Ginseng Reverses Established Cardiomyocyte Hypertrophy and Post Myocardial Infarction-Induced Hypertrophy and Heart Failure

Moey et al: Ginseng Reverses Cardiac Hypertrophy

Melissa Moey BScH†, Xiaohong T. Gan MSc†, Cathy Xiaoling Huang MD†,
Venkatesh Rajapurohitam PhD†, Eduardo Martínez-Abundis PhD†,
Edmund M.K. Lui PhD † and Morris Karmazyn PhD†

†Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario N6A 5C1, Canada

Correspondence to
Morris Karmazyn, PhD
Department of Physiology and Pharmacology
The University of Western Ontario
London, Ontario N6A 5C1
Canada
Tel: 519-661-3872
Fax 519-661-3827
Email: Morris.Karmazyn@schulich.uwo.ca

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Abstract

Background—A major challenge in the treatment of heart failure is the ability to reverse already established myocardial remodeling and ventricular dysfunction with few available pharmacological agents prescribed for the management of heart failure having demonstrated successful reversal of the remodeling and hypertrophic processes. North American ginseng (Panax quinquefolius) has previously been shown to effectively prevent cardiomyocyte hypertrophy and heart failure. Here, we determined whether North American ginseng can reverse established cardiomyocyte hypertrophy in cultured myocytes as well as hypertrophy and left ventricular dysfunction in experimental heart failure secondary to coronary artery occlusion.

Methods and Results—Ginseng was administered in drinking water (0.9 g/L) ad libitum to rats after 4 weeks of sustained coronary artery ligation when heart failure was established or to angiotensin II- (100 nM), endothelin-1- (10 nM) or phenylephrine- (10 μM) induced hypertrophic cultured neonatal ventricular cardiomyocytes. Echocardiographic and catheter-based measurements of hemodynamic parameters 4 weeks after starting ginseng treatment (8 weeks postinfarction) revealed nearly complete reversibility of systolic and diastolic abnormalities. Similarly, ginseng administration to hypertrophic cardiomyocytes resulted in a complete reversal to a normal phenotype after 24 hours as determined by cell surface area and expression of molecular markers. The effects of ginseng both in vivo as well as in cultured cardiomyocytes were associated with reversal of calcineurin activation and reduced nuclear translocation of the transcription factor NFAT3 in cultured myocytes. Moreover, the beneficial effect of ginseng was associated with normalization in the gene expression of profibrotic markers including collagen (I and III) and fibronectin.

Conclusions—Our study demonstrates a marked ability of ginseng to reverse cardiac hypertrophy, myocardial remodeling and heart failure which was associated with and likely mediated by reversal of calcineurin activation. Ginseng may offer a potentially effective approach to reverse the myocardial remodeling and heart failure processes particularly in combination with other treatment modalities.

Key Words: heart failure, cardiac hypertrophy, ginseng, reversal of remodeling, calcineurin/NFAT
Cardiovascular disease remains a major cause of mortality in the world.\textsuperscript{1} Although survival in patients with ischemic heart disease has markedly improved over the past number of years, the incidence of heart failure continues to rise.\textsuperscript{2} Current pharmacotherapies for the treatment of heart failure have proven to be of substantial benefit in prolonging and improving the quality of life for heart failure patients without markedly reducing mortality rates\textsuperscript{3,4} which are currently greater than 40\%, 5 years after diagnosis.\textsuperscript{5} The high mortality rate associated with heart failure likely reflects the complexity of the myocardial remodeling processes which contributes to heart failure and the difficulty in reversing these processes with current pharmacotherapy.\textsuperscript{6} Improvement in the treatment of heart failure will likely result as a consequence of better understanding of the underlying mechanisms for this complex syndrome which should ultimately lead to the development of novel and effective pharmacological agents.\textsuperscript{7} Another possible and generally unexplored route for the identification of new heart failure therapeutic approaches may lie with alternate and less conventional medications such as Traditional Chinese Medicines (TCMs) which have been used in Asian societies for treating a variety of disorders, including cardiovascular diseases, for thousands of years. Among these TCMs, North American (NA) ginseng (\textit{Panax quinquefolius}) as well as other ginseng varieties have been garnering increasing interest in Western societies for their salutary effects on the cardiovascular system (reviewed in\textsuperscript{8}). Recently, we have demonstrated a robust ability of NA ginseng to prevent cardiomyocyte hypertrophy and heart failure through a mechanism likely involving prevention of calcineurin...
activation, the latter representing a key factor for myocardial hypertrophy and remodelling. In view of a recent study indicating calcineurin-dependent cardiac hypertrophy as a reversible process, and the potential importance of calcineurin as a target for the antihypertrophic effect of ginseng, we determined whether the latter can reverse already established hypertrophy and heart failure using both in vitro and in vivo approaches.

Methods

The protocol for the use of animals were approved by the University of Western Ontario Animal Care and Use Committee and conformed to the guidelines in the Guide for the Care and use of Laboratory Animals published by the Canadian Council of Animal Care (Ottawa, ON, Canada).

In vivo Studies

The protocol for the in vivo study is summarized in Figure 1A. Forty male Sprague-Dawley rats with an average body weight of 228 ± 8.3 g (approximately 53-55 days of age) were randomly assigned to either a sham or a coronary artery ligated group without or with ginseng treatment (0.9 g/L in drinking water provided ad libitum) started 4 weeks after surgery and maintained for a further 4 weeks. Coronary artery ligation (CAL) was performed as previously described under sodium pentobarbital (50 mg/kg bw) anesthesia. Buprenorphine (0.03 mg/kg bw) was immediately administered to all animals after completion of surgery for pain management. The studies were completed after a total of 8 weeks of sustained CAL or sham surgery at which time animals were subjected to final echocardiography and catheter-based hemodynamic assessment.
prior to sacrifice. Blood samples were also obtained at this time to determine plasma glucose and creatinine levels using commercially available kits (Cayman Chemical Co., Ann Arbor, MI).

**Echocardiography**

Animals were subjected to echocardiographic analyses before surgery as well as 4 and 8 weeks after surgery (see online Supplement).

**Hemodynamic Measurements and Tissue Processing**

After 8 weeks of treatment rats were anesthetized with pentobarbital sodium (50 mg/kg bw) and an anterior thoracotomy was performed as previously described. A 2.0F P-V Mikro-Tip catheter (Millar Instruments, Houston, Texas) was retrogradely inserted into the left ventricle via the right carotid artery. Hemodynamic data were recorded and analyzed using the Notocord-Hem 4.2 Software (Notocord, Croissy-sur-Seine, France) digitized with a sampling rate of 1000 Hz. The hearts were then removed, blotted dry and weighed. Left ventricular weights (free wall without the septum) were also obtained. For subsequent tissue biochemical or molecular assessment the infarct area was removed and viable tissue from the left ventricular free wall remote from the infarct area was processed as described below.

**Cultured Cardiomyocyte Treatment and Experimental Groups**

Neonatal ventricular cardiomyocytes from one to three day old Sprague Dawley rats were isolated and cultured as previously described. Cells were grown in fetal bovine serum-
containing medium for the first 24 h after digestion after which the serum-containing medium was removed, the myocytes were washed and re-cultured in serum-free medium for 24 h prior to agonist administration and throughout the treatment period. For reversal experiments, cardiomyocytes were pre-treated with either angiotensin II (100 nM), endothelin-1 (ET-1, 10 nM) or the $\alpha_1$ adrenoceptor agonist phenylephrine (10 $\mu$M) (all from Sigma-Aldrich, Oakville, Ontario, Canada) for up to 24h followed by the addition of ginseng extract (10 $\mu$g/ml) for a further 24h, in the presence of continued exposure to the respective hypertrophic stimulus.

**Cell Surface Area Measurement**

A Leica microscope (Leica, Westzlar, Germany) equipped with an Infinity 1 camera was used to obtain cardiomyocyte images using 100x magnification. The surface area of a minimum of 50 cells per treatment group was measured using SigmaScan Pro 5 software (Systat, Richmond, CA) and averaged to produce one “N” value.

**Gene Expression Analyses**

To determine gene expression RNA was collected from either left ventricular tissue remote from the infarct area or cardiomyocytes, reverse transcribed to complementary DNA and the gene product was quantified by real time polymerase chain reaction as previously described$^{13}$ (see online Supplement).

**Calcineurin Activity Assay**
Calcineurin activity from cellular and tissue extracts collected with a lysis buffer and protease inhibitor cocktail was measured using a colorimetric Calcineurin Cellular Activity Assay Kit as per the manufacturer’s instructions (Enzo Life Sciences, Ann Arbor, MI). Calcineurin activity was measured by the amount of nmol phosphate released at 620nm using a SpectraMax 5 (Molecular Devices, Sunnyvale, CA) plate reader from tissue and cellular extracts.

**Immunofluorescence**

NFAT translocation was also visualized using immunofluorescence as previously described\(^1\) (see online Supplement).

**Ginseng Extract**

Ginseng extract was provided by the Ontario Ginseng Innovation and Research Consortium (University of Western Ontario). Four-year-old NA ginseng roots collected in 2007 from five different farms in Ontario, Canada were provided by the Ontario Ginseng Growers Association and the 75% ethanolic extracts were prepared by Naturex (South Hackensack, NJ) and characterized as described previously.\(^1\)

**Statistical Analysis**

Data were analyzed with a one-way ANOVA test followed by a post-hoc Tukey test to determine the effect of CAL and potential influence of ginseng. Echocardiographic data were analyzed using two-way ANOVA with repeated measures and a post-hoc Tukey’s test. P values of < 0.05 were considered statistically significant.
Results

Ginseng Reverses CAL-induced Cardiac Hypertrophy

We first determined whether ginseng administration 4 weeks after CAL reverses indices of myocardial remodelling and heart failure after a further 4 week follow-up with continued ligation. Animals were administered NA ginseng (0.9 g/L) dissolved in the drinking water 4 weeks after initiating CAL (Figure 1A). Water consumption was monitored daily and was found to be identical (≈50 mL/day/animal) in all groups studied. Sustained CAL produced no mortality during the 8 week post CAL period although 20% of the animals died within 24h after CAL. These animals were replaced to ensure the maintenance of 10 animals per group at the end of the study. All animals exhibited identical growth patterns throughout the 8 week post-surgery period (Figure 1B). CAL produced a significant increase in cardiac hypertrophy as evidenced by increased expression of α-skeletal actin (Figure 1C), myosin heavy chain (Figure 1D) and both total (Figure 1D) as well as left ventricular (Figure 1E) heart weights all of which were normalized by ginseng treatment. Plasma glucose or creatinine concentrations were unaffected by any treatment (see online Supplement Table 2).

Evidence of Reversibility of CAL-induced Left Ventricular Dysfunction by Serial Echocardiography

Reversibility of cardiac dysfunction by ginseng was assessed by serial echocardiography in which animals were analyzed before surgery, 4 weeks after surgery but before starting ginseng
treatment and finally, 8 weeks after surgery. As shown in Figure 2 animals subjected to 4 weeks of CAL had significantly depressed ejection fraction, fractional shortening, cardiac output and stroke volume with values progressively declining during the remaining 4 week follow-up period. However, administration of ginseng 4 weeks after CAL resulted in a dramatic reversal of left ventricular dysfunction as evidenced by restoration of all parameters to values not significantly different from baseline but significantly greater than those observed in animals not treated with ginseng.

Figure 3 shows echocardiograms and corresponding quantified data for cardiac parameters. As illustrated in M mode images (panel A) as well as in panels B and C, left ventricular internal diameters during systole (LVIDs) and diastole (LVIDd) were significantly increased by 4 weeks after CAL although ginseng significantly reversed both parameters. Furthermore, E/A ratios determined by Doppler echocardiography analysis were increased in CAL rats while although ginseng restored values to control levels (Figure 3D and E).

As summarized in the Table there were no statistically significant changes in echocardiographic parameters in rats subjected to sham surgery throughout the 8 week follow-up period nor was there any direct influence of ginseng on any parameter studied in sham-operated animals.

**Hemodynamic Parameters in 8 Week Postinfarcted Animals**

Catheter-based hemodynamic measurements were performed at the end of the study to further assess left ventricular systolic and diastolic function. Eight weeks of CAL resulted in
significantly increased diastolic blood pressure and decreased systolic function with respect which was accompanied by markedly enhanced left ventricular end-diastolic pressures and end-diastolic volumes (Figure 4). Figure 5 illustrates CAL-induced contractile and diastolic dysfunction as evidenced by a decreased slope in the ESPVR and an increased slope in the EDPVR. However, as shown in both Figures 4 and 5, animals treated with ginseng demonstrated almost complete normalization of hemodynamic parameters.

**Heart Rates**

Mean baseline heart rates for all experimental groups (pooled data) were 358±16 beats/min (N=40). In rats subjected to CAL without subsequent ginseng administration heart rates were 318±24 (N=10) and 328±17 (N=10) beats/min 4 and 8 weeks following CAL, respectively. Corresponding heart rates in rats treated with ginseng 4 weeks after CAL were 342±13 (N=10) and 358±20 beats/min (N=10). There were no significant differences in heart rates between any treatment groups.

**Expression of Pro-fibrotic Genes**

We examined changes in expression in myocardial pro-fibrotic genes at the end of the 8 week post surgery period. As shown in Figure 6, the increased expression levels of Collagen 1 and 3 as well as fibronectin were normalized by ginseng.

**Direct Reversal by Ginseng of Cardiomyocyte Hypertrophy in Culture**
To further assess the ability of ginseng to reverse cardiomyocyte hypertrophy and obtain insights into mechanisms we studied cultured ventricular myocytes exposed to either of three hypertrophic stimuli including ET-1, angiotensin II or phenylephrine. Ginseng was then added 24h after addition of the specific hypertrophic stimulus. Figure 7 illustrates individual hypertrophic responses to each of the stimuli after either 24h or 48h treatment. As shown in micrograph images (panel A) as well as quantified data (panel B) untreated myocytes significantly increased in size when culture duration increased from 24h to 48h, in the absence of ginseng, although this was not accompanied by any changes in α-skeletal actin expression (panel C). Each of the agonists significantly increased myocyte surface area by approximately 37% compared to 24h untreated myocytes whereas this was increased to approximately 55% in 48h treated myocytes. These changes in cell surface area were accompanied by increased α-skeletal actin expression although gene expression upregulation was relatively similar for 24h and 48h treatment values.

We next determined whether ginseng administration could reverse the hypertrophic response when added 24h after addition of the respective agonist. Myocytes were then maintained for a further 24h in the presence of the hypertrophic agonist under study in the absence or presence of ginseng. As shown in Figure 7, cells treated with ginseng according to this protocol demonstrated no evidence of agonist-induced hypertrophy when assessed by either by surface area (panel B) or gene expression levels of α-skeletal actin (panel C) or MHC (panel D).
Potential Role of Calcineurin as a Target for Ginseng-induced Reversal of the Hypertrophic Response

All three agonists significantly increased calcineurin activity at 24h, while significant activation of calcineurin was observed in ET-1 treated cells only at 48h (Figure 8A). Treatment with ginseng after 24 h administration of hypertrophic agonists completely abolished the increases as observed at 24h and 48h. Calcineurin activation in angiotensin II, ET-1 or phenylephrine-treated myocytes at 24h was not significantly different from activation at 48h. Similarly, upregulation of MCIP-1 gene expression at 24h and 48h by any of the three hypertrophy inducers was reversed by treatment with ginseng (Figure 8B). Increased MCIP-1 gene expression at 24h induced by any of the agonists was not significant from expression at 48h.

NFAT3 nuclear translocation was visualized using immunofluorescence (Figure 8C) and quantified according to the nuclear:cytosolic fraction (Figure 8D) using ImageJ software as described previously.14 Significant NFAT3 nuclear translocation was observed in angiotensin II, ET-1 and phenylephrine-treated myocytes at 24h and 48h whereas this effect was reversed by ginseng (Figure 8C and 8D). Ginseng alone was without effect on calcineurin activity, MCIP-1 gene expression or NFAT3 nuclear translocation.

We also determined potential similarities with respect to calcineurin activity in hearts subjected to CAL. As illustrated in Figure 8E, there was a strong trend for ginseng to inhibit CAL-induced increase in calcineurin activity after (p=0.05) as well as MCIP-1 expression (Figure 8F, p=0.06).
whereas ginseng treatment alone was without effect.

**Discussion**

Although the use of ginseng as a therapeutic tool dates back to more than 2000 years (reviewed in\textsuperscript{16,17}) its increasing use as well as that of other TCMs in Western societies has resulted in resurgence into their possible therapeutic properties for the treatment of cardiovascular diseases (reviewed in\textsuperscript{8}). This has been primarily evident in the case of ginseng as a cardioprotective agent for mitigating ischemic and reperfusion injury although emerging evidence suggests that ginseng may be effective in preventing cardiomyocyte hypertrophy and heart failure (reviewed in\textsuperscript{8}). Here, we show for the first time, that ginseng can reverse cardiomyocyte hypertrophy in cultured ventricular myocytes in response to three hypertrophic factors including ET-1, angiotensin II and the $\alpha_1$ adrenoceptor agonist phenylephrine. More importantly, ginseng administration 4 weeks following induction of CAL, at which time substantial left ventricular dysfunction and hypertrophy is clearly evident, produces substantial reversal resulting in the near-normalization of all parameters studied. It should be emphasized that at the dose studied, ginseng produced no effect on blood pressure nor were there any effects on either plasma glucose or creatinine concentrations. These observations, coupled with direct effects seen in cardiomyocytes, suggest a direct reversal of the hypertrophic program by ginseng.

*In vivo* reversal of postinfarction ventricular remodeling and heart failure was demonstrated first by serial echocardiography which revealed marked evidence of left ventricular diastolic and
systolic dysfunction after 4 weeks of CAL. These effects persisted for the duration of the 8 week post CAL period but were completely reversed by ginseng administration at the 4 week period. These results were reinforced by hemodynamic assessments. Serial catheter-based invasive hemodynamic determinations are difficult to perform in view of the invasive nature of the procedure and true reversal is impossible to demonstrate when using different animals. However, work from our laboratory and that of others has clearly demonstrated marked left ventricular systolic and diastolic dysfunction as early as one week after CAL which is well established at the 4 week period. We have confirmed this in the present study in rats (N=5) subjected to catheter-based left ventricular function assessment 4 weeks after CAL. These animals demonstrated a 3-fold increase in LVEDP and a 21% reduction in LVESP compared to rats subjected to sham procedure accompanied by a 43% increase in the left ventricular weight/body weight values (data not shown). As seen in the results, animals administered ginseng after 4 weeks of CAL demonstrated normalized hemodynamic properties that were nearly identical to sham animals demonstrating potent reversibility properties of ginseng.

A major contributing factor for ginseng’s ability to reverse remodeling and heart failure likely stems from its ability to reverse cardiomyocyte hypertrophy, potentially mediated by regression/reversal of calcineurin upregulation (discussed below). Indeed, reduction in cardiac hypertrophy represents an important component of the therapeutic strategy for treating heart failure (reviewed in6,21,22). Our study supports the underlying hypothesis that myocardial remodelling is a bidirectional process which can be reversed by pharmacotherapy independently
of load reduction such as that observed with therapeutic devices. The key role for cardiac hypertrophy as a target for ginseng-induced reversibility is supported by a direct reversal of hypertrophy in cultured myocytes exposed to three different prohypertrophic agents. Moreover, cardiac dysfunction in control animals subjected to CAL was accompanied by both gravimetric and gene analyses data indicating a hypertrophic phenotype. Additionally, echocardiographic analysis demonstrated significantly increased systolic and diastolic LVID values which were reversed by ginseng administration.

We assessed the possible underlying mechanisms for the ability of ginseng to reverse myocardial remodeling in general and hypertrophy in particular. Ginseng exerts a myriad of cell signalling effects which could contribute to its antihypertrophic properties (reviewed in ). For example, we have recently reported that ginseng can prevent hypertrophy produced by the satiety-inducer adipokine leptin by inhibiting the activation of the RhoA-ROCK pathway. In the present study we concentrated exclusively on the calcineurin pathway both in vivo as well as in cultured myocytes where experimental conditions can be much better controlled. Calcineurin is one of a number of key signaling pathway in the pathogenesis of cardiac hypertrophy and heart although it likely plays a particularly important role in carrying out the hypertrophic program. This reflects calcium-calmodulin dependent upregulation of calcineurin activity which results in dephosphorylation of the transcriptional factor NFAT3 and its subsequent translocation into the nucleus. A role for calcineurin in hypertrophy and heart failure is further supported by studies showing that pharmacological inhibition of this enzyme prevents cardiac hypertrophy and
improves cardiac function.\textsuperscript{23,24} We have previously shown that activation of calcineurin in the 4 week postinfarcted myocardium as well as in hypertrophied cultured ventricular myocytes is prevented by ginseng.\textsuperscript{9} We have assessed calcineurin activation by determining phosphatase activity as well as by expression levels of MCIP-1 which has been shown to be related to the degree of activation.\textsuperscript{25} Robust calcineurin activation was clearly seen in cultured myocytes 24h after initiation of hypertrophy which persisted at 48h although in the presence of ginseng added at 24h values returned to control levels. A similar strong trend was observed in hearts subjected to CAL although the inhibition in calcineurin activity was not statistically significant. We believe that the latter reflects the temporal kinetics of regression of peak calcineurin activation which clearly precedes the 8 week post CAL time point. Indeed, we have shown a two-fold increase in calcineurin activity in the 4 week post-infarcted rat heart.\textsuperscript{9} Interestingly, our results bear some conceptual similarity to a recent report demonstrating reversibility of calcineurin-dependent cardiac hypertrophy.\textsuperscript{12} That study showed that calcineurin-dependent hypertrophy produced by overexpression of a mutant calcineurin transgene precedes the development of heart failure, thus precluding the necessity of sustained calcineurin activation for the production of ventricular dysfunction associated with heart failure. Moreover, turning off calcineurin activity reversed the hypertrophic phenotype.\textsuperscript{12}

In conclusion, our study shows that NA ginseng reverses cardiomyocyte hypertrophy \textit{in vitro} as well as myocardial hypertrophy, remodeling and heart failure in rats subjected to sustained CAL. Reversal of cardiac hypertrophy remains a major therapeutic challenge but one which is critical
for the development of improved therapeutic strategies for treating heart failure.\textsuperscript{6} Our results based on findings in cultured myocytes and \textit{in vivo} heart failure as well as evidence in the literature\textsuperscript{12} suggest that reversal of calcineurin-dependent processes represents a viable mechanistic basis for the reversal of hypertrophy. The results therefore reinforce the concept that targeting the calcineurin pathway may represent a key approach towards developing effective therapeutic strategies although it is recognized that other potential intracellular targets for reversal of hypertrophy likely exist and cannot be excluded. However, it should be emphasized that adverse remodelling is mediated by a large number of other factors including apoptosis as well as changes in the extracellular matrix associated with fibrosis. Although it was beyond the scope of the present study to investigate additional aspects in depth, the ability of ginseng to normalize gene expression levels of collagen and fibronectin, which reflect myocardial fibrosis\textsuperscript{26,27} suggests that ginseng may also be effective in reversing extracellular remodelling in the postinfarcted myocardium although this needs to be confirmed with additional studies examining various aspects of the adverse remodelling process. A limitation of the present study is our inability to identify and implicate the constituent(s) responsible for the therapeutic properties of ginseng, a challenge rendered particularly difficult in view of the large number of bioactive compounds, including more than 100 ginsenosides alone (in addition to other bioactive compounds) which are present in ginseng.\textsuperscript{28,29} Interestingly, ginsenosides undergo metabolic biotransformation after oral administration especially under the influence of intestinal microflora and some of these metabolites are known to be pharmacologically active and have good systematic bioavailability.\textsuperscript{30,31} Thus, the ability of ginseng to reverse hypertrophy in
cardiomyocytes implies that this property is likely not dependent on its biotransformation. This observation may provide direction for future research to define ginseng bioactive compounds for treatment of heart failure especially adjunctive therapy with existing medications such as beta adrenergic blockers, which, at least with respect to metoprolol, have provided some evidence of reverse remodelling in clinical trials or in combination with LVAD support to improve efficacy. Lastly, our results may also be interpreted to suggest that in addition to ginseng, other naturally-occurring phytochemicals may be of value in the treatment of heart failure (and likely other cardiovascular diseases) and thus deserving of further studies.

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Disclosures
None.

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Table. Echocardiographic parameters in sham-operated animals without or with ginseng administered 4 weeks post-surgery

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<td>Week 8</td>
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<tr>
<td>Ejection fraction (%)</td>
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<td>80.4 ± 2.1</td>
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<tr>
<td>Fractional shortening (%)</td>
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<tr>
<td>Cardiac output (ml/min/kg)</td>
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<tr>
<td>Stroke volume (μL)</td>
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<td>E/A ratio</td>
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Values indicate means ± SE (N=10). There were no significant differences between the four groups for any parameter.
Figure Legends

Figure 1. Ginseng reverses gravimetric evidence of cardiac hypertrophy and expression of α-skeletal actin in hearts of animals subjected to 8 weeks of CAL. Panel A illustrates the experimental protocol used in the study. Panel B shows changes in body mass during the 8 week post-surgery period. Myocardial gene expression of α-skeletal actin and myosin heavy chain is shown in panel C and D whereas panels E and F summarize changes in total heart weights and left ventricular weights, respectively. Values indicate means ± SE (N=10). *p<0.05 vs. sham group; †p<0.05 vs. CAL no treatment group. BW, body weight; HW, heart weight; LV, left ventricle; αSA, α-skeletal actin; MHC, myosin heavy chain.

Figure 2. Indices of left ventricular function obtained from serial echocardiography in rats subjected to 8 weeks CAL. For the treated group ginseng was administered 4 weeks after induction of CAL. Values indicate means ± SE (N=10). *p<0.05 and **p<0.01 vs. week 0; †p<0.05 and ††p<0.01 vs. week 4. For all parameters, values between the two treatment groups were significantly different at 8 weeks (P<0.05).

Figure 3. Ginseng reverses CAL-induced increased left ventricular internal diameters during systole (LVIDs) and diastole (LVIDd) and changes in the E/A ratio. Echocardiographic recordings were measured before surgery (0 wk), 4 weeks after surgery (4 wk) and at the end of the study (8 wk). Panel A shows M-mode images whereas quantified data for LVID values are
shown in panels B and C. Panel D shows representative Doppler images for transmitral velocity with corresponding quantified results in panel D. *p<0.05 vs. week 0; †p<0.05 vs. week 4. For all parameters, values between the two treatment groups were significantly different at 8 weeks.

**Figure 4.** Normalization of hemodynamic parameters by ginseng. All values were obtained at the end of the study period (week 8) in sham operated animals or animals subjected to CAL with a Millar P-V catheter as described in Methods. LVES(D)P, left ventricular end-systolic (diastolic) pressure; LVES(D)V; left ventricular end-systolic(diastolic) pressure; dP/dtmax/min, peak and minimum values of rates of left ventricular pressure development. Values indicate means ± SE (N=10). *p<0.05 and **p<0.01 vs. sham group; †p<0.05 and ††p<0.01 vs. CAL no treatment group.

**Figure 5.** Pressure-volume relationships 8 weeks following CAL without or with ginseng treatments. Panels A and B, shows quantified data for end-systolic and end-diastolic pressure volume relationship (ESPVR and EDPVR) obtained through analysis of the pressure-volume loops. Representative pressure-volume loops are shown in panels C to F. Values in panels A and B indicate means ± SE (N=10).**p<0.01 vs. sham; ††p<0.01 vs. CAL no treatment group.

**Figure 6.** Normalization of upregulated myocardial expression levels of Collagen 1 and 3 as well as fibronectin by ginseng. Tissues were obtained at the end of the study period (week 8) in sham operated animals or animals subjected to CAL. Values indicate means ± SE (N=10).
*p<0.05 vs. sham untreated group; †p<0.05 vs. CAL no treatment group.

**Figure 7.** Reversal of hypertrophy by ginseng in cultured cardiomyocyte. Panel A shows representative micrographs of cardiomyocytes subjected to different treatments as indicated. Top row represents agonist treatment for 24h while the second and third row represent 48h treatment. The first and last column represents control myocytes without or with ginseng, respectively. The second, third and fourth column represent cells treated with angiotensin II (Ang II, 100 nM), endothelin-1 (ET-1, 10 nM) or phenylephrine (Phe, 10 μM), respectively, with (3rd row) or without (1st and 2nd row) ginseng. Panels B-D show that cardiomyocytes subjected to hypertrophic stimuli for 24h demonstrated a significant increase in cell surface area, α-skeletal actin and myosin heavy chain (MHC) gene expression, respectively which were further augmented at 48h. Addition of ginseng after 24h incubation with the hypertrophic agonists reversed all indices of hypertrophy although ginseng alone was without effect. Values in panels B-D indicate means ± SE (N=7). *p<0.05 vs. respective time controls; †p<0.05 vs. respective hypertrophic agent.

**Figure 8.** Indices of calcineurin activity. Panels A and B shows calcineurin activity and MCIP-1 gene expression in cardiomyocytes treated with angiotensin II (Ang II, 100 nM), endothelin-1 (ET-1, 10 nM) or phenylephrine (Phe, 10 μM), for 24h or 48h. Addition of ginseng after 24h incubation with the hypertrophic agonists reversed the upregulation in calcineurin activity and MCIP-1 gene expression. Ginseng alone was without effect. Panel C shows representative
images demonstrating increased NFAT3 nuclear translocation with different treatments at 48h. Top, middle and bottom rows represent NFAT3, the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) and merged images, respectively. The first and last columns represent control cells without and with ginseng, respectively. Panel D illustrates quantified data from fluorescence images. Panels E and F show calcineurin activity and MCIP-1 expression in rat hearts 8 weeks post-surgery. N=6-8 for data in panels A, B and D whereas N=8 for data in panels E and F.

AngII: angiotensin II; ET-1: endothelin-1; Phe: phenylephrine. *p<0.05 vs. control; †p<0.05 vs. respective hypertrophic agonist.
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SUPPLEMENTAL MATERIAL

Ginseng Reverses Established Cardiomyocyte Hypertrophy and Post Myocardial Infarction-Induced Hypertrophy and Heart Failure

Melissa Moey, Tracey Xiaohong Gan, Cathy Xiaoling Huang, Venkatesh Rajapurohitam, Eduardo Martínez-Abundis, Edmund M. K. Lui and Morris Karmazyn

Supplemental Methods

Echocardiography: Echocardiography measurements were taken at the following three different time points: baseline/before surgery (week 0), 4 weeks after surgery (week 4) and at the end of the study (week 8). Rats were prepared for echocardiography as previously described. Briefly, animals were anesthetized with 2% isofluorane and placed supine on a heated platform. Echocardiography measurements were performed using a Vevo 770 high-resolution in vivo microimaging system equipped with a real-time microvisualization scan head of 17.5 MHz (VisualSonics, Toronto, Ontario, Canada) to obtain M-Mode 2-dimensional and Doppler images. M-Mode was obtained from the parasternal short axis to analyze left ventricular dimensions during diastole and systole and Doppler mode was obtained from the parasternal long axis to determine the E/A ratio calculation. All images were analyzed using the Vevo 770 Protocol-Based Measurements software.

RNA Isolation, Reverse Transcription (RT) and Real-time Polymerase Chain Reaction (PCR)

RNA was collected from either left ventricular tissue or cardiomyocytes using QIAzol Reagent (Qiagen Canada, Toronto, Ontario, Canada) as per the manufacturer’s instructions and reverse transcribed to complementary DNA (cDNA) for real-time PCR analysis of α-skeletal actin and
modulatory calcineurin interacting protein-1 (MCIP-1) as previously described. cDNA was synthesized from 5 μg of total RNA using random primers (Invitrogen) and M-MLV Reverse Transcriptase (Invitrogen) as per the manufacturer’s protocol. The reaction was performed with a SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and the gene products quantified with a DNA Engine Opticon 2 thermal cycler (MJ Research, Waltham, MA). Primer sequences are summarized in Supplemental Table 1 below. PCR cycle conditions involved 40 cycles of denaturation at 95°C for 30 seconds, followed by annealing at 50°C and 60°C for 30s for 18S and α-skeletal actin and MCIP-1, respectively, ending with elongation at 72°C for 45s. The housekeeping gene, 18S, was measured and quantified to normalize cDNA levels.

**Immunofluorescence.** Cardiomyocytes were prepared for immunofluorescence on collagen coated (3 μl of collagen/1 ml of PBS A) glass cover slips and incubated at 37°C for a minimum of 30 minutes. Cells were allowed to attach to prepared cover slips in serum-containing medium for 24 hours followed by serum starvation for an additional 24h before appropriate treatments. Cardiomyocytes were pre-treated with angiotensin II, ET-1, or phenylephrine for 24h without ginseng or 48h with or without treatment of ginseng for an additional 24h. Cells were fixed with 2:5 acetone-methanol for 1 hour at 4°C followed by permeabilization of cells for 15 minutes with 0.1% (v/v) Triton X-100 and blocking with blocking solution (1% BSA in PBS A). Cells were incubated with NFAT3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution) in 2% BSA in PBS A overnight at 4°C. Cells were subsequently probed with the secondary antibody IgG anti-rabbit AlexFluor-596 (Invitrogen) (1:250 dilution) in 2% BSA in PBS A for 1h at room temperature under light-free conditions. Cells were mounted with Vectashield® mounting medium containing 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) for detection of nuclei onto microscope slides for image capture using a Zeiss (Oberkochen,
Germany) inverted fluorescence microscope at 630x magnification. Nuclear translocation was measured using ImageJ software as described previously.³

References


**Supplemental Tables**

**Supplemental Table 1.** Primer sequences of analyzed genes of interest

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-skeletal actin</td>
<td>5'-CACGGCATTATCACAACCTG-3'</td>
<td>5'-CCGGAGGCATAGAGAGACAG-3'</td>
</tr>
<tr>
<td>myosin heavy chain</td>
<td>5'-CATCACGGGAGAATCCGGAGC-3'</td>
<td>5'-CTATTGAGGCCACAGTCGTC-3'</td>
</tr>
<tr>
<td>18S</td>
<td>5'-GTAACCGTGGACCCATT-3'</td>
<td>5'-CATACCAATCGGTAGTAGCG-3'</td>
</tr>
<tr>
<td>MCIP-1</td>
<td>5'-TCTCCAAGCTGGACCAGAGA-3'</td>
<td>5'-ATCAGAAGCCTCGLGCTCGGT-3'</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>5'-GCGACTCTGACTGGCCTTAC-3'</td>
<td>5'-CCGTGTAAGGGTCAAGCAT-3'</td>
</tr>
<tr>
<td>Collagen I</td>
<td>5'-TGCTGCTTTCTGTTCTTT-3'</td>
<td>5'-AAGGTGCTGGGTAGGGAAGT-3'</td>
</tr>
<tr>
<td>Collagen III</td>
<td>5'-GTCCACGGAGGTGACAAGGT-3'</td>
<td>5'-CATCTTTTCCAGGAGGCTCA-3'</td>
</tr>
</tbody>
</table>

**Supplemental Table 2.** Plasma glucose and creatinine concentrations

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Glucose</th>
<th>Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham Untreated (N=8)</td>
<td>70.5 ± 10.4</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td>Sham + Ginseng (N=6)</td>
<td>75.2 ± 16.3</td>
<td>0.65 ± 0.11</td>
</tr>
<tr>
<td>CAL Untreated (N=9)</td>
<td>79.8 ± 10.7</td>
<td>0.52 ± 0.07</td>
</tr>
<tr>
<td>CAL + Ginseng (N=6)</td>
<td>65.1 ± 8.5</td>
<td>0.50 ± 0.07</td>
</tr>
</tbody>
</table>

Values indicates means ± SE with number of animals in parentheses. All values are given in mg/dL and were determined from samples obtained at the end of the 8 wk post-surgery period.