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Modulation of the Cardiomyocyte Hypertrophic Responses to Endothelin-1 by Adipocytes

Suresh Chandra Bairwa
The University of Western Ontario

Supervisor
Dr. Morris Karmazyn
The University of Western Ontario

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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Modulation of the Cardiomyocyte Hypertrophic Responses to Endothelin-1 by Adipocytes

(Thesis format: Monograph)

by

Suresh Chandra Bairwa

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Leptin and adiponectin are the adipokines that are shown to exert pro-hypertrophic and anti-hypertrophic effects respectively, in cardiomyocytes. We sought to determine the direct interaction between the adipocytes and cardiomyocytes during endothelin-1 (ET-1)-induced cardiomyocyte hypertrophy and determine the role of leptin and adiponectin.

The adipose tissue conditioned medium (ACM) inhibited the ET-1-induced hypertrophy in a concentration-dependent manner and this anti-hypertrophic effect was more potent or reversed in the presence of leptin receptor antagonist (LRA; 0.1 nM) or adiponectin receptor-1 antibody (ARA; 100 ng/mL), respectively. ACM from heart failure rats induced by coronary artery ligation or obese rats did not mitigate the ET-1 induced hypertrophy in cardiomyocytes.

These studies demonstrate that the net anti-hypertrophic activity of ACM in ET-1-induced cardiac hypertrophy is likely mediated by adiponectin due activation of AMPK and determined by the leptin to adiponectin ratio. Heart failure and obese-induced pathology changes the characteristics of adipocytes although the underlying mechanisms for this effect are not completely known.

Keywords

Cardiac hypertrophy, heart failure, cardiomyocytes, leptin, adiponectin, ACM, adipocytes, ET-1, obesity.
Co-Authorship Statement

The following manuscript is underdevelopment:

“Cardiac-specific excision of the jak2 gene causing cardiac hypertrophy, heart failure and mortality”. Xiaohong Tracey Gan, Venkatesh Rajapuhohitam, Cathy Huang, Jenny Xue, Suresh Chandra Bairwa, Jeffrey Tin-Yu Chow, Melissa Fung Wah Liu, Felix Chiu, Kay-Uwe Wagner¹, Morris Karmazyn (¹Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center 985950 Nebraska Medica Center, DRCII, Rm. 5033 Omaha, NE).

Heart failure model was prepared by Cathy Huang.

Adipose tissue conditioned medium from JCR rats was prepared and sent by Dr. Spencer Proctor from the University of Alberta. He also provided adipocyte pictures and heart characteristics data.

Dr. Venkatesh Rajapurohitam and Dr. Morris Karmazyn were responsible for designing the study and to prepare manuscript.
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Last but not the least, I would like to thank my family for their boundless love, for believing in me and encouraging me to follow my dreams against all odds.
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<td>Adipose tissue conditioned medium</td>
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<tr>
<td>AdipoR</td>
<td>Adiponectin receptor</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-Aminoimidazole-4-carboxamide-1-β-d-ribofuranoside</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ANP</td>
<td>Atrial natriuretic peptide</td>
</tr>
<tr>
<td>ARA</td>
<td>Adiponectin receptor-1 antibody</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>C.C</td>
<td>Compound C</td>
</tr>
<tr>
<td>CAL</td>
<td>Coronary artery ligation</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calmodulin-dependent kinase II</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase-2</td>
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<tr>
<td>CVDs</td>
<td>Cardiovascular diseases</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>fAD &amp; gAD</td>
<td>Full length adiponectin and globular adiponectin</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HW</td>
<td>Heart weight</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus activated kinase</td>
</tr>
<tr>
<td>LA</td>
<td>Left atrial</td>
</tr>
<tr>
<td>LKB1</td>
<td>Liver kinase B1</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
</tr>
<tr>
<td>LRA</td>
<td>Leptin receptor antagonist</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricular</td>
</tr>
<tr>
<td>LVW</td>
<td>Left ventricular weight</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappaB</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>ob gene</td>
<td>Obesity gene</td>
</tr>
<tr>
<td>OB-R</td>
<td>Leptin receptor</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>RhoA/ROCK</td>
<td>Ras homolog gene family, member A/ Rho-associated protein kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
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<tr>
<td>SEM</td>
<td>Standard error mean</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAC</td>
<td>Trans-aortic constriction</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TPT1</td>
<td>Tumor protein, translationally controlled 1</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
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<tr>
<td>β-MHC</td>
<td>β-myosin heavy chain</td>
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1.1 Introduction

Cardiovascular diseases (CVDs) are the number one cause of death worldwide (WHO 2011). In Canada, someone dies of CVDs every 7 minutes (Statistics Canada (2011c, October)). Although statistics show that CVD related deaths have declined over time among Canadians (Tu et al. 2009), there is an increase in CVDs due to risk factors such as hypertension, diabetes and obesity in all age groups, especially among younger Canadians (Lee et al. 2009).

1.2 Heart failure

Heart failure affects more than 500,000 Canadians and more than 50,000 new patients are diagnosed each year (Ross et al. 2006). Heart failure cases are rarely found amongst individuals younger than 50 years, however, in those older than 50 years, the prevalence and incidence of heart failure increases progressively with age (Mosterd and Hoes 2007). Heart failure is defined as the inability of the heart muscles to pump a sufficient amount of blood to meet the oxygen and blood demands of the body at normal filling pressure, which leads to dyspnoea, premature death and/or oedema. Heart failure is defined by the ejection fraction (EF) which is the ability of the heart to pump the blood out of the left and right ventricles on each beat. Based on EF, heart failure is categorised into two types: heart failure with preserved ejection fraction and heart failure with reduced ejection fraction. Heart failure with preserved EF is also known as diastolic heart failure whereas heart failure with reduced EF is also known as systolic heart failure. A number of epidemiology studies and clinical studies have examined the heart failure incidences in the population, it was observed that heart failure subjects were older people with a history of hypertension, obesity and type 2 diabetes mellitus (Lam et al. 2011, Brouwers et al. 2013). These risk factors were common in both types of heart failure (reduced and preserved EF) but incidence of heart failure and mortality were high for reduced EF (Brouwers et al. 2013).

Risk factors of heart failure include hypertension, diabetes, smoking, obesity and coronary heart disease (NIH 2011). Heart failure symptoms include fatigue, numbness,
drowsiness, insomnia, anxiety, depression and chest pain resulting in reduced living quality (Blinderman et al. 2008). Based on Framingham Heart Study in adults, incidence of heart failure was dependent on body mass index. Overweight and obese people were at a higher risk of heart failure and this trend was evident between in both male and female (Kenchaiah et al. 2002). One population based study found that the heart failure risks increased in older people with diabetes compared with those without diabetes, suggesting a relationship between diabetes and heart failure (Bertoni et al. 2004). With an increasing number of heart failure patients and a resulting increase in healthcare costs, heart failure will be an increasing burden on society (Blinderman et al. 2008). Except for heart transplants, no cure exists for heart failure and prognosis is poor, as one third of the patients die within a year of diagnosis (Bleumink et al. 2004, McMurray and Pfeffer 2005).

1.3 Heart failure & cardiac hypertrophy

Association of cardiac hypertrophy with almost all forms of heart failure (Levy et al. 1990) has aroused the great interest in understanding the molecular mechanism which leads to cardiac hypertrophy. The heart is composed of various types of cells such as myocytes and non-myocyte cells including fibroblasts, endothelial cells and vascular smooth muscle cells (Weber et al. 1992). When the normal heart is exposed to risk factors (chronic increases in pressure or volume overload), it first develops into compensatory phase cardiac hypertrophy in which cardiomyocytes grow in length and/or width in order to compensate for increased blood supply demand by improving cardiac pump function and reducing ventricular wall tension (compensated hypertrophy) (Haider et al. 1998, Berenji et al. 2005). However, if the wall tension is not relieved over time then this prolonged hypertrophy phase can progress towards heart failure as well as sudden death (Levy et al. 1990, Frey and Olson 2003). The presence of left ventricular hypertrophy is an important predictor for the development of heart failure (Maron 1997). Therefore, studies are focused on finding new therapeutic approaches to prevent cardiac hypertrophy and heart failure.
1.4 Cardiac hypertrophy

It has been two decades since studies linked cardiac hypertrophy and heart failure. Cardiac hypertrophy is an independent risk factor for myocardial infarction, angina pectoris, arrhythmia and cardiac sudden death (Levy et al. 1990). Several studies have proven that cardiac hypertrophy is positively associated with several risk factors such as diabetes (de Simone et al. 2002), high blood pressure and obesity (Falkner et al. 2013). Cardiac hypertrophy is a general term for the heart’s condition when it experiences increased hemodynamic workload or when exposed to neurohumoral factors. Consequently, the heart enters a cardiac remodelling phase called cardiac hypertrophy in which cardiomyocytes grow in size leading to thickening of the ventricular walls (Frey et al. 2004). Based on the various types of stimuli, the heart develops into physiological or pathological hypertrophy and based on the response of the cardiomyocytes it shows two phenotypes: (1) concentric hypertrophy that is caused by pressure overload, in which sarcomeres are added in parallel and cell width increases, and (2) eccentric hypertrophy that is caused by volume overload, in which sarcomeres are added in series and cell length increases (Dorn et al. 2003) (Figure 1).

1.4.1 Physiological hypertrophy

Physiological hypertrophy is a type of cardiac hypertrophy which involves healthy growth of the heart in response to pregnancy-induced or exercise-induced events. This hypertrophied heart shows a different phenotype. For example, isotonic exercises such as running, cycling and swimming, causes volume overload resulting in eccentric hypertrophy and whereas isometric exercise such as weight lifting, causes pressure overload resulting in concentric hypertrophy (Pluim et al. 2000). This hypertrophy does not develop into heart failure (Fagard 1997, Pluim et al. 2000) and benefits the heart by up-regulating cardiac outflow at normal pressure. Generally, the morphology consists of incremental increases in myocyte volume and the formation of new sarcomeres. The end result of this hypertrophy is that the heart has normal or improved cardiac function. This type of hypertrophy is reversible and it does not show fibrosis or fetal gene up-regulation which is seen in pathological hypertrophy (Beisvag et al. 2009).
Fig. 1. Physiological and pathological types of cardiac hypertrophy. Depending on the type of stimulus the normal heart responds differently. When the pressure overload stimuli are present, the heart develops into concentric hypertrophy and in the presence of volume overload stimuli; the heart develops into eccentric hypertrophy. “Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Maillet et al. 2013), copyright 2013.”
1.4.2 Pathological hypertrophy

The pathological hypertrophy is the unhealthy growth of the heart in the response to stimuli with different pathological stimuli resulting in different types of hypertrophy. Hypertension or aortic constriction leads to pressure overload hypertrophy (Grossman et al. 1975) which develops into concentric phenotypic hypertrophy (Figure 1). On the other hand aortic valve disease leads to volume overload hypertrophy (Grossman et al. 1975, Pluim et al. 2000) which develops into eccentric type of hypertrophy. This hypertrophy is initially compensatory where cardiomyocytes expand and the formation of new sarcomeres takes place which reduces wall stress. However, the hypertrophied heart does not remain in the compensatory phase and eventually transitions into a decompensatory phase leading to heart failure (Levy et al. 1990). Increased cardiac fibrosis, cardiomyocytes loss, cardiac dysfunction and risk of heart failure are associated with pathological hypertrophy (Weber et al. 1993, Cohn et al. 1997). The distinguished features of cardiac hypertrophy at the cellular level include increases in the cardiomyocytes size and enhanced rate of protein synthesis (Sugden and Clerk 1998). At the molecular level, hypertrophy is characterized by re-expression of the fetal genes, such as β-myosin heavy chain (β-MHC), α-skeletal actin and atrial natriuretic peptide (ANP) (Hefti et al. 1997).

Generally, left ventricular hypertrophy occurs due to the resistance of outgoing blood flow from left ventricle to the whole body. On the other hand right ventricular hypertrophy is due to pulmonary hypertension (pressure in the pulmonary arteries). Pulmonary hypertension is caused by various factors including pulmonary blood vessel constriction and blood clot formation in the arteries. We will be referring to pathological cardiac hypertrophy throughout this manuscript.

There are several mechanisms associated with cardiac hypertrophy. One pathway that plays an important role in cardiac hypertrophy is Ca$^{2+}$/calmodulin-dependent kinase II (CaMKII). Intracellular Ca$^{2+}$ is a cardiac contractility regulator and it is important to maintain appropriate levels in the heart, otherwise this could lead to pathological abnormalities such as arrhythmogenesis and prolonged tachycardia, eventually
culminating in heart failure (O'Rourke et al. 1999, Pogwizd et al. 1999). CaMKII are signal transducers of Ca\(^{2+}\) which regulate cellular actions mediated by Ca\(^{2+}\) (Braun and Schulman 1995). During cardiac hypertrophy, failing myocardium and heart failure expression and activity of CaMKII are increased (Netticadan et al. 2000, Hagemann et al. 2001, Anderson 2009). CaMKII overexpressing transgenic mice show dilated cardiomyopathy and cardiac hypertrophy accompanied by fibrosis (Zhang et al. 2003) which suggest a critical role of CaMKII during cardiac dysfunction. Another signalling mechanism is the calcium and calmodulin-activated protein phosphatase calcineurin. Elevation in intracellular calcium levels in cells activates calcineurin which then binds with the nuclear factor of activated T cells (NFAT), dephosphorylates NFAT, which causes NFAT activation and consequent translocation into the nucleus (Crabtree and Olson 2002). Studies in transgenic mice show that cardiac hypertrophy and heart failure are a result of either calcineurin or NFAT activation (Molkentin et al. 1998). Calcineurin/NFAT signalling is increased during cardiac hypertrophy and failing human heart. This signalling is absent during exercise induced hypertrophy which suggests that it is only associated with pathological hypertrophy and not with physiological hypertrophy (Wilkins et al. 2004).

### 1.5 Cardiac hypertrophy in response to endothelin-1 (ET-1)

ET-1 is a 21 amino acid peptide and a potent vasoconstrictor which has been shown to decrease heart rate and raise mean arterial blood pressure (Weitzberg et al. 1993). Its release is controlled in order to maintain homeostasis by means of blood pressure regulation (Somlyo and Somlyo 1993). This regulation is through Ca\(^{2+}\) channels (Miwa et al. 2005). ET-1 is produced from proendothelin-1 (38 amino acid precursor) by endothelin-converting enzyme (ECE). Obesity increases ET-1 protein and gene expression. During cardiac hypertrophy, both ET-1 and ET-1 binding sites are up-regulated (Arai et al. 1995). High circulating levels of ET-1 in plasma have been reported to be positively correlated with obesity, hypertension and type 2 diabetes mellitus (Saito et al. 1990, Takahashi et al. 1990, Ferri et al. 1995).
ET-1 is also a neurohumoral factor which is released by cardiomyocytes (Nunez et al. 1990, Suzuki et al. 1993, Yamazaki et al. 1996) during mechanical stretch-induced cardiac hypertrophy. ET-1 is also produced by vascular endothelial cells (Yanagisawa et al. 1988), cardiac fibroblast (Fujisaki et al. 1995) and macrophages (Fukuchi and Giaid 1998) suggesting the autocrine and paracrine role of ET-1 on regulating cardiac functions. Two ET-1 receptors have been identified, ET_A and ET_B. Studies show that the ET_A receptor exerts a vasoconstriction property (Zamora et al. 1993) while ET_B receptor shows both vasoconstriction and vasodilation properties (Sato et al. 1995). In the rat myocardium, the ET_A receptor is expressed widely (Hilal-Dandan et al. 1994). Cardiomyocyte secreted ET-1 in response to Angiotensin II (Ang II) treatment and blockage of ET_A receptor attenuated the protein expression which was induced by Ang II thus suggesting that ET-1 contributes in the hypertrophic signals of Ang II through the ET_A receptor in cardiomyocytes (Ito et al. 1993). Another study also found that in neonatal cardiomyocytes, ET-1 via ET_A receptor induces the hypertrophic effect which includes activation of mitogen activated protein kinases (MAPK) and increased rate of protein synthesis (Yamazaki et al. 1996).

1.6 Cardiac hypertrophy and obesity

The rate of obesity and subsequent metabolic disorders are increasing worldwide. According to The World Health Organisation, globally in 2008, obesity incidences have increased almost 2 fold since 1980, and in 2008 the numbers of obese women were 1.5 fold higher than men and total obese population was 11% (WHO 2008). Obesity is caused by accumulation of the adipose tissue (adiposity) in the different regions of the body which increases the likelihood of health problems and leads to reduced life expectancy (Haslam and James 2005). Obesity is defined by the body mass index (BMI) which is the person's body weight divided by the square of their height. According to distribution of the fat mass in the body, people are divided into 3 categories according to three BMI ranges; normal BMI ranges from 18.5 to 25 kg/m², overweight BMI ranges from 25 to 30 kg/m² and an obese BMI is equal to or more than 30 kg/m² (WHO 1990).
Obesity is a chronic metabolic disorder linked with numerous comorbidities such as type 2 diabetes mellitus, cancer, sleep apnea and ischemic stroke (Poirier and Eckel 2002, Suk et al. 2003, Forte et al. 2012). The alarming increase in the incidence of obesity has had a significant impact on the incidence of several cardiovascular abnormalities including hypertension (Lauer et al. 1992), cardiac hypertrophy (Turkbey et al. 2010) and heart failure (Kenchaiah et al. 2002).

Studies are being carried out to better understand the factors and mechanisms involved in the heart’s remodelling during obesity. Cardiac remodelling includes left ventricular (LV) hypertrophy, left atrial (LA) enlargement, and impairment of LV systolic and diastolic function (Bugger and Abel 2008). These changes are believed to be the indicators of more serious complications such as cardiac dysfunction and heart failure (Abel et al. 2008).

Many studies show link obesity with anatomical and physiological changes in the heart in both human and animal models (Abel et al. 2008). Association of obesity with the heart include LV hypertrophy evident in both human (Berkalp et al. 1995, Morricone et al. 2002, Avelar et al. 2007) and animal models (Barouch et al. 2003, Mazumder et al. 2004, Barouch et al. 2006). Recent human studies on obese patients showed that LV hypertrophy was due to excessive accumulation of epicardial adipose tissue (Erdogan et al. 2013, Lin et al. 2013). Human studies also show the right ventricular and left atrium wall thickness and size were associated with obesity (Alpert et al. 1985, Alpert et al. 1997, Wong et al. 2006). Animals study found triglyceride accumulation and cardiac fibrosis in the heart during obesity (Zhou et al. 2000, Toblli et al. 2005, Boudina and Abel 2006, Boudina et al. 2007). Impaired systolic function (Otto et al. 2004, Yue et al. 2007) and diastolic function (Morricone et al. 2002, Christoffersen et al. 2003) was also observed during obesity in both human and animal studies.

Although obesity is generally considered as increasing cardiovascular risk, a number of studies have reported that obese patients who are suffering from cardiovascular implications have better survival rate than normal weight people (Horwich et al. 2001, Lissin et al. 2002, Lavie and Milani 2003, Lavie et al. 2003, Hall et al. 2005). One study
showed that obese patients diagnosed with heart failure have improved life span in contrast with normal weight patients (Curtis et al. 2005). This obesity paradox is further supported by another study which suggested that patients hospitalized for heart failure and examined for 3 years showed significant lower mortality rate when they adjusted age, gender and severe illness (Hall et al. 2005). Similarly, obese subjects had either no effect or demonstrated improved life expectancy over both short term or and long term observation (Oreopoulos et al. 2008). Thus, some studies suggest that obesity may play a beneficial role in the patients who are diagnosed with cardiovascular disease.

1.7 Adipokines

Adipose tissue is widely distributed and major depots are found in both the visceral and subcutaneous regions. Adipose tissue is comprised of several types of cells including adipocytes, pre-adipocytes, endothelial cells and macrophages out of which adipocytes contributes about 50 to 70 percent (Hauner 2005). Adipocytes contain triglycerides which are a source of energy reservoir to be used when needed (Ferguson and Leese 2006).

Since the discovery of leptin in 1994, adipose tissue is now recognized not only as an insulator of the body but also as an endocrine organ which secretes many bioactive compounds known as adipokines. White adipose tissue (WAT) is the principal source of adipokines which play important roles in regulating many bodily functions such as lipid metabolism, glucose metabolism as well as neuroendocrine and cardiovascular functions (Trujillo and Scherer 2006). Adipokines also regulate adipose tissue metabolism and affect other organs like muscle, pancreas, liver and brain (Kershaw and Flier 2004). There are more than 100 known adipokines including cytokines, chemokines and growth factors. Adipose tissue secreted some well-known adipokines including leptin, adiponectin, apelin and visfatin. Adipocytes have also been shown to release steroid hormones and prostaglandins (Hauner 2004, Staiger and Haring 2005).

Adipocytes increase in size in obesity and their secretory properties changes which results in the release of more pro-inflammatory adipokines including leptin, TNF-alpha, some interleukins (IL-1, IL-6 and IL-8) and less anti-inflammatory adipokines such as
adiponectin (Jernas et al. 2006, Skurk et al. 2007) thus suggesting that size of adipocytes determines the release of adipokines. The number of macrophages in adipose tissue increases in obesity, and shows a positive relation with the size of adipocytes. Moreover, secretion of TNF-alpha has been discovered to be greater in macrophages than in adipocytes (Weisberg et al. 2003). Studies show that weight loss in obese patients increased anti-inflammatory factors and decreased both pro-inflammatory factors and macrophage infiltration (Clement et al. 2004, Cancell et al. 2005).

A number of studies have reported that adipokines play an important role in regulating cardiac functions as evident in obese animal and obese knockout animal studies (Dong et al. 2006, Ren and Ma 2008, Amin et al. 2010, Guo et al. 2013). It has also been shown that adipokines are a potential link between obesity and heart failure (Schulze et al. 2003, Ouchi et al. 2006, Frankel et al. 2009, Ebert and Fasshauer 2011). Heart depends on the energy to maintain contractile functions and energy required by the heart is dependent on the metabolism of fatty acids. Metabolism of fatty acids is essential in order to prevent storage of triglyceride in the heart (McGavock et al. 2006). During obesity and diabetes increased fatty acid uptake, oxidation and decrease in glucose metabolism has been reported (Stanley et al. 2005, Abel et al. 2008). In the liver and skeletal muscles, it has been shown that adipokines maintain glucose and fatty acid metabolism (Badman and Flier 2007) and leptin (Palanivel et al. 2006) and adiponectin can regulate glucose uptake and fatty acid metabolism in cardiomyocytes (Guo et al. 2007, Palanivel et al. 2007). This suggests that adipokines maintain fatty acid and glucose metabolism and regulate homeostasis. A recent study analyzed the interaction between adipocytes obtained from human and animal and cardiomyocytes. It reported that adipocytes increased lipid accumulation and promoted apoptosis in HL-1 cardiomyocytes with increased lipid droplets (Anan et al. 2011) which suggests that adipocytes induces lipotoxicity and apoptosis in the cardiomyocytes leading to heart failure. A study in 2006 reported that human adipocytes exert cardio-depressant activity in a dose-dependent manner and the molecular weights of the effective adipokines were between 10kDa and 30kDa, which suggests that adipokines in overweight patients may contribute to heart failure (Lamounier-Zepter et al. 2006). Another study revealed that adipose tissue derived from normal rats increased glucose uptake and fatty acid
oxidation in rat neonatal ventricular cardiomyocytes (Palanivel et al. 2008). However, adipose tissue derived from streptozotocin-induced diabetic rats had the ability to decrease oxidation of fatty acids and lower glucose uptake ability. These diabetic rats showed decreased levels of adiponectin and leptin expression and increased interleukin-6 expression (Palanivel et al. 2008). Taken together these studies suggest that adipokines play a critical role to maintain homeostasis of the heart and cardiomyocytes. However, during pathological condition such as obesity and diabetes, alteration in the levels of adipokines (leptin and adiponectin) negatively affect the heart. Leptin and adiponectin have been studied extensively in terms of their cardiac effect, specifically cardiac hypertrophy.

1.7.1 Leptin

Leptin is a 16 kDa peptide product of the obesity (ob) gene (Zhang et al. 1994) discovered in 1994. It is secreted mainly by adipose tissue (Scherer et al. 1995) but organs including stomach (Bado et al. 1998), heart and cardiomyocytes (Purdham et al. 2004, Rajapurohitam et al. 2006) also have the ability to produce leptin. Leptin secretion from heart and cardiomyocytes suggests that leptin could affect cardiac functions in autocrine and paracrine manner. Leptin circulates in the blood at concentrations between 5-15 ng/mL in lean individuals (Sinha et al. 1996). The major physiological function of leptin is to decrease food intake and increase the energy consumption via signalling in the central nervous system. Leptin in the circulatory system affects central nervous system (CNS) in order to regulate energy balance in the body (Elmquist et al. 1998, Friedman and Halaas 1998). It is well known that energy expenditure and food intake is regulated by hypothalamus. Animal studies show that the hypothalamus expresses leptin binding sites (Stephens et al. 1995) and studies using in situ hybridisation show that many hypothalamus nuclei in mouse brain expresses OB-Rb gene expression abundantly (Mercer et al. 1996) suggesting that in hypothalamus OB-Rb could be the main receptor for leptin signaling. Another study shows that injection of leptin in mice directly activates STAT3 in hypothalamus in a dose-dependent manner (Vaisse et al. 1996). These data suggest that leptin acts on hypothalamus and contributes in the regulation of
the energy expenditure and food intake. Therefore it is known as circulating satiety factor (Jacob et al. 1997).

Studies in both mice and humans suggest that the obese phenotype is associated with leptin gene mutation (Zhang et al. 1994, Montague et al. 1997, Strobel et al. 1998). Human subjects who had mutation in the leptin gene, observed low leptin levels regardless of their obesity (Montague et al. 1997, Strobel et al. 1998). These studies suggest that decreased production of leptin is due to improper leptin gene regulation which causes obesity. Leptin gene is essential for maintaining homeostasis through energy balance. However, plasma leptin levels are proportionate to adiposity; as adipose tissue abnormally accumulates in the body, the plasma leptin concentration also increases (Maffei et al. 1995, Considine et al. 1996). Obese subjects show high circulating leptin levels (Frederich et al. 1995). Though the levels of leptin in the plasma are high and leptin is known as a satiety hormone, leptin is unable to exert its effect during obesity. The possible cause for leptin’s inability to decrease weight in these patients might be due to hypothalamic leptin resistance (Seufert 2004). Some studies suggest the mechanism for leptin resistance. A study by Burguera et al found that obese rats had saturation of leptin receptors in the blood brain barriers (BBB) and leptin transport was decreased which could explain the leptin resistance in obese subjects (Burguera et al. 2000). Another study in diet-induced obesity in mice showed two reasons for the leptin resistance in hypothalamus, one was a defect in the leptin binding site in the hypothalamus which inhibited leptin-induced STAT3 signalling in the hypothalamus and the second was a defect in the intracellular signalling of leptin to activate STAT3 in the hypothalamus (El-Haschimi et al. 2000).

Elevated plasma leptin levels have been observed in patients with left ventricular hypertrophy (Kartal et al. 2008), hypertension (Shankar and Xiao 2010), myocardial infarction (Soderberg et al. 1999) ischemic heart disease (Wallace et al. 2001) as well as heart failure (Chan et al. 2003). Animal studies with regards to metabolic effects of leptin show that leptin addition to perfused heart elevates fatty acid oxidation, down-regulates triglyceride contents and up-regulates oxygen consumption in the myocardium which decreases efficiency of the heart (Atkinson et al. 2002). Another study showed
that elevation in fatty acid oxidation was dependent on STAT3, nitric oxide and p38 MAPK-dependent mechanism (Sharma et al. 2009) which are linked to the hypertrophic program. Long term treatment with leptin down-regulated fatty acid uptake which led to increased lipid accumulation in the HL-1 cardiomyocytes contradicts the result seen with short term leptin treatment (Palanivel et al. 2006). Direct effects of leptin in inducing hypertrophy in neonatal cardiomyocytes have been documented (Rajapurohitam et al. 2003, Zeidan et al. 2008). Leptin treatment increased reactive oxygen species (ROS) and ET-1 levels in neonatal cardiomyocytes which was inhibited by ET-1 receptor (ET\(_A\)) antagonists; leptin induced cardiomyocyte hypertrophy was shown to be mediated through ET-1-ROS mechanism (Xu et al. 2004).

1.7.1.1 Leptin signaling

Leptin produces effects by binding with its receptors termed as OBRs (or LEPR) and are expressed in many tissues including pancreas, foetus, placenta, spleen, kidney, ovaries, heart and adipose tissue (Zhang et al. 1994, Kieffer et al. 1996, Hoggard et al. 1997, Chen et al. 1999). Leptin receptors exist in 6 isoforms (OB-Ra to OB-Rf) (Figure 2), and only the OB-Rb isoform has full signalling capacity regarding transduction of signals intracellularly in order to maintain homeostasis. This is supported by the study that show that mice which lacked the long form of the receptor showed similar phenotype compared with mice and animals which were leptin-deficient (ob/ob) (Chua et al. 1997, Friedman and Halaas 1998). Being the long structure, OB-Rb has suitable binding site for Janus activated kinase 2/signal transducers and activators of transcription 3 (JAK2/STAT3) signaling (Tartaglia 1997, White et al. 1997). During leptin signaling, leptin binds with the leptin receptor which changes the structural conformation of leptin receptor (OB-Rb homodimer) and phosphorylates JAK2 and JAK2 becomes activated (Devos et al. 1997, Tartaglia 1997, Couturier and Jockers 2003). Studies suggest that there are three intracellular known activation sites on the OB-Rb domain which upon phosphorylation become activated and conduct downstream leptin signaling that are: Tyr\(_{985}, \) Tyr\(_{1077} \) and Tyr\(_{1138} \) (Tartaglia 1997, White et al. 1997, Banks et al. 2000, Hekerman et al. 2005, Gong et al. 2007). Only Tyr\(_{1138} \) creates binding site for STAT3
when phosphorylated which results in STAT3 to translocate to the nucleus and mediate the regulation of transcription (White et al. 1997, Banks et al. 2000).

Leptin receptors are present in all types of cells including cardiomyocytes (Rajapurohitam et al. 2006). Several mechanisms have been proposed for leptin-induced cardiomyocytes hypertrophy in which leptin binds with the OB-Rb receptor and this leads to several downstream signaling cascades resulting in hypertrophy. During leptin-induced hypertrophy mitogen activated protein kinase (MAPK) members such as extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 are activated by leptin but not c-Jun N-terminal kinase whereas only p38 is involved in cardiac hypertrophy inhibition (Rajapurohitam et al. 2003). Leptin activated RhoA/ROCK pathway during hypertrophic effect and by blocking the RhoA/ROCK signalling, leptin-induced hypertrophic effect was attenuated in neonatal cardiomyocytes (Zeidan et al. 2006, Zeidan et al. 2008). RhoA/ROCK also translocated p38 MAPK into nucleus which mediated leptin induced cardiomyocyte hypertrophy (Zeidan et al. 2008). Leptin has been shown to mediate the hypertrophic effects of ET-1 and Ang II in the neonatal rat ventricular cardiomyocytes (Rajapurohitam et al. 2006). It has also been shown that leptin production is associated with other pro-hypertrophic factors such as endothelin-1 and Ang II and these factors increase leptin production by activating NF-kB and p38 MAPK pathway which translocated both NF-kB and p38 MAPK into nucleus and phosphorylated the p38 MAPK (Rajapurohitam et al. 2012). Taken together these findings suggest that leptin has detrimental effects on the heart.
Fig. 2: Structure of the leptin receptor (OB-R) isoforms (Karmazyn et al. 2007).

“Reprinted from Trends in Cardiovascular Medicine, Volume 17, Karmazyn M., Purdham D. M., Rajapurohitam V., Zeidan A., Leptin as a cardiac hypertrophic factor: a potential target for therapeutics, Pages No. 206-11, Copyright 2007, with permission from Elsevier.”

All the leptin receptor isoforms contain extracellular region, transmembrane and intracellular region except OB-Re which does not have intracellular region. The extracellular region has two cytokine receptor (CK) domains separated by one fibrinoactin type III (F3) domain and followed by three more fibrinoactin type III domains. In the intracellular region, all isoforms (Ob-Ra-Ob-Rd and Ob-Rf) contain box 1 (B1). Only OB-Rb contains box 2 (B2) and box 3 (B3). In OB-Rb, box 1 and box 2 bind with JAK and box 3 binds with STAT. All the receptor isoforms vary in their amino acid length range from 805 to 1162.
1.7.2 Adiponectin

Adiponectin is a 30kDa protein also known as Acrp30 (adipocyte complement-related protein of 30 kDa). In addition to adipocytes, (Scherer et al. 1995) it is also synthesized by other organs such as placenta (Chen et al. 2006) and cardiomyocytes (Pineiro et al. 2005). It was first isolated as cDNA in 1995 (Scherer et al. 1995) and has been of substantial research interest since as it is a protein that possesses anti-inflammatory (Ouchi et al. 2003), anti-tumor (Brakenhielm et al. 2004), anti-angiogenesis (Brakenhielm et al. 2004) and insulin sensitizing functions (Hotta et al. 2000). It is the most abundant circulating adipokine in healthy individuals ranging from 2-30 μg/mL (Shimada et al. 2004). Adiponectin circulates in two forms, full-length adiponectin (fAd) and globular adiponectin (gAd). Full-length adiponectin has a molecular weight 30 kDa and exists in three forms trimer (low-molecular weight), hexamer (trimer-dimer) and multimer (high-molecular weight) (Nakano et al. 1996, Tsao et al. 2003, Waki et al. 2003) where globular adiponectin exists only as a trimer. Adiponectin does not exist in the monomeric form in plasma (Pajvani et al. 2003). Adiponectin levels are decreased during obesity (Arita et al. 1999), coronary artery disease (Nakamura et al. 2004) and in heart failure patients (Takano et al. 2009). In the liver, adiponectin improves insulin sensitivity (Nawrocki et al. 2006) and in skeletal muscle, fatty acid oxidation (Fruebis et al. 2001) by activating AMP-activated protein kinase (AMPK) which suggests that adiponectin plays an important role in regulating homeostasis.

Adiponectin’s cardio-protective effect has been studied extensively. Knockout studies on mice observed that adiponectin deficient mice had enhanced myocardial apoptosis and increased infarct size compared to wild type animals and upon administration of adiponectin’s globular form significantly reduces apoptosis and infarct size (Shibata et al. 2005, Tao et al. 2007). Globular adiponectin administration also attenuated the peroxynitrite formation which protected the heart from the ischemic reperfusion injury in the adiponectin deficit mice (Tao et al. 2007) and ischemic perfusion injury was also protected through the mechanism of AMPK and COX-2 (Shibata et al. 2005). Another knockout study found that adiponectin deficient mice showed cardiac remodelling phenotype following transverse aortic constriction (TAC) compared to wild type mice.
They also showed low protein expression of AMPK which suggests that adiponectin is important to protect the hearts from pressure overloaded cardiac remodeling via activation of AMPK (Liao et al. 2005). It has been shown that long term activation of AMPK through pharmacological activator AICAR (5-Aminoimidazole-4-carboxamide-1-β-d-ribofuranoside) attenuates cardiac hypertrophy induced by pressure overload (Li et al. 2007). AICAR, in cytosol, is phosphorylated by adenosine kinase and converted into ZMP (AICA-riboside monophosphate), which is an AMP analogue that acts as AMP and activates AMPK (Corton et al. 1995). AICAR also attenuates cardiac hypertrophy through activating AMPK in both in vivo and in vitro (Meng et al. 2011) suggesting the role of AMPK as an anti-hypertrophic agonist.

1.7.2.1 Adiponectin signaling

The discovery of adiponectin receptors (AdipoR1 and AdipoR2) (Yamauchi et al. 2003) has unfolded various molecular pathways mediating the effect of adiponectin. Both receptors are widely expressed in the body including adipose tissue (Bluher et al. 2005, Rasmussen et al. 2006), pancreas (Staiger et al. 2005, Gu et al. 2006), osteoblasts (Berner et al. 2004, Kanazawa et al. 2007), brain (Kubota et al. 2007), muscle (Beylot et al. 2006, Dai et al. 2006), liver (Bonnard et al. 2008, Felder et al. 2010), heart (Ding et al. 2007, Palanivel et al. 2007) and leukocytes (Alberti et al. 2007, Weigert et al. 2008). AdipoR1 is abundantly expressed in skeletal muscles and AdipoR2 in liver (Yamauchi et al. 2003). The structure of adiponectin receptors are related to G protein-coupled receptors (GPCRs) but their sequence homology is very low. Adiponectin receptors contain intracellular N terminal and extracellular C terminal as opposed to G protein-coupled receptors (Yamauchi et al. 2003) (figure 3).
Fig. 3: Adiponectin receptors (AdipoR1 & AdipoR2) structure (Yamauchi and Kadowaki 2013). “Reprinted from Cell Metabolism, Volume 17, Yamauchi, T., Kadowaki, T., Adiponectin Receptor as a Key Player in Healthy Longevity and Obesity-Related Diseases, Pages no. 185–196, Copyright 2013, with permission from Elsevier.”

The structure of AdipoR2 has 66.7% sequence homology with AdipoR1 and these receptors are different from G-protein coupled receptors. Unlike GPCRs these receptor have intracellular N terminal and extracellular C terminal.

AdipoR1 possesses high-affinity towards the globular form of adiponectin and low-affinity towards full-length form of adiponectin (Yamauchi et al. 2003). AdipoR1 in skeletal muscle has been shown to up-regulate glucose uptake and fatty acid oxidation by activation of AMPK (Yamauchi et al. 2003). AdipoR2 exerts intermediate affinity towards both globular form and full-length adiponectin and it mediates the effect of full-length adiponectin by activating AMPK in the liver (Yamauchi et al. 2003). It is reported that in the neonatal cardiomyocyte AdipoR1 receptor is expressed more than AdipoR2 receptor (Palanivel et al. 2007).

AdipoR1 mediates the protective effect of adiponectin against ischemic-reperfusion injury in mouse cardiomyocytes (Wang et al. 2010). Studies using mice show that adiponectin binds with APPL1 adaptor protein to induce and regulate downstream signaling (Mao et al. 2006, Wang et al. 2009). Pre-treatment with adiponectin protected rat embryonic cardiac myoblasts (H9c2) by reducing reactive oxygen species (ROS) production, which is up-regulated during hypoxia/reoxygenation injury, through AdipoR1 and APPL1 mediated signalling which suggests the anti-oxidative effect of
adiponectin (Park et al. 2011). APPL1 is expressed in a variety of tissues including skeletal muscle, brain, spleen and the heart (Mao et al. 2006). APPL1 is identified as an essential protein which interacts with AdipoR1 and AdipoR2 in adult rat cardiomyocytes (Fang et al. 2010). The coimmunoprecipitation study in the rat cardiomyocytes shows that adiponectin increases binding of AdipoR1 to APPL1 but not AdipoR2 which leads to translocation of liver kinase B1 (LKB1) from the nucleus to cytosol and it is then bound with the AdipoR1-APPL1 complex which activates downstream AMPK phosphorylation which in turn regulates fatty acid metabolism (Fang et al. 2010). Studies in animals show that adiponectin attenuates Ang II (Shibata et al. 2004) and leptin-induced cardiac hypertrophy (Rajapurohitam et al. 2013) which suggests that adiponectin acts as an anti-hypertrophic agent.

1.8 Hypothesis and objectives

**Rational:** Adipose tissue is present throughout the body and adipose tissue-derived adipokines exerts various effects on different organs. The effect of individual adipokines on the cardiovascular system are under study. It is important to consider the effects induced by individual and combined adipokines on different organs and how those effects can alter the physiological response of the tissues. During obesity and cardiac hypertrophy, blood plasma levels of leptin are upregulated and adiponectin levels decreased in comparison with healthy individual. Moreover, leptin possesses pro-hypertrophic properties and adiponectin possesses anti-hypertrophic properties. Thus, we believe that the leptin and adiponectin play a critical role in mediating the cardiac hypertrophic response.

**Hypothesis:** Adipocyte-derived leptin and adiponectin modulate ET-1-induced neonatal rat ventricular hypertrophy.

**Objective 1:** To examine the potential effect of ACM on ET-1-induced cardiomyocyte hypertrophy.

- To determine the effect of ACM we examine the role of leptin and adiponectin during hypertrophic response of ET-1 in cardiomyocytes.
**Objective 2:** To study the underlying signalling mechanism associated with the modulation of ET-induced cardiomyocyte hypertrophy by leptin/adiponectin.

- In order to explore the signalling mechanism we focus on AMPK signalling as AMPK is associated with cardio-protective effect of adiponectin.
Chapter 2: Materials and Methods
2. Materials and methods

2.1 Primary culture of neonatal rat ventricular cardiomyocytes

Neonatal hearts were extracted from 1-5 days old Sprague–Dawley rats (Charles River Canada, Montreal, Quebec, Canada). After sacrifice, hearts were isolated and rinsed in buffer containing 1x Hank’s balanced salt solution (HBSS) (Wisent Inc., St. Bruno, QC). Hearts were squeezed gently to remove residual blood and transferred to another dish containing fresh ice-cold 1x HBSS. Atrias were removed and ventricles were placed into another dish. Ventricles were minced into smaller pieces using a surgical blade. The heart pieces were then transferred into a water-jacketed Erlenmyer flask maintained at 37 °C after which digestion buffer was added for six sequential digestions. The digestion buffer contained 10% 10 x HBSS (Gibco, Life Technologies Burlington, ON, Canada), 2% 1 M HEPES (Gibco), 2% Penicillin/Streptomycin (Gibco), 0.11 mg/mL Collagenase (Worthington Corp., Lakewood, NJ, USA), 0.13 mg/mL Trypsin (Worthington) and 0.03mg/mL DNase II (Worthington). In some early experiments, hearts were digested with 0.33mg/mL concentration without trypsin and DNase II (Figure 1-11). After each digestion, the supernatant was removed from the flask and poured into an Eppendorf tube having same volume of stop buffer which consisted of 10% 10 x HBSS, 2% Penicillin/streptomycin and 20% Fetal Bovine Serum (FBS) to abolish collagenase activity. The solution containing both the supernatant and stop buffer was filtered in another Eppendorf tube using a 70 µm cell strainer and centrifuged at 2000 rpm at 4°C. The supernatant was discarded and pellets were re-suspended in the cell culture medium (Table 1). The suspension was subjected to pre-plating for one hour followed by another 30 min of pre-plating to ensure cardiomyocytes enrichment. Following pre-plating, Primaria™ (Falcon) culture dishes were plated with cardiomyocytes at an optimal density required for this type of experiment. Cells (1 x 10^4 cells) were plated to perform cell surface area analysis as this quantity ensures sufficient density to visualise growth while 6 x 10^4 cells were plated for RNA and protein isolation as this quantity ensures sufficient density for gene expression and protein expression.
The cells were grown in an incubator at 37°C 5% CO₂. After 48 hours, myocytes were washed with phosphate buffer saline-ABC (PBS-ABC) (Table 2) and cultured in serum-free medium (Table 3) for another 24 hours before starting the treatments. Tissue culture medium pH were prepared at 7.10 and all the reagents were filtered, sterilized and autoclaved.

2.2 Animal models for isolation of adipose tissue

All the rats used in the experiments were 3-6 months old.

After the rats were sacrificed, adipose tissue in normal Sprague-Dawley rats was isolated from the epididymal and perirenal region and in heart failure models and obese models adipose tissue was isolated from epididymal region.

**Heart failure model:** To prepare heart failure model rats, coronary artery ligation (CAL) surgery was performed in adult Sprague-Dawley rats. Surgical procedure commenced after the rats were anesthetized by pentobarbital sodium (50 mg/kg body weight) which was administered by intraperitoneal injection. Rats were kept on a respirator (Model 683, Harvard Apparatus). The thoracotomy was performed and a 5-0 braided silk suture was tied on the left main coronary artery at a distance of ~3 mm from the origin. To prepare heart failure control models (Sham), the ligature was positioned in a similar fashion and the silk suture was removed without tying. Four weeks after surgery, the rats were sacrificed and adipose tissue was isolated.

**Obese model:** To obtain adipose tissue from obese model rats, JCR strain rats were used as they provide a suitable model of metabolic syndrome which is similar to humans (Russell and Koeslag 1990). The metabolic syndromes they show are abdominal obesity, mild type II diabetes and high risk for cardiovascular diseases (Russell and Koeslag 1990). The JCR rats are a unique strain that carries the mutant autosomal recessive cp gene, therefore, these strains are leptin receptor deficient (Koletsky 1975). Rats that are homozygous for the gene (cp/cp) develop obesity, insulin resistance, hyperlipidemia, vasculopathy, and atherosclerosis and rats that are normal and
homozygous (+/+) or heterozygous (+/-) do not develop any metabolic disease and are lean (Russell et al. 1998).

2.3 Preparation of adipose tissue conditioned medium

Adipose tissue was isolated from 3-6 months old Sprague-Dawley rats, heart failure (Sham and CAL) rats and JCR (lean and obese) rats and transferred to culture dishes containing Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12). Adipose tissue was weighed and minced into small pieces with a surgical blade mounted on scalpel (about 50 strokes in criss-cross section) in the same culture dish. The tissue was cut with a pair of scissors to ensure that all pieces were cut uniformly and as small as possible; any visible blood capillaries and clots were removed. Then 3-dimentional collagen gel (Table 4) was added and mixed to make a homogenous mixture. The suspension was set aside for half an hour at room temperature to solidify. After solidification, serum free media was added onto the gel and put in the incubator at 37°C 5% CO₂ for 24 hours. Adipose tissue conditioned medium (ACM) was then collected to use in the experiment. Adipose tissue conditioned medium from JCR (lean and obese) rats was kindly provided by Dr. Spencer Proctor (the University of Alberta).

2.4 Cell surface area analysis of cardiomyocytes

Cardiomyocytes were allowed to grow in serum-containing medium for 24 hours then were washed with PBS-ABC and again allowed to grow in serum-free medium for 24 hours before treatment. Cells were treated with serum free medium (control) and various dilutions of ACM obtained from normal Sprague-Dawley, heart failure and JCR rats in the presence or absence of ET-1, Ovine super-active leptin antagonist (LRA), adiponectin receptor-1 antibody (ARA), AMPK activator AICAR, AMPK inhibitor dorsomorphine (Compound C) (table 5) for 24 hours. One hour time difference was kept before adding another chemical in the same culture dish. Cell surface area was analyzed using a Leica inverted microscope equipped with an infinity 1 camera at 100 x magnification. Cell surface area was measured using SigmaScan Software (Systat, Richmond, CA, USA).
2.5 RNA isolation and purification

Following the treatments, total RNA from cells was extracted using TRIZol® Reagent (Bio-Rad Laboratories Ltd., Mississauga, ON) according to the manufacturer’s instructions. Briefly, following 24 hours of treatment, growth media was removed from cardiomyocytes dish and washed with PBS ABC. TRIZol® Reagent was added to each culture dish and cells were lysed and homogenized by using a cell scraper. This homogenized sample was transferred to small Eppendorf tubes and kept at room temperature for 5 minutes to allow complete dissociation of the nucleo-protein complex. Chloroform was added and the tubes were shaken vigorously for 15 seconds by hand and incubated at room temperature. This mixture was centrifuged at 10,000 rpm for 30 minutes at 4 °C and separated in 3 phases: lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The aqueous phase was removed using a pipette and transferred to a new Eppendorf tube. Isopropanol (100%) was added to the aqueous phase and kept overnight at -20 °C. The following day, the sample was centrifuged at 10,000 rpm for 20 minutes at 4 °C. The RNA is often invisible before centrifugation, and forms a gel-like pellet on the side and bottom of the tube. Supernatant was discarded from the tube, leaving only the RNA pellet. Ethanol (75%) was added to the tube to wash the RNA. The tube was vortexed briefly, then centrifuged at 10,000 rpm for 10 minutes at 4 °C. The RNA pellet stuck to the bottom, the supernatant was removed and the pellet was air dried for only 10 minutes so to prevent loss of solubility. The RNA pellet was dissolved in Diethylpyrocarbonate (DEPC) treated water. The RNA was quantified using MD SpectraMax M5e Reader (Molecular devices, Sunnyvale, CA, USA).

2.6 First-strand cDNA synthesis

Four microgram RNA was used to synthesize the first strand of cDNA using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) according to the manufacturer’s protocol and was used as a template in the polymerase chain (PCR) reactions. The cDNA synthesis was performed in nuclease-free micro-centrifuge tube. First 4 µg of RNA was added in the tube and DEPC treated water was added to make up
a 10 µl volume. Then 1x random primer and 5 mM Deoxynucleoside triphosphates (dNTP) mix was added to the same tube (Table 6). This mixture was heated to 65 °C for 5 minutes and quickly chilled on ice. After brief centrifugation, the contents of the tube were collected and 5x FSB, 0.1 M DTT and M-MLV RT were added and mixed gently up and down using a pipette. The tube was incubated at 25 °C for 10 minutes followed by 37 °C for 50 minutes and the reaction was inactivated by heating it to 70 °C for 15 minutes. All the reagents were from Invitrogen, Life Technologies Inc, Burlington, ON, Canada.

2.7 Quantitative Real-time PCR

The expression of the genes was performed in 10 µl reaction volumes using EvaGreen qPCR Mastermix (Applied Biological Materials Inc., Richmond, BC, Canada) and fluorescence was measured and quantified using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). The Real-Time PCR reaction was carried out in 96 well plates from Bio-Rad and each well contained 10 µl mixture of components (Table 7). The PCR consists of amplification of gene for 35-40 cycles with temperature changes. The PCR reaction steps consists of initialization step (heating the samples at 95 °C for 3 minutes), denaturation step (samples are heated at 95 °C for 30 seconds as this step separates DNA templates by disrupting the hydrogen bonds thus producing single-stranded DNA), annealing step (samples are cooled to 50-60 °C for 20 seconds which results in primers binding to the single-stranded DNA) and elongation step (samples are heated at 72 °C so that taq polymerase enzyme synthesizes a new complementary DNA strand). The primer sequences used for TPT1 (tumor protein, translationally-controlled 1), ANP, α-skeletal actin, leptin, adiponectin OBRb and adipor1 are shown in table 8. TPT1 mRNA expression was used as a control.

2.8 Adipokines analysis in ACM

ACM from normal rats (except adiponectin concentration), JCR rats and heart failure rats was analyzed for adipokine concentrations by Eve Technologies, Calgary, AB. The
analysis is based on color-coded polystyrene beads. Red and infrared dyes are used to create unique coloured bead sets and each unique coloured bead is bound with different antibody. The bead analyzer identifies the unique colour and it contains dual laser system. Fluorescent dye that identifies the antibody, which is within the beads, is activated by one laser and the other laser stimulates the streptavidin-phycoerythrin (fluorescent conjugate) that binds with the beads in the essay. The conjugate is detected by the analyzer is proportionate to the target antibody and the results are obtained using standard curve.

2.9 ELISA analysis for adiponectin content in normal ACM

ACM was used to determine the adiponectin content using enzyme-linked immunosorbent assay kit (ELISA) according to manufacturer’s instructions (EMD Millipore, Etobicoke, ON, Canada). Briefly, all the samples were brought to room temperature before starting the assay. A 96-well plate in the kit was used, washed with wash buffer and the plate was inverted to remove the residue of wash buffer. Assay running buffer was added to each well and rat adiponectin standard was added in ascending concentration to the proper well. Rat adiponectin quality control was added in appropriate wells followed by the sample. The plate was covered with plate sealer, placed on the shaker and incubated at moderate speed. After incubation, the plate sealer was removed and tapped to remove residual solutions. Wells were washed, detection antibody was added to each well and the plate was covered with plate sealer. After incubation it was washed again. Enzyme solution was added, washed, substrate solution was added, covered with plate sealer and incubated for 5 to 25 minutes depending on the development of blue colour in the wells. After colour development, the stop solution was added to each well and plate was gently shaken to ensure complete mixing. The formation of yellow colour indicated complete reaction. The plate was then read for absorbance at 450 nm and 590 nm and the difference between these absorbances was recorded.
2.10 Western blotting

Cells were treated with appropriate compounds and washed with cold PBS-ABC. PBS-ABC was discarded completely without drying the cells and lysis buffer containing various compounds (Table 9) and protease inhibitors were added. The cells were scrapped using scrapper and transferred into small tubes which were then kept on ice. The lysates were pipetted several times to ensure complete breakage of the cells in the buffer. The tubes were centrifuged at 10,000 rpm for 30 minutes. The solution had two forms: clear solution at the top and pellets at the bottom. Clear solution was removed and transferred to other small tubes leaving the pellets in the tube. This clear solution was transferred to the 48-well plate and then appropriate Bio-Rad protein essay solution was added. Following the addition of protein essay, plates were read for absorbance at 495 nm and the amount of protein was determined using standard curve. 50 µg (adiponectin, AdipoR1, p-AMPK and OBRb) to 80 µg (leptin) protein was used to prepare samples and 6x sample buffer was added. Theses samples were heated at 100 °C for 10 minutes. Then sodium dodecyl sulfate (SDS) containing polyacrylamide gels were prepared. 7.5% (for OBRb), 10% (for adiponectin AdipoR1 and p-AMPK) and 12% (for leptin) gels were made.

Gels were transferred into the box and the box was filled with 1x Running Buffer (Table 10). 8 µL protein marker (Bio-Rad) was loaded in one well and samples were loaded into other wells of the gel. The whole box was connected to the power source and constant 100 mV electric current was provided. The proteins were then separated according to their molecular weight on to the gels; high molecular weight proteins were separated at the top of the gel and low molecular weight protein remained at the bottom of the gel. Electric current was stopped and the gels were transferred on to the cassette sandwich containing sponge, filter paper, nitrocellulose membrane (20 µm and 45 µm, according to the protein of interest), gel, filter paper and sponge.

This sandwich was then transferred to another box and filled with 1x Transfer Buffer which was then connected to a power unit. Constant 30 mA electric current was provided and the whole assembly was placed at 4 °C room temperature for overnight
transfer. The following day, membranes were removed, transferred to the box and the membranes were stained with Ponceau S solution to check transfer efficiency and protein quality. Following the staining, Ponceau S solution was discarded and membranes were washed with 1x Wash Buffer three times for 10 minutes each to remove residue from the solution completely. Membranes were blocked by 5% skim dry milk solution for 1 hour at room temperature on a plate shaker. The milk solution was discarded and the membranes were washed with wash buffer three times for 10 minutes each to remove the residue of the milk solution and then these membranes were probed with the primary antibody of leptin, OBRb, adiponectin, AdiopR1, p-AMPK, β-Actin and AMPK (Table 11). The boxes were kept on the shaker overnight at 4 °C. The following day, the membranes were kept on a shaker for one hour at room temperature. The membranes were then washed with wash buffer three times for 10 minutes each to remove the primary antibody completely from the membrane and proper secondary antibody was added. The boxes were kept on the shaker for one hour. After one hour the membrane was washed again with wash buffer thrice. The membranes were then read using Odyssey Clx Infrared Imaging System (Li-COR) (Lincoln, NE, United States). The blots were analyzed using FluorChem 8000 software.

2.11 Molecular weight cut-off filter

To filter the ACM we used Amicon® Ultra-15 Centrifugal Filter (3kDa and 30kDa) devices according to manufacturer’s instructions (EMD Millipore, Etobicoke, ON, Canada). Briefly, ACM was added in the tubes provided with kit and the tubes were put into fixed angle rotor centrifuge. The centrifuge was run at 5,000 x g for 30 minutes. At the end of the run, tubes were removed from centrifuge and the solution was withdrawn using pipette. This filtered and remained medium was used in the experiment.

2.12 Statistical analysis

All the experiments were analyzed using GraphPad Prism 6 and the data were transformed into fold change. Mean value of all the controls in each experiment was used to divide with each treatment value to get the fold change expression. The
statistical outcome was identical when raw values and fold change values were used as the fold change values were normally distributed. All data are represented as mean ± standard error mean (SEM).

Data for cell surface area and fetal gene expressions were analyzed using 1-way analysis of variance (ANOVA) followed by a post hoc Tukey test. Data for the concentration response curve of ACM in the presence or absence of LRA and ARA were analyzed by 2-way ANOVA followed by Bonferroni post hoc test. Data for JCR and heart failure rat’s characteristics and analysis of adipokines in ACM were analyzed using Student’s unpaired t-test. P < 0.05 was considered significant.
Chapter 3: Results
3. Results

3.1 ACM attenuates ET-1-induced cardiomyocyte hypertrophy

Cardiomyocytes were treated with ET-1 (10 nM) and in the presence or absence of different dilution of ACM (20x10^8, 10x10^8, 5x10^8, 2.5x10^8 and 1.25x10^8) for 24 hours. Higher dilution of ACM contains lower concentration of ACM and lower dilution of ACM contains higher concentration of ACM. Figure 4A shows the changes in the size of cardiomyocytes with the treatment of ET-1 with or without ACM. ET-1 increased cardiomyocytes surface area significantly (P < 0.001) compared with untreated cardiomyocytes and it was completely attenuated by higher concentrations of ACM (5x10^8, 2.5x10^8 and 1.25x10^8) treatment significantly (P < 0.05, 0.001 and 0.001, respectively) (Figure 4B and 4C). ET-1 significantly (P < 0.05) up-regulated expression of ANP and α-Skeletal actin which was significantly (P < 0.05) attenuated by higher concentration of ACM (1.25x10^8) treatment (Figure 1D and 1E). Lower concentration of ACM (20x10^8) did not show any significant effect on ET-1-induced cardiomyocyte hypertrophy (Figure 4B, 4C, 4D and 4E). In addition, cardiomyocytes treated only with the higher concentration of ACM (1.25x10^8) also did not show any difference on the cell surface area and fetal genes in contrast with untreated cardiomyocytes (Figure 4B, 4C, 4D and 4E).
Fig. 4. ET-1–induced hypertrophy in cardiomyocytes is attenuated by ACM. Panel A shows the phase-contrast micrographic images of cardiomyocytes (100X magnification) in the absence or presence of ET-1 with the treatment of different dilution of ACM for 24 hours. Panels B & C show cell surface area and % maximum hypertrophic response on cardiomyocyte cell surface area treated in the presence of ET-1 and with the treatment of different dilution of ACM for 24 h. Panel D and panel E show fetal gene (α-Skeletal actin and ANP respectively) expression on cardiomyocyte treated in the presence of ET-1 and with the treatment of ACM for 24 h. For cell surface area, the mean of 50 cells were used to provide one value. Values indicate mean ± SEM (n=6-10). *P<0.05 and ***P<0.001 from untreated cardiomyocytes, #P<0.05 and ###P<0.001 from ET-1.
3.2 Presence of adipokines in the ACM

Experiment was conducted using undiluted ACM (200 mg/mL). The graph represents concentration of Adipokines ranging from nanogram to picogram.

27 adipokines were analyzed in the ACM and it was observed that Adiponectin was the most secreted adipokine from adipose tissue among all and the concentration was at ~1.2 µg/mL (Figure 5 C). Leptin concentration was at ~3.5 ng/mL. The difference between these two adipokine is about 1000 fold and it is believed that the ratio is essential to exert adipose tissue’s effect.
Fig. 5. Adipokines present in the ACM. Values indicate mean ± SEM (n=12).
3.3 Concentration response of LRA and ARA

To determine the role of leptin and adiponectin in cardiomyocytes, we used the leptin receptor antagonist (LRA) to block the OBRb and AdipoR1 antibody (ARA) to block the AdipoR1.

We used four different concentrations of LRA (0.01 nM, 0.1 nM, 1 nM and 10 nM) to determine the optimum concentration response. We used LRA in the presence of ET-1 and treated cardiomyocytes for 24 hours. The cell surface analysis shows that the LRA concentrations of 0.1 nM, 1 nM and 10 nM significantly ($P < 0.05$, 0.01 & 0.001, respectively) reduced the cell surface area compared to ET-1 alone (Figure 6A). The LRA concentration of 0.01 nM did not attenuate cell surface area significantly.

To determine the optimum concentration response we used four different concentrations of ARA (1 ng/mL, 10 ng/mL, 100 ng/mL and 1000 ng/mL) and treated cardiomyocytes in the presence of ET-1. We used ACM ($1.25 \times 10^8$) in order to attenuate ET-1 effect so ARA could block the anti-hypertrophic effect of ACM in a concentration-dependent manner. The cell surface analysis show that ARA concentrations of 1 ng/mL, 10 ng/mL and 100 ng/mL significantly ($P < 0.001$, 0.01 and 0.05, respectively) blocked the cell surface area compared with ET-1 alone (Figure 6B). The ARA concentration of 1000 ng/mL did not attenuate ET-1 effect.

We used the optimum concentration of LRA (0.1 nM) and ARA (100 ng/mL), for determining the concentration response curve and EC$_{50}$ of ACM in the presence of LRA and ARA (Figure 7 and 10).
Fig. 6. ET-induced increase in cardiomyocyte cell surface area is attenuated by LRA and ACM-induced decrease in cardiomyocyte cell surface area is inhibited by ARA in a concentration-dependent manner. Panel A and B show % maximum hypertrophic response in cardiomyocyte cell surface area treated with 4 different concentrations of LRA and ARA (respectively) in the presence of ET-1 for 24 hours. Values indicate mean ± SEM (n=5-6). *P<0.05, **P<0.01 and ***P<0.001 from ET-1.
3.4 Leptin receptor blockade increases anti-hypertrophic effect of ACM

To analyze the concentration response curve and determine EC$_{50}$ of ACM in the presence of LRA, we treated cardiomyocytes with 0.1 nM concentration of LRA and with ET-1 in the presence of different dilution of ACM for 24 hours (Figure 7). The graph was plotted on the scale of percentage maximum hypertrophic response and hypertrophic assessment was performed. The cell surface area and α-Skeletal actin gene expression analysis show that anti-hypertrophic effect of ACM is increasing in the presence of LRA compared with ACM in the absence of LRA. EC$_{50}$ analysis show that cell surface area shifts EC$_{50}$ to the left from dilution of 3.125x10$^8$ to 8.75x10$^8$ (Figure 7A). Similarly, the α-Skeletal actin gene expression shows that the EC$_{50}$ shifts from the dilution 3.825x10$^8$ to 9.375x10$^8$ (Figure 7B).
Fig. 7. In the presence of LRA, the anti-hypertrophic effect of ACM increases in a concentration-dependent manner. Panel A shows the effect of LRA on cell surface area and EC$_{50}$ shifts from dilution $3.125 \times 10^8$ to $9.375 \times 10^8$. Panel B shows the effect of LRA on α.Skeletal actin gene expression and EC$_{50}$ shifts from dilution $3.825 \times 10^8$ to $9.375 \times 10^8$. Cardiomyocytes were treated with ET-1 with ACM and with and without LRA for 24 hours. Values indicate mean ± SEM (n=6). ###P<0.001 from ET-1+ACM.
3.5 ACM down-regulates gene expression of leptin and OBRb

We looked at leptin and OBRb genes expression to determine the change during ACM-induced anti-hypertrophic effect. Cardiomyocytes treated in the presence of ET-1 for 24 hours significantly ($P < 0.05$) up-regulated leptin and OBRb gene expression which was significantly ($P < 0.05$) down-regulated by higher concentration of ACM ($1.25 \times 10^8$) (Figure 8). Lower concentrations of ACM did not down-regulate leptin and OBRb gene expression compared with ET-1. Treatment of high concentration of ACM alone did not show any significant effect on these gene expressions in contrast with untreated cardiomyocytes.
Fig. 8. ET-1-induced up-regulation in leptin gene and leptin receptor (OBRb) gene are attenuated by ACM in concentration-dependent manner. Panel A and B show leptin and OBRb mRNA expression, respectively, treated in the presence or absence of ET-1 and with the treatment of ACM for 24 h. Values indicate mean ± SEM (n=6-8). *P<0.05 from untreated cardiomyocytes, #P<0.05 from ET-1.
3.6 ACM down-regulates protein expression of leptin and OBRb

To address the intracellular changes in the protein expressions during anti-hypertrophic effect of ACM, we carried out the protein expression of leptin (16kDa) and OBRb (120kDa). The treatment of ET-1 significantly ($P<0.01$) up-regulated leptin and OBRb protein expressions in cardiomyocytes which was significantly ($P<0.05$) down-regulated by higher concentrations of ACM ($2.5\times10^8$ and $1.25\times10^8$) (Figure 9). Lower concentrations of ACM did not show significant change in leptin and OBRb protein expressions compared with ET-1.
Fig. 9. ET-1-induced up-regulation of leptin and OBRb protein expression is abolished by ACM in a concentration-dependent manner. Panel A and B show the western blot and respective quantification data for leptin (16kDa) and OBRb (120kDa) protein expression, respectively. Cardiomyocytes were treated in the presence or absence of ACM with or without ET-1 for 24 hours. Values indicate mean ± SEM (n=5-7). **P<0.01 from control and #P<0.05 from ET-1.
3.7 AdipoR1 blockade decreases anti-hypertrophic effect of ACM

To analyze the concentration response curve of ACM in the presence of ARA, we treated cardiomyocytes with 100 ng/mL ARA and with ET-1 in the presence of different dilution of ACM for 24 hours. We analyzed the cell surface area and α-Skeletal actin gene expression. We observed that anti-hypertrophic effect of ACM was decreased in the presence of ARA compared with ACM which is evident by cell surface area and α.Skactin gene expression (Figure 10). EC$_{50}$ analysis could not be performed in these experiments.
Fig. 10. Blocking AdipoR1 by ARA decreases the anti-hypertrophic effect of ACM in cardiomyocyte in a concentration-dependent manner. Panel A shows the effect of ARA on cell surface area and panel B shows the effect of ARA on α-skeletal actin gene expression. Cardiomyocytes were treated with ET-1 with ACM and with or without ARA for 24 hours. Values indicate mean ± SEM (n=5-7). #P<0.05 & ###P<0.001 from ET-1+ACM.
3.8 ACM up-regulates adiponectin and AdipoR1 gene expression

To explore the intracellular changes associated with the anti-hypertrophic effect of ACM - such as adiponectin and AdipoR1 gene expression – the expression of the aforementioned genes were assessed in cardiomyocytes treated with ET-1 in the presence or absence of varying dilutions of ACM. 24 hours treatment with ET-1 significantly ($P < 0.05$) down-regulated adiponectin gene expression but had no effect on AdipoR1 mRNA expression (Figure 11). High concentration of ACM (1.25x10$^8$) significantly ($P < 0.05$) up-regulated the adiponectin gene expression compared with the ET-1 (Figure 11A). AdipoR1 mRNA expression was also significantly ($P < 0.01$) up-regulated compared with ET-1 (Figure 11B). Cardiomyocytes treated with ACM alone significantly ($P < 0.01$) up-regulated AdipoR1 mRNA expression (Figure 11B). There also is a trend in adiponectin gene.
Fig. 11. ACM up-regulates both adiponectin gene and AdipoR1 gene expression in a concentration-dependent manner. Panel A and B show adiponectin and AdipoR1 mRNA expression, respectively, treated in the presence or absence of ET-1 and with varying dilutions of ACM for 24 h. Values indicate mean ± SEM (n=5-7). *P<0.05 and **P<0.01 from control. #P <0.05 and ###P<0.01 from ET-1.
3.9 ACM up-regulates protein expression of adiponectin and AdipoR1

Protein expression of adiponectin (30kDa) and AdipoR1 (42kDa) during anti-hypertrophic effect of ACM was analyzed. 24 hours treatment of cardiomyocytes with ET-1 did not produce any significant change in adiponectin and AdipoR1 protein expression compared with untreated cardiomyocytes (Figure 12A and 12B). High concentrations of ACM (2.5x10^8 and 1.25x10^8) significantly up-regulated adiponectin (P< 0.05) and adipoR1 (P < 0.05 and 0.01 respectively) protein expressions compared with the ET-1 alone. Lower concentrations of ACM did not show any significant change.

We observed multiple bands in the western blots for both adiponectin and AdipoR1 and we quantified ~30kDa band for adiponectin and ~42kDa for AdipoR1. We also quantified other nonspecific bands, however, there was no significance difference.
Fig. 12. ACM up-regulates both adiponectin and AdipoR1 protein expression in a concentration-dependent manner. Panels A and B show the western blots and respective quantification data for adiponectin (30kDa) and AdipoR1 (42kDa) protein expression, respectively. Cardiomyocytes were treated in the presence or absence of ET-1 and with or without ACM for 24 hours. Values indicate mean ± SEM (n=5-7). #P<0.05 and ##P<0.01 from ET-1.
3.10 Anti-hypertrophic adipokines are present above 3kDa molecular weight

To determine the molecular weight of adipokines for the anti-hypertrophic effect of ACM, we filtered the high concentration of ACM (1.25x10^8) using 3kDa filter device which separated ACM into two phases, high molecular weight (HMW) 3kDa (>3kDa) and low molecular weight (LMW) 3kDa (<3kDa). We also boiled the original concentration of ACM (200ng/mL) to determine that the effect of ACM which is due to the presence of protein. We treated cardiomyocytes for 24 hours with HMW 3kDa, LMW 3kDa and boiled ACM in the presence of ET-1 and analyzed cell surface area (Figure 13). ET-1 significantly (P < 0.01) increased the cell surface area which was abolished by HMW 3kDa ACM (P < 0.05). LMW 3kDa and boiled ACM did not attenuate ET-1-induced increase in cell surface area. Cardiomyocytes treated only with HMW 3kDa, LMW 3kDa and boiled alone did not show any significant change in cell surface area compared with untreated cardiomyocytes.
Fig. 13. Anti-hypertrophic adipokines are present above 3kDa. The graph shows the cell surface area analysis of cardiomyocytes treated with LMW ACM (<3kDa), HMW ACM (>3kDa) and boiled ACM and in the presence or absence of ET-1 for 24 hours. Values indicate mean ± SEM (n=5-8). **P<0.01 from control and #P<0.05 from ET-1.
3.11 Anti-hypertrophic adipokines are present below 30kDa molecular weight

To determine the molecular weight range of adipokines which show anti-hypertrophic property during ACM treatment on ET-1-induced cardiomyocytes hypertrophy, we filtered high concentration ACM (1.25x10^8) using a 30kDa filtration device which separated ACM in HMW 30kDa and LMW 30kDa ACM. Cardiomyocytes were treated with ET-1 and in the presence or absence of HMW 30kDa and LMW 30kDa for 24 hours and were subjected to hypertrophic assessment. ET-1 significantly increased cell surface area (P < 0.05) which was significantly attenuated by LMW 30kDa ACM (P < 0.001) (Figure 14A). ET-1-induced significantly up-regulated α-Skeletal actin and ANP gene expression (P < 0.05 and 0.01, respectively) and it was down-regulated by LMW 30kDa ACM significantly (P < 0.05 and 0.01, respectively) (Figure 14A and 14B). HMW 30kDa ACM in the presence of ET-1 did not attenuate the increase in cell surface area and fetal gene expressions compared with ET-1. Moreover, treatment of cardiomyocytes with HMW 30kDa ACM and LMW 30kDa ACM alone did not show any change in cell surface area or in gene expression.
Fig. 14. Anti-hypertrophic adipokines are present below 30kDa. Panel A shows cardiomyocyte cell surface area analysis treated with ET-1 and with or without the treatment of HMW ACM (>30kDa) and LMW ACM (<30kDa) for 24 h. Panel B and panel C show fetal gene (α-Skeletal actin and ANP respectively) expression on cardiomyocytes treated in the presence or absence of ET-1 and with the treatment of HMW ACM and LMW ACM for 24 h. Values indicate mean ± SEM (n=6). *P<0.05, **P<0.01 & ***P<0.001 from Control, #P<0.05, ##P<0.01 from ET-1 & ¥P<0.05, ¥¥P<0.01 from ET-1 + >30kDa.
3.12 Effect of ACM isolated from epididymal and perirenal adipose tissue

Our previous results have suggested that ACM show anti-hypertrophic effect on ET-1-induced cardiomyocytes hypertrophy. We wanted to observe the effect of ACM isolated from different parts of the visceral region; epididymal and perirenal. We obtained the ACM from both region and carried out the hypertrophic assessment. Cardiomyocytes were treated with different dilutions of both ACM in the presence or absence of ET-1 for 24 hours. Hypertrophic assessment of both types of ACM show similar trends and the ability to attenuate ET-1-induced hypertrophic effect in concentration-dependent manner (Figure 15). Lower dilution of both ACM (20x10^8) did not affect the increased cell surface area whereas all the other dilutions showed significant inhibition in ET-1-induced increase in cell surface area (Figure 15A and 15B). Similarly, ANP and α.Skeletal actin gene expression was significantly (P < 0.05 and 0.01 wherever applicable) up-regulated in the presence of ET-1 which was significantly attenuated by higher concentration of both epididymal and perirenal ACM (1.25x10^8) (P < 0.05 and 0.01 wherever applicable) (Figure 15C, 15D, 15E and 15F).
Epididymal

**ET-1**

ANP/TPT1 mRNA (Fold change)

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Perirenal

**ET-1**

ANP/TPT1 mRNA (Fold change)

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Fig. 15. ET-1–induced cardiomyocyte hypertrophy is inhibited by both ACM (epididymal and perirenal) in a concentration-dependent manner. Panel A and B show cardiomyocyte cell surface area treated in the presence or absence of different dilutions of both ACM and with or without ET-1 for 24 h. Panel C & D and panel E & F show fetal gene (α-Skeletal actin and ANP respectively) expression on cardiomyocyte treated in the presence of ET-1 and with the treatment of both ACM (epididymal and perirenal) for 24 h. Values indicate mean ± SEM (n=6-8). *P<0.05, **P<0.01 & ***P<0.001 from untreated cardiomyocyte, #P<0.05, ##P<0.01 & ###P<0.001 from ET-1.
3.13 Effect of obesity on the ability of ACM to inhibit ET-1-induced cardiomyocyte hypertrophy

We obtained ACM from lean and obese JCR rat’s adipose tissue and treated cardiomyocytes with or without ET-1 in the presence of different dilutions of ACM (20x10^8, 1.25x10^8 and 1 (original concentration)) and hypertrophic assessment was carried out. ET-1-induced significantly increased in cell surface area ($P < 0.001$) was significantly attenuated by high concentrations of ACM from lean adipose tissue (1.25x10^8 and 1) ($P < 0.001$ and 0.01 respectively) (Figure 16A). Similarly α-Skeletal actin and ANP gene expression was significantly up-regulated by ET-1 ($P < 0.05$) which was significantly down-regulated by high concentrations of ACM from lean adipose tissue (1.25x10^8 and 1) ($P < 0.05$) (Figure 16A and 16B). Lower concentration of ACM from lean adipose tissue (20x10^8) did not show any significant difference compared with ET-1. Cardiomyocytes treated with ACM (1.25x10^8) from both types of adipose tissue in the absence of ET-1 did not show any difference in both cell surface area and fetal gene expression compared with untreated cardiomyocytes. Different dilutions of ACM from obese adipose tissue also did not show any significant difference in cell surface area and fetal gene expression compared with ET-1.
Fig. 16. ACM obtained from JCR obese rat’s adipose tissue does not attenuate ET-1-induced cardiomyocyte hypertrophy. Panel A shows cardiomyocyte cell surface area treated in the presence or absence of ET-1 and with the treatment of different dilution of ACM obtained from JCR lean and obese rat’s adipose tissue for 24 h. Panel B and panel C show fetal gene (α-Skeletal actin and ANP respectively) expression on cardiomyocytes with or without ET-1 and with the treatment of both types of ACM for 24 h. Values indicate mean ± SEM (n=6). *P<0.05 & ***P<0.001 from Control and #P<0.05, ##P<0.01 & ###P<0.001 from ET-1.
3.14 JCR rats: characteristics of the heart and adipokines released by adipose tissue in both lean and obese rats

The obese JCR adipocytes from epididymal region were bigger in size compared to lean JCR adipocytes (Figure 17A) and quantification data showed significant ($P < 0.01$) difference in the adipocyte size in obese JCR rats compared to lean JCR rats (Figure 17B) (Figure of adipocyte was kindly provided by Dr. Spencer Proctor, the University of Alberta).

The characteristics of these JCR rats showed that obese rats had significantly ($P < 0.001$) higher body weight (BW) and significantly lower heart weight (HW) ($P < 0.001$) than lean JCR rats (Figure 18). Left ventricular weight (LVW) had no significant effect in both lean and obese JCR rats.

Original concentration of ACM (200 mg/mL) from JCR (lean and obese) rat’s adipose tissue was used to determine the adipokines release. Leptin levels were significantly ($P < 0.0065$) higher, adiponectin levels were significantly ($P < 0.0001$) lower and leptin and adiponectin ratio (L/R ratio) was significantly higher ($P < 0.0065$) in the ACM from obese JCR rat’s adipose tissue compared with ACM from lean JCR rat’s adipose tissue (Figure 19). Other adipokines were at similar concentration in both lean and obese JCR ACM (Figure 20).
Fig. 17. JCR obese rat’s epididymal adipocytes have significantly bigger area than JCR lean rat’s epididymal adipocytes. Panel A and B shows representative images (20X magnification) of adipocytes and relative quantification of adipocyte area of lean and obese JCR rat’s epididymal adipose tissue, respectively. Histological slides were stained with hematoxylin and eosin stain (H&E stain). Image J was used to trace seven adipocytes on each section. Total area was analyzed and then divided by seven to get average adipocyte area. Values indicate mean ± SEM (n=3). **P< 0.01 lean JCR rats.
Fig. 18. JCR obese rats show significant increase in body weight compared with JCR lean rats. Panel A shows body weight, panel B shows HW, panel C shows LV weight, panel D shows HW/BW and panel E shows LVW/HW. Values indicate mean ± SEM (n=6). *P<0.05 & ***P<0.001 from JCR lean rats.
Fig. 19. Leptin and adiponectin levels are significantly higher in the ACM from obese JCR rat’s adipose tissue. The panel A shows leptin concentration, panel B shows adiponectin concentration and panel C shows leptin/adiponectin ratio in the ACM released by lean and obese JCR rat’s adipose tissue. Values indicate mean ± SEM (n=6). **P<0.01 & ***P<0.001 from lean JCR rats
Fig. 20. There is no difference between ACM from lean and obese JCR rat’s adipose tissue in the levels adipokines (other than leptin and adiponectin). The graphs show the adipokines levels in both types of ACM from lean and obese JCR rat’s adipose tissue. Values indicate mean ± SEM (n=6)
3.15 Effect of heart failure on the ability of ACM to inhibit ET-1-induced cardiomyocyte hypertrophy

ACM was obtained from both Sham and CAL rat’s adipose tissue. Cardiomyocytes were treated with or without ET-1 in the presence of different dilutions of ACM (20x10^8, 1.25x10^8 and 1 (original concentration)) from both rats and hypertrophic assessment was carried out. ET-1-induced significantly increased cell surface area ($P < 0.001$) had significantly decreased as a result of high concentration of ACM from Sham rat’s adipose tissue (1.25x10^8 and 1) ($P < 0.001$) (Figure 21A). Lower concentration of ACM (20x10^8) did not show any significant difference compared with ET-1. Similarly, $\alpha$-Skeletal actin and ANP gene expression was significantly up-regulated by ET-1 ($P < 0.05$) which was attenuated by high concentrations of ACM from Sham rat’s adipose tissue (1.25x10^8 and 1) ($P < 0.05$) (Figure 21B and 21C). ACM from adipose tissue obtained from the rats subjected to CAL had no effect on ET-1 induced hypertrophy.
Fig. 21. ACM from CAL rat’s adipose tissue does not attenuate ET-1-induced cardiomyocytes hypertrophy. Panel A shows cardiomyocyte cell surface area treated in the presence or absence of ET-1 and with the treatment of different dilution of ACM obtained from the adipose tissue of CAL rats and Sham rats for 24 h. Panel B and panel C show fetal gene (α-Skeletal actin and ANP respectively) expression on cardiomyocytes treated in the presence or absence of ET-1 and with the treatment of ACM obtained from Sham and CAL rats adipose tissue for 24 h. Values indicate mean ± SEM (n=6). *P<0.05 & ***P<0.001 from untreated cardiomyocytes and #P<0.05 & ###P<0.001 from ET-1. (CAL-coronary artery ligated)
3.16 Heart failure rats: characteristics of the heart and adipokines released by adipose tissue in both Sham and CAL rats

The characteristics of heart failure rats showed that Sham and CAL rat were not significantly different with each other in either BW, HW or LVW (Figure 2).

Original concentration (200 mg/mL) of ACM from Sham and CAL rat’s adipose tissue was used to determine the adipokines release. The analysis showed that leptin and adiponectin were at identical levels in both types of ACM (Sham and CAL rat’s adipose tissue) and L/A ratio was also not different in both types of ACM (Figure 23). Similarly, there was not any significant difference among all the other adipokines (Figure 24).
Fig. 22. There is no change in the characteristics of the BW, HW and LVW in both Sham and CAL rats. The graphs show the characteristics of heart failure rats (Sham and CAL). Panel A, B, C, D, E show body weight, HW, LVW, HW/BW and LVW/BW, respectively. Values indicate mean ± SEM (n=4-5). *P<0.05 from Sham rats.
Fig. 23. There is no significant difference between the ACM from both Sham and CAL rat’s adipose tissue in the levels of leptin and adiponectin release. Panel A shows leptin concentration, panel B shows adiponectin concentration and panel C shows leptin/adiponectin ratio in both Sham and CAL rats ACM. Values indicate mean ± SEM (n=6-7).
Fig. 24. There is no significant difference in the levels of adipokines release between both types of ACM form Sham and CAL rat’s adipose tissue. The panels show the adipokines levels in the ACM from Sham and CAL rat’s adipose tissue. Values indicate mean ± SEM (n=6-7)
3.17 ACM increases AMPK activation

To determine the signalling mechanism associated with anti-hypertrophic effect of ACM we focused on the signalling mechanism associated with adiponectin as it has been shown to attenuate cardiomyocytes hypertrophy. We focused on AMPK signalling mechanism. To assess the effect of ACM on p-AMPK (62kDa) we treated cardiomyocytes with or without ET-1 in the presence or absence of different dilution of ACM for 24 hours. ET-1 did not have any effect on p-AMPK protein expression (Figure 25). High concentrations of ACM (2.5x10^8 and 1.25x10^8) increased the p-AMPK protein expression significantly (P < 0.05) compared with ET-1. ACM-induced activation of AMPK was in a concentration-dependent manner.
Fig. 25. ACM increases p-AMPK protein expression in a concentration-dependent manner. Panel above illustrates western blots for p-AMPK (62kDa) and total AMPK (62kDa) protein expression with relative quantification data. Cardiomyocytes were treated with or without ET-1 in the presence or absence of varying dilutions of ACM and ARA (1000 ng/mL) for 24 h. Values indicate mean ± SEM (n=5-7). #P<0.05 from ET-1.
3.18 AdipoR1 blockade attenuates ACM-induced activation of AMPK

In order to assess the role of AdipoR1 in ACM-induced activation of AMPK, cardiomyocytes were treated with or without ET-1 in the presence or absence of ACM (1.25x10^8) and ARA (1000 ng/mL) for 24 hours. ACM increased the p-AMPK protein expression in the presence or absence of ET-1 significantly (P < 0.05) compared with ET-1 and untreated cardiomyocytes, respectively (Figure 26). In the presence of ARA and ACM, p-AMPK protein expression did not show any significant change from untreated cardiomyocytes. Similarly, Cardiomyocytes treated with ARA alone did alter the basal level of p-AMPK.
Fig. 26. AdipoR1 blockade decreases ACM-induced increase in p-AMPK protein expression. Panel above shows western blot of p-AMPK and total AMPK with respective quantification graph. Cardiomyocytes were treated in the presence of ET-1 and with and without ACM and AdipoR1 antibody (ARA- 1000 ng/mL) for 24 h. Values indicate mean ± SEM (n=6). *P<0.05 from untreated cardiomyocytes and #P<0.05 from ET-1.
3.19 Compound C (C.C) attenuates AICAR-induced anti-hypertrophic effect in cardiomyocytes

To determine the role of AMPK in the anti-hypertrophic effect of ACM we blocked the AMPK activity by using C.C which is a potent and selective AMPK inhibitor. We used four different concentration of C.C (0.1 µM, 1 µM, 5 µM and 10 µM) to determine the concentration response in ACM-induced anti-hypertrophic effect in cardiomyocytes. To up-regulate AMPK activation we used AICAR which is a known potent activator of AMPK and our lab has demonstrated that 1mM AICAR increased AMPK activity significantly (Javadov et al. 2009). Cardiomyocytes treated in the presence or absence of ET-1, AICAR and C.C for 24 hours. The cells were then subjected to hypertrophic assessment. ET-1 significantly increased cell surface area and α-Skeletal actin gene expression ($P < 0.001$ and 0.01, respectively) which was significantly attenuated by AICAR ($P < 0.001$ and 0.01, respectively) (Figure 27A and 27B). C.C attenuated the anti-hypertrophic effect of AICAR in a concentration-dependent manner. 10 µM concentration of C.C completely attenuated the anti-hypertrophic effect of AICAR which was evident by cell surface area analysis and α-Skeletal actin gene expression. Other concentrations of C.C did not show any significant change on α-Skeletal actin gene expression. Cardiomyocytes treated with 10 µM of C.C alone did not show any effect compared with untreated cardiomyocytes.
Fig. 27. Anti-hypertrophic effect of AICAR is attenuated by C.C. Panel A and B shows cardiomyocyte cell surface area analysis and α-Skeletal actin gene expression, respectively. Cardiomyocytes were treated with or without ET-1 in the presence or absence of AICAR (1 mM) and different concentrations of C.C (0.5-10 µM) for 24 hours. Values indicate mean ± SEM (n=6-8). *P<0.05, **P<0.01 & ***P<0.001 from Control and #P<0.05, ##P<0.01 & ###P<0.001 from ET-1.
Chapter 4: Discussion
4 Discussion

Annually, the number one cause of death worldwide is CVD (WHO 2011). Cardiovascular disease encompasses a multitude of pathological features. One of those features, which the focus of this study is dedicated to, is cardiac hypertrophy. Cardiac hypertrophy is a well-known risk factor for cardiovascular diseases such as heart failure, angina pectoris, arrhythmia and cardiac sudden death (Levy et al. 1990). Obesity is a disorder that accompanies many cardiovascular diseases. It is a common risk factor for, and thus often seen, in hypertension (Lauer et al. 1992), cardiac hypertrophy (Turkbey et al. 2010) and heart failure (Kenchaiah et al. 2002). Adipose tissue is distributed across different regions of the body, the major sites include: the visceral (epididymal, perirenal and mesenteric), epicardial and subcutaneous regions. Excessive accumulation of adipose tissue in these regions leads to obesity. This excess in adipose tissue is reported to be directly involved in facilitating CVD (Chess and Stanley 2008). Adipose tissue, after the discovery of leptin (Zhang et al. 1994), gained popularity as an endocrine organ which synthesises and releases adipokines. Adipokines include pro-inflammatory and anti-inflammatory factors (Ouchi et al. 2003, Berg and Scherer 2005, Zoccali et al. 2005). Studies have shown that these adipokines play a critical role in modulating homeostasis in an autocrine, paracrine and endocrine manner. These adipokines exert their effects on energy metabolism, the immune system, insulin sensitivity and inflammation (Trayhurn and Wood 2004, Otero et al. 2005). Two particular adipokines, leptin and adiponectin, are known to regulate cardiac function by being functionally antagonistic to one another: leptin is a hypertrophic (Rajapurohitam et al. 2003, Zeidan et al. 2008) agent whereas adiponectin is an anti-hypertrophic agent (Shibata et al. 2004, Rajapurohitam et al. 2013). The interplay between these two adipokines may represent the link between obesity and heart failure. The presumption is that increased levels of adipocyte-derived leptin and decreased levels of adipocyte-derived adiponectin promote cardiac hypertrophy – with eventual progression to heart failure.

Preventing cardiovascular implications is a global concern. Interaction of adipose tissue and the heart may allow researchers to target CVDs and explore new therapeutic approaches. Based on this hypothesis this thesis delves into the potential interaction
between adipokines and cardiomyocytes. We mimicked the pathological condition of the body where adipose tissue affects the heart via autocrine and paracrine mechanisms by using in vitro experiment techniques. To observe the interaction between adipose tissue and the heart we subject cardiomyocytes to ACM and assessed the degree of cardiomyocyte hypertrophy induced by ET-1.

4.1 The effect of ACM on ET-1-induced cardiomyocyte hypertrophy

Cardiac hypertrophy is characterized by increases in the cardiomyocyte cell surface area, enhanced rate of protein synthesis (Sugden and Clerk 1998) and re-expression of the fetal genes β- MHC, α-Sk.actin and ANP (Hefti et al. 1997). ET-1 is a vasoconstrictor hormone and it is not only produced by vascular endothelial cells (Yanagisawa et al. 1988) but also by cardiomyocytes (Nunez et al. 1990, Suzuki et al. 1993, Yamazaki et al. 1996), cardiac fibroblasts (Fujisaki et al. 1995) and macrophages (Fukuchi and Giaid 1998). The levels of ET-1 are elevated during obesity and cardiac hypertrophy (Barton et al. 2003). ET-1 acts via the ET_A and ET_B receptors. ET_A receptor is widely expressed by myocardium (Molenaar et al. 1993) and by cardiomyocytes (Ito et al. 1993). Production and expression of the ET-1 receptor by cardiac cells suggests the autocrine and paracrine role of ET-1 on the regulation of cardiac functions. Because of this positive association between plasma levels of ET-1 during obesity and cardiac hypertrophy, ET-1 was used in these cardiomyocyte in vitro experiments to induce cardiac hypertrophy and to study the modulation of this cardiac hypertrophy by adipose tissue. Studies, including those from our laboratory, have shown that a 10 nM concentration of ET-1 significantly induces hypertrophy in neonatal rat cardiomyocytes and it was marked by a significant increase in cardiomyocyte cell surface area and fetal gene (ANP and α.Skeletal actin) expression (Ito et al. 1991, Rajapurohitam et al. 2006, Kilic et al. 2010, Zeidan et al. 2014). Our results show that treatment of cardiomyocytes with a lower dilution of ACM (1.25 \times 10^8) completely attenuates cell surface area and increases fetal gene expression significantly. This suggests an anti-hypertrophic effect of ACM on ET-1-induced hypertrophy, an effect which is attributed to the presence of anti-hypertrophic adipokines. However, a higher dilution of ACM (20 \times 10^8) did not show
any effect which suggests the absence of anti-hypertrophic adipokines in this higher dilution ACM.

4.1.1 Role of leptin and OB-Rb in the anti-hypertrophic effect of ACM on cardiomyocytes

It is well documented that leptin is produced by adipose tissue (Scherer et al. 1995). Plasma levels of leptin are proportionate to adiposity thus high leptin levels are observed during obesity and the increased levels are also associated with increased congestive heart failure risk (Lieb et al. 2009). Leptin levels have been linked with increased risk of numerous CVDs suggesting the role of leptin levels as a marker for diagnosis of CVDs (Schulze and Kratzsch 2005). Leptin is also produced by the heart and cardiomyocytes. Furthermore, leptin receptors are expressed by the heart and cardiomyocytes (Purdham et al. 2004, Rajapurohitam et al. 2006) suggesting an effect of leptin on the heart through autocrine/paracrine mechanisms and through the endocrine system. Our study show that by-blocking the OB-Rb receptor using LRA, the anti-hypertrophic effect of ACM becomes more pronounce and effect of ACM is concentration-dependent. In addition, the result from both cell surface area and fetal gene expression shows that the concentration response curve shifts to the left side in the presence of LRA suggesting that the ACM’s anti-hypertrophic property is now more potent and cardiomyocytes are more sensitive to the effect of ACM. Analysis of EC50 suggests that the anti-hypertrophic effect can be achieved with a high dilution of ACM. This may suggest critical involvement of the leptin receptor in the anti-hypertrophic response of ACM. This result is in agreement with studies from our laboratory, displaying in vitro and in vivo data, which demonstrates the cardio-protective effect of blocking the OB-Rb receptor in the presence of leptin (Rajapurohitam et al. 2006, Zeidan et al. 2006, Purdham et al. 2008). ACM down-regulates leptin and OBRb gene and protein expression whereas ET-1 up-regulates leptin and OBRb gene and protein expression in cardiomyocytes.
4.1.2 Role of adiponectin and AdipoR1 in the anti-hypertrophic effect of ACM on cardiomyocytes

Adiponectin is a hormone produced by adipose tissue (Scherer et al. 1995) and it is the most abundant circulating plasma protein in healthy individuals (Arita et al. 1999). It possesses anti-inflammatory, (Ouchi et al. 2003) anti-tumor (Brakenhielm et al. 2004), anti-angiogenesis (Brakenhielm et al. 2004) and insulin sensitizing functions (Hotta et al. 2000). Reduced adiponectin levels are observed during obesity (Arita et al. 1999), coronary artery disease (Nakamura et al. 2004) and in heart failure patients (Takano et al. 2009). Plasma adiponectin levels are significantly up-regulated during weight loss in type 2 diabetes mellitus and in healthy subjects (Hotta et al. 2000). This suggests an inverse relationship between adiposity and adiponectin. Adiponectin has been known to regulate homeostasis because of its wide variety of functions in other organs including improving insulin sensitivity in the liver (Nawrocki et al. 2006) and fatty acid oxidation in skeletal muscle (Fruebis et al. 2001).

Adiponectin plays a cardio-protective role which is evident from a number of studies. A study on adiponectin deficient mice demonstrated that exogenous adiponectin administration protected the heart from myocardial apoptosis, increased infarct size and ischemic perfusion injury (Shibata et al. 2005, Tao et al. 2007). Another study demonstrated that adiponectin knockout mice showed significant cardiac remodelling induced by pressure overload on the heart compared with wild type mice (Liao et al. 2005). These studies suggest an anti-hypertrophic role for adiponectin. Neonatal cardiomyocytes express more AdipoR1 receptor than AdipoR2 receptor (Palanivel et al. 2007). The protective effect of adiponectin against ischemic reperfusion injury is mediated through the AdipoR1 receptor (Wang et al. 2010). Adiponectin has also been shown to protect rat embryonic cardiac myoblasts (H9c2) from hypoxia/reoxygenation injury via AdipoR1-mediated signaling (Park et al. 2011). A recent study shows that adiponectin protects atrial cardiomyocytes from Ang II-induced hypertrophy which was found to be mediated by the AdipoR1 receptor (Cao et al. 2014). Our results show that in the presence of ARA the concentration response curve of the ACM shifted to the right.
side which indicates a decrease in potency of the ACM in cardiomyocytes resulting in a desensitization of the cardiomyocytes to the anti-hypertrophic effect of the ACM. Which also displays that for the same anti-hypertrophic effect of ACM, a greater amount of ACM is needed in the presence of ARA. This result suggests that the anti-hypertrophic effect of ACM is dependent on signalling through the AdipoR1 receptor. ACM up-regulates adiponectin and AdipoR1 gene and protein expression during ET-1-induced hypertrophy and this up-regulation in gene and protein expression contributes towards the anti-hypertrophic effect of the ACM.

4.1.3 Levels of adipokines in ACM

Analysis of the ACM showed that adiponectin was secreted abundantly and concentration of leptin was ~3.5 ng/mL and adiponectin was ~1.2 µg/mL. This analysis of levels of adipokines in the ACM would help us to better appreciate the ACM obtained from the adipose tissue undergoing a pathological condition.

4.1.4 Molecular weight of anti-hypertrophic adipokines

Narrowing down the molecular weight range for the anti-hypertrophic effect of ACM lead us to filter the ACM and the results showed that the anti-hypertrophic adipokines were present between the molecular weight range of 3-30kDa. This suggests the possibility of involvement of leptin and adiponectin for the anti-hypertrophic effect. Results from the boiled medium showed no effect on cardiomyocytes with or without ET-1 which confirmed the involvement of proteins in the anti-hypertrophic effect of the ACM.

4.1.5 Effect of adipose tissue from different region

Studies have reported that adipose tissue from different regions of the body have different effects. There is a positive relation between abdominal obesity and increased likelihood of hypertension (Cassano et al. 1990), obstructive sleep apnea (Schafer et al. 2002) and type 2 diabetes mellitus (Chan et al. 1994). One study observed that increased adipose tissue around the legs was protective against impaired glucose levels (Snijder et
These studies suggest that different regions of adipose tissue may have different effects. Therefore we hypothesized that adipose tissue from epididymal and perirenal regions will exert different effects on neonatal rat cardiomyocyte hypertrophy. Results from our study showed that the ACM, regardless of the particular body region the adipose tissue was obtained from, exerted identical anti-hypertrophic effects. This result was analyzed by the hypertrophic assessment of both types of ACM during ET-1-induced cardiomyocytes hypertrophy. This suggests that the adipose tissue obtained from different regions of the body contains the same anti-hypertrophic adipokines and that the ACM remains cardiomyocyte-protective irrespective of the particular body region the adipose tissue was resected from.

4.1.6 Effect of ACM obtained from adipose tissue during pathological condition

Our aim was to better understand the ACM response and adipokine profile obtained from the adipose tissue of an animal in a pathological state. We hypothesize that the adipose tissue taken from an animal undergoing a pathological condition will modulate the hypertrophic response of ET-1. In addition we hypothesize that the levels of adipokines will also change. To explore this hypothesis we used an obese animal model and heart failure animal model.

4.1.7 Role of ACM from obese rats

It is well known that obesity exacerbates several CVDs including cardiac hypertrophy (Turkbey et al. 2010) and heart failure (Kenchaiah et al. 2002). To study the link between obesity and cardiac hypertrophy and the involvement of adipose tissue we used JCR strain rats. JCR (Jcr:LA-cp) is a unique strain that was originated from LA/N strain and fifth backcross with LA/N-cp and breeding stock from NIH, Maryland was sent to JC russell’s lab at the University of Alberta. These rats carries the mutant autosomal recessive cp gene, homozygous (cp/cp) rats develop obesity, insulin resistance, hyperlipidemia, vasculopathy, and atherosclerosis and the rats that are homozygous (+/+ ) or heterozygous (+/- ) are lean and do not develop any metabolic disease (Russell
et al. 1998). These rats provide a suitable model to study obesity, insulin resistance and CVDs which is similar to humans (Russell and Koeslag 1990).

Our results showed that the ACM from JCR obese animals did not protect cardiomyocytes against the hypertrophic response induced by ET-1. In contrast, the ACM from JCR lean animals displayed an anti-hypertrophic effect. We believed that this effect is due to the alteration in the release of adipokines in the ACM (more leptin and less adiponectin). Analysis of released adipokines in the JCR obese ACM shows that leptin levels are significantly up-regulated and levels of adiponectin are significantly down-regulated compared with JCR lean ACM. This analysis supports the studies that show that obese adipose tissue releases less adiponectin (Arita et al. 1999) and more leptin (Maffei et al. 1995, Considine et al. 1996). This phenomenon can be explained by the adipose tissue remodelling that occurs during obesity (Jernas et al. 2006, Skurk et al. 2007). This remodelling involves increases in adipocyte size (which is evident by images of the epididymal adipose tissue from JCR obese rats), and an increase in the release pro-inflammatory adipokines such as leptin, TNF-alpha which is accompanied by a decrease in anti-inflammatory adipokines such as adiponectin (Jernas et al. 2006, Skurk et al. 2007). Increased numbers of macrophages in adipose tissue are also observed in obesity which releases significant amounts of TNF-alpha (Weisberg et al. 2003). TNF-alpha has inhibitory effects on the release of adiponectin (Hector et al. 2007). With more leptin and less adiponectin released by the obese ACM we expect to observe a pro-hypertrophic effect of obese ACM on cardiomyocytes. Interestingly we did not see any effect of obese ACM treated without any hypertrophic agonist. We believed that the reason behind inability of ACM to modulate the hypertrophic response is that, though the levels of leptin are up-regulated, they are not sufficient enough to induce hypertrophic response on its own. This elevation in the levels of leptin may contribute towards the hypertrophic effect of ET-1 in cardiomyocytes. The result from this study suggests that the protective effect of ACM on cardiomyocytes is blunted during obesity and the modulation of hypertrophic response by adipocytes is dependent on leptin/adiponectin ratio that is significantly high during obese condition.
4.1.8 Role of ACM from heart failure rats

Studies have shown that adipokines are a potential link between obesity and heart failure (Schulze et al. 2003, Ouchi et al. 2006, Frankel et al. 2009, Ebert and Fasshauer 2011). Leptin levels are elevated whereas adiponectin levels are decreased during heart failure (Chan et al. 2003, Takano et al. 2009). Results from cardiomyocytes treated with ACM derived from the adipose tissue of both sham and CAL rats demonstrated that ACM from adipose tissue obtained from CAL rats did not attenuate ET-1-induced cardiomyocyte hypertrophy and, interestingly, this effect of both ACM could be due to the alteration in the release of adipokines levels. A recent study from our laboratory shows that CAL induced significant increases in plasma levels of leptin but there was no change in adiponectin levels compared with sham (Gan et al. 2014) which suggests that the heart failure condition increases leptin levels which may be due to adipose tissue remodelling. We performed the analysis to identify the levels of adipokines and surprisingly, we did not observe any change in the adipokine profile and both types of ACM showed identical levels of leptin and adiponectin and also other adipokines in ACM obtained from adipose tissue between Sham and CAL heart failure rats. However, the leptin levels in ACM were almost ten-fold higher, and the adiponectin levels were about 5-fold higher in both Sham and CAL heart failure rats compared with normal, healthy rats - suggesting the remodelling of adipose tissue in heart failure causes more release of leptin and adiponectin. These results raise the possibility for the involvement of different adipokines other than leptin and adiponectin that are playing a protective role in ACM obtained from adipose tissue of Sham rats and this protective effect is lost in ACM obtained from adipose tissue of CAL rats. A recent study by Yi at al. shows that a newly discovered adipokine “C1q TNF related protein-3 (CTRP3)” protects heart failure mice from ventricular cardiac remodelling (Yi et al. 2012). The heart failure condition attenuated CTRP3 gene expressions in adipose tissue resulting in decreased CTRP3 levels in the plasma and injecting exogenous recombinant CTRP3 with an intraperitoneal pump restored the plasma level of CTRP3 leading to improved left ventricular ejection fraction (LVEF) and survival rate. It is reported that CTRP3 regulates adiponectin release from adipose tissue (Wolffing et al. 2008). This study
suggests that the heart failure condition remolds adipose tissue which suppresses the release of protective adipokines. This further suggests the protective effect of ACM from adipose tissue of sham rats is abrogated in ACM from adipose tissue of CAL rats and this might be due to a decrease in CTRP3. All in all, further analysis is required to confirm the involvement of other adipokines.

4.2 Role of AMPK during ACM-induced anti-hypertrophic effect

As we observed the anti-hypertrophic effect of ACM during ET-1-induced cardiomyocyte hypertrophy, we were determined to understand the signalling mechanism regulating the protective effect of ACM. Our primary focus was adiponectin-mediated signalling, as a number of studies have shown the cardio-protection effect of adiponectin via an AMPK signalling mechanism. Adiponectin activates AMPK and protected the heart from ischemic perfusion injury (Shibata et al. 2005). Adiponectin knockout mice were unable to prevent cardiac remodelling induced by pressure overload conditions and they showed low expression of p-AMPK protein expression compared with wild type mice (Shimano et al. 2010). It has been reported that AMPK activates PPAR alpha to inhibit cardiac hypertrophy by down-regulating ERK1/2 protein expression (Meng et al. 2011). In addition, it inhibits Ang II-induced nuclear factor kappaB (NF-kB) activation (Wang et al. 2010) and inhibits protein synthesis resulting in inhibition of cardiac hypertrophy (Li et al. 2007). A recent study shows that the anti-hypertrophic effect of adiponectin against Ang II is mediated by AdipoR1 and AMPK (Cao et al. 2014). Our results show that the ACM increases p-AMPK levels in cardiomyocytes which suggests the anti-hypertrophic effect of ACM involves an AMPK dependent signalling mechanism.

As the protective effect of ACM is through activation of AdipoR1, blocking AdipoR1 may suggest the causative or correlative role of AMPK. To further investigate the involvement of AdipoR1 we hypothesized that blocking AdipoR1 would also block the increase in p-AMPK protein expression. The protein expression of p-AMPK showed that ARA attenuated the ACM-induced up-regulation p-AMPK. This effect may be
responsible for the abolishment of the anti-hypertrophic effect caused by ARA. ET-1 and ARA treatment alone failed to down-regulate the basal level of p-AMPK level. It is possible that a basal level of p-AMPK is maintained in the cardiomyocyte for normal function.

To further investigate the causative or correlative role of AMPK we used C.C to block the AMPK activation completely. In contrast, we used AICAR to increase AMPK activity. AICAR is a 5-Aminoimidazole-4-carboxamide-1-β-d-ribofuranoside which is phosphorylated by adenosine kinase and converted into ZMP (AICA-riboside monophosphate) in the cytosol, which is an AMP analogue that acts as AMP and activates AMPK (Corton et al. 1995). Hypertrophic assessment shows that the AICAR treatment blocked the ET-1-induced hypertrophy in cardiomyocytes. 10 µM concentration of C.C completely blocked the AICAR effect. This result suggests that the AICAR treatment increased AMPK activation resulting in an anti-hypertrophic effect. This anti-hypertrophic effect was then blocked by C.C. The current study demonstrates that AMPK plays a causative role when AdipoR1 is blocked during ACM-induced anti-hypertrophy. This study proceeds forward to establish a causative role for AMPK by use of AICAR and C.C. Future studies include assessing protein expression of p-AMPK in cardiomyocytes treated with ACM in the presence or absence of C.C.

4.3 Significance and clinical perspective of the study

As CVDs are accountable for more deaths than any other diseases worldwide, researchers and pharmaceutical companies are searching for new therapeutic approaches for CVDs to reduce the mortality rate. One leading cause of CVDs is obesity and the consequent secretion of adipose tissue-derived cytokines (adipokines). These adipokines have been the focus of research in this field because of their modulatory effect on homeostasis. To date there are some adipokines including leptin and adiponectin that are known to regulate cardiac function and their plasma levels can be considered as a strong predictor for CVDs. In order to better understand the relationship between CVDs and adipokines, they are currently being studied both in vitro and in vivo. Our study provides direct interaction between adipokines and cardiomyocytes and our results could
explain the cardio-protective role of adipokines. Adiponectin has been the centre of research for the therapeutic approach to treating CVDs because of adiponectin’s cardio-protective effects through its receptors and AMPK signalling (Gu and Li 2012, Nanayakkara et al. 2012). Our results also suggest that the protective effect of ACM was due to an adiponectin-dependent effect mediated by AdipoR1 and activation of AMPK. The results of this thesis have been demonstrated in an in vitro system and further in vivo evidence is necessary to gain further confidence. The findings of the study may explain the role and involvement of adipokines in CVDs during physiological and pathophysiological conditions and targeting adiponectin, AdipoR1 or AMPK will be helpful for finding new therapeutic approaches for the treatment of CVDs.

4.4 Conclusion

The major conclusion that can be drawn from the results obtained in this study is that the ACM from the adipose tissue obtained from normal rats contains anti-hypertrophic properties against ET-1. This anti-hypertrophic effect of ACM is due to activation of AdipoR1 and the net anti-hypertrophic effect is dependent on the interaction of leptin and adiponectin in cardiomyocytes.

The pathological situation such as obesity leads to adipose tissue remodelling resulting in increases in pro-inflammatory adipokine release and decreases in anti-inflammatory adipokine release from the tissue. Thus the anti-hypertrophic effect of ACM from adipose tissue obtained from JCR obese rats is abrogated due to significant up-regulation in leptin levels and down-regulation in adiponectin levels compared with ACM from adipose tissue obtained from JCR lean rats.

The signalling mechanism for the anti-hypertrophic effect of ACM is dependent upon up-regulation of p-AMPK though AdipoR1 signalling.

The findings from this study may explain the beneficial effect of staying within the normal and healthy weight range and the adverse effect of being overweight and obese.
References


ventricular hypertrophy in severe obesity: interactions among blood pressure, nocturnal hypoxemia, and body mass." Hypertension 49(1): 34-39.


cardiomyocytes and upregulated by activation of peroxisome proliferator-activated receptor gamma." J Mol Cell Cardiol 43(1): 73-84.


from pancreatic islet beta cells at high glucose concentrations." Endocrine 30(2): 217-221.


right patient at the right time: access to heart failure care." Can J Cardiol 22(9): 749-754.


ischemia-reperfusion injury through AMPK- and COX-2 dependent mechanisms."


196. Statistics (Canada (2011c, October)). "Mortality, summary list of causes, 2008."


## Appendices

### Appendix 1: Tables

**Table 1:** Composition of culture medium reagents for neonatal cardiomyocytes

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Concentration</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F12</td>
<td>1 pkg</td>
<td>Gibco (Life Technologies Burlington, ON, Canada)</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.4 mg/mL</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>10%</td>
<td>Gibco (Life Technologies Burlington, ON, Canada)</td>
</tr>
<tr>
<td>HEPES</td>
<td>30 mM</td>
<td>Gibco (Life Technologies Burlington, ON, Canada)</td>
</tr>
<tr>
<td>Bromodeoxyuridine</td>
<td>0.1 mM</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>5 µg/mL</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>Pyruvic Acid</td>
<td>3 mM</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>2 mg/mL</td>
<td>Gibco (Life Technologies Burlington, ON, Canada)</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>1%</td>
<td>Gibco (Life Technologies Burlington, ON, Canada)</td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td>10 µg/mL</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td><strong>Holo-Transferrin</strong></td>
<td>10 µg/mL</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td><strong>Sodium Selenite</strong></td>
<td>10 ng/mL</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td><strong>L-Ascorbic Acid</strong></td>
<td>100 µM</td>
<td>Gibco (Life Technologies Burlington, ON, Canada)</td>
</tr>
<tr>
<td><strong>MEM Non-essential Amino Acids</strong></td>
<td>1%</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td><strong>MEM Vitamins 100X</strong></td>
<td>0.1 %</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
</tbody>
</table>
Table 2: Composition of PBS-ABC solution for neonatal cardiomyocytes.

All chemicals are from Sigma-Aldrich (Oakville, ON, Canada).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Chemical</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS ABC</td>
<td>PBS A</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td>PBS B</td>
<td>10%</td>
</tr>
<tr>
<td></td>
<td>PBS C</td>
<td>10%</td>
</tr>
<tr>
<td>PBS A</td>
<td>NaCl</td>
<td>10 g/L</td>
</tr>
<tr>
<td></td>
<td>KCl</td>
<td>0.250 g/L</td>
</tr>
<tr>
<td></td>
<td>Na$_2$HPO$_4$.7$\text{H}_2$O</td>
<td>2.71 g/L</td>
</tr>
<tr>
<td></td>
<td>KH$_2$PO$_4$</td>
<td>0.250 g/L</td>
</tr>
<tr>
<td>PBS B</td>
<td>CaCl$_2$.2$\text{H}_2$O</td>
<td>1.32 g/L</td>
</tr>
<tr>
<td>PBS C</td>
<td>MgCl$_2$.6$\text{H}_2$O</td>
<td>2.13 g/L</td>
</tr>
</tbody>
</table>
Table 3: Serum-free medium reagents for neonatal cardiomyocytes

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Concentration</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM/F12</td>
<td>1 pkg</td>
<td>Gibco (Life Technologies Burlington, ON, Canada)</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.4 mg/mL</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>Bromodeoxyuridine</td>
<td>0.075 mM</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>1%</td>
<td>Gibco (Life Technologies Burlington, ON, Canada)</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>1%</td>
<td>Gibco (Life Technologies Burlington, ON, Canada)</td>
</tr>
<tr>
<td>Insulin</td>
<td>10 µg/mL</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>Holo-Transferrin</td>
<td>5 µg/mL</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>Sodium Selenite</td>
<td>10 ng/mL</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>Fetuin</td>
<td>250 µg/mL</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>80 µg/mL</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>L-Ascorbic Acid</td>
<td>100 µM</td>
<td>Gibco (Life Technologies Burlington, ON, Canada)</td>
</tr>
</tbody>
</table>
MEM Non-essential Amino Acids 1% Sigma-Aldrich (Oakville, ON, Canada)

MEM Vitamins 100X 0.1% Sigma-Aldrich (Oakville, ON, Canada)

BSA-Palmitate 0.2% Sigma-Aldrich (Oakville, ON, Canada)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen, type I from rat tail</td>
<td>60%</td>
</tr>
<tr>
<td>10 x DMEM F-12 medium</td>
<td>10%</td>
</tr>
<tr>
<td>*Reconstructive Buffer</td>
<td>10%</td>
</tr>
<tr>
<td>Sterile Water</td>
<td>20%</td>
</tr>
</tbody>
</table>

This mixture is kept on ice as it solidifies at room temperature

*Reconstructive Buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₂</td>
<td>2.2%</td>
</tr>
<tr>
<td>HEPES</td>
<td>4.7%</td>
</tr>
<tr>
<td>NaOH</td>
<td>0.05 M</td>
</tr>
</tbody>
</table>
Table 5: The table summarizes the chemical concentration and manufacturer.

<table>
<thead>
<tr>
<th>Chemical name</th>
<th>Concentration</th>
<th>manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>10 nM</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
<tr>
<td>LRA</td>
<td>0.01 nM – 10 nM</td>
<td>Protein Laboratories Rehovot (Rehovot, Israel)</td>
</tr>
<tr>
<td>ARA</td>
<td>1 ng/mL – 1000 ng/mL</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA, USA)</td>
</tr>
<tr>
<td>AICAR</td>
<td>1 mM</td>
<td>Cell Signaling Technology (Danvers, MA, USA)</td>
</tr>
<tr>
<td>C.C</td>
<td>0.5 µM – 10 µM</td>
<td>Sigma-Aldrich (Oakville, ON, Canada)</td>
</tr>
</tbody>
</table>

Table 6: Mixture composition for reverse transcription

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/reaction (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x Random Primer</td>
<td>1</td>
</tr>
<tr>
<td>5mM dNTP mix</td>
<td>2</td>
</tr>
<tr>
<td>5x First Stand Buffer (FSB)</td>
<td>4</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>2</td>
</tr>
<tr>
<td>MLV-RT</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 7: Real-Time PCR reaction mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/reaction (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EvaGreen qPCR Mastermix</td>
<td>5</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.5</td>
</tr>
<tr>
<td>cDNA</td>
<td>2</td>
</tr>
<tr>
<td>Double Distilled Water</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 8: Primer sequences and real-time RT-PCR conditions.

<table>
<thead>
<tr>
<th>Primers</th>
<th>PCR parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phase</td>
<td>Temp. °C</td>
</tr>
<tr>
<td>TPT1</td>
<td>Denaturation</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>72</td>
</tr>
<tr>
<td>Forward:5’-GGAGGGCAAGATGGTCAGTA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse:5’-AGGCCTCTTTTTGTAAGCTG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANP</td>
<td>Denaturation</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>72</td>
</tr>
<tr>
<td>Forward:5’-CTGCTAGACCACCTGGAGGA-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse:5’-AAGCTGTGTCAGCCTAGTCC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Primer (5′-3′)</td>
<td>Reverse Primer (5′-3′)</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>α-Skeletal actin</td>
<td>5′-CACGGCATTATCACCAACTG-3′</td>
<td>5′-CCGGAGGCATAGAGACAG-3′</td>
</tr>
<tr>
<td>Leptin</td>
<td>5′-TGATATCGCCAAACAGCAA-3′</td>
<td>5′-AGTGTCCGCTCTTTTGGA-3′</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>5′-AATCCTGCCCAGTCATGAAG-3′</td>
<td>5′-CCACAATGAGGCAGAGATG-3′</td>
</tr>
<tr>
<td>OBRb</td>
<td>5′-TGACCACTCCAGATTCCACA-3′</td>
<td>5′-CCACTGTTTTTCACGTTGCT-3′</td>
</tr>
<tr>
<td>AdipoR1</td>
<td>5′-TGGTCTTCGGGATGTTCTTC-3′</td>
<td>5′-CCACAATGAGGCAGAGATG-3′</td>
</tr>
</tbody>
</table>
Table 9: Composition of lysis buffer

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>50 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>Triton</td>
<td>1%</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10%</td>
</tr>
<tr>
<td>EDTA</td>
<td>2 mM</td>
</tr>
<tr>
<td>EGTA</td>
<td>2 mM</td>
</tr>
<tr>
<td>NaF</td>
<td>50 mM</td>
</tr>
<tr>
<td>Na₃V₀₄</td>
<td>200 µM</td>
</tr>
<tr>
<td>Na₄P₂O₇</td>
<td>10 mM</td>
</tr>
<tr>
<td>β-Glycerophosphate</td>
<td>40 mM</td>
</tr>
</tbody>
</table>
Table 10: Composition of buffers used in western blotting. The pH was adjusted to 7.5.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Running Buffer</th>
<th>Transfer Buffer</th>
<th>Wash Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>0.3%</td>
<td>0.4%</td>
<td>0.3%</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.44%</td>
<td>1.8%</td>
<td>---</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NaCl</td>
<td>---</td>
<td>---</td>
<td>0.9%</td>
</tr>
<tr>
<td>Tween20</td>
<td>---</td>
<td>---</td>
<td>0.1%</td>
</tr>
<tr>
<td>Methanol</td>
<td>---</td>
<td>25%</td>
<td>---</td>
</tr>
</tbody>
</table>
**Table 11: Details of antibodies, dilution ranges, types and manufacturers.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Type</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>leptin</td>
<td>1:500</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA, USA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>polyclonal</td>
<td></td>
</tr>
<tr>
<td>OBRb</td>
<td>1:1000</td>
<td>Mouse</td>
<td>Alpha Diagnostics International, (San Antonio, TX, USA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>monoclonal</td>
<td></td>
</tr>
<tr>
<td>Adiponectin</td>
<td>1:500</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA, USA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>polyclonal</td>
<td></td>
</tr>
<tr>
<td>AdipoR1</td>
<td>1:500</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA, USA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>polyclonal</td>
<td></td>
</tr>
<tr>
<td>α-AMPK</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>Cell Signaling Technology (Danvers, MA, USA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>polyclonal</td>
<td></td>
</tr>
<tr>
<td>AMPK</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>Cell Signaling Technology (Danvers, MA, USA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>polyclonal</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>1:1000</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA, USA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>polyclonal</td>
<td></td>
</tr>
</tbody>
</table>
Appendix 2: Animal use protocol approval from the Animal Use Subcommittee of the University Council on Animal Care

AUP Number: 2013-031
PI Name: Karmazyn, Morris
AUP Title: Heart failure

Official Notification of AUS Approval: A MODIFICATION to Animal Use Protocol 2013-031 has been approved.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
Curriculum Vitae
Name: Suresh Chandra Bairwa

Post-secondary Education and Degrees:

Rajasthan University of Health Sciences Jaipur, Rajasthan, India
2006-2010, B. Pharmacy

The University of Western Ontario
London, Ontario, Canada
2012-2014, M.Sc.

Honours and Awards:

Western Graduate Research Stipend
The University of Western Ontario
2012-2014

Schulich Graduate Scholarship
The University of Western Ontario
2012-2014

Poster Presentation Award in Circulatory and Respiratory Health
London Health Research Day, London, Ontario, Canada
2014, Cash $500

Related Work Experience:

Physiology 1021 Teaching Assistant
The University of Western Ontario
2014

Scientific Meetings Attended:

Charles W. Gowdey Lecture and Research Day
The University of Western Ontario
2013
London Health Research Day
London, Ontario, Canada
2014

James A.F. Stevenson Distinguished Lecture and Research Day
The University of Western Ontario
2014

Oral Cardiac Biology Journal Club
Preseantation: The University of Western Ontario
2014

Publications:
Xiaohong Tracey Gan, Venkatesh Rajapuhohitam, Cathy Huang, Jenny Xue, Suresh Chandra Bairwa, Jeffrey Tin-Yu Chow, Melissa Fung Wah Liu, Felix Chiu, Kay-Uwe Wagner1, Morris Karmazyn. Cardiac-specific excision of the jak2 gene causing cardiac hypertrophy, heart failure and mortality. Manuscript in preparation

Suresh Chandra Bairwa, Venkatesh Rajapurohitam and Morris Karmazyn. Modulation of the Cardiomyocyte Hypertrophic Responses to Endothelin-1 by Adipocytes. Manuscript in preparation