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STUDIES INTO THE MECHANISMS UNDERLYING THE CARDIAC ANTI- HYPERTROPHIC AND ANTI-REMODELLING EFFECTS OF GINSENG

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STUDIES INTO THE MECHANISMS UNDERLYING THE CARDIAC ANTI-HYPERTROPHIC AND ANTI-REMODELLING EFFECTS OF GINSENG

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Abstract

Ginseng is a widely prescribed herbal drug that has been used for over 2000 years in Asia for the treatment of several different disorders of the body including those of the cardiovascular system. Advances in ginseng research have identified the bioactive constituents considered responsible for eliciting its pharmacological effects known as 'ginsenosides'. Several published reports have demonstrated the potential ability of ginseng and their isolated ginsenosides in the prevention and treatment of heart disease. In the study presented here the effects of ginseng on agonist-induced cardiac hypertrophy in isolated cardiomyocytes (**Chapter 2 – 4**) as well as an *in vivo* model of heart failure (**Chapter 4**) were investigated.

In our first study (**Chapter 2**), the ability of ginseng to prevent leptin-induced ventricular cardiac hypertrophy by inhibiting p115RhoGEF-RhoA/ROCK-dependent MAPK activation was investigated. Leptin (50 ng/ml, which is a concentration representative of plasma levels found in obese individuals) 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 globular to filamentous actin ratio. These effects were prevented by North American ginseng (alcoholic extract; 10 μg/ml), hereon referred to as "ginseng". The stimulation of RhoA/ROCK by leptin was associated with significantly increased p115RhoGEF gene and protein expression and exchange activity, all of which were inhibited by ginseng. The attenuation of leptin-induced activation of RhoA/ROCK by ginseng was further associated with diminished p38 MAPK activation and nuclear translocation.

In a follow-up study (**Chapter 3**), the ability of ginseng to reverse leptin-induced cardiac hypertrophy by enhancing Rnd3-p190RhoGAP-mediated downregulation of RhoA/ROCK

activation, was investigated. Cardiomyocytes incubated with leptin for 48 h displayed significantly increased cell surface area, which was accompanied by an increase in the expression of the fetal gene α-skeletal actin. A decrease in the G/F actin ratio, most likely as a result of RhoA/ROCK cofilin-2 phosphorylation was observed in hypertrophied cells treated with leptin. Treatment with ginseng however reversed these effects. In leptin-treated cells, Rnd3 gene and protein expression were decreased however treatment with ginseng reversed these effects by leptin. In the left ventricular tissues of rats subjected to four weeks of sustained myocardial infarction (MI), Rnd3 protein expression was markedly reduced while p63RhoGEF and ROCK expressions, which reflect upregulation of RhoA, were increased. These MI-induced effects however were restored by ginseng to expressions as observed in sham.

For our third study **(Chapter 4)**, we investigated the ability of ginseng to reverse already established cardiac dysfunction as well as hypertrophy both *in vitro* and *in vivo* by inhibition of calcineurin/NFAT3 activation. The ability of a pharmacological agent to *reverse* HF is of particular importance as the majority of current treatments are unable to reverse already established myocardial remodelling and ventricular dysfunction. Accordingly, ginseng was administered in drinking water *ad libitum* to rats after 4 weeks of sustained coronary artery ligation (CAL) when hypertrophy and HF were established or to hypertrophic neonatal ventricular myocytes treated with angiotensin II, endothelin-1 or phenylephrine. Echocardiographic and catheter-based measurements of hemodynamic parameters revealed complete reversibility of systolic and diastolic abnormalities as well as increased myocardial collagen gene expression in CAL-rats after treatment with ginseng. Similarly, ginseng administration to hypertrophic cardiomyocytes resulted in complete reversal to a normal phenotype after 24 h as determined by cell surface area and α-skeletal gene expression. The

effects of ginseng *in vivo* were associated with a tendency to attenuate calcineurin activation and MCIP-1 gene expression. In the cultured cardiomyocytes however, ginseng completely reversed agonist-induced calcineurin activation and NFAT3 nuclear translocation. Taken together, results from our studies demonstrate a marked anti-hypertrophic and anti-remodelling ability of ginseng in the prevention and treatment of cardiovascular disease.

Keywords: cardiac hypertrophy, leptin, p115RhoGEF, RhoA/ROCK, ginseng, ginsenosides, heart failure, reversal, calcineurin/NFAT3

Co-Authorship

The experimental studies included in this thesis were all performed in the laboratory of Dr. Morris Karmazyn who supervised the experimental design, provided knowledgeable insight to the interpretation of results and the preparation and writing of the manuscripts. Dr. Asad Zeidan (**Chapter 2**) and Dr. Venkatesh Rajapurohitam (**Chapter 2 – 4**) additionally assisted with the experimental design and carrying out of the experiments. All *in vivo* animal surgeries were performed by Ms. Cathy Xiaoling Huang (**Chapter 3** and **4**) and all echocardiographic data were obtained by Ms. Tracey Xiaohong Gan (**Chapter 4**). Bi-weekly blood collections for the *in vivo* project were obtained with the assistance of Dr. Venkatesh Rajapurohitam and Dr. Eduardo Martinez-Abundis (**Chapter 4**).

Dedication

For God so loved the world, that he gave his only Son, that whoever believes in Him should not perish but have eternal life. For God did not send his Son into the world to condemn the world, but in order that the world might be saved through him.

John 3:16-17

And we know that for those who love God all things work together for good, for those who are called according to his purpose.

Romans 8:28

"For I know the plans I have for you," declares the LORD, plans to prosper you and not to harm you, plans to give you hope and a future."

Jeremiah 29:11

Praise the Lord! Oh give thanks to the Lord, for he is good, for his steadfast love endures forever!

Psalm 106:1

Mum and Dad

Thank you for your endearing encouragement, sacrifice and belief in everything I chose to pursue. You have showered me with love and care of which I am so am thankful to be blessed with. I love you guys.

Tubs aka Brotha Mo

Thanks buds. Love, love, love. You're the real scavenger. 171668.

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LIST OF ABBREVIATIONS, SYMBOLS AND NOMECLATURE

$[Ca^{2+}]_i$: intracellular calcium concentration

$[Na^{+}]_i$: intracellular sodium concentration

ACC/AHA: American College of Cardiology/American Heart Association

ACE-I: angiotensin converting enzyme inhibitor

AngII: angiotensin II

ANP: atrial natriuretic peptide

BSA: bovine serum albumin

CAM: complementary and alternative medicine

CO: cardiac output

Con: control

CVD: cardiovascular disease

E/A: early/ (late) atrial

ECM: extracellular matrix

EDP: end-diastolic pressure

EDPVR: end-diastolic pressure volume relationship

EDV: end diastolic volume

EF: ejection fraction

EGTA: ethylene glycol tetraacetic acid

ERK: extracellular signal-regulated kinase

ESP: end-systolic pressure

ESPVR: end-systolic pressure volume relationship

ESV: end-systolic volume

ET-1: endothelin-1

G/F actin: globular/filamentous actin

GAP: GTPase activation protein

GDI: guanosine nucleotide dissociation inhibitor

GDP: guanine diphosphate

GEF: guanine nucleotide exchange factor

Gin: ginseng

GPCR: G-protein coupled receptor

GST: glutathione S-transferase

GTP: guanine triphosphate

JNK: c-Jun N-terminal kinase

Lep: Leptin

LIMK: LIM domain kinase

LVAD: left ventricular assist device

LVIDd: end diastolic left ventricular internal diameter

LVIDs: end systolic left ventricular internal diameter

MAPK: mitogen activated protein kinase

MAPKK: MAPK kinase

MAPKKK: MAPK kinase kinase

MCIP-1: modulatory calcineurin interacting protein-1

MHC: myosin heavy chain

MLC: myosin light chain

M-MLV: moloney murine leukemia virus

mTOR: mammalian target of rapamycin

NCX: sodium calcium exchanger

NFκB: nuclear factor kappa-light-chain enhancer of activated B cells

NHE-1: sodium hydrogen exchanger

NYHA: New York Heart Association

Ob-R: leptin receptor

PCNA: proliferating cell nuclear antigen

Phe: phenylephrine

PI3K: phosphatidylinositol 3-kinase

PPAR: peroxisome proliferator-activated receptors (PPARs)

PPD: protopanaxadiol

PPT: protopanaxatriol

RAAS: renin-angiotensin-aldosterone system

RGS: regulator of G protein signaling

ROCK: Rho-associated, coiled-coil containing protein kinase

RTK: receptor tyrosine kinase

SAPK: stress-activated protein kinase

SV: stroke volume

TCM: traditional Chinese medicine

Y-27632: ROCK inhibitor

αSA: α-skeletal actin

CHAPTER 1:

Literature Review: *Ginseng, Cardiovascular Disease and Leptin*

A version of section 1.3 has been published elsewhere:
Karmazyn M, **Moey M** and Gan XT. Therapeutic potential of ginseng in the management of cardiovascular disorders. Drugs 2011;71:1989-2008

1.1 The Heart

A general overview of the anatomical and physiological characteristics of the heart has been provided in the subsequent sections as supporting background information for the material discussed in this thesis. These concepts include a basic description of the structural (section 1.1.1.1) and cellular (section 1.1.1.2) anatomy followed by the physiological (section 1.1.1.3) function of the heart relevant to the content of the thesis.

1.1.1 *Anatomy of the Heart*

1.1.1.1 Structural Anatomy of the Heart

Weighing at 250 to 300 g and the size of one's own fist, the human heart is a powerful muscular pump that provides oxygenated blood to the whole body. Enclosed and protected in a double-layered (fibrous/outer and visceral/inner layer) sac known as the pericardium, the heart is located in the thoracic chamber where it sits anterior to the spinal cord and posterior to the sternum.

The heart is a four chamber pump consisting of the right and left atria (RA and LA, respectively) which are located at the apex (top) and the right and left ventricles (RV and LV, respectively) toward the base (bottom) of the heart. The RA, located superior to the RV, receives oxygen depleted blood from the tissues *via* the superior and inferior vena cava (SVC and IVC, respectively). Deoxygenated blood collected in the RA is ejected through the tricuspid valve into the RV and subsequently out through the semilunar pulmonary valve. Deoxygenated blood continues to travel through the pulmonary arteries towards the lungs where oxygen is diffused into the blood *via* capillary diffusion. Oxygenated blood returns to the heart *via* the pulmonary veins and into the LA where it is directed through the mitral or bicuspid valve into the LV. The

blood is subsequently ejected through the semilunar aortic valve into the aorta to provide oxygen to the tissues of the body (reviewed in Cabin, 2002; Klabunde, 2005).

Coordinated and synchronized contraction of the heart is initiated by an intricate electrical network that consists of the sino atrial (SA) node located near the superior portion of the RA close to the SVC, followed by the atrial ventricular (AV) node between the RA and RV. The electrical network is further branched out at the base of the heart into the bundle of His which extends upward throughout the RV and LV. Efficient communication beginning at the SA node towards the bundle of His branches allows synchronized contraction of the heart to provide oxygenated blood to the rest of the body (reviewed in Cabin, 2002; Klabunde, 2005).

1.1.1.2 Cellular Anatomy of the Heart

The heart is essentially made up of three layers which include the epicardium, myocardium and endocardium (reviewed in Cabin, 2002). The epicardium, which is also fused to the visceral pericardium, is the most exterior layer of the heart and is predominantly made up of connective tissue that serves as a protective barrier. Immediately adjacent to the epicardium is the myocardium or middle layer which is composed of cardiac muscle responsible for producing contraction of the heart. The endocardium is the innermost layer of the heart consisting mainly of endothelial cells lining the chambers and has also been shown to contribute to myocardial contraction (reviewed in Cabin, 2002; Klabunde, 2005).

The myocardium is composed of striated cardiac muscle fibers, which are fundamentally comprised of heart cells termed cardiomyocytes. Unlike skeletal myocytes which may contain several nuclei, cardiomyocytes are typically mono or dinucleated. Cardiomyocytes are similar to skeletal myocytes as they both contain contractile apparati known as myofibrils. Continuous chains of sarcomeric segments which contain thick and thin actin filaments that slide between

each other are the functional units that comprise the myofibrils responsible for producing cardiomyocyte contraction (Solaro, 2010). Most importantly, the cellular and membranous components of a cardiomyocyte which are multitudinous, significantly contribute to the intracellular homeostatic environment. Changes in intracellular ion concentration, pH or extracellular stimuli can lead to significant changes in the molecular signalling of the cardiomyocyte which may negatively affect cardiomyocyte contraction as well as the cellular morphology. The cellular signalling pathways of the cardiomyocyte have been summarized in detail in section 1.2.2.

1.1.2 *Physiology of the Heart*

1.1.2.1 The Cardiac Cycle

The cardiac cycle describes the stages of cardiac relaxation (diastole) in which blood fills the ventricle and contraction (systole) in which blood is ejected from the heart in a single beat. The events within a cardiac cycle can be visualized using a pressure sensitive catheter that is inserted into the LV, which can record pressure changes while the heart is beating. Figure 1.1 demonstrates a standard example of the changes in pressure and volume of the LV as a function of time. In the RV, the pattern of pressure and volume changes is generally similar to that observed in the LV (reviewed in Cabin, 2002; Klabunde, 2005).

The cardiac cycle is comprised of five phases which are (1) atrial contraction, (2) isovolumetric contraction, (3) ventricular contraction, (4) isovolumetric relaxation and (5) ventricular filling. Phase 1 or atrial contraction: in atrial contraction, the atrium becomes depolarized and begins to contract causing an increase in atrial pressure, which pushes the blood into the ventricle. Most of the ventricular filling however occurs as a result of passive blood flow into the ventricle, which is dependent on venous return. At this point in time, the ventricle is

maximally filled and is represented on Figure 1.1A as the end-diastolic volume (EDV). Phase 2 or isovolumetric contraction: the end of phase 1 marks the beginning of isovolumetric contraction where electrical depolarization of the ventricle initiates contraction while maintaining a constant volume. Phase 3 or ventricular contraction: as the ventricular pressure begins to rapidly rise, it exceeds the pressure in the aorta and contraction occurs as blood is rapidly ejected from the LV through the aortic valve. Phase 4 or isovolumetric relaxation: immediately after rapid ejection of the blood from the LV, the ventricle experiences repolarization and begins to relax. The intraventricular pressure of the LV continues to gradually decline and consequently forces the aortic valves close. All the while, ventricular volume remains constant and the residual volume at the end of this phase is the end-systolic volume (ESV) (Figure 1.1A). Phase 5 or ventricular filling: as a result of continuous venous return into the atrium, the atrial pressure begins to steadily increase and consequently establishes a pressure gradient between the atrium and ventricle. This consequently favours movement of blood into the ventricle, thereby restarting the cardiac cycle (reviewed in Cabin 2002; Klabunde 2005).

1.1.2.2 Pressure-Volume Loops

The measurements of pressure and volume during one cardiac cycle can be translated into a graphical representation known as a pressure-volume (PV) loop (Figure 1.1B), which can provide an abundant amount of information for assessing cardiac function (Sagawa, 1978; Katz, 1988). The ESV and EDV are indicated on the PV loop of Figure 1.1B at the bottom left and right, respectively. The difference between EDV and ESV, which can be visualized by a line drawn across the PV loop or the width of the PV loop, is the stroke volume (SV). In a healthy individual, more than 60% of the EDV is ejected during systole (reviewed in Klabunde, 2005). The SV divided by EDV provides an important marker known as the ejection fraction (EF) to

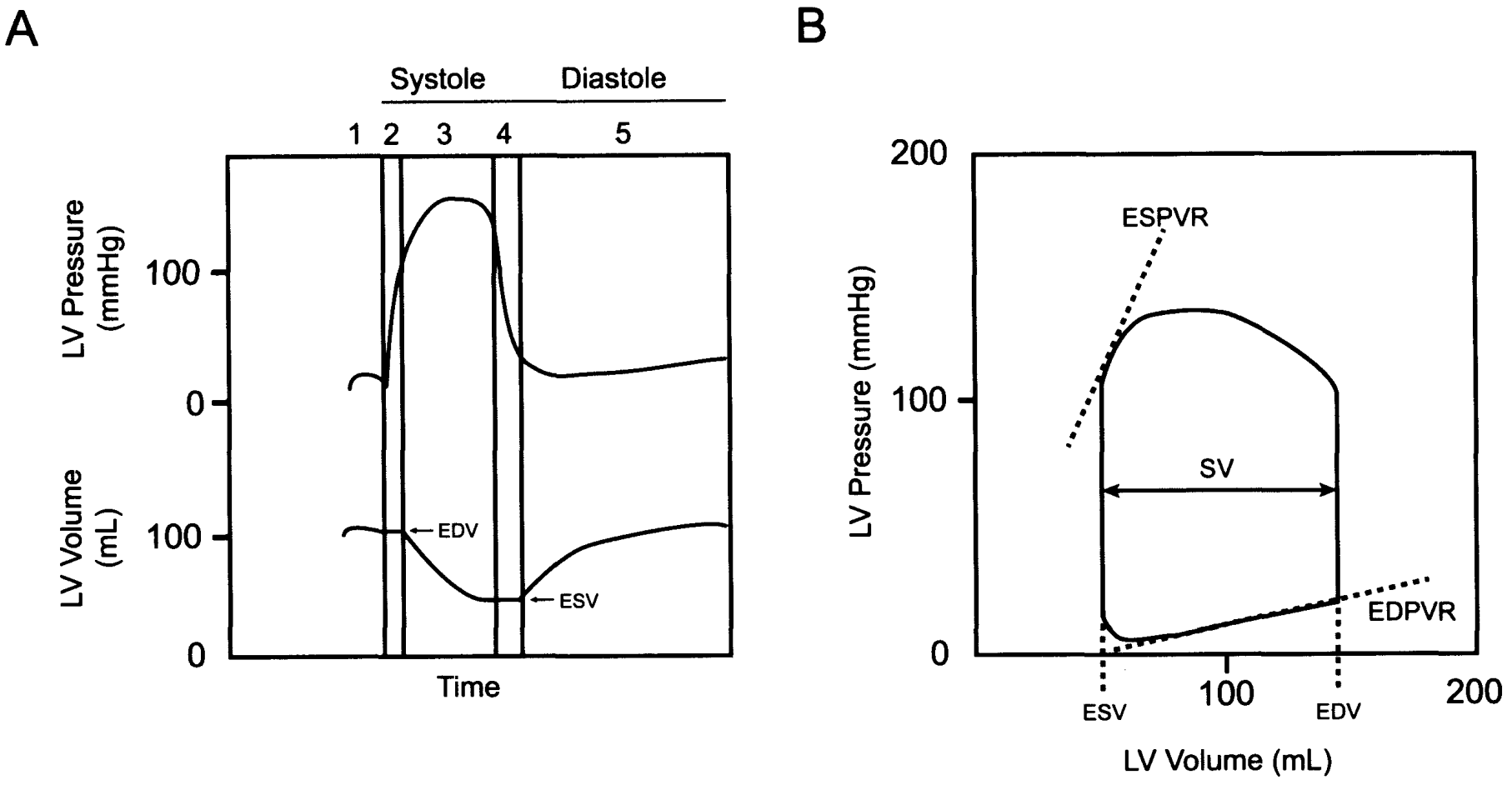


Figure 1.1. Cardiac cycle and pressure-volume loop. Graphical representations of the changes in pressure and volume of the LV within a single heart beat. Graph A represents the cardiac cycle. Numbers above indicate the cardiac phases as described in the text. Graph B represents the changes in pressure as a function of volume. Image has been recreated and modified from Klabunde, 2005. EDV: end-diastolic volume; ESV: end-systolic volume; EDPVR: end-diastolic pressure volume relationship; ESPVR: end-systolic pressure volume relationship; SV: stroke volume.

represent cardiac function. In addition to EF, the SV is essential in calculating cardiac output (CO), which is the amount of blood ejected from the ventricle per minute (mL/min or L/min), or SV multiplied by heart rate (HR) (reviewed in Klabunde, 2005).

Mathematical measurements such as the PV relationship slopes at the end of systole and diastole (ESPVR and EDPVR, respectively) from the PV loop may also be obtained. ESPVR is drawn on the top-left hand side of the PV loop, which indicates the contractile efficiency of the ventricle, while the EDPVR is drawn on the bottom-right hand side of the PV loop, representing ventricular compliance (reviewed in Klabunde, 2005). Pathological changes to the heart, such as heart failure (HF) which is discussed in detail in section 1.2, result in abnormal pressure and volume measurements that are translated as shifts of the PV loop along the pressure and (y) and volume (x) axis (Katz, 1988). These aberrations consequently affect the ESPVR and EDPVR slopes providing valuable information for clinicians in the assessment of ventricular function.

1.2 Cardiovascular Disease

Cardiovascular disease (CVD) describes a disorder of the heart (cardio) and blood vessels (vascular) as well as any other organs that may be affected by the circulatory system namely, but not limited to, the brain, lungs and kidney (Canada, 2010). The specific types of CVD are termed and categorized by the origin of the disease (reviewed in Nabel, 2003).

The predominant vascular-specific CVD types include cerebrovascular disease (stroke) which is an impediment of blood supply in the vasculature of the brain that can be further classified as a transient ischemic attack (blockage less than 24 hours) or a cerebrovascular thrombosis/clot (chronic blockage) and peripheral vascular disease, which is a disease in the vasculature of the legs and arms. (Canada, 2010).

Cardio-originating CVDs includes ischemic heart disease as a result of a myocardial infarction (MI) or impediment of the blood supply to the cardiac muscle; congenital heart disease, which is the result of an anatomical birth defect of the heart or genetic mutation; rheumatic heart disease, which is most common in younger individuals as a result of a bacterial infection of the heart valves; inflammatory heart disease that specifically includes endocarditis or pericarditis, which is the inflammation of the inner cardiac muscle and outer lining sac of the heart, respectively; and HF, which is considered the end stage result of CVDs where the pumping efficiency of the heart to supply adequate blood to the rest of the body is impaired.

1.2.1 Heart Failure

1.2.1.1 Definition and Types of Heart Failure

HF has been described as the 'state of any heart disease in which [...] the heart's output is decreased or in which the heart is unable to pump blood at a rate adequate for satisfying the requirements of the tissues with function parameters remaining within normal limits' (Denolin et al., 1983). As discussed below HF can be described as either acute or chronic and can also be classified as either systolic or diastolic (HF with preserved ejection fraction) (Bhatia et al., 2006; Owan et al., 2006). Classification systems such as the New York Heart Association (NYHA) (Criteria Committee, New York Heart Association, 1964) as well as the American College of Cardiology/American Heart Association (ACC/AHA) have been developed to rate the severity of HF in patients and to provide appropriate recommendations for therapy (Hunt et al., 2002).

Acute HF, which may manifest after short-term stimuli such as cardiac surgery, acute sepsis or MI, refers to the rapid onset of decreased cardiac function/output in the absence of compensatory mechanisms that lasts for hours to days. In contrast however, chronic HF is the

development of cardiac dysfunction involving adaptive responses at the global and molecular level over a longer period of time (Dickstein et al., 2008).

Diastolic (DHF) and systolic (SHF) HF are classical types of chronic HF that may manifest as individual syndromes, however these are often clinically presented together where distinct molecular and structural remodelling of the heart have occurred (van Heerebeek et al., 2006). DHF refers to an impairment of the elasticity or an increased stiffness of the myocardium resulting in abnormal ventricular filling during diastole while SHF refers to abnormal ventricular contraction of the heart (reviewed in Chatterjee and Massey, 2007).

1.2.1.2 Diagnosis and Classification of Heart Failure

Clinical diagnosis of HF is determined by echocardiography, which is a technique that uses ultrasound waves at high frequencies to provide two-dimensional images of the heart (Oh et al., 1997). An echocardiogram can provide essential information for the analysis of ventricular function such as EDV and ESV to calculate SV, CO and EF and can additionally provide morphological recordings to visualize any abnormal changes in chamber dimension.

While there are many methods of classifying HF, such as whether the disorder is predominantly left or right-sided, or if it pertains to depressed contraction or relaxation of the heart (SHF and DHF, respectively), the commonly applied system of categorizing is the NYHA classification. The NYHA scale rates the degree of abnormal function of the heart based on presenting symptoms and quality of life, which essentially describes the severity of the disease (Criteria Committee, New York Heart Association, 1964). Class I is the least severe which describes a patient with no limitation or associated symptoms during ordinary activities and up to Class IV, which is the most severe where the minimalist of physical activity inflicts discomfort and symptoms manifest even at rest. Class IV patients have a very low predicted survival

outcome and may require more invasive treatment options such as heart transplantation as the patients are most often non-responsive to conventional pharmacotherapy (Criteria Committee, New York Heart Association, 1964).

In comparison, the AHA/ACC classification system of HF is arranged by stages from A to B which describes the progression of the disorder. It has an advantage over the NYHA classification as it includes a pre-HF stage A which describes patients who are absent of the disease but who demonstrate a high risk factor for the development of HF. Stage B is equivalent to NYHA class I and includes patients with structural damage with no associated symptoms present. Stage D is comparable to NYHA class IV and describes the most advanced HF with severe symptoms that are challenging to manage with conventional therapy (Hunt et al., 2001). Detection of HF at the earliest stage is fundamental for success in treatment of the disorder (reviewed in Jessup and Brozena, 2001).

1.2.1.3 Etiology and Pathophysiology of Heart Failure

The causes of HF are numerous and habitually coexist with one another. The predominant factors leading to HF namely include ischemic heart disease, which is the number one cause of mortality worldwide (World Health Organization, 2011), followed by cigarette smoking, hypertension, obesity, diabetes and valvular heart disease (reviewed in McMurray and Stewart, 2000). Less common causes of HF are arrhythmias, viral infections, and damages to the cellular structures of the heart such as amyloidosis or systemic lupus erythematosus (Canada, 2008).

These initiating stimuli compromises the normal function of the heart, which may be manifested by an EF of less than 40%, increased EDV and ESV, increased end-diastolic pressure (EDP), decreased end-systolic pressure (ESP), an early to late (atrial) ventricular filling velocity

measured across the mitral valve (E/A ratio) of greater than 1.5 (Bella et al., 2002) and/or significant enlargements of the ventricular chambers.

A decrease in CO from the failing heart signals the body to appropriately adapt to the compromised circumstance by increasing sympathetic stimulation. For example, in an effort to counterbalance the decrease in arterial blood pressure, the brain signals the release of catecholamines to induce systemic vasoconstriction or directly enhances the release of antidiuretic hormone or vasopressin to promote fluid retention in the kidneys (Clark and Cleland, 2000; Charkoudian and Rabbits, 2009; Floras, 2009). Decreased systemic perfusion to the kidney upregulates the renin-aldosterone-angiotensin system (RAAS) and consequently results in vasoconstriction in an effort to increase blood pressure and volume. Long term effects of this compensatory mechanism however leads to an increase in peripheral resistance and therefore a greater afterload producing a marked increase in resistance to left ventricular outflow. In addition to the changes in blood pressure, significant modifications to the heart termed cardiac remodelling also occur further aggravating the HF syndrome.

1.2.2 Cardiac Remodelling and Hypertrophy

Cardiac remodelling may refer to either the structural changes, such as shape and size, of the heart (global remodelling) or to the changes in the molecular signalling of the cardiomyocyte (cellular remodelling) as well as modifications to the extracellular matrix (ECM) of the myocardium (extracellular remodelling) (reviewed in Fedak et al., 2005). The most characteristic feature in the cardiac remodelling process is the development of cardiac hypertrophy that is considered a maladaptive compensatory mechanism which results in decreased pumping efficiency of the heart.

1.2.2.1 Types of Hypertrophy: Physiological vs. Pathological and Concentric vs. Eccentric

Cardiac hypertrophy is classified as either physiological or pathological (reviewed in MucMullen and Jennings, 2007; Bernardo et al., 2010). In cases of physiological hypertrophy, which mainly occur during fetal growth, pregnancy or as a result of chronic exercise (athlete's heart), there is a proportional increase in chamber sizes. In contrast, pathological hypertrophy is detrimental because it involves abnormal structural and cellular remodelling that may lead to necrosis and apoptosis of viable myocardium resulting in cardiac dysfunction and ultimately HF (reviewed in Bernardo et al., 2010). Evidently, there are significant differences in the diagnostic markers which classify physiological and pathological hypertrophy as the stimuli and global and cellular remodelling processes are unique to both classifications of hypertrophy. For example, the reexpression of fetal genes such as α-skeletal actin (αSA) or atrial natriuretic peptide (ANP) (Ueno et al., 1999) are present in pathological but are absent in physiological hypertrophy (reviewed in Bernardo et al., 2010). In addition, activation of specific cellular signalling pathways are upregulated in pathological hypertrophy while it has no contribution in the physiological hypertrophic process. An example of this phenomenon is the activation of the calcineurin/NFAT3 signalling pathway (discussed in section 1.2.3.3) that was upregulated only in mice subjected to pressure-overload or MI-induced pathological hypertrophy and not in exercise training-induced physiological hypertrophy (Wilkins et al., 2004).

Cardiac hypertrophy can additionally be classified as concentric or eccentric hypertrophy which is based on the structural arrangement of the cardiomyocytes (reviewed in Jessup and Brozena, 2003). In concentric hypertrophy, cardiac cells increase along the horizontal axis and are stacked in parallel therefore resulting in an increase in the mass and width of the ventricular

wall and a decrease in the ventricular cavity. Pressure overload stimuli such as strength training/heavy lifting in athletes (Mihl et al., 2008) or hypertension and aortic stenosis are examples of causes of concentric hypertrophy (reviewed in Bernardo et al., 2010). Conversely, eccentric hypertrophy involves an elongation of the cardiomyocyte arranged in series which produces a thinner ventricular wall and an increase in ventricular cavity size. Eccentric hypertrophy usually arises from volume overload stimuli which may include endurance training (Mihl et al., 2008) or valvular disease (reviewed in Bernado et al., 2010).

1.2.3 Signalling Pathways in Cardiac Hypertrophy

As many humoral factors are released in the circulation in response to a prohypertrophic stimuli, there are consequently multiple and complex signalling pathways that may contribute to the pathogenesis of cardiac hypertrophy (reviewed in Frey and Olson, 2003). Accordingly, select molecular mechanisms which have been studied in this thesis, particularly mitogen activation of protein kinases (MAPK) and small GTPases protein signalling pathways are discussed in detail in section 1.2.3.1 and 1.2.3.2, respectively.

1.2.3.1 Mitogen-activating Protein Kinase (MAPK) Pathway: p38 and ERK1/2

One of the cellular signalling pathways that has been shown to be upregulated by several different classes of extracellular hypertrophic stimuli is the MAPK pathway (reviewed in Rose et al., 2010). Communication between the initial external stimuli and the nucleus of the cardiomyocyte is appropriately coordinated and transmitted *via* the MAPKs to induce an upregulation of transcriptional factors which lead to hypertrophy.

MAPKs are specific serine/threonine-protein kinases that are activated by upstream protein kinases, which include a three-step MAPK cascade in response to inflammatory cytokines, growth factors or G-protein coupled receptors (GPCRs) agonists (reviewed in Rose et

al., 2010). First in response to the external stimuli are MAP kinase kinase kinases (MAPKKK, MAP3K, MEKK or MKKK) such as MLK3 (Chadee and Kyriakis, 2004) or MEK2,3 (Xiao et al., 2001; Shama et al., 2008). MAPKKKs subsequently phosphorylate the MAP kinase kinases (MAPKK MAP2K, MEK or MKK) on serine or threonine residues, which are second in line of the three-tier cascade. Activation of MAPKKs phosphorylate threonine and tyrosine residues (*Thr-X-Tyr* or T-X-Y motif) (Canagarajah et al., 1997) of their respective MAPKs. The cascade is complete when activated MAPKs phosphorylate the appropriate nuclear and cellular substrates downstream of the specific MAPK pathway (reviewed in Rose et al., 2010).

The three major MAPKs that have been implicated in cardiac hypertrophy and HF are the extracellular signal-regulated kinases (ERK) phosphorylated at the *Thr-Glu-Tyr* sites, c-Jun N-terminal kinases (JNKs) also known as stress-activated protein kinases (SAPKs) phosphorylated at *Thr-Pro-Tyr* motif and the p38 isoforms (α, β, γ and δ), also a type of SAPK, which are phosphorylated at *Thr-Gly-Tyr* (reviewed in Rose et al., 2010). Although there are unique characteristics of each MAPK signalling cascade they are also very much intertwined with each another.

1.2.3.1.1 ERK1/2 MAPK Signalling in Cardiac Hypertrophy

ERK 1 and 2 (ERK1/2) were the first to be identified and cloned in the ERK MAPK signalling cascade in 1991 (Boulton et al., 1991) and are mainly responsive to mitogenic growth factors that stimulate receptor tyrosine kinases (RTKs) and GPCRs (reviewed in Rose et al., 2010). ERK 1 and 2 are nearly identical to each other as they proceed via similar signalling mechanisms and have consequently been referred to as simply ERK1/2.

The ERK MAPK cascade has been demonstrated to be involved in several other pathological disorders such as cancer (Roberts and Der, 2007; Wu, 2007), diabetes (Mokhtari et

al., 2008; Pan et al., 2011) and CVDs (Ruwhof and van der Laarse, 2000; Muslin, 2008) as it has been shown to affect several different cellular processes regulating cell cycle progression, gap junctions, cellular adhesions and cytoskeleton dynamics by activation of more than 100 substrates (Yoon and Seger, 2006). External stimuli which activates RTKs or GPCRs such as platelet-derived growth factor (PDGF) (Reusch et al., 2001; Yamada et al. 2002; Beeser et al., 2005) or angiotensin II (AngII) (Zou et al., 1996; Hong et al., 2004), respectively, activates Ras in the cytosol subsequently recruiting the MAP3K, Raf. Activated Raf phosphorylates the corresponding MAP2K, MEK1/2, which activates the final component of the cascade, ERK1/2. Phosphorylated or activated ERK1/2 activates downstream substrates that have the ability to increase the transcription of prohypertrophic factors (reviewed in Rose et al., 2010; Kolch, 2000) such as GATA-4 (Liang et al., 2001). In addition, protein tyrosine phosphatase Shp2 and increases in intracellular calcium levels ($[Ca^{2+}]_i$) have been demonstrated to upregulate protein kinase C (PKC) activity that is also linked to activation of the Ras/Raf/MEK/ERK1/2 cascade (Hagiwara et al., 2007).

There are several *in vitro* and *in vivo* studies that have demonstrated the role of the Ras/Raf/MEK1/ERK1/2 cascade in cardiac hypertrophy (reviewed in Rose et al., 2010). By compromising expression and activity of the components of the ERK1/2 MAPK cascade, cardiac hypertrophy, ventricular dysfunction and cardiac fibrosis were inhibited. For example, attenuation of hypertrophy was observed by expression of a dominant negative Raf (Harris et al., 2004). Elsewhere, in transgenic animal studies where cardiac Ras was constitutively active, such as in the MLC-2v-H-Ras-V12 (Zheng et al., 2004) and α-MHC-H-Ras-V12 (Mitchell et al., 2006) mouse mutant models, significant LV hypertrophy and cardiac dysfunction were observed. Constitutive activation of MEK1 has similarly been demonstrated to induce cardiac hypertrophy

(Bueno et al., 2000; Ueyama et al., 2000). However attenuation of MEK1 via direct inhibition by a dominant negative expression of MEK1 in cardiomyocytes prevents the hypertrophic program (Ueyama et al., 2000). Attenuation of the ERK1/2 cascade can also be achieved by inhibition of the upstream components of Raf such as *via* prevention of PKC activation (Schonwasser et al., 1998; Pan et al., 2005).

Direct upregulation of ERK1/2 in cultured cardiomyocytes by classical hypertrophic agonists such as AngII, endothelin-1 (ET-1) and phenylephrine (Phe) as well as other growth and stress factors have likewise been observed (reviewed in Rose et al., 2010). In a clinical aspect, upregulation of ERK1/2 activation has been detected in tissues of HF patients (Takeishi et al., 2002) and in another study was correspondingly attenuated after mechanical unloading using a left ventricular assist device (LVAD) (Baba et al., 2003). Interestingly however, in a transgenic model investigating the role of ERK1/2 in pressure-overload-induced cardiac hypertrophy of mutant mice that either lacked ERK1/2 or overexpressed the ERK1/2 inhibiting protein dual-specific phosphatase 6 (DUSP-6), pressure-loaded or neuroendocrine agonist-induced cardiac hypertrophy was not attenuated (Purcell et al., 2007). Similarly, in the case of leptin-induced cardiac hypertrophy, the ERK1/2 inhibitor, PD98509, only partially attenuated leptin-induced cell surface area and protein synthesis (Rajapurohitam et al., 2003). These results may propose that although the ERK1/2 MAPK cascade is upregulated in the pathogenesis of cardiac hypertrophy, it may be secondary to additional pathways that have a more contributive role in the disorder.

1.2.3.1.2 p38 MAPK Signalling in Cardiac Hypertrophy

The p38 MAPK cascade may refer to any of the four isoforms (α, β, γ and δ) and is induced by stress-like stimuli such as UV radiation and oxidative stress (reviewed in Rose et al.,

2010). Although the p38 isoforms share very similar homologies, deletion of the p38 α in mutant mice is lethal while knockout of the other three isoforms produce viable progeny (Mudgett et al., 2000; Tamura et al., 2000). Activation of p38 can also be upregulated by inflammatory cytokines via the gp130 cytokine membrane receptor and is therefore typically implicated in chronic inflammatory diseases (Langdon et al., 2003). The p38 signalling cascade generally involves MEKK1-4, MKK2/3 and ASK1/2 as MAPKKKs which have the potential to phosphorylate the MKKs that include MKK4 and MKK3/6 (reviewed Rose et al., 2010). p38 may be additionally directly activated by factors separate from the MAPK cascade such as via GPCR-agonist-induced TAO1/2/3 activation (Chen et al., 2003; Raman et al., 2007) as well as by the growth factor TGF-β-induced TAK1 (Kimura et al., 2000). Activation of p38 involves phosphorylation or translocation into the nucleus where it upregulates several transcription factors such as *c-fos* (FBJ murine osteosarcoma viral oncogene) and serum response factor (SRF) contributing to the development of hypertrophy (Lee et al., 2008; Ely et al., 2011).

Although the specific role of p38 in the heart has not been fully detailed, several *in vitro* and *in vivo* work have demonstrated its robust role in the hypertrophic program (reviewed in Rose et al., 2010). It appears as though p38 may be upregulated *via* its prototypic MAPK signalling cascade, but similar to the ERK1/2 pathway as previously discussed, additional cross-talk with other associated pathways in hypertrophy exists. In isolated cardiomyocytes, the specific inhibitors of p38, SB203580 (Rajapurohitam et al., 2003) or SB202190 (Kinugawa et al., 2005) in addition to adenoviruses expressing a dominant negative form of p38 (Wang et al., 1998) inhibited agonist-induced cardiac hypertrophy. In leptin-induced cardiac hypertrophy, p38 inhibition by SB203580 exhibited a more marked reduction in molecular indices of hypertrophy

in comparison to the inhibition of ERK1/2, further indicating the significance of the p38 MAPK cascade in cardiac hypertrophy (Rajapurohitam et al., 2003).

In transgenic animal models with a dominant negative form of p38 (Watanabe et al., 2007), cardiac hypertrophy and ventricular dysfunction were similarly attenuated further supporting *in vitro* results that implicated a more robust role of p38 in comparison to ERK1/2 in the hypertrophic program. While inhibition of MKK3 and MKK6 in isolated cardiomyocytes has been shown to attenuate cardiac hypertrophy (Wang et al., 1998), results from *in vivo* studies demonstrate that dominant negative forms of MKK3/6 can result in compensatory upregulation of other pro-hypertrophic pathways (Braz et al., 2003). Conversely however, others have demonstrated that constitutively active cardiac MKK 3 and 6 contributes to the development of interstitial fibrosis and ventricular dysfunction (Liao et al., 2001). The *in vivo* evidence demonstrating the ability to prevent cardiac hypertrophy by inhibiting p38 activation remains non-conclusive and consequently is still an area of active research.

1.2.3.2 Small GTPase Proteins – RhoA/ROCK Pathway

There is evidence of cross-talk between MAPK signalling cascades and non-MAPK pathways such as the small guanine triphosphatase (GTPase) binding protein signalling pathways, which is a unique cascade that eventually leads to the development of cardiac hypertrophy (reviewed in Brown et al., 2006). Small GTPases are small monomeric proteins (20 to 25 kDa) of which there are over 100 members that are further divided into 5 main groups: Ras, Rho, Rab, Arf and Ran (reviewed in Lezoualc'h et al., 2008). In addition to their difference in molecular weights, they are uniquely distinct from heterotrimeric GTPases as they do not interact with GPCRs or with β/γ G-protein subunits. Small GTPases act as molecular switches transitioning from its inactive to active state when it is bound to GDP and GTP, respectively. Once activated,

the small G-protein-GTP bound member acts on its effector molecules eliciting the appropriate cellular responses (reviewed in Brown et al., 2006).

While there is minimal literature on the effects of the Rab, Arf and Ran groups in CVD, there have been reviews which discuss Ras-mediated hypertrophy (reviewed in Sugden and Clerk, 2000; Proud, 2004) and particularly the subfamily members, Rac1 and RhoA, as they were the first of the groups to be linked to cardiac hypertrophy. After the first publication by Ridley and Hall, which described the role of RhoA and Rac1 in cytoskeleton actin dynamics in fibroblasts (Ridley and Hall, 1992), several studies subsequently followed, providing further insight into the cellular processes affected by RhoA. These include, but are not exclusive to, cell proliferation and transformation, smooth muscle contraction, ion channel activity and reactive oxygen species production (reviewed in Brown et al., 2005; Lezoualc'h et al., 2008). For the purpose of this thesis, only RhoA activation and its role in cardiac hypertrophy are reviewed.

Several hypertrophic stimuli, namely GPCR-agonists, result in the activation of RhoA. The most acknowledged effector molecules downstream of RhoA are Rho-associated coiled-coil protein kinase (ROCK) and mammalian diaphanous (mDia). The regulation and function of ROCK and its implication in hypertension and HF has also been reviewed extensively elsewhere (reviewed in Loirand et al., 2006; Noma et al., 2006). In cardiac hypertrophy, activation of RhoA results in the activation of ROCK, which phosphorylates the secondary effector enzyme, lim kinase (LIMK). Activation of LIMK will subsequently phosphorylate the ubiquitous actin depolymerizing agent, cofilin-2, resulting in a decrease in the globular (G) to filamentous (F) actin.

In cultured neonatal ventricular cardiomyocytes, RhoA activation is upregulated by classic agonists of the heterotrimeric G protein Gαq such as AngII, ET-1, phenylephrine and

prostaglandin F2α (reviewed in Brown et al., 2006; Lezoualc'h et al., 2008). Activation of RhoA may also result from stretch stimuli (Torsoni et al., 2005), activation of $G_{12/13}$ (Maruyama et al,. 2002) and by a non-classical agonist, leptin, acting on the leptin receptor (Ob-Rb), which has been studied extensively in our laboratory (Rajapurohitam et al., 2003; Rajapurohitam et al., 2004; Purdham et al., 2008; Zeidan et al., 2006; Zeidan et al., 2008; Moey et al., 2011; Zeidan et al., 2011).

RhoA/ROCK activation appears to be a critical pathway in the hypertrophic program as studies have shown that by inhibiting RhoA/ROCK activation with C3 exoenzyme or Y-27632, inhibitors of RhoA and ROCK, respectively, phenylephrine- (Yanazume et al., 2002), ET-1- (Kuwahara et al., 1999; Porchia et al., 2008; Hunter et al., 2009), AngII- (Yamakawa et al., 2000; Guilluy et al., 2010), LPA- (Hilal-Dandan et al., 2004) and leptin- (Zeidan et al., 2008; Zeidan et al., 2006) induced cardiac hypertrophy was significantly attenuated. Interaction between the RhoA/ROCK and other associated pathways such as the MAPK cascades has also been observed in the hypertrophic phenotype (Zeidan et al., 2006; Zeidan et al., 2008; Moey et al., 2011; Zeidan et al., 2011) as inhibition of RhoA/ROCK correspondingly blunts p38 MAPK activation as evidenced by decreased phosphorylation and nuclear translocation (Zeidan et al., 2008; Moey et al,. 2011).

Results from *in vivo* studies inhibiting RhoA activation are in concordance with the *in vitro* literature such that cardiac hypertrophy and dysfunction are attenuated with RhoA inhibitor treatment. RhoA inactivation *in vivo* has been mainly studied using inhibitors of its downstream effector molecule, ROCK. For example, in hypertensive rats receiving Y-27632, myocardial hypertrophy was reduced and cardiac function was significantly improved (Kobayashi et al., 2002; Satoh et al., 2003). Cross talk of RhoA/ROCK signalling with additional pathways such as

the oxidative stress-induced LOX-1 pathway *in vivo* has also been observed where cardiac hypertrophy, remodelling and dysfunction in hypertensive salt-sensitive rats were inhibited by Y-27632 (Mita et al., 2005). HF induced by isoproternol (ISO) in rats (Wang et al., 2011) has also been shown to be prevented with subcutaneous treatment of fasidil hydrochloride hydrate, which is a ROCK inhibitor. ISO-induced ROCK-mediated upregulation of ERK1/2 and JNK MAPK were additionally reduced in the animals receiving fasidil (Wang et al., 2011). In contrast however, there has been one report which demonstrated no effect on the regression of cardiac hypertrophy following haploinsufficient ROCK suppression in transgenic mice, although there was evidence of attenuated fibrosis (Rikitake et al., 2005). Complete deletion of ROCK resulted in unviable litters suggesting a protective role of RhoA for the survival of fibroblasts during the early developmental stages of the heart. Results from this study allowed the authors to conclude a more contributive role of RhoA/ROCK activation to the fibrotic response than cardiac hypertrophy (Rikitake et al., 2005). Although multiple agonists have been shown to elicit hypertrophic responses through the activation of RhoA/ROCK signalling, the specific mechanism by which RhoA/ROCK is upregulated has not been fully elucidated. Consequently more consideration has been directed towards the positive and negative regulators of small G-proteins.

1.2.3.2.1 Regulators of Small GTPase-Proteins

Activation of RhoA proteins are mediated by regulators that are classified as guanine nucleotide exchange factors (GEFs), guanine nucleotide-dissociation inhibitors (GDIs) and GTPase-activating proteins (GAPs) (reviewed in Lezoualc'h et al., 2008). RhoA proteins have a high affinity for GDP molecules and thus GEFs promote the activation of RhoA by facilitating the exchange of GDP for GTP (reviewed in Rossman et al., 2005). Negative regulators of the

RhoA pathway include GAPs, which promote the hydrolysis of GTP while GDIs sequester inactive RhoA-GDP in the cellular compartment distancing them from the membrane where GDP for GTP exchange occurs (reviewed in Schmidt and Hall, 2002; Rossman et al., 2005; Bos et al., 2007).

There are more than 70 RhoGEFs which have been identified in the human body (reviewed in Rossman et al., 2005). GEFs specifically recognize distinct members of the five small GTPase proteins. The Dbl (DH) and pleckstrin (PH) homology are highly conserved among the GEF members and are known to regulate its activity (reviewed in Rossman et al., 2005). The DH domain is responsible for binding of the RhoGEF to the RhoGTPase as well as catalyzing the exchange of GDP for GTP on the Rho molecule as mutations in the amino acid sequence of this conserved domain negatively affects nucleotide exchange activity (reviewed in Rossman et al., 2005; Bos et al., 2007). A detailed description of the mechanism of nucleotide exchange can be found in the review by Rossman and colleagues (Rossman et al., 2005). Conversely, the PH domain of the RhoGEF serves as an anchor by interacting with the phospholipids comprising the cellular membrane to bring the RhoGEF and RhoGTPase in close proximity with the activated receptor or heterotrimeric G proteins such as $G_{q/11}$ (Lutz et al., 2005) and $G_{12/13}$ (Suzuki et al., 2003; Siehler, 2009). RTKs are another example of receptors that associate with RhoGEFs as they have been suggested to phosphorylate tyrosine residues on RhoGEFs to regulate its nucleotide exchange activity (reviewed in Schiller, 2006). Indeed site mutation at Tyr^{738} of p115RhoGEF, a RhoGEF which has been indicated in high blood pressure, reduced its nucleotide exchange activity and subsequent RhoA activation (Guilluy et al., 2010).

Although the activation of RhoGEFs in cardiac hypertrophy has not been extensively studied, there have been a few reports which have demonstrated the roles of p115RhoGEF

(Porchia et al., 2008; Moey et al., 2011), p63RhoGEF (Porchia et al., 2008), PDZ-RhoGEF (Ying et al., 2009), and A-kinase anchoring protein (AKAP)-Lbc (Appert-Collin A et al., 2007) in the hypertrophic disease process. p115RhoGEF and p63RhoGEF expression has also been demonstrated to be upregulated in hypertensive conditions (Guilluy et al,. 2010) while p63RhoGEF (Souchet et al., 2002) and AKAP-Lbc (Ruehr et al., 2004) are expressed in the human myocardium. Activation of small G proteins may be alternatively controlled by GAPs which promote the hydrolysis of GTP thereby downregulating RhoA activation (reviewed in Brown et al., 2005). While there has been much focus on the regulators of G-protein signalling (RGS) such as RGS2, RGS4 and RGS5 in attenuating cardiac hypertrophy by enhancing GTPase activity of heterotrimeric G proteins (reviewed in Sugden, 2001), there is no study to date which has investigated the involvement of small GTPase GAPs. There was some effort however in our laboratory to assess the role of an endogenous activator of p190RhoGAP, Rnd3 (Wennerberg 2003; Riou et al., 2010), to downregulate leptin-induced RhoA activation (**Chapter 3**).

1.2.3.3 Calcineurin/NFAT3 Pathway

The calcineurin/NFAT3 signalling pathway is considered one of the key processes involved in ventricular remodelling as it results in the induction of hypertrophy (reviewed in Frey and Olson, 2003). Calcineurin (CaN), a phosphatase enzyme which dephosphorylates NFAT3 (nuclear factor of activated T cells) allowing nuclear translocation and upregulation of the transcriptional product MCIP-1 (modulatory calcineurin interacting protein-1), has been shown to be activated by several humoral factors released in HF such as phenylephrine (Molkentin et al., 1998; Taigen et al., 2000; Guo et al., 2011), AngII (Molekintin et al., 1998; Taigen et al., 2000; Fu et al., 2001) and ET-1 (Zhu et al., 2000; Bao et al., 2008). Administration of the CaN inhibitors cyclosporine A and FK506 in mice has been shown to prevent pressure-overload-

induced cardiac hypertrophy (Sussman et al., 1998) and upregulation of CaN in human patients with HF has likewise been observed (Lim et al,. 1999). Indeed there have been additional studies demonstrating the significant role of CaN/NFAT3 signalling in ventricular remodelling and heart failure such that inhibition leads to improved echocardiographic and hemodynamic parameters and, notably, increased survival (Sakata et al., 2000; Wilkins et al., 2004).

Activation of CaN has also been shown to be intricately associated with the upregulation of the sodium hydrogen exchanger isoform 1 (NHE-1), which is the dominant cellular membrane regulator of internal cardiomyocyte pH that expels protons (H^+) for sodium (Na^+) ions (reviewed in Karmazyn et al., 2008b). Activation of NHE-1 by the α_1-adrenergic agonist, phenylephrine, was proposed to upregulate CaN activation by increasing $[Ca^{2+}]_i$ secondary to the increases in intracellular Na^+ ($[Na^+]_i$) (Guo et al., 2011). Observed increases in $[Ca^{2+}]_i$ as a result of NHE-1-induced elevation of $[Na^+]_i$ in the hypertrophic program and HF has been suggested to initiate reverse mode activation of the Na^+Ca^{2+} exchanger (NCX) (reviewed in Karmazyn et al., 2008b; Guo et al., 2011). Increased protein and gene expression of NHE-1 in phenylephrine-induced cardiac hypertrophy was concomitantly observed with upregulation of CaN activity most likely via increases of $[Na^+]_i$ and $[Ca^{2+}]_i$ (Guo et al., 2011). Upregulation of the CaN activation and downstream MCIP-1 gene production in MI-induced rats were also subsequently observed and was inhibited by treatment with the ginseng, further indicating a pivotal role of CaN in the development of cardiac hypertrophy and remodelling *in vitro* and *in vivo* (Guo et al., 2011). Although several studies have already demonstrated that inhibition of CaN has the ability to attenuate cardiac hypertrophy and HF, the potential for reversal of CaN activation for the treatment of HF has only been recently demonstrated (Berry et al., 2011). Reversal of cardiac remodelling is the most challenging obstacle in the treatment of HF however, by demonstrating

the ability to treat cardiac hypertrophy and dysfunction by the reversal of CaN proposes the potential to target the reversal of molecular pathways and specifically CaN upregulated in cardiac remodelling for the management of HF.

1.2.4 Obesity and Cardiovascular Disease

Obesity, which is defined by a body mass index (BMI) > 30 kg/m^2 (Guh et al., 2009) or a waist circumference > 88 cm (Health Canada, 2003) is a prevalent and insidious disease in today's society. Studies have shown a correlation between mortality and obesity (Horwhich et al., 2001) as obesity is typically accompanied by pathological conditions such as type II diabetes, osteoarthritis, cancer, sleep apnea and CVD (Sader et al., 2003; Patel et al., 2008; Guh et al., 2009). While there is a well acknowledged relationship between obesity and CVD (Sowers, 2003), the particular mechanisms linking the two still remain unclear. A particular adipokine encoded by the obesity (*ob*) gene, leptin, however has become the highlight of obesity-associated CVD research (reviewed in Karmazyn et al., 2008a) as studies have suggested its contributive role in the development of obesity-associated CVD.

1.2.4.1 Leptin

1.2.4.1.1 Discovery and Physiological Functions of Leptin

Although it was generally understood through studies involving lesions of the hypothalamus that changes in body weight resulted in appropriate balancing of energy consumption and metabolic processes initiated by signals from the brain, the connection between both signal points had not yet been identified. Parabiotic experiments, which surgically link two animals together to observe the physiological effects of factors within the blood such as hormones, between obese (*ob/ob*), diabetic obese (*db/db*) and normal mice allowed Coleman in 1973 to demonstrate that a particular, though unidentified, circulatory factor was responsible for

regulating body weight (Coleman, 1973). This was shown by the parabiosis of *ob/ob* or normal mice with *db/db* mice where the obese or normal mice became hypoglycemic, lost weight and eventually died of starvation, while the *db/db* mice exhibited no changes and survived. Interestingly however, when *ob/ob* mice were paired with normal mice, the weight loss was not as drastic and the obese mice survived. The results from these parabiotic experiments demonstrated that the *ob/ob* mice were not producing sufficient amounts of this unidentified factor affecting satiety while the *db/db* mice were unresponsive to this satiety factor (Coleman, 1973). It was not until 20 years later through cloning of the *ob* gene where the factor responsible for regulating satiety was identified and termed 'leptin', stemming from the Greek language, *leptos*, meaning 'thin' (Zhang et al., 1994).

1.2.4.1.2 Production and Structure of Leptin

Leptin is a 167 amino-acid polypeptide encoded by the *ob* gene on chromosome 7q31.1, 4 and 6 of humans, rat and mouse, respectively (Zhang et al., 1994). The amino acid sequence of leptin from all three species is highly conserved (approximately 80% homologous), particularly the disulphide bond between Cys^{96} and Cys^{146} at the C-terminal region (Kline et al., 1997; Zhang et al., 1997). In fact, compromises to the amino acid sequence coding the disulphide bond or C-terminal region have been suggested to inhibit the biological activity of leptin by impairing its structural folding and receptor binding capacity (Imagawa et al., 1998). Its structural properties, specifically the long chain helical cytokine fold, are comparable to the type I cytokine receptor family including leukemia inhibitory factor (LIF), ciliary neutrotrophic factor (CNTF), cardiotrophin-1 (CT-1) and also members of the interleukin family, IL-6, -11 and -12 (reviewed in Frühbeck, 2006). Although leptin is predominantly produced by and in proportion to white adipose tissue, leptin has been shown to be found in several other tissues including the heart

(Purdham et al., 2004), liver, skeletal muscle, stomach, hypothalamus, placenta and ovaries. Leptin may be found either bound to the specific leptin receptor (Ob-R) isoform e (see section 1.2.4.2.1), but may also remain unbound and free floating. The latter is often increased in the plasma of obese individuals.

1.2.4.1.3 Role of Leptin in Obesity-associated Cardiovascular Disease

The discovery of this satiety factor especially in the perspective as a regulator of body weight sparked great interest in studying this peptide for the treatment of obesity and potentially against its associated co-morbidities. Early studies targeting leptin for the treatment of obesity however were unsuccessful and were considered to be a result of the 'leptin resistance' phenomenon (Schwartz et al., 1996). Leptin resistance describes the syndrome where obese individuals with chronic hyperleptinemia tend to be non-responsive to treatment with leptin as a result of decreased leptin sensitivity at the hypothalamus. In the obese condition, other peripheral tissues may likewise become resistant or even more sensitive (reviewed in Sweeney, 2010). Indeed a non-resistant, but rather enhanced, response to leptin has been observed in both the vascular system of mice where hyperleptinemia was induced by a high fat diet (Belin de Chantemèle et al., 2011). Although central leptin resistance was present, leptin-mediated stimulation of the heart and vasculature was still active (Belin de Chantemèle et al., 2011).The opposite has also been observed where ventricular cardiomyocytes isolated from rats on a sucrose diet demonstrated impaired leptin signalling in addition to the development of hyperleptinemia and insulin resistance (Hintz et al., 2003).

Leptin has been suggested as a contributory factor to the pathogenesis of obesity-associated CVD by potentiating sympathetic tone and catecholamine release (reviewed in Hall et al., 2010; Kotsis et al., 2010; Rahmouni, 2010), though paradoxical effects of leptin, where it has

been considered as a cardioprotective agent in the 'obesity paradox phenomenon, has also been observed (reviewed in Abel et al., 2008). Enhanced sympathetic activation results in increased blood pressure, while catecholamine release would aggravate the hypertension in addition to directly promoting abnormal biochemical and molecular changes within the cardiac tissue. Hypertension caused by leptin-induced increase in sympathetic tone has indeed been demonstrated (Belin de Chantemèle et al., 2009; Morgan et al., 2008) and may be a considerable target for the treatment of obesity-associated CVDs. Alternatively, leptin-induced increased production and release of catecholamines have been proposed to occur by direct stimulation of the adrenal medullary cells via MAPK activation (Shibuya et al., 2002). In addition to an increased risk of hypertension in obese conditions, leptin may contribute to atherothrombosis (Ciccone et al., 2001; Singh et al., 2010), endothelial dysfunction (Bouloumie et al., 1999; Korda et al., 2008; Cirillo et al., 2010) and the formation of atherosclerotic plaques (Park et al., 2001) possibly by upregulating the activity of inflammatory cells (Schneiderman et al., 2008).

1.2.4.2 Leptin Signalling in the Cardiovascular System

Leptin administration to cultured cardiomyocytes generally elicits a prohypertrophic effect, although there has been one study stating that leptin does not induce hypertrophy (Pineiro et al., 2005). In human subjects, elevated plasma leptin levels of $\geq$ 30 ng/ml has been shown as an indicator of cardiac dysfunction independently of obesity (Schulze et al., 2003). Direct leptin-induced cardiomyocyte hypertrophy has been suggested to occur through several different signalling mechanisms, which mainly include JAK/STAT3 (Janus kinase/signal transducer and activator of transcription), MAPK, RhoA/ROCK (Ras homolog gene family, member A/Rho-associated, coiled-coil containing protein kinase), PI3K/PD3B/cAMP (phosphoinositide 3-kinase/phosphodiesterase 3B) and AMPK (5'-AMP-activated protein kinase) (reviewed in

Karmazyn et al., 2008a). Although these pathways are unique in the molecular events that ensue, it is important to note that they are very much intertwined with each other. For the purpose of this thesis, leptin-induced activation of MAPK and RhoA/ROCK-activation will be discussed, however detailed information on leptin-induced activation of PI3K/PD3B/cAMP and AMPK can be read elsewhere (reviewed in Frühbeck, 2006; Yang and Barouch, 2007; Karmazyn et al,. 2008a). A summary of the activated pathways discussed is illustrated in Figure 1.2.

1.2.4.2.1 Leptin Receptors: Isoforms and Expression

Leptin elicits its physiological effects by binding to its receptors (Lep-R or Ob-R) of which there are five identified spliced variants. These include the short-intracellular domain isoforms (Ob-Ra, Ob-Rc, Ob-Rd and Ob-Re) and its long-intracellular domain isoform (Ob-Rb), which are encoded by the *db* gene (Lee et al,. 1996) located on chromosome 1, 4 and 5 in humans, mice and rats, respectively. Although there has been less focus on the function of the shorter leptin receptor isoforms, Ob-Re, which lack an intracellular domain, has been shown to primarily bind to leptin in plasma for the regulation of free leptin. Ob-Ra typically heterodimerizes with the long-domain isoform receptor, Ob-Rb (reviewed in Frühbeck, 2006), where the latter has also been shown to homodimerize to produce a post-receptor response (Devos et al., 1997), which is linked to a multitude of downstream signalling processes discussed in subsequent sections 1.2.4.2.2 to 1.2.4.2.4. It is the long intracellular domain containing the STAT box of Ob-Rb which predominantly results in leptin signalling via JAK2 recruitment as Ob-Ra, c, d and e lack this essential binding region (Tartaglia et al., 1995; Tartaglia, 1997). The extracellular domains (800 amino acids) of all five isoforms however are highly conserved, which consists of two cytokine (four Cys residues and WSXWS) and four fibronectin III domains (Tartaglia et al., 1995). Within cardiac tissue, all isoforms except Ob-Rc have been

detected, though Ob-Ra and Ob-Rb are dominantly expressed (Purdham et al., 2004). Distribution of the specific receptor isoforms has been shown to be dependent on the specific chamber of the heart where expression of Ob-R was greater in the atria than the ventricles and also varied depending on gender (Purdham et al., 2004).

1.2.4.2.2 Leptin in JAK/STAT3 Signalling

Shortly after the discovery of leptin and its role in obesity, studies into the post-receptor signalling mechanisms subsequently followed which detailed the molecular events attributing to the physiological effects of leptin. It is to no surprise that the first leptin-activated signalling pathway identified was JAK/STAT because of the functional similarity of the dominant leptin receptor isoform, Ob-Rb, to the type I cytokine receptor family (reviewed in Frühbeck, 2006). Among the four JAK members, which include, JAK 1, JAK2, JAK3 and tyrosine kinase 2 (TYK2), the intracellular domain of Ob-Rb contains box motifs which bind predominantly to JAK2 (Tartaglia et al., 1995). Upon binding of leptin to a homodimer formation of Ob-Rb, the activated receptor recruits JAK2 whereby JAK2 undergoes autophosphorylation. Subsequent phosphorylation of Tyr^{985}, Tyr^{1077} and Tyr^{1138} on Ob-Rb by JAK2 occurs (Eyckerman et al., 2000), however it is the specific phosphorylation of Tyr^{985} that promotes the recruitment of STAT3, which dimerizes and translocates to the nucleus, activating transcription of its respective genes (reviewed in Myers, 2004). Phosphorylation of Tyr^{1077} and Tyr^{1138} has been shown to recruit STAT5 and STAT1/3/5, respectively, independently of JAK2-Ob-Rb association (Hekerman et al., 2005) (Figure 1.2).

1.2.4.2.2.1 Leptin-induced Activation of JAK/STAT3 Signalling in Cardiac Hypertrophy and Heart Failure

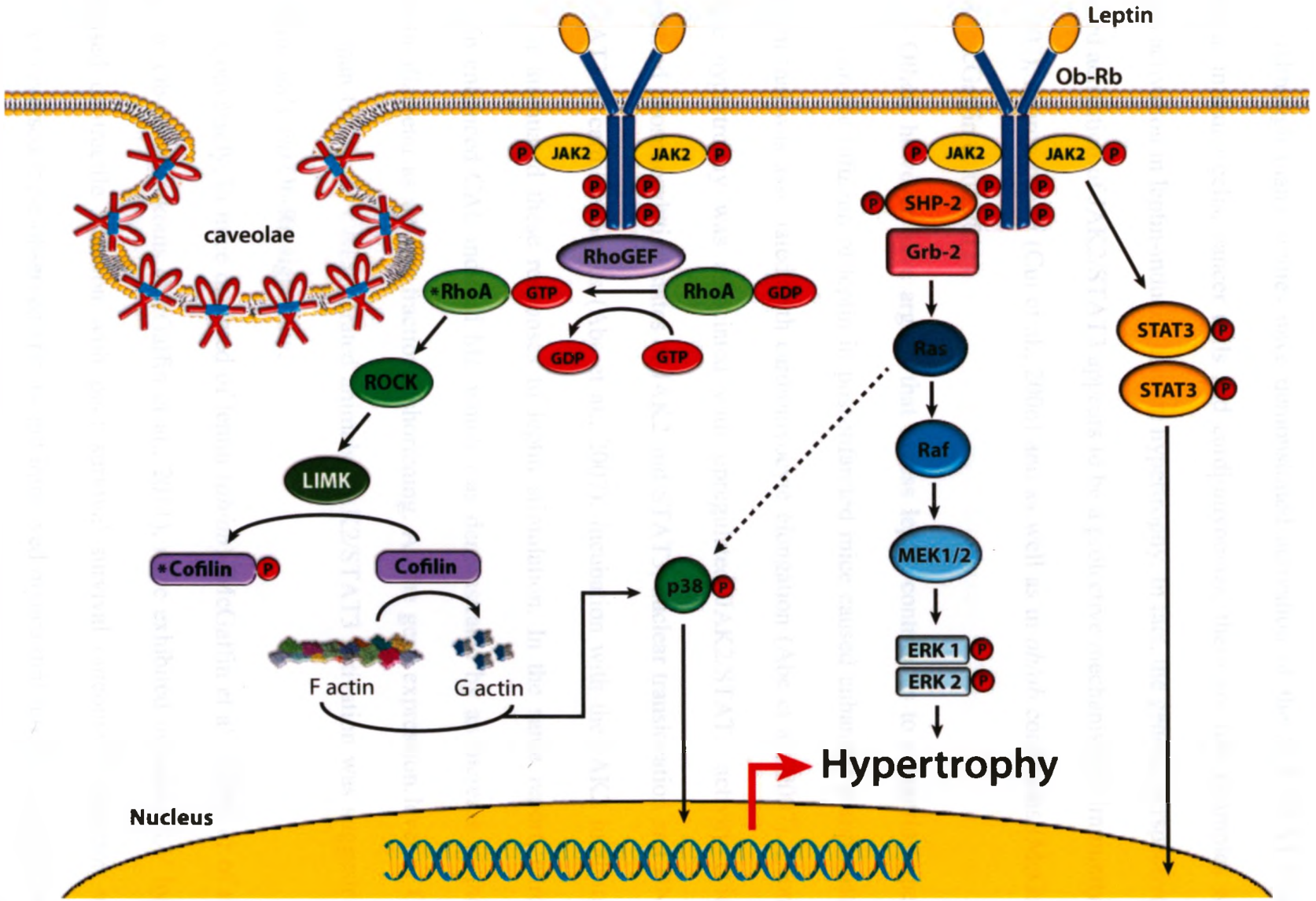


Figure 1.2. Leptin-induced activation of signalling pathways in cardiac hypertrophy. Leptin binds to the leptin-receptor (Ob-Rb) and may activate several signalling cascades though which namely include RhoA/ROCK, p38 and ERK1/2 MAPK as well as JAK/STAT3. Leptin-induced signalling is mainly due to the phosphorylation of the receptor by JAK2. Activaton of these pathways leads to the uregulation of several transcription factors leading to hypertrophy. See text for more details.

Although many studies have demonstrated activation of the JAK/STAT3 pathway by leptin in immune cells, cancer cells and cardiomyocytes, there are few examples which have shown activation in leptin-induced cardiac hypertrophy. In fact, the general perspective of leptin-induced activation of JAK2/STAT3 appears to be a protective mechanism in immunity (Gruen et al., 2007), neuron health (Cui et al., 2006) and as well as in *ob/ob* conditions (McGaffin et al., 2008; McGaffin et al., 2011).

Others however have argued that excess leptin contributes to eccentric hypertrophy *in vivo* as chronic infusion of leptin in post-infarcted mice caused enhanced compensated cardiac function that was associated with cardiomyocyte elongation (Abe et al., 2007). Leptin-induced cardiac hypertrophy was associated with upregulated JAK2/STAT3 activity evidenced by augmented phosphorylation states of JAK2 and STAT3, nuclear translocation and DNA binding of STAT3 in cardiomyocytes (Abe et al., 2007). Incubation with the JAK2 inhibitor, AG490, however attenuated these responses to leptin stimulation. In the same report, chronic leptin infusion enhanced CAL-induced MI, which was demonstrated by an increase in the LV end-diastolic diameter as well as fractional shortening. As the gene expression level of Ob-Rb was higher than Ob-Ra in CAL-operated animals, JAK2/STAT3 activation was suggested to occur predominantly *via* Ob-Rb signalling.

Conversely, in mice depleted of leptin (*ob/ob*) (McGaffin et al., 2008) or of response to leptin in Ob-Rb knockouts (McGaffin et al., 2011), mice exhibited increased LV hypertrophy, decreased contractile function with poor survival survival outcomes. Repletion with leptin however reversed these observed effects and improved myocardial function therefore suggesting leptin as an anti-hypertrophic agent. It is important to note however that the *ob/ob* model

includes a plethora of metabolic abnormalities that is compensated by systemic alterations resulting in cardiac hypertrophy and dysfunction. The *ob/ob* model has been regarded as a non-ideal model for studying the direct effects of leptin as the anti-hypertrophic effects of leptin observed after leptin infusion are considered to correct the physiological compensations in *ob/ob* conditions that occurred as a result of a chronic lack of leptin. These statements however have been disputed by a study which demonstrated that leptin exerted a direct antihypertrophic effect in *ob/ob* and *db/db* mice as weight loss induced by caloric restriction did not decrease wall thickness or cardiomyocyte hypertrophy, while leptin infusion completely reversed ventricular hypertrophy (Barouch et al., 2003). Consequently there is still much to understand about the effects of leptin *in vivo*.

1.2.4.2.3 Leptin and MAPK Signalling

Phosphorylation of Tyr^{985} on Ob-Rb has additionally been shown to be associated with activation of MAPK signalling pathways that are upregulated in cardiac hypertrophy (reviewed in Myers, 2004). Leptin-induced MAPK activation, specifically ERK1/2 and p38, has been demonstrated to occur via JAK2-dependent or independent phosphorylation of Tyr^{985} (Bjørbaek et al., 2001). In JAK2 phosphorylation of Tyr^{985}, the *src* homology 2 (SH2) domain of the *src* homology 2-containing tyrosine phosphatase (SHP-2) is recruited and phosphorylated by JAK2. Activation of SHP-2 results in the association of growth factor receptor binding-2 (Grb-2), which leads to the subsequent activation of ERK1/2 via the Ras/Raf/MEK1/2 cascade (reviewed in Frühbeck, 2006) (Figure 1.2). Leptin-induced MAPK activation independent of receptor phosphorylation occurs via direct interaction of JAK2 with the SHP-2 domain (Bjørbaek et al., 2001). Expression of *c-fos* and *egr-1* transcriptional factors, which contributes to cell proliferation and differentiation, is subsequently upregulated after ERK1/2 activation.

Although leptin-induced p38 activation has been previously demonstrated (Madani et al., 2006; Zeidan et al., 2006; Zeidan et al., 2008), the exact mechanism of activation still remains unidentified, however this may involve upstream activation of RhoA (Zeidan et al., 2006; Zeidan et al., 2008). Activation of p38 is observed by increased phosphorylation as well as translocation of total p38 forms into the nucleus to induce the expression of transcriptional factors contributing to cardiac hypertrophy. Another downstream target of p38 implicated in leptin-induced cardiac hypertrophy may be NFκB (nuclear factor kappa B) as it is essential in the induction of pro-inflammatory cytokines TNFα (tumour necrosis factor α) and IL-1β (interleukin 1-beta), which have been shown to be increased in CVD (Napoleone et al., 2007).

1.2.4.2.3.1 Leptin-induced Activation of MAPK Signalling in Cardiac Hypertrophy

In cultured cardiomyocytes, leptin-induced activation of the p38 and ERK1/2 MAPK signalling cascade has been shown to elicit a pro-hypertrophic effect (Rajapurohitam et al., 2003; Madani et al., 2006; Zeidan et al., 2006; Zeidan et al., 2008; Schram et al., 2010; Moey et al., 2011). Although there was significant activation of both p38 and ERK1/2 in leptin-treated cells, p38 upregulation in cardiac hypertrophy has been considered to be more robust than ERK1/2 activation as incubation with the ERK1/2 inhibitor PD98059 did not inhibit leptin-induced cardiac hypertrophy measured by cell surface area, αSA and myosin light chain-2 (MLC-2) fetal gene expression and protein synthesis (Rajapurohitam et al., 2003), while the p38 inhibitor completely prevented leptin-induced effects. A similar observation was also demonstrated in leptin-treated hypertrophied cardiomyocytes where only total p38 nuclear translocation was observed (Zeidan et al., 2008; Moey et al., 2011).

Activation of MAPK pathways in leptin-induced cardiac hypertrophy has also been demonstrated in human pediatric ventricular myocytes (Madani et al., 2006) and in HL-1 murine cardiomyocytes (Schram et al., 2010). Both studies focused on the contribution of leptin to ECM remodelling in the pathogenesis of HF via JAK (Madani et al., 2006) and MAPK pathways (Schram et al., 2010; Madani et al 2006). Increased cell surface area, protein synthesis and fetal gene expression were observed with marked fibrosis and collagen deposition in leptin-treated cells. Incubation with inhibitors of the JAK, p38 and ERK1/2 pathways, AG490, SB203580 and PD96059, respectively, however significantly attenuated leptin-induced cardiac hypertrophy and ECM remodelling (Madani et al., 2006; Schram et al., 2010), indicating a pivotal role of MAPK in leptin-induced cardiac remodelling. Activation of the MAPK pathways however appears to be dependent on the upregulation of the classic small G-protein RhoA/ROCK signalling pathway as inhibitors of the RhoA pathway attenuated p38 activation (Zeidan et al., 2006; Zeidan et al., 2008).

1.2.4.2.4 Leptin and RhoA/ROCK Signalling

Leptin-induced activation of the RhoA/ROCK pathway has been a relatively recent finding as leptin is not a member of the GPCR group of classical RhoA/ROCK upregulators that include AngII, ET-1 and phenylephrine (reviewed in Brown et al., 2006). Activation of RhoA/ROCK by leptin may however be comparable to cytokine upregulation of RhoA such as by IL-6 (Campos et al., 2009). Upregulation of RhoA by leptin has been observed in several different tissues of the body such as chondrocytes (Liang et al., 2011), endothelial cells (De Rosa S et al., 2009), liver (Jiang et al., 2008), kidney (Attoub et al., 2000), vascular smooth muscle (Zeidan et al., 2007) and cardiomyocytes (Zeidan et al., 2006; Zeidan et al. 2008; Moey et al., 2011; Zeidan et al., 2011) and is associated with the pathogenesis of a variety of disorders.

1.2.4.2.4.1 Leptin-induced Activation of RhoA/ROCK Signalling in Cardiac Hypertrophy and Heart Failure

Although direct administration of leptin in isolated cardiomyocytes has been shown to induce hypertrophy via MAPK pathways, activation of the p38 and ERK1/2 cascade appears to be dependent on the upregulation of the RhoA/ROCK pathway (Zeidan et al., 2006; Zeidan et al., 2008). Zeidan and colleagues showed that leptin-induced increase in cell surface area and protein synthesis was associated with a robust activation of RhoA and its downstream components, particularly cofilin-2 phosphorylation and a decrease in the G/F actin ratio (Zeidan et al. 2006; Zeidan et al., 2008). Incubation with the RhoA inhibitor C3 exoenzyme, ROCK inhibitor Y-27632 or actin depolymerizing agent latrunclin B attenuated leptin-induced cardiac hypertrophy, RhoA signalling and additionally p38 and ERK1/2 MAPK activation (Zeidan et al., 2008). The signalling events in RhoA activation was further investigated in a subsequent study by the same authors where the importance of caveolae, which are invaginations of the cellular membrane (Das and Das, 2011), was identified in post-receptor Ob-Rb RhoA signalling (Zeidan et al., 2008). Furthermore, the dependency of p38 MAPK upregulation on caveolae formation and RhoA activation was additionally demonstrated as p38 nuclear translocation was inhibited by the cholesterol chelator agent methyl-beta-cyclodextrin (MβCD), C3 exoenzyme and Y-27632 compounds (Zeidan et al., 2008). Recently, further downstream events as well as association of RhoA/ROCK signalling with the PI3K/mTOR/p70(S6K) (phosphotidylinositol 3-kinase/mammalian target of rapamycin/serine/threonine p70 S6 kinase) cascade and transcriptional activation by GATA4 in cardiac hypertrophy have been additionally identified (Zeidan et al., 2011). Crosstalk between RhoA/ROCK and the PI3K/PKB (protein kinase B) axis

has been observed in leptin-induced hypertrophy of vascular smooth muscle cells of the rat portal vein (Zeidan et al., 2007).

The specific mechanism of leptin-induced activation of RhoA/ROCK in cardiac hypertrophy however at the time had not yet been fully elucidated. Recently published work from our laboratory has suggested the role of RhoGEFs in this signalling cascade and our findings have been summarized in detail in **Chapter 2**.

1.2.5 Pharmacotherapy for the Prevention and Treatment of Heart Failure

The goals of pharmacotherapy for the prevention and treatment of HF are primarily to improve survival outcomes, alleviate associated symptoms and reduce incidences of hospitalization (reviewed in Tamargo and López, 2011). The difficulty however with treating HF is to reverse the characteristic accompanying feature of cardiac remodelling where detrimental cellular changes have occurred. By understanding the molecular mechanisms that are involved in the cardiac remodelling process, new efficient drugs which target specific signalling pathways are being produced in an attempt to completely abrogate and reverse the disease.

1.2.5.1 Pharmacological Agents in the Treatment of Heart Failure

The appropriate line of treatment is determined by the HF symptoms manifested at the time of clinical presentation. Although a single drug may be used, these are often prescribed in combination with additional pharmacological agents. The conventional drug regimen for the treatment of HF may include one or more of the following: inhibitors of the RAAS (Ma et al., 2010) which include angiotensin converting enzyme (ACE) inhibitors, AngII type 1 receptor blockers (ARBs) and aldosterone blockers, β-adrenergic blockers, calcium (Ca^{2+}) channel blockers (CCBs), loop diuretics and positive ionotropic agents (reviewed in Hellawel and Margulies, 2010; Tamargo and López, 2011).

The RAAS is an important physiological system that regulates blood pressure by sensing pressure changes, receiving information from the central nervous system and relaying information to the rest of the body to promote or inhibit the release of factors regulating blood pressure (Skeggs et al., 1976). ACE inhibitors prevent the conversion of angiotensin I to angiotensin II (Erdös, 1976), of which the latter is a robust humoral factor released during states of cardiac stress that contributes to the pathogenesis of cardiac hypertrophy and HF (Wollert and Drexler, 1999; Blaufarb and Sonnenblick, 1996). ACE inhibitors are usually prescribed to reduce afterload or an increase in systemic arterial pressure (Brown and Vaughn, 1998). Captopril, enalapril and lisonopril are a few examples of ACE inhibitors that have been shown to elicit antihypertrophic and antiremodelling effects (Tamura et al., 2000; Juan et al., 2003; Gagnon et al., 2004; Brower et al., 2007) and have proven efficacious in several clinical trials (Cohn et al., 1991; Konstam et al., 1992; Yusuf et al., 1992). Generally, ACE inhibitors produce optimal outcomes for patients as trials have shown a reduction in all-cause mortality, reduction in hospitalizations as well as improvement in NYHA functional class (The CONSENSUS Trial Study Group, 1987). Appropriate dosages of ACE inhibitors must be taken into careful consideration as higher doses can easily result in hypotension and worsening cardiac dysfunction (Phillips et al., 2007). Compliance may also become an issue because of the ACE inhibitor-induced cough as a result of excessive build up of bradykinin (Dicpinigaitis, 2006), a product secondary to angiotensin II from angiotensin I. Consequently, ARBs such as losartan and cadesartan, which act essentially via the same inhibitory pathway of ACE-I by blocking angiotesin II binding, may be preferred over ACE-I (O'Meara et al., 2004; Young et al., 2004; Konstam et al., 2009). While ARBs may be the preferred prescription, there is conflictive discussion as to which of the two RAAS inhibitors is more effective. Aldosterone blockers which

include spirinolactone and epelernone are the third common drug prescribed in the treatment of HF that acts in close proximity with the RAAS (Guglin et al., 2011). There are a multitude of published articles which have shown direct inhibition of agonist-induced hypertrophy where aldosterone antagonism attenuated cardiac hypertrophy (Yoshida et al., 2010; Veliotes et al., 2010). In clinical trials assessing the efficiency of aldosterone antagonism in the treatment of HF, significant decrease in all cause mortalities and hospitalization incidences were observed in patients receiving aldosterone blocker treatment (Pitt et al., 2003; Chan et al., 2007).

Treatment of HF may include sympathetic blockade such as β-adrenergic receptor blockers, which include practolol, atenolol, metopolol, carvedilol, bisoprolol as well as others (Ambrosioni et al., 2001; Foody et al., 2002). The use of β-blockers in HF almost appears contradictory however, in HF patients taking β-blockers there was a marked improvement in cardiac function as well as a decrease in the chamber enlargement (Waagstein et al., 1975). The success of β-blockers were measured based on all-cause mortality and rate of hospitalization which were in fact significantly reduced in large randomized clinical trials (Hjalmarson et al., 2000; Packer et al., 2002). The remaining drugs include diuretics such as furosemide, torsemide and bumetanide (Jentzer et al., 2010) and the calcium channel receptor blockers (Opie et al., 1995), which are used for the treatment of HF by reducing preload and afterload, respectively. While diuretics and calcium channel blockers have received less attention in clinical trials, results from trials investigating the latter are less convincing for the treatment of HF (Packer et al., 1987). Consequently there is an increased need to discover new pharmacotherapy that would prove efficacious in the treatment of HF.

1.2.5.2 Natural Products for the Treatment of Heart Failure

There has been a reported 15 million number of complementary and alternative medicine (CAM) users in the United States (Eisenberg et al., 1998), which includes herbs, relaxation techniques, chiropractic, yoga, massage, special diet, megavitamins, homeopathy and tai chi (Eisenberg et al., 1993). Among the list of CAM therapeutics, herbal supplements rank at the top of the list and are particularly common in patients with already prescribed conventional medications and in individuals who are older in age (Eisenberg 1998; Wood et al., 2003). The rise in CAM use has been seen in patients suffering from CVDs, where in fact in one example, 64% of the patients from the Improving Cardiovascular Outcomes in Nova Scotia (ICONS) group reported the use of CAM. Fourty percent of these patients were taking herbal supplements and referred to their CVD as the main reason for the use of CAM as they believed CAM would "potentially improve their condition", that CAM had "proven beneficial" or that they were "dissatisfied with their current treatment regimen" (Wood et al., 2003). Among the list of herbal supplements that has been increasing in usage is NA ginseng.

1.3 North American Ginseng (*Panax quinquefolius*)

1.3.1 History of Ginseng and its First Application in Cardiovascular Disease

Ginseng, considered a prized possession in Asian communities and often prescribed in TCM has a rich history dating more than 2000 years. The medicinal properties of ginseng, known by the Chinese as '*rén shēn*' (meaning 'essence of man', as well as other colloquial terms such as 'spirit of the earth', 'fat of the sea' and 'the remedy that dispenses immortality'), were first recorded in an ancient Chinese book of agriculture and medicinal plant collections of TCM practices between 300 B.C. and 200 A.D. This book describes ginseng as having the ability to "repair the five visceras, harmonize energies, strengthen the soul, allay fears, remove toxic

substances, brighten the eyes, open the heart and improve thought" such that "consistent use will invigorate the body and prolong life." (Chu and Zhang, 2009) This proposed all-curative property of ginseng, also referred to by its genus name *Panax*, stemming from *panacea* derived from the Greek goddess of healing (Greek, Panakeia), was considered to be due to the close resemblance of the ginseng root, considered the most valuable portion of the ginseng plant, to the shape of a human being. Traditionally, it was believed that healing of a diseased area of the body or a specific organ could be accomplished by ingesting the portion of the root reflecting that diseased area (Goldstein, 1975; Hu, 1976; Chu and Zhang, 2009), e.g. the middle portion would be prescribed for cardiopulmonary diseases. Sprouting from the top of the root are the stems, leaves (Ligor et al., 2005; Liu et al., 2010) and berries (Attele et al., 2002; Dey et al., 2003), which have also been used for therapeutic purposes.

Ginseng belongs to the *Araliaceae* family and has several different species, namely *Panax ginseng, Panax japonicus,* and *Panax quinquefolius*, among others, which differ in both physical appearance and chemical constitution (Chan et al., 2000). There have been several claims of additional ginseng species such as Siberian ginseng; however, authentic ginseng species are characterized by the presence of ginsenosides, which in fact are absent in Siberian ginseng (Mar and Bent, 1999). The two major species of ginseng that have been well documented in history and used as therapeutic agents are *P. ginseng C.A. Meyer* (named after the Russian botanist C.A. Meyer, who identified and separated the different ginseng species in 1842), which is found naturally in hardwood forests of large shady trees in dense and nutrient-limited soil in China and Korea, and *P. quinquefolius*, which is located in similar climates and environments in North America, particularly southern Canada (Ontario and Quebec) and the US (Wisconsin, Minnesota, Oklahoma and Georgia).

The use of ginseng in traditional Chinese medicine is based on the belief that there exists an appropriate balance of the *yin* and *yang* (loosely defined as the interrelationship and interdependence of seemingly opposite forces) of the individual human being (Goldstein, 1975; Hu, 1976). This was, and still is, an unfamiliar concept in the West, rendering it particularly difficult to comprehend and practice. The popularity of ginseng as a medicinal drug, not to mention a high trade commodity item, after its introduction into New France in North America in the early 1700s, however, was quite remarkable (Carlson, 1986). By late 1700 there were several accounts as well as refuted claims of the therapeutic effects of ginseng, although these were generally based on individual cases or the effects experienced by the researcher or physician themselves (Appleby, 1983).

A PubMed database search of ginseng- or ginsenoside-related research articles (Figure 1.3) shows minimal research interest as depicted by the low number of publications in the late 1950s, with a significant upward trend nearing the end of the 1990s and a particularly sharp rise during the past 10 years. The first accessible research publication in English demonstrating quantitative data of the effects of ginseng on the cardiovascular system originated from Wood and colleagues in 1964, who assessed the effect of alcoholic *P. ginseng* extract (10–20 mg/kg) on blood pressure, ventricular contraction, blood flow and heart rate in dogs (Wood et al., 1964). They showed that ginseng exerts a biphasic influence on blood pressure with an initial hypotensive effect followed by a slightly prolonged hypertensive response, which occurred in the absence of significant cardiac responses. Although these authors suggested a lack of cardiac influence of ginseng *in vivo*, *in vitro* studies using guinea pig isolated left atrial myocytes revealed a direct effect of *P. ginseng* as manifested by a significant reduction of both the Bowditch effect (positive inotropic response with increasing heart rates, 'positive staircase') and

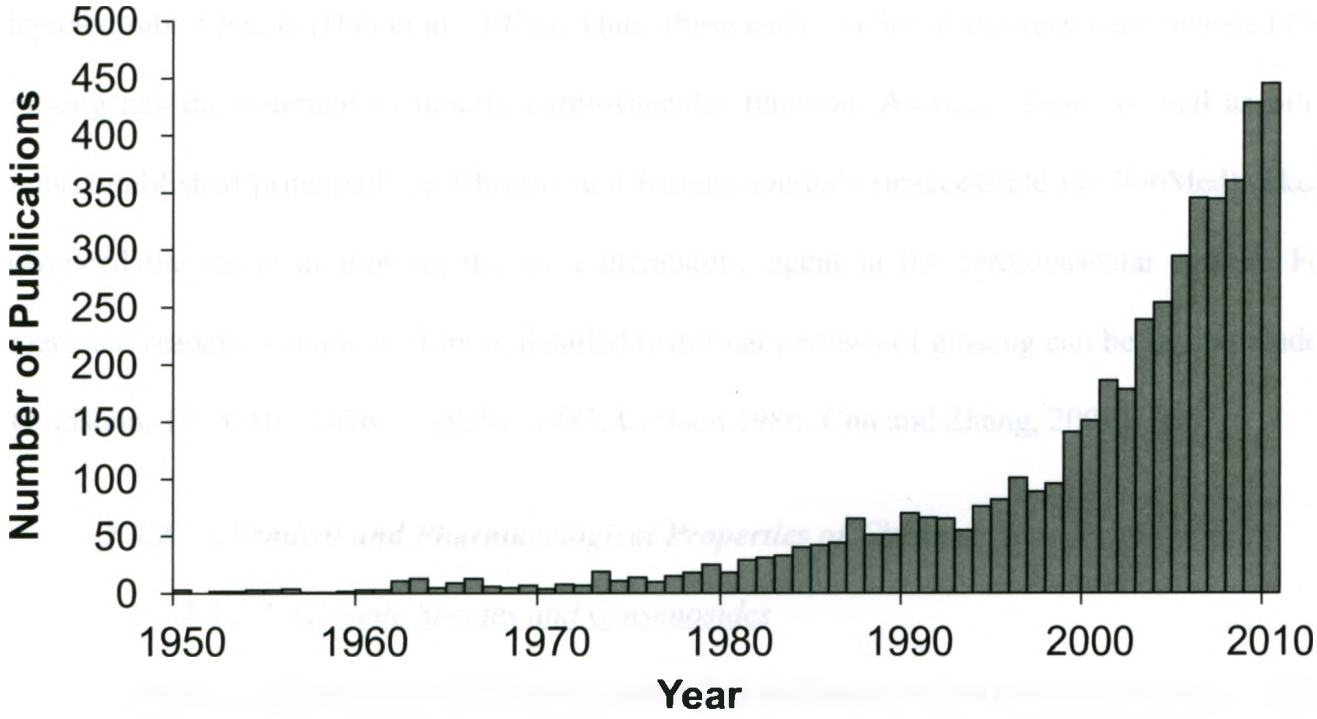


Figure 1.3 Number of ginseng publications from 1950 to 2010. The yearly number of publications concerning ginseng-related research since 1950, when the first ginseng-related publication was identification. Data were obtained from the PubMed database using the keywords 'ginseng' or 'ginsenoside' in the search strategy. Note particularly the large surge in publications since the year 2010.

Note: this figure was published in **Karmazyn M, Moey M** and Gan XT. Therapeutic potential of ginseng in the management of cardiovascular diseases. Drugs 2011;71:1989-2008.

Woodworth effect ('negative staircase'), and prevention of calcium reuptake in calcium-depleted rabbit hearts (Hah et al., 1978). Thus, these early studies at the very least revealed that ginseng has the potential to modify cardiovascular function. As such, these, as well as other reports published principally in Chinese and Korean journals (inaccessible via PubMed), likely advanced the cause of ginseng use as a therapeutic agent in the cardiovascular system. For interested readers, a number of more detailed historical reviews of ginseng can be recommended (Goldstein, 1975; Hu, 1976; Appleby, 1983; Carlson 1986; Chu and Zhang, 2009).

1.3.2 Chemical and Pharmacological Properties of Ginseng

1.3.2.1 Ginseng Species and Ginsenosides

Ginseng exists as several different species that are based on their country of origin which namely includes *Panax quinquefolius* (North American ginseng), *Panax notoginseng* (Chinese ginseng), *Panax ginseng* (Korean or Red ginseng) and *Panax japonicus* (Japanese ginseng). In addition to the species' variation in shape and size, their chemical profiles are uniquely distinct resulting in diverse physiological responses (Chan et al., 2000). For example, as previously mentioned, the goal of TCM is to achieve a balance between the *yin* and *yang* or the 'cold' and 'hot' Qi of the body. TCM practitioners believe that Asian ginseng provides more *yin* to the body which is manifested by decreasing blood pressure. These anti-hypertensive effects of Asian ginseng have now been considered due to NO-mediated vasodilation (Jeon et al., 2000; Sung et al., 2000). In comparison, North American ginseng has not been shown to elicit any effects on blood pressure (Stavro et al., 2005; Stavro et al., 2006). Use of high pressure liquid chromatography (HPLC) technique has allowed scientists to reveal the bioactive constituents considered responsible for eliciting these physiological responses to ginseng known as 'ginsenosides', a type of triterpene saponin (reviewed in Attele et al., 1999).

Ginsenoside nomenclature is based on a simple formula, Rx, where 'R' represents 'root' and 'x' indicates the polarity of the ginsenoside based on HPLC in alphabetic order (Shibata et al., 1963). For example, Rg is more polar (hydrophyllic) than Ra which is the least polar and therefore more hydrophobic of all the ginsenosides. Consequently the degree of polarity of ginsenosides would not only discernibly elicit varied responses in different biological systems, but would also be an important consideration in optimizing ginsenoside extraction techniques for purification processes in the use of basic or clinical studies as discussed below in section 1.3.2.2.

There are currently more than 100 different identified ginsenosides (Jia and Zhao, 2009) that have been further categorized into classes which catalogue each ginsenoside based on the type, position and number of sugar moieties at the C-3 and C-20 positions of the dammarane (tetracyclic triterpine) skeleton (reviewed in Attele et al., 1999; Jia and Zhao, 2009; Jian et al., 2009; Lü et al., 2009; Qi et al., 2011). The major two groups include the 20-(S) protopanaxadiol ginsenosides (PPD) (Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, and Rs1) and the 20-(S) protopanaxatriol ginsenosides (PPT) (Re, Rf, Rg1, Rg2, and Rh1) which differ from PPDs because of an additional carboxyl group at the C-6 position (reviewed in Leung and Wong, 2010) (Figure 1.4) Additional groups also consist of the ocotillol group (pseudoginsenoside F11, majoniside R1 and R2) (Namba et al., 1986) and the oleanane group (Ro or chikusetsusaponin V) (Sanada et al., 1974). As indicated earlier, ginseng species vary in chemical constitution which is predominantly defined by the different ginsenosides present. NA ginseng has been regarded as a higher grade ginseng in comparison to its Asian counterparts as a higher amount of ginsenosides, particularly Rb1, Rb3, Rc, Rd, Re and Rg1, has been reported (Assinewe et al., 2003). Ginsenoside composition in the different ginseng species are provided in Table 1.

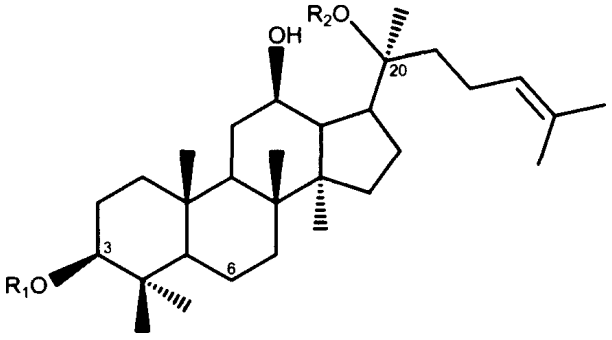
Although many of the salutary effects of ginseng have been attributed to ginsenosides, the ginseng phytochemical content actually contains less than 10% ginsenoside while the remaining 90% is composed of polysaccharides, amino acids, fatty acids and vitamins. Indeed non-ginsenoside components such as trilinolein (Chen et al., 2005; Chen et al., 2010), a fatty acid extract from *P. ginseng* extracted from *P. quinquefolius* have proven to elicit beneficial health effects. Similarly, although much attention has been focused on the whole ginseng root or individual ginsenosides, compounds extracted from other portions of the herb such as the leaves (Ligor et al., 2005; Liu et al., 2010) and berries (Attele et al., 2002; Dey et al., 2003) have additionally been demonstrated to exhibit promising therapeutic effects, if not more pronounced.

1.3.2.2 Biotransformation and Bioavailability of Ginsenosides

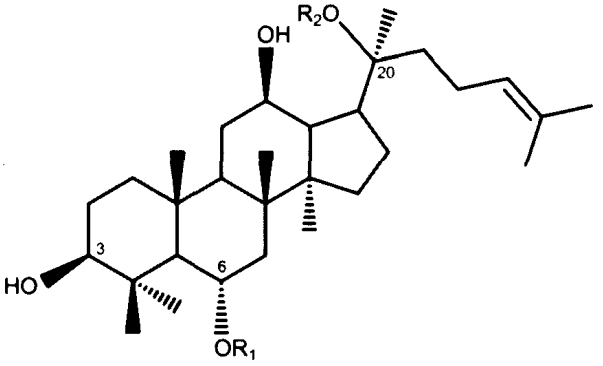
Pharmacokinetics describes the time-related absorption, distribution, metabolism and excretion (ADME) of compounds in the body, which defines the capacity of the drug to elicit its pharmacological actions. The ADME process of a certain compound can be affected by several extraneous and non-extraneous factors such as variation in drug dosage or site of administration and individual genetic variability, respectively. The gastrointestinal (GI) tract and liver play significant roles in the biotransformation and bioavailability of ginsenosides after oral consumption of ginseng (Tawab et al., 2003; Ling et al., 2006; Lee et al., 2008; Leung and Wong, 2010). In fact although ginsenosides have been regarded as the responsible constituents for eliciting its pharmacological effects, several *in vitro* as well as *in vivo* studies in both animal and human subjects have revealed that these effects are in fact due to the metabolites of ginsenosides (Bae et al., 2004; Liu et al., 2006). After oral consumption of ginseng, usually prepared in the form of a tea, ginsenosides enter into the human GI tract where they are broken down by either the acidic environment or the microflora into its respective metabolites (reviewed

in Leung and Wong, 2010). The main intestinal bacteria that have been identified in the metabolic breakdown of ginsenosides include species of *Bifidobacterium* (Int-57, K-103, K-506, and SJ32), *Aspergillus* (*A. niger* and *A. usamii*), *Bacteroides* JY6, *Lactobacillus, Fusobacterium* K-60, *Eubacterium A-44, Streptococcus,* and *Prevotella oris* (reviewed in Leung and Wong, 2010) Generally, the production of metabolites is a result of deglycosylation of ginsenosides at the C-3 or C-20 positions by both acid and human intestinal microfloral however may also include other alterations such as epimerization of the C-20 sugar moiety (Ling et al., 2006). Ginsenosides may follow a number of different metabolic pathways, which have been determined from AMDE *in vivo* and *in vitro* animal and human studies and have been summarized elsewhere (reviewed in Ling et al., 2006).

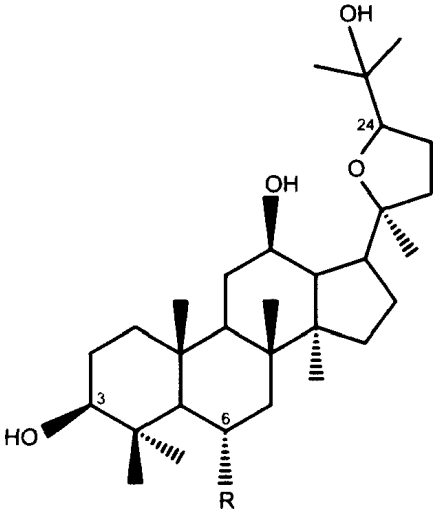
While the mechanisms of absorption and transportation of ginsenosides and metabolites have yet to be detailed, studies have revealed poor absorption of ginsenosides most likely due to its large bulky structure (Ling et al., 2006). Transportation of ginsenosides may involve energy-dependent mechanisms and therefore absorption of their respective metabolites is favoured. Consequently, the bioavailability of ginsenosides is extremely low as previous studies measuring ginsenoside content in rat plasma as well as urine were less than 5% recovery of the starting dose (Odani et al., 1983a; Odani et al., 1983b; Tanizawa et al., 1993; Xu et al., 2003) and in some cases undetectable. For example, in the first pharmacokinetic studies of single ginsenosides in the rat by Odani and colleagues, oral bioavailabilities of Rb1 and Rg1 were only 0.1% and 1.9%, respectively (Odani et al., 1983a, Odani et al., 1983b). In contrast however, the absorption of primary ginsenoside metabolites such as C-K and Rh1 is considered to be much higher than their original structures as a greater percentage of these metabolites were detected in both rat and human serum and urine (Tawab et al., 2003). In addition to poor absorption of ginsenosides, the



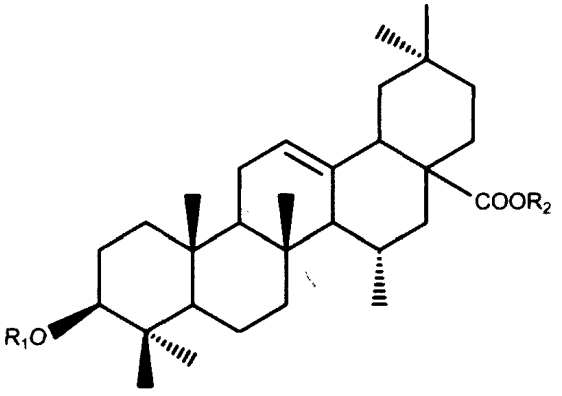
Protopanaxadiol (PPD)



Protopanaxatriol (PPT)



Octolilol-type Saponin



Oleanic Acid

Figure 1.4 Chemical structures of ginsenosides. Chemical structures were drawn with eMolecules software edited appropriately using Adobe Illustrator. The two main types of ginsenosides include the protopanaxadiols (PPDs) and the protopanaxatriols (PPTs). The main difference between PPDs and PPTs is the presence of carboxyl groups at C-6. Other ginsenoside-like compounds include the octolilol-type saponin and oleanic acid. The octoliol-type saponins include pseudoginsenoside F11 and majoniside R1 that are unique based on the stereisomeric orientation at C-24. R_1: radical group 1, R_2: radical group 2; #s: carbon numbering.

Table 1.1. Primary differences in ginsenoside composition between North American and Asian ginseng

Ginsenosides	American ginseng (*Panax quinquefolius*)	Asian ginseng (*Panax ginseng*)
Total ginsenosides (g/kg)	40 – 60	20 – 40
Major ginsenosides	Rb1, Re, Rd, Rb2, Rc, Rg1, Rb3	Rb1, Rg1, Rc, Rb2, Rd, Re, Rf, Rb3
PPD Group	Rb1, Rb2, Rb3, Rc, Rd	Rb1, Rb2, Rb3, Rc, Rd
PPT Group	Re, Rg1, F_{11}	Re, Rg1, Rg2, Rf
Pseudoginsenosides F_{11}	Present	Absent

PPD = protopanaxadiol; **PPT** = protopanaxatriol

Note: Table has been published in Karmazyn M, Moey M and **Gan XT**. Therapeutic potential of ginseng in the management of cardiovascular disorders. Drugs 2011;71:1989-2008

half-lives of the major ginsenosides such as Rg1, Rb1, Rh1 and Rh2 are relatively short (<24 hours) (Karikura et al., 1990; Xu et al., 2003). Studies are currently ongoing to enhance the bioavailability of ginsenosides in order to appropriately assess the pharmacological actions of ginseng as well as to determine the optimal clinical dosage.

1.3.2.3 Effects of Processing on Ginseng Properties

Advances in technology have provided available and accessible means to analyze the chemical constituents within the ginseng root (Qi et al., 2011). This capability is critical to the eventual better understanding of both the pharmacodynamic and pharmacokinetic properties of ginseng. Although the majority of studies have focused on the roots of different ginseng species, it is important to mention that the leaf (Ligor et al., 2005; Liu et al., 2010) and berry (Attele et al., 2002; Dey L et al., 2003) of the ginseng root have also been shown to contain ginsenosides along with additional compounds that are considered responsible for mediating therapeutic effects.

Growth, maintenance, cultivation and harvesting of ginseng are long and tedious processes, such that when performed haphazardly will yield poor ginseng root quality as well as value. Post-processing of ginseng for commercial sale by means of skin peeling and air drying (North American and Chinese ginseng) or heating via steaming while keeping the skin intact (Korean/red ginseng) has also been shown to affect the chemical profile, particularly with respect to the ginsenoside species present. Recent studies have found improved and more efficient methods for the extraction of the ginsenosides known to elicit these therapeutic effects (Kim et al., 2007; Kim et al., 2010; Lee et al., 2011).

There have been various reports demonstrating the effects of different chemical ginseng processing procedures, such as increasing temperature and pressure, which result in the

appearance of new ginsenoside species (Kim et al., 2000; Park et al., 2002; Lee et al., 2009) in addition to chemically altered ginsenoside species (Kang et al., 2007) not originally present in the raw root. These studies have shown that heat and steam processing, a characteristic method in Korean/red ginseng, results in a more pronounced biological effect than raw air-dried ginseng, potentially as a result of the appearance of new functionally active ginsenosides (Kim et al., 2000), as previously noted, or as a result of chemical modification of ginsenosides yielding a new stereoisomer or the addition/deletion of a functional group (Park et al., 2002) thus eliciting an enhanced physiological response (Tung et al., 2010). An example of the latter can be provided with respect to both *P. ginseng* and the ginsenoside Rb2, whose hydroxyl radical scavenging is enhanced by heat processing (Kang et al., 2007).

The use of different solvents such as alcohol and water for ginsenoside extraction has also been shown to play a significant role in the amount of ginsenosides isolated, which may certainly result in different biological properties (Lee et al., 1981; Gafner et al., 2004). There is consensus that a higher ginsenoside yield is produced in the alcoholic versus the aqueous ginseng extract. The pharmacological effects of different solvent ginseng extractions on the cardiovascular system were, in fact, demonstrated as early as 1981; the ether, ethanol and aqueous *P. ginseng* extracts administered to anaesthetized dogs had differing effects on several cardiac variables, including cardiac output, stroke volume, heart rate, mean arterial pressure and total peripheral resistance (Lee et al., 1981). In other systems of the body, alcoholic and aqueous ginseng extracts also elicit diverse responses. For example, King and colleagues showed that an alcoholic ginseng extract exhibited a greater response on estrogen receptors in the estrogen-receptor-positive MC-7 human breast cancer cell line than the aqueous *P. quinquefolius* extract (King et al., 2006). Elsewhere, the aqueous ginseng extract has been shown to induce apoptosis

in several cancer cell lines, suggesting it may be useful in cancer therapy (Kim et al., 2003; Peralta et al., 2009). Conversely, ginseng has been shown to promote angiogenesis in human umbilical vein endothelial cells and may therefore be of potential use for revascularization therapy (Huang et al., 2005; Morisaki et al., 1995; Kim et al., 2007). The different pharmacological profiles of aqueous versus alcoholic extracts are likely explained by the different ginsenosides and compounds present in these extracts and their distinct effects in different biological systems. Evidently, the preparation processes and extraction methods have a significant effect on the physiological responses in different biological systems and should, therefore, be taken into particular consideration when choosing the appropriate ginseng species for pharmacological studies.

1.3.3 Ginseng in Cardiac Hypertrophy and Heart Failure

Cardiac hypertrophy, as described in detail in section 1.1.2, is a fundamental contributor to cardiac remodeling which eventually leads to HF. One of the major challenges in treating HF is the difficulty in reversing already established pathological cardiac hypertrophy as it involves a plethora of complex molecular mechanisms (reviewed in Frey and Olson, 2003). Consequently, the focus of research in discovering new therapeutic agents for the management of HF involves the identification of the underlying causes by targeting signalling pathways in cardiac hypertrophy. Ginseng has recently become an increasingly popular candidate for the prevention and treatment of HF as several studies have demonstrated its anti-hypertrophic and anti-remodelling effects both *in vitro* (Liu et al., 2004; Chen et al., 2005; Yang et al., 2005; Jiang et al., 2007; Guo et al., 2011; Moey et al., 2011) and *in vivo* (You et al., 2005; Jiang et al., 2007; Deng et al., 2009; Deng et al., 2010; Guo et al., 2011).

The whole ginseng root as well as individual ginsenosides, such as Rb1 (Jiang et al., 2007) have been shown to exert anti-hypertrophic effects in isolated cardiomyocytes subjected to classical hypertrophic agonists which include AngII (Liu et al., 2004; Guo et al., 2011), ET-1 (Chen et al., 2005; Yang et al., 2005), phenylephrine (Guo et al., 2011) as well as leptin (Moey et al., 2011) and PGF2α (Jiang et al., 2007). Prevention of agonist-induced cardiac hypertrophy *in vivo* by whole NA ginseng alcoholic extracts (Moey et al., 2011; Guo et al., 2011) and Rb1 (Jiang et al., 2007) were evidenced by decreases in cell size and gene expression of fetal gene markers such as ANP, αSA and myosin heavy chain (MHC). In addition, non-ginsenoside components such as trilinolein (a triacylglycerol extracted from *P. ginseng*) has been shown to inhibit both AngII- (Liu et al., 2004) and ET-1 (Chen et al., 2005; Yang et al., 2005) induced cardiac hypertrophy potentially through an antioxidant mechanism *via* MAPK inhibition.

While studies using cultured myocytes have demonstrated the potential of ginseng as an anti-hypertrophic agent, the more critical issue is to determine if these results are translatable to *in vivo* models of HF. Indeed there have been many publications that have assessed the ability of ginseng (You et al., 2005; Guo et al., 2011) and ginsenosides Rb1 (Jiang et al., 2007; Zhao et al., 2010) and Rg1 (Deng et al., 2009; Deng et al., 2010) in various *in vivo* models of cardiac hypertrophy. For example, 30-day treatment with *P. ginseng* at a dose of 150 mg/kg has been shown to prevent adriamycin-induced HF (You et al., 2005) *via* an antioxidant effect. Ginsenoside Rb1 administration to rats subjected to monocrotaline-induced right ventricular cardiac hypertrophy prevented ventricular remodeling and HF (Jiang et al., 2007), which was considered to be a result of the inhibition of the CaN/NFAT3 pathway (see section 1.2.3.3). In a recent study, Rb1 similarly exhibited anti-hypertrophic effects in a genetically engineered mouse model that had a missense mutation (R141W) in the strong tropomyosin-binding region of the

cardiac troponin T (cTnT) model, which is considered responsible for the development of familial dilated cardiomyopathy. The proposed mechanism of action of Rb1 however was proposed to be via attenuation of the heparin-binding epidermal growth factor (HB-EGF) involved in the activation of STAT3 (Zhao et al., 2010) in cardiac hypertrophy. The postulated anti-hypertrophic mechanisms of action by Rg1 have been shown to involve the inhibition of CaN and MAPK (p38, ERK1/2 and JNK) activation (Deng et al., 2009) as well as through NO (nitric oxide)-related pathways (Deng et al., 2010). Most recently, using an alcoholic *P. quinquefolius* extract, Guo and colleagues similarly showed the inhibition of HF and cardiac hypertrophy both *in vitro* and *in vivo* by attenuating NHE-1 related CaN/NFAT3 activation (Guo et al., 2011). As discussed in **Chapter 3** and **Chapter 4**, we sought to assess the ability of ginseng to treat HF by administration of ginseng in drinking water after well-established cardiac remodeling and HF. Results demonstrated the marked ability of ginseng to reverse multiple agonist-induced cardiac hypertrophy and ventricular dysfunction by attenuation of CaN/NFAT3 activity as well as RhoA/ROCK-dependent MAPK activation. The following tables summarize the experimental conditions, measured variables and proposed mechanisms in the studies of the antihypertrophic effects of ginseng, Rb1 and Rg1 *in vitro* (Table 1.2) and *in vivo* (Table 1.3).

1.4 Study Objectives

Heart failure is a complex syndrome that involves the upregulation of several cellular signalling pathways. By identifying the molecular mechanisms that are key to the cardiac remodelling process in the development of heart failure, these particular signalling cascades can be targeted for the management of HF. NA ginseng has demonstrated significant potential for the prevention of CVDs however its ability to prevent and treat cardiac hypertrophy and remodelling in leptin-associated conditions have not been studied. Accordingly the objectives of these studies

were to determine the antihypertrophic and antiremodelling effects of ginseng in agonist-induced cardiac hypertrophy *in vitro* (**Chapter 2** and **3**) and in an HF animal model (**Chapter 4**).

Table 1.2 Evidence for the antihypertrophic and antiremodelling effects of ginseng *in vitro*.

Ginseng Preparation	Ginseng Dose	Model of Hypertrophy	Parameters	Proposed Mechanisms	Reference
Trilinolein (*P. ginseng*)	0.1 – 10 μM	Angiotensin II (1 – 1000 nM) Endothelin-1 (10 nM)	Decreased: ^{3}[H] leucine incorporation β-MHC promoter activity ROS generation ERK1/2, p38, JNK activation	Antioxidant effect via MAPK	Liu et al., 2004 Chen et al., 2005
	1 and 10 μM	Endothelin-1 (10 nM)	Decreased: *c-fos* gene expression NADPH oxidase activity ROS and superoxide levels ERK1/2 and JNK activation NFκB promoter activity	Antioxidant effect via MAPK	Yang et al., 2005
Ginsenoside Rb1	50, 100, 200 μg	Prostaglandin F2α (100 nM)	Decreased: Cell diameter and protein level ANP gene expression $[Ca^{2+}]_i$ CaN/NFAT3/GATA4 protein	CaN/NFAT3	Jiang et al., 2007
Total ginsenosides (*P. quinquefolius*)	10 μg/mL	Angiotensin II (100 nM) Endothelin-1 (10 nM) Phenylephrine (10 μM)	Decreased: Cell surface area and ANP gene $[Ca^{2+}]_i$ and CaN activity NFAT3 nuclear translocation GATA4 binding Intracellular pH NHE-1 protein and gene expression	NHE-1 related CaN/NFAT3	Guo et al., 2011
	10 μg/mL	Leptin (3.1 nM)	Decreased: Cell surface area and ^{3}[H] leucine αSA and MHC gene expression p115RhoGEF-RhoA/ROCK activity p115RhoGEF gene and protein cofilin-2 activation and F/G actin p38 and ERK1/2 activation	p115RhoGEF-RhoA/ROCK-dependent p38 MAPK	Moey et al., 2011

$[Ca^{2+}]_i$ = intracellular calcium concentration; **ANP** = atrial natriuretic peptide; **c-fos** = FBJ murine osteosarcoma viral oncogene homolog; **ERK1/2** = extracellular regulator kinase 1/2; **GEF** = guanine nucleotide exchange factor; **JNK** = c-jun N-terminal kinase; **MHC** = myosin heavy chain; **NADPH** = *nicotinamide adenine dinucleotide phosphate*; **NHE-1** = *sodium hydrogen exchanger-1*; **NFAT** = *nuclear factor of activated T cells*; **NFκB** = *nuclear factor* kappa-light-chain-enhancer of activated B cells; **RhoA** = Ras homolog member A; **ROCK** = Rho-associated, coiled-coil containing protein kinase 1; **ROS** = reactive oxygen species; **αSA** = alpha-skeletal actin

Table 1.3 Evidence for improved cardiac function and antihypertrophic effects of ginseng *in vivo*.

Ginseng Preparation	Ginseng Dose	Model of Hypertrophy	Parameters	Proposed Mechanisms	Reference
Panax ginseng	150 g/kg, gavage (1 month)	Wistar Rats Adriamycin (15 mg/kg) 6 i.p injections for 2 weeks	Decreased: Mortality, HW:BW and ascites Protein, RNA and DNA expression GSHPx, SOD and MDA levels	Antioxidant effect	You et al., 2005
Panax quinquefolius	100 mg/kg, gavage (1 month)	Sprague-Dawley Rats CAL of the LADA 4 weeks	Decreased: Cardiac dysfunction HW:BW and LV:BW ratios ANP, NHE-1 and MCIP-1 expression CaN activity	NHE-1 related CaN/NFAT3	Guo et al., 2011
Ginsenoside Rb1	70 mg/kg, *ad libitum* (7 months)	$cTnT^{R141W}$ mice FDCM	Decreased: Mortality, HW:BW and fibrosis Cardiac dysfunction, degeneration and intercalated disk remodeling HB-EGF and STAT3 activation	JNK/STAT3	Zhao et al., 2010
	10 and 40 mg/kg (2 weeks)	Sprague-Dawley Rats Monocrotaline (60 mg/kg) 2 to 4 weeks	Decreased: HW:BW, RV:BW and LV:BW Cardiomyocyte injuries ANP, CaN, NFAT3 and GATA4	CaN/NFAT3	Jiang et al., 2007
Ginsenoside Rg1	3.75, 7.5 and 15 mg/kg/day, i.p (3 weeks)	Sprague-Dawley Rats Abdominal aorta coarctation (3 weeks)	Decreased: LV:BW and fibrosis ANP, CaN and ERK1gene expression CaN and MAPK-1 protein expression	CaN/NFAT-3 ERK1-MAPK	Deng et al., 2009
	15 mg/kg/day (3 weeks)	Sprague-Dawley Rats Abdominal aorta coarctation (3 weeks)	Decreased: LV:BW and fibrosis Cardiac dysfunction ANP and eNOS gene expression	eNOS and NO	Deng et al., 2010

ANP = atrial natriuretic peptide; **BW** = body weight; **CAL** = coronary artery ligation; **CaN** = calcineurin; **cTnT** = cardiac troponin T; **DNA** = deoxyribonucleic acid; **eNOS** = endothelial nitric oxide synthase; **ERK1** = extracellular regulator kinase-1; **FDCM** = familial dilated cardiomyopathy; **GSHPx** = glutathione peroxidase activity; **HB-EGF** = *heparin-binding epidermal-growth factor*; **HW** = *heart weight*; **i. p** = *intraperitoneal*; **LADA** = left anterior descending artery; **LV** = left ventricle; **MDA** = malondialdehyde; **MCIP-1** = modulatory calcineurin interacting protein-1; **NFAT3** = nuclear factor of transcription-3; **NHE-1** = sodium hydrogen exchanger-1; **RNA** = ribonucleic acid; **RV** = right ventricle; **SOD** = superoxide dismutase

1.5 References

Abe Y, Ono K, Kawamura T, Wada H, Kita T, Shimatsu A and Hasegawa K. Leptin induces elongation of cardiac myocytes and causes eccentric left ventricular dilatation with compensation. Am J Physiol Heart Circ Physiol 2007;292:H2387-2396.

Abel ED, Litwin SE and Sweeney G. Cardiac remodelling in obesity. Physiol Rev 2008;88:369-419

Ambrosioni E, Bacchelli S, Esposti DD and Borghi C. Beta-blockade in hypertension and congestive heart failure. J Cardiovasc Pharmacol 2011;38 Suppl 3:S25-31

Appert-Collin A, Cotecchia S, Nenniger-Tosato M, Pedrazzini T and Diviani D. The A-kinase anchoring protein (AKAP)-Lbc-signaling complex mediates alpha1 adrenergic receptor-induced cardiomyocyte hypertrophy. Proc Natl Acad Sci USA 2007;104:10140-10145

Appleby JH. Ginseng and the Royal Society. Notes Rec R Soc Lond 1983; 37: 121-45

Assinewe VA, Baum BR, Gagnon D and Arnason JT. Phytochemistry of wild populations of Panax quinquefolius L. (North American ginseng). J Agric Food Chem 2003;51:4549-4553

Attele AS, Wu JA and Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. Biochem Pharmacol 1999;58:1685-1693.

Attele AS, Zhou YP, Xie JT, Wu JA, Zhang L, Dey L, Pugh W, Rue PA, Polonsky KS and Yuan CS. Antidiabetic effects of Panax ginseng berry extract and the identification of an effective component. Diabetes 2002; 51: 1851-8

Baba HA, Stypmann J, Grabellus F, Kirchhof P, Sokoll A, Schäfers M, Takeda A, Wilhelm MJ, Scheld HH, Takeda N, Breithardt G and Levkau B. Dynamic regulation of MEK/Erks and Akt/GSK-3beta in human end-stage heart failure after left ventricular mechanical support: myocardial mechanotransduction-sensitivity as a possible molecular mechanism. Cardiovasc Res 2003;59:390-399

Bae EA, Han MJ, Kim EJ and Kim DH. Transformation of ginseng saponins to ginsenoside Rh2 by acids and human intestinal bacteria and biological activities of their transformants. Arch Pharm Res 2004;27:61-67

Bao Y, Li R, Jiang J, Cai B, Gao J, Le K, Zhang F, Chen S and Liu P. Activation of peroxisome proliferator-activated receptor gamma inhibits endothelin-1-induced cardiac hypertrophy via the calcineurin/NFAT signaling pathway. Mol Cell Biochem 2008;317:189-196

Barouch LA, Berkowitz DE, Harrison RW, O'Donnell CP and Hare JM. Disruption of leptin signaling contributes to cardiac hypertrophy independently of body weight in mice. Circ 2003;108:754-759

Beeser A, Jaffer ZM, Hofmann C and Chernoff J. Role of group A p21-activated kinases in activation of extracellular-regulated kinase by growth factors. J Biol Chem 2005;280:36609-36615

Belin de Chantemèle EJ, Mintz JD, Rainey WE and Stepp DW. Impact of leptin-mediated sympatho-activation on cardiovascular function in obese mice. Hypertension 2011;58:271-279

Belin de Chantemèle EJ, Muta K, Mintz J, Tremblay ML, Marrero MB, Fulton DJ and Stepp DW. Protein tyrosine phosphatase 1B, a major regulator of leptin-mediated control of cardiovascular function. Circ 2009;120:753-763

Bella JN, Palmieri V, Roman MJ, Liu JE, Welty TK, Lee ET, Fabsitz RR, Howard BV and Devereux RB. Mitral ratio of peak early to late diastolic filling velocity as a predictor of mortality in middle-aged and elderly adults: the strong heart study. Circ 2002;105:1928-1933

Bernardo BC, Weeks KL, Pretorius L and McMullen JR. Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. Pharmacol and Ther 2010;128:191-227

Berry JM, Le V, Rotter D, Battiprolu PK, Grinsfelder B, Tannous P, Burchield JS, Czubryt M, Backs J, Olson EN, Rothermel BA and Hill JA. Reversibility of adverse, calcineurin-dependent cardiac remodeling. Circ Res 2011;109:407-417

Bhatia RS, Tu JV, Lee DS, Austin PC, Fang J, Haozi A, Gong Y and Liu PP. Outcome of heart failure with preserved ejection fraction in a population-based study. N Engl J Med 2006;355:260-269

Bjørbaek C, Buchholz RM, Davis SM, Bates SH, Pierroz DD, Gu H, Neel BG, Myers MG Jr and Flier JS. Divergent roles of SHP-2 in ERK activation by leptin receptors. J Biol Chem 2001;276:4747-4755

Blaufarb IS and Sonnenblick EH. The renin-angiotensin system in left ventricular remodeling. Am J Cardiol 1996;77:8C-16C

Bos JL, Rehmann H and Wittinghofer A. GEFs and GAPs: critical elements in the control of small G proteins. Cell 2007;129:865-877.

Bouloumie A, Marumo T, Lafontan M and Busse R. Leptin induces oxidative stress in human endothelial cells. FASEB J 1999;13:1231-1238

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH and Yancopoulos GD. ERK's: a family of protein serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 1991;65:663-675

Braz JC, Bueno OF, Liang Q, Wilkins BJ, Dai YS, Parsons S, Braunwart J, Glascock BJ, Klevitsky R, Kimball TF, Hewett TE and Molkentin JD. Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling. J Clin Invest 2003;111:1475-1486

Brower GL, Levick SP and Janicki JS. Inhibition of matrix metalloproteinase activity by ACE inhibitors prevents left ventricular remodeling in a rat model of heart failure. Am J Physiol Heart Circ Physil 2007;292:H3057-3064

Brown JH, Del Re DP and Sussman MA. The Rac and Rho hall of fame: a decade of hypertrophic signaling hits. Circ Res 2006;98:730-742

Brown NJ and Vaughn DE. Angiotensin-converting enzyme inhibitors. Circ 1998;97:1411-1420

Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Klevitsky R, Hewett TE, Jones SP, Lefer DJ, Peng CF, Kitsis RN and Molkentin JD. The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. EMBO J 2000;19:6341-6350

Cabin HS. The Heart and Circulation, in *Yale University School of Medicine Heart Book* (Zaret BL, Moser M and Cohen LS eds) pp 3-10, William Morrow and Company, Inc, 2002

Campos SB, Ashworth SL, Wean S, Hosford M, Sandoval RM, Hallett MA, Atkinson SJ and Molitoris BA. Cytokine-induced F-actin reorganization in endothelial cells involves RhoA activation. Am J Physiol Renal Physiol 2009;296:F487-95

Canada. Public Health Agency of Canada. Cardiovascular Disease, 2010.

Canagarajah B, Khokhlatchev A, Cobb M and Goldsmith E. Activation mechanism of the MAPK kinase ERK2 by dual phosphorylation. Cell 1997;90:859-869

Carlson AW. Ginseng: America's botanical drug connection to the orient. Econ Bot 1986; 40: 233-49

Chadee DN and Kyriakis JM. MLK3 is required for mitogen activation of B-Raf, ERK and cell proliferation. Nat Cell Biol 2004;6:770-776

Chan AK, Sanderson JE, Wang T, Lam W, Yip G, Wang M, Lam YY, Zhang Y, Yeung L, Wu EB, Chan WW, Wong JT, So N and Yu CM. Aldosterone receptor antagonism induces reverse remodeling when added to angiotensin receptor blockade in chronic heart failure. J Am Coll Cardiol 2007;50:591-596

Chan TWD, But PPH, Cheng SW, Kwok IM, Lau FW and Xu HX. Differentiation and authentication of Panax ginseng, Panax quinquefolius and ginseng products by using HPLC/MS. Anal Chem 2000;72:1281-7

Charkoudian N and Rabbitts JA. Sympathetic neural mechanisms in human cardiovascular health and disease. Mayo Clin Proc 2009;84:822-830

Chatterjee K and Massie B. Systolic and diastolic heart failure: differences and similarities. J Card Fail 2007;13:569-576

Chen SC, Cheng JJ, Hsieh MH, Chu YL, Kao PF, Cheng TZ and Chan P. Molecular mechanism of the inhibitory effect of trilinolein on endothelin-1-induced hypertrophy of cultured neonatal rat cardiomyocytes. Planta Med. 2005;71:525-529

Chen Z, Raman M, Chen L, Lee SF, Gilman AG and Cobb MH. TAO (Thousand-and-one Amino Acid) protein kinases mediate signaling from carbochol to p38 mitogen-activated protein kinase and ternary complex factors. J Biol Chem 2003;278:22278-22283

Chu SF and Zhang JT. New achievements in ginseng research and its future prospects. Chin J Integr Med 2009; 15: 403-8

Ciccone M, Vettor R, Pannacciulli N, Minenna A, Bellacicco M, Rizzon P, Giorgino R and De Pergola G. Plasma leptin is independently associated with the intima-media thickness of the common carotid artery. Int J Obes Relat Metab Disord 2001;25:805-810

Cirillo P, Angri V, De Rosa S, Cali G, Petrillo G, Maresca F, D'Ascoli GL, Maietta P, Brevetti L and Chiariello M. Pro-atherothrombotic effects of leptin in human coronary endothelial cells. Thromb Haemost 2010;103:1065-1075

Clark AL and Cleland JGF. The control of adrenergic function in heart failure: therapeutic intervention. Heart Failure Rev 2000;5:101-114

Cohn JN, Johnson G, Ziesche S Cobb F, Francis G, Tristani F, Smith R, Dunkman WB, Loeb H, Wong M, Bhat G, Goldman S, Fletcher RD, Doherty J, Hughes CV, Carson P, Cintron G, Shabetai R and Haakenson C. A comparison of enalapril with hydralazine-isosorbide

dinitrate int he treatment of chronic congestive heart failure. N Engl J Med 1991;325:303-310

Coleman DL. Effects of parabiosis of obese with diabetes and normal mice. Diabetologia 1973;9:294-298

Criteria Committee, New York Heart Association. *Diseases of the heart and blood vessels. Nomenclature and criteria for diagnosis, 6th ed.* Boston: Little, Brown and co, 1964;114.

Cui H, Cai F and Belsham DD. Leptin signaling in neurotensin neurons involves STAT, MAPK kinases ERK1/2 and p38 through c-Fos and ATF1. FASEB J 2006;20:2654-2656

Das M and Das DK. Caveolae, caveolin and cavins: Potential targets for the treatment of cardiac disease. Ann Med 2011; Jun 29, Epub ahead of print.

Deng J, Lv XT, Wu Q and Huang XN. Ginsenoside Rg1 inhibits rat left ventricular hypertrophy induced by abdominal aorta coarctation: involvement of calcineurin and MAPK signaling. Eur J Pharmacol. 2009;608:42-47

Deng J, Wang YW, Chen WM, Wu Q and Huang XN. Role of nitric oxide in ginsenoside Rg1-induced protection against left ventricular hypertrophy produced by abdominal coarctation in rats. Biol Pharm Bull. 2010;33:631-635

Denolin H, Kuhn K, Krayenbuehl HP, Loogen F and Reale A. The definition of heart failure. Eur Heart J 1983;7:445-448

Devos R, Guisez Y, Van der Heyden J, White DW, Kalai M, Fountoulakis M and Plaetinck G. Ligand-indepdent dimerization of the extracellular domain of the leptin receptor and determination of the stoichiometry of leptin binding. J Biol Chem 1997;272:18304-18310

Dey L, Xie JT, Wang A, Maleckar SA and Yuan CS. Anti-hyperglycemic effects of ginseng: comparison between root and berry. Phytomedicine 2003; 10: 600-5

Dickstein K, Cohen-Solal A, Filippatos G, McMurray JJ, Ponikowski P, Poole-Wilson PA, Strömberg A, van Veldhuisen DJ, Atar D, Hoes AW, Keren A, Mebazaa A, Nieminen M, Priori SG and Swedberg K; ESC Committee for Practice Guidelines (CPG). ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the diagnosis and treatment of acute and chronic heart failure 2008 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association of the ESC (HFA) and endorsed by the European Society of Intensive Care Medicine (ESICM). Eur J Heart Fail 2008;10:933-989

Dicpinigaitis PV. Angiotensin-converting enzyme inhibitor-induced cough. Chest 2006;129:169S-173S

Eisenberg DM, Davis RB, Ettner SL, Appel S, Wilkey S, Van Rompay M and Kessler RC. Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. JAMA 1998;280:1569-1575

Eisenberg DM, Kessler RC, Foster C, Norlock FE, Calkins DR and Delbanco TL. Unconventional medicine in the United States: prevalence, costs and patterns of use. N Engl J Med 1993;328:246-252

Ely HA, Mellon PL and Coss D. GnRH induces the c-Fos gene via phosphorylation of SRF by the calcium/calmodulin kinase II pathway. Mol Endocrinol 2011;25:669-680

Erdös EG. Conversion of angiotensin I to angiotensin II. Am J Med 1976;60:749-759

Eyckerman S, Broaekaert D, Verhee A, Vandekerckhove J and Tavernier J. Identification of the Y985 and Y1077 motifs as SOCS3 recruitment sites in the murine leptin receptor. FEBS Lett;2000;486:33-37

Fedak PWM, Verma S, Weisel RD and Li RK. Cardiac remodeling and failure: from molecules to man (part I). Cardiovasc Pathol 2005;14:1-11

Floras JS. Sympathetic nervous system activation in human heart failure: clinical implications of an updated model. J Am Coll Cardiol 2009;54:375-385

Foody JM, Farrell MH and Krumholz HM. Beta-blocker therapy in heart failure: scientific review. JAMA 2002;287:883-889

Frey N and Olson EN. Cardiac hypertrophy: the good, the bad and the ugly. Annu Rev Physiol 2003;65:45-79

Frühbeck G. Intracellular signalling pathways activated by leptin. Biochem J 2006;393:7-20

Fu M, Zhang J, Xu S, Pang Y, Liu N and Tang C. Role of calcineurin in angiotensin II-induced cardiac myocyte hypertrophy of rats. Chin Med Sci J 2001;16:1-4

Gafner S, Bergeron C, McCollom MM, Cooper LM, McPhail KL, Gerwick WH and Angerhofer CK. Evaluation of the efficiency of three different solvent systems to extract triterpene saponins from roots of Panax quinquefolius using high-performance liquid chromatography. J Agric Food Chem 2004; 52: 1546-50

Gagnon C, Legault F, Geraldes P, Tanguay JF and Lambert C. Diverse effects of Ace inhibitors and angiotensin II receptor antagonists on prevention of cardiac hypertrophy and collagen distribution in spontaneously hypertensive rats. Int J Cardiol 2004;97:373-381

Goldstein B. Ginseng: its history, dispersion and folk tradition. Am J Chin Med 1975; 3: 223-34

Gruen ML, Hao M, Piston DW and Hasty AH. Leptin requires canonical migratory signaling pathways for induction of monocyte and macrophage chemotaxis. Am J Physiol Cell PHysiol 2007;293:C1481-C1488

Guglin M, Kristof-Kuteyeva O, Novotorova I and Pratap P. Aldosterone antagonists in heart failure. J Cardiovasc Pharmacol Ther 2011;16:150-19

Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL and Anis AH. The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. BMC Public Health 2009;9:88-108

Guo J, Gan XT, Haist JV, Rajapurohitam V, Zeidan A, Faruq NS and Karmazyn M. Ginseng inhibits cardiomyocyte hypertrophy and heart failure via NHE-1 inhibition and attenuation of calcineurin activation. Circ Heart Fail 2011;4:79-88

Hagiwara Y, Miyoshi S, Fukuda K, Nishiyama N, Ikegami Y, Tanimoto K, Murata M, Takahashi E, Shimoda K, Hirano T, Mitamura H and Ogawa S. SHP-2 mediated signaling cascade through gp130 is essential for LIF-dependent ICaL, $[Ca^{2+}]_i$ transient, APD increase in cardiomyocytes. J Mol Cell Cardiol 2007;43:710-716

Hah JS, Kang BS and Kang DH. Effect of Panax ginseng alcohol extract on cardiovascular ginseng. Yonsei Med J 1978; 19: 11-8

Hall JE, da Silva AA, do Carmo JM, Dubinion J, Hamza S, Munusamy S, Smith G and Stec DE. Obesity-induced hypertension: role of sympathetic nervous system, leptin and melanocortins. J Biol Chem 2010;285:17271-17276

Harris IS, Zhang S, Treskov I, Kovacs A, Weinheimer C and Muslin AJ. Raf-1 kinase is required for cardiac hypertrophy and cardiomyocyte survival in response to pressure overload. Circ 2004;110:718-723

Health Canada. (2003) *Canadian guidelines for body weight classification in adults.* Ottawa, 2003, p.3

Hekerman P, Zeidler J, Bamberg-Lemper S, Knobelspies H, Lavens D, Tavernier J, Joost HG and Becker W. Pleiotropy of leptin receptor signalling is defined by distinct roles of the intracellular tyrosines. FEBS J 2005;272:109-119

Hilal-Dandan R, Means CK, Gustafsson AB, Morissette MR, Adams JW, Brunton LL and Heller Brown J. Lysophosphatidic acid induces hypertrophy of neonatal cardiac myocytes via activation of G_i and Rho. J Mol Cell Cardiol 2004;36:481-493

Hintz KK, Aberle NS and Ren J. Insulin resistance induces hyperleptinemia, cardiac contractile dysfunction but not cardiac leptin resistance in ventricular myocytes. Int J Obes Relat Metab Disord 2003;27:1196-1203

Hong HJ, Chan P, Liu JC, Juan SH, Huang MT, Lin JG and Cheng TH. Angiotensin II induces endothelin-1 gene expression via extracellular signal-regulated kinase pathway in rat aortic smooth muscle cells. Cardiovasc Res 2005;161:159-168

Horwich TB, Fonarow GC, Hamilton MA, MacLellan WR, Woo MA and Tillisch JH. The relationship between obesity and mortality in patients with heart failure. J Am Coll Cardiol. 2001;38:789-795

Hu SY. The genus *Panax* (ginseng) in Chinese medicine. Econ Bot 1976; 30: 11-28

Huang YC, Chen CT, Chen SC, Lai PH, Liang HC, Chang Y, Yu LC and Sung HW. A natural compound (ginsenoside Re) isolated from Panax ginseng as a novel angiogenic agent for tissue regeneration. Pharm Res 2005; 22: 636-46

Hunt SA, Abraham WT, Chin MH, Feldman AM, Francis GS, Ganiats TG, Jessup M, Konstam MA, Mancini DM, Michl K, Oates JA, Rahko PS, Silver MA, Stevenson LW, Yancy CW, Antman EM, Smith SC Jr, Adams CD, Anderson JL, Faxon DP, Fuster V, Halperin JL, Hiratzka LF, Jacobs AK, Nishimura R, Ornato JP, Page RL, Riegel B; American College of Cardiology; American Heart Association Task Force on Practice Guidelines; American College of Chest Physicians; International Society for Heart and Lung Transplantation; Heart Rhythm Society. ACC/AHA 2005 Guideline Update for the Diagnosis and Management of Chronic Heart Failure in the Adult: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2001 Guidelines for the Evaluation and Management of Heart Failure): developed in collaboration with the American College of Chest Physicians and the International Society for Heart and Lung Transplantation: endorsed by the Heart Rhythm Society. Circulation 2005;112:e154-235

Hunter JC, Zeidan A, Javadov S, Kilić A, Rajapurohitam V and Karmazyn M. Nitric oxide inhibits endothelin-1-induced neonatal cardiomyocyte hypertrophy via a RhoA-ROCK-dependent pathway. J Mol Cell Cardiol 2009;47:810-818

Imagawa K, Numata Y, Katsura G, Sakaguchi I, Morita A, Kikuoka S, Matuomto Y, Tsuji T, Tamaki M, Sasakura K, Teraoka H, Hosoda K, Ogawa Y and Nako K. Structure-function studies of human leptin. J Biol Chem 1998;273:35245-35249

Jentzer JC, DeWald TA and Hernandez AF. Combination of loop diuretics with thiazide-type diuretics in heart failure. J Am Coll Cardiol 2010;56:1527-34

Jeon BH, Kim CS, Park KS, Lee JW, Park JB, Kim KJ, Kim SH, Chang SJ and Nam KY. Effect of Korea red ginseng on the blood pressure in conscious hypertensive rats. Gen Pharmacol 2000;35:135-141

Jessup M and Brozena S. Heart Failure. N Engl J Med 2003;348:2007-2018

Jia L and Zhao Y. Current evaluation of the millennium phytomedicine. Ginseng (I): etymology, pharmacognosy, phytochemistry, market and regulations. Curr Med Chem 2009;16: 2475-84

Jia L, Zhao Y and Liang XJ. Current evaluation of the millennium phytomedicine. Ginseng (II): collected chemical entities, modern pharmacology, and clinical applications emanated from traditional Chinese medicine. Curr Med Chem 2009; 16: 2924-42

Jiang QS, Huang XN, Dai ZK, Yang GZ, Zhou QX, Shi JS and Qin W. Inhibitory effect of ginsenoside Rb1 on cardiac hypertrophy induced by monocrotaline in rat. *J* Ethnopharmacol 2007;111:567-572

Jiang QS, Huang XN, Yang GZ, Jiang XY and Zhou QX. Inhibitory effect of ginsenoside Rb1 on calcineurin signal pathway in cardiomyocyte hypertrophy induced by prostaglandin F2a. Acta Pharmacol Si. 2007;8:1149-1154

Juan W, Nakazawa M, Watanabe K, Ma M, Wahed MI, Hasegawa G, Naito M, Yamamoto T, Fuse K, Kato K, Kodama M and Aizawa Y. Quinapril inhibits progression of heart failure and fibrosis in rats with dilated cardiomyopathy after myocarditis. Mol Cell Biochem 2003;251:77-82

Kang KS, Kim HY, Baek SH, Yoo HH, Park JH and Yokozawa T. Study on the hydroxyl radical scavenging activity changes of ginseng and ginsenoside-Rb2 by heat processing. Biol Pharm Bull 2007;30:724-8

Karmazyn M, Kilić A and Javadov S. The role of NHE-1 in myocardial hypertrophy and remodelling. J Mol Cell Cardiol 2008a;44:647-653

Karmazyn M, Purdham DM, Rajapurohitam V and Zeidan A (2008b) Leptin Signaling in the Cardiovascular System, in *Signal Transduction in the Cardiovascular System in Health and Disease* (Srivastava AK and Anand-Srivastava MB eds) pp 377-395, Springer US.

Katz AM. Influence of altered inotropy and lusitropy on ventricular pressure-volume loops. J Am Coll Cardiol 1988;11:438-435

Kim EH, Jang MH, Shin CH, Shin MS and Kim CJ. Protective effect of aqueous extract of Ginseng radix against 1-methyl-4-phenylpyridinium-induced apoptosis in PC12 cells. Biol Pharm Bull 2003;26:1668-73

Kim GN, Lee JS, Song JH, Oh CH, Kwon YI and Jang HD. Heat processing decreases Amadori products and increases total phenolic content and antioxidant activity of Korean red ginseng. J Med Food 2010;13:1478-1484

Kim HY, Kang KS, Yamabe N and Yokozawa T. Comparison of the effects of Korean ginseng and heat-processed Korean ginseng on diabetic oxidative stress. Am J Chin Med 2008;36:989-1004

Kim WY, Kim JM, Han SB, Lee SK, Kim ND, Park MK, Kim CK and Park JH. Steaming of ginseng at high temperature enhances biological activity. J Nat Prod 2000;63:1702-4

Kim YM, Namkoong S, Yun YG, Hong HD, Lee YC, Ha KS, Lee H, Kwon HJ, Kwon YG and Kim YM. Water extract of Korean read ginseng stimulates angiogenesis by activating the PI3K/Akt-dependent ERK1/2 and eNOS pathways in human umbilical vein endothelial cells. Biol Pharm Bull 2007;30:1674-9

Kimura N, Matsuo R, Shibuya H, Nakashima N and Taga T. BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6. J Biol Chem 2000;275:17647-17652

King ML, Adler SR and Murphy LL. Extraction-dependent effects of American ginseng (Panax quinquefolium) on human breast cancer cell proliferation and estrogen receptor activation. Integr Cancer Ther 2006;5:236-43

Kinugawa K, Jeong MY, Bristow MR and Long CS. Thyroid hormone induces cardiac myocyte hypertrophy in a thyroid hormone receptor alpha1-specific manner that requires TAK1 and p38 mitogen-activated protein kinase. Mol Endocrinol 2005;1618-1628

Klabunde RE. *Cardiovascular physiology concepts*. Philadelphia: Lippincott Williams & Wilkins, 2005.

Kline AD, Becker GW, Churgay LM, Landen BE, Martin DK, Muth WL, Rathnachalam R, Richardson JM, Schoner B, Ulmer M and Hale JE. Leptin is a four-helix bunde: secondary structure by NMR. FEBS Lett 1997;407:239-242

Kobayashi N, Horinaka S, Mita S, Nakano S, Honda T, Yoshida K, Kobayashi T and Matsuoka H. Critical role of Rho-kinase pathway for cardiac performance and remodeling in failing rat hearts. Cardiovasc Res 2002;55:757-67

Kolch W. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J 2000;351:289-305

Konstam MA, Neaton JD, Dickstein K, Drexler H, Komajda M, Martinez FA, Riegger GA, Malbecg W, Smith RD, Guptha S and Poole-Wilson PA; HEAAL Investigators. Effects of high-dose versus low-dose losartan on clinical outcomes with heart failure (HEEAL study): a randomized, double-blinded trial. Lancet 2009;374:1840-1848

Konstam MA, Rousseau MF, Kronenberg MW, Udelson JE, Melin J, Stewart D, Dolan N, Edens TR, Ahn S and Kinan D.. Effects of the angiotensin converting enzyme inhibitor enalapril on the long-term progression of left ventricular dysfunction in patients with heart failure. SOLVD Investigators. Circ 1992;86:431-438

Korda M, Kubant R, Patton S and Malinski T. Leptin induced endothelial dysfunction in obesity. Am J Physiol Heart Circ Physiol 2008;295:H1514-h1521

Kotsis V, Stabouli S, Papakatsika S, Rizos Z and Parati G. Mechanisms of obesity-induced hypertension. Hypertens Res 2010;33:386-393

Kuwahara K, Saito Y, Nakagawa O, Kishimoto I, Harada M, Ogawa E, Miyamoto Y, Hamanaka I, Kajiyama N, Takahashi N, Izumi T, Kawakami R, Tamura N, Ogawa Y and Nakao K. The effects of the selective ROCK inhibitor, Y27632, on ET-1-induced hypertrophic response in neonatal rat cardiac myocytes--possible involvement of Rho/ROCK pathway in cardiac muscle cell hypertrophy. FEBS Lett 1999;452:314-318

Langdon C, Kerr C, Tong L and Richards CD. Oncostatin M regulates eotaxin expression in fibroblasts and eosinophilic inflammation in C57BL/6 mice. J Immunol 2003;170:548-555

Lee CW, Kim NH, Cho HK, Sun Y, Nam JS, Rhee HJ, Chun J and Huh SO. Lysophosphatidic acid-induced c-fos up-regulation involves cyclic AMP response element-binding protein activated by mitogen- and stress-activated protein kinase-1. J Cell Biochem 2008;104:785-794

Lee DC, Lee MO, Kim CY and Clifford DH. Effect of ether, ethanol, and aqueous extracts of ginseng on cardiovascular function in dogs. Can J Comp Med 1981; 45: 182-7

Lee GH, Proenca R, Montez JM, Carrol KM, Darvishzadeh JG, Lee JI and Friedman JM. Abnormal slicing of the leptin receptor in diabetic mice. Nature 1996;379:632-635

Lee HS, Lee HJ, Yu HJ, Ju do W, KimY, Kim CT, Kim CJ, Cho YJ, Kim N, Choi SY and Suh HJ. A comparison between high hydrostatic pressure extraction and heat extraction of ginsenosides from ginseng (Panax ginseng CA Meyer). J Sci Food Agric 2011;91:1466-1473

Lee L, Lee E, Kim D, Lee J, Yoo J and Koh B. Studies on absorption, distribution and metabolism of ginseng in humans after oral administration. J Ethnopharmacol 2009;143-148

Lee SM, Shon HJ, Choi CS, Hung TM, Min BS and Bae K. Ginsenosides from heat processed ginseng. Chem Pharm Bull (Tokyo) 2009;57:92-4

Leung KW and Wong AST. Pharmacology of ginsenosides: a literature review. Chin Med 2010;5:20-27

Lezoualc'h F, Métrich M, Hmitou I, Duquesnes N and Morel E. Small GTP-binding proteins and their regulators in cardiac hypertrophy. J Mol Cell Cardiol 2008;44:623-632

Liang Q, Wiese RJ, Bueno OF, Dai YS, Markham BE and Molkentin JD. The transcription factor GATA4 is activated by extracellular signal-regulated kinase 1- and 2-mediated phosphorylation of serine 105 in cardiomyocytes. Mol Cell Biol 2001;21:7460-7469

Liao P, Georgakopoulos D, Kovacs A, Zheng M, Lerner D, Pu H, Saffitz J, Chien K, Xiao RP, Kass DA and Wang Y. The in vivo role of p38 MAP kinases in cardiac remodeling and restrictive cardiomyopathy. Proc Natl Acad Sci USA 2001;98:12283-12288

Ligor T, Ludwiczuk A, Wolski T and Buszewski B. Isolation and determination of ginsenosides in American ginseng leaves and root extracts by LC-MS. Anal Bioanal Chem 2005;383:1098-105

Lim HW and Molkentin JD. Calcineurin and human heart failure. Nat Med 1999;5:246-247

Ling Y, Yong L and Chang-Xiao L. Metabolism and pharmacokinetics of ginsenosides. Asian J Pharmacodynamics and Pharmacokinetics 2006;6:103-120

Liu GY, Li XW, Wang NB, Zhou HY, Wei W, Gui MY, Yang B and Jin YR. Three new dammarane-type triterpene saponins from the leaves of Panax ginseng C.A. Meyer. J Asian Nat Prod Res 2010;12:865-73

Liu JC, Cheng TH, Lee HM, Lee SW, Shih NL, Chen YL, Chen JJ and Chan P. Inhibitory effect of trilinolein on angiotensin II-induced cardiomyocyte hypertrophy. *Eur J Pharm.* 2004;484:1-8

Liu Y, Zhang JW, Li W, Ma H, Sun J, Deng MC and Yang L. Ginsenoside metabolites, rather than naturally occurring ginsenosides, lead to inhibition of human cytochrome P450 enzymes. Toxicol Sci 2006;91:356-364

Loirand G, Guérin P and Pacaud P. Rho kinases in cardiovascular physiology and pathophysiology. Circ Res 2006;98:322-334

Lü JM, Yao Q and Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. Current Vasc Pharmacol 2009;7:293-302

Lutz S, Blomquist-Freichel A, Yang Y, Rümenapp U, Jakobs KH, Schmidt M and Wieland T. The guanine nucleotide exchange factor p63RhoGEF, a specific link between $G_{q/11}$-coupled receptor signaling and RhoA. J Biol Chem 2005;280:11134-11139

Ma TK, Kam KK, Yan BP and Lam YY. Renin-angiotensin-aldosterone system blockade for cardiovascular diseases: current status. Br J Pharmacol 2010;160:1273-92

Madani S, De giorlamo S, Munoz DM, Li RK and Sweeney G. Direct effects of leptin on size and extracellular matrix components of human pediatric ventricular myocytes. Cardiovasc Res 2006;69:716-725

Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA and Friedman JM. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1995;1:1155-1161

Mar C and Bent S. An evidence-based review of the 10 most commonly used herbs. West J Med 1999;171:168-71

Maruyama Y, Nishida M, Sugimoto Y, Tanabe S, Turner JH, Kozasa T, Wada T, Nagao T and Kurose H. $G\alpha_{12/13}$ mediates α_1-adrenergic receptor-induced cardiac hypertrophy. Circ Res 2002;91:961-969

Maulik SK and Katiyar CK. Terminalia arjuna in cardiovascular diseases: making the transition from traditional to modern medicine in Iindia. Curr Pharm Biotechnol 2010;11:855-860

McGaffin KR, Witham WG, Yester KA, Romano LC, O'Doherty RM, McTiernan CF and O'Donnell CP. Cardiac-specific leptin receptor deletion exacerbates ischaemic heart failure in mice. Cardiovasc Res 2011;89:60-71

McMullen JR and Jennings GL. Differences between pathological and physiological cardiac hypertrophy: novel therapeutic strategies to treat heart failure. Clin Exp Pharmacol Physiol 2007;34:255-262

McMurray JJ and Stewart S. Epidemiology, aetiology and prognosis of heart failure. Heart 2000;83:596-602

Mihl C, Dassen WR and Kuipers H. Cardiac remodelling: concentric versus eccentric hypertrophy strength and endurance athletes. Neth Heart J 2008;16:129-133

Mita S, Kobayashi N, Yoshida K, Nakano S and Matsuoka H. Cardioprotective mechanisms of Rho-kinase inhibition associated with eNOS and oxidative stress-LOX-1 pathway in Dahl salt-sensitive hypertensive rats. J Hypertens 2005;23:87-96

Mitchell S, Ota A, Foster W, Zhang B, Fang Z, Patel S, Nelson SF, Horvath S and Wang Y. Distinct gene expression profiles in adult mouse heart following targeted MAP kinase activation. Physiol Genomics 2006;25:50-59

Moey M, Rajapurohitam V, Zeidan A and Karmazyn M. Ginseng (*Panax quinquefolius*) attenuates leptin-induced cardiac hypertrophy via inhibition of p115RhoGEF-RhoA/ROCK-dependent MAPK signalling pathways. J Pharm Exp Ther 2011;339:746-756

Mokhtari D, Myers JW and Welsh N. The MAPK kinase kinase-1 is essential for stress-induced pancreatic islet cell death. Endocrinology 2008;149:3046-3053

Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR and Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 1998;93:215-228

Morgan DA, Thedens DR, Weiss R and Rahmouni K. Mechanisms mediating renal sympathetic activation to leptin in obesity. Am J Physiol Regul Integr Comp Physiol 2008;295:R1730-R1736

Morisaki N, Watanabe S, Tezuka M, Zenibayashi M, Shiina R, Koyama N, Kanzaki T and Saito Y. Mechanism of angiogenic effects of saponin from ginseng Radix rubra in human umbilical vein endothelial cells. Br J Pharmacol 1995;115:1188-93

Morrissey RP, Czer L and Shah PK. Chronic heart failure: current evidence, challenges to therapy and future directions. Am J Cardiovasc Drugs 2011;11:153-171

Mudgett J, Ding J, Guh-Siesel L, Chartrain N, Yang L, Gopal S and Chen M. Essential role for p38α mitogen-activated protein kinase in placental angiogenesis. Proc Natl Acad Sci USA 2000;97:10454-10459

Muslin AJ. MAPK signalling in cardiovascular health and disease: molecular mechanisms and therapeutic targets. Clin Sci (Lond) 2008;115:203-218

Myers MG Jr. Leptin receptor signaling and the regulation of mammalian physiology. Recent Prog Horm Res 2004;59:287-304

Nabel EG. Cardiovascular disease. N Engl J Med 2003;349:60-72

Namba T, Matsushige K, Morita T and Tanaka O. Saponins of plants of Panax species collected in central Nepal and their chemotaxonomical significance. Chem Pharm Bull (Tokyo) 1986;34:730-738

Napoleone E, DI Santo A, Amore C, Baccante G, di Febbo C, Porreca E, de Gaetano G, Donati MB and Lorenzet R. Leptin induces tissue factor expression in human peripheral blood mononuclear cells: a possible link between obesity and cardiovascular risk? J Thromb Haemost 2007;5:1462-1468

Noma K, Oyama N and Liao JK. Physiological role of ROCKs in the cardiovascular system. Am J Physiol Cell Physiol 2006;290:C661-C668

O'Meara E, Solomon S, McMurray J, Pfeffer M, Yusuf S, Michelson E, Granger C, Olofsson B, Young JB and Swedberg K. Effect of candesartan on New York Heart Assocation functional class. Results of the Candesartan in Heart failure: Assessment of Reduction in Mortality and morbidity (CHARM) programme. Eur Heart J 2004;25:1920-1926

Oh JK, Appleton CP, Hatle LK, Nishimura RA, Sweard JB and Tajik AJ. The noninvasive assessment of left ventricular diastolic function with two-dimensional and Doppler echocardiography. J Am Soc Echo 1997;10:246-270

Opie LH, Frishman WH. Thadani U. (1995) Calcium channel antagonists. In: Opie LH, ed. *Drugs for the Heart*, 4th edn. W.B. Saunders, Philadelphia: pp. 50-82

Owan TE, Hodge DO, Herges RM, Jacobsen SJ, Roger VL and Redfield MM. Trends in prevalence and outcome of heart failure with preserved ejection. N Engl J Med 2006;355:251-259

Packer M, Kessler PD and Lee WH. Calcium-channel blockade in the management of severe chronic congestive heart failure: a bridge too far. Circ 1987;75:V56-V64

Pan B, Ren H, Ma Y, Liu D, Yu B, Ji L, Pan L, Li J, Yang L, Lv X, Shen X, Chen B, Zhang Y, Willard B, He Y and Zhang L. HDL of patients with type 2 diabetes mellitus elevates the capability of promoting migration and invasion of breast cancer cells. Int J Cancer 2011; Jun 29, Epub ahead of print.

Pan J, Singh UH, Takahashi T, Okay Y, Palm-Leis A, Herbelin BS and Baker KM. PKC mediates cyclic stretch-induced cardiac hypertrophy through Rho family GTPases and mitogen-activated protein kinases in cardiomyocytes. J Cell Physiol 2005;202:536-553

Park IH, Kim NY, Han SB, Kim JM, Kwon SW, Kim HJ, Park MK and Park JH. Three new dammarane glycosides from heat processed ginseng. Arch Pharm Res 2002;25:428-32

Patel SB, Reams GP, Spear RM, Freeman RH and Villarreal D. Leptin: linking obesity, the metabolic syndrome and cardiovascular disease. Curr Hypertension Rep 2008;10:131-137

Peralta EA, Murphy LL, Minnis J, Louis S and Dunnington GL. American ginseng inhibits induced COX-2 and NFKB activation in breast cancer cells. J Surg Res 2009;157:261-7

Phillips CO, Kashani A, Ko DK, Francsi G and Krumholz HM. Adverse effects of combination angiotensin II receptor blockers plus angiotensin-converting enzyme inhibitors for left ventricular dysfunction: a quantitative review of data from randomized clinical trials. Arch Intern Med 2007;167:1930-1936

Piñieiro R, Iglesias MJ, Eiras S, Viñuela J, Lago F and González-Juanatey JR. Leptin does not induce hypertrophy, cell cycle alterations, or production of MCP-1 in cultured rat and mouse cardiomyocytes. Endocr Res 2005;31:375-386

Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, Bittman R, Hurley S, Kleiman J, Gatlin M; Eplerenone Post-acute Myocardial infarction Heart Failure Efficacy and Survival Study Investigators. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. N Engl J Med 2003;348:1309-21

Porchia F, Papucci M, Gargini C, Asta A, De Marco G, Agretti P, Tonacchera M and Mazzoni MR. Endothelin-1 up-regulates p115RhoGEF in embryonic rat cardiomyocytes during the hypertrophic response. J Recept Signal Transduct Res 2008;28:265-283.

Proud CG. Ras, PI3-kinase and mTOR signaling in cardiac hypertrophy. Cardiovasc Res 2004;63:403-413

Purcell N, Wilkins BJ, York A, Saba-El-Leil M, Meloche S, Robbins J and Molkentin J. Generic inhibition of cardiac ERK1/2 promotes stress-induced apoptosis and heart failure but has no effect on hypertrophy in vivo. Proc Natl Acad Sci USA 2007;104:14074-14079

Purdham DM, Zou MX, Rajapurohitam V and Karmazyn M. Rat heart is a site of leptin production and action. Am J Physiol Heart Circ Physiol 2004;287:H2877-2284

Qi LW, Wang CZ and Yuan CS. Isolation and analysis of ginseng: advances and challenges. Nat Prod Rep 2011;28:467-495

Rahmouni K. Leptin-induced sympathetic nerve activation: signalling mechanisms and cardiovascular consequences in obesity. Curr Hypertens Rev 2010;6:104-209

Rajapurohitam V, Gan XT, Kirshenbaum LA and Karmazyn M. The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. Circ Res 2003;93:277-279.

Raman M, Earnest S, Zhang K, Zhao Y and Cobb MH. TAO kinases mediate activation of p38 in response to DNA damage. EMBO J 2007;26:2005-2014

Resuch HP, Zimmermann S, Schaefer M, Paul M and Moelling K. Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. J Biol Chem 2011;276:33630-33637

Ridley AJ and Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992;70:389-399

Rikitake Y, Oyama N, Wang CYC, Noma K, Satoh M, Kim HH and Liao JK. Decreased perivascular fibrosis but not cardiac hypertrophy in ROCK1$^{+/-}$ haploinsufficient mice. Circ 2005;112:2959-2965

Roberts PJ and Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 2007;26:3291-3310

Rose BA, Force T and Wang Y. Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale. Physiol Rev 2010;1507-1546

Rossman KL, Der CJ and Sondek J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nat Rev Mol Cell Biol 2005;6:167-180.

Ruehr ML, Russell MA and Bond M. A-kinase anchoring protein targeting of protein kinase A in the heart. J Mol Cell Cardiol 2004;37:653-665

Ruwhof C and van der Laarse A. Mechanical stress-induced cardiac hypertrophy: mechanisms and signal transduction pathways. Cardiovasc Res 2000;47:23-37

Sader S, Nian M and Liu P. Leptin: a novel link between obesity, diabetes, cardiovascular risk and ventricular hypertrophy. Circ 2003;108:644-646

Sagawa K. The ventricular pressure-volume diagram revisited. Circ Res 1978;43:677-687

Sakata Y, Masuyama T, Yamamoto K, Nishikawa N, Yamamoto H, Kondo H, Ono K, Otsu K, Kuzuya T, Miwa T, Takeda H, Miyamoto E and Hori M. Calcineurin inhibitor attenuates left ventricular hypertrophy, leading to prevention of heart failure in hypertensive rats. Circulation 2000;102:2269-2275

Sanada S, Kondon N, Shoji J, Tanaka O and Shibata S. Studies on the saponin of ginseng I. Structures of ginsenoside-Ro, -Rb1, -Rc, and –Rd. Chem Pharm Bull (Tokyo) 1974;22:421-428

Satoh S, Ueda Y, Koyanagi M, Kadokami T, Sugano M, Yoshikawa Y and Makino N. Chronic inhibition of Rho kinase blunts the process of left ventricular hypertrophy leading to cardiac contractile dysfunction in hypertension-induced heart failure. J Mol Cell Cardiol. 2003;35:59-70

Schiller MR. Coupling receptor tyrosine kinases to Rho GTPases-GEFs: what's the link. Cell Signal 2006;181834-43

Schmidt A and Hall A. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev 2002;16:1587-1609.

Schneiderman J, Simon AJ, Schroeter MR, Flugelman MY, Konstantinides S and Schaefer K. Leptin receptor is elevated in carotid plaques from neurologically symptomatic patients and positively correlated with augments macrophage density. J Vasc Surg 2008;48:1146-1155

Schonwasser DC, Marais RM, Marshall CJ and Parker PJ. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel and atypical protein kinase C isotypes. Mol Cel Biol 1998;18:790-798

Schram K, De girolamo S, Madani S, Munoz D, Thong F and Sweeney G. Leptin regulates MMP-2, TIMP-1 and collagen synthesis via p38 MAPK in HL-1 murine cardiomyocytes. Cell Mol Biol let 2010;15:551-563

Schulze PC, Kratzsch J, Linke A, Schoene N, Adams V, Gielen S, Erbs S, Moebius-Winkler S and Schuler G. Elevated serum levels of leptin and soluble leptin receptor in patients with advanced chronic heart failure. Eur J Heart Fail 2003;5:33-40.

Schwartz MW, Peskind E, Raskind M, Boyko EJ and Porte D Jr. Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. Nat Med 1996;2:589-593

Shama J, Garcia-Medina R, Pouysségur J and Vial E. Major contribution of MEK1 to the activation of ERK1/ERK2 and to the growth of LS174T colon carcinoma cells. Biochem Biophys Res Commun 2008;372:845-849

Shibata S, Fujita M, Itokawa H, Tanko O and Ishii T. Studies on the constituents of Japanese and Chinese Crude Drugs. XI. Panaxadiol, a sapogenin of ginseng roots (1). Chem Pharm Bull (Tokyo) 1963;11:759-761

Shibuya I, Utsunomiya K, Toyohira Y, Ueno S, Tsutsui M, Cheah TB, Ueta Y, Izumi F and Yanagihara N. Regulation of catecholamine synthesis by leptin. Ann NY Acad Sci 2002;971:522-527

Siehler S. Regulation of RhoGEF proteins by $G_{12/13}$-coupled receptors. Br J Pharmacol 2009;158:41-49

Singh P, Peterson TE, Barber KR, Kuniyoshi FS, Jensen A, Hoffmann M, Shamsuzzaman AS and Somers VK. Leptin upregualted the expression of plasminogen activator of inhibitor-1 in human vascular endothelial cells. Biochem Biophys Res Commun 2010;392:47-52

Skeggs LT, Dorer F, Kahn JR, Lentz KE and Levine M. The biochemistry of the renin-angiotensin system and its role in hypertension. Am J Med 1976;60:737-748

Solaro RJ. Sarcomere control mechanisms and the dynamics of the cardiac cycle. J Biomed Biotechnol 2010;2010:1-8

Souchet M, Portales-Casamar E, Mazurais D, Schmidt S, Léger I, Javré JL, Robert P, Berrebi-Bertrand I, Bril A, Gout B, Debant A and Calmels TP. Human p63RhoGEF, a novel RhoA-specific guanine nucleotide exchange factor, is localized in cardiac sarcomere. J Cell Sci 2002;115:629-640

Sowers JR. Obesity as a cardiovascular risk factor. Am J Med 2003;8:37-41

Stavro PM, Woo M, Heim TF, Leiter LA and Vuksan V. North American ginseng exerts a neutral effect on blood pressure in individuals with hypertension. Hypertension 2005;46:406-411

Stavro PM, Woo M, Leiter LA, Heim TF, Sievenpiper JL and Vuksan V. Long-term intake of North American ginseng has no effect on 24-hour blood pressure and renal function. Hypertension 2006;47:791-796

Sugden PH. RGS proteins to the rescue. J Mol Cell Cardiol 2001;33:189-195

Sugden PH and Clerk A. Activation of the small GTP-binding proteins Ras in the heart by hypertrophic agonists. Trends Cardiovasc Med 2000;10:1-8

Sung J, Han KH, Zo JH, Park HJ, Kim CH and Oh BH. Effects of red ginseng upon vascular endothelial function in patients with essential hypertension. Am J Chin Med 2000;28:205-216

Sussman MA, Lim HW, Gude N, Taigen T, Olson EN, Robbins J, Colbert MC, Gualberto A, Wieczorek DF and Molkentin JD. Prevention of cardiac hypertrophy in mice by calcineurin inhibition. Science 1198;281:1690-1693

Suzuki N, Nakamura S, Mano H and Kozasa T. Gα12 activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. PNAS 2003;100:733-738

Sweeney G. Cardiovascular effects of leptin. Nat Rev Cardiol 2010;7:22-29

Taigen T, De Windt LJ, Lim HW and Molkentin JD. Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. Proc Natl Acad Sci USA 2000;97:1196-1201

Tartaglia LA. The leptin receptor. J Biol Chem 1997;272:6093-6093

Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA and Tepper RI. Identification and expression cloning of a leptin receptor, OB-R. Cell 83:1263-1271

Takeishi Y, Huang Q, Abe J, Che W, Lee JD, Kawakatsu H, Hoit BD, Berk BC and Walsh RA. Activation of mitogen-activated protein kinases and p90 ribosomal S6 kinase in failing human hearts with dilated cardiomyopathy. Cardiovasc Res 2002;53:131-137

Tamura K, Sudo T, Senftleben U, Dadak A, Johnson R and Karin M. Requirement for p38alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. Cell 2000;102:221-231

Tamura T, Said S, Harris J, Lu W and Gerdes M. Reverse remodeling of cardiac myocytes hypertrophy in hypertension and failure by targeting of the renin-angiotensin system. Circ 2000;102:253-259

Tawab MA, Bahr U, Karas M, Wurglics M and Schubert-Zsilavecz M. Degradation of ginsenosides in humans after oral administration. Drug Metabol and Disp 2003;31:1065-1071

The CONSENSUS Trial Study Group. Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). N Engl J Med 1987;316:1429-1435

Torsoni AS, Marin TM, Velloso LA and Franchini KG. RhoA/ROCK signaling is critical to FAK activation by cyclic stretch in cardiac myocytes. Am J Physiol Heart Circ Physiol 2005;289:H1488-1496

Tung NH, Song GY, Minh CV, Kiem PV, Jin LG, Boo HJ, Kang HK and Kim YH. Steamed ginseng-leaf components enhance cytotoxic effects on human leukemia HL-60 cells. Chem Pharm Bull (Tokyo) 2010;58:1111-1115

Ueno M, Miyauchi T, Sakai S, Kobayashi T, Goto K and Yamaguchi I. Effects of physiological or pathological pressure load in vivo on myocardial expression on ET-1 and receptors. Am J Physiol 1999;277:R1321R-1320

Ueyama T, Kawashima S, Sakoda T, Rikitake Y, Ishida T, Kawai M, Yamashita T, Ishido S, Hotta H and Yokoyama M. Requirement of activation of the extracellular signal-regulated kinase cascade in myocardial cell hypertrophy. J Mol Cell Cardiol 2000;32:947-960

van Heerebeek L, Borbély A, Niessen HWM, Bronzwaer JGF, van der Velden J, Stienen GJM, Linke WA, Laarman GJ and Paulus WJ. Myocardial structure and function differ in systolic and diastolic heart failure. Circ 2006;113:1966-1973

Veliotes DG, Norton GR, Correia RJ, Strijdom H, Badenhorst D, Brooksbank R and Woodiwiss AJ. Impact of aldosterone receptor blockade on the deleterious cardiac effects of adrenergic activation in hypertensive rats. J Cardiovasc Pharmacol 2010;56:203-211

Wang N, Guan P, Zhang JP, Li YQ, Chang YZ, Shi ZH, Wang FY and Chu L. Fasudil hydrochloride hydrate, a Rho-kinase inhibitor, suppresses isoproterenol-induced heart failure in rats via JNK and ERK1/2 pathways. J Cell Biochem 2011;112:1920-1929

Wang Y, Huang S, Sah VP, Ross J Jr, Brown JH, Han J and Chien KR. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. J Biol Chem 1998;273:2161-2168

Watanabe K, Ma M, Hirabayashi K, Gurusamy N, Veeraveedu PT, Prakash P, Zhang S, Muslin AJ, Kodama M and Aizawa Y. Swimming stress in DN 14-3-3 mice triggers maladaptive cardiac remodeling: role of p38 MAPK. Am J Physiol Heart Circ Physiol 2007;292:H1269-1277

Wilkins BJ, Dai Y, Bueno O, Parsons SA, Xu J, Plank DM, Jones F, Kimball R and Molkentin JD. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. Circ Res 2004;94:110-118

Wollert KC and Drexler H. The renin-angiotensin system and experimental heart failure. Cardiovasc Res 1999;43:838-849

Wood MJ, Stewart RL, Merry H, Johnstone DE, and Cox JI. Use of complementary and alternative medical therapies in patients with cardiovascular disease. Am Heart J 2003;145:806-12

Wood WB, Roh BL and White RP. Cardiovascular actions of Panax ginseng in dogs. Jpn J Pharmacol 1964;14:284-94

Wu GS. Role of mitogen-activated protein kinase phosphatases (MKPs) in cancer. Cancer Metastasis Rev 2007;26:579-585

Xiao L, Pimental DR, Amin JK, Singh K, Sawyer DB and Colucci WS. MEK1/2-ERK1/2 mediates alpha1-adrenergic receptor-stimulated hypertrophy in adult rat ventricular myocytes. J Mol Cell Cardiol 2011;33:779-787

Yamada H, Tsushima T, Murakami H, Uchigata Y and Iwamoto Y. Potentiation of mitogenic activity of platelet-derived growth factor by physiological concentrations of insulin via the MAP kinase cascade in rat A10 vascular smooth muscle cells. Int J Exp Diabetes Res 2002;3:131-144

Yamakawa T, Tanaka S, Numaguchi K, Yamakawa Y, Motley ED, Ichihara S and Inagami T. Involvement of Rho-kinase in angiotensin II-induced hypertrophy of rat vascular smooth muscle cells. Hypertension 2000;35:313-318

Yanazume T, Hasegawa K, Wada H, Morimoto T, Abe M, Kawamura T and Sasayama S. Rho/ROCK pathway contributes to the activation of extracellular signal-regulated kinase/GATA-4 during myocardial cell hypertrophy. J Biol Chem 2002;277:8618-8625

Yang HY, Liu JC, Chen YL, Chen CH, Lin H, Lin JW, Chiu WT, Chen JJ and Cheng TH. Inhibitory effect of trilinolein on ET-1-induced *c-fos* gene expression in cultured neonatal rat cardiomyocytes. Naunyn-Schmiedeberg's Arch Pharmacol 2005;372:160-167

Yang R and Barouch LA. Leptin signaling and obesity: cardiovascular consequences. Circ Res 2008;101:545-559

Ying Z, Yue P, Xu X, Zhong M, Sun Q, Mikolaj M, Wang A, Brook RD, Chen LC and Rajagopalan S. Air pollution and cardiac remodeling: a role for RhoA/Rho-kinase. Am J Physiol Heart Circ Physiol 2009;296:H1540-1550

Yoon S and Seger R. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors 2006;21:21-44

You JS, Huang HF and Chang YL. *Panax ginseng* reduces adriamycin-induced heart failure in rats. Phytother Res 2005;19:1018-1022

Young JB, Dunlap ME, Pfeffer MA, Probstfield JL, Cohen-Solal A, Dietz R, Granger CB, Hradec J, Kuh J, McKelvie RS, McMurray JJ, Michelson EL, Olofsson B, Ostergen J, Held P, Solomon SD, Yusuf S and Swedberg K; Candesartan in Heart failure Assessment of Reduction in Mortality and morbidity (CHARM) Investigators and Committees. Mortality and morbidity reduction with Candesartan in patients with chronic heart failure and left ventricular systolic dysfunction: results of the CHARM low-left ventricular ejection fraction trials. Circ 2004;110:2618-2626

Yusuf S, Pepine CJ, Garces C, Pouleur H, Salem D, Kostis J, Benedict C, Rousseau M, Bourassa M and Pitt B. Effect of enalapril on myocardial infarction and unstable angina in patients with low ejection fractions. Lancet 1992;340:1173-1178

Zeidan A, Hunter JC, Javadov S and Karmazyn M. mTOR mediates RhoA-dependent leptin-induced cardiomyocyte hypertrophy. Mol Cell Biochem 2011;352;99-108

Zeidan A, Javadov S and Karmazyn M. Essential role of Rho/ROCK-dependent processes and actin dynamics in mediating leptin-induced hypertrophy in rat neonatal ventricular myocytes. Cardiovasc Res 2006;72:101-111.

Zeidan A, Javadov S, Chakrabarti S and Karmazyn M. Leptin-induced cardiomyocyte hypertrophy involves selective caveolae and RhoA/ROCK-dependent p38 MAPK translocation to nuclei. Cardiovasc Res 2008;77:64-72.

Zeidan A, Paylor B, Steinhoff KJ, Javadov S, Rajapurohitam V, Chakrabarti S and Karmazyn M. Actin cytoskeleton dynamics promotes leptin-induced vascular smooth muscle

hypertrophy via RhoA/ROCK and phosphatidylinositol 3-kinase/protein kinase B-dependent pathways. J Pharmacol Exp Ther 2007;322;1110-1116

Zhang F, Basinski MB, Beals JM, Briggs SL, Churgay LM, Clawson DK, DiMarchi RD, Furman TC, Hale JE, Hsiung HM, Schoner BE, Smith DP, Zhang XY, Wery JP and Schevitz RW. Crystal structure of the *obese* protein leptin-E100. Nature (London) 1997;387:206-209

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L and Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994;372:425-432

Zhao H, Lv Dan, Zhang W, Dong W, Feng J, Xiang Z, Huang L, Qin C and Zhang L. Ginsenoside-Rb1 attenuates dilated cardiomyopathy in cTnTR141w transgenic mouse. *J* Pharmacol Sci 2010;112:214-222

Zheng M, Dilly K, Santos Cruz JD, Li M, Gu Y, Ursitti JA, Chen J, Ross J, Chien KR, Lederer JW and Wang Y. Sarcoplasmic reticulum calcium defect in Ras-induced hypertrophic cardiomyopathy heart. Am J Physiol 2004;286:H424-H433

Zhu W, Zou Y, Shiojima I, Kudoh S, Aikawa R, Hayashi D, Mizukami M, Toko H, Shibasaki F, Yazaki Y, Nagai R and Komuro I. Ca^{2+}/calmodulin-dependent kinase II and calcineurin play critical roles in endothelin-1-induced cardiomyocyte hypertrophy. J Biol Chem 2000;275:15239-15245

Zou Y, Komuro I, Yamazaki T, Aikawa R, Kudoh S, Shiojima I, Hiroi Y, Mizuno T and Yazaki Y. Protein kinase C, but not tyrosine kinases or Ras, plays a critical role in angiotensin II-induced activation of Raf-1 kinase and extracellular signal-regulated protein kinases in cardiac myocytes. J Biol Chem 1996;271:33592-33597

CHAPTER 2:

Ginseng (*Panax quinquefolius*) attenuates leptin-induced ventricular cardiac hypertrophy through inhibition of the p115RhoGEF-RhoA/ROCK-dependent MAPK pathway activation

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2.1 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 which 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). Interestingly, plasma levels of leptin are elevated in patients with heart failure independently of obesity (Schulze et al., 2003).

Several molecular signalling pathways upregulated by leptin in cardiac hypertrophy have been identified namely the RhoA/ROCK and p38 and ERK1/2 pathways (Frühbeck, 2006; Zeidan et al., 2006; Zeidan et al., 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; Zeidan et al., 2008). Although we have previously demonstrated the potent stimulation of the RhoA/ROCK pathway by leptin, the specific mechanism of activation however has not yet been defined. Regulators of small G-proteins such as RhoA include GTP-ase activating proteins, which hydrolyze GTP to GDP thereby

deactivating RhoA, guanine nucleotide dissociation inhibitors which sequester GDP-bound small G-proteins in the cytoplasm and guanine nucleotide exchange factors (GEFs) which 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 upregulation of RhoA/ROCK in cardiac hypertrophy in response to GPCR-linked hypertrophic agonists such as ET-1 (Porchia et al, 2008) and AngII (Guilluy et al., 2010).

Ginseng (genus *Panax*) is a medicinal herb which 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 (*P. 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 following leptin addition and the role of GEFs. In addition, we studied the relationship between RhoA and MAPK pathway activation.

2.2 Materials and Methods

2.2.1 Treatment and Experimental Groups

Neonatal ventricular cardiomyocytes were isolated and cultured from one to three day old Sprague Dawley rats as previously described (Rajapurohitam et al., 2006). Cells were grown in fetal bovine serum (FBS) medium for up to 48 hours following 24 hour serum starvation. For cell size and Western blotting time-course experiments, cardiomyocytes were pre-treated with the alcoholic extract of North American ginseng (*Panax quinquefolius*). Extracts were prepared by Naturex (South Hackensack, NJ) using ginseng roots supplied from 5 different farms in Ontario, Canada (provided by Dr. Edmund Lui's lab at the University of Western Ontario) as previously described (Guo et al,. 2011) and were studied at concentrations of 0.1, 1, 10 and 100 μg/ml for up to 24 hours 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 (Maffei et al., 1995). For all subsequent experiments, cells were pre-treated with a ginseng concentration of 10 μg/ml for 1 hour in the presence or absence of leptin for up to 24 hours. 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 published by the US National Institutes of Health and the Canadian Council of Animal Care (Ottawa, Ontario, Canada).

2.2.2 Cell Surface Area Measurement

Cardiomyocyte images were taken with a Leica microscope (Leica, Westzlar, Germany) equipped with an Infinity 1 camera at 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.

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

RNA was collected from cultured and treated neonatal ventricular cardiomyocytes using Trizol Reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions and reverse transcribed to complementary DNA (cDNA) for real-time polymerase chain reaction analysis of αSA, myosin heavy chain α, p115RhoGEF and p63RhoGEF. Briefly, cDNA was synthesized from 4 μg of total RNA using random primers (Invitrogen, Carlsbad, CA) and M-MLV (Moloney Murine Leukemia Virus) Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. The reaction was performed with a SYBR® (Synergy Brands) 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 (Invitrogen) for the genes of interest are listed in Table 2.1. PCR cycle conditions involved 40 cycles of denaturation at 95°C for 30s, followed by annealing at 60°C for 30s and finally by elongation at 72°C for 30s. The housekeeping gene, 18S, was measured and quantified to normalize cDNA levels.

2.2.4 [^{3}H] Leucine Incorporation Measurement

Leucine incorporation was performed as previously described (Zeidan et al., 2006) to analyze protein synthesis under different experimental conditions. Cardiomyocytes were cultured in 24-well Primaria culture plates for 48 hours in serum media following by 24 hour serum starvation. Leptin was administered with or without ginseng pre-treatment in the presence of 2 μCi of [^{3}H]-leucine for 24 hours. Myocytes were washed the following day with cold PBS and

proteins were precipitated with 5% trichloroacetic acid (TCA) for 30 minutes on ice. Protein precipitates were dissolved with 0.5N NaOH following with two washes of cold 5% TCA. 0.5N HCl was used to neutralize the precipitates and the total radioactivity was measured by liquid scintillation.

2.2.5 Isolation of Cytosolic-Enriched and Membrane Fractions

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

2.2.6 Western Blotting

Total cellular lysates were collected using a lysis buffer and protease cocktail inhibitor mixture as previously described (Zeidan et al., 2006; Zeidan et al., 2008) for the measurement of proteins of interest. Proteins were loaded equally on either 7.5%, 10% or 15% SDS (sodium dodecyl sulfate) gels as appropriate after protein quantification *via* BioRad Reagent (BioRad, Hercules, CA) per the manufacturer's instructions. For time course experiments, ventricular cardiomyocytes were treated for 5, 10, 15, 30 or 60 minutes with leptin in the presence or absence of ginseng (10 μg/ml). For the quantification of cofilin-2 phosphorylation, cells were pre-treated with ginseng for 1 hour followed by administration of leptin for 10 minutes. For all

additional protein measurements, cells were pre-treated with ginseng for 1 hour in the presence or absence of leptin for 24 hours. The primary antibodies and respective dilutions used in this study include total (1:1000 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and phosphorylated p38 (Thr180/Tyr182) forms (1:1000 dilution, Cell Signaling, Danvers, MA), total (1:1000 dilution, Santa Cruz) and phosphorylated ERK1/2 (Thr202/Tyr204) forms (1:1000 dilution, Cell Signaling), actin (1:1000 dilution, Cytoskeleton Inc., Denver, CO), p115RhoGEF (1:250 dilution, Santa Cruz), p63RhoGEF (1:200 dilution, Santa Cruz) and phosphorylated (1:1000 dilution, Santa Cruz) and total cofilin-2 (1:1000 dilution, Millipore, Billerica, MA). Goat-anti-rabbit IgG (immunoglobulin) and goat-anti-mouse IgG HRP (horseradish peroxidase) conjugate (BioRad) were used at 1:5000 dilution and donkey-anti-goat IgG HRP conjugate (Santa Cruz) was used at 1:10,000 dilution as appropriate. β-actin (1:1000 dilution, Cytoskeleton) and PCNA (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.

2.2.7 GST-RhoG17A Bead Preparation

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

2.2.8 Co-immunoprecipitation 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 previously described (Garcia-Mata et al., 2006; Kakiashvili et al., 2009).

2.2.9 Immunofluorescence

Cells 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 media for 24 hours followed by serum-free media starvation for an additional 24 hours before appropriate treatment. Immunofluorescence measurements from cells administered leptin for 3 hours were performed for total p38 and total ERK1/2, 10 minutes for p115RhoGEF and RhoA and 24 hours for G/F actin with or without ginseng pre-treatment.

Total p38, total ERK1/2, p115RhoGEF and RhoA immunofluorescence. Cells were fixed with 2:5 acetone-methanol for 1 hour at 4°C followed by permeabilization of cells for 15 minutes with 0.2% (v/v) Triton X-100 and blocking with blocking solution (1% BSA, 0.1% Triton X-100) for 1 hour. 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 AlexFluor-488 (Invitrogen) or IgG anti-rabbit AlexFluor-596 (Invitrogen) (1:250 dilution) in 2% BSA in PBS A for 1 hour at room temperature under light-free conditions. For detection of the nucleus, cells were incubated with Hoechst dye for 30 minutes prior to mounting on microscope slides (VWR, West Chester, PA) for image capture using a Zeiss (Oberkochen, Germany) inverted fluorescence microscope at 630X magnification.

G and F actin immunofluorescence. Cells were prepared as previously described (Albinsson et al., 2004). Cells were fixed with 3.7% (w/v) paraformaldehyde in PBS A for 1 hour 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-FITC and 10 μg/ml deoxyribonuclease I (DNase I), Texas Red conjugate in 2% BSA with 0.1% Triton X-100 in PBS A for 1 hour at room temperature under light-free conditions. Similarly, Hoechst dye was used to detect the nucleus and glass cover slips were subsequently mounted onto microscope slides (VWR) for visualization of G/F actin. All immunofluorescence images shown in results are representative of a minimum of 3 independent experiments.

2.2.10 Measurement of p115RhoGEF and RhoA Co-localization

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

2.2.11 Measurement of RhoA Activity

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

2.2.12 p115RhoGEF Activity Assay

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

2.2.13 G/F Actin Measurement

Globular (G) and filamentous (F) actin were isolated using ultracentrifugation as previously described (Albinsson et al., 2004). Briefly cell lysates were collected and homogenized at 37°C in a lysis and F-actin stabilizing buffer (50mM PIPES, 50mM NaCl, 5mM $MgCl_2$, 5mM EGTA, 1mM ATP, 5% glycerol, 0.1% Nonidet-P40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β-mercaptoethanol, 1:100 protease inhibitor cocktail and 0.0001% anti-foam). Cell lysates were ultracentrifuged at 100,000g at 30°C for 1 hour and the supernatant (soluble, G-actin) was collected and diluted 1:2 in Laemmli buffer. The remaining pellet (F-actin) was resuspended in cold distilled water with 1 μM cytochalasin D. The resuspended pellet was kept on ice for 45 to 60 minutes to dissociate the F-actin and was then diluted 1:4 with 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 (Cytoskeleton) and quantified using spot densitometry.

2.2.14 Statistics

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

Table 2.1. Gene-specific primer sequences of analyzed genes of interest.

Gene	Forward Primer Sequence	Reverse Primer Sequence
α-skeletal actin	5'-CACGGCATTATCACCAACTG-3'	5'-CCGGAGGCATAGAGAGACAG-3'
myosin heavy chain	5'-CATCACCGGAGAATCCGGAGC-3'	5'-CTATTGAGGCCACAGTCGTC-3'
p115RhoGEF	5'-TAGAGGACTTCCGCTCCAAA-3'	5'-CAGTGACCACAGCAGCACTT-3'
p63RhoGEF	5'-TATGTGGACGACTTGGGACA-3'	5'-TGATGAACAGCTGAGCCAAC-3'
18S	5'-GTAACCCGTTGAACCCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3'

2.3 Results

2.3.1 ***Ginseng inhibits leptin-induced myocyte 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 hour prior to the addition of leptin for a total incubation time of 24 hours (Figure 2.1). As shown in Figure 2.1, leptin induced a significant increase ($p<0.05$) in cell size which was attenuated by ginseng in a concentration-dependent manner. A ginseng concentration of 10 μg/ml of ginseng was used for all subsequent experiments as this represented the lowest concentration which completely abrogated the hypertrophic response to leptin (Figure 2.1).

An increase in cell surface area in leptin treated cells after 24 hours was additionally associated with a significant increase ($p<0.05$) in protein synthesis as indicated by increased [^{3}H] leucine incorporation as well as (Figure 2.1, left panel) MYHCα (Figure 2.1, middle panel) and α-SA (2.1, right panel) gene expression, as quantified by real-time PCR. As shown in Figure 1 ginseng alone had no effect any parameter.

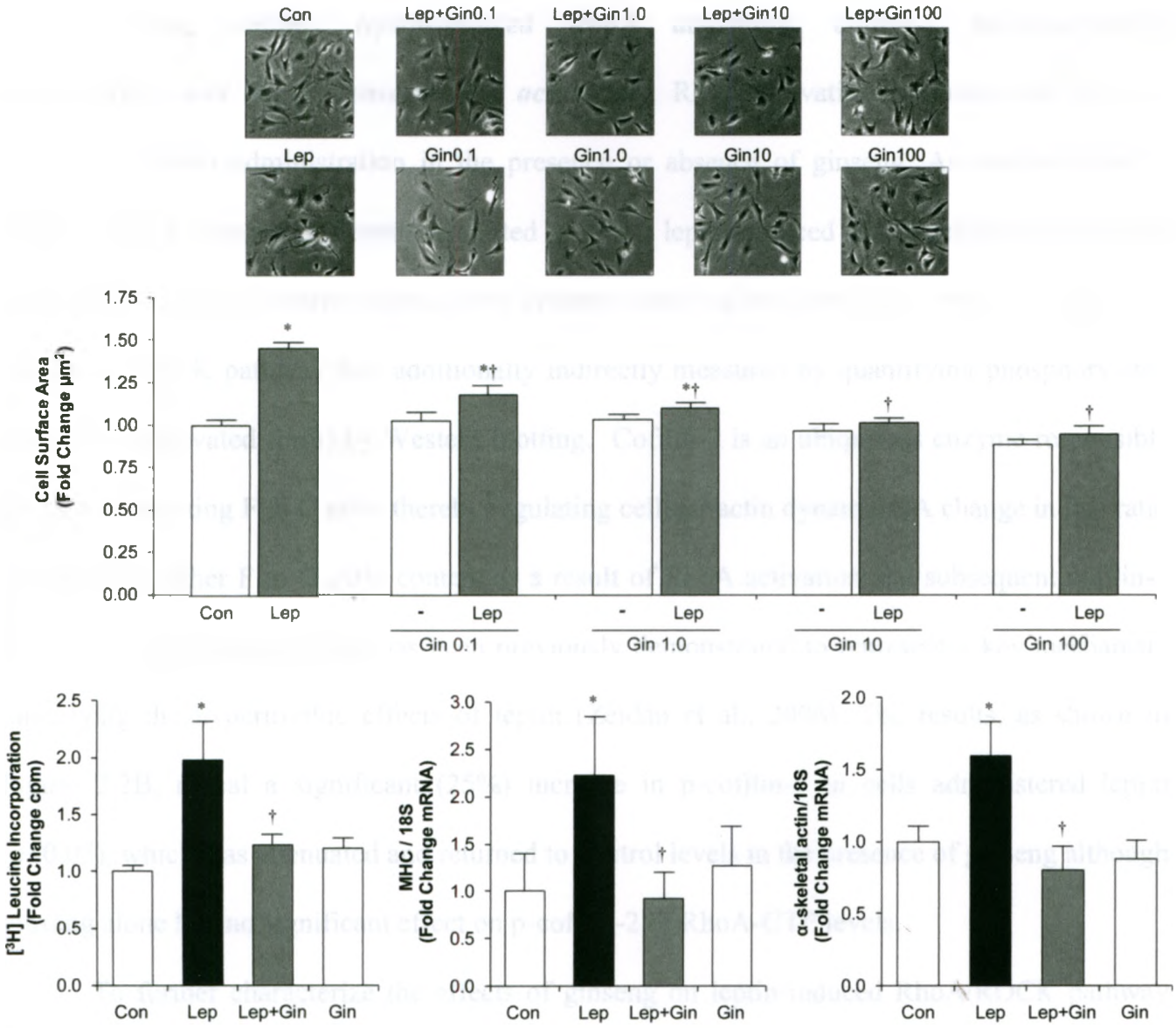

Figure 2.1. Ginseng inhibits leptin-induced increase in cell surface area, [^{3}H] leucine incorporation and expression of the gene markers of cardiac hypertrophy, α-skeletal actin and myosin heavy chain. Micrographs show representative images of neonatal ventricular cardiomyocytes with or without pre-treatment with increasing ginseng (Gin) concentrations (0.1, 1.0, 10 and 100 µg/ml, respectively) in the absence (top row) or presence (bottom row) of leptin (3.1 nM) pretreatment. Middle row shows surface area whereas bottom row shows [^{3}H] leucine incorporation (left panel), myosin heavy chain (MHC) (middle panel) and α-skeletal actin (α-SA) (right panel) with different treatments. Data represent means ± S.E.M. N=8-10 for surface area, N=6 for leucine incorporation and N=6-8 for molecular markers of hypertrophy. *p<0.05 vs. control; †p <0.05 vs. leptin. Con, control; Lep, leptin; Gin, ginseng.

2.3.2 Ginseng inhibits leptin-induced RhoA activation, cofilin-2 phosphorylation (inactivation) and the decrease in G/F actin ratio. RhoA activation was measured after 10 minutes of leptin administration in the presence or absence of ginseng. As demonstrated in Figure 2.2A, ginseng significantly inhibited ($p<0.05$) leptin-induced RhoA activation, returning RhoA-GTP levels to control values, while 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 an ubiquitous enzyme responsible for depolymerizing F to G actin thereby regulating cellular actin dynamics. A change in this ratio favouring 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 (Zeidan et al., 2006). The results, as shown in Figure 2.2B, 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 (Figure 2.3A) as well as visualization using immunofluorescence (Figure 2.3C) after 24 hours. In leptin-treated cells, the G/F actin ratio was decreased as measured by quantification of G actin (S; supernatant) and F actin (P; pellet) using Western blotting (Figure 2.3A), while pre-treatment with ginseng restored this ratio to control values (Figure 2.3B). This was similarly observed in the representative immunofluorescence images (Figure 2.3C, *2nd 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 pre-treatment with ginseng (Figure 2.3C, *3rd column*). Treatment with ginseng alone had no direct effect on the G/F actin dynamics.

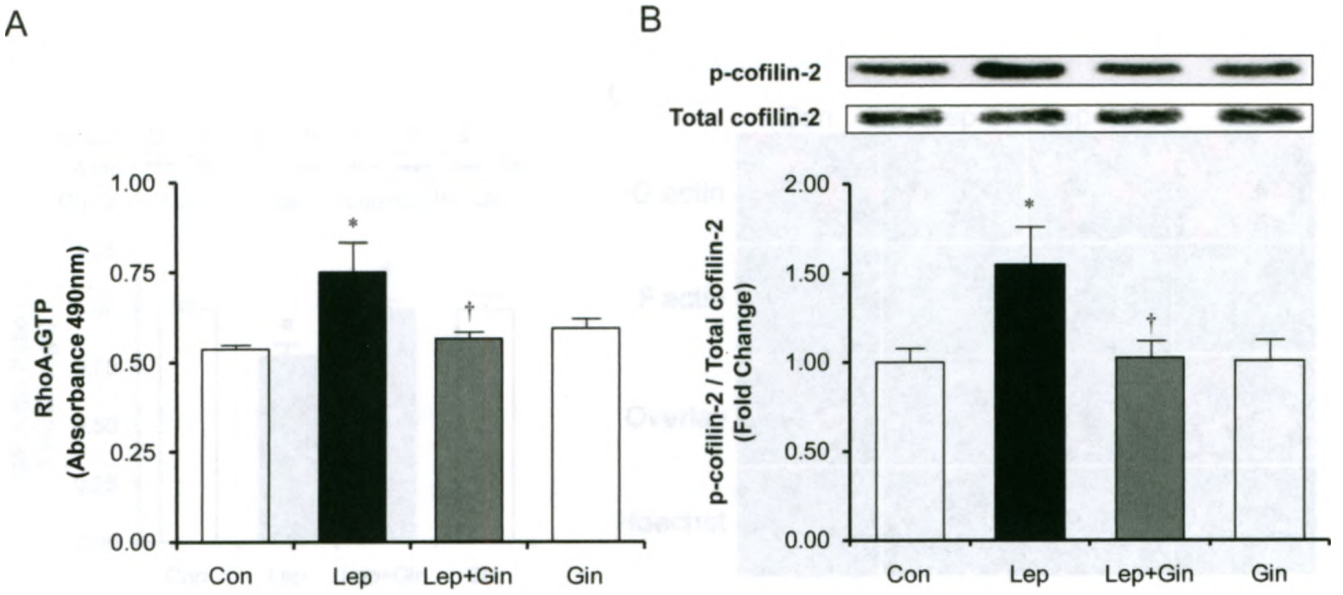

Figure 2.2. Ginseng inhibits leptin-induced RhoA activation and phosphorylation (inactivation) of cofilin-2. Panel A shows RhoA-GTP (activated RhoA) levels whereas panel B demonstrates Western blots and densitometric values for phosphorylated cofilin-2. Data represent means ± S.E.M. N=6. *p<0.05 vs. control; †p <0.05 vs. leptin. Con, control; Lep, leptin; Gin, ginseng

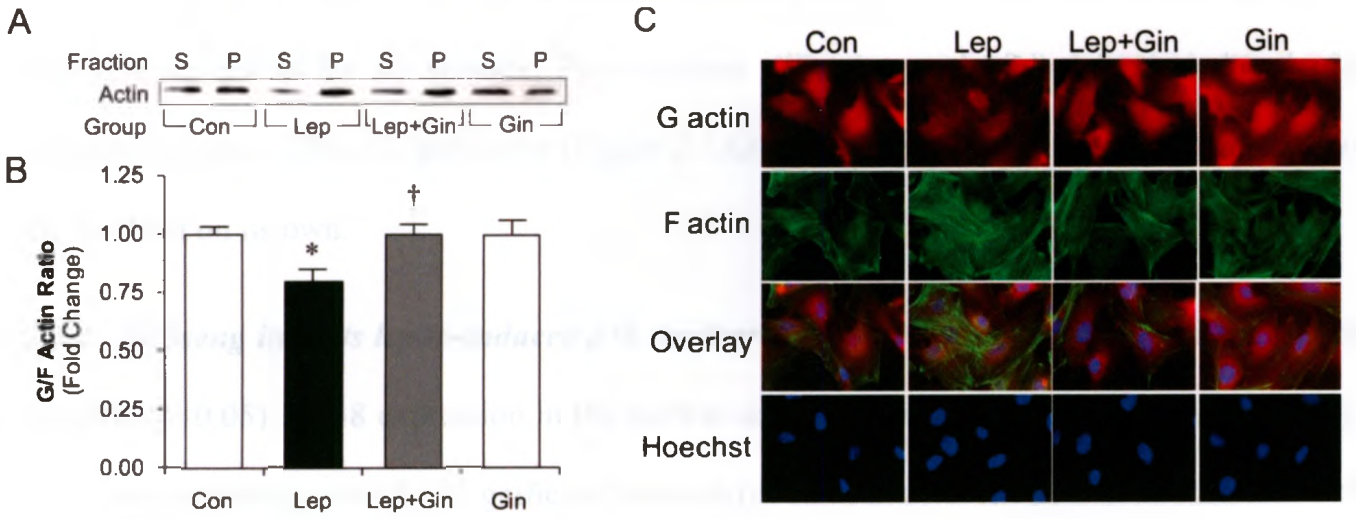

Figure 2.3. Ginseng inhibits leptin-induced decrease in G/F actin. Panel A shows Western blots for actin dynamics with respect to G actin in supernatant (S) and F actin in pellet fraction (P) with different treatments. Densitometric values are shown in panel B. Representative fluorescence images of cardiomyocytes are shown in panel C. For these studies cells were fixed on collagen-coated glass cover slips and G actin (1st row) and F actin (2nd row) were visualized with DNAse-I Texas Red Conjugate and Phalloidin-FITC, respectively. Hoechst staining was used to detect nuclei whereas overlay depicts all three stains merged. For panel B, data represent means ± S.E.M. N=6-9. *p<0.05 vs. control; †p <0.05 vs. leptin. Con, control; Lep, leptin; Gin, ginseng.

2.3.3 Ginseng inhibits leptin-induced p38 and ERK1/2 MAPK phosphorylation. Leptin significantly induced both p38 (Figure 2.4A) and ERK1/2 (Figure 2.5A) phosphorylation as early as 5 minutes after addition with maximum activation seen at 15 minutes followed by values returning to control by 30 minutes. Pre-treatment with ginseng inhibited leptin-induced p38 (Figure 2.4A) and ERK1/2 activation (Figure 2.5A) at all time points although ginseng had no direct effect on its own.

2.3.4 Ginseng inhibits leptin-induced p38 nuclear translocation. Leptin induced a significant increase ($p<0.05$) in p38 expression in the nuclear-containing membrane fraction (Figure 2.4C), which was complemented by a significant decrease ($p<0.05$) in cytosolic p38 levels, indicative of nuclear translocation in p38 in leptin-treated cells (Figure 2.4B). Nuclear translocation of p38 was further visualized by immunofluorescence (Figure 2.4D) 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 Figure 5 (panels B-D), leptin had no effect on ERK1/2 nuclear translocation.

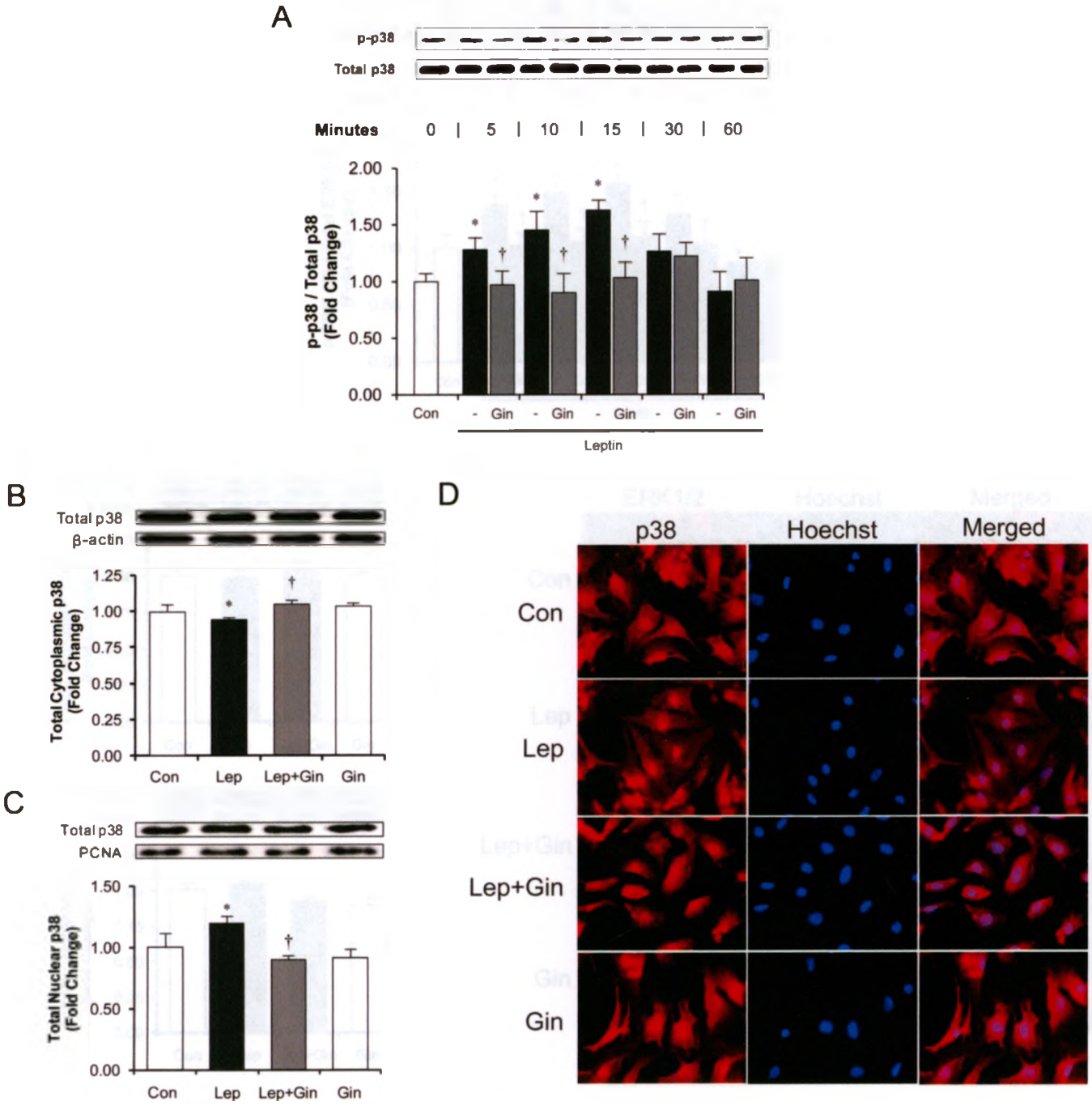

Figure 2.4. Ginseng inhibits leptin-induced phosphorylation and p38 nuclear translocation Panel A shows Western blots and corresponding densitometric values for phosphorylated p38 whereas panels B and C show Western blots and corresponding densitometric values for p38 MAPK content in cytoplasmic-enriched and nuclear-containing membrane fractions, respectively. Panel D shows representative immunofluorescence images measuring total p38 indicated by red staining (IgG AlexFluor-596) in the left column, Hoechst nuclear staining in the middle column and merged staining in the right column. Data in panels A-C represent means ± S.E.M. N=5-8. *$p<0.05$ vs. control; †$p<0.05$ vs. leptin. Con, control; Lep, leptin; Gin, ginseng. Minutes indicate time after leptin addition.

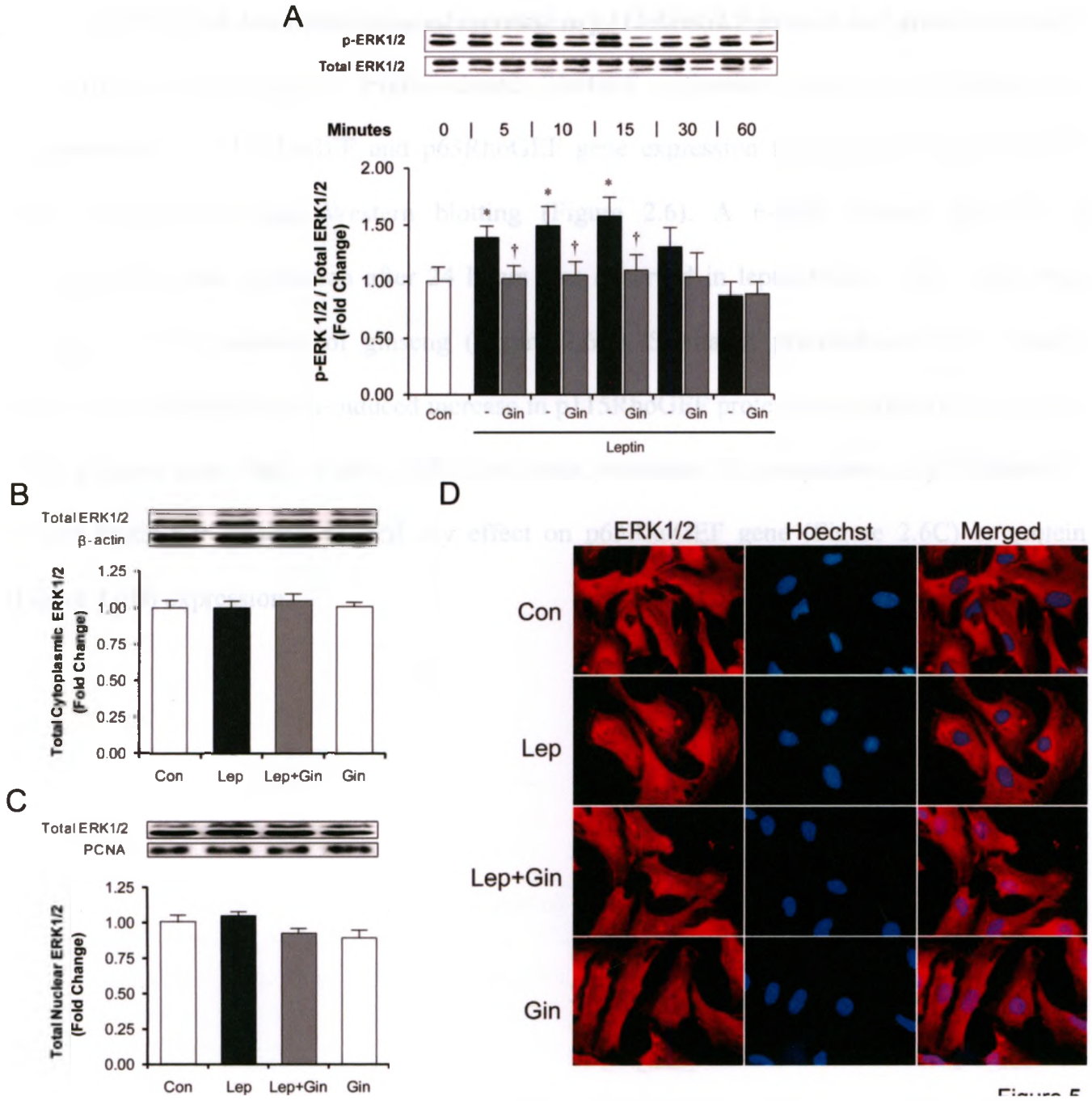

Figure 2.5. Ginseng inhibits leptin-induced ERK1/2 phosphorylation with no effect on nuclear translocation. Panel A shows Western blots and corresponding densitometric values for phosphorylated ERK1/2 whereas panels B and C show Western blots and corresponding densitometric values for ERK1/2 MAPK content in cytoplasmic-enriched and nuclear-containing membrane fractions, respectively. Panel D shows representative immunofluorescence images measuring total ERK1/2 indicated by red staining (IgG AlexFluor-596) in the left column, Hoechst nuclear staining in the middle column and merged staining in the right column. Data in panels A-C represent means ± S.E.M. N=5-8. *$p<0.05$ vs. control; †$p<0.05$ vs. leptin. Con, control; Lep, leptin; Gin, ginseng. Minutes indicate time after leptin addition.

2.3.5 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 (Figure 2.6). A 6-fold increase ($p<0.05$) in p115RhoGEF gene expression after 24 hours was observed in leptin-treated cells, which was abolished in the presence of ginseng (Figure 2.6A). Similarly, pre-treatment with ginseng significantly inhibited leptin-induced increase in p115RhoGEF protein expression (Figure 2.6B), while ginseng alone had no direct effect on either parameter. In comparison to p115RhoGEF, neither leptin nor ginseng exerted any effect on p63RhoGEF gene (Figure 2.6C) or protein (Figure 2.6D) expression.

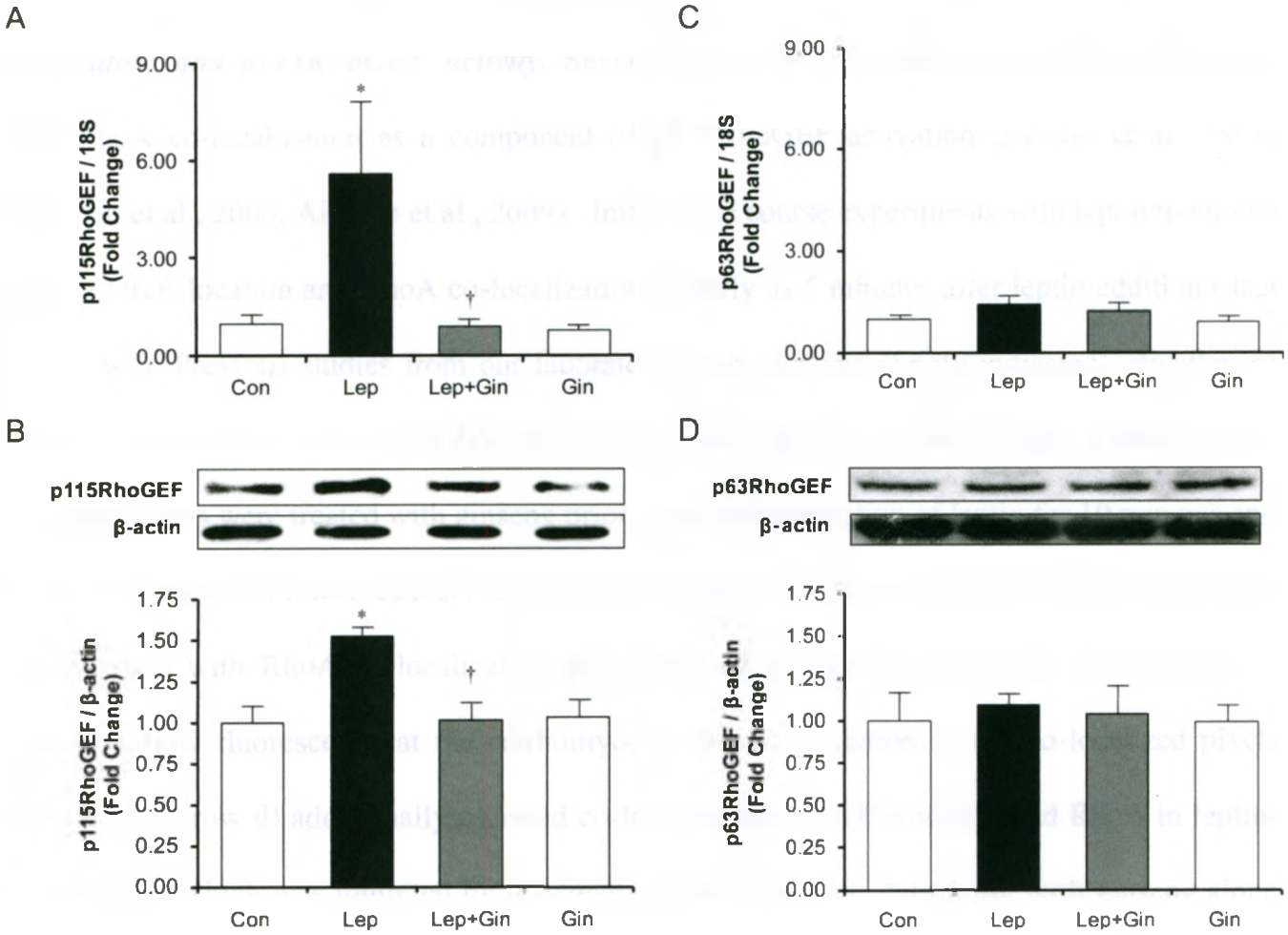

Figure 2.6. Ginseng inhibits leptin-induced upregulation of p115RhoGEF but not p63RhoGEF protein and gene expression. Gene expression (panels A and C) and protein levels (panels B and D) for p115RhoGEF and p63RhoGEF in cardiomyocytes treated with leptin in the absence or presence of ginseng. Data represent means ± S.E.M. N=8. *p<0.05 vs. control; †p <0.05 vs. leptin. Con, control; Lep, leptin; Gin, ginseng.

2.3.6 Ginseng inhibits leptin-induced p115RhoGEF membrane translocation with RhoA co-localization and p115RhoGEF activity. Several studies have shown membrane translocation with RhoA co-localization as a component of p115RhoGEF activation (Kozasa et al., 1998; Rossman et al., 2005; Aittaleb et al., 2009). Initial time course experiments with leptin treatment revealed translocation and RhoA co-localization as early as 5 minutes after leptin addition (data not shown). Previous studies from our laboratory have correspondingly indicated activation of RhoA as early as 10 minutes (Zeidan et al,. 2006; Zeidan et al., 2008). Consequently, cardiomyocytes were treated with ginseng prior to the administration of leptin for 10 minutes and prepared for immunofluorescence. As depicted in Figure 2.7A (row c), p115RhoGEF membrane translocation with RhoA co-localization was observed in leptin-treated cells as indicated by evident yellow fluorescence at the cardiomyocyte border. Isolation of the co-localized pixels (Figure 2.7A, row d) additionally revealed co-localization of p115RhoGEF and RhoA in leptin-treated cells, which was inhibited by pre-treatment with ginseng. Treatment with ginseng alone was without any effect (Figure 2.7A, 4^{th} *column*).

Using the same treatment protocol, cell lysates were collected at 10 minutes after leptin administration and incubated with GST-RhoG17A, a nucleotide-free RhoA mutant that has a high affinity for active RhoGEFs (Garcia-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 GTP-ase RhoA was then performed. p115RhoGEF activation was increased 2-fold in leptin-treated cells in comparison to control (Figure 2.7B). Particularly of interest, this observed increase in guanine nucleotide exchange activity was significantly abolished in cells pre-treated with ginseng. p115RhoGEF activity was unaffected by ginseng alone.

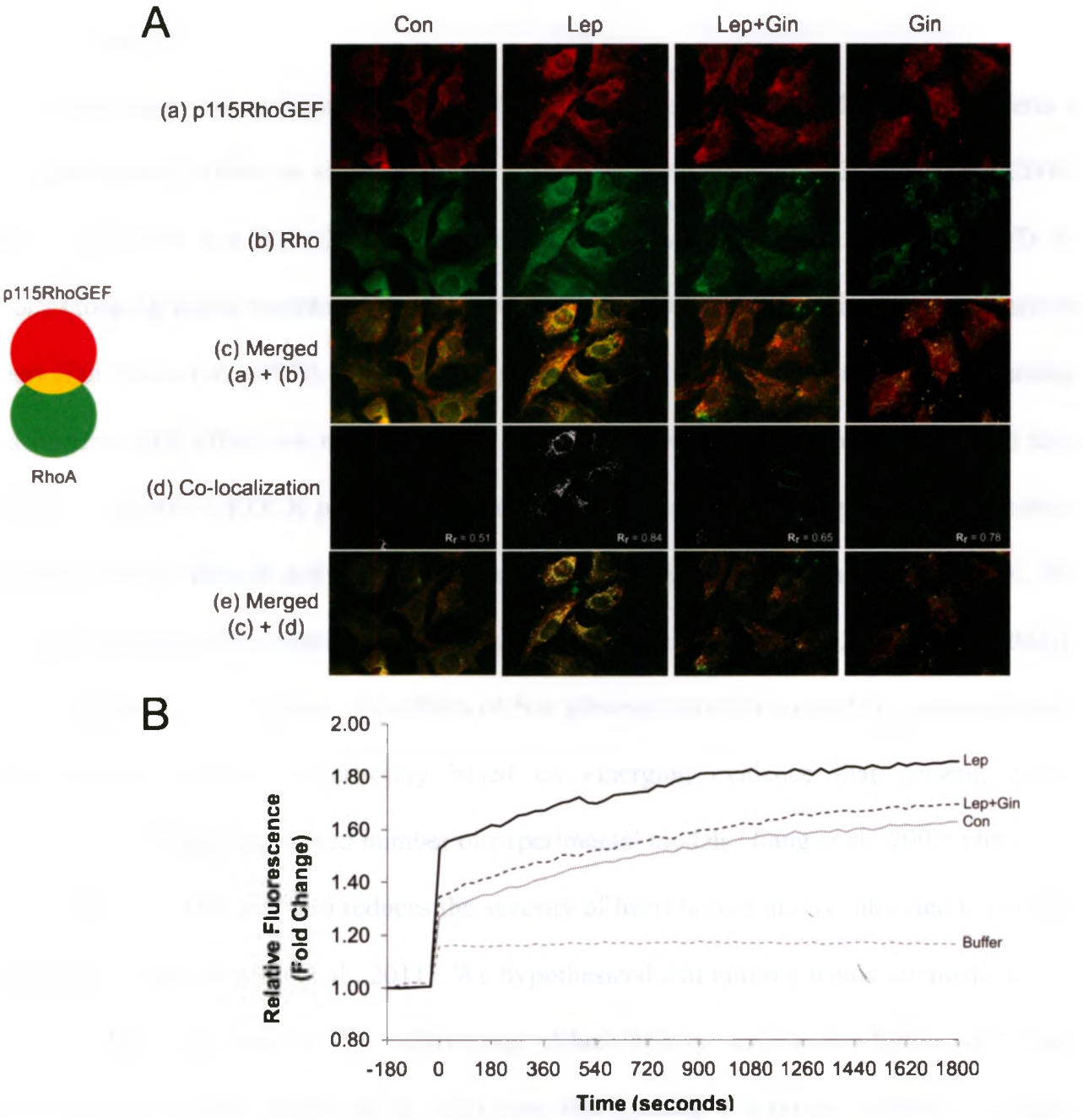

Figure 2.7. Ginseng inhibits leptin-induced p115RhoGEF co-localization with RhoA and guanine nucleotide exchange activity. Panel A shows representative immunofluorescence images of p115RhoGEF translocation and co-localization with RhoA after administration and incubation with leptin for 10 minutes in the presence or absence of ginseng. Row (a) shows p115RhoGEF (IgG AlexFluor-594) stained in red; row (b) represents RhoA (IgG AlexFluor-488) stained in green, row (c) represents merged images of p115RhoGEF and RhoA; row (d) shows isolated co-localized pixels of p115RhoGEF and RhoA and row (e) represents isolated co-localized pixels of merged images of p115RhoGEF and RhoA. The Pearson's correlation coefficient (R_r) as calculated by the co-localization plugin of ImageJ software is represented at the bottom right of row (d). Panel B shows p115RhoGEF activity determined by fluorescence assay at an excitation of 360 nm and emission at 440 nm. Buffer group depicts results using exchange buffer alone + RhoA.

2.4 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) as well as intact myocardium *in vivo* (Abe et al., 2007). In addition, blocking leptin receptors attenuates remodeling and heart failure in the post-infarcted rat heart (Purdham et al., 2008). Although the precise mechanism of action of leptin accounting for its hypertrophic effect are not completely understood, we have previously suggested that activation of the RhoA/ROCK pathway plays a critical role in mediating leptin-induced cardiac hypertrophy, likely through activation and subsequent nuclear translocation of p38 MAPK, the latter effect dependent on alterations in actin dynamics (Zeidan et al., 2006; Zeidan et al., 2008). In the present study, we assessed the effects of NA ginseng alcoholic extract (*P. quinquefolius*) on leptin-induced cardiac hypertrophy based on emerging evidence that ginseng exerts antihypertrophic 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 following 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 [^{3}H] leucine incorporation, as well as increased expression of two molecular hypertrophic gene markers, α-SA and MHC, 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; Zeidan et al., 2008). RhoA/ROCK activation was further demonstrated by increased phosphorylation (inactivation) of cofilin-2, an ubiquitous enzyme downstream of RhoA which depolymerizes actin, resulting in a decrease in the G/F actin ratio. We have previously reported that leptin-induced RhoA/ROCK activation was critical for p38 MAPK, but not ERK1/2 MAPK nuclear translocation and the subsequent hypertrophic response, a response likely dependent on the changes in actin dynamics (Zeidan et al., 2008). When taken 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 antihypertrophic 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 extensively studied. Although a number of RhoGEFs have been identified, p115RhoGEF and p63RhoGEF were considered of particular interest since their expression have 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 co-localization with RhoA (Kozasa et al., 1998; Rossman et al., 2005; Aittaleb et al., 2009). Our results revealed membrane translocation of p115RhoGEF with RhoA co-localization after 10 minutes of leptin stimulation and also demonstrate an increase p115RhoGEF activity.

The ability of ginseng to inhibit p115RhoGEF activation suggest this as a target for the ability of ginseng to inhibit leptin-induced cardiomyocyte hypertrophy and it 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 pathway. Further studies are required to confirm this particularly by determining the effect of p115RhoGEF downregulation on the ability of leptin to activate RhoA.

As previously reported (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 likely mediated by changes in actin dynamics since 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 following 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 triterpine saponins and which are considered the primary active constituents contributing to the medicinal effects of ginseng (Attele et al., 1999), have been compared to steroidal structures such as estrogen. Recently, Leung and colleagues alluded to the competitive binding of the specific ginsenoside, Rb1 (the predominant ginsenoside of North American ginseng), selective to the estrogen receptor (ER) 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 anti-angiogenic factor (Leung et al., 2007). Indeed, we have previously shown that estrogen (as 17β estradiol) exerts a prohypertrophic effect on cultured ventricular myocytes at very low (1 pM) concentrations but has antihypertrophic actions at nanomolar concentrations (Kilic et al., 2009). The observation of the ability of ginsenoside Rb1 to bind to ERs is intriguing and raises the question of potential gender specific effects of ginseng. Although it appears that ERs 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 cannot be currently identified.

Another potential mechanism for the observed inhibition of these pathways by ginseng may occur extracellularly at the level of Ob-Rb, considered the principle receptor mediating the biological effects of leptin (reviewed in Villanueva and Myers, 2008), potentially as a result of

early binding and antagonism of Ob-Rb, consequently down-regulating further leptin signalling (Figure 2.8). 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 previously noted, further work is necessary to demonstrate a precise causal relationship between leptin-induced p115RhoGEF activation and subsequent activation of downstream pathways. Also, since ginseng exerts numerous and diverse effects on the heart (reviewed in 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.

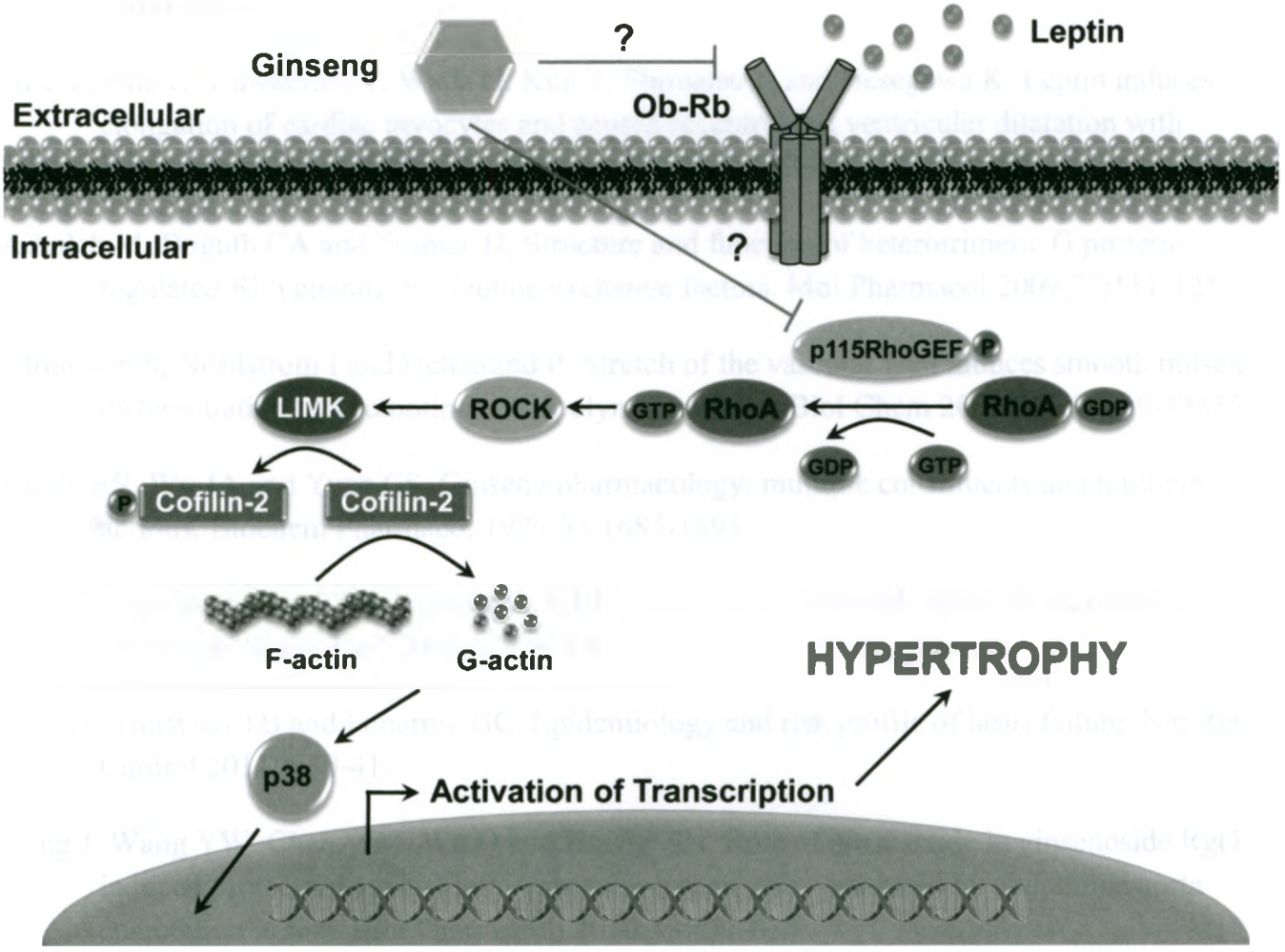

Figure 2.8. Proposed mechanism of the attenuation of leptin-induced ventricular cardiac hypertrophy by ginseng via inhibition of the p115RhoGEF-RhoA/ROCK-dependent MAPK pathway. Leptin binds to its receptor (Ob-Rb) resulting in activation of p115RhoGEF, which subsequently facilitates the exchange of GDP for GTP on RhoA after co-localization and translocation to the membrane. Activated RhoA (RhoA-GTP) results in the activation of ROCK and LIMK, which consequently phosphorylates (inactivates) cofilin-2 resulting in an increase in the F to G actin ratio. Changes in actin dynamics results in activation of p38 and ERK1/2 *via* phosphorylation and nuclear translocation of the former thus leading to an increase in transcriptional growth factors of hypertrophy. Pre-treatment with ginseng attenuates the leptin-induced ventricular cardiac hypertrophy by inhibiting the p115RhoGEF-RhoA/ROCK-dependent MAPK pathways potentially through down regulating leptin signalling by competitive binding at the receptor or by additional mechanisms, currently undefined, via direct entry into the cell.

2.5 References

Abe Y, Ono K, Kawamura T, Wada H, Kita T, Shimatsu A and Hasegawa K. Leptin induces elongation of cardiac myocytes and causes eccentric left ventricular dilatation with compensation. Am J Physiol Heart Circ Physiol 2007;292:H2387-2396.

Aittaleb M, Boguth CA and Tesmer JJ. Structure and function of heterotrimeric G protein-regulated Rho guanine nucleotide exchange factors. Mol Pharmacol 2009;77:111-125.

Albinsson S, Nordstrom I and Hellstrand P. Stretch of the vascular wall induces smooth muscle differentiation by promoting actin polymerization. J Biol Chem 2004;279:34849-34855.

Attele AS, Wu JA and Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. Biochem Pharmacol 1999;58:1685-1693.

Bos JL, Rehmann H and Wittinghofer A. GEFs and GAPs: critical elements in the control of small G proteins. Cell 2007;129:865-877.

Bui AL, Horwich TB and Fonarow GC. Epidemiology and risk profile of heart failure. Nat Rev Cardiol 2011;8:30-41.

Deng J, Wang YW, Chen WM, Wu Q and Huang XN. Role of nitric oxide in ginsenoside Rg(1)-induced protection against left ventricular hypertrophy produced by abdominal aorta coarctation in rats. Biol Pharm Bull 2010;33:631-635.

Frühbeck G. Intracellular signalling pathways activated by leptin. Biochem J 2006;393:7-20.

Garcia-Mata R, Wennerberg K, Arthur WT, Noren NK, Ellerbroek SM and Burridge K. Analysis of activated GAPs and GEFs in cell lysates. Methods Enzymol 2006;406:425-437.

Goldstein B. Ginseng: its history, dispersion, and folk tradition. Am J Chin Med (Gard City N Y) 1975;3:223-234.

Guilluy C, Bregeon J, Toumaniantz G, Rolli-Derkinderen M, Retailleau K, Loufrani L, Henrion D, Scalbert E, Bril A, Torres RM, Offermanns S, Pacaud P and Loirand G. The Rho exchange factor Arhgef1 mediates the effects of angiotensin II on vascular tone and blood pressure. Nat Med 2010;16:183-190.

Guo J, Gan XT, Haist JV, Rajapurohitam V, Zeidan A, Faruq NS and Karmazyn M. Ginseng inhibits cardiomyocyte hypertrophy and heart failure via NHE-1 inhibition and attenuation of calcineurin activation. Circ Heart Fail 2011;4:79-88.

Hou N, Luo MS, Liu SM, Zhang HN, Xiao Q, Sun P, Zhang GS, Luo JD and Chen MS. Leptin induces hypertrophy through activating the peroxisome proliferator-activated receptor

alpha pathway in cultured neonatal rat cardiomyocytes. Clin Exp Pharmacol Physiol 2010;37:1087-1095.

Jeon BH, Kim CS, Park KS, Lee JW, Park JB, Kim KJ, Kim SH, Chang SJ and Nam KY. Effect of Korea red ginseng on the blood pressure in conscious hypertensive rats. Gen Pharmacol 2000;35:135-141.

Jiang QS, Huang XN, Dai ZK, Yang GZ, Zhou QX, Shi JS and Wu Q. Inhibitory effect of ginsenoside Rb1 on cardiac hypertrophy induced by monocrotaline in rat. J Ethnopharmacol 2007;111:567-572.

Kakiashvili E, Speight P, Waheed F, Seth R, Lodyga M, Tanimura S, Kohno M, Rotstein OD, Kapus A and Szaszi K. GEF-H1 mediates tumor necrosis factor-alpha-induced Rho activation and myosin phosphorylation: role in the regulation of tubular paracellular permeability. J Biol Chem 2009;284:11454-11466.

Karmazyn M, Purdham DM, Rajapurohitam V and Zeidan A. (2008) Leptin Signaling in the Cardiovascular System, in Signal Transduction in the Cardiovascular System in Health and Disease (Srivastava AK and Anand-Srivastava MB eds) pp 377-395, Springer US.

Karmazyn M, Moey M, and Gan XT. Therapeutic potential of ginseng in the management of cardiovascular disorders. Drugs 2011;71:1989-2008

Kilic A, Javadov S and Karmazyn M. Estrogen exerts concentration-dependent pro-and anti-hypertrophic effects on adult cultured ventricular myocytes. Role of NHE-1 in estrogen-induced hypertrophy. J Mol Cell Cardiol 2009;46:360-369.

Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG, Bollag G and Sternweis PC. p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. Science 1998;280:2109-2111.

Leung KW, Cheung LW, Pon YL, Wong RN, Mak NK, Fan TP, Au SC, Tombran-Tink J and Wong AS. Ginsenoside Rb1 inhibits tube-like structure formation of endothelial cells by regulating pigment epithelium-derived factor through the oestrogen beta receptor. Br J Pharmacol 2007;152:207-215.

Li J, Xie ZZ, Tang YB, Zhou JG and Guan YY. Ginsenoside-Rd, a purified component from panax notoginseng saponins, prevents atherosclerosis in apoE knockout mice. Eur J Pharmacol 2011;652:104-110.

Li Q, Lau A, Morris TJ, Guo L, Fordyce CB and Stanley EF. A syntaxin 1, Galpha(o), and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolocalization. J Neurosci 2004;24:4070-4081.

Lu JM, Yao Q and Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. Curr Vasc Pharmacol 2009;7:293-302.

Luo W, Morrison H, de Groh M, Waters C, DesMeules M, Jones-McLean E, Ugnat AM, Desjardins S, Lim M and Mao Y. The burden of adult obesity in Canada. Chronic Dis Can 2007;27:135-144.

Madani S, De Girolamo S, Munoz DM, Li RK and Sweeney G. Direct effects of leptin on size and extracellular matrix components of human pediatric ventricular myocytes. Cardiovasc Res 2006;69:716-725.

Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA and Friedman JM. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1995;1:1155-1161.

Porchia F, Papucci M, Gargini C, Asta A, De Marco G, Agretti P, Tonacchera M and Mazzoni MR. Endothelin-1 up-regulates p115RhoGEF in embryonic rat cardiomyocytes during the hypertrophic response. J Recept Signal Transduct Res 2008;28:265-283.

Purdham DM, Rajapurohitam V, Zeidan A, Huang C, Gross GJ and Karmazyn M. A neutralizing leptin receptor antibody mitigates hypertrophy and hemodynamic dysfunction in the postinfarcted rat heart. Am J Physiol Heart Circ Physiol 2008;295:H441-446.

Purdham DM, Zou MX, Rajapurohitam V and Karmazyn M. Rat heart is a site of leptin production and action. Am J Physiol Heart Circ Physiol 2004;287:H2877-2884.

Qin N, Gong QH, Wei LW, Wu Q and Huang XN. Total ginsenosides inhibit the right ventricular hypertrophy induced by monocrotaline in rats. Biol Pharm Bull 2008;31:1530-1535.

Rajapurohitam V, Gan XT, Kirshenbaum LA and Karmazyn M. The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. Circ Res 2003;93:277-279.

Rajapurohitam V, Javadov S, Purdham DM, Kirshenbaum LA and Karmazyn M. An autocrine role for leptin in mediating the cardiomyocyte hypertrophic effects of angiotensin II and endothelin-1. J Mol Cell Cardiol 2006;41:265-274.

Rossman KL, Der CJ and Sondek J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nat Rev Mol Cell Biol 2005;6:167-180.

Schmidt A and Hall A. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev 2002;16:1587-1609.

Schulze PC, Kratzsch J, Linke A, Schoene N, Adams V, Gielen S, Erbs S, Moebius-Winkler S and Schuler G. Elevated serum levels of leptin and soluble leptin receptor in patients with advanced chronic heart failure. Eur J Heart Fail 2003;5:33-40.

Villanueva EC and Myers MG, Jr. Leptin receptor signaling and the regulation of mammalian physiology. Int J Obes (Lond) 32 Suppl 2008;7:S8-12.

Zeidan A, Javadov S, Chakrabarti S and Karmazyn M. Leptin-induced cardiomyocyte hypertrophy involves selective caveolae and RhoA/ROCK-dependent p38 MAPK translocation to nuclei. Cardiovasc Res 2008;77:64-72.

Zeidan A, Javadov S and Karmazyn M. Essential role of Rho/ROCK-dependent processes and actin dynamics in mediating leptin-induced hypertrophy in rat neonatal ventricular myocytes. Cardiovasc Res 2006;72:101-111.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L and Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994;372:425-432.

CHAPTER 3:

Ginseng reverses leptin-induced ventricular cardiac hypertrophy *via* Rnd3-mediated inhibition of RhoA/ROCK pathway

3.1 Introduction

Pathological cardiac hypertrophy, which involves the abnormal structural and molecular changes of the heart, is a classical feature in myocardial remodelling leading to the development of HF (reviewed in Bernado et al., 2010). Once cardiac remodelling has been established however, the success for reversal of HF decreases drastically and becomes exceptionally challenging to treat. As a result, current research on therapeutic options has focused on targeting the molecular mechanisms involved in the development of cardiac hypertrophy.

The cardiac remodelling process can manifest as early as 24 hours after a cardiac insult such as after an MI, whereby additional hypertrophic humoral factors are released further aggravating the disease. One of these factors includes the 16kDa adipokine peptide, leptin, which has been demonstrated to be elevated in plasma (>30 ng/ml) of HF patients independently of obesity (Schulze et al., 2003). Indeed elevated plasma leptin levels have been shown to be associated with an increased risk of acute MI (Soderberg et al., 1999).

In cultured ventricular cardiomyocytes, several laboratories including our own, have demonstrated the marked ability of leptin to directly induce cardiac hypertrophy both *in vitro* (Rajapurohitam et al., 2003; Madani et al., 2006; Zeidan et al., 2005; Zeidan et al., 2008; Hou et al., 2010; Schram et al., 2010; Moey et al., 2011) and *in vivo* (Abe et al., 2007). In a four week sustained infarction rat model produced by coronary artery ligation (CAL) of the left anterior descending artery, treatment with leptin and Ob-Rb antibodies significantly inhibited cardiac hypertrophy and improved ventricular function (Purdham et al., 2008). A key signalling mechanism that has been shown to facilitate leptin-induced cardiac hypertrophy is the activation of RhoA/ROCK-dependent MAPK pathway (Zeidan et al., 2006; Zeidan et al., 2006).

RhoA/ROCK is a small G-protein signaling pathway that is essential for the management of the cellular actin dynamics (reviewed in Brown et al., 2006) in several different cellular

systems including cardiomyocytes. RhoA is activated upon the exchange of GTP for its bound GDP molecule, which is facilitated by factors known as Rho guanine nucleotide exchange factors (RhoGEFs) (reviewed in Schmidt and Hall, 2002; Rossman et al., 2005; Bos et al., 2007) Recently, our laboratory demonstrated the critical role of p115RhoGEF (also known as ARHGEF1) in the facilitation of leptin-induced RhoA activation which leads to a decrease in the G to F cellular actin ratio and induction of cardiac hypertrophy (Moey et al,. 2011). In addition to RhoGEFs, RhoA may be negatively regulated by GTPase activating proteins (GAPs) which promotes the hydrolysis of GTP to GDP. A major GAP protein and downregulator of RhoA is p190RhoGAP (Wennerberg et al., 2003), which itself is regulated by other small G-protein members belonging to the Rho family known as Rnd (Rnd1/2/3) proteins (Riou et al., 2010). Little however is known about Rnd expression in cardiomyocytes and its potential role in preventing cardiac hypertrophy through downregulation of the RhoA/ROCK signaling pathway.

The present study was therefore carried out to determine the potential role of Rnd3 in leptin-induced cardiomyocyte hypertrophy and its involvement in the antihypertrophic effect of ginseng. Specifically, we hypothesized that ginseng can reverse leptin-induced RhoA/ROCK activation by upregulation of the p190RhoGAP endogenous activator Rnd3 and by inhibition of p115RhoGEF facilitated activation of RhoA/ROCK.

3.2 Materials and Methods

3.2.1 Treatment and Experimental Groups

Neonatal ventricular cardiomyocytes from one to three day old Sprague Dawley rats were isolated and cultured as previously described (Moey et al., 2011). Cells were grown in FBS containing medium for up to 48 h following 24 h starvation. For reversal experiments, cardiomyocytes were pre-treated with 50 ng/ml leptin (3.1 nM) for up to 24 h followed by the addition of NA ginseng alcoholic extract provided by Dr. Edmund Lui's lab at the University of Western Ontario (Gin, 10 μg/ml; a concentration originally tested to be optimal in attenuating cardiac hypertrophy) (Moey et al., 2011) (Naturex, South Hackensack, NJ) for up to 24 h. Leptin and ginseng were present throughout the entire treatment duration of up to 48 h. 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 US National Institutes of Health and the Canadian Council of Animal Care (Ottawa, Ontario, Canada).

3.2.2 Cell Surface Area Measurement

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

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

RNA was collected from treated cardiomyocytes using Qiazol Reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions and reverse transcribed to complementary DNA

(cDNA) for real-time PCR analysis of αSA and Rnd3 gene expression as previously described (Moey et al., 2011). cDNA was synthesized from 5μg of total RNA using random primers (Invitrogen) and M-MLV Reverse Transcriptase (Invitrogen) 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 (Invitrogen) used the PCR analyses are summarized in Table 3.1 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 αSA, MCIP-1, p63RhoGEF and Rnd3, respectively, ending with elongation at 72°C for 45s. The housekeeping gene, 18S, was measured and quantified to normalize cDNA levels.

3.2.4 Western Blotting

Total cellular lysates were collected using a lysis buffer and protease cocktail inhibitor mixture as a previously described (Moey et al., 2011) for the measurement of proteins of interest. Proteins were loaded equally on either 10% or 15% SDS gels as appropriate after protein quantification via BioRad reagent (BioRad, Hercules, CA) following the manufacturer's protocol. Cardiomyocytes were treated with leptin and collected at either 5 or 10 minutes to measure phosphorylated (p)-p38 (1:1000 dilution, Cell Signaling), p-ERK1/2 (1:1000 dilution, Cell Signaling) and normalized to total p38 (1:1000 dilution, Santa Cruz) and total ERK1/2 (1:1000 dilution, Santa Cruz). Ginseng was administered after 10 minutes of leptin incubation and collected after 5 minutes. For the measurement of all additional proteins were, cardiomyocytes were pre-treated with the hypertrophic agonist for 24 hours followed by the administration of ginseng for an additional 24 hours. The primary antibodies and respective dilutions used in this study include total and phosphorylated p38 forms (1:1000 dilution, Santa Cruz Biotechnology

Inc., Santa Cruz, CA, USA), total and phosphorylated ERK1/2 forms (1:1000 dilution, Santa Cruz), actin (1:1000 dilution, Cytoskeleton Inc., Denver, CO), phosphorylated and total cofilin-2 (1:1000 dilution, Upstate) and Rnd3 (1:200 dilution, Santa Cruz). Cells were treated for 24 hours with leptin followed by treatment with ginseng for an additional 24 hours to measure Rnd3, p63RhoGEF and ROCK protein expression. Membranes were incubated with either Rnd3 (1:250 dilution, Santa Cruz), p63RhoGEF (1:250 dilution, Santa Cruz) or ROCK (1:500 dilution, Santa Cruz). Goat-anti-rabbit IgG and goat-anti-mouse IgG HRP conjugate (BioRad) were used at 1:5000 dilution and donkey-anti-goat IgG HRP conjugate (Santa Cruz) was used at 1:10,000 dilution as appropriate. Spot densitometry using FluorChem (Alpha Innotech Corporation, Santa Clara, CA) software was performed to quantify protein.

3.2.5 In vivo Studies

Six to ten male Sprague-Dawley rats weighing between 215 and 250 g were randomly assigned to the following four treatment groups: sham or CAL with drinking water or sham or CAL with NA ginseng (0.9 g/L) *ad libitum* treatment 4 weeks after surgery. Coronary artery ligation was performed as previously described (Guo et al., 2011). Buprenorphine (0.03 mg/kg) was immediately administered to all animals after completion of surgery for pain management. Studies were completed for a total of 8 weeks.

3.2.6 Statistical Analysis

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

Table 3.1. Gene-specific primer sequences of analyzed genes of interest.

Gene	Forward Primer Sequence	Reverse Primer Sequence
18S	5'-GTAACCCGTTGAACCCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3'
α-skeletal actin	5'-CACGGCATTATCACCAACTG-3'	5'-CCGGAGGCATAGAGAGACAG-3'
p63RhoGEF	5'-TATGTGGACGACTTGGGACA-3'	5'-TGATGAACAGCTGAGCCAAC-3'
Rnd3	5'-CGGACAGATGTCAGCACACT-3'	5'-GTGGCCCTCTGTGATTTGTT-3'

3.3 Results

3.3.1 Ginseng reverses leptin-induced cardiac hypertrophy and increase in α-skeletal actin gene expression.

Serum-starved isolated neonatal ventricular cardiomyocytes were treated with leptin for 24 h followed by administration of ginseng for an additional 24 h. At the 48 h time point, images of the treated cardiomyocytes were captured or RNA was collected to measure cell surface area or αSA, a fetal gene reexpressed during cardiac hypertrophy, gene expression, respectively. Leptin-induced increase in cell size (Figure 3.1B) and αSA gene expression (Figure 3.1C) observed at 48 h was reversed after administration with ginseng. Leptin induced an increase in both measured indices of cardiac hypertrophy at 24 h (data not shown). Treatment with ginseng alone was without effect on cell surface area and αSA gene expression.

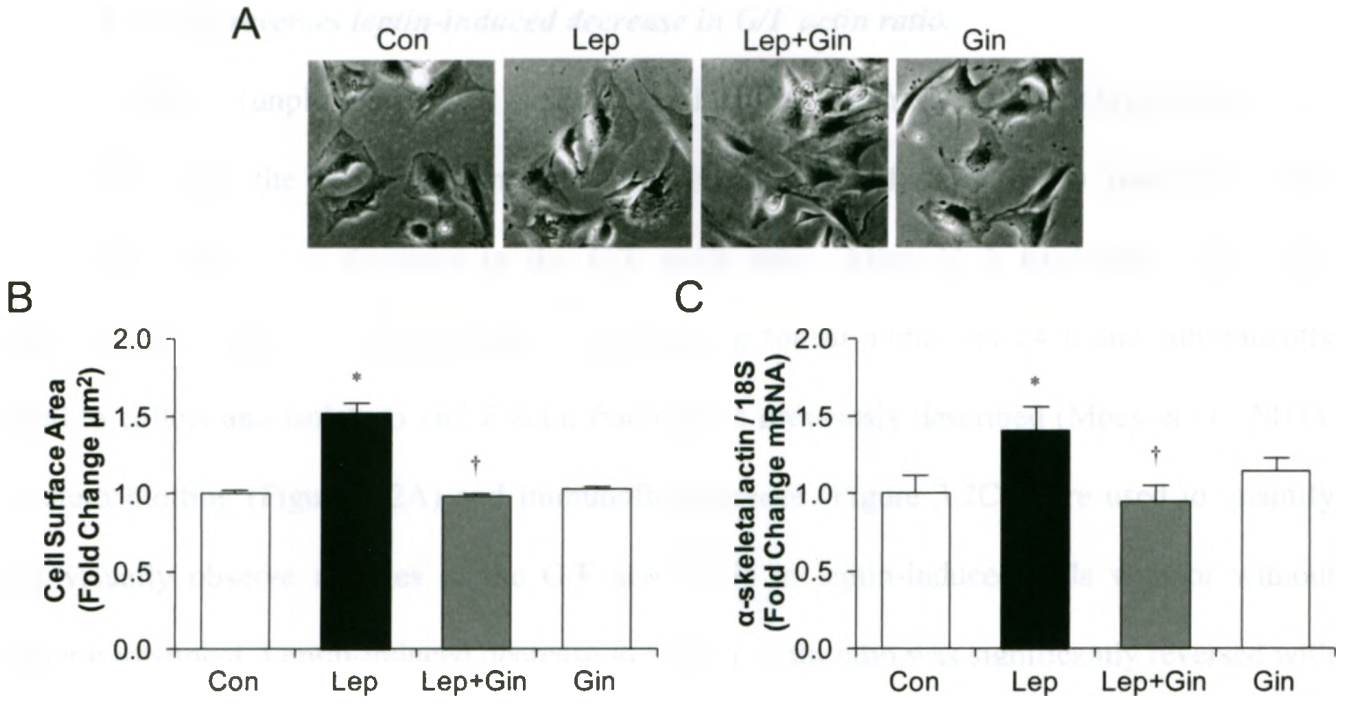

Figure 3.1. Ginseng reverses leptin-induced ventricular cardiac hypertrophy and increase in α-skeletal actin gene expression. Micrographs show representative images (A) of neonatal ventricular cardiomyocytes in the presence or absence of leptin (3.1 nM) with or without treatment with ginseng (10 µg/ml) at 48 h. Panel B shows cell surface area and panel C shows expression of αSA, respectively, with different treatments. Data represent means ± S.E.M. N=8 for surface area and N=6 for α-SA gene expression. *p<0.05 vs. control; †p <0.05 vs. leptin. Con, control; Lep, leptin; Gin, ginseng.

3.3.2 Ginseng reverses leptin-induced decrease in G/F actin ratio.

Cofilin-2 (unphosphorylated state) is a ubiquitous enzyme which depolymerizes F to G actin controlling the cellular actin dynamics. Inactivation of cofilin-2 by upstream RhoA activation results in a decrease in the G/F actin ratio. After 24 h treatment with leptin, cardiomyocytes were further incubated with ginseng for an additional 24 h and subsequently lysed to collect and isolate G and F actin fractions as previously described (Moey et al., 2011). Western blotting (Figure 3.2A) and immunofluorescence (Figure 3.2C) were used to quantify and visually observe changes in the G/F actin ratio in leptin-induced cells with or without ginseng treatment. Leptin-induced decrease in the G/F actin ratio was significantly reversed with ginseng treatment while ginseng alone was without any effect (Figure 3.2A to C).

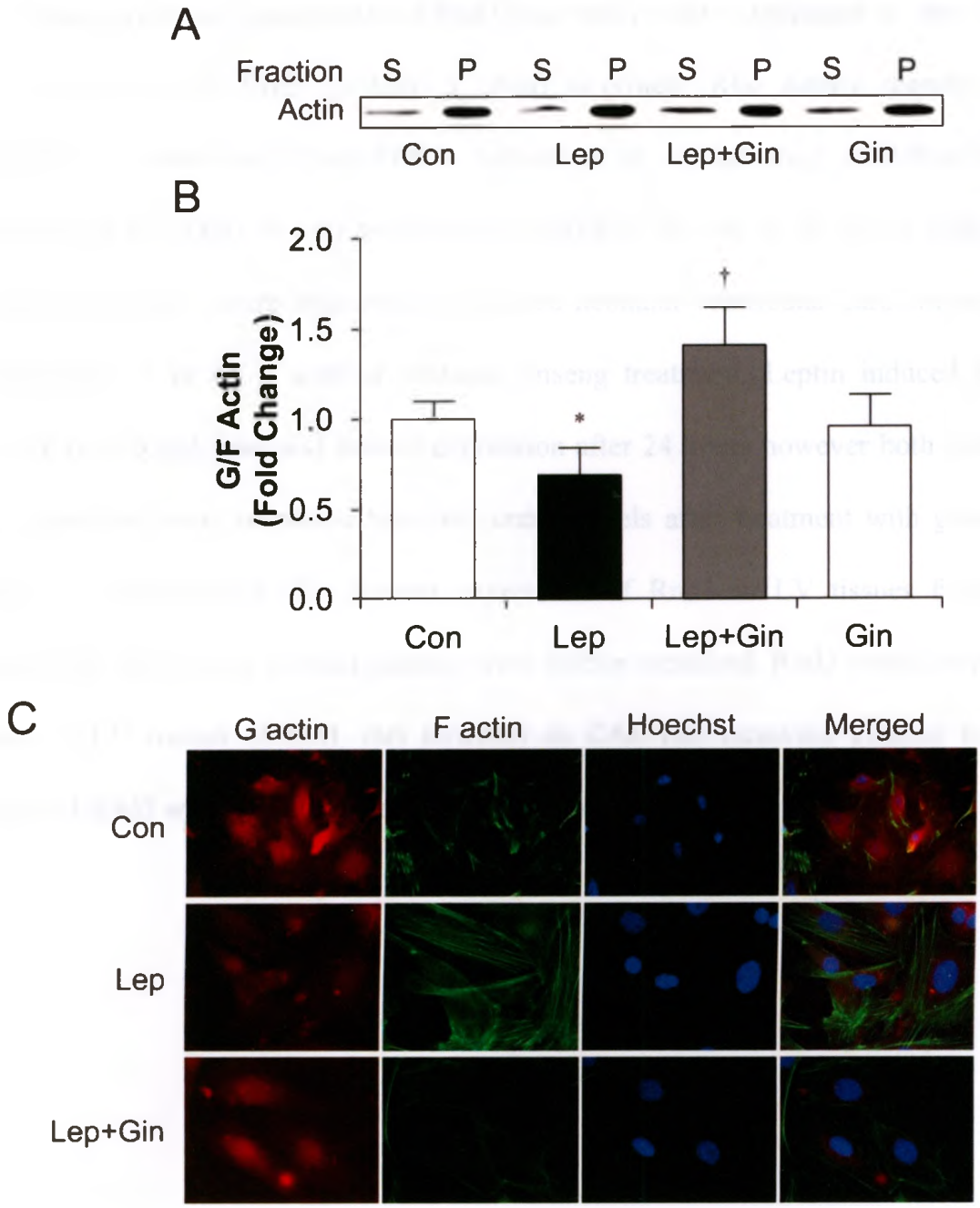

Figure 3.2. Ginseng reverses leptin-induced decrease in G/F actin. Panel A shows Western blots for actin dynamics with respect to G actin in supernatant (S) and F actin in pellet fraction (P) from cells treated with leptin in the presence or absence of ginseng. Densitometric values are shown in panel B. Representative fluorescence images of cardiomyocytes are shown in panel C. For these studies cells were fixed on collagen-coated glass cover slips and G actin (1st column) and F actin (2nd column) were detected with DNAse-I Texas Red Conjugate and Phalloidin-FITC, respectively. Hoechst staining was used to detect nuclei (3rd column) whereas the merged (4th column) depicts all three stains combined. For panel B, data represent means ± S.E.M. N=7. *$p<0.05$ vs. control; †$p<0.05$ vs. leptin. Con, control; Lep, leptin; Gin, ginseng.

3.3.3 Ginseng reverses leptin-induced Rnd3 gene and protein expression in vitro and in vivo.

Upregulation of Rnd3 protein, a small G-protein Rho family member, has been demonstrated to inhibiting RhoA/ROCK activation by upregulating p190RhoGAP activity (Wennerberg et al., 2003) as well as inhibition of ROCK (Riento et al., 2003). Both Rnd3 gene and protein expression were measured in cultured neonatal ventricular cardiomyocytes treated with leptin for 24 or 48 h with or without ginseng treatment. Leptin induced a significant decrease in both Rnd3 gene and protein expression after 24 hours however both Rnd3 gene and protein expression were increased back to control levels after treatment with ginseng (Figure 3.3B and A, respectively). The protein expression of Rnd3 in LV tissues from sham and sustained CAL rats with or without ginseng were further measured. Rnd3 protein expression was decreased in LV tissues of CAL rats however in CAL rats receiving ginseng treatment the expression of Rnd3 was markedly upregulated (Figure 3.3C).

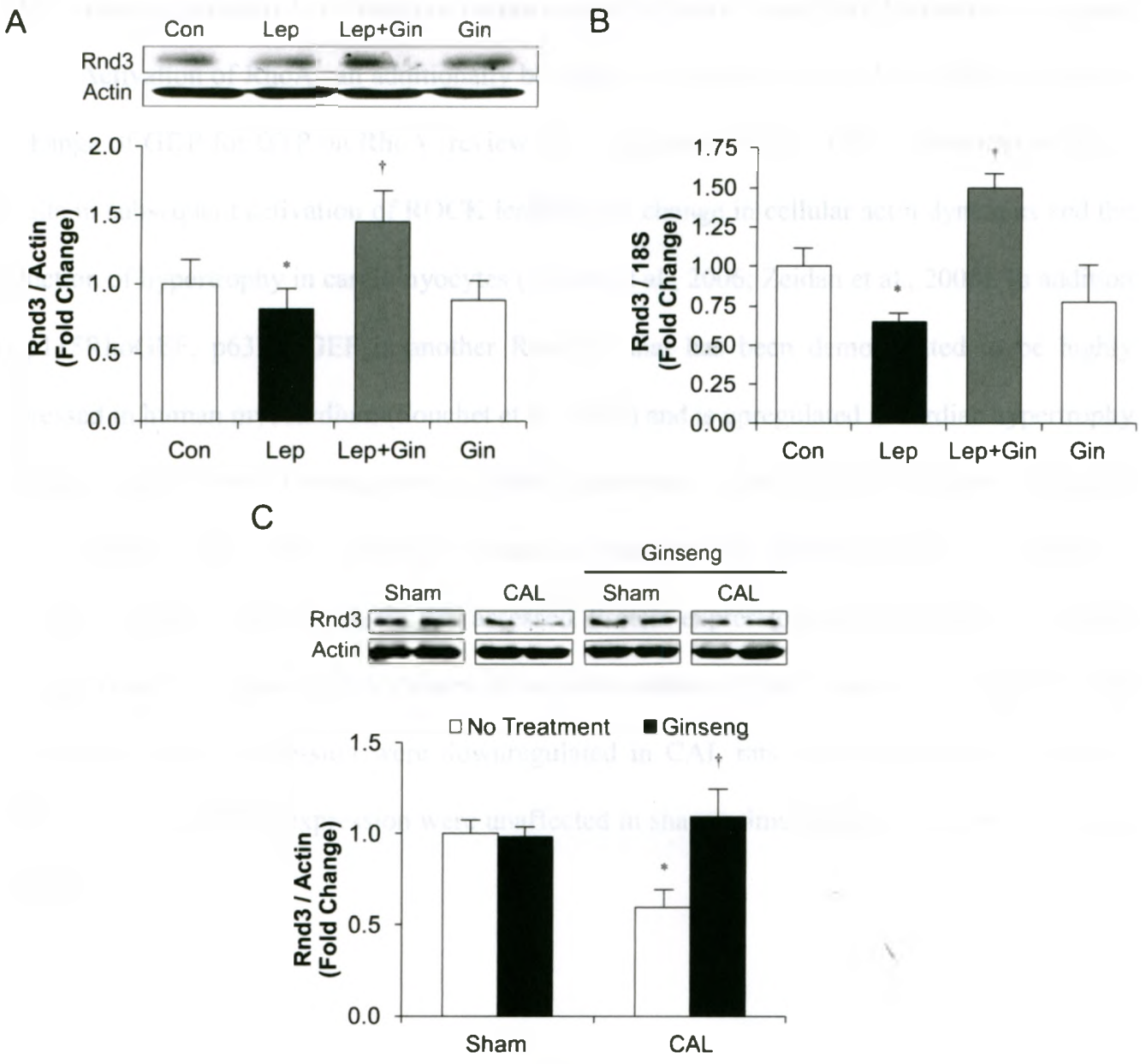

Figure 3.3. Ginseng reverses leptin-induced decrease in Rnd3 expression *in vitro* and *in vivo*. Panels A and B represent Rnd3 protein and gene expression, respectively in cultured neonatal ventricular cardiomyocytes. Panel A shows Western blots of Rnd3 and actin as control with respective densitometric values immediately below. In panel C, Western blots represent Rnd3 protein expression and respective densitometric values from two different LV samples of either sham or CAL animals as appropriately labeled with or without ginseng treatment. For panels A-C, data represent means ± S.E.M. N=6. *p<0.05 vs. control or sham; †p <0.05 vs. leptin or CAL. Con, control; Lep, leptin; Gin, ginseng; CAL, coronary artery ligation.

3.3.4 Ginseng prevents CAL-induced increase in p63RhoGEF and ROCK protein expression.

Activation of RhoA can additionally be positively regulated by GEFs which facilitate the exchange of GDP for GTP on RhoA (reviewed in Lezoualc'h et al., 2008). Activation of RhoA results in subsequent activation of ROCK leading to a change in cellular actin dynamics and the induction of hypertrophy in cardiomyocytes (Zeidan et al., 2006; Zeidan et al., 2008). In addition to p115RhoGEF, p63RhoGEF is another RhoGEF that has been demonstrated to be highly expressed in human myocardium (Souchet et al., 2002) and is upregulated in cardiac hypertrophy (Porchia et al., 2008). Consequently, protein expression of p63RhoGEF (Figure 3.4A) and ROCK (Figure 3.4B) from isolated LV tissues of sham or CAL animals with or without post-treatment of ginseng for four weeks was assessed. Protein expression of p63RhoGEF and ROCK was significantly increased in LV tissues of rats with sustained CAL, however both p63RhoGEF and ROCK protein expression were downregulated in CAL rats receiving ginseng treatment. p63RhoGEF and ROCK expression were unaffected in sham animals receiving water or ginseng treatment.

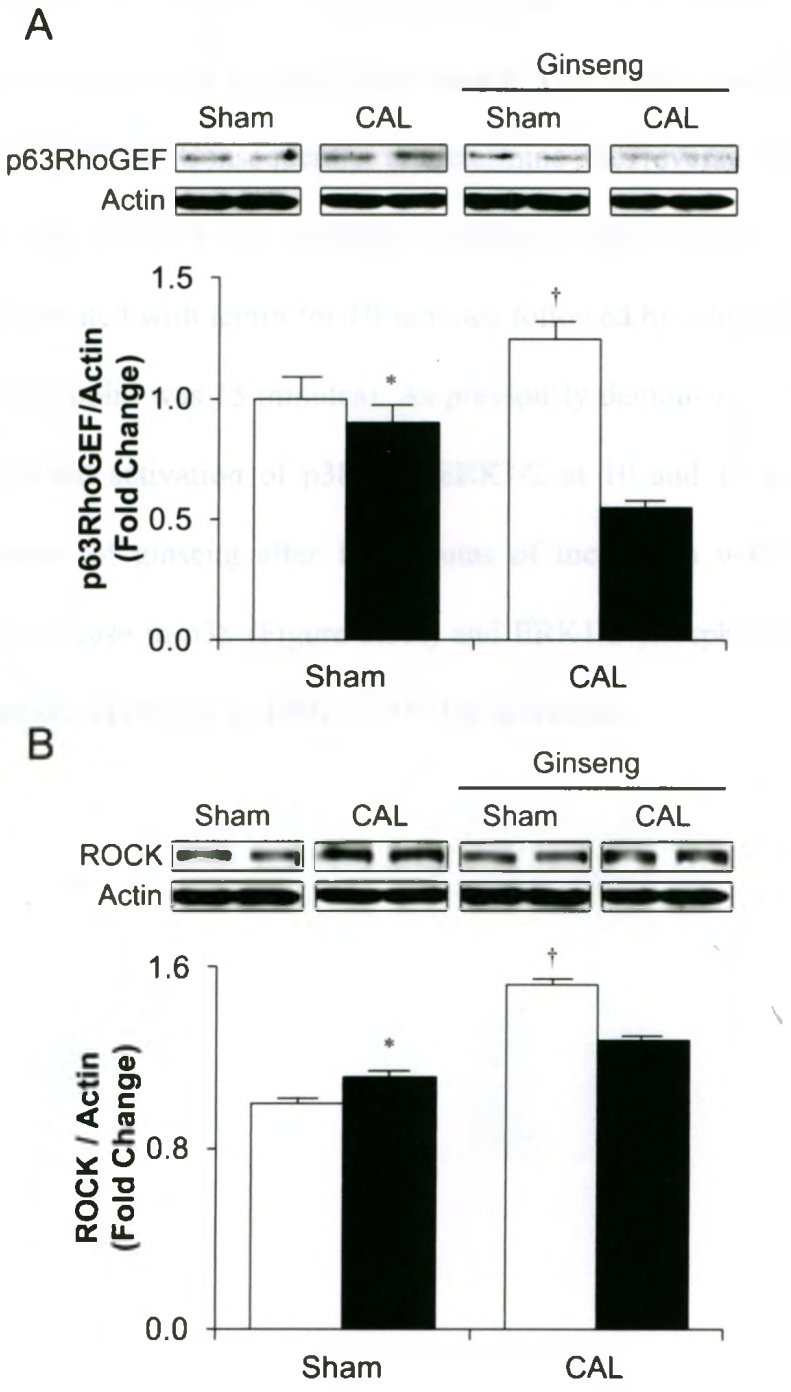

Figure 3.4 Ginseng prevents CAL-induced p63RhoGEF and ROCK increased protein expression. Western blots and respective densitometric values in (A) and (B) represent protein expression of p63RhoGEF and ROCK from two different LV samples of either sham or CAL animals as appropriately labeled. For panels A and B, data represent means ± S.E.M. N=6. *p<0.05 vs. sham; †p <0.05 vs. CAL. CAL, coronary artery ligation.

3.3.5 Ginseng does not reverse leptin-induced p38 and ERK1/2 MAPK activation.

In our previous study, peak activation/phosphorylation of p38 and ERK1/2 by leptin was observed at 10 minutes, continued to remain elevated at 15 minutes and declined at 30 and 60 minutes (Moey et al., 2011). Consequently to determine the reversibility of leptin-induced activation of p38 and ERK1/2 by ginseng, serum-starved isolated neonatal ventricular cardiomyocytes were treated with leptin for 10 minutes followed by administration with ginseng for 5 minutes (end time point was 15 minutes). As previously demonstrated (Moey et al., 2011), leptin induced significant activation of p38 and ERK1/2 at 10 and 15 minutes. Interestingly however, administration of ginseng after 10 minutes of incubation with leptin resulted in a marked 2- to 3-fold increase in p38 (Figure 3.5A) and ERK1/2 phosphorylation (Figure 3.5B). Ginseng alone had no effect on p38 or ERK1/2 MAPK activation.

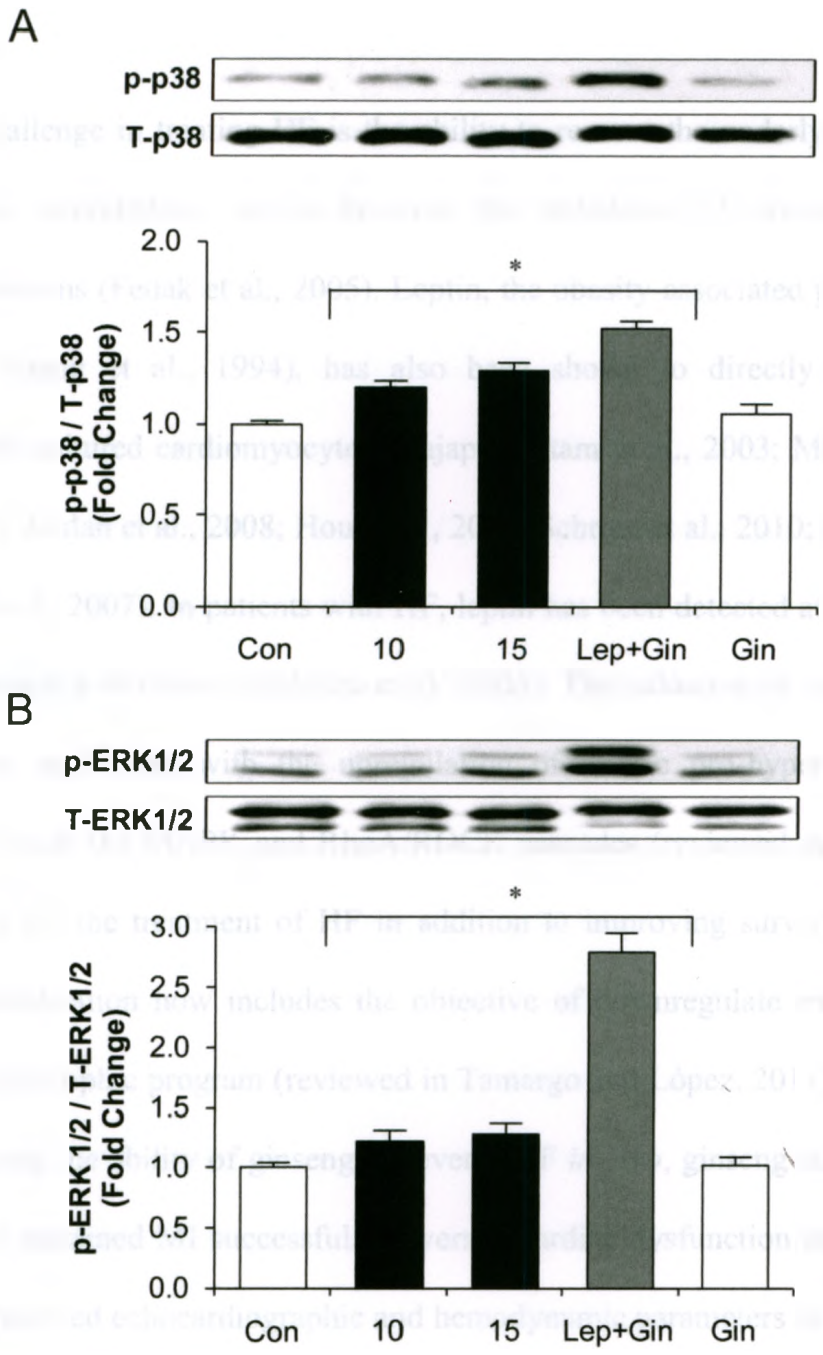

Figure 3.5. Ginseng does not reverse leptin-induced increase in p38 and ERK1/2 phosphorylation/activation. Panel A shows Western blots and corresponding densitometric values for phosphorylated p38 whereas panel B show Western blots and corresponding densitometric values for phosphorylated ERK1/2 from cell lysates in the presence of leptin with or without ginseng treatment. Data in panels A and B represent means ± S.E.M. N=6. *$p<0.05$ vs. control; †$p<0.05$ vs. leptin. Con, control; Lep, leptin; Gin, ginseng.

3.4 Discussion

A major challenge in treating HF is the ability to reverse the underlying cause of well-established cardiac remodelling, which involves the imbalance of diverse molecular and biochemical mechanisms (Fedak et al., 2005). Leptin, the obesity-associated peptide that acts as a satiety factor (Zhang et al., 1994), has also been shown to directly stimulate cardiac hypertrophy in both cultured cardiomyocytes (Rajapurohitam et al., 2003; Madani et al., 2006; Zeidan et al., 2005; Zeidan et al., 2008; Hou et al., 2010; Schram et al., 2010; Moey et al., 2011) and *in vivo* (Abe et al., 2007). In patients with HF, leptin has been detected at elevated levels (≥ 30 ng/mL) independently of obesity (Schulze et al., 2003). The induction of cardiac hypertrophy by leptin has been associated with the upregulation of classic pro-hypertrophic signalling pathways which include the MAPK and RhoA/ROCK cascades (reviewed in Karmazyn et al., 2008b). The goals for the treatment of HF in addition to improving survival and decreasing incidences of hospitalization now includes the objective of downregulate enhanced signalling pathways of the hypertrophic program (reviewed in Tamargo and López, 2011). In another study (**Chapter 4**) assessing the ability of ginseng to reverse HF *in vivo*, ginseng administered to rats after four weeks of sustained MI successfully reversed cardiac dysfunction and hypertrophy as demonstrated by improved echocardiographic and hemodynamic parameters as well as decreased heart weights and expression of the fetal gene marker, αSA. Results from this study were of particular significance as we showed for the first time, the potential for ginseng to reverse cardiac hypertrophy and HF by inhibiting calcineurin (CaN) activation *in vivo* and *in vitro*. The reversal of cardiac hypertrophy may not be limited to CaN activation, although it is considered to be a key player in the remodelling process (Wilkins et al., 2004) unique to pathological cardiac hypertrophy, as there are a multitude of pathways that may be concomitantly upregulated

(reviewed in Frey and Olson, 2003). In the present study, the ability of ginseng to reverse leptin-induced cardiac hypertrophy was demonstrated. Moreover, the mechanisms likely involve the reversal of RhoA/ROCK activation by upregulation of Rnd3.

In cultured ventricular cardiomyocytes, significant increases in surface area as well as αSA gene expression were observed in cardiomyocytes incubated with leptin as early as 24 h (data not shown) and were further increased after 48 h. Ginseng treatment however returned these increased markers of hypertrophy to control phenotype. A major signalling pathway that has been implicated in leptin-induced cardiac hypertrophy is the activation of the RhoA/ROCK pathway (Zeidan et al., 2006; reviewed in Karmazyn et al., 2008; Zeidan et al., 2008; Karmazyn et al., 2008; Moey et al., 2011; Zeidan et al., 2011) which is required for the subsequent activation of the classic pro-hypertrophic p38 and ERK1/2 MAPK pathways. Activation of the RhoA/ROCK pathway was indirectly measured by the changes in the G to F actin ratio using Western blotting and immunofluorescence in cells incubated with leptin at 48 h with or without ginseng treatment. Administration of leptin for 24 and 48 h resulted in a significant increase the F actin component and consequently decreased the G/F actin ratio. However, ginseng treatment for 24 h after initial incubation with leptin for 24 h completely reversed these effects.

RhoA/ROCK activation has been shown to mediate upregulation of p38 and ERK1/2 MAPK (Zeidan et al., 2008; Moey et al., 2011) pathways, that eventually result in the expression of transcription factors for cell proliferation and differentiation (reviewed in Rose et al., 2010). Peak activation (phosphorylation) of p38 and ERK1/2 by leptin has been previously observed at 10 minutes and plateaus at 30 minutes onwards (Moey et al., 2011). Accordingly, phosphorylation of p38 and ERK1/2 was measured after treatment of ginseng in cardiomyocytes that were already exposed to 10 minutes of leptin incubation. In agreement with our previously

published data (Moey et al., 2011), leptin induced significant activation of both p38 and ERK1/2 at 10 and 15 minutes. The inhibition of RhoA activation by ginseng however did not reverse these responses and instead caused a marked 2-fold increase in the phosphorylation of both p38 and ERK1/2. These results are rather intriguing as RhoA and ROCK inhibitors, C3 exoenzyme and Y-278, respectively have been shown to directly inhibit p38 phosphorylation and nuclear translocation (Zeidan et al., 2008). The robust increase in p38 and ERK1/2 activation may potentially be due to increased external stress factors caused by the administration of ginseng, which therefore potentiates the hypertrophic stimuli of leptin. Indeed, stress stimulators are known to activate MAPK (reviewed in Rose et al., 2010). In addition, it was previously reported in the hypertrophied hearts of renovascular hypertensive rats that p38 and ERK1/2 activation, which was measured by mRNA expression, was maintained at an elevated level while expression of CaN and JNK were reversed by the CaN inhibitor cyclosporin A (Liang et al., 2006). This may implicate the more predominant role of other pathways, such as CaN or RhoA, rather than the MAPK cascade as targets for the reversal of cardiac hypertrophy. Alternatively, this may provide insight into the mechanism by which ginseng inhibits RhoA/ROCK-dependent p38 MAPK activation at the extracellular level by preventing binding of leptin at the receptor.

Accordingly, the regulation of RhoA was investigated in cultured cardiomyocytes and LV tissues of rats treated with ginseng after four weeks of sustained MI. In **Chapter 2**, leptin-induced RhoA activation was demonstrated to be mediated by p115RhoGEF, which along with p63RhoGEF that is expressed in the myocardium (Souchet et al., 2002), are positive regulators of RhoA activity linked to hypertension (Guilluy et al., 2010; Wuertz et al., 2010) as well as cardiac hypertrophy (Porchia et al., 2008). RhoA activation may also be downregulated by p190RhoGAP of which its GTP hydrolysis activity is promoted by binding of Rnd proteins

(Rnd1 and 3) (Wennerberg et al., 2003). Research of the effect of Rnd proteins on RhoA activation in the CV system is limited, however has been extensively studied in the area of cancer and contraction of the myometrium (Cario-Toumaniantz C et al., 2004; Lartey et al., 2006). Regulation of Rnd is primarily observed by gene transcription (reviewed in Riou et al., 2010) followed by protein expression and RhoGAP activity. Here we showed for the first time expression of Rnd3 in cardiomyocytes, the effects of leptin and ginseng on Rnd3 gene and protein expression as well as Rnd3 protein expression in LV tissues of sham and CAL rats. Leptin at 24 h in cardiomyocytes caused a significant decrease in Rnd3 gene and protein expression which were restored by treatment with ginseng. These results are in agreement with the previously observed leptin-induced increase in RhoA activation, which may therefore be a result of decreased downregulation of RhoA by Rnd3. Importantly, our *in vitro* data corresponded with Rnd3 protein expression measured from LV tissues of CAL-operated rats. Rnd3 protein expression in rats with sustained MI for 4 weeks was significantly depressed however in CAL rats that were administered ginseng protein expression values were similar to expression in sham animals. Hyperleptinemia has been associated with HF patients (Schulze et al., 2003) and consequently these observed decreases in Rnd3 protein expression in the LV tissues of CAL rats may be a result of elevated plasma leptin levels in HF rats. There may however have been additional circulatory levels of prohypertrophic factors such as AngII, ET-1 or inflammatory markers that are typically upregulated in HF, which may have also contributed to the observed decrease in Rnd3 protein expression in CAL rats.

Changes in the regulation of enhancers of RhoA activation in leptin-induced cardiac hypertrophy were additionally studied by measurement of the positive regulators, p63RhoGEF and ROCK, in the LV tissues of sham and CAL rats. Both p63RhoGEF and ROCK protein

expression were significantly increased in rats with sustained MI however these effects were attenuated in LV tissues of rats treated with ginseng. Interestingly, in our previous study, leptin administration to cultured ventricular cardiomyocytes did not induce a significant change in p63RhoGEF gene and protein expression whereas a 4-fold increase in the expression of p115RhoGEF was observed (Moey et al., 2011). This likely suggests differences between hypertrophy in cultured myocytes versus changes occurring in the intact remodelled myocardium.

In summary, here we have shown for the first time the reversibility of leptin-induced cardiac hypertrophy by ginseng via an increase Rnd3-mediated downregulation of RhoA/ROCK. Results from our study provide prospective insight into targeting the RhoA/ROCK pathway as potential therapy for the treatment of cardiac hypertrophy and HF associated with hyperleptinemia.

3.5 References

Abe Y, Ono K, Kawamura T, Wada H, Kita T, Shimatsu A and Hasegawa K. Leptin induces elongation of cardiac myocytes and causes eccentric left ventricular dilatation with compensation. Am J Physiol Heart Circ Physiol 2007;292:H2387-2396.

Attele AS, Wu JA and Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. Biochem Pharmacol 1999;58:1685-1693.

Bernardo BC, Weeks KL, Pretorius L and McMullen JR. Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. Pharmacol and Ther 2010;128:191-227

Bos JL, Rehmann H and Wittinghofer A. GEFs and GAPs: critical elements in the control of small G proteins. Cell 2007;129:865-877.

Brown JH, Del Re DP and Sussman MA. The Rac and Rho hall of fame: a decade of hypertrophic signaling hits. Circ Res 2006;98:730-742

Cario-Toumaniantz C, Reillaudoux G, Sauzeau V, Heutte F, Vaillant N, Finet M, Chardin P, Loirand G and Pacaud P. Modulation of RhoA-Rho kinase-mediated Ca^{2+} sensitization of rabbit myometrium during pregnancy-role of Rnd3. J Physiol 2003;552:403-413

Deng J, Wang YW, Chen WM, Wu Q and Huang XN. Role of nitric oxide in ginsenoside Rg(1)-induced protection against left ventricular hypertrophy produced by abdominal aorta coarctation in rats. Biol Pharm Bull 2010;33:631-635.

Fedak PWM, Verma S, Weisel RD and Li RK. Cardiac remodeling and failure: from molecules to man (part I). Cardiovasc Pathol 2005;14:1-11

Frey N and Olson EN. Cardiac hypertrophy: the good, the bad and the ugly. Annu Rev Physiol 2003;65:45-79

Guilluy C, Bregeon J, Toumaniantz G, Rolli-Derkinderen M, Retailleau K, Loufrani L, Henrion D, Scalbert E, Bril A, Torres RM, Offermanns S, Pacaud P and Loirand G. The Rho exchange factor Arhgef1 mediates the effects of angiotensin II on vascular tone and blood pressure. Nat Med 2010;16:183-190.

Guo J, Gan XT, Haist JV, Rajapurohitam V, Zeidan A, Faruq NS and Karmazyn M. Ginseng inhibits cardiomyocyte hypertrophy and heart failure via NHE-1 inhibition and attenuation of calcineurin activation. Circ Heart Fail 2011;4:79-88.

Hou N, Luo MS, Liu SM, Zhang HN, Xiao Q, Sun P, Zhang GS, Luo JD and Chen MS. Leptin induces hypertrophy through activating the peroxisome proliferator-activated receptor alpha pathway in cultured neonatal rat cardiomyocytes. Clin Exp Pharmacol Physiol 2010;37:1087-1095.

Jiang QS, Huang XN, Dai ZK, Yang GZ, Zhou QX, Shi JS and Wu Q. Inhibitory effect of ginsenoside Rb1 on cardiac hypertrophy induced by monocrotaline in rat. J Ethnopharmacol 2007;111:567-572

Karmazyn M, Moey M and Gan XT. Therapeutic potential of ginseng in the management of cardiovascular disorders. Drugs 2011;71:1989-2008

Karmazyn M, Purdham DM, Rajapurohitam V and Zeidan A (2008) Leptin Signaling in the Cardiovascular System, in *Signal Transduction in the Cardiovascular System in Health and Disease* (Srivastava AK and Anand-Srivastava MB eds) pp 377-395, Springer US.

Lartey J, Gampel A, Pawade J, Mellor H and López Bernal A. Expression of RND proteins in human myometrium. Biol Repro 2006;75:452-461

Liang Q, Elson AC and Gerdes AM. P38 MAP kinase activity is correlated with angiotensin II type 1 receptor blocker-induced left ventricular reverse remodeling in spontaneously hypertensive heart failure rats. J Cardiac Fail 2006;12:479-486

Madani S, De Girolamo S, Munoz DM, Li RK and Sweeney G. Direct effects of leptin on size and extracellular matrix components of human pediatric ventricular myocytes. Cardiovasc Res 2006;69:716-725

Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA and Friedman JM. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1995;1:1155-1161.

Moey M, Rajapurohitam V, Zeidan A and Karmazyn M. Ginseng (Panax quinquefolius) attenuates leptin-induced cardiac hypertrophy through inhibition of p115RhoGEF-RhoA/ROCK-dependent MAPK pathway activation. J Pharmacol Exp Ther 2011; 2011;339:746-756

Porchia F, Papucci M, Gargini C, Asta A, De Marco G, Agretti P, Tonacchera M and Mazzoni MR. Endothelin-1 up-regulates p115RhoGEF in embryonic rat cardiomyocytes during the hypertrophic response. J Recept Signal Transduct Res 2008;28:265-283

Predy GN, Goel V, Lovlin R, Donner A, Stitt L and Basu TK. Efficacy of an extract of North American ginseng containing poly-furanosyl-pyranosyl-saccharides for preventing upper respiratory tract infections: a randomized controlled trial. CMAJ 2005;173:1043-8

Purdham DM, Rajapurohitam V, Zeidan A, Huang C, Gross GJ and Karmazyn M. A neutralizing leptin receptor antibody mitigates hypertrophy and hemodynamic dysfunction in the postinfarcted rat heart. Am J Physiol Heart Circ Physiol 2008;295:H441-446.

Qin N, Gong QH, Wei LW, Wu Q and Huang XN. Total ginsenosides inhibit the right ventricular hypertrophy induced by monocrotaline in rats. Biol Pharm Bull 2008;31:1530-1535.

Rajapurohitam V, Gan XT, Kirshenbaum LA and Karmazyn M. The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. Circ Res 2003;93:277-279.

Riento K, Guasch RM, Garg R, Jin B and Ridley AJ. RhoE binds to ROCK I and inhibits downstream signaling. Mol Cell Biol 2003;23:4219-4229

Riou P, Villalong P and Ridley AJ. Rnd proteins: multifunctional regulators of the cytoskeleton and cell cycle progression. Bioessays 2010;32:986-992

Rose BA, Force T and Wang Y. Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale. Physiol Rev 2010;1507-1546

Rossman KL, Der CJ and Sondek J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nat Rev Mol Cell Biol 2005;6:167-180.

Schmidt A and Hall A. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev 2002;16:1587-1609.

Schram K, De Girolamo, Madani S, Munoz D, Thong F and Sweeney G. Leptin regulates MMP-2, TIMP-1 and collagen synthesis via p38 MAPK in HL-1 murine cardiomyocytes. Cell Mol Biol Lett 2010;15:551-563

Schulze PC, Kratzsch J, Linke A, Schoene N, Adams V, Gielen S, Erbs S, Moebius-Winkler S and Schuler G. Elevated serum levels of leptin and soluble leptin receptor in patients with advanced chronic heart failure. Eur J Heart Fail 2003;5:33-40

Söderberg S, Ahrén B, Jansson JH, Johnson O, Hallmans G, Asplund K and Olsson T. Leptin is associated with increased risk of myocardial infarction. J Intern Med 1999;246:409-418

Souchet M, Casamar-Portales E, Mazurais D, Schmidt S, Léger I, Javré JL, Robert P, Berrebi-Bertrand I, Bril A, Gout B, Debant A and Calmels TPG. Human p63RhoGEF, a novel RhoA-specific guanine nucleotide exchange factor, is localized in cardiac sarcomere. J Cell Sci 2002;115:629-640

Tamargo J and López-Sendón J. Novel therapeutic targets for the treatment of heart failure. Nat Rev Drug Discov 2011;10:536-555

Wennerberg K, Forget MA, Ellerbroek SM, Arthur WT, Burridge K, Settleman J, Der CJ and Hansen SH. Rnd proteins function as RhoA antagonists by activating p190RhoGAP. Curr Biol 2003;1106-1115

Wuertz CM, Lorincz A, Vettel C, Thomas MA, Wieland T and Lutz S. p63RhoGEF-a key mediator of angiotensin II-dependent signaling and processes in vascular smooth muscle cells. FASEB J 2010;24:4865-4876

Zeidan A, Javadov S and Karmazyn M. Essential role of Rho/ROCK-dependent processes and actin dynamics in mediating leptin-induced hypertrophy in rat neonatal ventricular myocytes. Cardiovasc Res 2006;72:101-111.

Zeidan A, Javadov S, Chakrabarti S and Karmazyn M. Leptin-induced cardiomyocyte hypertrophy involves selective caveolae and RhoA/ROCK-dependent p38 MAPK translocation to nuclei. Cardiovasc Res 2008;**77**:64-72.

Zeidan A, Hunter JC, Javadov S and Karmazyn M. mTOR mediates RhoA-dependent leptin-induced cardiomyocyte hypertrophy. Mol Cell Biochem 2011;352;99-108

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L and Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994;372:425-432

CHAPTER 4:

Ginseng reverses cardiac hypertrophy *in vitro* and heart failure *in vivo*

4.1 Introduction

Cardiovascular disease (CVD) continues to remain the number one cause of mortality in the world (World Health Organization, 2011). Although ischemic heart disease predominates as the most prevalent CVD, mortality from ischemic heart disease has been on the decline whereas, based on American Heart Association statistics (Lloyd-Jones et al., 2010), the incidence of heart failure continues to rise. Current pharmacotherapies for the treatment of HF, including β-adrenergic blockers, RAAS blockers such as AngII receptor blockers (ARBs), angiotensin converting enzyme (ACE) inhibitors and aldosterone blockers (Marian 2009; Hellawel and Margulies, 2010) have been proven to be of substantial benefit in prolonging and improving quality of life for HF patients without markedly reducing mortality rates (Krum and Teerlink, 2011; Rouleau, 2011) which are currently greater than 40%, 5 years after diagnosis (Jessup and Brozena, 2003). The high mortality rate associated with HF likely reflects the complexity of the myocardial remodelling process which contributes to HF and the difficulty in reversing this process with current pharmacotherapy (Marian 2009; Hellawel and Margulies, 2010).

As recently reviewed, improvement in the treatment of HF will likely result as a consequence of better understanding of the underlying mechanisms for this complex syndrome which should lead to the development of new and effective pharmacological agents (Tamargo and López, 2011). Another possible route for the identification of novel therapeutic approaches for treating HF may lie with alternate and less conventional medications such as TCMs which have been used for treating a variety of disorders including CVDs for thousands of years. Among these TCMs, NA ginseng as well as other ginseng varieties have been garnering increasing interest for their salutary effects in the CV system specifically as a cardioprotective agent in ischemia and reperfusion as well as prevention of cardiac hypertrophy and HF (reviewed in

Karmazyn et al., 2011). Recently, we have demonstrated a robust ability of NA ginseng to prevent cardiomyocyte hypertrophy and heart failure through a mechanism likely involving prevention of CaN activation (Guo et al., 2011), the latter representing a key factor for myocardial hypertrophy and remodelling (Molkentin, 1998; Frey and Olson, 2003). A recent intriguing study has shown that CaN-dependent cardiac hypertrophy is a reversible process (Berry et al., 2011). Accordingly, in view of the potential importance of CaN 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.

4.2 Methods

4.2.1 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 (Moey et al., 2011). Cells were grown in FBS containing medium for up to 48h following 24h starvation. For reversal experiments, cardiomyocytes were pre-treated with either AngII (100 nM), ET-1 (10 nM) or the α1 adrenoceptor agonist Phe (10 μM) (Sigma Aldrich, St. Louis, Missouri) for up to 24h followed by the addition of ginseng extract (Gin, 10 μg/ml) for a further 24h, in the presence of the hypertrophic stimuli. The extract was prepared from four-year-old NA ginseng (Panax quinquefolius) roots collected from 5 farms in Ontario, Canada (provided by Dr. Edmund Lui's lab at the University of Western Ontario) and shipped to Naturex (South Hackensack, NJ) for ginsenoside extraction with use of a hydroalcoholic process (Naturex, South Hackensack, NJ), as previously described (Guo et al., 2011). 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 US National Institutes of Health and the Canadian Council of Animal Care (Ottawa, Ontario, Canada).

4.2.2 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.

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

RNA was collected from treated cardiomyocytes using Qiazol Reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions and reverse transcribed to complementary DNA (cDNA) for real-time PCR analysis of αSA and modulatory calcineurin interacting protein-1 (MCIP-1) as previously described (Moey et al., 2011). cDNA was synthesized from 5 μg of total RNA using random primers (Invitrogen) and M-MLV Reverse Transcriptase (Invitrogen) 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 (Invitrogen) included in the PCR analyses are summarized Table 4.1. 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 αSA and MCIP-1, respectively, ending with elongation at 72°C for 45s. The housekeeping gene, 18S, was measured and quantified to normalize cDNA levels.

4.2.4 Calcineurin Activity Assay

CaN activity from cellular and tissue extracts collected with a lysis buffer and protease inhibitor cocktail was measured following the colorimetric Calcineurin Cellular Activity Assay Kit as per the manufacturer's instructions (Enzo Life Sciences, Ann Arbor, MI). CaN activity was measured by the amount of nmol phosphate (PO_4) released at 620nm using the SpectraMax 5 (Molecular Devices, Sunnyvale, CA) plate reader from tissue and cellular extracts.

4.2.5 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 Ang II, ET-1, or Phe 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 (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 1 hour at room temperature under light-free conditions. Cells were mounted with Vector Staining medium that also detects the nucleus onto microscope slides (VWR, West Chester, PA) for image capture using a Zeiss (Oberkochen, Germany) inverted fluorescence microscope at 630X magnification. Nuclear translocation was measured using ImageJ (Noursadeghi et al., 2008).

4.2.6 In vivo Studies

Fourty male Sprague-Dawley rats weighing between 200 and 250 g were randomly assigned to either a sham or CAL group without or with ginseng treatment (0.9 g/L in drinking water provided *ad libitum*) started four weeks after surgery and maintained for a further four weeks. CAL was performed as previously described under sodium pentobarbital (50 mg/kg bw) anesthesia (Guo et al., 2011). Buprenorphine (0.03 mg/kg) was immediately administered to all animals after completion of surgery for pain management. Studies were completed for a total of 8 weeks.

4.2.8 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 (Guo et al., 2011). Briefly, rats 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 for the E/A ratio calculation. All images were analyzed using the Vevo 770 Protocol-Based Measurements software.

4.2.9 Hemodynamic Measurements

After 8 weeks of treatment (end of the study), rats were anesthetized with pentobarbital sodium (50 mg/kg) and an anterior thoractomy was performed as previously described (Guo et al., 2011). A 2.0F P-V Mikro-Tip catheter (Millar Instruments, Houston, Texas) was retrogradely inserted into the LV via the right carotid artery as previously described. 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.

4.2.10 Statistical Analysis

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

Table 4.1. Gene-specific primer sequences of analyzed genes of interest.

Gene	Forward Primer Sequence	Reverse Primer Sequence
18S	5'-GTAACCCGTTGAACCCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3'
α-skeletal actin	5'-CACGGCATTATCACCAACTG-3'	5'-CCGGAGGCATAGAGAGACAG-3'
MCIP-1	5'-TCTCCAAGCTGGGACCAGGAGAGA-3'	5'-ATCAGAACGCGCGTGTCGGT-3'

4.3 Results

4.3.1 Ginseng reverses CAL-induced cardiac hypertrophy

We first determined whether ginseng administration four weeks after CAL reverses indices of myocardial remodelling and heart failure after a further four week follow-up with continued ligation. Forty animals were initially recruited into the study as described in Materials and Methods with an average body weight of 230 ± 4.4 g. Treated animals were administered NA ginseng (900 mg/L) dissolved in the drinking water four weeks after CAL (Figure 4.1A). Water consumption was monitored for the following four weeks and was found to be identical (approx 50 mL/day/animal at week 4) in all groups studied. Sustained CAL produced no mortality during the eight week post CAL period although 20% of the animals died within 24 h after CAL. All animals exhibited identical growth patterns throughout the eight week post-surgery period (Figure 4.1B). CAL produced a significant increase in all indices of cardiac hypertrophy as evidenced by increased expression of αSA(Figure 4.1C) and both total (Figure 4.1D) as well as left ventricular (Figure 4.1E) heart weights. As shown in Figure 1, all markers of hypertrophy were normalized by ginseng treatment.

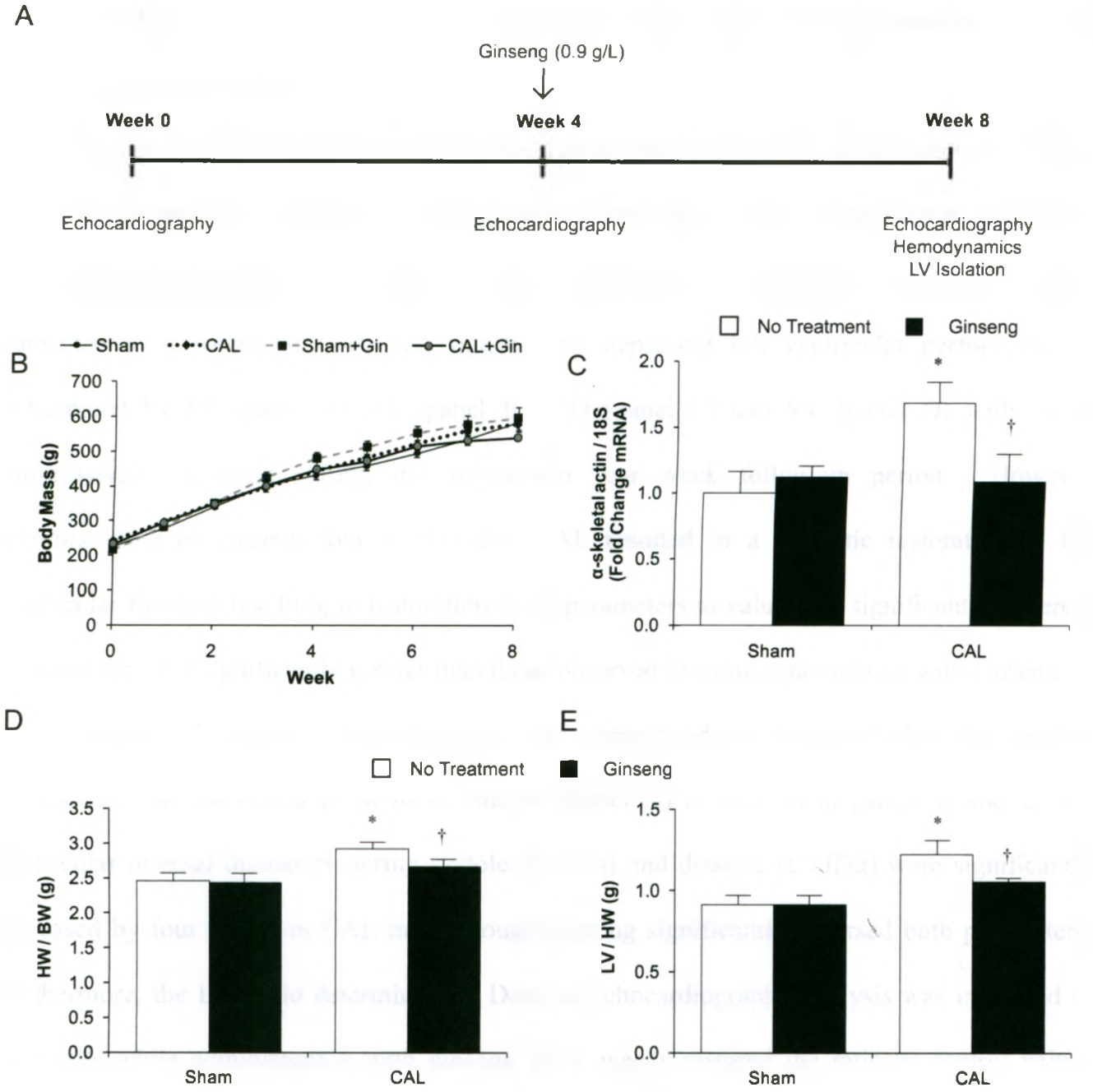

Figure 4.1. Ginseng reverses cardiac hypertrophy and α-skeletal gene expression *in vivo* without any effect on body mass changes. (A) Experimental design time as described in methods. Ginseng was administered to animals four weeks after sham or CAL surgery. (B) Sprague-Dawley rats averaging between 215 to 250 g were randomized to either sham or CAL administered water or ginseng. Body mass changes over the course of the eight-week study in all experimental groups were not significantly different from each other. CAL-induced cardiac hypertrophy demonstrated by increases in αSA gene expression (C), HW/BW (D) and LV/HW ratios (E) were significantly reduced with ginseng. Treatment with ginseng alone was without effect on any of the above measured parameters. $^{*}p<0.05$ vs. sham; $^{\dagger}p<0.05$ vs. CAL. BW: body weight; HW: heart weight; LV: left ventricle; αSA: α-skeletal actin.

4.3.2 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 (week 0), 4 weeks after surgery but before starting ginseng treatment and finally, 8 weeks after surgery. As shown in Figure 4.2 animals subjected to 4 weeks of CAL had significantly depressed left ventricular performance as determined by EF (panel A), FS (panel B), CO (panel C) and SV (panel D) with values progressively declining during the subsequent four week follow-up period. However, administration of ginseng four weeks after CAL resulted in a dramatic restoration in left ventricular function resulting in restoration in all parameters to values not significantly different from baseline but significantly greater than those observed in animals not treated with ginseng.

Figure 3.3 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 four weeks in CAL rats although ginseng significantly reversed both parameters. Furthermore, the E/A ratio determined by Doppler echocardiography analysis was increased in CAL rats while administration with ginseng for 4 weeks restored the ratio to control values (Figure 4.3D and E).

Echocardiographic parameters in rats subjected to sham surgery remained unchanged throughout the 8 week follow-up period nor was there any influence of ginseng on any parameter studied in sham-operated animals. These data are summarized in Table 4.1

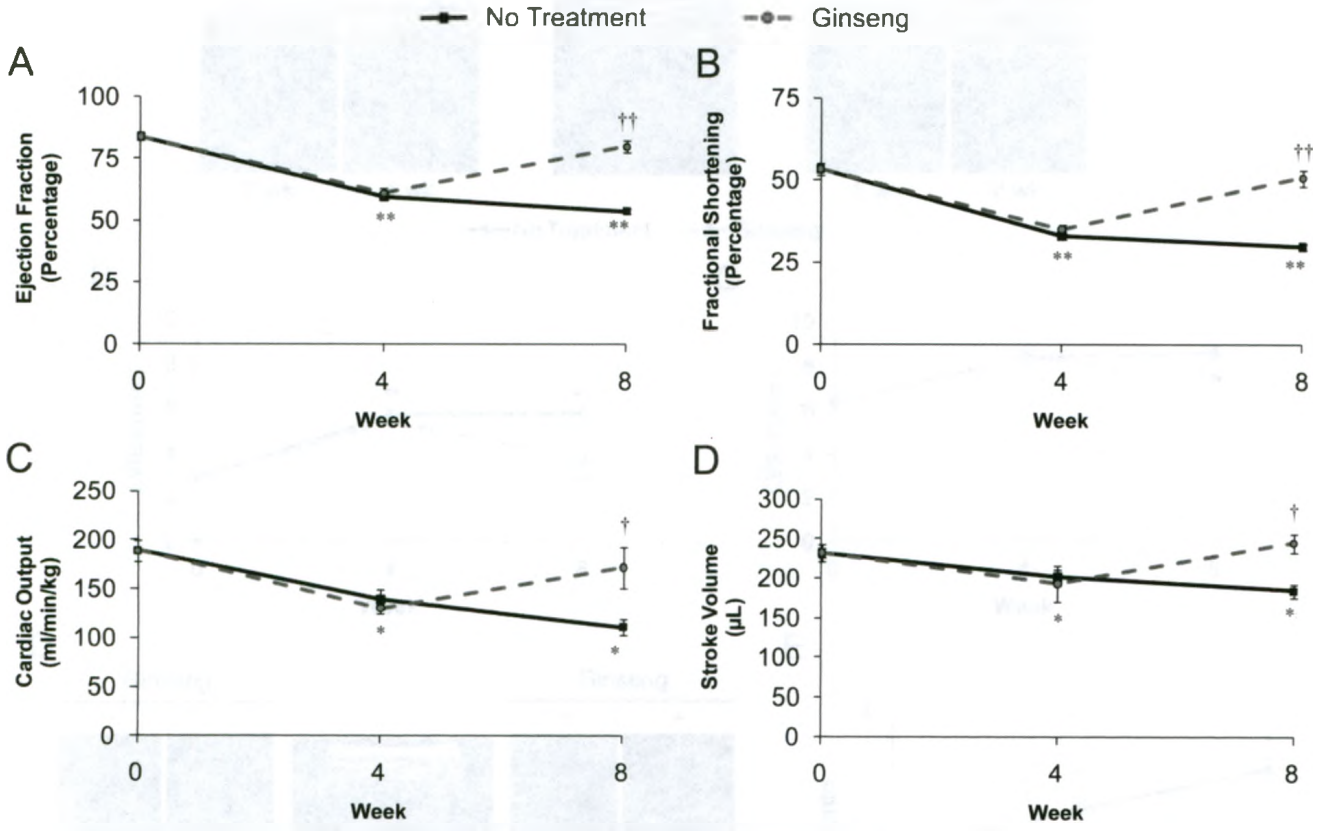

Figure 4.2. Ginseng reverses CAL-induced decreased ejection fraction (EF), fractional shortening (FS), cardiac output (CO) and stroke volume (SV). Echocardiographic recordings were measured before the surgery (week 0), four weeks after surgery (week 4) and at the end of the study (week 8) to assess ejection fraction (EF) (A), fractional shortening (FS) (B), (C) cardiac output (C) and stroke volume (SV) (D) as indicators of cardiac function. CAL (solid black line) induced significant decreases in EF, FS, CO and SV four weeks post-surgery and further worsened these parameters at eight weeks. Four week treatment with ginseng (dotted grey line) however returned EF, FS, CO and SV to control values at week 0. The EF, FS, CO and SV values for sham rats receiving water or ginseng were not significantly different from values shown at week 0. (N=10; *p<0.05 and **p<0.01 vs. week 0; †p<0.05 and ††p<*0.01* vs. week 4)

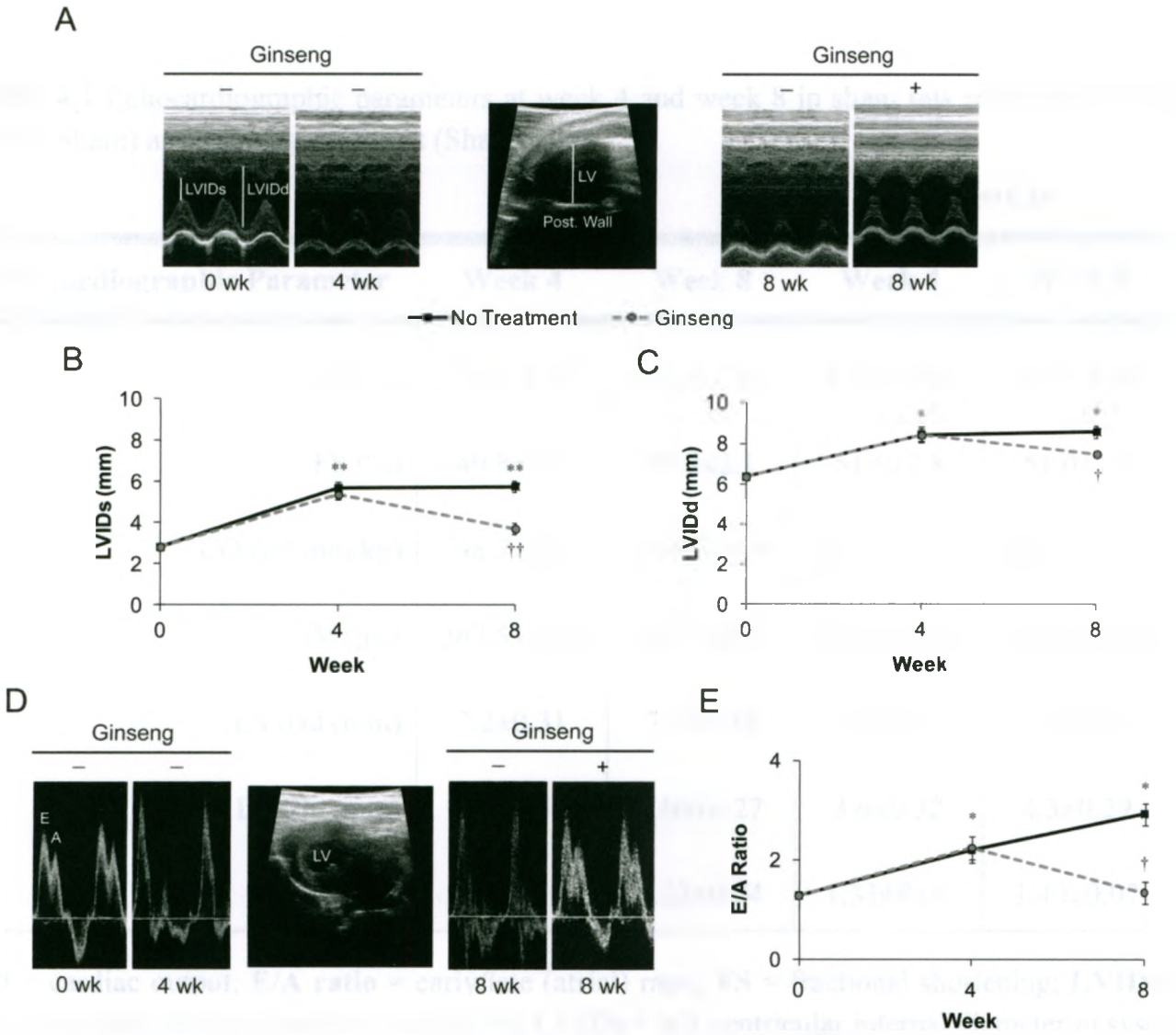

Figure 4.3. Ginseng reverses CAL-induced increased left ventricular internal diameters during systole (LVIDs) and diastole (LVIDd) and E/A ratio. Echocardiographic recordings were measured before surgery (week 0), four weeks after surgery (week 4) and at the end of the study (week 8) to assess changes in left ventricular internal diameter during systole (LVIDs) and diastole (LVIDd) as well as the early and late (atrial) ventricular filling velocity (E/A). CAL induced (solid black line) significant increases during both systole (B) and diastole (C) at week 4 which were returned to normal however with ginseng treatment (dotted grey line) at week 8. Changes in LVIDs and LVIDd at week 8 were not significantly different from week 4 measurements. Ginseng treatment further reversed the significant increase in E/A ratio (E) at week 4 and 8 to control values. The LVIDs, LVIDd and E/A ratio values of sham rats receiving water and ginseng were consistent control values similar to week 0 throughout the duration of the study. Representative echocardiographic M-Mode images exhibit increases in LVIDs and LVIDd (A) in CAL rats at week 4 (2^{nd} image panel) and 8 (3^{rd} image panel) and the reversal of these increases with ginseng treatment at week 8 (4^{th} image panel). Doppler echocardiographic images demonstrate an increased and decreased amplitude in the E and A wave (D), respectively, in CAL rats at week 4 (2^{nd} image panel) and 8 (3^{rd} image panel), however this effect was returned to normal with ginseng treatment (4^{th} image panel). (N=10; *p<0.05 vs. week 0; †p<0.05 vs. week 4).

Table 4.1 Echocardiographic parameters at week 4 and week 8 in sham rats receiving drinking water (Sham) and ginseng treatment (Sham+Gin).

	Sham		Sham+Gin	
Echocardiographic Parameter	**Week 4**	**Week 8**	**Week 4**	**Week 8**
EF (%)	79.5±1.67	80.4±2.08	80.7±2.44	81.1±1.48
FS (%)	49.8±1.9	49.8±2.1	51.6±2.8	51.0±1.5
CO (ml/min/kg)	198.7±21.1	166.7±4.9	181.3±13.8	161.5±12.1
SV (μL)	267.5±26.9	247.9±11	251.2±17.6	248.9±13.9
LVIDd (mm)	7.2±0.31	7.6±0.34	7.4±0.35	7.8±0.25
LVIDs (mm)	3.9±0.35	4.06±0.27	3.6±0.32	4.3±0.29
E/A ratio	1.4±0.14	1.33±0.04	1.31±0.05	1.40±0.07

CO = cardiac output; **E/A ratio** = early/late (atrial) ratio; **FS** = fractional shortening; **LVIDd** = left ventricular internal diameter in diastole; **LVIDs** = left ventricular internal diameter in systole; **SV** = stroke volume

4.3.3 Hemodynamic parameters in eight week postinfarcted animals

Catheter-based hemodynamic measurements were performed at the end of the study to further assess additional left ventricular systolic and diastolic function. As shown in Figure 3.4, eight weeks of CAL resulted in significantly decreased systolic function with respect to all parameters studied which was accompanied by markedly enhanced left ventricular end-diastolic pressures and end-diastolic volumes. Figure 3.5 further illustrates CAL-induced contractile and diastolic dysfunction as evidenced by a decreased slope in the ESPVR and an increased slope in the EDVR. However, as shown in both Figures 3.4 and 3.5, animals treated with ginseng four weeks after CAL demonstrated almost complete normalization of hemodynamic parameters.

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

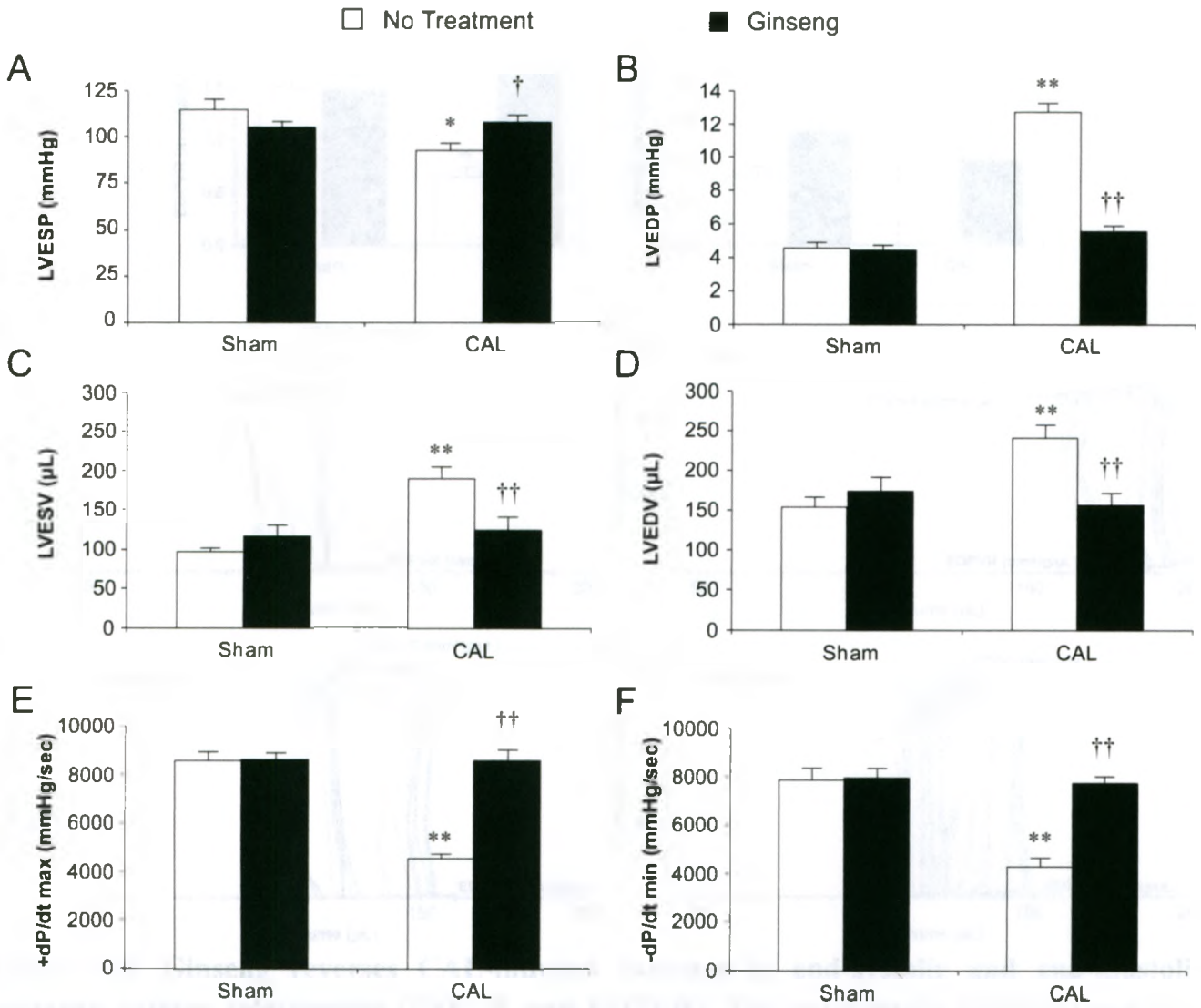

Figure 4.4. Ginseng reverses CAL-induced cardiac dysfunction. Hemodynamic measurements at the end of the study (week 8) were recorded as previously described (see Methods) to obtain left ventricular end systolic and diastolic pressures and volumes as well as dP/dT max and min. CAL significantly reduced LVESP (A), dP/dT max (E) and dP/dT min (F) which were prevented with ginseng treatment. LVEDP, LVESV and LVEDV increases in CAL rats were similarly prevented after four weeks of treatment with ginseng. All hemodynamic parameters in sham rats receiving ginseng treatment were not significantly different from sham rats receiving water. (N=10; *p<0.05 and **p<0.01 vs. sham; †p<0.05 and ††p<0.01 vs. CAL)

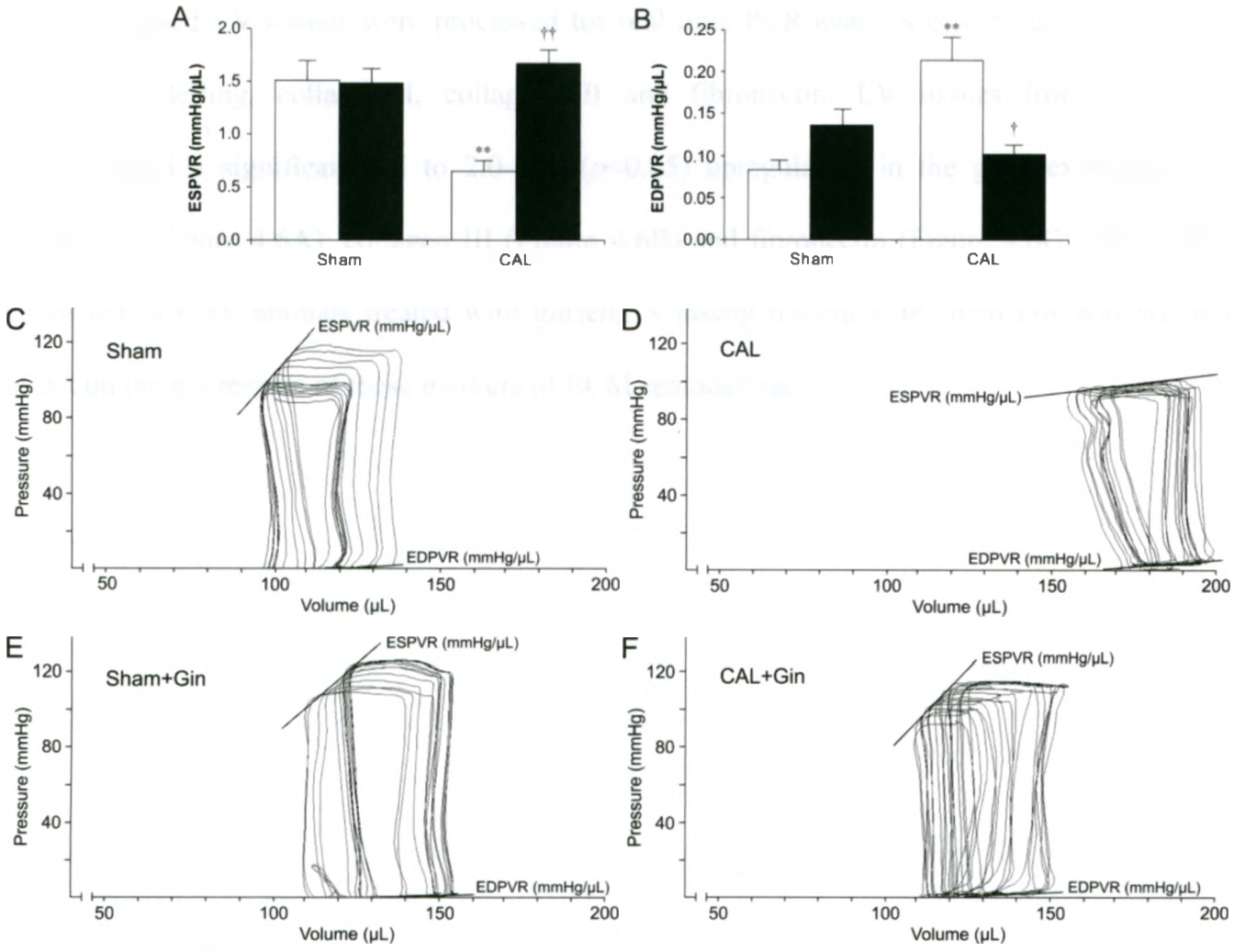

Figure 4.5. Ginseng reverses CAL-induced increase in end-systolic and end-diastolic pressure volume relationship (ESPVR and EDPVR). The end-diastolic (EDPVR) and end-systolic (ESPVR) pressure relationship slope from each experimental group was determined through analysis of the pressure-volume (PV) loops obtained from hemodynamic analysis. CAL-induced decrease and increase in ESPVR (A) and EDPVR (B), respectively, was significantly ($p<0.05$) prevented with four week ginseng treatment. EDPVR and ESPVR values of sham rats receiving ginseng treatment were not significantly different from sham control values. Representative PV loops from sham (C), CAL (D), sham with ginseng (E) and CAL with ginseng (F). Slope lines of the ESPVR (top left of loop) and EDPVR (bottom right of loop) were drawn using Adobe Photoshop CS. The slope of ESPVR was evidently decreased while EDVPR was increased in CAL rats (D) however treatment with ginseng (F) restored these parameters to sham control values. (N=10; **$p<0.01$ vs. sham; ††$p<0.01$ vs. CAL)

4.3.4 Ginseng prevents indices of extracellular matrix remodelling/cardiac fibrosis

Isolated LV tissues were processed for real-time PCR analysis of the gene markers for ECM remodelling, collagen I, collagen III and fibronectin. LV tissues from CAL rats demonstrated a significant 1.5 to 2.0-fold ($p<0.05$) upregulation in the gene expressions of collagen I (Figure 4.6A), collagen III (Figure 4.6B) and fibronectin (Figure 4.6C) which were abolished in CAL animals treated with ginseng. Ginseng treatment in sham rats was without effect on the expression of these markers of ECM remodelling.

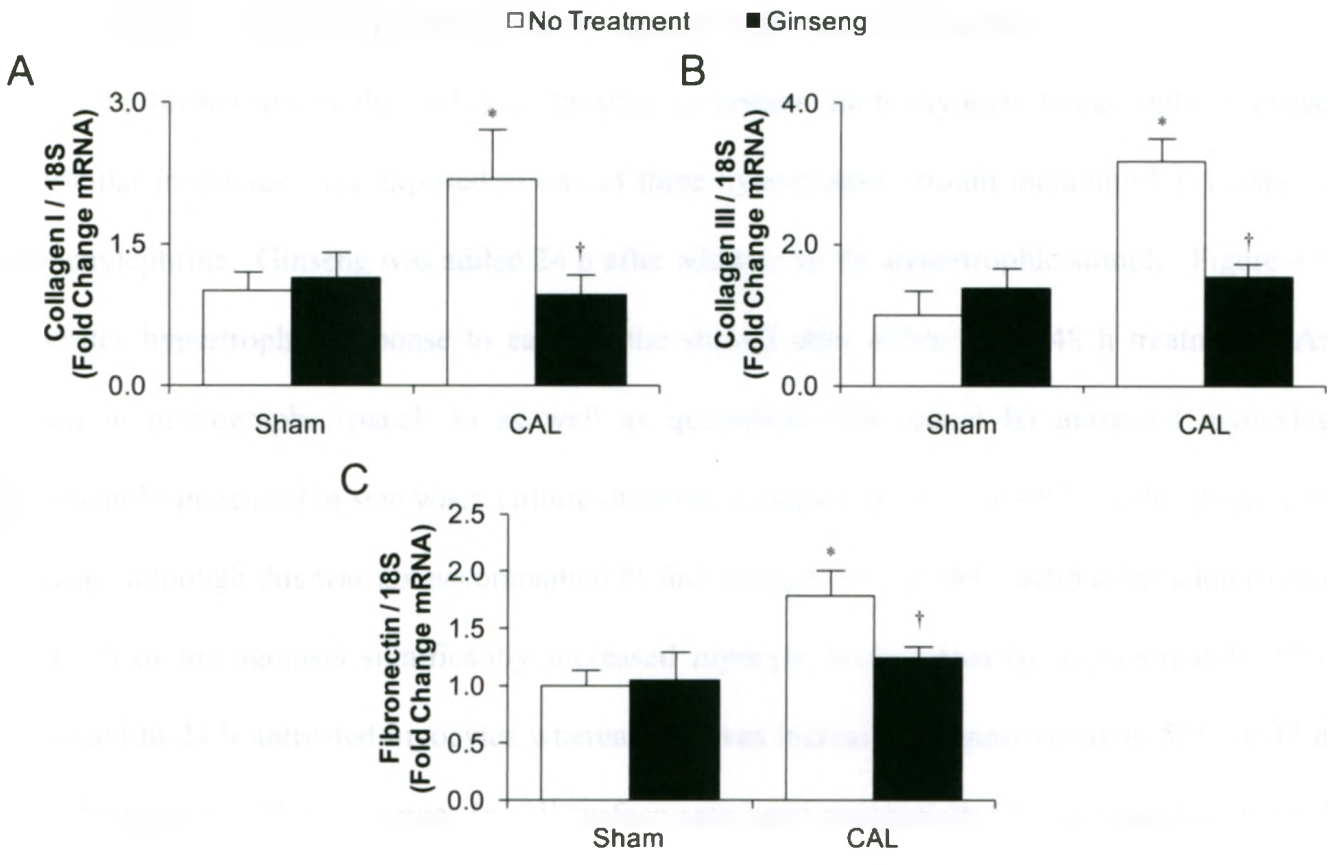

Figure 4.6. Ginseng prevents upregulation of markers of extracellular matrix remodeling. Gene expression of extracellular matrix (ECM) remodeling markers, collagen I, collagen II and fibronectin were quantified using real-time PCR from isolated LV tissues of the different experimental groups. CAL-induced significant increases in collagen I (A), collagen III (B) and fibronectin (C) gene expression were significantly ($p<0.05$) reduced in CAL animals receiving ginseng treatment. Expression of these gene markers of ECM were not affected by ginseng treatment alone. (N=6-8; *$p<0.05$ vs. sham; †$p<0.05$ vs. CAL)

4.3.5 Direct reversal by ginseng of cardiomyocyte hypertrophy in culture

To further assess the ability of ginseng to reverse cardiomyocyte hypertrophy cultured ventricular myocytes were exposed to one of three hypertrophic stimuli including ET-1, Ang II or phenylephrine. Ginseng was added 24 h after addition of the hypertrophic stimuli. Figure 4.7 illustrates hypertrophic response to each of the stimuli after either 24 or 48 h treatment. As shown in micrographs (panel A) as well as quantified data (panel B) untreated myocytes significantly increased in size when culture duration increased from 24 to 48 h, 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 24 h untreated myocytes whereas this was increased to approximately 55% in 48 h treated myocytes. These changes in cell surface area were accompanied by increased α-skeletal actin expression although gene expression upregulation was relatively similar for 24 and 48 h treatment values.

We next determined whether ginseng administration could reverse the hypertrophic response when added 24 h after addition of the respective agonist. Cells were then maintained for a further 24 h in the presence of the hypertrophic agonist under study in the absence or presence of ginseng. As shown in Figure 4.7, 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).

4.3.6 Ginseng reverses AngII, ET-1 and Phe-induced ventricular cardiac hypertrophy and α-skeletal actin gene expression in vitro.

Cultured neonatal ventricular cardiomyocytes were treated accordingly with AngII, ET-1 and phenylephrine (see Methods), which as alluded to earlier are classical humoral factors

released in HF to assess the ability of ginseng to reverse hypertrophy *in vitro*. Cardiac hypertrophy was evident in cultured cardiomyocytes treated with the hypertrophic agonists at 24 h and was further increased after 48 h (Figure 4.7A) Treatment with ginseng however significantly ($p<0.05$) abrogated these effects and returned cell size to control values at 48 h. An increase in expression of αSA was concomitantly observed in cells subjected to AngII, ET-1 and phenylephrine at 24 and 48 h (Figure 4.7B). Ginseng similarly reversed these observed increases in αSA expression. Treatment with ginseng alone was without effect.

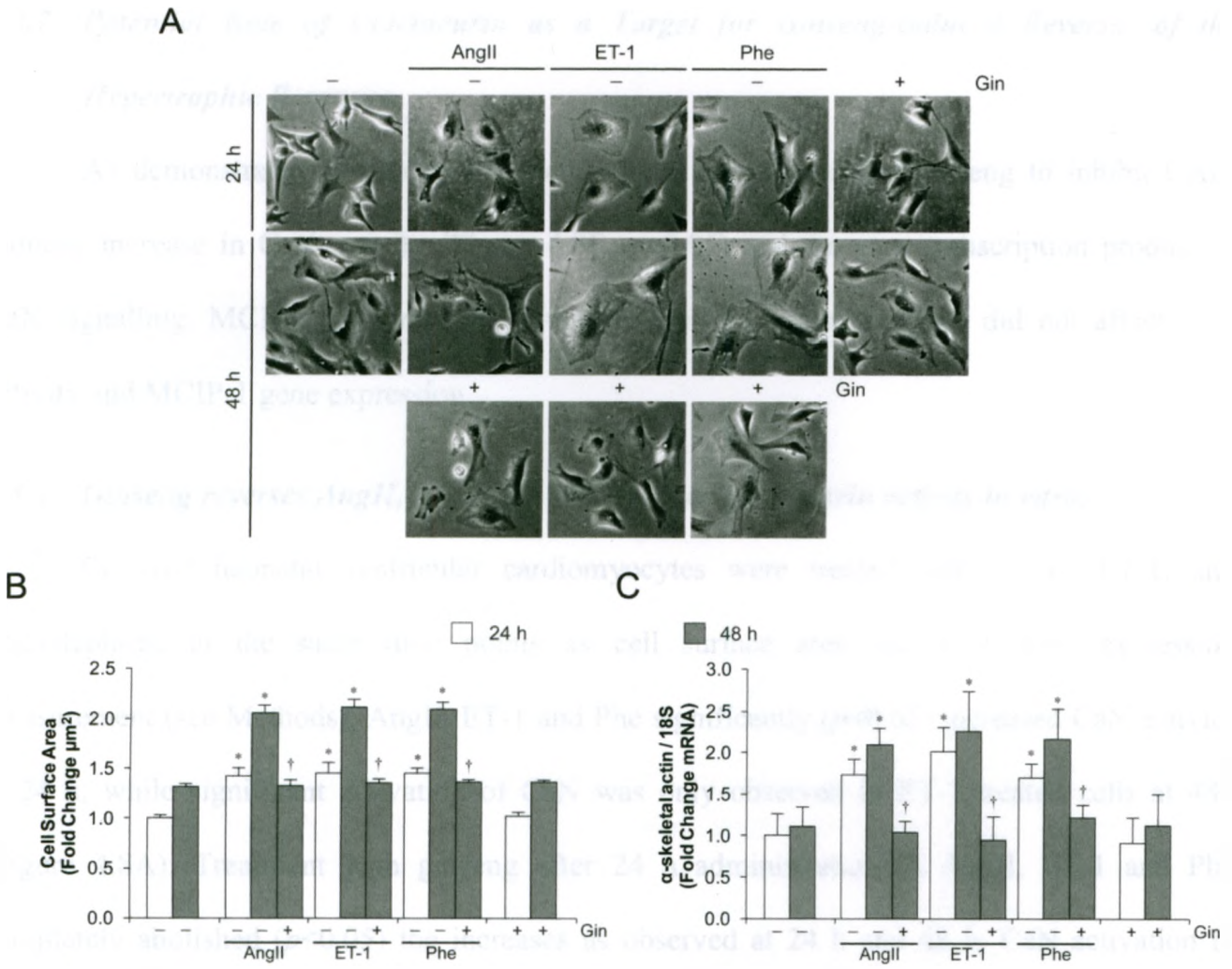

Figure 4.7. Ginseng reverses cardiac hypertrophy and α-skeletal gene expression *in vitro.* (A) Representative cell size images of neonatal ventricular cardiomyocytes subjected to different treatments as indicated. Top row represents an incubation time period of 24h while the second and third row represent 48h incubation. The first and last column represents control conditions of cells without and with ginseng, respectively. Second, third and fourth column represent cells treated with AngII, ET-1 and Phe, respectively, with (*3rd row*) or without (*1st* and *2nd row*) ginseng. Isolated neonatal ventricular cardiomyocytes subjected to AngII, ET-1 and Phe incubation for 24h demonstrated a significant increase in cell surface area (B) as well as αSA gene expression (C) which were further augmented at 48h. Treatment with ginseng after 24h incubation with the hypertrophic agonists reversed these observed increases at 24h and 48h in cell surface and αSA. Ginseng alone was without effect. αSA: α-skeletal actin; AngII: angiotensin II; ET-1: endothelin-1; Phe: phenylephrine; Gin: ginseng; +: with; -: without. (N=7; *$p<0.05$ vs. respective time controls; †$p<0.05$ vs. respective hypertrophic agent).

4.3.7 Potential Role of Calcineurin as a Target for Ginseng-induced Reversal of the Hypertrophic Response

As demonstrated in Figure 4.8, there was a strong trend for ginseng to inhibit CAL-induced increase in CaN activity (Figure 4.8E, p=0.05) and the gene transcription product of CaN signalling, MCIP-1 (Figure 4.8F, p=0.06). Ginseng treatment alone did not affect CaN activity and MCIP-1 gene expression.

4.3.8 Ginseng reverses AngII, ET-1 and Phe-induced calcineurin activity in vitro.

Cultured neonatal ventricular cardiomyocytes were treated with AngII, ET-1, and phenylephrine at the same time points as cell surface area and αSA gene expression measurement (see Methods). AngII, ET-1 and Phe significantly (p<0.05) increased CaN activity at 24 h, while significant activation of CaN was only observed in ET-1 treated cells at 48h (Figure 4.8A). Treatment with ginseng after 24 h administration of AngII, ET-1 and Phe completely abolished (p<0.05) the increases as observed at 24 h and 48 h. CaN activation in AngII, ET-1 and Phe treated at 24 h were not significantly different from activation at 48 h. Similarly, upregulation of MCIP-1 gene expression at 24 h and 48 h by AngII, ET-1 and Phe was reversed by treatment with ginseng (Figure 4.8B). Increased MCIP-1 gene expression at 24 h induced by AngII, ET-1 and Phe was not significant from expression at 48 h. Upstream NFAT3 nuclear translocation of MCIP-1 gene expression was visualized using immunofluorescence (Figure 4.8C) and quantified by the nuclear:cytosolic fraction (Figure 4.8D) using ImageJ as described previously (Noursadeghi et al., 2008). Significant NFAT3 nuclear translocation observed in AngII, ET-1 and Phe treated cells at 24h and 48h was reversed with ginseng (Figure 4.8C and 4.8D). Ginseng alone was without effect on CaN activation, MCIP-1 gene expression or NFAT3 nuclear translocation.

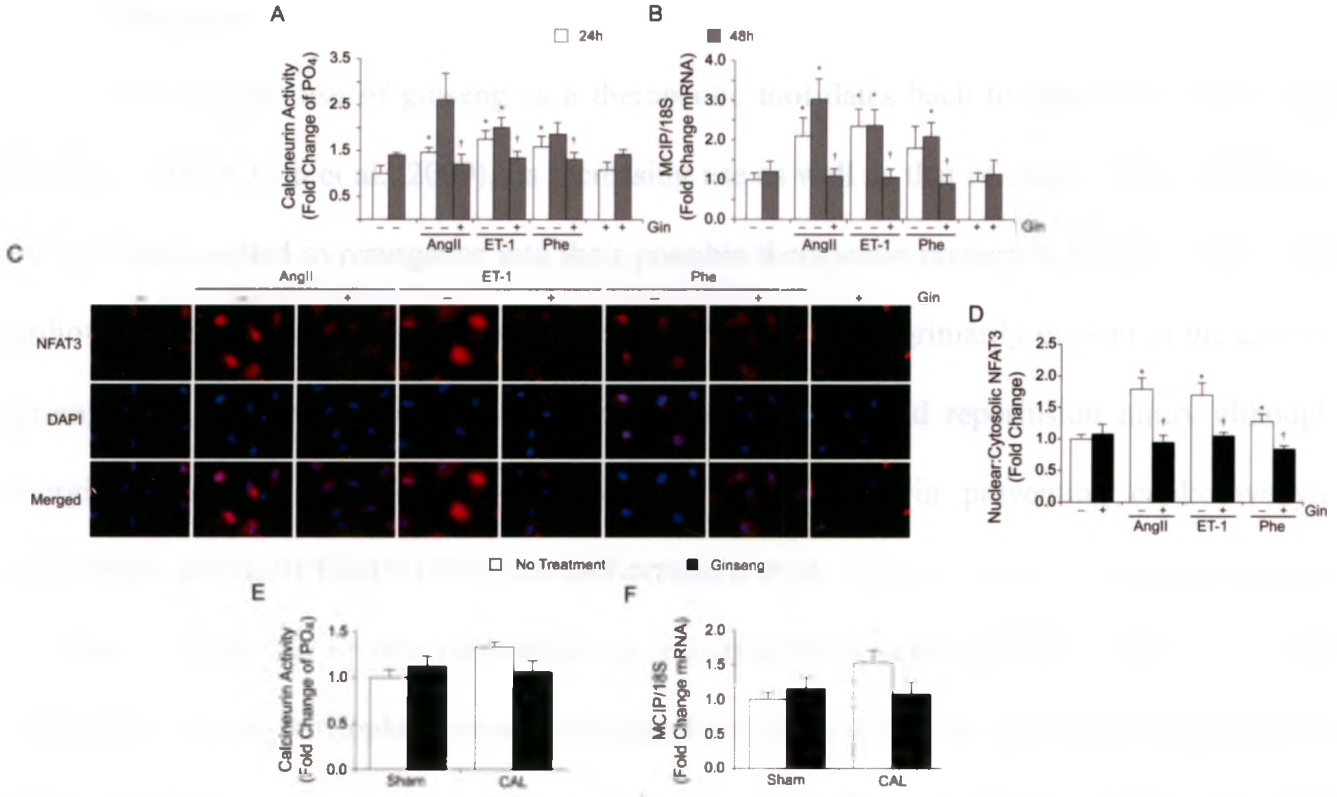

Figure 4.8. Ginseng reverses calcineurin/NFAT3 signaling pathway *in vitro* and *in vivo*. Calcineurin activity (A) and MCIP-1 (B) was significantly increased in ventricular cardiomyocytes subjected to AngII, ET-1 and Phe incubation for 24h and 48h. Treatment with ginseng after 24h however significantly reversed these effects. Ginseng alone was without effect. (N=7). (C) Representative images demonstrating increased NFAT3 nuclear translocation, an essential component of calcineurin signaling, after administration of AngII, ET-1 and Phe at both 24h and 48h with or without ginseng. Selected images exhibit stimulation of NFAT3 nuclear translocation at 48h. Top, middle and bottom row represents NFAT3, Hoechst and merged images, respectively. First and last column represent control cells without and with ginseng, respectively. (D) Nuclear translocation from three independent fluorescent sets was analyzed using a nuclear:cytosolic fraction quantification method as previously described via ImageJ. AngII, ET-1 and Phe-induced nuclear translocation was significantly reversed by treatment with ginseng. (N=3) Calcineurin activity (E) (p=0.05) and MCIP-1 (F) (p=0.09) increased gene expression measured in LV tissues (N=6-8) from CAL rats were similarly prevented by ginseng. AngII: angiotensin II; ET-1: endothelin-1; Phe: phenylephrine; Gin: ginseng; +: with; -: without. (*p<0.05 vs. control; †p<0.05 vs. respective hypertrophic agonist).

4.4 Discussion

Although the use of ginseng as a therapeutic tool dates back to more than 2000 years (Goldstein, 1975; Chu et al., 2009), 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 (Karmazyn et al., 2011). 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 Karmazyn et al., 2011). 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, Ang II and the α_1 adrenoceptor agonist, phenylephrine. More importantly, ginseng administration for 4 weeks following induction of MI, at which time substantial LV dysfunction and hypertrophy is clearly evident, produces substantial reversal resulting in the near-normalization of all parameters studied. It should be noted that at the dose studied, ginseng produced no effect on blood pressure. This, coupled with direct effects seen in cardiomyocytes, suggests a direct reversal of the hypertrophic program by ginseng.

In vivo reversal of postinfarction ventricular remodelling and HF was demonstrated first by serial echocardiography which revealed marked evidence of LV dysfunction after four weeks of CAL as demonstrated by reductions in EF, FS, SV and CO. These effects persisted for the duration of the 8 week post CAL period but were completely reversed by ginseng administration at the 8 week period. These results were reinforced by hemodynamic assessments. Although serial catheter-based invasive hemodynamic determinations are difficult to perform in view of the invasive nature of the procedure, work from our laboratory and that of others has clearly

demonstrated marked LV systolic and diastolic dysfunction four weeks after CAL (Guo et al., 2011; Purdham et al., 2008). As seen in the results, animals with ginseng after four weeks of CAL demonstrated normalized hemodynamic properties that were in some cases completely identical to sham animals demonstrating potent reversibility properties of ginseng. An additional unexpected benefit seen in ginseng-treated rats is the reduction in myocardial collagen gene expression levels thus suggesting mitigation of myocardial fibrosis which can contribute to ventricular dysfunction in HF (Zannad et al., 2010; de Jong et al 2011; Konstam et al 2011). Indeed, this represents a major component in the pathogenesis of cardiac dysfunction since a disruption in the equilibrium between the tensile and elastic fibers constituting the collagen network of the myocardial ECM, particularly collagen I and collagen III, contribute to the myocardial remodelling process (Chapman et al., 1990; Brower et al., 2006). Myocardial collagen content represents a balance between synthesis and degradation although at present (Chapman et al., 1990), the specific mechanism by which ginseng reduces collagen formation in the postinfarcted heart is not known. Although the antifibrotic effect of ginseng in the heart has not been previously studied, ginseng and its bioactive ginsenosides have been shown to reduce fibrosis both *in vitro* and *in vivo* in various models (Peng et al., 2009; Ahn et al., 2011; Lo et al., 2011; Tsai et al., 2011). Irrespective of precise mechanisms, the possibility exists that the antifibrotic effect of ginseng may contribute to its salutary effect seen in the present study. This therefore represents a potentially important area of future research which may help to reinforce a possible antifibrotic role of ginseng in remodelled myocardium.

We believe that a major contributing factor of ginseng to reversal of remodelling and HF stems from its ability to reverse cardiomyocyte hypertrophy, most likely by a direct effect potentially mediated by reversal of CaN upregulation (discussed below). Indeed, reduction in

cardiac hypertrophy represents an important component of the therapeutic strategy for treating HF (Tamargo and López, 2011; Jessup and Brozena, 2003). The key role of cardiac hypertrophy as a target for ginseng-induced reversibility is supported in our study 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 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 remodelling in general and hypertrophy in specifics. Ginseng exerts a myriad of cell signalling effects which could contribute to its antihypertrophic properties (reviewed in Karmazyn et al., 2011). For example, we have recently reported that ginseng can prevent leptin-induced hypertrophy by inhibiting the activation of the RhoA-ROCK pathway (Moey et al., 2011). In the present study we concentrated exclusively on the CaN pathway both *in vivo* as well as in cultured myocytes where experimental conditions can be much better controlled. CaN is a key signalling pathway in the pathogenesis of cardiac hypertrophy and HF (Molkentin et al., 1998; Frey and Olson, 2003). This reflects calcium-calmodulin dependent upregulation of CaN which results in dephosphorylation of NFAT3 and its subsequent translocation into the nucleus (Molkentin et al., 1998). A role for CaN in hypertrophy and HF is further supported by studies showing that pharmacological inhibition of CaN prevents cardiac hypertrophy (Taigen et al., 2000; Takeda et al., 2002; Obata et al., 2005) as well as improving cardiac function *in vivo* (Takeda et al., 2002; Obata et al., 2005). We have previously shown that activation of CaN in the four week postinfarcted myocardium as well as in hypertrophied cultured ventricular myocytes is

prevented by ginseng (Guo et al., 2011). We have assessed CaN 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 CaN activation (Molkentin et al., 1998). Robust CaN activation was clearly seen in cultured myocytes 24 h after initiation of hypertrophy which persisted for a further 24 h although in the presence of ginseng added at 24 h values returned to control levels. A similar strong trend was observed in hearts subjected to CAL, although CaN activation was markedly reduced at eight weeks compared to four week values. The latter likely reflects the temporal kinetics of regression of peak CaN activation which clearly precedes the eight week post CAL time point. Interestingly, our results bear some conceptual similarity to a recent report demonstrating reversibility of CaN-dependent cardiac hypertrophy (Berry et al., 2011). That study showed that CaN-dependent hypertrophy produced by overexpression of a mutant CaN transgene precedes the development of HF, thus precluding the necessity of sustained CaN activation for the production of ventricular dysfunction associated with HF. Moreover, turning off CaN activity reversed the hypertrophic phenotype (Berry et al., 2011).

In conclusion, our study shows that NA ginseng reverses cardiomyocyte hypertrophy *in vitro* as well as myocardial hypertrophy, remodelling and HF 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 HF (Marian, 2009; Hellawel and Margulies, 2011). Our results based on findings in cultured myocytes and HF as well as evidence in the literature (Berry et al., 2011) suggest that reversing of CaN-dependent processes represents a viable mechanistic basis for the reversal of hypertrophy by ginseng and therefore the results reinforce the concept that targeting the CaN/NFAT3 pathway may represent a key approach towards developing effective therapeutic strategies. At present, we do not know 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 which are present in ginseng (Jiao and Zhao, 2009). While results from animal experimental models should be treated with some degree of caution, our results suggest that administration of ginseng for the treatment of HF, particularly as adjunctive therapy with established medications, may offer substantial additional benefit particularly with respect to reversing the remodelling process.

4.5 References

Attele AS, Wu JA and Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. Biochem Pharmacol 1999;58:1685-1693

Ahn JY, Kim MH, Lim J, Park S, Lee SL, Yun YS and Song JY. The inhibitory effect of ginsan on TGF-β mediated fibrotic process. J Cell Physiol 2011;226:1241-1247

Berry JM, Le V, Rotter D, Battiprolu PK, Grinsfelder B, Tannous P, Burchield JS, Czubryt M, Backs J, Olson EN, Rothermel BA and Hill JA. Reversibility of adverse, calcineurin-dependent cardiac remodeling. Circ Res 2011;109:407-417

Brower GJ, Gardner JD, Forman MF, Murray DB, Voloshenyuk T, Levick SP and Janicki JS. The relationship between myocardial extracellular matrix remodeling and ventricular function. Eur J Cardio-thor Surg 2006;30:604-610

Chapman D, Weber KT and Eghbali M. Regulation of fibrillar collagen types I and III and basement membrane type IV collagen gene expression in pressure overloaded rat myocardium. Circ Res 1990;67:787-794

de Jong S, van Veen TA, de Bakker JM, Vos MA and van Rijen HV. Biomarkers of myocardial fibrosis. J Cardiovasc Pharmacol 2011;57:522-535

Frey N and Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. Annu Rev Physiol 2003;65:45-79

Guo J, Gan XT, Haist JV, Rajapurohitam V, Zeidan A, Faruq NS and Karmazyn M. Ginseng inhibits cardiomyocyte hypertrophy and heart failure via NHE-1 inhibition and attenuation of calcineurin activation. Circ Heart Fail 2011;4:79-88

Hellawel JL and Margulies KB. Myocardial reverse remodeling. Cardiovasc Ther. 2010; 00:1-10

Jessup M and Brozena M. Heart Failure. N Engl J Med 2003;348:2007-2018

Jia L and Zhao Y. Current evaluation of the millennium phytomedicine--ginseng (I): etymology, pharmacognosy, phytochemistry, market and regulations. Curr Med Chem 2009;16:2475-2484.

Karmazyn M, Moey M and Gan XT. Therapeutic potential of ginseng for the management of cardiovascular disorders. Drugs 2011;71:1989-2008

Konstam MA, Kramer DG, Patel AR, Maron MS and Udelson JE. Left ventricular remodeling in heart failure: current concepts in clinical significance and assessment. JACC Cardiovasc Imaging 2011;4:98-108

Krum H and Teerlink JR. Medical therapy for chronic heart failure. Lancet 2011;378;713-721

Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott MM, Meigs J, Mozaffarian D, Mussolino M, Nichol G, Roger VL, Rosamond W, Sacco R, Sorlie P, Roger VL, Thom T, Wasserthiel-Smoller S, Wong ND and Wylie-Rosett J; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke stastics-2010 update: a report from the American Heart Association. Circ 2010;121:e46-e215

Lo YT, Tsai YH, Wu SJ, Chen JR and Chao JC. Ginsenoside rb1 inhibits cell activation and liver fibrosis in rat hepatic stellate cells. J Med Food 2011;14:1135-1143

Lu JM, Yao Q and Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. Curr Vasc Pharmacol 2009;7:293-302

Marian AJ. Experimental therapies in hypertrophic cardiomyopathy. J Cardiovasc Transl Res 2009;4:483-492

Moey M, Rajapurohitam V, Zeidan A and Karmazyn M. Ginseng (Panax quinquefolius) attenuates leptin-induced ventricular cardiac hypertrophy through inhibition of the p115RhoGEF-RhoA/ROCK-dependent MAPK pathway activation. J Pharm Exp Ther 2011;339:746-756

Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR and Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 1998;93 :215-228

Noursadeghi M, Tsang J, Haustein T, Miller RF, Chain BM and Katz Dr. Quantitative imaging assay for NF-kappaB nuclear translocation in primary human macrophages. J Immunol Methods 2008;329:194-200

Obata K, Nagata K, Iwase M, Odashima M, Nagasaka T, Izawa H, Murohara T, Yamada Y and Yokota M. Overexpression of calmodulin induces cardiac hypertrophy by a calcineurin-dependent pathway. Biochem Biophys Res Commun 2005;338:1299-1305

Peng XD, Dai LL, Huang CQ, He CM, Yang B and Chen LJ. Relationship between anti-fibrotic effect of Panax notoginseng saponins and serum cytokines in rat hepatic fibrosis. Biochem Biophys Res Commun 2009;388:31-4

Purdham DM, Rajapurohitam V, Zeidan A, Huang C, Gross GJ and Karmazyn M. A neutralizing leptin receptor antibody mitigates hypertrophy and hemodynamic dysfunction in the postinfarcted rat heart. Am J Physiol Heart Circ Physiol 2008;295:H441-H446

Rouleau JL. New and emerging drugs and device therapies for chronic heart failure in patients with systolic ventricular dysfunction. Can J Cardiol 2011; 27:296-301

Taigen T, De Windt LJ, Lim HW and Molkentin JD. Targeted inhibition of calcineurin prevents agonist-induced cardiomyocyte hypertrophy. Proc Natl Acad Sci USA 2000;97:1196-1201

Takeda Y, Yoneda T, Demura M, Usukura M and Mabuchi H. Calcineurin inhibition attenuates mineralocorticoid-induced cardiac hypertrophy. Circulation 2002;105:677-679

Tamargo J and López-Sendón J. Novel therapeutic targets for the treatment of heart failure. Nat Rev Drug Discov 2011;10:536-555

Tsai KD, Yang SM, Lee JC, Wong HY, Shih CM, Lin TH, Tseng MJ and Chen W. Panax notoginseng attenuates bleomycin-induced pulmonary fibrosis in mice. Evid Based Complement Alternat Med 2011;2011:404761

World Health Organization. (2011) Ten leading causes of death in 2008: high income and low- and middle-income families.

Zannad F, Rossignol P and Iraqi W. Extracellular matrix fibrotic markers in heart failure. Heart Fail Rev 2010;15:319-329

CHAPTER 5:

General Discussion

5.1 Overall Findings

The main objective of these studies (**Chapter 2 to 4**) was to determine the molecular mechanisms involved in the antihypertrophic and antiremodelling effects of ginseng in agonist-induced cardiomyocyte hypertrophy as well to determine if these effects could be observed in an *in vivo* model. Results from our first (**Chapter 2**) and second (**Chapter 3**) study demonstrated the ability of ginseng to prevent and treat leptin-induced cardiac hypertrophy, respectively, by attenuating RhoA/ROCK-dependent MAPK activation via inhibition of the positive RhoA regulator, p115RhoGEF, as well as restoring expression of Rnd3 to downregulate RhoA activation. These antihypertrophic and antiremodelling effects of ginseng were further observed in HF rats such that animals receiving ginseng treatment demonstrated marked improvements in echocardiographic and hemodynamic parameters (**Chapter 4**). Restoration of cardiac function by ginseng was observed to involve the reversal of CaN activation. Overall, it is evident from our studies that ginseng has a potent antihypertrophic and antiremodelling effect in HF because of its ability to target several key signalling mechanisms which contribute to the pathogenesis of HF.

5.2 Ginseng Reverses Heart Failure and Hypertrophy: Insights into Mechanisms

As mentioned earlier, failure to treat HF is mainly due to the inability of conventional drugs to reverse already established cardiac hypertrophy (reviewed in Marian, 2009; Hellawel and Margulies, 2010; Tamargo and López, 2011). As demonstrated in our studies however, ginseng may be a potential therapeutic agent for the treatment of HF as ginseng was able to reverse cardiac remodelling and HF in cultured cardiomyocytes as well as in rats with sustained MI. Our results propose that these reversal effects of ginseng in the hypertrophic program are due to the inhibition of the CaN/NFAT3 and RhoA/ROCK signalling pathway.

The importance of CaN/NFAT3 activation unique to pathological hypertrophy (Wilkins et al., 2004) has been observed by several researchers (Kilić et al., 2010; Yang et al., 2010; Zhou et al., 2010; Guo et al., 2011). Moreover, the reversibility of CaN activation (Berry et al., 2011) in cardiac hypertrophy and dysfunction further indicates this mechanism as a potential target for the treatment of HF. In our third study (**Chapter 4**), a similar trend was observed such that CAL-induced upregulation of CaN activation, which was prevented by treatment with ginseng. Additionally in cultured cardiomyocytes, agonist-induced CaN activation was markedly reversed after the administration of ginseng. The tendency of ginseng to prevent CaN activation in CAL-rats was reflected in the improvement of cardiac function measured by echocardiography and hemodynamic analysis at eight weeks. Improvement in cardiac function was additionally associated with decreases in the gravimetric measurements of the heart as well as the expression of collagen gene expressions, which indicated a reversal of cardiac remodelling and hypertrophy in CAL-induced HF by ginseng.

Reversal of HF and hypertrophy by ginseng may also involve Rnd3-mediated RhoA/ROCK downregulation as reported in **Chapter 3**. These experiments stemmed from the observation that RhoA/ROCK signalling is another key mechanism in the development of pathological cardiac hypertrophy both *in vivo* (Phrommintikul et al., 2008; Ying et al., 2009) and *in vitro* (Aikawa et al., 1999; Zeidan et al., 2006; Zeidan et al., 2008). By inhibiting the RhoA/ROCK pathway via inhibition of RhoA (Phrommintikul et al., 2008; Ying et al., 2009), cardiac hypertrophy was consequently attenuated. Inhibition of RhoA/ROCK activation by increasing the expression of downregulating factors such as Rnd3 or p190RhoGAP (Wennerberg et al., 2003; Riou et al., 2010) to attenuate cardiac hypertrophy however has not yet been investigated. In our experiments, the reversal of leptin-induced cardiac hypertrophy by ginseng

(**Chapter 3**) was considered to be facilitated by the upregulation of Rnd3-mediated RhoA/ROCK downregulation. Indeed, Rnd proteins, particularly Rnd1 and Rnd3 have been shown to directly activate p190RhoGAP, a specific GAP of RhoA, as well as inhibit the kinase activity of ROCK (Wennerberg et al., 2003; Riou et al., 2010). Although reversal of leptin-induced RhoA activation was observed, leptin-induced activation of p38 and ERK1/2 was not reversed by ginseng further supporting previously published data which have suggested MAPK pathways as secondary to RhoA in the hypertrophic program (Zeidan et al., 2008). Results from these studies suggest that ginseng exerts antihypertrophic and antiremodelling effects via inhibition of CaN/NFAT3 activation or by downregulating RhoA/ROCK activation.

5.3 Implications of the Beneficial Effects of Ginseng against Leptin-induced Hypertrophy

Ginseng may additionally have an important role in the prevention and treatment of hypertrophy in obesity-associated conditions by inhibiting leptin-induced activation of the prohypertrophic RhoA/ROCK pathway. The novelty of this project in comparison to our previously published data, which had already demonstrated RhoA/ROCK-dependent activation of downstream p38 and ERK1/2 MAPK signalling (Zeidan et al., 2006; Zeidan et al., 2008), was the identification of p115RhoGEF in mediating leptin-induced RhoA activation. Although small G-proteins typically respond to GPCR agonists such as phenylephrine, AngII and ET-1, activation of RhoGEFs has also been linked to phosphorylation by RTKs (Schiller, 2006). Leptin receptors lack intrinsic kinase activity however they do recruit JAK2 molecules which may phosphorylate and subsequently activate RhoGEFs (reviewed in Frühbeck, 2006). In fact, Guilluy and colleagues demonstrated that JAK2 phosphorylates Tyr^{738} on RhoGEF in rat aortic smooth muscle that leads to increased vasoconstriction (Guilluy et al., 2010). Results from this

study not only provided new insight into the mechanistic basis of RhoA activation by leptin, but additionally supported previously cited work which described a dependency of p38 MAPK activation on RhoA activation. Importantly, pre-treatment with ginseng was able to completely abolish leptin-induced cardiac hypertrophy along with increased RhoA/ROCK activation and its downstream components. Taken together, our results demonstrate that ginseng is able to attenuate leptin-induced cardiac hypertrophy by attenuating RhoA/ROCK-dependent MAPK signalling. In concordance with the previous work summarized in **Chapter 3**, the data from both studies implicate the potential for ginseng to both prevent as well reverse hypertrophy and remodelling in obesity-associated conditions most likely *via* inhibition of positive upregulators as well as by increasing expression of downregulating factors of RhoA/ROCK signalling.

5.4 Conclusions and Future Directions

The increased interest in ginseng as a result of its claimed all cure effects and as potential for the management of CVDs has prompted researchers to identify and elucidate the mechanisms by which ginseng elicits its antihypertrophic and antiremodelling effects. Results from our studies **(Chapter 2 and 3)** have shown the ability of ginseng to both prevent and treat obesity-associated CVD via attenuation of leptin-induced p115RhoGEF upregulation and decreased Rnd3 downregulation of RhoA/ROCK-dependent MAPK signalling pathway. Ginseng may not be exclusive to inhibition of RhoA/ROCK in obesity-associated hypertrophic conditions, however may also act as an inhibitor of the CaN/NFAT3 signalling pathway in classic representations of cardiac hypertrophy **(Chapter 4)**. Based on our observations, there is a clear indication of the pharmaceutical value of ginseng as adjunctive therapy for HF and further studies investigating additional molecular pathways are encouraged.

5.5 References

Aikawa R, Komuro I, Yamazaki T, Zou Y, Kudoh S, Zhu W, Kadowaki T and Yazaki Y. Rho family small G proteins play critical roles in mechanical stress-induced hypertrophic responses in cardiac myocytes. Circ Res 1999;84:458-466

Berry JM, Le V, Rotter D, Battiprolu PK, Grinsfelder B, Tannous F, Burchfield JS, Czubryt M, Backs J, Olson EN, Rothermel BA and Hill JA. Reversibility of adverse, calcineurin-dependent cardiac remodeling. Circ Res 2011;109:407-417

Frühbeck G. Intracellular signalling pathways activated by leptin. Biochem J 2006;393:7-20

Guilluy C, Bregeon J, Toumaniantz G, Rolli-Derkinderen M, Retailleau K, Loufrani L, Henrion D, Scalbert E, Bril A, Torres RM, Offermanns S, Pacaud P and Loirand G. The Rho exchange factor Arhgef1 mediates the effects of angiotensin II on vascular tone and blood pressure. Nat Med 2010;16:183-190

Guo J, Gan XT, Haist JV, Rajapurohitam V, Zeidan A, Faruq NS and Karmazyn M. Ginseng inhibits cardiomyocyte hypertrophy and heart failure via NHE-1 inhibition and attenuation of calcineurin activation. Circ Heart Fail 2011;4:79-88

Hellawel JL, Margulies KB. Myocardial reverse remodeling. Cardiovasc Ther 2010; 00:1-10

Kilic A, Rajapurohitam V, Sandberg SM, Zeidan A, Hunter JC, Said Faruq N, Lee CY, Burnett JC Jr, and Karmazyn M. A novel chimeric natriuretic peptide reduces cardiomyocyte hypertrophy through the NHE-1-calcineurin pathway. Cardiovasc Res 2010;88:434-442

Marian AJ. Experimental therapies in hypertrophic cardiomyopathy. J Cardiovasc Transl Res 2009;4:483-492

Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant S and Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 1998;93 :215-228

Phrommintikul A, Tran L, Kompa A, Wang B, Adrahtas A, Cantwell D, Kelly DJ and Krum H. Effects of a Rho kinase inhibitor on pressure overload induced cardiac hypertrophy and associated diastolic dysfunction. Am J Physiol Heart Circ Physiol 2008;294:H1804-H1814

Riou P, Villalong P and Ridley AJ. Rnd proteins: multifunctional regulators of the cytoskeleton and cell cycle progression. Bioessays 2010;32:986-992

Schiller MR. Coupling receptor tyrosine kinases to Rho GTPases-GEFs: what's the link. Cell Signal 2006;181834-43

Schulze PC, Kratzsch J, Linke A, Schoene N, Adams V, Gielen S, Erbs S, Moebius-Winkler S and Schuler G. Elevated serum levels of leptin and soluble leptin receptor in patients with advanced chronic heart failure. Eur J Heart Fail 2003;5:33-40

Wennerberg K, Forget MA, Ellerbrock SM, Arthur WT, Burridge K, Settleman J, Der CJ and Hansen SH. Rnd proteins function as RhoA antagonists by activating p190RhoGAP. Curr Biol 2003;1106-1115

Yang D, Ma S, Tan Y, Li D, Tang B, Chen J, Su X, Li G, Zhang X and Yang Y. Adrenergic receptor blockade-induced regression of pressure-overload cardiac h ypertrophy is associated with inhibition of the calcineurin/NFAT3/GATA4 pathway. Mol Med Report 2010;3:497-501

Ying Z, Yue P, Xu X, Zhong M, Sun Q, Mikolaj M, Wang A, Brook RD, Chen LC and Rajagopalan S. Air pollution and cardiac remodeling: a role for RhoA/Rho-kinase. Am J Physiol Heart and Circ Physiol. 2009;296:H1540-H1550

Zeidan A, Javadov S and Karmazyn M. Essential role of Rho/ROCK-dependent processes and actin dynamics in mediating leptin-induced hypertrophy in rat neonatal ventricular myocytes. Cardiovasc Res 2006;72:101-111.

Zeidan A, Javadov S, Chakrabarti S and Karmazyn M. Leptin-induced cardiomyocyte hypertrophy involves selective caveolae and RhoA/ROCK-dependent p38 MAPK translocation to nuclei. Cardiovasc Res 2008;77:64-72.

Zhou N, Li L, Wu J, Gong H, Niu Y, Sun A, Ge J and Zou Y. Mechanical stress-evoked but angiotensin II-independent activation of angiotensin II type 1 receptor induces cardiac hypertrophy through calcineurin pathway. Biochem Biophys Res Commun 2010;397:263-269

APPENDIX

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