THE AKT/FOXO3A/ATROGIN-1 SIGNLAING PATHWAYS UNDERLYING
CARDIAC REGRESSION AFTER DETRAINING IN A MOUSE HEART.

by

Wanseok Lee, B.Ed.

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Approved

Eunhee Chung, PhD
Chair of Committee

Yoonjung Park, PhD

Joaquin U. Gonzales, PhD

Dominick Casadonte
Dean of the Graduate School

December, 2013

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ABSTRACT

Exercise-induced cardiac hypertrophy reverses after cessation of exercise training. The Akt/FoxO3a/Atrogin-1 pathways have been implicated as important players in skeletal and cardiac muscle atrophy. However, little is known about the role of these pathways in cardiac muscle regression after exercise cessation. Thus, the purpose of this study was to determine the activities of Akt, FoxO, and to measure the levels of the muscle-specific E3 ligase, Atrogin-1 in cardiac muscle which has undergone regression of mass after cessation of exercise training. Three to four month old male C57Bl/6 mice were randomly divided into six groups: sedentary controls, 21 days of exercise, and 4 different time points of detraining after 21 days of voluntary wheel running (ie. 3 days, 5 days, 14 days, and 21 days). Body weight was not significantly different among groups. Exercise training significantly increased the heart weight/body weight ratio compared to sedentary controls (13% increase in HW/BW), but this growth was regressed after 21 days of detraining. We found that phosphorylation of Akt normalized to total Akt was significantly increased in the exercise group, and also in the 3-, 5-, and 14 days of detraining groups, but was significantly decreased in the 21 days of detraining group compared to the sedentary control group. However, the phospho-FoxO3a to total FoxO3a ratio was not different among groups. Interestingly, the level of Atrogin-1 was significantly decreased in 14-, and 21 days of detraining groups. Our results suggest that cardiac regression following exercise cessation is not mediated by up-regulation of Atrogin-1. Taken together, this study demonstrates that cardiac regression occurs after 21 days of exercise cessation and it may be partially mediated by inactivation of Akt.

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CHAPTER I

INTRODUCTION

Heart disease is a major health problem and the leading cause of death in the United States. Approximately 6 million patients currently suffer from heart failure, and over 170 per 100,000 people are dying due to heart disease every year (Murphy, Xu, & Kochanek, 2012; Velez, Kohli, & Sabbah, 2013). Left ventricular hypertrophy (LVH) is a strong predictor of sudden death (Haider, Larson, Benjamin, & Levy, 1998; Kearney et al., 2004) and often leads to heart failure (Benjamin & Levy, 1999; Levy, Garrison, Savage, Kannel, & Castelli, 1990; Zhong et al., 2012). One third of the patients are dying within a year after diagnosis of heart failure, and the long term survival rates are very low (Bleumink et al., 2004; Cowie et al., 2000).

Currently, there is no effective cure for heart failure. It has been suggested that reduction of heart mass in patients with pathological cardiac hypertrophy may significantly diminish the risk of death and mortality (Devereux et al., 2004; Koren, Ulin, Koren, Laragh, & Devereux, 2002; Okin et al., 2004; Verdecchia et al., 2003). Thus, in the past, treatments for heart failure were focused on reducing heart muscle mass by blocking the progress of prohypertrophic factors with pharmacological interventions (Razeghi & Taegtmeier, 2006). However, prevention of prohypertrophic signaling to convert pathological hypertrophy does not significantly reduce the heart mass due to large excess of prohypertrophic signaling pathways (Frey, Katus, Olson, & Hill, 2004; Razeghi & Taegtmeier, 2006). It is believed that reversing cardiac hypertrophy is a better strategy compared to inhibiting prohypertrophic signaling since this method is not

affected by the presence of prohypertrophic molecules, and is able to rebalance the protein turnover rate (Razeghi & Taegtmeyer, 2006). Therefore, discovering regulators of cardiac atrophy in order to reduce cardiac hypertrophy could be an effective strategy in heart failure patients.

Cardiac hypertrophy can be subdivided into two different types, pathological and physiological cardiac hypertrophy. Cardiac hypertrophy induced by pathological stimuli increases heart size and mass with decreased cardiac function. According to early studies, it has been known that the pathological hypertrophy is not reversible (Donaldson et al., 1982; Krayenbuehl, Hess, Schneider, & Turina, 1983). However, some studies have demonstrated that the pathological hypertrophy can be reversible with improved patient outcomes (Frazier et al., 1996; Heerdt et al., 2000; Loebe, Müller, & Hetzer, 1999; Ogletree-Hughes et al., 2001). Thus, it is important to understand the roles of pro-atrophic genes that mediate cardiac regression.

The levels of pro-atrophic proteins have been measured and as a result, the pro-atrophic proteins and transcriptional genes have shown to play important roles in cardiac atrophy. As shown in Figure 1, forkhead box O (FoxO), a downstream target of Akt, is an important mediator of protein degradation and apoptosis (Accili & Arden, 2004; Skurk et al., 2005). The FoxO activity is down-regulated by activation of Akt, a well-known protein for increasing cardiac mass. Phosphorylation of Akt leads to phosphorylation of FoxO, and phosphorylated FoxO remains in the cytosol of cardiomyocyte and incapable of turning on the FoxO transcriptional factors (Schisler, Willis, & Patterson, 2008). Under atrophy conditions, such as starvation, FoxO is localized to the nucleus and

decreases cell size (Sengupta, Molkentin, & Yutzey, 2009) by activating transcriptional atrogene such as Atrogin-1.

Figure 1. Schematic model of addressed cardiac regression signaling pathway.

Atrogin-1 is cardiac- and skeletal- muscle specific F-box protein that negatively regulates pathologic and physiologic cardiac hypertrophy (Li et al., 2004; Li et al., 2007). Recent studies demonstrate that Atrogin-1 inhibits calcineurin-dependent pathological cardiac hypertrophy (Li et al., 2004) and also shows cardiac regression by disrupting the Akt pathways (Li et al., 2007). Abundance of Atrogin-1 in the mouse heart has shown to increase FoxO activation, that suppresses cardiac hypertrophy (Li et al., 2004). In physiological cardiac hypertrophy, Akt-dependent cardiac hypertrophy is prevented by Atrogin-1 via FoxO activation (Li et al., 2007). Taken together, activation of Atrogin-1 prevents cardiac hypertrophy by the increased activity of FoxO transcription factors.

While signaling pathways underlying cardiac hypertrophy have been extensively studied, those leading to cardiac regression are poorly understood. Previous studies demonstrate that regression of cardiac hypertrophy occurs during detraining (Mujika & Padilla, 2000a, 2000b). For example, after cessation of exercise training, increased heart

size and mass start to decrease and return back to normal range after three weeks of detraining (Mujika & Padilla, 2000a, 2000b). Although detraining is one of the important conditions that regulates cardiac remodeling, the signaling pathways of pro-atrophic proteins during cardiac muscle regression after exercise cessation has not been well understood.

Significance of the study

There is a great deal to be learned about the mechanisms of the cardiac regression following exercise-induced cardiac hypertrophy. Moreover, most of our understanding of signaling pathways leading to cardiac regression is derived from pathological models. In considering regression of pathological hypertrophy as a potential therapeutic approach, it is important to understand whether the signaling pathway that causes cardiac regression of physiological hypertrophy is distinct from regression of pathological hypertrophy.

Statement of the purpose

The Akt and FoxO/Atrogin-1 pathways have been implicated important players in skeletal and cardiac muscle atrophy in disease and fasting models (Arya et al., 2004; Gomes, Lecker, Jagoe, Navon, & Goldberg, 2001; Sandri et al., 2004). However, the role of these pathways in cardiac muscle regression following exercise cessation is not clearly understood. Thus, the purpose of this study is to determine the activities of Akt, FoxO, and Atrogin-1 in regressed cardiac muscle which is induced by cessation of exercise training.

Hypothesis

Detraining would activate FoxO and Atrogin-1 via inactivation of Akt in hearts of detraining mice.

1. I tested the hypothesis that the activity of Akt in LV samples would be decreased along with increase of detraining period.
2. I tested the hypothesis that the activity of FoxO in LV samples would be increased along with increase of detraining period.
3. I tested the hypothesis that the levels of the muscle-specific E3 ligase, Atrogin-1 in LV samples would be increased along with increase of detraining period.

Delimitations

1. This study delimited to the use of male C57Bl/6 mice, although, there is sex difference in cardiac hypertrophy and regression.
2. This study delimited to the use of different time points of detraining such as 3, 5, 14 and 21 days of detraining.
3. This study delimited to the use of western blot to analyze pro-atrophic proteins.

Definition of terms

Hypertrophy. Cardiac remodeling occurs when the work load of the heart changes. Cardiac hypertrophy is often referred as the increase size or muscle mass, and reported as increases in either absolute or relative heart weight, and heart weight normalized to body weight or tibial length (Yin, Spurgeon, Rakusan, Weisfeldt, & Lakatta, 1982). Some studies report the changes of the wall thickness from echocardiography

(Foppa, Duncan, & Rohde, 2005). Hypertrophy is subdivided into two different types: physiological and pathological cardiac hypertrophy. Physiological cardiac hypertrophy occurs as a result of development, exercise training, and pregnancy. Physiological hypertrophy is characterized by normal or enhanced cardiac function.

Pathological cardiac hypertrophy is resulted from several different stimuli such as; pressure overload, blood volume overload, inflammatory disease (myocarditis), myocardial infarction, and mutation of sarcomeric proteins (Hou & Kang, 2012; Kehat & Molkentin, 2010; Weeks & McMullen, 2011). In response to pathological stimuli, the heart initially becomes larger to compensate the workload, but often leads to cardiac dysfunction and heart failure. Moreover pathological hypertrophy is accompanied by interstitial fibrosis that contributes to mechanical stiffness of the heart and cardiac dysfunction (Hein et al., 2003).

Protein synthesis. Protein synthesis is the process that generates new proteins by the biological cells (Koshland Jr, 1958).

Proteolysis. Proteolysis, also known as protein degradation is the chemical reaction that breakdowns the peptide bond by hydrolysis. Protein breaks down into smaller polypeptides and amino acids by activation of protein digestive enzymes.

Intracellular proteins are constantly being broken down into amino acids (Goldstein, Brunschede, & Brown, 1975).

Ubiquitination (ubiquitylation). Ubiquitin is a small molecule which acts as a tag to transport the protein of interest to the proteasome machinery for degradation (Pickart, 2001). In ubiquitination, a protein is inactivated by attaching ubiquitin to it.

Phosphorylation. Phosphorylation is the post-translational modification, which

occurs by the attachment of a phosphate (PO_4^{3-}) group to a protein or other organic molecule. Many protein enzymes and receptors are turned on and off, and altering their function and activity by phosphorylation and dephosphorylation (Cieřła, Frączyk, & Rode, 2011). Kinases phosphorylate proteins and phosphatases dephosphorylate proteins from targeted molecules. Particularly, protein phosphorylation plays a significant role in a wide range of cellular processes.

Fibrosis. The amount of cardio myocytes starts to decrease and collagen accumulation takes place in the area of cardiomyocytes' death and reduces the contractility of the cardiac muscle (Hein et al., 2003).

Atrophy. Cardiac atrophy is defined as a decrease in heart muscle mass compare to the normal heart (Razeghi et al., 2006). Atrophy occurs in response to fasting, disease, prolonged bed rest, and microgravity (Burch, Phillips, & Ansari, 1968).

Cardiac regression. Increased heart weight and protein content of the heart start to decrease and return to initial base-line values after cessation of stimuli. Exercise-induced cardiac hypertrophy returned to normal sized heart after termination of exercise training, but further decrease in the heart size does not occur (Weiner et al., 2012).

Left ventricular assist device (LVAD). LVAD helps to reduce the load of pathologically hypertrophied heart by withdrawing blood from the left ventricle and returning it to the arterial system. This technique has been used to bridge patients who are waiting for heart transplantation (Zafeiridis, Jeevanandam, Houser, & Margulies, 1998). Although this technique is not intended to recover the heart failure, significant functional improvements have been observed in 5% of the patients so the heart transplantation was not needed any longer (Mancini et al., 1998).

Heterotopic heart transplantation. Heterotopic transplantation of the heart is one of the techniques to induce left ventricular unloading (Ono & Lindsey, 1969). This technique is used to develop cardiac atrophy. The weight of transplanted hearts decreases by 55% compared with control hearts, during the first two weeks of transplantation, and no further reduction occurs (Depre et al., 1998).

Transverse aortic constriction (TAC). Transverse aortic constriction (TAC) is a microsurgical technique that causes cardiac hypertrophy artificially by manipulating the blood pressure. In this technique, a stable 35- to 45-mmHg pressure gradient is applied across the thoracic aorta of the mouse. The aorta was constricted to 65-70% of normal vessel to cause pressure overload. The mouse heart was increased 33% after 7 days of TAC (Howard A Rockman et al., 1991). After four weeks, the TAC was released and the heart size returns to normal (Willis et al., 2009).

Atrogenes. Atrogenes are atrophy-regulating genes, and their expression increases during atrophy-related stimuli. Muscle Ring-finger protein-1 (MuRF-1) and Atrogin-1 are well known atrogenes, and play important roles in cardiac atrophy (Sandri et al., 2004).

Akt. Serine/threonine kinase (Akt), is a well characterized downstream target of phosphoinositide 3-kinase (PI3K) which plays a key role in physiological cardiac hypertrophy (DeBosch et al., 2006).

FoxO. Forkhead box O (FoxO) protein is a downstream target of PI3K/Akt signaling pathway that play a role in regulating cardiac mass and apoptosis (Skurk et al., 2005).

Atrogin-1. Atrogin-1 is F-box protein ubiquitin E3 ligases (MAFbx), but the

roles of Atrogin-1 in cardiac hypertrophy and regression has not been clearly identified. In several studies, overexpression of Atrogin-1 reduces cardiac hypertrophy (Li et al., 2004; Li et al., 2007; Schisler et al., 2008). In contrast, other studies show that during cardiac atrophy, Atrogin-1 activity is decreased (Razeghi et al., 2006; Razeghi et al., 2003).

Detraining. Detraining is the partial or complete cessation of exercise training. After exercise training, many physiological functions are improved as an adaptation. These enhanced anatomical, physiological, and performance are returned back to the basal levels after detraining (Mujika & Padilla, 2000a).

Calcineurin. The protein phosphatase which binds to the Z-disc in cardiomyocytes via α -actinin, promotes cardiac hypertrophy in response to numerous pathologic stimuli.

Abbreviations

Ang II, angiotensin II; Akt, Serine/threonine kinase; BV, Blood volume; CO, Cardiac output; EDV; End diastolic volume; ET-1, Endotheline-1; FoxO, Forkhead box O; GPCR, G protein-coupled receptors; HR, Heart rate; IGF, Insulin like growth factor; IGF1R, Insulin like growth factor 1 receptor; KO, Knockout; LV, Left ventricular; LVAD, Left ventricular assist device; MAFbx, F-box protein ubiquitin E3 ligases; NFAT, Nuclear factor of activated T-cells; PI3K, Phosphoinositide 3-kinase; SV, Stroke volume; TAC, Transverse aortic constriction; UPS, ubiquitin proteasome system.

CHAPTER II

REVIEW OF LITERATURE

Cardiac remodeling

Cardiac remodeling refers to the alteration of size, shape, and function of the heart. The heart has the ability to change size by either getting larger or becoming smaller, as a result of adaptation to environmental demands and response to stimuli. Hypertrophy, atrophy, and regression of the heart are examples of cardiac remodeling. **Cardiac**

hypertrophy. Hypertrophy is one of the common types of cardiac remodeling. Cardiac hypertrophy can be simply explained as the augmentation of the size and mass of the heart. Its functional, mechanical, and histological features are distinguishable from normal embryonic and postnatal myocardial growth by changes in cardiac myocyte shape and volume (Dorn II, 2007). The increased size and mass in hypertrophic heart is due to the greater protein synthesis rather than protein degradation in cardiomyocytes (Razeghi & Taegtmeier, 2006). At the cellular level, cardiac hypertrophy is the outcome of augmented cardiomyocyte volume (Li et al., 2007; Weeks & McMullen, 2011).

Hypertrophy has two different types: pathological and physiological hypertrophy. Cardiac hypertrophy that associated with cardiovascular disease or myocardial injury is called pathological hypertrophy. In contrast to pathological cardiac hypertrophy, physiological cardiac hypertrophy does not progress to cardiac dysfunction, and it returns to normal heart size following cessation of stimuli. Exercise-induced cardiac hypertrophy is one example of physiological hypertrophy. Physiological hypertrophic growth also occurs in untrained females during pregnancy (M. Eghbali et al., 2005), without any

myocardial injury. In these two conditions, the heart maintains normal or even enhanced cardiac contractile function. In addition to functional difference, there are differences in structural, metabolic, biochemical and molecular attributes between pathological and physiological hypertrophy. Pathological hypertrophy is associated with an increase in interstitial fibrosis from the accumulation of collagen deposition in the area of cell death. These structural changes cause a reduction of cardiac function. In contrast, physiological hypertrophy does not induce fibrosis and increases contractile capacity of the heart (Ritter & Neyses, 2003).

A normal heart mainly uses ATP, from fatty acid oxidation, to generate energy and approximately 30% of the ATP is synthesized from glucose and lactate metabolism (Van der Vusse, Glatz, Stam, & Reneman, 1992). In the change of workload and relative levels of fuel molecules, the heart can flexibly control the ratio of energy substrates (Van der Vusse et al., 1992). This adaptive mechanism is the key system that the heart can constantly maintain ATP supply, even under diverse physiological conditions. Pathological cardiac hypertrophy is associated with increased rates of glucose metabolism and decreased rates of fatty acid oxidation (Vedala et al., 2002). This may be a protective mechanism resulting in limitation of oxygen supply and blockage of the fatty acid utilization. Interestingly, pathological hypertrophy progresses, glucose metabolism also diminishes due to increased insulin resistance in heart. This results in metabolic impairment of the heart to generate sufficient amount of ATP (Neubauer, 2007). On the contrary, physiological cardiac hypertrophy induced by exercise training is associated with enhanced metabolic rate of fatty acid oxidation and glucose utilization (Gertz, Wisneski, Stanley, & Neese, 1988).

Numerous previous studies have shown that pathological and physiological cardiac hypertrophy have different biochemical and molecular features (M. Iemitsu et al., 2001; Ritter & Neyses, 2003). Myosin ATPase activity is highly correlated to the contractile properties, such as increased shortening velocity and power output (Herron & McDonald, 2002). A decrease in myosin ATPase activity is seen in pathological hypertrophy, while an increase in myosin ATPase activity is observed in physiological hypertrophy (Rupp, 1981). mRNA expression in response to pathological and physiological hypertrophy, shows that those two settings have a distinct gene expression profiles (Mansoureh Eghbali, Wang, Toro, & Stefani, 2006; M. Iemitsu et al., 2001). Pathological cardiac hypertrophy results in protein alterations and decreases the ratio of α - and β -myosin heavy chain (MHC). In addition, genes that are abundantly expressed in fetal heart (e.g. atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and α -skeletal actin) typically re-express in response to pathological stimuli, but not physiological stimuli. Moreover calcium-handling proteins (e.g. sarcoplasmic reticulum Ca^{2+} -ATPase 2a (SERCA2a)) decrease in response to pathological stimuli, while SERCA2a gene expressions in exercised (physiological hypertrophy) and sedentary controls are comparatively unchanged (Bucks et al., 2009; Danzi & Klein, 2002; Zhang et al., 2005).

Signaling pathways underlying physiological and pathological hypertrophy. Previous studies suggest that insulin like growth factor (IGF1)-phosphoinositide 3-kinase (PI3K, (p110 α))-serine/threonine kinase (Akt) pathways are important in physiological cardiac hypertrophy (Bernardo, Weeks, Pretorius, & McMullen, 2010; McMullen et al., 2007; McMullen et al., 2004b; T. Shioi et al., 2000).

Importance of IGF1 signaling in physiological cardiac hypertrophy has confirmed through several studies with transgenic mouse models. Mice express cardiac specific overexpression of IGF1 receptor (IGF1R) have greater heart size and enhanced systolic function at baseline (McMullen et al., 2004a). In addition, these mice have greater hypertrophic response after swim training compared to non-transgenic littermates (McMullen et al., 2004a). On the other hand, IGF1R gene knockout mice block to exercise-induced cardiac hypertrophy (Kim et al., 2008).

PI3K(p110 α) is a downstream enzyme of IGF1. PI3K has three different classes, class I, II, and III based on structure and substrate specificity (Kok, Geering, & Vanhaesebroeck, 2009). Each class is divided into many different catalytic subunits. Among these subunits, two isoforms of class I PI3K, p110 α and p110 γ are abundantly expressed in the heart (Crackower et al., 2002). The constitutively active PI3K(p110 α) mutant mice, which elevates activity of PI3K(p110 α) in the heart, develops physiological hypertrophy with maintained cardiac function (McMullen et al., 2003). Other transgenic studies show that reduced expression of PI3K(p110 α) activity induces a smaller heart mass compared with non-transgenic littermates (T. Shioi et al., 2000). In contrast to the PI3K(p110 α), activation of PI3K(p110 γ) does not affect the heart size, but it couples with G protein-coupled receptors (GPCRs) and deleteriously affect the heart function, such as a decrease in cardiac contractility (Gavin Y Oudit et al., 2004a). PI3K(p110 γ) knockout mice display enhanced contractile function (Crackower et al., 2002). Taken together, PI3K(p110 α) is the important regulator in exercise-induced physiological hypertrophy.

Akt, a serine/threonine kinase, is a well characterized target of phosphoinositide

3-kinase (PI3K). Akt plays a key role in cellular processes, including cell survival, cell cycle, metabolism and induction of protein synthesis (Bernardo et al., 2010). Akt has three isoforms (Akt1, Akt2 and Akt3) that are encoded by distinct genetic loci. In the heart, Akt1 and Akt2 are highly expressed but not Akt3 (Matsui & Rosenzweig, 2005). Akt is activated by phospholipid binding and phosphorylated at Thr-308 and Ser-473. The activity of Akt, commonly expressed as phosphorylated form of Akt normalized by total protein level (Ni et al., 2007; Skurk et al., 2005), and is increased by multiple physiological and pathological pro-hypertrophic stimuli. For example, the activity of Akt increases after swimming training (McMullen et al., 2003) and voluntary wheel running (Konhilas et al., 2004). However, the activity of Akt also increased by pathological stimuli, such as pressure overload (Skurk et al., 2005) and aortic-banding in mice (Ha et al., 2005). These results suggest that Akt activation seems likely involved in both physiological and pathological hypertrophy.

To clarify the roles of signaling molecules in physiological hypertrophy, a number of studies have been conducted on mouse models that express activated or inactivated forms of Akt. The results from mouse models that express inactivated forms of Akt1 and Akt2 demonstrate that Akt1 and Akt2 have distinct roles. For example, the results from Akt1 knockout mice demonstrate that Akt1 is essential for both fetal and postnatal growth (Cho, Thorvaldsen, Chu, Feng, & Birnbaum, 2001). Further, Akt1 knockout mice study shows that Akt1 is required for physiological rather than pathological cardiac hypertrophy. Akt1 knock-out mice have a blunted hypertrophic response to swim exercise training, but not pathological stimuli which is induced by pressure overload (DeBosch, Treskov et al., 2006). Cardiac specific constitutively active

Akt1 mice induces cardiac hypertrophy with preserved function (Shioi et al., 2002). On the other hand, results from Akt2 knockout mice suggest that Akt2 is important for insulin metabolism (Cho et al., 2001), but not essential for the development of cardiac hypertrophy (DeBosch, Sambandam, Weinheimer, Courtois, & Muslin, 2006). Another study of Akt2 knockout mice shows normal cardiac growth response to pressure overload by TAC provocative stimulation. These results implicate that Akt1 regulates cardiac cell growth whereas Akt2 is important for cardiac metabolism and cardiomyocyte survival (DeBosch, Sambandam et al., 2006; DeBosch, Treskov et al., 2006). Other signaling pathways include gp130/JAK/STAT pathway, thyroid hormone signaling, and heat shock transcription factor 1 (HSF1) are also associated with physiological cardiac hypertrophy (Franke, Kaplan, Cantley, & Toker, 1997; Ole Johan Kemi et al., 2008; O'Neill et al., 2007).

Pathological cardiac hypertrophy has distinct signaling pathways compared to physiological cardiac hypertrophy, which is able to be explained from the Gαq signaling pathway (downstream of G protein-coupled receptors (GPCRs) activated by angiotensin II (Ang II), endotheline-1 (ET-1) and catecholamines) (H.A. Rockman, Koch, & Lefkowitz, 2002; Yayama et al., 2004). Ang II and ET-1 are pro-hypertrophic hormones that are secreted from cardiac myocytes during pressure overload (Weeks & McMullen, 2011). Ang II and ET-1 binds to Gαq protein-coupled receptors (GPCR) and activate Gαq protein. Mice that express the constitutive active mutant of Gαq protein develop significant pathological cardiac hypertrophy and cardiac dysfunction (D'Angelo et al., 1997; Mende et al., 1998). On the other hand, mice with cardiac specific inhibition of Gαq signaling blocks pressure overload-induced hypertrophy (Thomas et al., 2002). Gαq

regulates pathological cardiac hypertrophy via downstream effector such as calcineurin (Mende et al., 1998). Calcineurin is a Ca^{2+} -dependent phosphatase that regulates hypertrophic gene by activating transcription factors such as nuclear factor of activated T-cells (NFAT) (Wilkins & Molkentin, 2004). Many studies of pressure overload induced-heart failure mouse model demonstrate the major role of calcineurin pathways in pathological cardiac hypertrophy (Wilkins et al., 2004). In the heart of patients with LV hypertrophy and heart failure, calcineurin activity is elevated (Haq et al., 2001). Mice with cardiac specific overexpression of calcineurin induce pathological cardiac hypertrophy with rapid progression of cardiomyopathy (Molkentin et al., 1998). Further, cardiac hypertrophy is prevented by inhibition of calcineurin in the settings of cardiomyopathy and pressure overload (Sussman et al., 1998). Abnormally increased PI3K(p110 γ) (G.Y. Oudit et al., 2004b), mitogen activated protein kinases (MAPKs) (Pearson et al., 2001), and calcineurin have also been correlated to pathological hypertrophy (Rothermel et al., 2001).

Cardiac atrophy and cardiac regression. Cardiac atrophy has been observed in several diseases and experiment models in both humans and animals. Cancer induced cardiac cachexia patients have smaller heart size and mass (Burch et al., 1968). Although specific cardiac abnormality is not observed in cachectic patients, a significant loss of LV mass (>20%) occurs in the cachexia patients (Florea et al., 2002). In another study, after 15 days of tumor cell injection, cardiac mass is reduced 8.3% in male mice. After 27 days, heart mass decreases by 21.8% from the normal heart mass (Casper & Leinwand, 2011). Moreover, prolonged bed rest causes cardiac atrophy (Perhonen et al., 2001). After 60 days of prolonged bed rest, the significant reduction in LV volume, LV mass, and

adjusted LV mass is observed in both male and female subjects (Dorfman et al., 2007).

The regression of cardiac hypertrophy occurs in several situations such as using left ventricle assist device (LVAD), TAC reversal technique, and dexamethasone-induced atrophy experiment (Willis et al., 2007; Willis et al., 2009; Zafeiridis et al., 1998).

Circulatory support with LVAD before heart transplantation leads to regression of cellular hypertrophy. The patients with 75 ± 15 days of LVAD support result in a 28% reduction in myocyte volume, 20% reduction in cell length, 20% reduction in cell width, and 32% reduction in cell length-to-thickness ratio. These results of cellular changes are associated with decreases in left ventricular size and mass. Therefore, regression of cellular hypertrophy is the major factor of left ventricular regression after LVAD implantation (Zafeiridis et al., 1998).

Four weeks of TAC, results in cardiac hypertrophy and TAC-induced cardiac hypertrophy returns to base-line after four weeks of removal of TAC (Willis et al., 2009). Detraining is one other condition that leads to cardiac regression. Increased heart weight and protein content of the heart start to decrease and return to initial values after cessation of exercise (Mujika & Padilla, 2000b). Enhanced left ventricular systolic function of the heart in response to exercise training also returns to normal level (Bocalini, Carvalho, de Sousa, Levy, & Tucci, 2010). The most important distinction between cardiac atrophy and cardiac regression is the changes of cardiac function. Cardiac regression comes along with no greater decrease than base-line of the cardiac function while cardiac atrophy is associated with a greater reduction of cardiac function as compared to basal levels (Hill & Olson, 2008; O.J. Kemi, Haram, Wisløff, & Ellingsen, 2004).

Signaling pathway underlying cardiac atrophy. The regulation of heart size on a cellular level depends on the balance between pro-hypertrophic and pro-atrophic signaling pathways within cardiomyocytes. Relatively little is known about signaling pathways regulating atrophy in the heart than in the skeletal muscle. In skeletal muscle, three different proteolytic pathway systems have been identified for protein degradation: (1) the calcium-dependent calpain system, (2) the lysosomal protease system, and (3) the ubiquitin proteasome system (UPS) (Glickman & Ciechanover, 2002). Although calpain system and lysosomal system do not contribute to protein degradation as large as UPS, previous studies suggest that the calpain system is activated in both unloaded normal rat heart and unloaded failing human heart (Razeghi & Taegtmeyer, 2006) and the lysosomal system correlates to cathepsin D activity during regression of thyrotoxic cardiac hypertrophy (Wildenthal & Crie, 1980). Recent studies show that UPS pathway is also involved in cardiac atrophy (Razeghi et al., 2006; Razeghi et al., 2003). Ubiquitin is activated through three different enzymatic actions, an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligating enzyme which is the rate-limiting step for UPS system (Mearini, Schlossarek, Willis, & Carrier, 2008). These three steps are essential to activate the ubiquitin and degrade peptides to amino acids (Glass, 2003; Razeghi & Taegtmeyer, 2006). Atrogin-1 is muscle-specific F-box protein ubiquitin E3 ligase (also called MAFbx), and it has been shown to block cardiac hypertrophy (Li et al., 2007). Atrogin-1 is used as a marker in studies of muscle atrophy due to its dramatic induction in nearly every form of muscle atrophy (Lee & Goldberg, 2011). Atrogin-1 was initially identified in skeletal muscle atrophy studies. During skeletal muscle atrophy, the level of Atrogin-1 is abundantly expressed (Bodine et al., 2001; Gomes et al., 2001).

Based on series of recent studies, in cardiac muscle, pathologic and physiologic cardiac hypertrophy can be negatively regulated by Atrogin-1. Atrogin-1 inhibits pathological hypertrophy by attenuating agonist-induced calcineurin activity and represses calcineurin-dependent transactivation. For example, cardiac specific over expression of Atrogin-1 reduces calcineurin protein level and reduces cardiac hypertrophy (Li et al., 2004). Atrogin-1 decreases levels of calcineurin-A and promotes its ubiquitination. The regulating role of Atrogin-1 in physiological hypertrophy has also been analyzed. Atrogin-1 inhibits Akt-dependent cardiac hypertrophy via dephosphorylation of FoxO (Li et al., 2007). Treatment of IGF-1 which activates Akt decreases Atrogin-1 mRNA expression in cardiac myocytes (Skurk et al., 2005). Cardiac specific overexpression of Atrogin-1 mice attenuates IGF-1/GH administration-induced cardiac hypertrophy (Skurk et al., 2005). On the other hand, Atrogin-1 null mice augments cardiac hypertrophy after three weeks of voluntary wheel running exercise compared to wild type mice (Schisler et al., 2008).

However, there are conflicting reports regarding the role of Atrogin-1 signaling in cardiac atrophy (Table 1). For example, several studies show that the expression of Atrogin-1 does not change or even decreases during cardiac atrophy (Razeghi et al., 2006; Razeghi et al., 2003). Mechanical unloading executed by heterotopic transplantation induces cardiac atrophy, but the expression of Atrogin-1 is not significantly increased (Razeghi et al., 2003). In addition, Atrogin-1 is significantly increased in response to TAC. When Atrogin-1 knockout mice are subjected to TAC, cardiac hypertrophy is attenuated compared to wild-type mice. These results suggest that Atrogin-1 may not be specific to atrophy, but plays an important role in mediating cardiac hypertrophy in

response to pressure overload (Usui et al., 2011). The existing data on the roles of Atrogin-1 in cardiac atrophy and hypertrophy are inconclusive, and suggest that stimuli dependent. Thus, it is noteworthy to understand the role of Atrogin-1 in cardiac regression following cessation of exercise-training.

Upstream regulator of Atrogin-1 is forkhead box O (FoxO). Atrogin-1 is enhanced by the increased activity of FoxO transcription factors. For example, when FoxO1 and FoxO3a expression is reduced, the activity of Atrogin-1 promoter is blocked. When FoxO3a protein is activated, the activity of Atrogin-1 promoter is increased (Schisler et al., 2008). FoxO is a subfamily group of forkhead transcription factors, which play an important role in regulating muscle mass. In human, FoxO1 (FKHR), FoxO3a (FKHRL-1), and FoxO4 (AFX) isoforms are contained in every kind of cells (Biggs III & Cavenee, 2001). FoxO1 is the gene that regulates atrophy and induced in every type of muscle atrophy. In various muscle atrophy condition (e.g., disease and fasting), FoxO1 and FoxO3 mRNA levels are increased (Sandri et al., 2004). Moreover, decreased FoxO1 and FoxO3 protein or mRNA attenuates starvation-induced Atrogin-1 expression in vivo model (Sandri et al., 2004; Zhao, Brault, Schild, Cao, Sandri, Schiaffino, Lecker, & Alfred L. Goldberg, 2007). In contrast, activation of FoxO3a inhibits cardiac hypertrophy induced by IGF-1 (Skurk et al., 2005). FoxO3a gene transfer in cultured rat neonatal cardiomyocyte, interrupts both IGF and stretch-induced hypertrophy.

FoxO activity is regulated by Akt signaling. The increased activation of Akt promotes the FoxO3a phosphorylation. Therefore, this FoxO phosphorylation status could be defined as 'inactive' state (Ni et al., 2007). Likewise, decreased activity of Akt

promotes FoxO translocation to the nucleus. Increased FoxO activity in nucleus stimulates the transcriptional activity of FoxO target gene such as Atrogin-1 (Willis & Patterson, 2006). Thus, Akt activation contributes to cardiac hypertrophy by inhibiting the FoxO3a-mediated atrogene transcriptional program in cardiomyocytes. FoxO3a has 3 different phosphorylation sites Ser-253, Ser-315, and Thr-32 (Brunet et al., 1999), and phosphorylation status at Thr-32 site is used in study of Skurk et al. to determine activation of FoxO3a (Skurk et al., 2005).

Table 1. Summary of prior studies that presented conflict results on the impact of Atrogin-1 in cardiac regression.

	Model	Response	Reference
Cardiac specific Atrogin-1 overexpression	TAC	Reduced cardiac hypertrophy	Li, et al. 2004
Cardiac specific Atrogin-1 overexpression	Subcutaneous injection of IGF-1 for 2 weeks	Inhibits cardiac hypertrophy	Li, et al. 2007
Akt1 overexpression in NRVMs	Cardiac myocyte culture	Cellular hypertrophy with decreased Atrogin-1 mRNA expression	Skurk et al., 2005
Atrogin-1 KO	Exercise	Exaggerated exercise-induced cardiac hypertrophy	Schisler et al., 2008 Li et al., 2007
Male Wister rat	Heterotopic transplantation induced cardiac atrophy	Cardiac atrophy with no change in Atrogin-1 mRNA expression	Razeghi et al., 2003
Atrogin-1 KO	TAC	Decreased cardiac hypertrophy	Razeghi et al., 2006

IGF-1, insulin like growth factor-1; Akt, protein kinase B; KO, knock out; TAC, transvers aortic constriction; NRVMs, neonatal rat ventricular myocytes.

Effects of aerobic training in the heart

Oxygen consumption (VO_2) measurement is most widely used technique to evaluate the aerobic capacity of an individual. (McArdle, Katch, & Katch, 2009). VO_2 is calculated by cardiac output (CO) and the amount of oxygen that extracted in the tissue (arteriovenous oxygen difference) as stated by Fick's equation:

$$\text{VO}_2 = \text{CO} \times \text{av-O}_{2\text{difference}}$$

Numerous human and animal studies have shown that aerobic training increases $\text{VO}_{2\text{max}}$ (Mostert & Kesselring, 2002; Mujika & Padilla, 2000a; Park, Park, Kwon, Yoon, & Kim, 2003). Increased $\text{VO}_{2\text{max}}$ after training is a result of increases in both CO and $\text{av-O}_{2\text{difference}}$ (Holloszy & Booth, 1976) and it is mainly due to greater increase in CO. The term 'cardiac output' describes the amount of blood that pumped out from the heart for one-minute period. Cardiac output is calculated by:

$$\text{Cardiac output (CO)} = \text{Heart rate (HR)} \times \text{Stroke volume (SV)}$$

SV is the volume of blood that is pumped out by the left ventricle by one heart beat and is calculated by subtracting the blood volume after ejection (end systolic volume or afterload) from the volume before ejection (end diastolic volume or preload). After exercise training, resting HR decreases as a result of an increase in parasympathetic tone

and the heart rate is lower during sub-maximal exercise at a given intensity. Increased SV is the major factor to increase CO in trained individuals. Thus, increased SV after training is the major factor of improved $\text{VO}_{2\text{max}}$ in trained individuals (Higginbotham et al., 1986). Training-induced increases in SV can be attained by decreased afterload, increased preload (EDV), cardiac hypertrophy, and increase in myocardial contractile function. End systolic volume decrease, mainly due to reduction of total peripheral resistance, results in an increase of aortic compliance. EDV is the major factor that changes SV. Increased plasma volume leads to an increase in total blood volume which enlarges venous return. Cardiac hypertrophy correlates to increases of EDV (Hambrecht et al., 2000; Vella & Robergs, 2005)

Effects of detraining

The definition of detraining is the effect of training in anatomical, physiological, and performance adaptations are partially or completely gone by cessation or reduction of training (Mujika & Padilla, 2000a). Several physiological and functional changes are observed as a consequence of detraining.

$\text{VO}_{2\text{max}}$ is declined with cessation of training (O.J. Kemi et al., 2004). The percentage of decreasing rate is various by studies from 4% to 14% (Ghosh, Paliwal, Sam, & Ahuja, 1987; Houmard et al., 1992; Moore et al., 1987). Greater diminish of $\text{VO}_{2\text{max}}$ occurs in long term (more than 4-weeks) detraining than short term (less than 4-weeks) detraining. The blood volume starts to decrease within 2 days of detraining. Plasma volume decreases and indeed total blood volume also reduces. A decrease in total blood volume is the main factor of decline in $\text{VO}_{2\text{max}}$ and CO (Coyle, Hemmert, & Coggan,

1986). Cardiac output decreases 8% after 3-weeks of detraining (Coyle et al., 1984).

Although the heart rate increases to maintain cardiac output, reduction of SV is greater than increase in heart rate. For example, the resting heart rate after short term detraining does not change significantly (Wibom et al., 1992). However, heart rate at sub-maximal (Madsen, Pedersen, Djurhuus, & Klitgaard, 1993) and maximal (Houmard et al., 1992) intensities of exercise is increased to compensate the detraining-associated decrease in SV. SV decreases linearly after cessation of training mainly due to detraining-associated reduction in blood volume. 10 to 17% of decline has been reported after short term of detraining (Coyle et al., 1986) and 14 to 17% decline during long term detraining (Martin, Coyle, Bloomfield, & Ehsani, 1986). No significant changes in cardiac dimensions are observed after 10 days of detraining in distance runners (Cullinane, Sady, Vadeboncoeur, Burke, & Thompson, 1986). On the other hand, 19.5% of decrease in left ventricular mass and 25% reduction in left ventricular wall thickness after 3-weeks of exercise cessation are reported (Martin et al., 1986).

In other studies, biometrical parameter (i.e., the cardiac weight), echocardiographic parameters (i.e., LV end-diastolic dimension, LV end-systolic dimension, and LV posterior wall thickness at diastole), and papillary muscle mechanics parameters (i.e., developed tension, maximum rate of tension development, and maximum rate of tension decline) are improved after 8-weeks of training and regressed back to the normal baseline value after four weeks of detraining (Bocalini et al., 2010).

Training protocol

According to recent studies, both voluntary wheel running and forced treadmill

running advantageously affect the physiological adaptation (Burghardt, Fulk, Hand, & Wilson, 2004; A. Moraska, Deak, Spencer, Roth, & Fleshner, 2000). Negative stress responses in forced physical exercise such as treadmill running and forced swimming are frequently accompanied by activation of corticotropin-releasing hormone (CRH) neurons (Cullinan, Herman, Battaglia, Akil, & Watson, 1995; White-Welkley et al., 1996). CRH-containing neurons in the hypothalamic paraventricular nucleus (PVN) work as central activator of stress response. CRH neurons in the PVN activated during physical exercise play an important role in psychological alteration. During forced physical exercise, the activity of CRH neurons in PVN is greater, which indicates more stress materials are produced, compared with spontaneous physical exercise even though the running distance of two groups are equivalent (Yanagita, Amemiya, Suzuki, & Kita, 2007).

Previous studies demonstrate that negative physiological adaptations of training such as adrenal hypertrophy, elevation of lymphocyte nitrite concentrations, thymic involution, suppression of antigen-specific IgM, suppression of lymphocyte proliferation, and decreased serum corticosteroid binding globulin occurred after 8 weeks of treadmill running (A. Moraska et al., 2000). While forced treadmill exercise, the animals should run according to the experimenters demand and this procedure activates more production of stress materials than voluntary wheel running. Exposure to chronic stress commonly resulted in the occurrence of potential problems in not only physiological, but also psychological conditions (Selye, 1936, 1956). Moreover, it has been documented that physiological systems including nerve, endocrine, and immune systems, are affected by certain intensity of noxious agents and stressors (Pacak et al., 1998). Many recent studies have suggested that, spontaneous physical exercise could be more beneficial to

physiological adaptation and psychological health rather than forced physical exercise (Burghardt et al., 2004; Greenwood et al., 2003; Albert Moraska & Fleshner, 2001). After considering all these studies, the voluntary wheel running exercise protocol was used in this study to reduce the effect of stressors and noxious agents which affects physiological responses.

Several studies acknowledge the limitation of voluntary exercise training that intensity of exercise training which performed spontaneously is too low to induce cardiac hypertrophy. Allen et al., compared the running distance between rat and mice during training period. The mice run a significantly farther distance when it compares to the distance that the rats run throughout either involuntary or voluntary exercise. Base on this result, it is safe to state that there is a species differences and the limitation of voluntary exercise is mostly derived from the study that uses rats as the animal. This study also demonstrates that after 4 weeks of voluntary wheel running, the absolute heart weight is increased approximately 15% when it is compared to the control group (Allen et al., 2001). Moreover, when they normalize the augmentation of heart to the body mass of the animals, heart weight relative to body mass is significantly increased after 2 weeks of voluntary running but the body mass is not significantly different among groups. More recently, Leinwand lab (University of Colorado, Boulder) in collaboration with the NHLBI-sponsored PGA at Harvard Medical School, already has expression array data on hearts from FVB female mice which underwent voluntary wheel running (21 days), swimming (7 days), and forced treadmill running (14 days). The time points were selected because they resulted in similar amounts of cardiac hypertrophy. These studies demonstrate that mice are able to run a sufficient amount of distance in a voluntary model

of exercise training to induce physiological cardiac hypertrophy.

CHAPTER III

RESEARCH DESIGN AND METHODS

Akt, FoxO, and Atrogin-1 have been implicated as important players for cardiac growth and regression (Li et al., 2007; Shioi et al., 2002; Skurk et al., 2005). For example, Akt and FoxO levels are significantly increased in mouse heart during cardiac atrophy induced by fasting (Skurk et al., 2005). In addition, mice with over expression of Atrogin-1 inhibit pressure overload induced cardiac hypertrophy (Li et al., 2004). However, how these pathways are regulated during detraining and are completely unexplored. Thus, the overall objective of this proposed study is to determine the signaling pathways of Akt, FoxO, and Atrogin-1 in a mouse heart following the cessation of exercise training by Western blot analysis. In addition, we investigated the signaling pathways more extensive time course since the proteins regulating cardiac growth are activated rapidly and precede the phenotype of the heart in response to exercise (Motoyuki Iemitsu et al., 2006; Konhilas et al., 2004).

Experimental animals and training/detraining protocol

All of the animals were handled and euthanized under the guidelines of University of Colorado Animal Use and Care Committee, consistent with regulations for vertebrate animal research outlined by the National Institutes of Health.

Animals. Three- to four-month old male C57Bl/6 mice (Taconic) were used for this study (N=30). Mice were randomly assigned into 6 groups: Sedentary controls (C), 21days of voluntary wheel running (21EX), 3 days of detraining (DT3), 5 days of

detaining (DT5), 14 days of detraining (DT14), and 21 days of detraining (DT21). All mice were housed in a temperature- and light-controlled room with food and water available *ad libitum*.

Training protocol. The voluntary wheel running exercise protocol was used in this study. Mouse-sized metal cage wheels with a diameter of 11.5 cm (model no. 6208; Petsmart, Phoenix, AZ) were fitted with digital magnetic counters (model BC 600, Sigma Sport, Olney, IL) and were placed into 4 × 26 × 14.5-cm cages for voluntary exercise. The counters measured maximum running speed, total distance run, and total time run. Each morning, these data were collected and recorded for each animal and the counter was reset (Allen et al., 2001). This training protocol was repeated for 21 days in both exercise group and detraining groups (n=24). After the last exercise session, mice were randomly divided into 21 days exercise group (21EX), and detraining group (DT). The exercised group was sacrificed right after 21 days of voluntary wheel running exercise.

Detraining protocol. For the detraining group, after 21 days of voluntary wheel running, the mice were taken out from the wheel running cage and housed individually so that they remained sedentary for certain time period (3, 5, 14 and 21 days n=3-6 mice per group) after exercise cessation. Mice were weighed and then sacrificed at certain time points by isoflurane overdose followed by cervical dislocation. Harvested hearts were washed in phosphate buffered saline (PBS) to allow blood to be pumped out from the cardiac chambers and coronary vessels. Connective tissue, vascular tissue, atria, and right ventricle were removed and the left ventricles were weighed after blotted dry. The left ventricle of mice were frozen in liquid nitrogen immediately and stored at -80°C for total

RNA isolation.

Akt-Foxo-Atrogin1 signaling pathways

The signaling pathways following exercise cessation are relatively unexplored. Thus, we carried out several time points in order to observe changes over the entire course of the regression.

Homogenization and protein assay

To detect and evaluate the protein expression levels, left ventricular tissues were homogenized by using a tissue homogenizer (PRO200 Bio-Gen Motor, Pro Scientific, Oxford, CT) in standard lysis buffer (1% NP-40, 10% glycerol, 137mM NaCl, 20mM Tris-HCl pH7.4, Halt protease and phosphatase inhibitor single-use cocktail (Thermo Scientific, 78442)). Protein concentration was measured by using Beckman Coulter DTX 880 multimodal detector (Fullerton, CA, USA) at 660 nm absorbance. Lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) was applied to the homogenized samples and the samples were incubated for 10 min at 70°C.

Western blotting

Gel casting. SDS–polyacrylamide gel electrophoresis (PAGE) was performed under reducing conditions in precast 4% to 10% gradient mini-gels applying the NuPAGE Bis-Tris buffer system (Invitrogen).

Separating gel. Molecular weight for GAPDH, Akt, FoxO, and Atrogin-1 are 37 kDa, 60 kDa, 97 kDa, and 38kDa, respectively. 25ml of 10% gel was used for separating due to the protein's molecular weight. 25 ml of 10% gel (mixture of separating gel buffer,

50% acrylamide/BIS, 50% sucrose, 10% SDS, catalyst, TEMED, H₂O) was poured into the gel casting cassette and stay for 1 hour at 23°C to be polymerized.

Stacking gel. 1.25ml of 4% stacking gel (mixture of stacking buffer, 50% acrylamide/BIS, 10% SDS, catalyst, TEMED, H₂O) was poured onto the top of polymerized separating gel. The comb was inserted to make each well and pour the gel at room temperature for 1 hour until the stacking gel becomes completely polymerized.

Gel running (separation). Protein separation was carried out using a PowerPac 200 power supply (Bio-Rad, Hercules, CA) and XCell Sure Lock mini cell electrophoresis apparatus (Invitrogen, Carlsbad, CA). 5 µl of precision plus protein western C molecular weight standard (Bio-Rad) and 15-35 µg of each protein samples were loaded onto the gel. Inner and outer tank (X cell II Blot Module, Invitrogen) of electrophoresis kit was filled with 1X running buffer (NuPAGE MES SDS Running buffer, 50mM MES, 50 mM Tris base, 0.1% SDS, 1mM EDTA, pH7.3). 500µL NuPAGE Antioxidant was applied at inner tank. Gel was run for 1 to 2 hours depending on the molecular weight of interest proteins at 150V running voltage with 100-125mA of starting current.

Transferring. The separated proteins in the gel were transferred to immune-blot treated nitrocellulose membrane (Hybond –P, Amersham Pharmacia Biotech). The membrane and chromatography filter paper (Whatman) were equilibrated for 15 minutes, and 20 minutes of equilibration for gel by soaking in the transfer buffer (48mM Tris, 39 mM Glycine, 200ml of 20% methanol, pH 9.2). Transferring materials were placed onto the platinum anode of transfer equipment in the following orders of pre-soaked sheet of filter paper, membrane, gel, and another filter paper to make a transfer sandwich. After

building the transfer sandwich, plug the unit into the power supply and gels were transferred for one hour at 15 V. Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad, Hercules, CA) was used for these transferring procedures. After transferring procedure is finished, the membrane was washed for 5 minutes with de-ionized water.

Developing. The membrane was blocked with 50ml 5% blocking buffer consisting of TBS T (1M Tris-HCl, pH8, 5M NaCl, 0.2g KCl, 5ml Tween 20) with 2.5g of nonfat dry milk for 30 minutes at 37°C. After half an hour, the membrane was washed three times with TBST for 5 minutes each. All antibodies were obtained from commercial companies and were applied according to the manufacturer's protocol. The following affinity-purified antibodies were used for the western blot analyses: primary GAPDH antibody (1:5,000 dilution in 5% w/v BSA, Cell Signaling), primary phospho-Ser473 Akt antibody (1:2,000 dilution in 5% w/v BSA, Cell Signaling), Akt antibody (1:1,000 dilution in 5% w/v BSA, Cell Signaling), phospho-FoxO3a-Ser253 antibody (1:1,000 dilution in 5% w/v BSA, Cell Signaling), FoxO3a antibody (1:1,000 dilution in 5% w/v BSA, Cell Signaling), Atrogin-1 antibody (1:1,000 dilution in 5% nonfat dry milk PBS, LifeSpan BioSciences). The membrane was stored at 4°C cold-room overnight with gentle shaking. Next day, the membrane was washed three times with TBST for 5 minutes per wash and then incubated in the diluted rabbit secondary goat anti-rabbit IgG antibody (1:5,000 ratio) in 5% nonfat dry milk TBST for 30 minutes at 37°C. After rinsing four times with TBST for 10 minutes each, the membrane was developed by using Clarity Western Chemi-Luminescence (ECL) substrate (Bio-Rad) for western blotting detection. Total 1.6 ml of peroxide solution and luminol/enhancer solution substrate components were mixed in 1:1 ratio and applied for 5 minutes. After 5 minutes,

quantification of the chemi-luminescent signals was acquired by using a Chemi-Doc digital imaging system (Bio-Rad, Hercules, CA). Chemi hi-sensitivity was used as protocol and the software was automatically optimize exposure time for the blot. When the band was not detected by the automatic optimize exposure, signal accumulation mode was setup and different exposure times were used until the band was detected. The clearest image of the bands on membrane was used for densitometric analysis which was performed with Quantity One software 4.6.8 (Bio-Rad). After imaging, the membrane was stripped for 15 minutes at 37 °C with Restore Western blot Stripping Buffer (Thermo Scientific) and reprobed for different target protein. The intensity of proteins was expressed as relative fold difference ratio compared to control group. The Western blot image is representative of two or more experiments. For Akt and FoxO, total protein content was analyzed after stripping the phosphor-blot and the ratio of phosphoprotein-to-total protein was used to represent the activity of Akt and FoxO. Atrogin-1 levels were normalized to total proteins loading that were stained with Novex Reversible membrane protein stain kit (Invitrogen: IB7710). Four to seven mice were used per group with two or more technical replicates per mouse.

Data analysis

Unpaired two tailed t-tests were used to test for difference in heart weight between control group and 21 days of exercise group. One-way analysis of variance (ANOVA) followed by Dunnett's least significant difference (LSD) was performed for multiple group comparisons by using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). All values were expressed as means $\pm$ SEM, and $p < 0.05$ was considered as

significant.

CHAPTER IV

RESULTS

The effect of exercise on body and heart weight

There were no significant differences in exercise hours and average distance run per day (km/day) among groups (Figure 2, Figure 3). Body weight was not significantly different among groups (Figure 4). In agreement with previous studies (Allen et al., 2001), 21 days of voluntary wheel running significantly made the heart weight greater when compared to the sedentary control group ($n=7$) (control = 115.60 ± 3.40 vs 21EX = 128.50 ± 1.50 mg; $p<0.05$). Heart weight remained higher than the control group during detraining at 3 days (125.70 ± 2.19 mg; $p<0.05$), 5 days (129.20 ± 4.18 mg; $p<0.05$), 14 days (124.10 ± 3.25 mg; $p<0.05$), and 21 days (128.00 ± 2.98 mg; $p<0.05$). Greater heart weight was also observed in 21 days of exercise group when the heart weight normalized to the body weight (control = 4.34 ± 0.05 vs 21EX = 4.90 ± 0.09 mg/g; $p<0.05$). Heart weight normalized to body weight remained larger than the sedentary control group during detraining at 3 days (4.69 ± 0.05 mg/g; $p<0.05$), 5 days (4.76 ± 0.06 mg/g; $p<0.05$), and 14 days (4.67 ± 0.07 mg/g; $p<0.05$). There was no difference in heart weight normalized to body weight between control and 21 days of detraining (4.48 ± 0.11 mg/g; $p>0.05$).

The effect of detraining on body and heart weight

Figure 4 and 5 shows the results for body weight and heart weight across the detraining period, respectively. There was no significant change in body weight during 21 days of detraining (Figure 4). Heart weight normalized to body weight did not

significantly change during 14 days of detraining, but showed significant ($p < 0.05$) regression at 21 days of detraining (Figure 5).

Figure 2. Average exercised hours per day. There are no significant differences among groups. 21EX (n=6), DT3 (n=5), DT5 (n=5), DT14 (n=3), DT21 (n=4). 21EX, 21days of voluntary wheel running exercise; DT3, 3 days of detraining after 21 days of exercise; DT5, 5 days detraining after 21 days of exercise; DT14, 14 days detraining after 21 days of exercise; DT21, 21 days detraining after 21 days of exercise. Values are mean $\pm$ SEM.

Figure 3. Average running distance per day was not significant different among groups. 21EX (n=6), DT3 (n=5), DT5 (n=5), DT14 (n=3), DT21 (n=4). 21EX, 21days of voluntary wheel running exercise; DT3, 3 days of detraining after 21 days of exercise; DT5, 5 days detraining after 21 days of exercise; DT14, 14 days detraining after 21 days of exercise; DT21, 21 days detraining after 21 days of exercise. Values are mean $\pm$ SEM.

Figure 4. Body weight was not significant different among groups. 21EX (n=6), DT3 (n=5), DT5 (n=5), DT14 (n=3), DT21 (n=4). 21EX, 21days of voluntary wheel running exercise; DT3, 3 days of detraining after 21 days of exercise; DT5, 5 days detraining after 21 days of exercise; DT14, 14 days detraining after 21 days of exercise; DT21, 21 days detraining after 21 days of exercise. Values are mean $\pm$ SEM.

Figure 5. Heart weight (HW) normalized to body weight (BW) in response to exercise training and detraining. 21EX (n=6), DT3 (n=5), DT5 (n=5), DT14 (n=3), DT21 (n=4). 21EX, 21days of voluntary wheel running exercise; DT3, 3 days of detraining after 21 days of exercise; DT5, 5 days detraining after 21 days of exercise; DT14, 14 days detraining after 21 days of exercise; DT21, 21 days detraining after 21 days of exercise. Values are mean $\pm$ SEM. * $p < 0.05$, significantly different from 21EX.

The phosphorylation of Akt Ser473 in each group

Akt phosphorylation to total Akt ratio was significantly higher in the exercise group compared to the sedentary control group (1.79 ± 0.29 -fold difference vs control; $p < 0.05$; Figure 6). Akt phosphorylation to total Akt ratio was still higher in 3- (2.55 ± 0.45 -fold difference vs control; $p < 0.05$; Figure 6), 5- (2.64 ± 0.79 -fold difference vs control; $p < 0.05$; Figure 6), and 14 days of detraining groups (3.62 ± 1.05 -fold difference vs control; $p < 0.05$; Figure 6). However, Akt phosphorylation to total Akt ratio was significantly decreased by 21 days of detraining (0.64 ± 0.11 -fold difference vs control; $p < 0.05$; Figure 6).

Figure 6. Western blot analysis of Akt phosphorylation in each group. The graph was presented as fold difference versus control in response to exercise training and detraining. C (n=7), 21EX (n=6), DT3 (n=5), DT5 (n=5), DT14 (n=3), DT21 (n=4). C, sedentary control; 21EX, 21days of voluntary wheel running exercise; DT3, 3 days of detraining after 21 days of exercise; DT5, 5 days detraining after 21 days of exercise; DT14, 14 days detraining after 21 days of exercise; DT21, 21 days detraining after 21 days of exercise. Values are mean $\pm$ SEM. * $p < 0.05$, significantly different from C.

The phosphorylation of FoxO3a in each group

FoxO3a is one of downstream targets of Akt (Skurk et al., 2005). During hypertrophic stimuli, increased activity of Akt leads to phosphorylation of FoxO, and phosphorylated FoxO remains in the cytosol of cardiomyocyte and incapable of turning on the FoxO transcriptional factors (Schisler et al., 2008). Under atrophy conditions, FoxO is dephosphorylated and localized to the nucleus. Nuclear translocation of FoxO activates transcriptional atrogenes, including the muscle-specific E3 ligase Atrogin-1 and results in decreased cell size (Sengupta et al., 2009). Thus, we measured phosphorylation status of FoxO3a and Total FoxO3a that has been shown to be important role in cardiac hypertrophy and atrophy (Zhao, Brault, Schild, Cao, Sandri, Schiaffino, Lecker, & Goldberg, 2007). The western blot results of p-FoxO3a and total FoxO3a showed similar patterns in each group (Figure 7). Therefore when the FoxO3a phosphorylation was normalized to total FoxO3a, no significant difference was observed among groups (Figure 7).

The amount of Atrogin-1 in each group

The level of Atrogin-1 was not significantly different in 21EX, DT3 and DT5 groups as compared to the sedentary control group. However, it was significantly decreased in 14- (0.35 ± 0.13 -fold difference vs control; $p < 0.05$; Figure 8), and 21 days of detraining groups (0.52 ± 0.13 -fold difference vs control; $p < 0.05$; Figure 8). Housekeeping proteins, such as GAPDH, Histone H3, β -Actin, and α -Tubulin (Ferguson et al., 2005) have been tested to check the loading control to normalize the Atrogin-1 levels, but all of the antibodies showed great fluctuation among groups. Therefore, the

membrane was stained with protein stain kit to check the loading control on the blot. The amount of total protein that loaded on the gel was not different among lanes (Figure 8).

Figure 7. Western blot analysis of FoxO3a phosphorylation status and total FoxO3a in each group. The graph was presented as fold difference versus control in response to exercise training and detraining. C (n=7), 21EX (n=6), DT3 (n=5), DT5 (n=5), DT14 (n=3), DT21 (n=4). C, sedentary control; 21EX, 21days of voluntary wheel running exercise; DT3, 3 days of detraining after 21 days of exercise; DT5, 5 days detraining after 21 days of exercise; DT14, 14 days detraining after 21 days of exercise; DT21, 21 days detraining after 21 days of exercise. Values are mean $\pm$ SEM.

Figure 8. Western blot analysis of Atrogin-1 in each group and the image of loading control. The graph was presented as fold difference versus control in response to exercise

training and detraining. The first and the last lanes are molecular markers.

C (n=7), 21EX (n=6), DT3 (n=5), DT5 (n=5), DT14 (n=3), DT21 (n=4). C, sedentary control; 21EX, 21days of voluntary wheel running exercise; DT3, 3 days of detraining after 21 days of exercise; DT5, 5 days detraining after 21 days of exercise; DT14, 14 days detraining after 21 days of exercise; DT21, 21 days detraining after 21 days of exercise. Values are mean $\pm$ SEM. * $p < 0.05$, significantly different from C.

CHAPTER V

DISCUSSION

The purpose of this study was to determine the roles of Akt, FoxO, and Atrogin-1 in cardiac muscle which has undergone regression of mass after cessation of exercise training. Although detraining induces cardiac regression (Mujika & Padilla, 2000a, 2000b), no study has been focused on cardiac regression regulatory proteins in the hearts after cessation of exercise. We found that the heart weight to body weight ratio (HW/BW) was significantly greater than sedentary control after 21 days of exercise training and remained higher after 3-, 5- and 14 days of detraining groups whereas HW/BW ratio regressed to the values of sedentary control group in 21 days of detraining group.

Previous studies show that Akt is an important player in exercise-induced cardiac hypertrophy (Konhilas et al., 2004; McMullen et al., 2003; Skurk et al., 2005). In agreement with previous studies, we found that the Akt activity, as assessed by the ratio of phospho-Akt to total Akt, was significantly higher in 21 days of exercise group. Although exercise stimulus was ceased, Akt activities were still significantly higher after 3-, 5-, and 14 days of detraining. However, Akt activity was significantly decreased when the heart mass regressed to the control group level.

FoxO is one of the downstream targets of Akt (see Figure 1). Decreased activity of Akt promotes de-phosphorylation of FoxO and FoxO translocates into the nucleus. Previous studies demonstrate that nuclear translocation of FoxO stimulates the transcriptional activity of FoxO target genes such as Atrogin-1 and results in cardiac atrophy (Skurk et al., 2005). However, we did not find any changes in phospho-FoxO3a

to total Foxo3a ratio among groups. This could be partly due to our experimental limitations. The activity of FoxO is mainly determined by localization of FoxO (ie., cytosol vs. nuclear) (Hedhli, Pelat, & Depre, 2005), but total tissue lysis samples for Western blot may not be sensitive enough to measure activity of FoxO3a. Therefore, quantification of the localization of FoxO3a whether it is in cytoplasmic or in nucleus by immunostaining needs to be done in future studies.

Atrogin-1 has been shown to be an important mediator of hypertrophy and atrophy (Schisler et al., 2008), and the expression of Atrogin-1 is regulated by FoxO3a activity (Sandri et al., 2004). The role of Atrogin-1 in skeletal muscle atrophy has been well established (Gomes et al., 2001; Sandri et al., 2004), but there are conflicting reports regarding the role of Atrogin-1 in cardiac atrophy and hypertrophy (Table 1). For example, Atrogin-1 knockout mice exhibits exaggerated cardiac hypertrophy in response to voluntary wheel running (Li et al., 2007). In contrast, Atrogin-1 mRNA levels were significantly increased in pathological cardiac hypertrophy induced by transverse aortic constriction (Usui et al., 2011) and heart failure (Adams et al., 2008; Galasso et al., 2010). However, exercise training in a rat model of heart failure significantly improves cardiac function with attenuation of Atrogin-1 expression levels. In addition, Galasso et al., demonstrate that the expression of Atrogin-1 is inversely related to cardiac function in human heart failure models (Galasso et al., 2010). Our results show that the levels of Atrogin-1 were significantly decreased in regression phase (14- and 21 days of detraining). It is currently not clear whether decreased Atrogin-1 in 14- and 21 days of detraining antagonizes cardiac hypertrophy as described in unloading-induced atrophy (Razeghi & Taegtmeier, 2006).

Future Direction

Our results suggest that other pathways may also contribute to cardiac regression after cessation of exercise training. Previous studies demonstrate that both ubiquitin-proteasome system (i.e Foxo/Atrogin-1) and the autophagic mechanism regulate skeletal and cardiac muscle atrophy (Ferdous, Battiprolu, Ni, Rothermel, & Hill, 2010; Sengupta et al., 2009). Autophagy is a lysosome-dependent cellular homeostatic process that cleans up the damaged proteins and organelles, which is necessary for survival, differentiation, development, and homeostasis (Gustafsson & Gottlieb, 2009; Levine & Kroemer, 2008). Three different major types of autophagy are macroautophagy, microautophagy, and chaperone-mediated autophagy (Levine & Kroemer, 2008; Maria Cuervo, 2004). The most common form of the catabolic mechanism that degrades proteins and organelles in eukaryotic cells are macroautophagy. More than 30 autophagy-related genes (ATGs) have been discovered through previous studies (Ferdous et al., 2010). Autophagy is activated by various stimuli including hypoxia, nutrient deprivation and growth factor withdrawal, and hyperthermia (Levine & Kroemer, 2008). In addition, autophagy is significantly increased in hearts from various pathological conditions, including fasting, cardiac hypertrophy, cardiomyopathy, myocardial ischemia/reperfusion and heart failure (Hamacher-Brady, Brady, & Gottlieb, 2006; Knaapen et al., 2001; Pattingre et al., 2005; Zhu et al., 2007). An increased number of autophagic vesicles has also been observed in isolated cardiac cells that are exposed to hypoxia/reoxygenation or simulated ischemia and reperfusion (Ferdous et al., 2010). Autophagy plays two different roles either beneficial or detrimental in the myocardium, depending on the levels of activation and the types of stimulation. In normal physiological conditions, autophagy activity

contributes to maintain myocyte function by resourcefully eliminating misfolded proteins and damaged organelles. On the other hand, autophagy activity is increased in the setting of severe or chronic stress, including biomechanical load (e.g. hypertension), starvation, chronic ischemia, and ischemia/reperfusion injury. In most cases these conditions are detrimental to cardiac cell survival. Absence of autophagic activity causes inadequate protein quality control, accumulation of toxic protein and accumulation of defective mitochondria (Ferdous et al., 2010). Taken together, these studies suggest that normal autophagy activity is essential to maintain homeostasis and the regulation of cardiac myocyte function, either excessive or absence of autophagic activity is deleterious to the heart. A possible extension of this study would be the examination of the role of autophagy on cardiac regression following exercise cessation.

Limitations

Although randomization is the most common way to divide groups, we felt random grouping is one of the major limitations in current study. Running times and total distance per day were not statistically different among groups, but 21 days exercise group showed shortest running distance among groups (Figure 3), while 14 days of detraining group showed longest hours and running distance among groups (Figure 3, Figure 4). Moreover, the activity of Akt was both increased in 21 days of exercise and 14 days of detraining groups. The 14 days of detraining group showed the greatest fold change (3.62-fold change) and 21 days of exercise group showed the smallest fold change (1.78-fold change) compared to the sedentary control group. The result of phosphorylation of Akt could have been affected by their running time and distance. Therefore to minimize

the variations of morphometry and signaling pathways, strictly controlled block design needs to be done in future study.

Furthermore physical activity in control mice and mice that underwent detraining was not measured in this study. Unless we did not compare the activity of sedentary control mice and detraining group mice during their detraining period, it is hard to claim that the physical activity was completely ceased. Therefore in future studies, using chamber system to examine their ambulation in control group and during detraining period may be a solid approach to compare the activity of control mice and detraining group mice to determine the sedentary activity of the mice.

Another limitation of this study could be the training and detraining period which may not be sufficient enough in time to induced cardiac hypertrophy and regression. Mice were exercised for 21 days with voluntary wheel running and the results of this study showed that running distance and hours are sufficient to induce physiological cardiac hypertrophy. However, we cannot totally exclude the possibility that training period was not sufficient enough to see a significant difference in signaling pathway among groups.

Mostly, cellular response occurs prior to morphological changes (Konhilas et al., 2004; Rosenkranz et al., 2002), therefore we expected that the expression and change of regulating proteins would have appeared in advance of heart weight changes. The 4 different detraining time points (3-, 5-, 14-, and 21 days) were used to verify this premise and to assess the exact occurrence time of cardiac regression regulatory proteins. However, HW/BW ratio was still significantly higher than sedentary control in 3-, 5-, and

14 days of detraining groups but this growth was regressed after 21 days of detraining. Therefore, based on these results we can speculate that the detraining periods (3 days, 5 days, 14 days, and 21 days) we used in this study might be too short to expect significant differences compared with control and exercise groups.

Training and detraining protocols that have been used in previous studies vary in duration. Argawal et al., trained rats for 42 days in training group, whereas their detraining group underwent 28 days of training and 14 days of cessation of training (Agarwal, Dange, Vila, Otamendi, & Francis, 2012). In another study, rats were trained for 8 weeks and detrained for 4 weeks (Carneiro-Júnior et al., 2013). After 4 weeks of detraining, the myocytes mechanical and molecular parameters turn back to normal. Similarly, Boclini et al., trained female rats for 8 weeks with swimming, and divided the animals into two detraining groups for 2 weeks and 4 weeks (Bocalini et al., 2010). The result of this study demonstrated that physical capacity in 2 weeks of detraining group was not significantly different compared with baseline, but still showed little higher physical capacity than 4 weeks of detraining group (Bocalini et al., 2010). Taken together, previous studies suggest that longer than 28 days of exercise training and longer than 4 weeks of detraining would be a sufficient amount of time for the heart to regress back to normal weight.

In future study, expanded training and detraining period may allow significant changes in exercise induced cardiac hypertrophy and cardiac regression in mice heart. This may result in greater chance to determine the signaling pathways underlying cardiac regression after exercise cessation. Finally, investigation of alternative signaling

mechanism, such as autophagy pathway may provide some insight into cardiac regression after detraining.

Conclusion

Our results showed that cardiac regression occurred after 21 days of detraining. We hypothesized that the activities of Akt would be markedly decreased and FoxO3a would be significantly increased during detraining. In addition, we hypothesized that the levels of Atrogin-1 would be increased during detraining. We found that decreased Akt activity may partially contribute to cardiac regression after 21 days of detraining. Unexpectedly, FoxO3a activity did not change as a result of training or detraining as compared to sedentary controls. In addition, Atrogin-1 levels were significantly decreased in 14- and 21 days of detraining.

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