy is related to preventing the activation/upregulation of NHE-1, which has been extensively implicated in the hypertrophic and heart failure process.\textsuperscript{17,18} This likely occurs subsequent to receptor-dependent NHE-1 activation. NHE-1 activation results in a number of intracellular alterations which can contribute to the hypertrophic program\textsuperscript{10,19,20} although a particularly important consequence of NHE-1 activation is the elevation in intracellular Na\textsuperscript{+} concentrations which is followed by increases in intracellular Ca\textsuperscript{2+} concentrations via reverse mode Na-Ca exchange activity.\textsuperscript{21} As discussed below, this in turn would induce hypertrophy by activating key factors in the hypertrophic program, especially the phosphatase calcineurin which results in transcriptional changes due to NFAT3 dephosphorylation and its translocation into nuclei. The ability of ginsenosides to attenuate the hypertrophic effects of both angiotensin II and endothelin-1, two NHE-1 activators,\textsuperscript{22} further supports NHE-1 as a target for their salutary effects. Nonetheless, the possibility that ginsenosides are acting through other or additional nonspecific
mechanisms cannot be ruled out and requires further studies.

In the present study we used an alcoholic extract of ginsenosides to demonstrate a potent antihypertrophic effect in vitro, as well as a highly effective ability to reduce hypertrophy and heart failure in vivo, through what appears to be identical mechanisms. The ability of ginsenosides to block the hypertrophic response to the $\alpha_1$ adrenoceptor agonist PE at the highest concentration equaled the antihypertrophic effect observed with the NHE-1 inhibitor cariporide (data not shown). The antihypertrophic effect of ginseng in vitro is in partial agreement with a previous study demonstrating that ginsenosides inhibit prostaglandin F2α-induced hypertrophy through a mechanism involving attenuation in the increased expression levels of calcineurin and various transcriptional factors. We were unable to observe any changes in abundance (either gene or protein) of calcineurin (data not shown) but rather activation of calcineurin was the primary response to hypertrophic stimuli. Ginseng prevented the upregulation of NHE-1 gene and protein abundance and depressed NHE-1 activity 24 h after PE treatment. Addition of PE produced a rapid elevation of intracellular Na$^+$ and Ca$^{2+}$ concentrations and calcineurin activation. Interestingly, the early upregulation of these factors was unaffected by ginseng up to 6 h following PE administration, except for a significant inhibition in intracellular Na$^+$ concentrations at 6 h, whereas all factors were significantly inhibited 12 and 24 h after PE administration. Since NFAT3 translocation into nuclei and activation of the transcriptional factor GATA-4 24 h after PE administration were markedly inhibited by ginseng, the results suggest that late inhibition of calcineurin activation via Na$^+$ and Ca$^{2+}$-dependent mechanisms is important in attenuating the hypertrophic response to PE, at least with respect to the antihypertrophic effect of ginsenosides.
Using a rat CAL model we also show that oral administration of ginseng reduces hypertrophy and hemodynamic abnormalities in this well-established model of heart failure. We used a ginseng dose of 100 mg/kg which produced optimal salutary effect in this model. Comparison of this dose with the clinical use of ginseng is difficult due to a lack of standardization in terms of dosing or the paucity of well-controlled clinical trials. Moreover, different ginseng varieties exhibit varied biological profiles. Nonetheless, the dose in our study would roughly approximate the mid-high end of the dosing spectrum in humans which can range between 0.5 and 15 g per day. The salutary effect of ginseng was evident in terms of substantial attenuation and in some cases near normalization of hemodynamic dysfunction as assessed by both invasive catheter-based determinations of hemodynamic parameters as well as by echocardiography. Moreover, the improved hemodynamic function was associated with diminished hypertrophy determined by gravimetric analysis and molecular markers. Although mechanistic insights are more difficult to establish using in vivo approaches, the beneficial effect of ginseng in this model demonstrated substantial concordance with the effect seen in cultured myocytes vis-à-vis NHE-1 and calcineurin in that the improved cardiovascular properties were associated with diminished expression of NHE-1 and prevention of calcineurin activation, the latter determined by measurement of phosphatase activity as well as expression levels of MCIP-1, an index of calcineurin activation.

Based on current knowledge, the ability of ginseng to inhibit NHE-1 activity may represent its pivotal effect in terms of attenuating the hypertrophic response. As previously noted, from a general perspective, there is substantial evidence implicating NHE-1 as a key contributor to
myocardial hypertrophy, remodeling and heart failure as the antiporter is upregulated in heart failure and its inhibition reduces the severity of the hypertrophic response and the development of cardiovascular dysfunction.\textsuperscript{10,15,25} The nature of NHE-1 involvement in the hypertrophic response is not completely understood although NHE-1 activation could result in a number of intracellular changes resulting in stimulation of the hypertrophic program.\textsuperscript{10,19,20} Ca\textsuperscript{2+} mediated signaling has gained substantial attention among many molecular mechanisms that are known to coordinate development of pathological hypertrophy.\textsuperscript{26} In particular, Ca\textsuperscript{2+}-dependent activation of calcineurin leads to dephosphorylation of NFAT3 which subsequently translocates to the nucleus where it acts with other transcription factors including GATA-4 to initiate gene transcription. The ability of ginseng to attenuate calcineurin activation, NFAT3 translocation as well as GATA-4 phosphorylation and DNA-binding activity suggest that this represents the target for its antihypertrophic effect. Among these responses is the elevation in intracellular Ca\textsuperscript{2+} concentrations, secondary to elevations in intracellular Na\textsuperscript{+} concentrations resulting in reverse mode Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activation which results in the active import of Ca\textsuperscript{2+} into the cell.\textsuperscript{27-31} There is emerging strong evidence in the literature that Ca\textsuperscript{2+}-dependent initiation of the hypertrophic program is NHE-1 dependent. For example, the antihypertrophic effect of NHE-1 inhibition in spontaneously hypertensive rats has been shown to be associated with normalization of the calcineurin pathway in the hearts of these animals.\textsuperscript{32} Moreover, a recent study demonstrated that cardiac NHE-1 overexpression in transgenic mice activates the Ca\textsuperscript{2+}-dependent hypertrophic program as manifested by increased intracellular Ca\textsuperscript{2+} concentrations, calcineurin activation and establishment of cardiac hypertrophy and heart failure, in the absence of insult.\textsuperscript{33} Importantly, the activation of the Ca\textsuperscript{2+}-dependent hypertrophic cell signaling process and the hypertrophy itself can be abrogated by the NHE-1 specific inhibitor cariporide.\textsuperscript{34}
In conclusion, we have used an integrative approach to demonstrate a robust antihypertrophic effect of ginseng in cultured neonatal myocytes exposed to hypertrophic stimuli as well as an in vivo model of heart failure secondary to sustained CAL. The effects of ginseng in vivo translate to improved hemodynamic status 4 weeks after CAL). Our results further suggest a common underlying mechanism involving NHE-1 inhibition resulting in the attenuation of calcineurin activation. A present limitation of our study is our inability to identify the nature of the specific ginsenoside which may account for the salutary effects on hypertrophy and heart failure. As alluded to previously, more than 40 ginsenosides have thus far been identified. Using HPLC analysis, the primary ginsenosides identified in the extract used in the present study were Rb1 and Re (each approximately 45% of total ginsenosides) whereas the content of minor ginsenosides (Rg1, Rb2, Rd and Re) was between 1 and 7% (Dr EMK Lui, University of Western Ontario and Dr JT Arnason, University of Ottawa, personal communication). From a quantitative perspective, it is attractive to speculate that Rb1 and Re represent the principal ginsenosides accounting for the antihypertrophic/antiremodelling effects although this needs to be determined with further studies. It is also possible that the beneficial effects represent the combined actions of a number of these compounds present in the ginseng extract. This limitation notwithstanding, our study suggests that administration of ginseng may represent an effective adjunct therapy for the limitation of myocardial hypertrophic response and for the treatment of heart failure. Indeed, interaction between ginseng and therapeutic agents used for the treatment of heart failure would be valuable future studies to explore.
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Disclosures

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### Table. Hemodynamic Data

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All results are shown as means ± SEM. CAL, coronary artery ligated; Gins, ginsenosides (100 mg/kg); LVESP, left ventricular end systolic pressure; LVEDP, left ventricular end diastolic pressure; +dP/dt and -dP/dt, rate of left ventricular pressure development and relaxation, respectively; HR, heart rate; ESV, the end systolic volume; EDV, the end diastolic volume; SV, stroke volume; EF, ejection fraction; CO, cardiac output. *P<0.05 vs sham; † P<0.05 vs CAL.
Figure 1. Effect of different concentrations of ginsenosides (Gins, 0.1-10 μg/ml) on cell surface area and ANP gene expression in myocytes treated with phenylephrine (PE, 10 μM) for 24 h. Panel (A) shows representative micrographs of cardiomyocytes. Panel (B) shows cell surface area and panel (C) shows expression of ANP gene. Data are shown as means ± SEM. *P<0.05 vs control; #P<0.05 vs PE; n=6-7. Ctl=control.

Figure 2. Effect of 10 μg/ml ginsenosides on cell surface area (A) and ANP gene expression (B) in myocytes treated with 100 nM angiotensin II or 10 nM endothelin-1. Data are shown as means ± SEM. *P<0.05 vs control; #P<0.05 vs respective agonist alone. n=5. Ctl=control.

Figure 3. Effect of different concentrations of ginsenosides (Gins, 0.1-10 μg/ml) on NHE-1 gene and protein expression and 10μg/ml Gins on NHE-1 activity in the cells treated with phenylephrine (PE, 10 μM) for 24 h. Quantification of NHE-1 gene expression is shown in panel (A). Representative Western blot and quantification of NHE-1 protein expression are shown in panel (B). Panel (C) shows pH recovery after intracellular acidosis. Data are shown as means±SEM. *P<0.05 vs control; #P<0.05 vs PE; n=5-9. Ctl=control.

Figure 4. Effect of phenylephrine (PE, 10 μM) treatment for indicated time points on intracellular Na⁺ and Ca²⁺ concentrations in presence or absence of 10 μg/ml ginsenosides (Gins). The cells were treated with PE in the presence or absence of ginsenosides for 15 min, 3 h, 6 h, 12 h and 24 h. Panel A and B represents the quantification of intracellular Na⁺ and Ca²⁺ levels measured by using CoroNa-Red dye and Furo-2 dye, respectively. Data are shown as means±
SEM.*$P<0.05$ vs control; $^\#P<0.05$ vs PE; $n=8$.

Figure 5. Effect of phenylephrine (PE, 10 μM) treatment on calcineurin activity, NFAT3 translocation and activation and DNA-binding activity of GATA4 in the presence or absence of 10 μg/ml ginsenosides (Gins). Panel (A) shows the quantification of calcineurin activity in the cells treated with PE in the presence or absence of ginsenosides for 15 min, 3 h, 6 h, 12 h and 24 h. Panel (B) shows the immunofluorescent images of NFAT3 translocation into nucleus in the cells treated with PE in the presence or absence of ginsenosides for 24 h. Representative Western blots and quantification of GATA4 phosphorylation are shown in Panel (C) and Panel (D) represents GATA4/DNA-binding activity in the cells treated with PE in the presence or absence of ginsenosides. Data are shown as means± SEM, *$P<0.05$ vs control; $^\#P<0.05$ vs PE, $n=3-5$. Ctl=control.

Figure 6. Effect of ginsenosides on CAL-induced changes in left ventricular internal diameters (LVID) and transmitral velocity. Animals were subjected to either CAL or sham operation, with or without daily ginsenoside treatment (Gins, 100 mg/kg) for 4 weeks. Panel A and B show short-axis biventricular M-mode images and quantification of end-diastolic (LVIDd) and end-systolic (LVIDs) diameter, respectively. Panel C and D shows representative images of long-axis transmitral velocity and E/A ratio, respectively. Data are shown as means± SEM, *$P<0.05$ vs respective sham; $^\#P<0.05$ vs CAL, $n=6-10$.

Figure 7. Body weights (Panel A) and indices of cardiac hypertrophy in animals subjected to either CAL or sham operation, with or without daily ginsenoside treatment (Gins, 100 mg/kg) for
4 weeks. Gravimetric data for heart weight and left ventricular weight and quantification of ANP gene expression levels are shown in panels B, C and D, respectively. Data are shown as means ± SEM, n= 6-10. *P < 0.05 vs respective sham; #P < 0.05 vs CAL.

Figure 8. Attenuation of CAL-induced elevation in NHE-1, MCIP1 gene expression and calcineurin activation by ginsenosides. Animals were subjected to either CAL or sham procedure, with or without daily ginsenoside treatment (Gins, 100 mg/kg) for 4 weeks. Panels A and B show the quantification of NHE-1 and MCIP1 gene expression levels, respectively whereas panel C shows calcineurin phosphatase activity. Data are shown as means ± SEM, n= 6-10. *P < 0.05 vs respective sham; #P < 0.05 vs CAL.
A

Control  PE  PE+0.1 Gins  PE+1.0 Gins  PE+10.0 Gins

B

Cell surface area (μm²)

0  200  400  600  800  1000  1200

Ctl PE PE+0.1 Gins PE+1.0 Gins PE+10.0 Gins

C

ANP/18S (fold change)

0  1.0  2.0  3.0

Ctl PE - PE +0.1 Gins PE +1.0 Gins PE +10.0 Gins
A

Cell surface area (µm²)

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B

ANP/18S (fold change)

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A

NHE-1/18S (fold change)

B

MCIP-1/18S (fold change)

C

Calcineurin activity (nmol phosphate)

Sham  Sham+Gins  CAL  CAL+Gins

*  *#  *

#  #  #  #  #  #
Ginseng Inhibits Cardiomyocyte Hypertrophy and Heart Failure via NHE-1 Inhibition and Attenuation of Calcineurin Activation
Juan Guo, Xiaohong Tracey Gan, James V. Haist, Venkatesh Rajapurohitam, Asad Zeidan, Nazo Said Faruq and Morris Karmazyn

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