Ginseng (Panax quinquefolius) Attenuates Leptin-Induced Cardiac Hypertrophy through Inhibition of p115Rho Guanine Nucleotide Exchange Factor-RhoA/Rho-Associated, Coiled-Coil Containing Protein Kinase-Dependent Mitogen-Activated Protein Kinase Pathway Activation

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ABSTRACT

Leptin is a 16-kDa peptide primarily derived from white adipocytes and is typically elevated in plasma of obese individuals. Although leptin plays a critical role in appetite regulation, leptin receptors have been identified in numerous tissues including the heart and have been shown to directly mediate cardiac hypertrophy through RhoA/ROCK (Ras homolog gene family, member A/Rho-associated, coiled-coil containing protein kinase)-dependent p38 mitogen-activated protein kinase (MAPK) activation; however, the basis for RhoA stimulation is unknown. Rho guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP resulting in Rho activation and may be the potential upstream factors mediating leptin-induced RhoA activation and therefore a potential target for inhibition. We investigated the effects of North American ginseng (Panax quinquefolius), reported to reduce cardiac hypertrophy, on RhoA/ROCK and MAPK activation in ventricular cardiomyocytes exposed to leptin (50 ng/ml) and the possible role of p115RhoGEF and p63RhoGEF in these responses. Leptin produced a robust hypertrophic response that was associated with RhoA/ROCK activation resulting in a significant increase in cofilin-2 phosphorylation and actin polymerization, the latter evidenced by a reduction in the G/F actin ratio. These effects were prevented by ginseng (10^{-6} M). The stimulation of RhoA/ROCK by leptin was associated with significantly increased p115RhoGEF gene and protein expression and exchange activity, all of which were completely prevented by ginseng. The ability of ginseng to prevent leptin-induced activation of RhoA/ROCK was further associated with diminished p38 MAPK activation and nuclear translocation. These results demonstrate a potent inhibitory effect of ginseng against leptin-induced cardiac hypertrophy, an effect associated with prevention of p115RhoGEF-RhoA/ROCK-dependent p38 MAPK activation.

Introduction

The prevalence of obesity in North America has significantly increased over the past 5 years (Luo et al., 2007) along with the detrimental accompanying risks for the development of cardiovascular disease (Bui et al., 2011). The underlying mechanisms for obesity-associated cardiovascular disease are not well understood although emerging evidence implicates a potential role of leptin, a member of the family of peptides known as adipokines, which is produced by the ob (obesity) gene (Zhang et al., 1994) and has been reported by a number of investigators to produce a direct hypertrophic effect on the heart (reviewed by Karmazyn et al., 2008). The primary source of leptin is white adipose tissue, and plasma levels of the peptide have been shown to be closely correlated to the degree of adiposity (Maffei et al., 1994) and has been reported by a number of investigators to produce a direct hypertrophic effect on the heart (reviewed by Karmazyn et al., 2008). The primary source of leptin is white adipose tissue, and plasma levels of the peptide have been shown to be closely correlated to the degree of adiposity (Maffei et al., 1995). We have previously shown that the rat heart produces leptin within the cardiomyocyte and also expresses leptin receptors, suggesting that the heart is a target for leptin's
effects, potentially in a paracrine/autocrine manner (Purdham et al., 2004). It is noteworthy that plasma levels of leptin are elevated in patients with heart failure independently of obesity (Schulze et al., 2003).

Several molecular signaling pathways up-regulated by leptin in cardiac hypertrophy have been identified, namely the RhoA/ROCK (Ras homolog gene family, member A/Rho-associated, coiled-coil containing protein kinase), p38, and extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) pathways (Fruhbeck, 2006; Zeidan et al., 2006, 2008), all of which can be activated by leptin. A particular importance of the RhoA/ROCK pathway is that its activation, and subsequent changes in actin dynamics, is critical for selective p38 MAPK translocation into nuclei, thus initiating the hypertrophic process (Zeidan et al., 2006, 2008). Although we have previously demonstrated the potent stimulation of the RhoA/ROCK pathway by leptin, the specific mechanism of activation has not yet been defined. Regulators of small G proteins such as RhoA include GTPase-activating proteins that hydrolyze GTP to GDP, thereby deactivating RhoA, guanine nucleotide dissociation inhibitors that sequester GDP-bound small G proteins in the cytoplasm, and guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP to GTP resulting in activation of RhoA (Schmidt and Hall, 2002; Rossman et al., 2005; Bos et al., 2007). RhoA GEFs (RhoGEFs), specifically p115RhoGEF and p63RhoGEF, have been shown to be involved in the up-regulation of RhoA/ROCK in cardiac hypertrophy in response to G-protein-coupled, receptor-linked hypertrophic agonists such as endothelin-1 (Porchia et al., 2008) and angiotensin II (Guilley et al., 2010).

Ginseng (genus Panax) is a medicinal herb that has been used widely in Asia for more than 2000 years (Goldstein, 1975; Lu et al., 2009). Its principal bioactive components are ginsenosides, which are triterpene saponins and are considered the main constituents responsible for ginseng’s medicinal effects (Attele et al., 1999). Emerging understanding of the chemistry of ginseng and its potential therapeutic use has resulted in increasing interest in western countries for the use of ginseng as a pharmacological agent for the treatment of a number of diseases. With respect to the cardiovascular system, ginsenosides have been shown to inhibit the development of atherosclerosis (Li et al., 2011), hypertension (Jeon et al., 2000), and cardiac hypertrophy, the latter effect being seen in a number of experimental models (Jiang et al., 2007; Qin et al., 2008; Deng et al., 2010; Guo et al., 2011). Whether ginseng affects leptin-induced hypertrophy has not been demonstrated. Accordingly, in this study, we determined the effect of North American ginseng (Panax quinquefolius) on leptin-induced cardiomyocyte hypertrophy and studied the potential underlying mechanisms for these effects. Our study centered on the possible modulatory effect of ginseng on the RhoA/ROCK pathway after leptin addition and the role of GEFs. In addition, we studied the relationship between RhoA and MAPK pathway activation.

Materials and Methods

Treatment and Experimental Groups. Neonatal ventricular cardiomyocytes were isolated and cultured from 1- to 3-day-old Sprague-Dawley rats as described previously (Rajapurohitam et al., 2006). Cells were grown in fetal bovine serum medium for up to 48 h after 24-h serum starvation. For cell size and Western blotting time-course experiments, cardiomyocytes were pretreated with the alcoholic extract of North American ginseng (P. quinquefolius). Extracts were prepared by Naturex (South Hackensack, NJ) using ginseng roots supplied from five different farms in Ontario, Canada, as described previously (Guo et al., 2011) and studied at concentrations of 0.1, 1, 10, and 100 μg/ml for up to 24 h in the presence or absence of 3.1 nM (50 ng/ml) leptin (Sigma-Aldrich, Oakville, Ontario, Canada), a concentration representative of plasma levels in obese individuals (Mafiei et al., 1995). For all subsequent experiments, cells were pretreated with a ginseng concentration of 10 μg/ml for 1 h in the presence or absence of leptin for up to 24 h. Treatment durations reflected the period of peak activation of the parameter under study. The protocols for the use of animals were approved by the University of Western Ontario Animal Care and Use Committee and conformed to guidelines in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and the Canadian Council of Animal Care (Ottawa, Ontario, Canada).

Cell-Surface Area Measurement. Cardiomyocyte images were taken with a microscope (Leica, Wetzlar, Germany) equipped with an Infinity 1 camera at magnification 100×. The surface area of a minimum of 50 cells per treatment group was measured using SigmaScan Pro 5 software (Systat Software, Inc., San Jose, CA) and averaged.

RNA Isolation, Reverse Transcription, and Real-Time Polymerase Chain Reaction. RNA was collected from cultured and treated neonatal ventricular cardiomyocytes using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions, and reverse-transcribed to cDNA for real-time polymerase chain reaction (PCR) analysis of α-skeletal actin (α-SA), myosin heavy chain α, p115RhoGEF, and p63RhoGEF. In brief, cDNA was synthesized from 4 μg of total RNA using random primers (Invitrogen) and Moloney murine leukemia virus reverse transcriptase (Invitrogen) following the manufacturer's protocol. The reaction was performed with a SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and the gene products were quantified with a DNA Engine Opticon 2 thermal cycler (MJ Research, Watertown, MA). Primer sequences (Invitrogen) for the genes of interest are listed in Table 1. PCR cycle conditions involved 40 cycles of denaturation at 95°C for 30 s, followed by annealing at 60°C for 30 s, and finally by elongation at 72°C for 30 s. The housekeeping gene 18S was measured and quantified to normalize cDNA levels.

[3H]Leucine Incorporation Measurement. Leucine incorporation was performed as described previously (Zeidan et al., 2006) to analyze protein synthesis under different experimental conditions. Cardiomyocytes were cultured in 24-well Primaria culture plates for 48 h in serum medium, followed by 24-h serum starvation. Leptin

## Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
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<tbody>
<tr>
<td>α-Skeletal actin</td>
<td>5′-CACCGGACTTAGCACCACTG-3′</td>
<td>5′-CCGGAGGCATAGAGAGACAG-3′</td>
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<tr>
<td>Myosin heavy chain</td>
<td>5′-CATCACGGGAAACTCGGAG-3′</td>
<td>5′-CATTTGAGCAGACGCGTTT-3′</td>
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<tr>
<td>p115RhoGEF</td>
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<td>5′-TGGTGAGAGCACTGACCA-3′</td>
</tr>
<tr>
<td>p63RhoGEF</td>
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<td>5′-TGATGACAGCTGGACCA-3′</td>
</tr>
<tr>
<td>18S</td>
<td>5′-GTACCCGTTGAAACCCATT-3′</td>
<td>5′-CCATCCATTGGTACGGTC-3′</td>
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was administered with or without ginseng pretreatment in the presence of 2 μCi of [3H]leucine for 24 h. Myocytes were washed the following day with ice-cold PBS, and proteins were precipitated with 5% trichloroacetic acid for 30 min on ice followed by two washes of ice-cold trichloroacetic acid (5%). Protein precipitates were resuspended in 0.5 N NaOH and neutralized with 0.5 N HCl. Total radioactivity was measured by liquid scintillation counting.

Isolation of Cytosolic-Enriched and Membrane Fractions. Cytosolic-enriched and membrane fractions from treated cell lysates were prepared using differential centrifugation as described previously (Zeidan et al., 2008). In brief, cell lysates were collected and homogenized in an ice-cold buffer containing 20 mM Tris-HCl, 2 mM EDTA, 137 mM NaCl, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM 4-(2-aminomethyl)-benzenesulfon- nyl fluoride, and 10 mg/ml leupeptin (buffer A). After clarification of the homogenate by centrifugation at 750g for 20 min at 4°C, the collected lysate was further centrifuged at 10,000g for 20 min at 4°C, and the cytosolic-enriched fraction (supernatant) was obtained. The remaining pellet was resuspended in a second ice-cold buffer B (buffer A with 2% SDS) and kept on ice to be used as the nuclear-containing membrane fraction.

Western Blotting. Total cellular lysates were collected using a lysis buffer and protease cocktail inhibitor mixture as described previously (Zeidan et al., 2006, 2008) for the measurement of proteins of interest. Proteins were loaded equally on 7.5, 10, or 15% SDS gels as appropriate after protein quantification via dye reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. For time-course experiments, ventricular cardiomyocytes were treated for 5, 10, 15, 30, or 60 min with leptin (50 ng/ml) in the presence or absence of ginseng (10 μg/ml). For the quantification of cofilin-2 phosphorylation, cells were pretreated with ginseng for 1 h followed by administration of leptin for 10 min. For all additional protein measurements, cells were pretreated with ginseng for 1 h in the presence or absence of leptin for 24 h. The primary antibodies and respective dilutions used in this study include total (1:1000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and phosphorylated (Thr180/Tyr182) p38 forms (1:1000 dilution; Cell Signaling Technology, Danvers, MA), total (1:1000 dilution; Santa Cruz Biotechnology, Inc.) and phosphorylated ERK1/2 (Thr202/Tyr204) forms (1:1000 dilution; Cell Signaling Technology, actin (1:1000 dilution; Cytoskeleton Inc., Denver, CO), p115RhoGEF (1:250 dilution; Santa Cruz Biotechnology, Inc.), p63RhoGEF (1:200 dilution; Santa Cruz Biotechnology, Inc.), and phosphorylated (1:1000 dilution; Santa Cruz Biotechnology, Inc.) and total (1:1000 dilution; Millipore Corporation, Billerica, MA) cofilin-2. Goat anti-rabbit IgG and goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad Laboratories) were used at 1:5000 dilution, and donkey anti-goat IgG horseradish peroxidase conjugate (Santa Cruz Biotechnology, Inc.) was used at 1:10,000 dilution as appropriate. β-Actin (1:1000 dilution; Cytoskeleton Inc.) and proliferating cell nuclear antigen (1:1000 dilution) were used for cytosolic and nuclear loading controls, respectively. Spot densitometry using FluorChem (Alpha Innotech Corporation, Santa Clara, CA) software was performed to quantify protein.

GST-RhoG17A Bead Preparation. GST-RhoG17A beads were prepared as described previously (Garcia-Mata et al., 2006; Kakishivili et al., 2009). The nucleotide-free RhoG17A cDNA construct mutant was generously provided by Dr. Katalin Szasz (St. Michael’s Hospital, Toronto, ON, Canada) and Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC).

Coimmunoprecipitation of p115RhoGEF. Active p115RhoGEF from cells treated with leptin in the presence or absence of leptin were immunoprecipitated with the nucleotide-free (does not bind to GTP or GDP) GST-RhoG17A-prepared beads, which has a high affinity for active RhoGEFs, including p115RhoGEF, as described previously (Garcia-Mata et al., 2006; Kakishivili et al., 2009).

Immunofluorescence. Cells were prepared for immunofluorescence on collagen-coated (3 μl of collagen/1 ml of PBS A) glass coverslips and incubated at 37°C for a minimum of 30 min. Cells were allowed to attach to prepared coverslips in serum medium for 24 h followed by serum-free medium starvation for an additional 24 h before appropriate treatment. Immunofluorescence measurements from cells administered leptin were performed for 3 h for total p38 and total ERK1/2, for 10 min for p115RhoGEF and RhoA, and for 24 h for globular (G/filamentous (F) actin with or without ginseng pretreatment.

Total p38, Total ERK1/2, p115RhoGEF, and RhoA Immunofluorescence. Cells were fixed with 2.5% acetone/methanol for 1 h at 4°C followed by permeabilization of cells for 15 min with 0.2% (v/v) Triton X-100 and blocking with blocking solution (1% BSA, 0.1% Triton X-100) for 1 h. Cells were incubated with the primary antibody of interest (1:100 dilution) in 2% BSA in PBS A overnight at 4°C. Cells were subsequently probed with the appropriate secondary antibody, IgG anti-mouse Alex Fluor-488 (Invitrogen) or IgG anti-rabbit Alex Fluor-596 (Invitrogen) (1:250 dilution) in 2% BSA in PBS A for 1 h at room temperature under light-free conditions. For detection of the nucleus, cells were incubated with Hoechst dye for 30 min before mounting on microscope slides (VWR, Westbury, CA) for image capture using a Carl Zeiss Inc. (Jena, Germany) inverted fluorescence microscope at magnification 630×.

G and F Actin Immunofluorescence. Cells were prepared as described previously (Albinsson et al., 2004). Cells were fixed with 3.7% (w/v) paraformaldehyde in PBS A for 1 h followed by a similar protocol for permeabilization and blocking as indicated above. To detect G and F actin, cells were incubated with 1 μg/ml phalloidin-fluorescein isothiocyanate, 10 μg/ml deoxyribonuclease I, and Texas Red conjugate in 2% BSA with 0.1% Triton X-100 in PBS A for 1 h at room temperature under light-free conditions. Likewise, Hoechst dye was used to detect the nucleus, and glass coverslips were subsequently mounted onto microscope slides (VWR) for visualization of G/F actin. All immunofluorescence images shown under Results are representative of a minimum of three independent experiments.

Measurement of p115RhoGEF and RhoA Colocalization. Co-localization of p115RhoGEF and RhoA was measured from merged immunofluorescence images detecting p115RhoGEF and RhoA under different experimental conditions using a built-in colocalization plug-in (Li et al., 2004) of ImageJ (National Institute of Mental Health, Bethesda, MD), which is quantitatively represented by Pearson’s correlation coefficient (Rc).

Measurement of RhoA Activity. RhoA activity, measured by RhoA-GTP levels, was quantified using the RhoA G-LISA Activity Biochem Assay kit (Cytoskeleton Inc.) according to the manufacturer’s protocol. Measurements were performed using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA) plate reader at an absorbance of 490 nm.

p115RhoGEF Activity Assay. Measurement of immunoprecipitated p115RhoGEF activity was measured according to the manufacturer’s protocol using the RhoGEF Biochem Exchange Assay (Cytoskeleton Inc.). Fluorescence was measured using a SpectraMax M5 (Molecular Devices) plate reader at an excitation of 360 nm and emission of 440 nm.

G/F Actin Measurement. G and F actin were isolated using ultracentrifugation as described previously (Albinsson et al., 2004). In brief, cell lysates were collected and homogenized at 37°C in a lysis and F actin stabilizing buffer (50 mM PIPES, 50 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, 1 mM ATP, 5% glycerol, 0.1% Nonidet-P40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β-mercaptoethanol, 1:1000 protease inhibitor cocktail, and 0.0001% anti-faoam). Cell lysates were ultracentrifuged at 100,000g at 30°C for 1 h, and the supernatant (soluble, G actin) was collected at diluted 1:2 in Laemmli buffer. The remaining pellet (F actin) was resuspended at 100,000g at 30°C for 1 h, and the supernatant (soluble, G actin) was collected at diluted 1:2 in Laemmli buffer. Protein concentrations were then quantified, equalized, and loaded onto 12.5% bis-acrylamide gels for Western
blotting. Membranes were probed with anti-actin antibody (Cyto-
skeleton Inc.) and quantified using spot densitometry.

**Statistics.** Data were analyzed with one-way analysis of variance
followed by a post hoc Student’s t test. P values of <0.05 were
considered statistically significant.

**Results**

**Ginseng Inhibits Leptin-Induced Cardiomyocyte Hypertrophy.** To first determine an appropriate concentration for studying the effects of ginseng on leptin-induced cardiac hypertrophy, cardiomyocytes were subjected to increasing concentrations of ginseng (0.1, 1, 10, and 100 µg/ml) for 1 h before the addition of leptin for a total incubation time of 24 h (Fig. 1, top). As shown in Fig. 1 (middle), leptin induced a significant increase (p < 0.05) in cell size, which was attenuated by ginseng in a concentration-dependent manner. A concentration of 10 µg/ml ginseng was used for all subsequent experiments because this represented the lowest concentration that completely abrogated the hypertrophic response to leptin (Fig. 1, middle).

An increase in cell-surface area in leptin-treated cells after 24 h was additionally associated with a significant increase (p < 0.05) in protein synthesis as indicated by increased [³H]leucine incorporation (Fig. 1, bottom left) and myosin heavy chain α (Fig. 1, bottom center) and α-SA (Fig. 1, bottom right) gene expression, as quantified by real-time PCR. As shown in Fig. 1, ginseng alone had no effect on any parameter.

**Ginseng Inhibits Leptin-Induced RhoA Activation, Cofilin-2 Phosphorylation (Inactivation), and the Decrease in G/F Actin Ratio.** RhoA activation was measured after 10 min of leptin administration in the presence or absence of ginseng. As demonstrated in Fig. 2A, ginseng significantly inhibited (p < 0.05) leptin-induced RhoA activation, returning RhoA-GTP levels to control values, whereas ginseng alone had no effect on its own. Activation of the RhoA/ROCK pathway was additionally indirectly measured by quantifying phosphorylated cofilin-2 (inactivated form) by Western blotting. Cofilin-2 is a ubiquitous enzyme responsible for depolymerizing F to G actin, thereby regulating cellular actin dynamics. A change in this ratio favoring a higher F-to-G actin content as a result of RhoA activation and subsequent cofilin-2 phosphorylation (inactivation) has been previously demonstrated to represent a key mechanism underlying the hypertrophic effects of leptin.

**Fig. 1.** Ginseng inhibits leptin-induced increase in cell-surface area, [³H]leucine incorporation, and expression of the gene markers of cardiac hypertrophy, α-skeletal actin, and myosin heavy chain. Top, micrographs show representative images of neonatal ventricular cardiomyocytes with or without treatment with increasing ginseng concentrations (0.1, 1.0, 10, and 100 µg/ml, respectively) in the presence (top row) or absence (bottom row) of leptin (3.1 nM) pretreatment. Middle, surface area. Bottom, [³H]leucine incorporation (left) and expression of myosin heavy chain (MHC; center) and α-skeletal actin (right) with different treatments. Data represent means ± S.E.M. n = 8 to 10 for surface area, n = 6 for leucine incorporation, and n = 6 to 8 for molecular markers of hypertrophy. *, p < 0.05 versus control; †, p < 0.05 versus leptin. Con, control; Lep, leptin; Gin, ginseng.
leptin (Zeidan et al., 2006). The results, as shown in Fig. 2B, reveal a significant (25%) increase in p-cofilin-2 in cells administered leptin (p < 0.05), which was attenuated and returned to control levels in the presence of ginseng although ginseng alone had no significant effect on p-cofilin-2 or RhoA-GTP levels.

To further characterize the effects of ginseng on leptin-induced RhoA/ROCK pathway activation, the G/F actin ratio was assessed by Western blotting of isolated G and F actin fractions (Fig. 3A) and visualization using immunofluorescence (Fig. 3C) after 24 h. In leptin-treated cells, the G/F actin ratio was decreased as measured by quantification of G actin (supernatant) and F actin (pellet) using Western blotting (Fig. 3A), whereas pretreatment with ginseng restored this ratio to control values (Fig. 3B). This was similarly observed in the representative immunofluorescence images (Fig. 3C, Lep column) of leptin-treated cells as depicted by a lighter red staining of G actin and intensified green staining of F actin, which was returned to control conditions by pretreatment with ginseng (Fig. 3C, Lep+Gin column). Treatment with ginseng alone had no direct effect on the G/F actin dynamics.

**Ginseng Inhibits Leptin-Induced p38 and ERK1/2 MAPK Phosphorylation.** Leptin significantly induced both p38 (Fig. 4A) and ERK1/2 (Fig. 5A) phosphorylation as early as 5 min after addition, with maximum activation seen at 15 min followed by values returning to control by 30 min. Pretreatment with ginseng inhibited leptin-induced p38 (Fig. 4A) and ERK1/2 (Fig. 5A) activation at all time points although ginseng had no direct effect on its own.

**Ginseng Inhibits Leptin-Induced p38 Nuclear Translocation.** Leptin induced a significant increase (p < 0.05) in p38 expression in the nuclear-containing membrane fraction (Fig. 4C), which was complemented by a significant decrease (p < 0.05) in cytosolic p38 levels, indicative of nuclear translocation in p38 in leptin-treated cells (Fig. 4B). Nuclear translocation of p38 was further visualized by immunofluorescence (Fig. 4D) where total p38, indicated by red fluorescence, was much more centralized in the nuclear region of leptin-treated cells. The ability of leptin to induce p38 translocation was significantly inhibited by ginseng. As summarized in Fig. 5, B–D, leptin had no effect on ERK1/2 nuclear translocation.

![Fig. 2](image1.png) Ginseng inhibits leptin-induced RhoA activation and phosphorylation (inactivation) of cofilin-2. A, RhoA-GTP (activated RhoA) levels. B, Western blots and densitometric values for phosphorylated cofilin-2. Data represent means ± S.E.M. n = 6. *, p < 0.05 versus control; †, p < 0.05 versus leptin. Con, control; Lep, leptin; Gin, ginseng.

![Fig. 3](image2.png) Ginseng inhibits leptin-induced decrease in G/F actin. A, Western blots for actin dynamics with respect to G actin in supernatant (S) and F actin in pellet fraction (P) with different treatments. B, densitometric values. C, representative fluorescence images of cardiomyocytes. For these studies, cells were fixed on collagen-coated glass coverslips, and G actin (first row) and F actin (second row) were visualized with deoxyribonuclease I Texas Red conjugate and phallolidin-fluorescein isothiocyanate, respectively. Hoechst staining was used to detect nuclei, whereas the overlay depicts all three stains merged. In B, data represent means ± S.E.M. n = 6 to 9. *, p < 0.05 versus control; †, p < 0.05 versus leptin. Con, control; Lep, leptin; Gin, ginseng.
Ginseng Inhibits Leptin-Induced Increase in p115RhoGEF Protein and Gene Expression. The effects of ginseng on leptin-induced RhoGEF activation was first determined by measurement of p115RhoGEF and p63RhoGEF gene expression through real-time PCR and protein expression using Western blotting (Fig. 6). A 6-fold increase ($p < 0.05$) in p115RhoGEF gene expression after 24 h was observed in leptin-treated cells, which was abolished in the presence of ginseng (Fig. 6A). Likewise, pretreatment with ginseng significantly inhibited leptin-induced increase in p115RhoGEF protein expression (Fig. 6B), whereas ginseng alone had no direct effect on either parameter. Compared with p115RhoGEF, neither leptin nor ginseng exerted any effect on p63RhoGEF gene (Fig. 6C) or protein (Fig. 6D) expression.

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Ginseng Inhibits Leptin-Induced Increase in p115RhoGEF Protein and Gene Expression. The effects of ginseng on leptin-induced RhoGEF activation was first determined by measurement of p115RhoGEF and p63RhoGEF gene expression through real-time PCR and protein expression using Western blotting (Fig. 6). A 6-fold increase ($p < 0.05$) in p115RhoGEF gene expression after 24 h was observed in leptin-treated cells, which was abolished in the presence of ginseng (Fig. 6A). Likewise, pretreatment with ginseng significantly inhibited leptin-induced increase in p115RhoGEF protein expression (Fig. 6B), whereas ginseng alone had no direct effect on either parameter. Compared with p115RhoGEF, neither leptin nor ginseng exerted any effect on p63RhoGEF gene (Fig. 6C) or protein (Fig. 6D) expression.

Ginseng Inhibits Leptin-Induced p115RhoGEF Membrane Translocation with RhoA Colocalization and p115RhoGEF Activity. Several studies have shown membrane translocation with RhoA colocalization as a component of p115RhoGEF activation (Kozasa et al., 1998; Rossman et al., 2005; Aittaleb et al., 2010). Initial time-course experiments with leptin treatment revealed translocation and RhoA colocalization as early as 5 min after leptin addition (data not shown). Our previous studies have correspondingly indicated activation of RhoA as early as 10 min (Zeidan et al., 2006, 2008). Consequently, cardiomyocytes were treated with ginseng before the administration of leptin for 10 min and prepared for immunofluorescence. As depicted in Fig. 7A (row c), p115RhoGEF membrane translocation with RhoA colocalization was observed in leptin-treated cells as indicated by evident yellow fluorescence at the cardiomyocyte border. In addition, isolation of the colocalized pixels (Fig. 7A, row d) revealed colocalization of p115RhoGEF and RhoA in leptin-treated cells, which was inhibited by pretreatment with ginseng. Treatment with ginseng alone was without any effect (Fig. 7A, Gin column).

Using the same treatment protocol, cell lysates were col-
lected at 10 min after leptin administration and incubated with GST-RhoG17A, a nucleotide-free RhoA mutant that has a high affinity for active RhoGEFs (García-Mata et al., 2006), to immunoprecipitate p115RhoGEF. Measurement of activated p115RhoGEF from leptin-treated cells in the presence or absence of ginseng to facilitate guanine nucleotide exchange activity by purified small GTPase RhoA was then performed. p115RhoGEF activation was increased 2-fold in leptin-treated cells compared with control (Fig. 7B). Particularly of interest, this observed increase in guanine nucleotide exchange activity was significantly abolished in cells pretreated with ginseng. p115RhoGEF activity was unaffected by ginseng alone.

Discussion

Increasing evidence from a number of laboratories has demonstrated that leptin exerts a direct hypertrophic effect on cardiomyocytes (Rajapurohitam et al., 2003; Xu et al., 2004; Madani et al., 2006; Hou et al., 2010) and intact myocardium in vivo (Abe et al., 2007). In addition, blocking leptin receptors attenuates remodeling and heart failure in the postinfarcted rat heart (Purdham et al., 2008). Although the precise mechanism of action of leptin accounting for its hypertrophic effect is not completely understood, we have previously suggested that activation of the RhoA/ROCK pathway plays a critical role in mediating leptin-induced cardiac hypertrophy, possibly through activation and subsequent nuclear translocation of p38 MAPK, the latter effect dependent on alterations in actin dynamics (Zeidan et al., 2006, 2008). In the present study, we assessed the effects of North American ginseng (P. quinquefolius) on leptin-induced cardiac hypertrophy based on emerging evidence that ginseng exerts anti-hypertrophic effects in a varied number of experimental models (Jiang et al., 2007; Qin et al., 2008; Deng et al., 2010).
and also reduces the severity of heart failure in rats subjected to chronic coronary artery ligation (Guo et al., 2011). We hypothesized that ginseng would attenuate leptin-induced cardiac hypertrophy by attenuating RhoA/ROCK activation after leptin administration. Our study shows for the first time that ginseng is a potent inhibitor of leptin-induced hypertrophy, and indeed this occurs through a mechanism associated with the abrogation of RhoA/ROCK activation. Moreover, we identified a potential key role of p115RhoGEF in facilitating RhoA/ROCK-dependent p38 and ERK1/2 MAPK pathway activation in leptin-induced cardiac hypertrophy and, critically, the ability of ginseng to target p115RhoGEF as a mechanism for its ability to prevent RhoA/ROCK activation, thus preventing cardiomyocyte hypertrophy.

Leptin-induced hypertrophy was manifested by an increase in cell-surface area, a 2-fold increase in [3H]leucine incorporation, and increased expression of two molecular hypertrophic gene markers, α-SA and myosin heavy chain, all of which were significantly attenuated by ginseng. Moreover, the hypertrophic effect of leptin was associated with an activation of the RhoA/ROCK pathway as exhibited by an increase in RhoA-GTP levels, in support of our previous findings (Zeidan et al., 2006, 2008). RhoA/ROCK activation was further demonstrated by increased phosphorylation (activation) of cofilin-2, a ubiquitous enzyme downstream of RhoA that depolymerizes actin, resulting in a decrease in the G/F actin ratio. We previously reported that leptin-induced RhoA/ROCK activation was critical for p38 MAPK and ERK1/2 MAPK nuclear translocation and the subsequent hypertrophic response, a response probably dependent on the changes in actin dynamics (Zeidan et al., 2008). Together, the ability of ginseng to completely prevent activation of the RhoA/ROCK pathway, p38 translocation, and the associated hypertrophic response strongly suggests that inhibition of RhoA/ROCK represents a key mechanism for the anti-hypertrophic effect of ginseng as seen in our study.

We next assessed the potential target mediating the ability of ginseng to inhibit RhoA/ROCK activation. Our study centered primarily on the potential role of RhoGEFs, which are critical for downstream RhoA activation (Rossman et al., 2005). However, the role of RhoGEFs in the cardiac hypertrophic program has not been studied extensively. Although a number of RhoGEFs have been identified, p115RhoGEF and p63RhoGEF were considered of particular interest because their expression has been demonstrated in cardiovascular tissues including the heart (Souchet et al., 2002; Porchia et al., 2008; Wuertz et al., 2010) and their activation has been shown in response to various hypertrophic agonists including angiotensin II (Guilluy et al., 2010) and endothelin-1 (Porchia et al., 2008). Our study shows for the first time that leptin induced a significant increase in p115RhoGEF gene and protein expression without affecting p63RhoGEF. We next studied whether increased expression of p115RhoGEF is also associated with increased GEF activity. Activation of p115RhoGEF involves receptor-mediated membrane translocation and colocalization with RhoA (Kozasa et al., 1998; Rossman et al., 2005; Aittaleb et al., 2010). Our results revealed membrane translocation of p115RhoGEF with RhoA colocalization after 10 min of leptin stimulation and also demonstrated an increase in p115RhoGEF activity.

The ability of ginseng to inhibit p115RhoGEF activation suggests this as a target for the ability of ginseng to inhibit leptin-induced cardiomyocyte hypertrophy, and it is reasonable to assume that p115RhoGEF activation by leptin and inhibition by ginseng represent the main regulatory sites influencing subsequent p38 and ERK1/2 phosphorylation or translocation of the former. However, a limitation of our study is that it does not establish a direct causal relationship between p115RhoGEF activation by leptin and stimulation of the RhoA

![Graphs showing the effects of ginseng on p115RhoGEF and p63RhoGEF expression](https://example.com/graphs)

**Fig. 6.** Ginseng inhibits leptin-induced up-regulation of p115RhoGEF but not p63RhoGEF protein and gene expression. Gene expression (A and C) and protein levels (B and D) for p115RhoGEF and p63RhoGEF in cardiomyocytes treated with leptin in the absence or presence of ginseng are shown. Data represent means ± S.E.M. n = 8, †, p < 0.05 versus control; †, p < 0.05 versus leptin. Con, control; Lep, leptin; Gin, ginseng.
pathway. Additional studies are required to confirm this, particularly by determining the effect of p115RhoGEF down-regulation on the ability of leptin to activate RhoA.

As reported previously (Zeidan et al., 2008), leptin induces phosphorylation of both p38 and ERK1/2 although only the former is translocated into nuclei, thus suggesting that phosphorylation is not a precondition for nuclear transport. Although the precise mechanisms for selective p38 translocation into nuclei are not known, the phenomenon is possibly mediated by changes in actin dynamics because the effect is prevented by latrunculin B, which prevents actin polymerization as a result of RhoA activation (Zeidan et al., 2008). The selective translocation of p38 into nuclei after leptin addition also helps to explain our initial finding that pharmacological inhibition of p38, but not ERK1/2, prevents leptin-induced hypertrophy (Rajapurohitam et al., 2003). The specific mechanism of p115RhoGEF-RhoA/ROCK-dependent p38 and ERK1/2 MAPK inhibition by ginseng, however, still remains unclear. The chemical structures of ginsenosides, which are triterpene saponins and are considered the primary active constituents contributing to the medicinal effects of ginseng (Attele et al., 1999), have been compared with steroidal structures such as estrogen. Leung et al. (2007) alluded to the competitive binding of the specific ginsenoside Rb1 (the predominant ginsenoside of North American ginseng), selective to the estrogen receptor β, where it was theorized to be engulfed with the bound ginsenoside through endocytosis into the cytoplasm in which it translocates into the nucleus to bind to transcription factors eliciting its effects as an antiangiogenic factor. Indeed, we previously have shown that estrogen (as 17β-estradiol) exerts a pro-hypertrophic effect on cultured ventricular myocytes at very low (1 pM) concentrations but has antihypertrophic actions at nanomolar concentrations (Kılıç et al., 2009). The observation of the ability of ginsenoside Rb1 to bind to estrogen receptors is intriguing and raises the question of potential gender-specific effects of ginseng. Although it seems that estrogen receptors are expressed in ventricular myocytes of both male and female rats, nonetheless, potential gender-dependent effects of ginseng are deserving of further study. In the present study, an alcoholic ginseng extract containing a large number of ginsenosides was used, and, consequently, the specific ginsenoside(s) responsible for the observed inhibition of leptin-induced effects currently cannot be identified.

Another potential mechanism for the observed inhibition of
these pathways by ginseng may occur extracellularly at the level of the leptin-receptor long isoform (Ob-Rb), considered the principal receptor mediating the biological effects of leptin (reviewed by Villanueva and Myers, 2008), potentially as a result of early binding and antagonism of Ob-Rb, consequently down-regulating further leptin signaling (Fig. 8). Although it was not an aim of the current study, competitive binding studies between leptin and ginseng at the Ob-Rb are deserving of further investigation.

In conclusion, our results show for the first time that ginseng markedly attenuates the direct hypertrophic effect of leptin. Moreover, our results are strongly supportive of the concept that this effect of ginseng against leptin-induced hypertrophy occurs via inhibition of p115RhoGEF expression and activity, thus abrogating RhoA/ROCK activation. The latter results in diminished p38 MAPK phosphorylation and translocation into nuclei, thus attenuating transcription and reducing the hypertrophic response. As noted previously, further work is necessary to demonstrate a precise causal relationship between leptin-induced p115RhoGEF activation and subsequent activation of downstream pathways. In addition, because ginseng exerts numerous and diverse effects on the heart (reviewed by Karmazyn et al., 2011), the contribution of other pathways as targets for the antihypertrophic effects of ginseng cannot be excluded. Moreover, it is important to point out that the present study was carried out using neonatal ventricular myocytes and therefore extrapolation of these results to the adult myocardium, particularly under in vivo conditions, should be done cautiously. Although the role of leptin in cardiac pathology still remains to be fully determined, our overall results suggest that ginseng could be an effective therapeutic approach aimed at mitigating potential deleterious cardiovascular complications associated with hyperleptinemia, particularly those involving a cardiac hypertrophic phenotype.

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Authorship Contributions
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Contributed new reagents or analytic tools: Moey.

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References


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