Western University

Western&Graduate&PostdoctoralStudies

Western University

Scholarship@Western

Electronic Thesis and Dissertation Repository

9-24-2015 12:00 AM

Studies into the Regulation of FTO by Angiotensin II in Cardiomyocyte Hypertrophy

Kwame N. Boakye-Ansah, *The University of Western Ontario*

Supervisor: Morris Karmazyn, *The University of Western Ontario*
A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology and Pharmacology
© Kwame N. Boakye-Ansah 2015

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Part of the Medicine and Health Sciences Commons

Recommended Citation

STUDIES INTO THE REGULATION OF FTO BY ANGIOTENSIN II IN
CARDIOMYOCYTE HYPERTROPHY

(Thesis format: Monograph)

by

Kwame Boakye-Ansah

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

Abstract

Leptin has been shown to upregulate the fat mass and obesity-associated protein (FTO) in the cardiomyocyte, an effect that has been linked to cardiomyocyte hypertrophy. In this current study, we sought to determine if other cardiomyocyte hypertrophic agonists could upregulate FTO. Angiotensin II displayed the ability to significantly upregulate mRNA expression and protein expression of FTO. Thus, in this study, we explored the cellular mechanisms behind angiotensin II-induced FTO upregulation.

Angiotensin II-induced FTO upregulation was attenuated by 10 µM valsartan, an AT-1 receptor antagonist, 5 µM EMD87580, a sodium-hydrogen exchanger isoform 1 (NHE-1) inhibitor, and 2 nM FK506, a calcineurin inhibitor. This data suggests that angiotensin II-induced FTO upregulation is AT-1 receptor mediated and has a signal transduction pathway involving the NHE-1/calcineurin system. All the above agents also abolished angiotensin II-induced hypertrophy.

In addition, our studies show that a super-ovine leptin receptor antagonist (LRA; 100 nM) and JAK2 inhibitor, 50 µM AG490, also attenuate angiotensin II-induced FTO upregulation. These results implicate the leptin-JAK2-STAT3 signalling system in angiotensin II-induced FTO upregulation. The above agents also abolished angiotensin II-induced hypertrophy.

The data suggests that NHE-1/calcineurin as well as leptin-mediated JAK2/STAT3 signalling play a critical role in angiotensin II-induced FTO upregulation.

Keywords: Cardiac hypertrophy, heart failure, cardiomyocytes, angiotensin II, sodium-hydrogen exchanger, calcineurin, leptin, JAK2, fat mass and obesity-associated protein

Acknowledgments

It is with great gratitude that I thank Dr. Morris Karmazyn for providing me the opportunity to pursue my master's degree at Western University. I am especially appreciative of his choice to not give up on me at times where there appeared to be little hope. Although I stretched his patience thin, and frustrated him multiple times, he found the inner resolve to keep me as a trainee and guide me to a successful completion of the Masters of Science program. I would like to acknowledge my committee members Dr. Qingping Feng, Dr. Doug Jones and Dr. Timothy Regnault for attending my committee meetings, providing me with tough constructive criticism and advice and most importantly encouragement. Dr. Regnault served as a great graduate student representative. He was always there when I needed him, and helped me tremendously to navigate through tough times with attentive listening, great advice and morale-boosting conversations. I am forever grateful.

I would also like to thank Dr. John DiGuglielmo for allowing me to use his fluorescence microscope. He exuded an immense level of patience when showing me how to use the microscope and displayed that even more so when he helped me troubleshoot my immunofluorescence experiments at a time where I was under enormous pressure. Dr. DiGuglielmo was not in my field of research and did not have to help me in the way he did, which speaks to the type of man he is. Without him, I am positive I am not where I am today. I will not forget the actions of Dr. DiGuglielmo and I am forever grateful.

I also extend my gratitude to my lab members, Tracey Xiaohong Gan and Dr. Venkatesh Rajapurohitam for guiding me with experimental procedures and their moral support. I also thank Cathy Huang, Dr. Dukgyu Lee and Dr. Chian Ju for their guidance and support. I would also like to thank Dr. Xilan Tang for her support and presence in the lab. She would always

brighten up my day and served as a great friend in the lab. I also extend my gratitude to Dr. Bo Li and Suresh Bairwa for being tremendously supportive and great friends. These two individuals were amazing support structures not only in the lab, but also in my life during the two years of my masters.

Lastly, but most importantly, I would like to thank my family for the encouragement and motivation they provided me with during a tumultuous two years. I would like to especially thank my parents for their emotional, mental and financial support. None of this is possible without you.

Table of Contents

List of Tables

List of Figures

List of Appendices

.

Chapter 1

1 Introduction

Cardiovascular disease is among the leading causes of death in Canada. Since the end of the late 1970s, hospital discharges for heart failure have been progressively increasing (Roger et al., 2011). This increase has been attributed to the development of new and effective therapeutic strategies for dealing with heart attacks. To clarify, more patients are surviving heart attacks and other acute ischemic diseases and as a result, these patients go on to develop heart failure due to the compromised myocardial function caused by the initial ischemic event (McMurray & Pfeffer, 2005). As well, heart failure is a disease that is most prevalent among the aged population. With the advancement of medicine, and beneficial lifestyle modifications, Canadians are living longer (McMurray & Pfeffer, 2005). This increased life expectancy has also increased the incidence of heart failure. Given that we are in the midst of an aging baby boomer population—as more than 14.8% of Canada's population is over the age of 65 (Statistics Canada, 2011)—heart failure is and will continue to be an inevitable burden on patients, their families and the health care system. Therefore, heart failure is a significant health concern to Canadians, and an urgent health issue that must be actively researched.

1.1 Cardiac hypertrophy

Cardiac hypertrophy is one of the few mechanisms employed by the heart when responding to a hemodynamic burden. Essentially the heart increases in mass through the growth of existing cells rather than division of these cells as would occur in hyperplasia.

This increase in mass serves to bear the extra load on the heart (Lorell & Carabello, 2000). Depending on the nature of the hemodynamic load, hypertrophy of the heart can manifest in two morphologically distinct ways; concentric hypertrophy and eccentric hypertrophy. Pressure overload constitutes one of the two major hemodynamic loads that can burden the heart. Pressure overload results in an increase in synthesis of sarcomeres—the smallest functional unit of the cardiomyocyte responsible for its contractile properties. These sarcomeres are arranged in a parallel orientation, resulting in an increase in width of the cardiomyocyte and an increase in the thickness of the myocardial wall. This is known as concentric hypertrophy. Volume overload, the other major hemodynamic load, also results in an increase in synthesis of sarcomeres though the arrangement of the sarcomeres differs in comparison to the arrangement in a pressure overload situation. During volume overload, the newly synthesized sarcomeres are organized in a series resulting in an increase in ventricular volume (Lorell & Carabello, 2000). Pathophysiological conditions in which pressure overload occurs are hypertension or aortic stenosis. These are conditions where the systolic stress otherwise known as afterload rises to supraphysiological levels. This pressure overload induces a thickening of the wall which, according to LaPlace's Law, will normalize the systolic stress thus preserving the ejection fraction of the heart (Frey et al., 2004). Volume overload occurs in pathophysiological conditions such as mitral regurgitation and aortic regurgitation (Lorell & Carabello, 2000). In both of these pathological conditions, the valves responsible for facilitating the unidirectional flow of blood through the heart to the rest of the body are dysfunctional, thus allowing blood to flow back into the heart. The result is an accumulation of blood in the heart otherwise known as volume overload. The volume overload serves as a biomechanical signal,

resulting in the longitudinal growth of the cardiomyocytes (Frey et al., 2004). This results in the ventricular cavity increasing in volume in a process called ventricular dilation (Lorell & Carabello, 2000). This change in structure will allow the heart to restore stroke volume (Lorell & Carabello, 2000).

There is an abundance of intracellular signaling pathways that induce cardiac hypertrophy, perhaps suggesting the importance of the hypertrophic process. Some of the main pathways that have been studied extensively are the mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol 3 kinase-Akt pathway and calcineurin-nuclear factor of activated T cells (NFAT) pathway (Bernardo et al., 2010). The two mechanisms responsible for activating these intracellular pathways are; biomechanical/stretch-sensitive mechanisms and/or neurohumoral mechanisms (Heineke & Molkentin, 2006). Although not fully understood, biomechanical signals such as stretch can be sensed by membrane receptors that are intrinsically unique in structure such that they respond to the stretch signal by activating some of these intracellular signalling pathways. The neurohumoral mechanisms are associated with the release of chemokines, growth factors and hormones that are sensed by receptors on the cell membrane (Heineke & Molkentin, 2006). These receptors in turn, activate second messengers and effectors that will participate in an intracellular signaling cascade culminating in the hypertrophic response. The signaling pathways activated by both mechanisms activate the hypertrophic growth process by modulating the expression of specific genes in the nucleus and increasing protein synthesis while decreasing protein degradation (Heineke & Molkentin, 2006). Cardiac hypertrophy is employed to be an adaptive physiologic process, but can develop into a maladaptive pathologic process as well.

1.2   Physiological Hypertrophy

Physiological hypertrophy is growth of the heart under physiological or disease-free conditions. Post-natal heart growth, pregnancy-induced heart growth and exercise-induced heart growth are all examples of physiological hypertrophy (Bernardo et al., 2010). This physiological growth of the heart is associated with normal cardiac structure and normal or improved cardiac function (Bernardo et al., 2010). Physiological hypertrophy can result in either concentric or eccentric hypertrophy depending on the inciting stimulus and physiological situation. Aerobic exercise increases venous return of blood to the heart resulting in a volume overload on the heart. As described in the earlier sections above this volume overload will lead to chamber dilation/enlargement otherwise known as eccentric hypertrophy (Pluim et al., 2000). Static exercise such as weight lifting results in a pressure overload leading to wall thickening of the myocardium otherwise known as concentric hypertrophy (Bernardo et al., 2010). Metabolically, a heart undergoing physiological hypertrophy demonstrates enhanced fatty acid and glucose oxidation to generate ATP (Gertz et al., 1988). In regards to cell signalling, the insulin-like growth factor (IGF1)-phosphoinositide 3-kinase (PI3K)-Akt signalling pathway has been implicated in physiological hypertrophy. A study was able to demonstrate that cardiac-specific ablation of the IGF1 receptor in mice resulted in the attenuation of the hypertrophic response seen in swim-exercised mice compared to the control mice (Kim et al., 2008). In addition Akt, the downstream target substrate of phosphoinositide 3-kinase was shown to play a major role in physiological hypertrophy but not in pathological hypertrophy. A study demonstrated that Akt1 knockout mice—mice which had normal heart sizes in basal conditions—showed attenuation of the hypertrophic response in response to swim training

compared to control mice. However these Akt1 knockout mice still developed hypertrophy in response to pressure overload via transverse aortic constriction (DeBosch et al., 2006)

In physiological hypertrophy, the cardiomyocyte grows in length more than it grows in width (Heineke & Molkentin, 2006). Thus there is an increase in chamber dimension along with a proportional increase in myocardial wall thickness (Heineke & Molkentin, 2006). Fibrosis does not exist in physiological hypertrophy because there tends to be no cell death. All in all, no cardiac dysfunction results from physiological hypertrophy.

1.3 Pathological Hypertrophy

Pathological cardiac hypertrophy results from growth of the heart caused by disease. Chronic pressure or volume overload leads to myocyte death, fibrotic remodeling and a perpetual hypertrophic program that concludes in cardiac dysfunction and heart failure (Bernardo et al., 2010), which will be discussed in later sections. Examples of pathological conditions that result in chronic pressure or volume overload are hypertension and chronic aortic regurgitation respectively (Lorell & Carabello, 2000). In a chronic pressure overload condition such hypertension or aortic stenosis, there is unrelenting wall stress on the myocardium. In efforts to normalize the wall stress there is an increase in wall thickness, otherwise known as concentric hypertrophy. This process differs from physiological concentric hypertrophy because the inciting stimulus for pathological hypertrophy is usually perpetual and excessive, leading to myocyte death and fibrotic replacement, which further compromises the function of the heart (Levy et al., 1990). Aortic or mitral regurgitations represent chronic volume overload situations where there is

constant accumulation of blood in the heart leading to diastolic wall stress (Grossman et al., 1975). This results in ventricular chamber dilation otherwise known as eccentric hypertrophy. The feature that differentiates physiological eccentric hypertrophy from pathological eccentric hypertrophy is that the inciting stimulus (aortic regurgitation or myocardial infarction) persists leading to perpetual chamber dilation and myocyte death (Bernardo et al., 2010).

The metabolism of the heart during pathological cardiac hypertrophy also differs from the metabolism of a physiologically hypertrophied heart. There is a decrease in fatty acid oxidation and concomitantly an increase in glucose oxidation relative to the normal heart (Vedala et al., 2002). This switch in metabolism may be an energetically favourable process employed by the heart with the intention of allowing the heart to produce more ATP per molecule of oxygen.

There is a multitude of intracellular signalling pathways responsible for activating the pathological hypertrophic program. Heterotrimeric G-protein signalling, MAPK signalling and calcium signalling are just some of the many pathways that participate in the manifestation of pathological hypertrophy (Bernardo et al., 2010). Among calcium signalling pathways, calcineurin-NFAT signalling appears to be the major pathway for the hypertrophic program. Calcineurin is a phosphatase that dephosphorylates NFAT (Haq et al., 2001) allowing for its translocation from the cytoplasm to the nucleus where it transcribe specific genes. A study conducted in 2001 was able to demonstrate that calcineurin activity was increased in the hypertrophied and failing hearts of human patients

(Haq et al., 2001). Another study revealed increases in calcineurin activity in the hypertrophied hearts of rodents following aortic banding (Saito et al., 2003).

As evidenced in figure 1, pathological hypertrophy also differs from physiological hypertrophy in morphology. The myocytes grow in width more than they do in length (Heineke & Molkentin, 2006). As well there is excessive fibrosis due to the perpetual deposition of fibrillary type 1 collagen (Heineke & Molkentin, 2006).
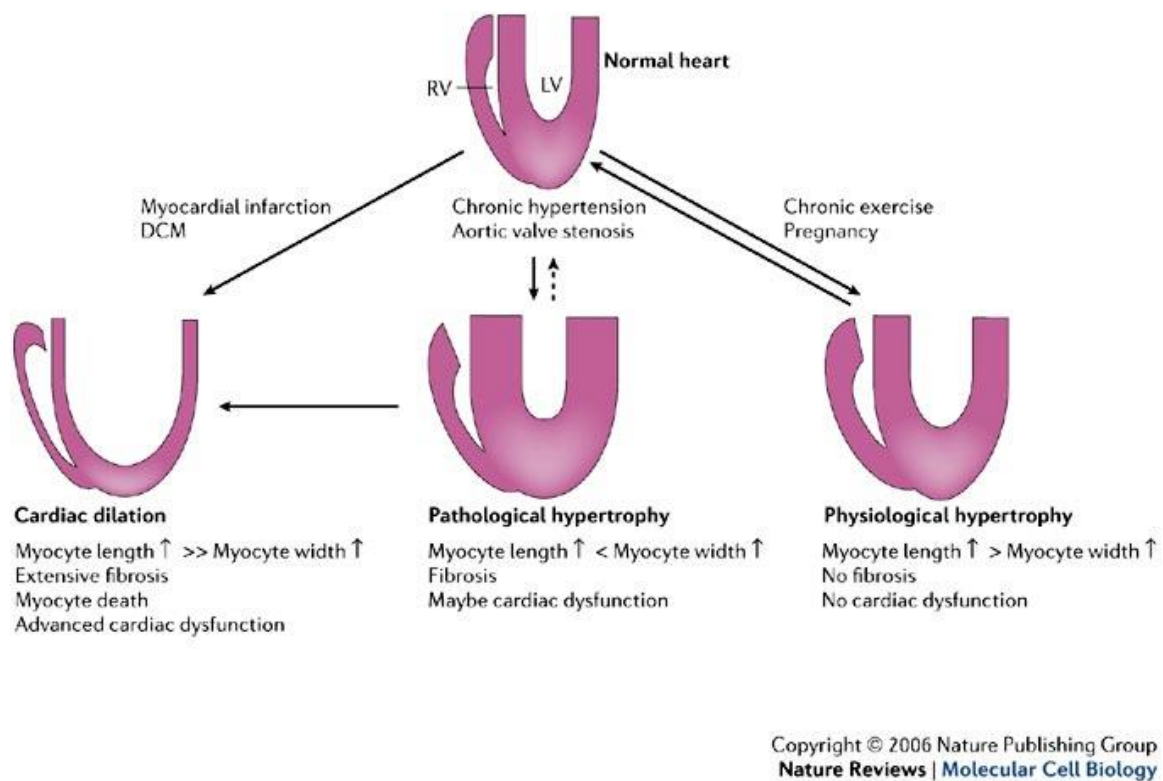
Figure 1: Physiological and pathological types of cardiac hypertrophy. The three manifestations of cardiac hypertrophy depending on the inciting stimulus. " Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology (Heineke & Molkentin, 2006), copyright 2006.

1.4 Heart failure and cardiac hypertrophy

Heart failure is a syndrome characterized by progressively declining left ventricular function leading to inadequate tissue perfusion. This syndrome represents a common final course that is subsequent to an initial stressor on the heart. Multiple prospective studies have elucidated the primary aetiologies of heart failure. One such study is the Hillingdon heart failure population-based study, where the authors categorized 220 new cases of heart failure—identified from a population of 151, 000 patients—into different aetiological groups based on the medical history of the patient and echocardiographic assessment (Cowie et al., 1999). Coronary artery disease, in particular acute myocardial infarction, was the single most common aetiology of heart failure making up 36% of cases. Hypertension was the second most common primary etiology making up 14% of the 220 new cases (Cowie et al., 1999). It is important to note that the 36% of cases attributed to coronary artery disease frequently co-existed with a history of hypertension, but the hypertension was not independent of the coronary artery disease and was therefore not considered as the primary aetiology. Similar to these findings, the Framingham Heart Study identified hypertension and myocardial infarction as the most common attributable risks for congestive heart failure (Kannel, 2000). The mechanisms by which these two aetiological factors cause heart failure will be discussed.

Heart failure constitutes a number of important defining features such as cardiomyocyte hypertrophy, systolic and/or diastolic dysfunction and defective cardiomyocyte calcium homeostasis (Frey et al., 2004) (Peterson et al., 1978) (Beuckelmann et al., 1992). Cardiomyocyte hypertrophy is one of the biological processes

that, both precedes and is involved in, the cardiac remodelling process that takes place during heart failure. To reiterate, there are two types of cardiac hypertrophy; concentric and eccentric hypertrophy. As discussed in previous sections, pressure-overload results in concentric hypertrophy and volume overload results in eccentric hypertrophy. Molecularly, both phenotypic hypertrophies present with a reversion to the fetal gene program. The re-installment of this fetal gene program—involving a re-expression and blunting of genes— modifies the myofilament machinery and energy metabolism of the cardiomyocyte (Swynghedauw, 1999). Atrial natriuretic peptide (ANP) and embryonic myosin light chain are some of the genes that are re-expressed while genes such as the sarco-endoplasmic reticulum calcium ATPase (SERCA) gene are blunted (Swynghedauw, 1999). After a myocardial infarction occurs, there is a loss of functional cardiac tissue. As a result there is a reduction in regional wall motion and a consequential reduction in stroke volume (Pfeffer, 1995). The remaining cardiomyocyte population faces a volume-overload (Pfeffer, 1995) because the number of contractile cells capable of handling normal volumes of blood is decreased. The volume-overload is also a consequence of sympathetic stimulation. The reduction in stroke volume will result in a reduction in blood flow which will trigger the sympathetic nervous system to stimulate the juxtaglomerular cells in the kidneys. These cells will release renin, an enzyme that will cleave angiotensinogen to angiotensin I. Angiotensin I will then be converted to angiotensin II, the effector peptide that will constrict the blood vessels and increase sodium and water reabsorption via the kidneys (Moon, 2013). Vasoconstriction of the veins and an increased fluid volume contribute to an increased preload or volume- overload on the heart (Florea & Cohn, 2014). In addition, these cardiomyocytes also face a pressure overload because the remaining

cardiomyocytes must now generate a greater amount of pressure to match the systolic blood pressure in order to open the aortic valve and subsequently eject blood out of the left ventricle. Neurohumoral responses to decreases in blood flow also result in an increased afterload, which contributes to the pressure overload. Therefore, the heart displays both a dilated ventricular cavity and ventricular wall thickening. This is a compensatory hypertrophy in which the heart tries to rescue the reduction in stroke volume by increasing both chamber size and the number of force generating units within the myocyte (Pfeffer, 1995). Yet, unlike the transient and/or intermittent loads that induce cardiac hypertrophy in physiological conditions, the increased load on the heart after a myocardial infarction is sustained (Lorell & Carabello, 2000). The increased load is further perpetuated by compensatory neurohumoral responses that increase systemic blood pressure. As time progresses, this results in decompensatory hypertrophy. To elaborate, the hypertrophy actually impairs left ventricular function by weakening the ventricular wall and disrupting systolic and diastolic function (Peterson et al., 1978) which ultimately leads to heart failure.

Therefore, attenuation of the hypertrophic response subsequent to cardiac damage is essential to not only the treatment but the prevention of heart failure.

1.5 Angiotensin II and cardiac hypertrophy

Angiotensin II is an octapeptide that exerts vasoconstrictive effects primarily through the AT-1 receptor. This peptide is formed through a series of proteolytic cleavages in a system call the renin-angiotensin-aldosterone system (Griendling et al., 1993). Angiotensinogen, a peptide produced by the liver, is cleaved by renin, a peptide produced by the kidneys. This cleavage results in the production of angiotensin I, a decapeptide.

Angiotensin I is then cleaved to angiotensin II by an enzyme called angiotensin-converting enzyme (Griendling et al., 1993). Angiotensin II circulates in the blood at a concentration of 50-100 pmol/L (Moon, 2013). The physiological function of this peptide is to increase blood pressure by stimulating contraction of the smooth muscle cells in the vasculature, as well as by increasing sodium and water retention (de Gasparo et al., 2000).

Angiotensin II also has direct hypertrophic effects on the heart. This effect is also mediated through the AT-1 receptor. The AT-1 receptor is a seven transmembrane G protein-coupled receptor that is coupled to the Gq protein. Intracellular signalling pathways such as MAPK (Sadoshima et al., 1995), increased intracellular calcium/protein kinase C (PKC) activity (Miyata & Haneda, 1994) and increased leptin autocrine signalling (Rajapurohitam et al., 2006) have been shown to be activated once angiotensin II binds to its AT-1 receptor. As discussed previously in the sections above, these pathways are associated with pathological hypertrophy. Cardiac specific over-expression of the AT-1 receptor in mice resulted in pathological cardiac hypertrophy and subsequent heart failure (Paradis et al., 2000). Another study revealed that mice with a gain of function mutant AT-1 subtype A receptor displayed cardiac fibrosis and diastolic dysfunction (Billet et al., 2007).

The MAPK family consists of a group of serine/threonine kinases that participate in the signal transduction for cell growth. When activated, these MAPKs phosphorylate nuclear transcription factors such as JUN and MYC, thus activating and allowing these transcription factors to regulate the transcription of specific genes (Bernardo et al., 2010). These MAPKs are rapidly activated in response to the stimulation of growth receptors. These growth receptors are tyrosine kinase receptors which when activated, initiate a chain

of tyrosine phosphorylation that will result in the eventual phosphorylation of these MAPKs. However G-protein coupled receptors have also been proven capable of activating this cascade of tyrosine phosphorylation and MAPK activation. One such receptor is the AT-1 receptor. In 1995, a study demonstrated that angiotensin II was able to induce tyrosine phosphorylation of many intracellular proteins and able to activate MAPKs (Sadoshima et al., 1995). The angiotensin II- induced tyrosine phosphorylation was found to be dependent on the increase in intracellular calcium.

Angiotensin II also increases in intracellular calcium and PKC activity by the Gq protein signalling pathway (Mehta & Griendling, 2007). Once angiotensin II binds to the AT-1 receptor, the receptor undergoes a conformational change that results in liberation of the Gq protein from its heterotrimeric G-protein complex. This protein will then activate phospholipase C, an enzyme that will cleave phosphatidyl inositol bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). These products will lead to calcium liberation from the endoplasmic reticulum and the eventual activation of PKC (Mehta & Griendling, 2007).

One of the downstream targets of PKC is the sodium hydrogen exchanger (NHE-1), an antiporter located in the plasma membrane that plays a role in regulating intracellular pH (Leem et al., 1999). It functions by extruding one proton out of the cell while simultaneously bringing in one sodium ion. Thus when proton concentrations exceed physiological levels, this antiporter is activated and starts to extrude out protons. At the very same instant, it brings in sodium ions thus increasing the intracellular sodium ion concentration. An interesting feature of this antiporter is that its sensitivity to proton concentrations can be regulated. When PKC is activated, via a Gq dependent mechanism,

it phosphorylates NHE-1 thus sensitizing the exchanger to proton concentrations in the cell (Karmazyn, 2001). As a result, the exchanger becomes more active at alkaline pHs. Sodium concentrations consequently rise resulting in activation of the sodium-calcium exchanger (NCX), a transporter that will respond to the increase in sodium by removing it from the cell while simultaneously bringing in calcium (Karmazyn et al., 1999). Increases in calcium concentrations result in calcium/calmodulin binding, which primes calmodulin to bind to calcineurin, a phosphatase that is responsible for dephosphorylating NFAT. NFAT will then translocate into the nucleus where it will regulate gene transcription that will induce the hypertrophic program. This signalling cascade will be further discussed in upcoming sections.

Angiotensin II has also been shown to increase leptin autocrine signalling. Furthermore this increase in leptin signalling has been shown to be critical for angiotensin II's ability to induce hypertrophy (Rajapurohitam et al., 2006). To clarify, this study demonstrated that neonatal rat cardiomyocytes treated with angiotensin II exhibited hypertrophy, however when a leptin triple mutant receptor antagonist was used in the presence of angiotensin II, hypertrophy was abolished (Rajapurohitam et al., 2006). The study found that the ability of the leptin antagonist to blunt angiotensin II-induced hypertrophy was not due to an interruption in MAPK signalling. In fact the intracellular mechanism for this phenomenon is not yet clear (Rajapurohitam et al., 2006).

1.6   Leptin

Leptin is a 16 kDa peptide that is the product of the ob gene (Zhang et al., 1994). This peptide is most notably recognized as a satiety factor or appetite suppressant, an effect

caused by its action on the hypothalamus. Leptin circulates in the blood at physiological concentrations of 5-15 ng/mL (Sinha et al., 1996). Leptin plasma levels have been shown to correlate with risk of coronary heart disease (Wallace et al., 2001). Adipose tissue is the main secretor of leptin (Scherer et al., 1995) although other tissues such as cardiac tissue also secrete the hormone (Purdham et al., 2004). Leptin has been shown to directly exert hypertrophic effects on isolated neonatal rat cardiomyocytes (Rajapurohitam et al., 2003). OB-Ra to OB- Rf represent the 6 isoforms of the leptin receptor through which leptin can signal through. However, OB-Rb is distinct from the rest of the five in that structurally it is longer than the other 5 isoforms. In addition, functionally, it has full signalling ability (Chua et al., 1997).

Our lab has demonstrated an interesting interaction between angiotensin II and leptin in the cardiomyocyte. Angiotensin II induced hypertrophy is abrogated by inhibiting the leptin signaling system (Rajapurohitam et al., 2006). To clarify, our lab discovered that stimulation of the cardiomyocyte by angiotensin II lead to an increase in leptin secretion and thus leptin-autocrine signaling. When a leptin receptor blocker or leptin neutralizing antibody was used in the presence of angiotensin II, cardiomyocyte hypertrophy was nullified.

1.7 Fat mass and obesity-associated protein

The fat-mass and obesity associated protein, abbreviated as FTO, became significant in obesity related research after a genome wide association study for type II diabetes was conducted in 2007. The researchers were able to find that for the 1924 type II diabetes patients they screened, there was a common single nucleotide polymorphism SNP

(rs9939609) within the FTO gene on chromosome 16. The strong association between this SNP and type II diabetes was later abolished after an adjustment for body mass index (BMI) was made, suggesting that the actual correlation existed between the SNP and BMI—a risk factor for type II diabetes (Frayling et al., 2007). The study further demonstrated with a separate cohort that those who were homozygous for the risk allele/SNP (16% of the cohort) had a 1.67 increased odds of obesity and weighed about 3 kilograms more (Frayling et al., 2007). The FTO SNPs that are showing strong associations with BMI exist within the intron of this gene. Thus they may alter the transcription of FTO resulting in an altered mRNA transcript and hence an altered FTO translated protein (Gerken et al., 2007).

FTO is a 50 kilodalton peptide that shares sequence motifs with 2-oxoglutarate oxygenases (Gerken et al., 2007). 2-oxoglutarate oxygenases exhibit a diverse range of functions such as DNA repair and histone lysine demethylation. In vitro assays of a recombinant murine FTO protein have revealed the protein's ability to demethylate methyl-thymine nucleic acids in single stranded DNA as well as 3-methyl uridine in single stranded RNA but with relatively lower efficiency (Gerken et al., 2007). The recombinant murine FTO's activity was dependent on the presence of iron and 2-oxoglutarate. As with other 2-oxoglutarate oxygenases, the iron serves as a cofactor for the murine recombinant FTO, while the 2-oxoglutarate serves as a cosubstrate (Gerken et al., 2007). It is quite plausible that given its relevance to obesity, FTO may be regulating the transcription of genes necessary for metabolism and energy balance. FTO mRNA was found to be expressed in all murine tissues with the highest expression found in the brain (Gerken et al., 2007). Specifically, the arcuate nuclei, paraventricular nuclei, dorsomedial nuclei and

ventromedial nuclei of hypothalamus was found to have high levels of FTO mRNA transcripts. This was another finding that bolstered the proposition that FTO could be involved in energy regulation given that the hypothalamus is the primary center in the brain for energy regulation and balance. To further test this proposition, the aforementioned study observed FTO mRNA levels in dissected hypothalamic nuclei from three groups of mice; freely feeding, 48-hour fasted mice and 48-hour fasted mice with daily intraperitoneal injections of leptin. The latter group was included to determine if any changes caused by the fasting were mediated by a reduction in circulating levels of leptin. The researchers observed that there was a 60% reduction of FTO mRNA in the arcuate nucleus of the fasted mice. In addition this reduction was not rescued by leptin supplementation (Gerken et al., 2007). Based on this study FTO levels in the hypothalamus appear to be nutritionally regulated. Another study has shown that a global knockout of the FTO gene in mice results in a lean phenotype (Fischer et al., 2009). These mice exhibit growth retardation from post-natal day 2 onwards and an average weight reduction of about 30-40% six weeks after birth compared to wild-type mice. Magnetic resonance imaging showed a 60% reduction and 23% reduction in fat mass of male and female respectively when compared to the wild-type. There was a significant reduction in epigonadal white adipose tissue with marked decrease in adipocyte size (Fischer et al., 2009). Furthermore, the FTO $^{-/-}$, FTO $^{+/-}$ and FTO $^{+/+}$ mice were subjected to a 12-week high fat diet. After the 12 weeks FTO $^{-/-}$ and FTO $^{+/-}$ mice showed a significantly reduced weight compared to FTO $^{+/+}$ mice (Fischer et al., 2009). This lean phenotype was attributed to an increase in the basal metabolic activity of the mice as food intake does not differ between the knockout mice and the wildtype mice (Fischer et al., 2009).

Wang et al. have demonstrated that leptin, a satiety factor involved in regulating eating behaviour, downregulates FTO protein expression in the hypothalamus (Wang et al., 2011). This study demonstrated this by subjecting mice to energy restriction (60% of the ad libitum food consumption) for 8 weeks and 50 weeks. At both time points, FTO mRNA transcripts and protein expression had significantly decreased. Conversely, in leptin receptor (OB-Rb) deficient mice, db/db, which were also subjected to energy restriction for the same time periods mentioned above there was no decrease in either gene or protein expression of FTO (Wang et al., 2011). Moreover, arcuate nucleus cultures were treated with leptin directly, and a STAT3-dependent FTO downregulation was observed. Thus the authors of the study concluded that energy restriction induced-FTO downregulation in the hypothalamus was being mediated by leptin-STAT3 signalling (Wang et al., 2011). This interaction with leptin, an important metabolic hormone, bolsters the conclusion that FTO regulates metabolism and energy expenditure.

Our lab has dedicated efforts towards trying to elucidate direct mechanisms explaining the association between obesity and cardiac disease. We identified leptin as one of those direct mechanisms, as leptin levels are significantly elevated in obesity. Moreover leptin has been shown to exert hypertrophic effects on the heart via its long-form receptor OB-Rb (Rajapurohitam et al., 2003). Given leptin's STAT3-dependent effect on FTO in the hypothalamus demonstrated by Wang in 2011, our lab was interested in assessing FTO expression in cardiomyocytes in response to leptin. Our lab has been the first, to not only identify FTO in the cardiomyocyte, but also to demonstrate its nuclear localization within the cardiomyocyte, a finding that is concordant with FTO's demethylating functions (Gan

et al., 2013). In addition our lab has shown that leptin also regulates FTO protein expression in the cardiomyocyte.

In contrast to the finding of Wang concerning the interaction between leptin and FTO in the hypothalamus, our lab has shown that leptin upregulates FTO in the cardiomyocyte. Specifically, leptin binds to OB-Rb and results in the dimerization and subsequent translocation of STAT3 into the nucleus. Once in the nucleus, dimerized STAT3 activates the protease cathepsin L resulting in the proteolytic cleavage of p 200 CUX into p 75 and p 110 CUX 1 proteins that are transcription factors. The active transcription factor p 110 upregulates the transcription of the FTO gene (Figure 2). Moreover, this upregulation is responsible for leptin's hypertrophic effect on the cardiomyocyte. When FTO is silenced using small interfering RNA (siRNA), the hypertrophic response of the cardiomyocyte to leptin is abolished (Gan et al., 2013). Thus

FTO upregulation in the cardiomyocyte is necessary for leptin-induced cardiomyocyte hypertrophy.
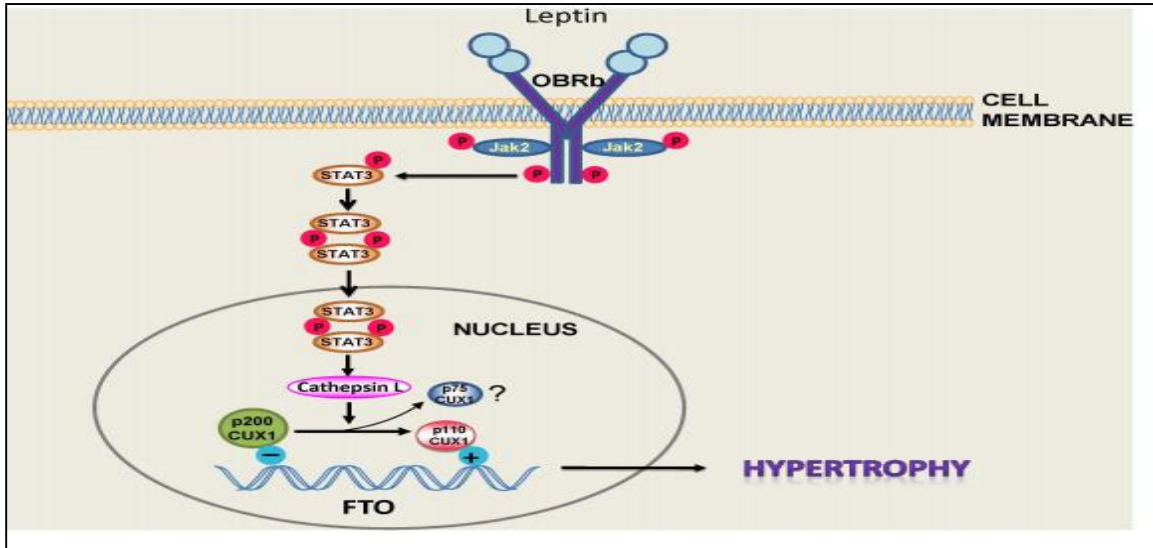
Figure 2: Proposed mechanism for leptin-induced JAK2/STAT3-dependent FTO upregulation. Activation of the long form receptor (OB-Rb) results in the dimerization and translocation of STAT3 into the nucleus where it activates the protease cathepsin L resulting in proteolytic processing of p200 CUX to the shorter p75 and p110 CUX1 proteins. The latter upregulates FTO resulting in the hypertrophic response (Gan et al., 2013).

1.8 Hypothesis and objectives

Rationale: In our lab, we study mechanisms that lead to cardiac hypertrophy and identify specific probable molecular targets for pharmacological intervention. We have identified that leptin, a satiety factor produced in our body induces cardiac hypertrophy. Leptin's hypertrophic effect appears to be mediated through multiple intracellular

signalling pathways. One of these pathways involves an increase in calcineurin activity. When calcineurin is inhibited, using calcineurin inhibitor FK506, the leptin-induced hypertrophy is abolished (Rajapurohitam et al., 2012). As mentioned previously, FTO upregulation also mediates leptin's hypertrophic effect on the cardiomyocyte. When FTO is silenced using siRNA, leptin's hypertrophic effect is abolished. Thus it appears that FTO upregulation and calcineurin activation are necessary molecular events for leptin-induced cardiomyocyte hypertrophy. This may suggest that FTO and calcineurin share a common pathway. In this current study, we were interested in determining which other hypertrophic agonists had the ability to regulate FTO expression in the cardiomyocyte. In the first objective of this current study, we observed that angiotensin II was able to significantly regulate FTO in the cardiomyocyte. Angiotensin II has been demonstrated to activate the NHE-1/calcineurin signalling pathway via the Gq protein signalling pathway, resulting in hypertrophy. This bolsters the hypothetical concept that the activation of calcineurin and the upregulation of FTO may take place in a common pathway. As well, angiotensin II has been demonstrated to increase leptin synthesis and secretion from the cardiomyocyte leading to autocrine effects of leptin on the cardiomyocyte. When leptin signaling is disrupted in the presence of angiotensin II, angiotensin II induced hypertrophy is abrogated. Therefore I have formulated two hypotheses; **1. Angiotensin II-induced FTO upregulation is mediated by NHE-1/calcineurin activation** and **2. Angiotensin II-induced leptin secretion and autocrine signalling leads to FTO upregulation** in neonatal rat ventricular myocytes. To carry out my investigation of this hypothesis, I have created a tentative list of objectives phrased in the form of major questions I sought to answer.

Objectives for Hypothesis 1:

a) **Do other hypertrophic agonists (apart from leptin) upregulate FTO?**

- To assess the effects of angiotensin II and phenylephrine on FTO expression

- To formulate a hypothesis based on the results of the above sub-aim

b) **Does NHE-1 and Calcineurin play a critical role in angiotensin II-induced FTO upregulation?**

- To assess the effects of NHE-1 inhibitor, EMD87580, and calcineurin inhibitor, FK506, on angiotensin II-induced FTO regulation

Objectives for Hypothesis 2:

a) **Does angiotensin II increase JAK2-STAT3 signalling**

- To assess jak2-stat3 activation after angiotensin II treatment with or without quadruple mutant leptin receptor antagonist

b) **Is angiotensin II-induced FTO upregulation JAK2 dependent?**

- To assess FTO expression after angiotensin II treatment with or without jak2 inhibitor AG490.

c) **Does angiotensin II- induced FTO upregulation involve leptin signaling?**

- To assess FTO expression after angiotensin II treatment with or without quadruple mutant leptin receptor antagonist

Chapter 2

2 Materials and Methods

2.1 Primary culture of rat neonatal ventricular cardiomyocytes

Neonatal hearts were extracted from 1-5 days old Sprague–Dawley rats (Charles River Canada, Montreal, Quebec, Canada). After the neonates were sacrificed, 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. Atria 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 Erlenmeyer 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). After each digestion, the supernatant was removed from the flask and poured into an Eppendorf tube containing the same volume of stop buffer which consisted of 10% 10 x HBSS, 2% Penicillin/streptomycin and 20% Fetal Bovine Serum (FBS) to abolish enzyme 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 514 g at 4°C. The supernatant was discarded and pellets were

re-suspended in the cell culture medium. The suspension was subjected to pre-plating for one hour followed by another 30 min of pre-plating to ensure cardiomyocyte enrichment. Following pre-plating, Primaria™ (Falcon) culture dishes were plated with cardiomyocytes at an optimal density, a density dependent on the type of experiment being conducted. Cells (1×10^4 cells) were plated to perform cell surface area analysis as this quantity ensured sufficient density to visualize growth while 6×10^4 cells were plated for RNA and protein isolation as this quantity ensured sufficient density for gene expression and protein expression. Cells were counted using Countess Automated Cell Counter (Life Technologies Inc, Burlington, Ontario, Canada). 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) and cultured in serum-free medium for another 24 hours before starting the treatments. All the reagents were filtered, sterilized and autoclaved.

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

Chemical Name	Concentration	Manufacturer
DMEM/F12	1 pkg	Gibco
NaHCO₃	2.4 mg/ml	Sigma-Aldrich
Fetal Bovine Serum (FBS)	10 %	Gibco
Hepes	30 mM	Gibco
Bromodeoxyuridine	0.1 mM	Sigma-Aldrich
Linoleic Acid	5 µg/ml	Sigma-Aldrich
Pyruvic Acid	3 mM	Sigma-Aldrich

Bovine Serum Albumin (BSA)	2 mg/ml	Gibco
Penicillin/Streptomycin	1 %	Gibco
Insulin	10 µg/ml	Sigma-Aldrich
Holo-Transferrin	10 µg/ml	Sigma-Aldrich
Sodium Selenite	10 ng/ml	Sigma-Aldrich
L-Ascorbic Acid	100 µM	Gibco
MEM Non-essential Amino Acids	1 %	Sigma-Aldrich
MEM Vitamins 100X	0.1 %	Sigma-Aldrich

Table 2: **Composition of PBS-ABC solution for neonatal rat cardiomyocytes. All chemicals are from Sigma-Aldrich (Oakville, ON, Canada)**

Reagent	Chemical	Concentration
PBS A	NaCl	10 g/L
	KCl	0.250 g/L
	$Na_2HPO_4 \cdot 7H_2O$	2.71 g/L
	KH_2PO_4	0.250 g/L
PBS B	$CaCl_2 \cdot 2H_2O$	1.32 g/L

PBS C	MgCl$_2$·6H$_2$O	2.13 g/L

PBS ABC	PBS A	80 %
	PBS B	10 %
	PBS C	10 %

Table 3: **Serum-free medium reagents for neonatal rat cardiomyocytes**

Chemical Name	Concentration	Manufacturer
DMEM/F12	1 pkg	Gibco
NaHCO$_3$	2.4 mg/ml	Sigma-Aldrich
Bromodeoxyuridine	0.075 mM	Sigma-Aldrich
Bovine Serum Albumin (BSA)	10 mg/ml	Gibco
Penicillin/Streptomycin	1 %	Gibco
Insulin	10 µg/ml	Sigma-Aldrich
Holo-Transferrin	5 µg/ml	Sigma-Aldrich
Sodium	10 ng/ml	Sigma-Aldrich

Selenite		
Fetuin	250 µg/ml	Sigma-Aldrich
CaCl$_2$	80 µg/ml	Sigma-Aldrich
L-Ascorbic Acid	100 µM	Gibco
MEM Non-essential Amino Acids	1 %	Sigma-Aldrich
MEM Vitamins 100X	0.1 %	Sigma-Aldrich
BSA-Palmitate	0.2 %	Sigma-Aldrich

2.2 Cell surface area analysis of cardiomyocytes

Cardiomyocytes were allowed to grow in serum-containing medium for 24 hours. They were then washed with PBS-ABC before allowing them to grow again in serum-free medium for 24 hours before treatment. Cells were treated with angiotensin II (100 nM), phenylephrine (10 µM) or leptin (3.1 nM) in the presence or absence of NHE-1 inhibitor EMD87580 (5 µM), calcineurin inhibitor FK506 (2 nM), Valsartan (10 µM), super-ovine leptin antagonist (LRA) (100 nM) and JAK2 inhibitor AG490 for 24 hours. For cell surface area, the mean of 50 cells were used to provide one value. 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).

Table 4: **Chemicals used, their concentrations and their manufacturers**

Chemical name	Concentration	Manufacturer
Angiotensin II	100 nM	Sigma-Aldrich
Phenylephrine	10 µM	Sigma-Aldrich
Valsartan	10 µM	Sigma-Aldrich
EMD87580	5 µM	Merck
FK506	2 nM	Cell Signalling Technology
LRA	100 nM	Protein Laboratories
Leptin	3.1 nM	Sigma-Aldrich
AG490	50 µM	Santa Cruz

2.3 RNA isolation and purification

Following the treatments, total RNA from cells was extracted using TRIzol® Reagent (BioRad 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 g 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 g for 20 minutes at 4 °C. Often, a gel-like pellet formed on the side and bottom of the tube. This pellet was the RNA. The supernatant was discarded from the tube, leaving only the RNA pellet. Ethanol (75%) in diethylpyrocarbonate (DEPC) water was added to the tube to wash the RNA. The tube was vortexed briefly, then centrifuged at 10,000 g for 20 minutes at 4 °C. Consequently the RNA pellet attached to the bottom of the tube, and the supernatant was removed. The pellet was air dried for only 10 minutes to prevent loss of solubility. The RNA pellet was

dissolved in DEPC treated water. The RNA was quantified using MD SpectraMax M5e Reader (Molecular devices, Sunnyvale, CA, USA).

2.4 First strand cDNA synthesis

1-5 µg 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 reactions (PCR). The cDNA synthesis was performed in a nuclease-free micro-centrifuge tube. 1-5 µg of RNA was added in the tube followed by DEPC water to make up the volume to 11 µL. Then 1x random primer and 5 mM deoxynucleoside triphosphates (dNTP) mix was added to the same tube. 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 first strand buffer, 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 followed by termination of the reaction by heating it to 70 °C for 15 minutes. All the reagents were from Invitrogen, Life Technologies Inc, Burlington, ON, Canada.

Table 5: **Mixture composition for reverse transcription**

Component	Volume/reaction (µL)
1x Random Primer	1
5mM dNTP mix	2
5x First Stand Buffer (FSB)	4

0.1M DTT	2
MLV-RT	1

2.5 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 (BioRad 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. PCR entailed amplification of gene for 35-40 cycles with temperature changes. The PCR reaction steps consists of the initiation 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 the Taq polymerase enzyme synthesizes a new complementary DNA strand). The primer sequences used for 18s, ANP, α-skeletal actin, FTO, CUX1, Cathepsin L and MCIP are shown in table 7. 18S mRNA expression was used as a control.

Table 6: **Real Time PCR reaction mixture**

Component	Volume/reaction (µL)
EvaGreen qPCR Mastermix	5
Forward Primer	0.5
Reverse Primer	0.5
cDNA	2
Double Distilled Water	2

Table 7: **Primer sequences and Real Time PCR conditions**

Primers	PCR parameters	
	Phase	**Temperature in ºC**
18s Forward: 5'-GTATCCCGTTGAACCCCATT-3' Reverse: 5'-CCATCCAATCGGTAGTAGCG-3'	Denaturation Annealing Extension	95 58 72
ANP Forward:	Denaturation Annealing	95 58

5'-CTGCTAGACCACCTGGAGGA-3'	Extension	72
Reverse:		
5'-AAGCTGTTGCAGCCTAGTCC-3'		
α-skeletal actin	Denaturation	95
Forward:	Annealing	58
5′-CACGGCATTATCACCAACTG-3′	Extension	72
Reverse:		
5′-CCGGAGGCATAGAGAGACAG-3′		
FTO	Denaturation	95
Forward:	Annealing	58
5'- AGGGCTGCACCATCAATTAC-3'	Extension	72
Reverse:		
5'- TCACGTTGTAGGCTGCTCTG-3'		
MCIP	Denaturation	95
Forward:	Annealing	58
5'-GCCCAATCCAGACAAACAGT -3'	Extension	72
Reverse:		
5'-TGATTTTTGGCTTGGGTCTC -3'		
Cathepsin L	Denaturation	95

Forward:	Annealing	58
5'-GAGAGCAGTGTGGGAGAAGA-3'	Extension	72
Reverse:		
5'-ATCTGCAGCATCAGAGGTTC-3'		
CUX1	Denaturation	95
Forward:	Annealing	58
5'-GGAGGAAGCTGAGCACAAAC-3'	Extension	72
Reverse:		
5'-GAGGCCAGTTGGAGAGAGTG-3'		

2.6 Western Blotting

Cells were treated with appropriate compounds and washed with cold PBS. PBS was discarded completely without drying the cells and lysis buffer containing various compounds and protease inhibitors were added. The cells were scraped using a dish scraper 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 g for 10 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 assay, plates were read for absorbance at 595 nm and the amount of protein was determined using a BSA standard curve. 30 to 50 µg of protein was used to prepare samples and 6x sample buffer containing β-mercaptoethanol (1:10 dilution) was added. These samples were heated at 100 °C for 10 minutes. Then sodium dodecyl sulfate (SDS) containing polyacrylamide gels were prepared. 10% gels were made.

Gels were transferred into the box and the box was filled with 1x Running Buffer. Samples were loaded into the wells of the gel and 9 µL protein marker (Bio-Rad) was loaded in one well. The whole box was connected to the power source and a constant 100 mV electric current was provided. The proteins were then separated according to their molecular weights 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 (0.45 µm), 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 with 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 FTO, β-actin NFAT3, PCNA, P-JAK2, JAK2, P-STAT3, STAT3. 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 antibodies (goat-anti-mouse, goat-anti-rabbit and donkey-anti-goat) were added. The boxes were kept on the shaker for one hour. After one hour the membranes were washed again with wash buffer three times. 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. Due to the use of different software for the imaging of the blots and the quantification of the blots, the densitometric measurements may not exactly reflect the density of the blots seen in the images.

Table 8: **Composition of lysis buffer**

Chemical	Concentration	Gm/ml
Tris-HCl	50 mM	1.212
NaCl	150 mM	1.740
Triton	1%	2 ml
Glycerol	10%	20 ml
EDTA	2 mM	0.117
EGTA	2 mM	0.152

NaF	50 mM	0.42
Na_3VO_4	200 μM	0.0072
$Na_4P_2O_7$	10 mM	0.892
β-Glycerophosphate	40 mM	1.782

Table 9: **Composition of buffers used in western blotting. The pH was adjusted to 7.5**

Chemical	Running Buffer	Transfer Buffer	Wash Buffer
Tris base	3 gm	3 gm	3 gm
Glycine	14.4 gm	14.4 gm	---
SDS	1gm	---	---
NaCl	---	---	9 gm
Tween20	---	---	1 ml
Methanol	---	200 ml	---
Water	1 liter	800 ml	1 liter

Table 10: **Details of antibodies, dilution ranges, types and manufacturers**

Antibody	Dilution range	Type	Manufacturer

NFAT3	1:500	Rabbit polyclonal	Santa-cruz
FTO	1:500	Mouse monoclonal	Abcam
p-JAK2	1:500	Goat polyclonal	Santa-cruz
JAK2	1:500	Mouse monoclonal	Santa-cruz
p-STAT3	1:1000	Rabbit polyclonal	Cell signalling
STAT3	1:1000	Rabbit polyclonal	Santa-cruz
β-Actin	1:1000	Goat polyclonal	Santa-cruz
PCNA	1:500	Mouse monoclonal	Santa-cruz

2.7 Immunofluorescence

Cardiomyocytes were grown on collagen-pre-coated glass coverslips in medium.
Following 24 hour incubation with treatments, cells were fixed in 20:80 acetone: methanol
for 20 min at 4°C. After 5 minutes of permeabilization in 0.3% Triton X-100 PBS,
myocytes were blocked for 1 h in 1% BSA in PBS A buffer solution and then incubated
overnight with the primary antibody against FTO (1:50 dilution) 1% BSA in PBS A buffer,
then for 1 hour with a secondary antibody (goat anti-mouse). Nuclei were counterstained
with the nuclear stain Hoechst 33342 (1 mg/ml, SigmaAldrich) for 5 minutes.
Cardiomyocytes were visualized using an Olympus IX81 inverted fluorescence
microscope (Olympus, Shinjuku Tokyo, Japan) and quantification performed using
SigmaScan Pro v.5 software (Systat Software Inc., San Jose, CA, USA). Due to the use of

different software for the imaging of the cells and quantification of the fluorescence, the fluorescent measurements may not exactly reflect the fluorescence intensity seen in the images.

2.8   Statistical analysis

All the experiments were analyzed using GraphPad Prism 6. All data are represented as mean ± standard error mean (SEM).

Data for cell surface area, gene expression, protein expression and immunofluorescence were analyzed using one-way analysis of variance (ANOVA) followed by a post hoc Tukey test.

Chapter 3

3 Results

3.1 Angiotensin II and phenylephrine induces cardiomyocyte hypertrophy

As evident from Figure 3A and 3B, both phenylephrine and angiotensin II treatments result in hypertrophic responses from neonatal rat cardiomyocytes. Phenylephrine treatment resulted in an increase in cell surface area (Figure 3A) and an increase in ANP gene expression ($P < 0.05$) (Figure 3B). Angiotensin II treatment also resulted in an increase in cell surface area and an increase in ANP gene expression ($P < 0.05$).
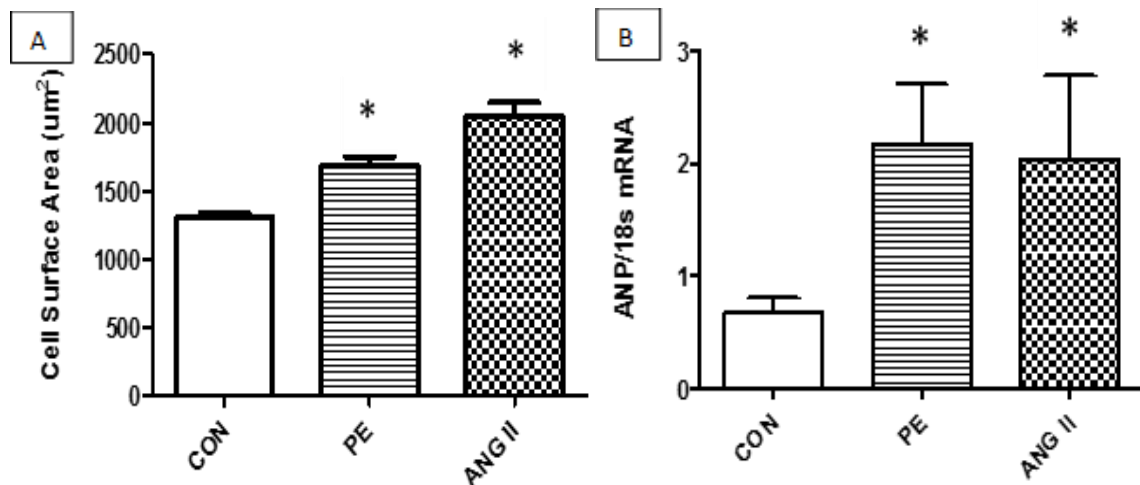

Figure 3: Angiotensin II & phenylephrine induce cardiomyocyte hypertrophy. Panel A displays the quantified assessment for cell surface area of neonatal rat cardiomyocytes after 24 h treatment with phenylephrine (PE) or angiotensin II (ANG II). Panel B displays fetal gene atrial natriuretic peptide (ANP) expression after 24 h treatment with PE or ANG II. Cell surface area and ANP gene expression data are presented as mean ± SEM. (n=8). *p<0.05 from control (CON) group.

3.2 Angiotensin II upregulates FTO in the cardiomyocyte

Only angiotensin II displays the ability to significantly increase the expression of FTO, demonstrating an increase in FTO gene expression (Figure 4A) ($P < 0.05$) and an increase in FTO protein expression (Figure 4B) ($P < 0.05$). Phenylephrine did not significantly increase FTO expression.
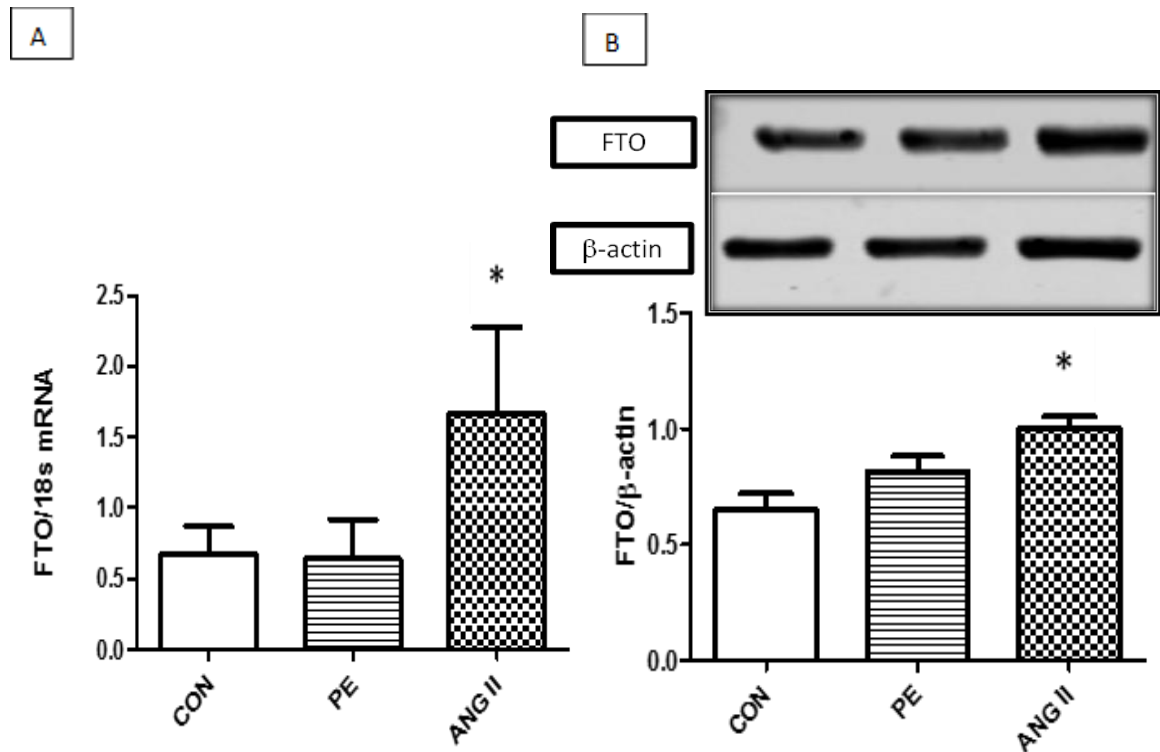

Figure 4: Angiotensin II upregulates FTO in the cardiomyocyte. Panel A displays FTO gene expression after 24 hour treatment with phenylephrine (PE) or angiotensin II. Panel B displays FTO protein expression after 24 hour treatment with phenylephrine or angiotensin II. FTO gene expression and FTO protein expression data are presented as mean ± SEM (n=5-8). *p<0.05 from CON group.

3.3 Angiotensin II-induced hypertrophy is attenuated by AT-1 receptor antagonist valsartan, NHE-1 inhibitor EMD87580 and calcineurin inhibitor FK506

As evident from Figure 5A and 5B the hypertrophic effect of angiotensin II is attenuated by AT-1 receptor antagonist valsartan, NHE-1 inhibitor EMD87580 and calcineurin inhibitor (FK506). Specifically, in panel A, angiotensin II significantly increases cell surface area, which is normalized back to control levels by the AT-1 receptor antagonist valsartan, the NHE-1 inhibitor EMD87580 and the calcineurin inhibitor FK506 ($P<0.05$). In panel B, alpha-skeletal actin gene expression, which is also significantly increased by angiotensin II is also normalized back to control levels by valsartan, EMD87580 and calcineurin inhibitor FK506 ($P<0.05$).
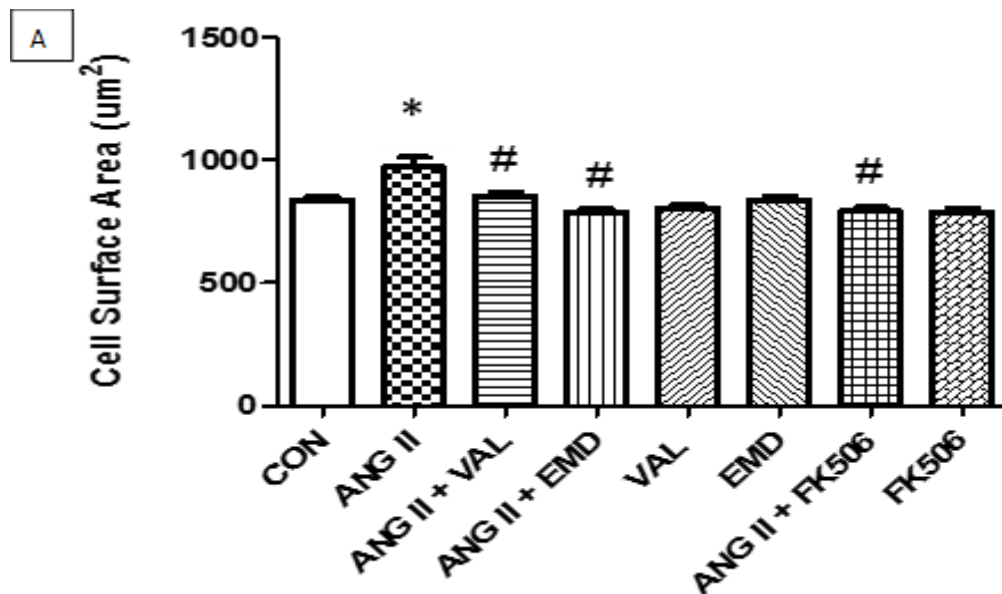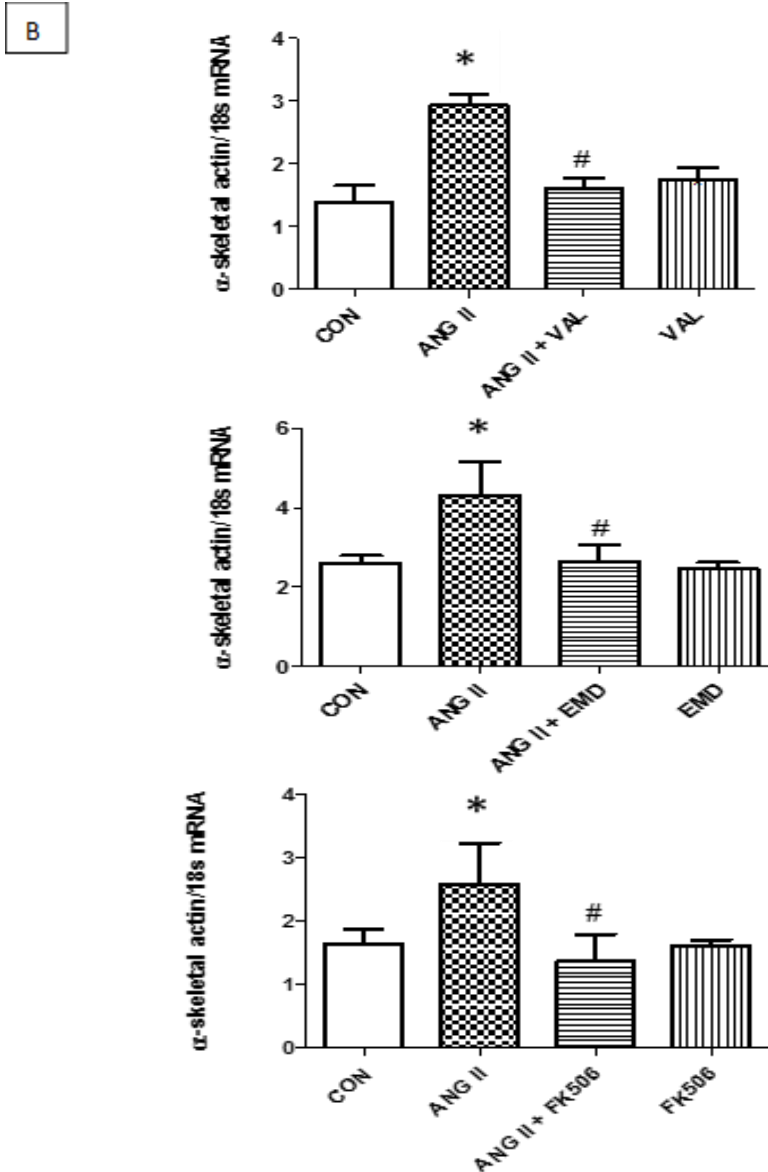
Figure 5: Angiotensin II-induced hypertrophy is attenuated by valsartan, EMD87580 and FK506. Panel A displays cell surface area of cardiomyocyte with ANG II and/or AT-1 receptor blocker valsartan (VAL) and/or NHE-1 inhibitor EMD87580 (EMD) and/or calcineurin inhibitor (FK506). Panel B displays α-skeletal actin gene expression in the cardiomyocyte with the same treatments mentioned above. Cell surface area and α-skeletal actin gene expression data are presented as mean ± SEM (n=6-8). *$p<0.05$ from CON group. #$p<0.05$ from ANG II group**.**

3.4 Angiotensin II-induced FTO upregulation is attenuated by valsartan, EMD87580 and FK506

The ability of angiotensin II to increase FTO levels in neonatal rat cardiomyocytes is significantly attenuated by valsartan, EMD87580 and FK506. As evident in Figure 6A, the increase in FTO gene expression is abolished by all the three agents mentioned above ($P<0.05$). In addition, as seen in Figure 6B, increase in FTO protein expression is also abolished by all the three agents mentioned above ($P<0.05$).

Figure 7 (A, B, C) reiterates the effect of these three agents on angiotensin II-induced FTO upregulation through immunofluorescence. Angiotensin II demonstrated a significant increase in nuclear fluorescence intensity that was attenuated by valsartan, EMD87580 and FK506 ($P<0.05$).
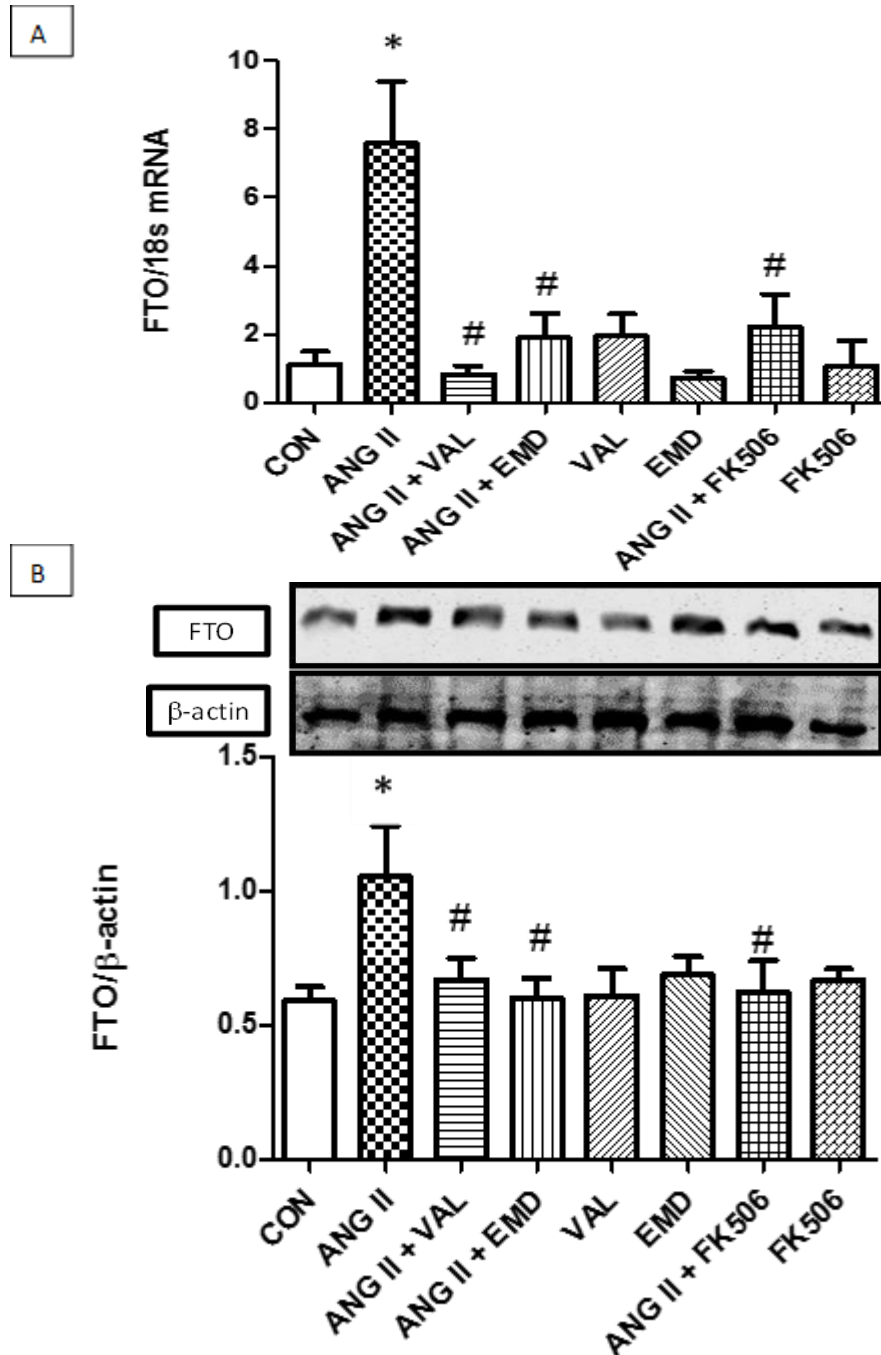
Figure 6: Angiotensin II-induced FTO upregulation is attenuated by valsartan, EMD87580 and FK506. FTO gene expression (A) and western blot with respective quantitative analysis of FTO protein expression (B) data in response to 24 h treatments of neonatal rat cardiomyocytes with ANG II and/or VAL and/or EMD and/or FK506. Quantitative data presented as mean ± SEM (n=6-8). *p<0.05 from CON group. #p<0.05 from ANG II group

A

	CON	ANG II	ANG II + VAL	VAL
FTO				
DAPI				
MERGED				
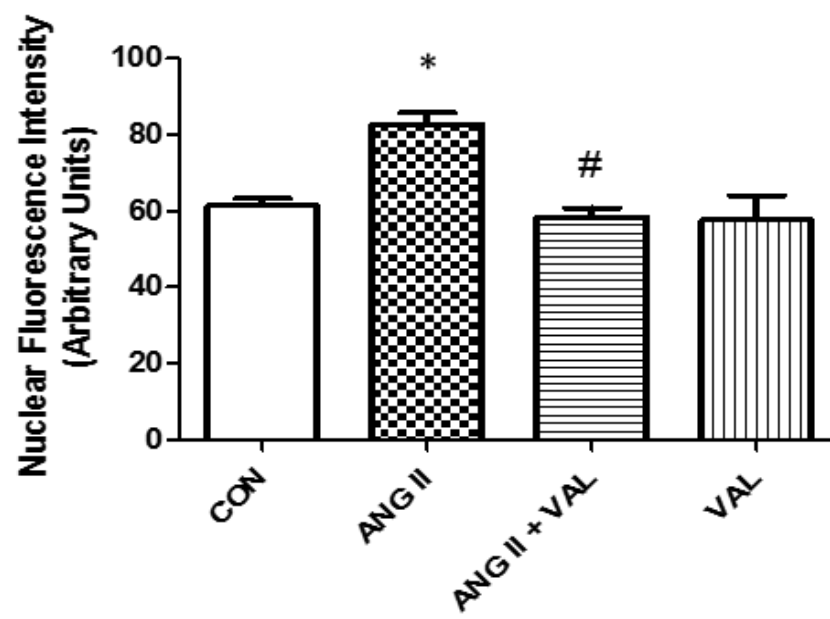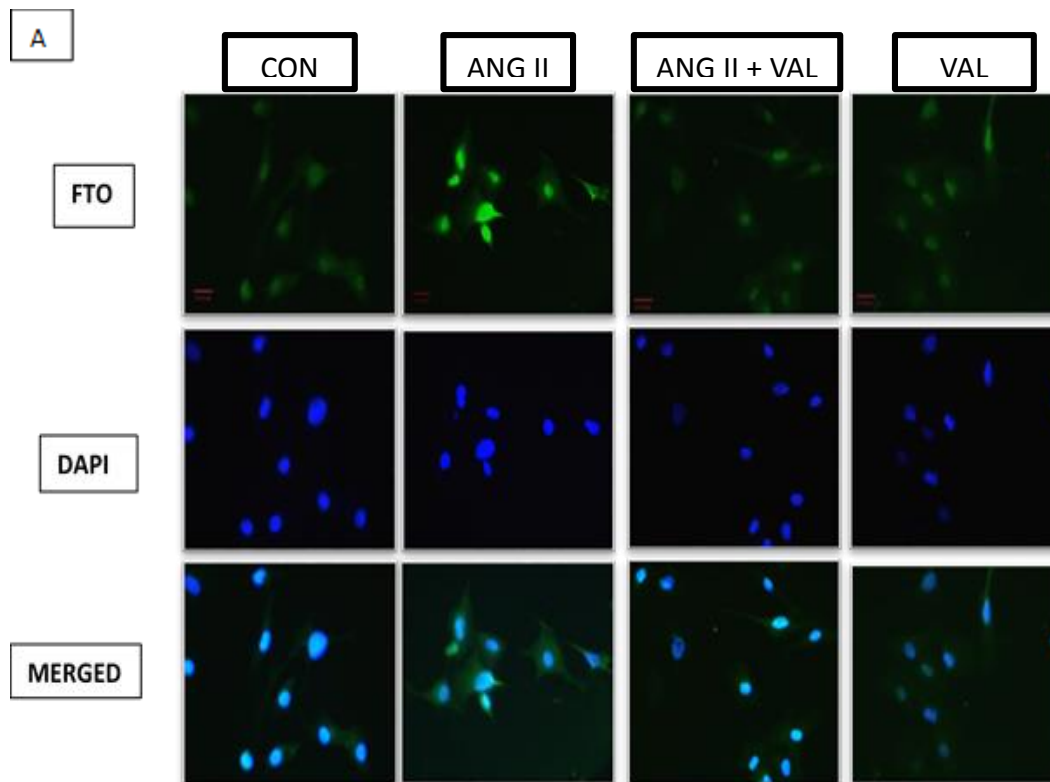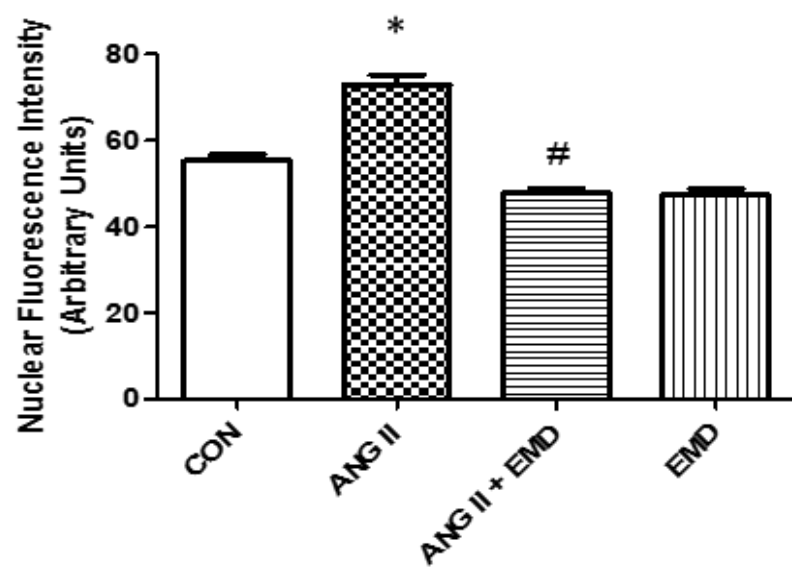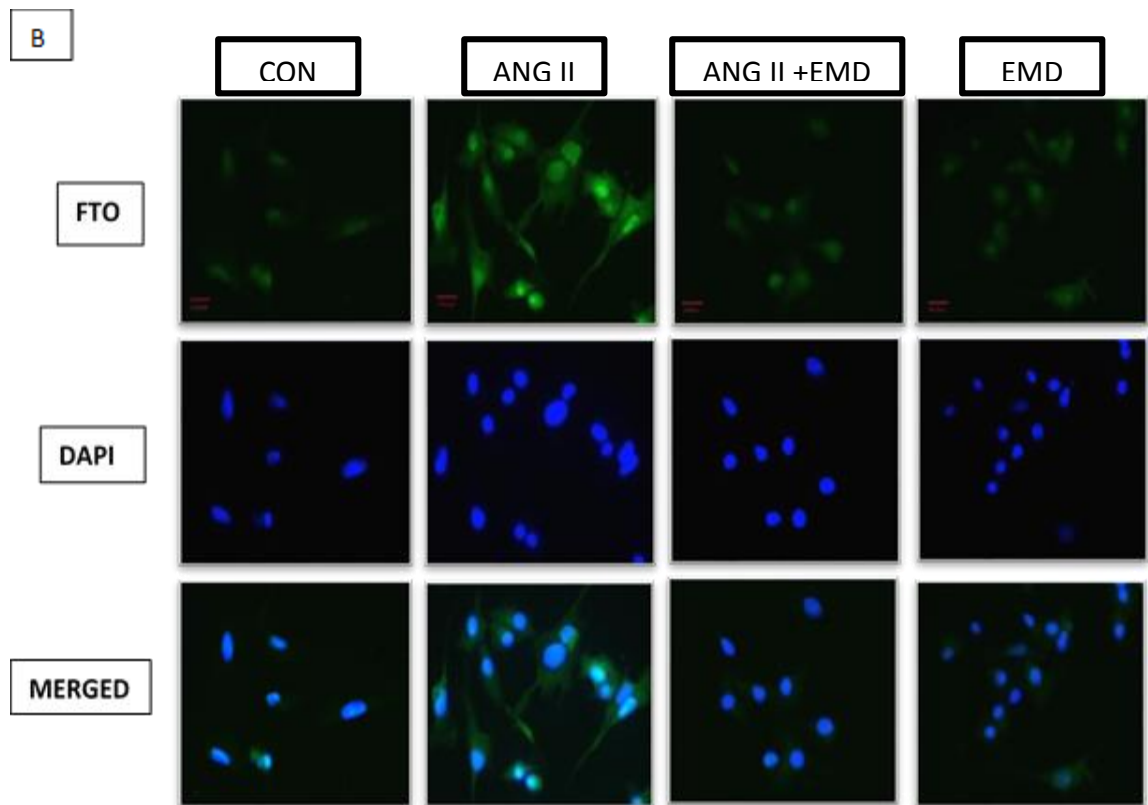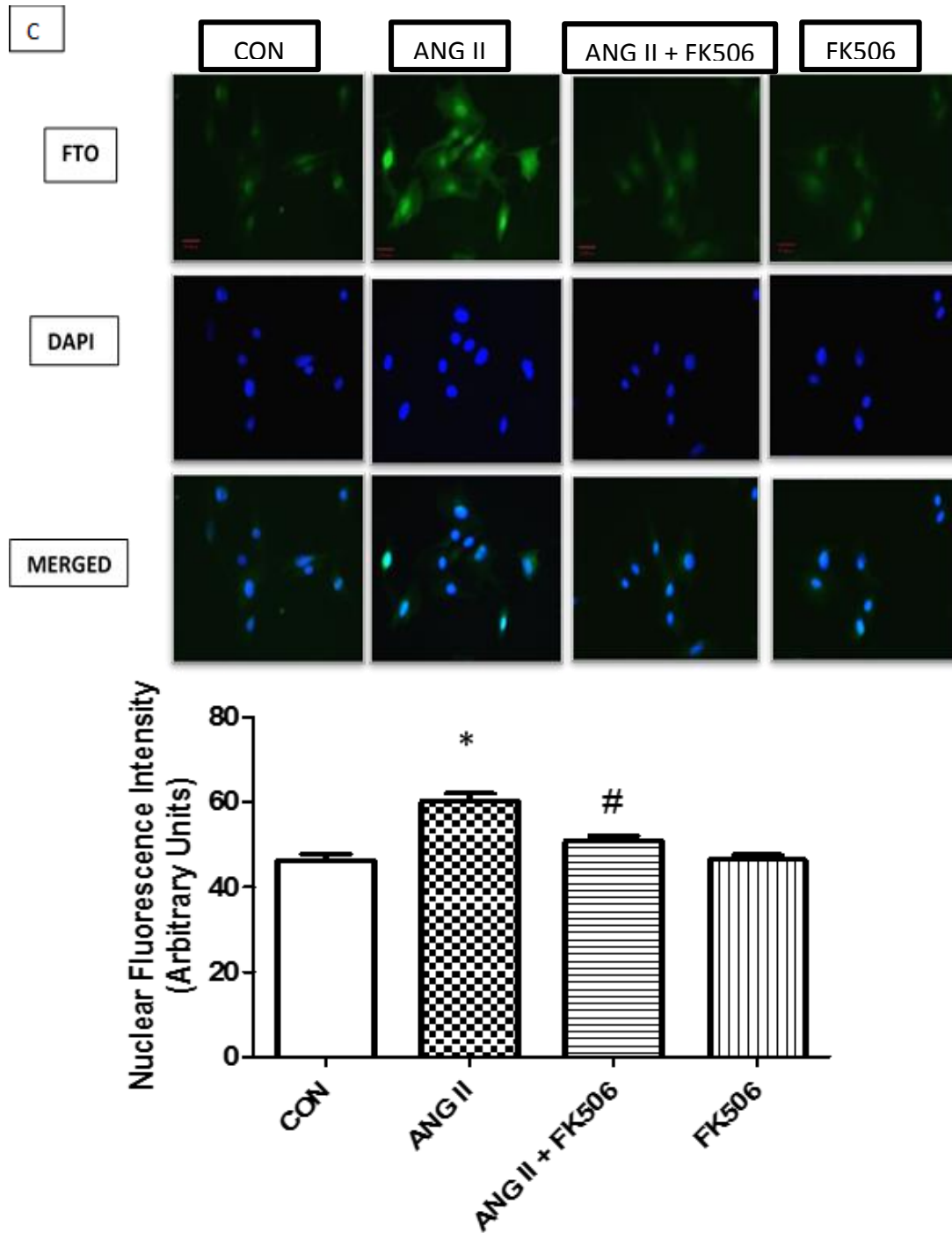
Figure 7: Nuclear fluorescence images of FTO in fixed cardiomyocytes and respective quantitative analysis. Nuclear fluorescence of FTO in the cardiomyocyte treated with ANG II and/or VAL (A) and/or EMD (B) and/or FK506 (C) and respective quantitative analysis (200 ms exposure time). Quantitative data presented as mean ± SEM (n=3). *p<0.05 from CON group. #p<0.05 from ANG II group.

3.5 Evidence of NHE-1/calcineurin activation

Figure 8 displays MCIP gene expression data in response to the treatment groups discussed above. Angiotensin II significantly increases MCIP expression. This is evidence of angiotensin II's ability to activate calcineurin. This effect is attenuated by valsartan, EMD87580 and FK506 ($P<0.05$).

As evident in Figure 9, angiotensin II also significantly increases NFAT 3 translocation to the nucleus after 24 h treatment. This translocation is abolished by FK506 ($P<0.05$).
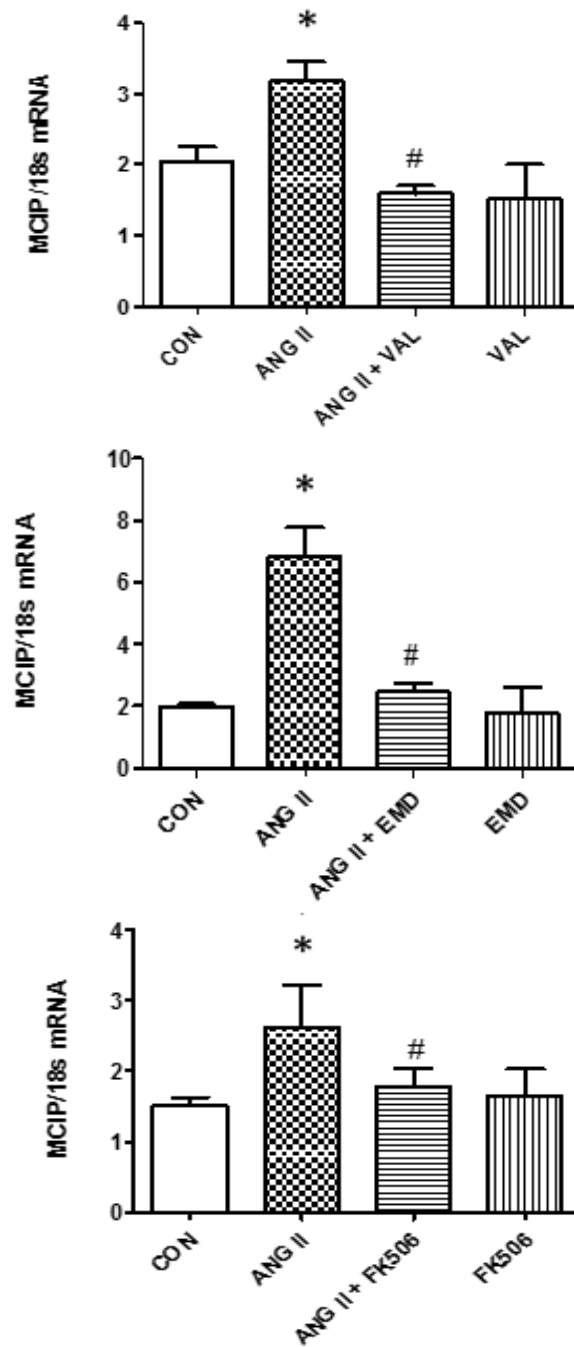
Figure 8: Evidence of calcineurin activation; Modulatory Calcineurin Interacting Protein (MCIP). MCIP gene expression in response to 24 h treatments cardiomyocytes with ANG II and/or VAL and/or EMD and/or FK506. MCIP gene expression data are presented as mean ± SEM (n=6). *p<0.05 from CON group. #p<0.05 from ANG II group.
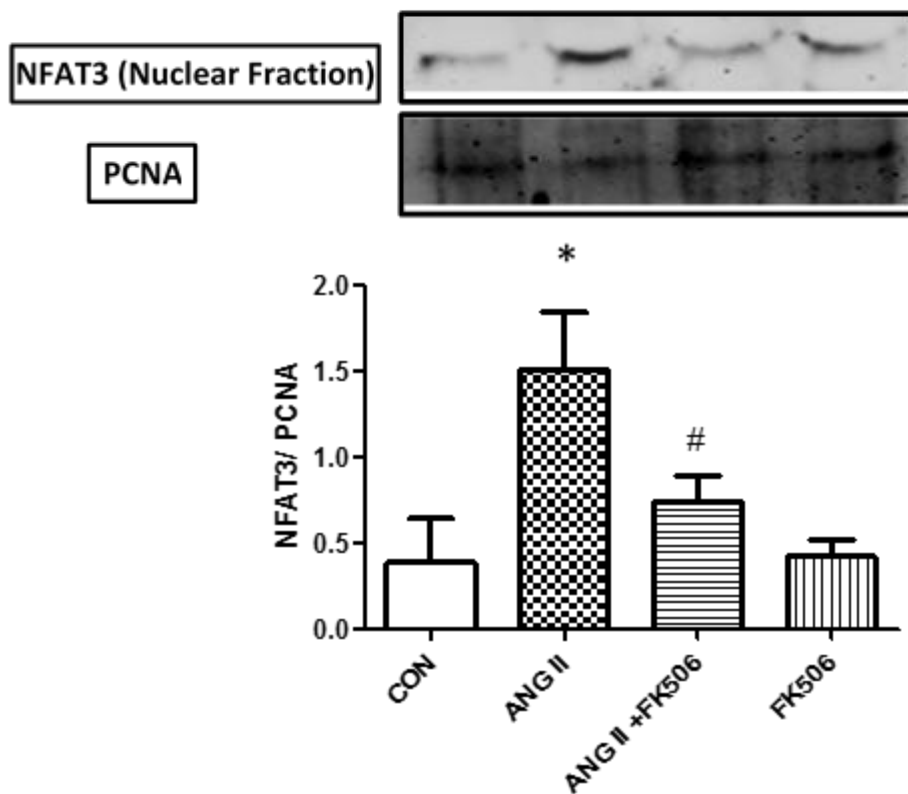
Figure 9: Evidence of calcineurin activation; Nuclear Factor of Activated T-cells isoform 3 (NFAT3). Western blot and respective quantitative analysis of NFAT3 translocation to the nucleus after 24 h treatment with ANG II and/or FK506. NFAT3 protein expression gene expression data are presented as mean ± SEM (n=3). *p<0.05 from CON group. #p<0.05 from ANG II group.

3.6 Angiotensin II-induced hypertrophy is attenuated by super-ovine leptin receptor antagonist (LRA)

Angiotensin II-induced hypertrophy is significantly attenuated by the super-ovine leptin receptor antagonist (LRA). The significant increase in cell surface area (Figure 10A) and α-skeletal actin gene expression (Figure 10B) caused by angiotensin II is attenuated by the leptin receptor antagonist ($P<0.05$).
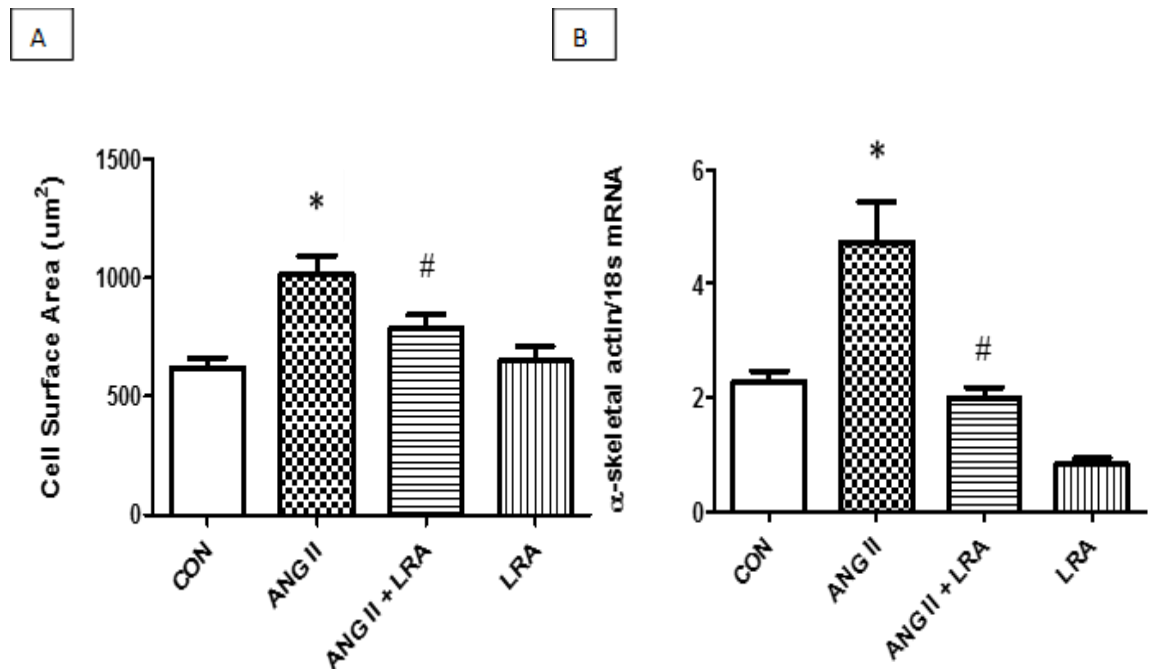
Figure 10: Angiotensin II-induced hypertrophy is attenuated by LRA. Panel A displays cell surface area of cardiomyocyte with ANG II and/or LRA 24 h treatment. Panel B displays α-skeletal actin gene expression after 24h treatment with ANG II and/or LRA. Cell surface area and α-skeletal actin gene expression data are presented as mean ± SEM (n=5-8). *$p<0.05$ from CON group. #$p<0.05$ from ANG II group.

3.7 Angiotensin II-induced FTO upregulation is attenuated by LRA

LRA was also able to attenuate angiotensin II-induced FTO upregulation. As evidenced in Figure 9C and 9D, angiotensin II-induced FTO upregulation is attenuated by LRA ($P<0.05$). Attenuation of cathepsin L and CUX1 gene expression increases by LRA was also observed ($P<0.05$).
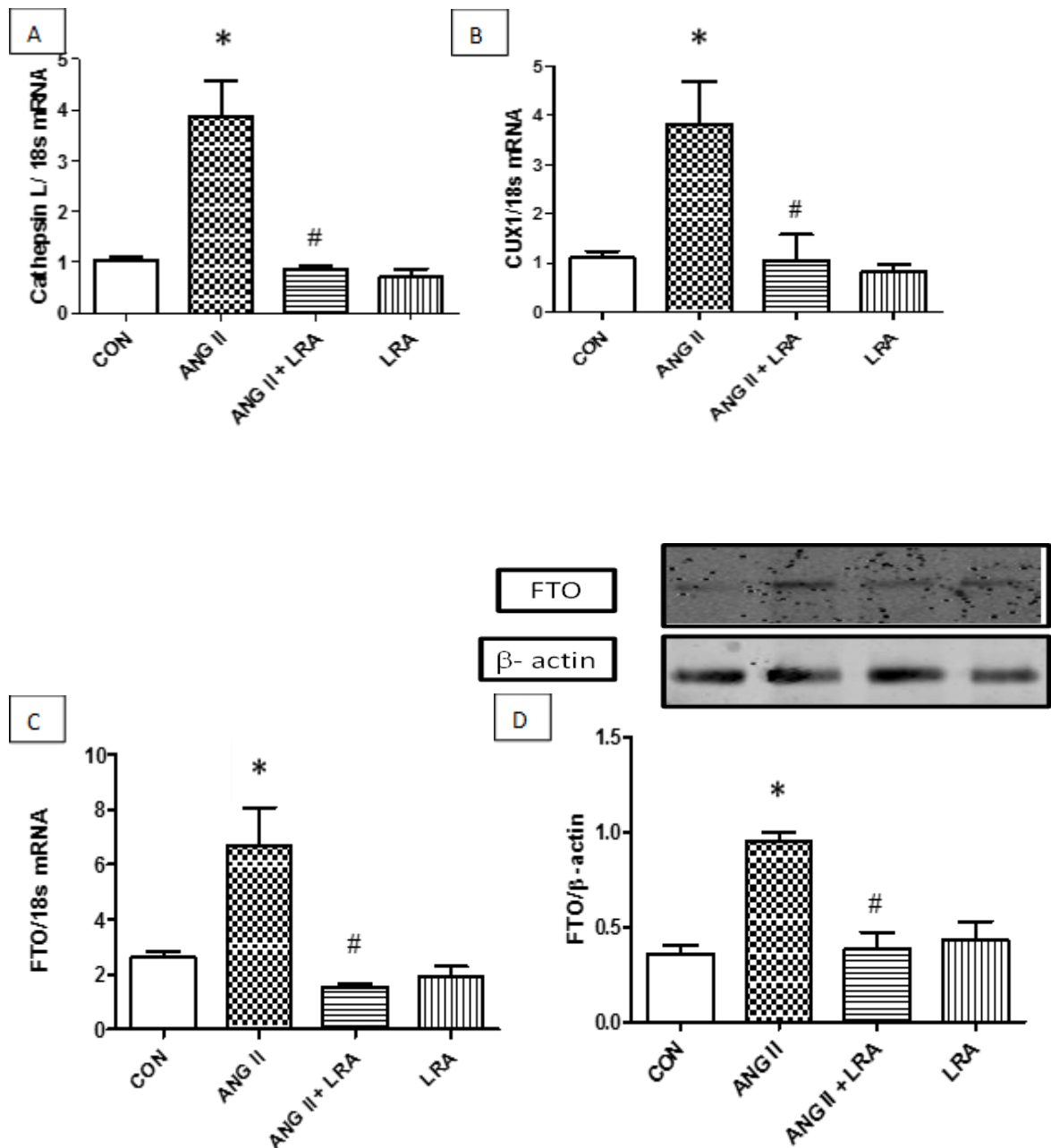
Figure 11: Angiotensin II-induced FTO upregulation is attenuated by LRA. Panels A and B displays cathepsin L gene expression and CUX1 gene expression respectively after ANG II and/or LRA 24 h treatment. Panels C and D displays FTO gene and protein expression respectively after ANG II and/or LRA 24 h treatment. FTO gene and protein expression data, cathepsin L and CUX1 gene expression data are presented as mean ± SEM (n=6-7). *p<0.05 from CON group. #p<0.05 from ANG II group.

3.8   Angiotensin II activates JAK2 and STAT3

As demonstrated in Figure 12, JAK2 is significantly phosphorylated after 15 minutes of angiotensin II treatment ($P < 0.05$). In Figure 13 we observe that STAT3 is significantly activated after 60 minutes of angiotensin II treatment ($P < 0.05$).
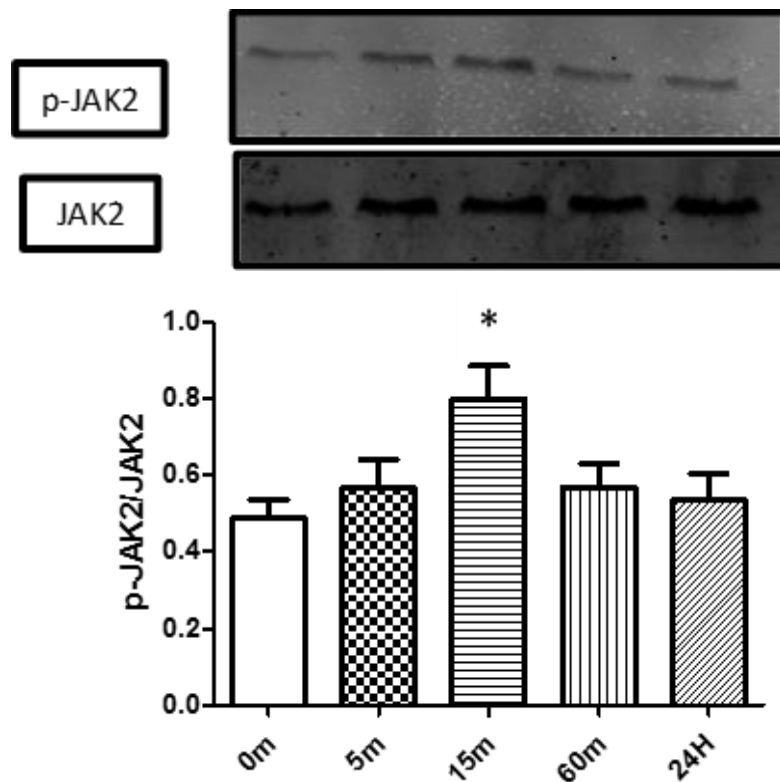
Figure 12: Angiotensin II activates JAK2. Time course of JAK2 phosphorylation after ANG II treatment at 0 minutes (m), 5 m, 15 m, 60 m, and 24 hours (H). Quantitative data presented as mean ± SEM. *$p < 0.05$ from 0 time point (n=5).
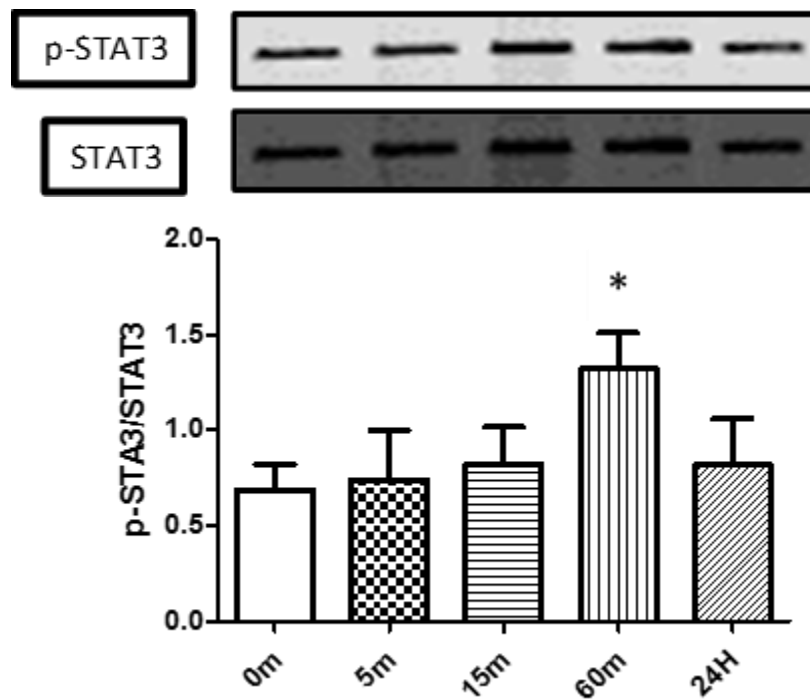
Figure 13: Angiotensin II activates STAT3. Time course of STAT3 phosphorylation after ANG II treatment at 0 m, 5 m, 15 m, 60 m, and 24 H. Quantitative data presented as mean ± SEM. *$p<0.05$ from 0 time point (n=5).

3.9 Inhibition of JAK2 attenuates angiotensin II-induced hypertrophy

Angiotensin II significantly increased cell surface area. This significant increase was abolished by JAK2 inhibitor AG490 (P<0.05). In addition the significant increase in α-skeletal actin caused by angiotensin II was also abolished by AG490 (P<0.05).
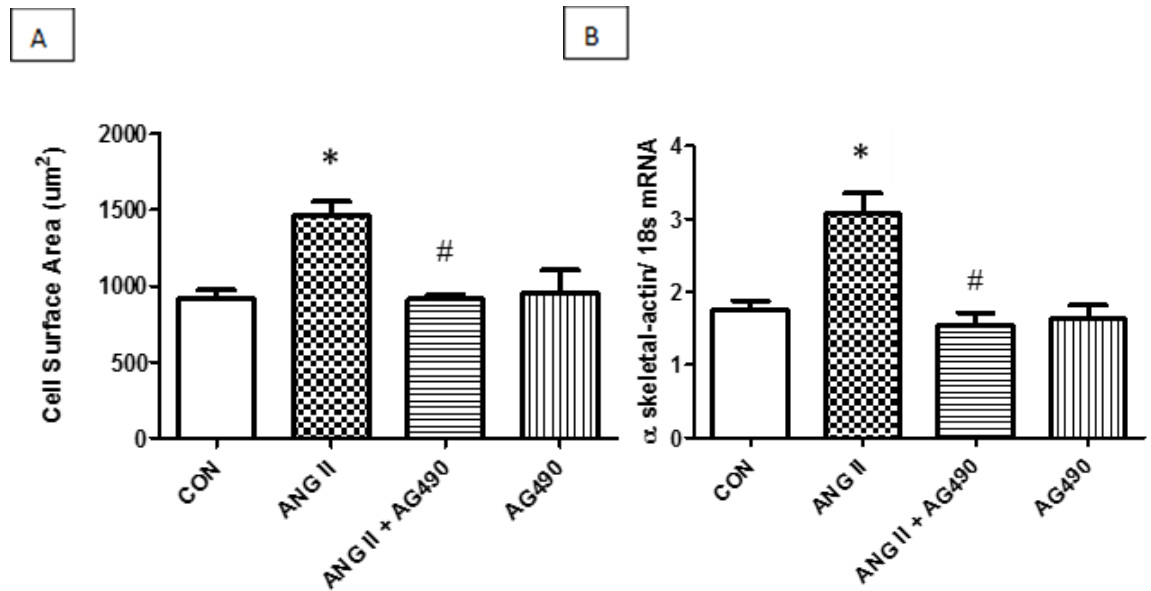
Figure 14: Angiotensin II-induced hypertrophy is attenuated by AG490. Panel A displays cell surface area of cardiomyocyte with ANG II and/or AG490 24 h treatment. Panel B displays α-skeletal actin gene expression after 24 h treatment with ANG II and/or AG490. Cell surface area and α-skeletal actin gene expression data are presented as mean ± SEM (n=5-6). *p<0.05 from CON group. #p<0.05 from ANG II group.

3.10 Inhibition of JAK2 attenuates angiotensin-II induced FTO upregulation

Angiotensin II-induced increases in CUX1 and cathepsin L gene expression are significantly attenuated by JAK2 inhibitor AG490 (Figure A, B) (P<0.05). Moreover, angiotensin II induced increases in FTO expression are also significantly attenuated by JAK2 inhibitor AG490 (Figure 14 C, D) (P<0.05).
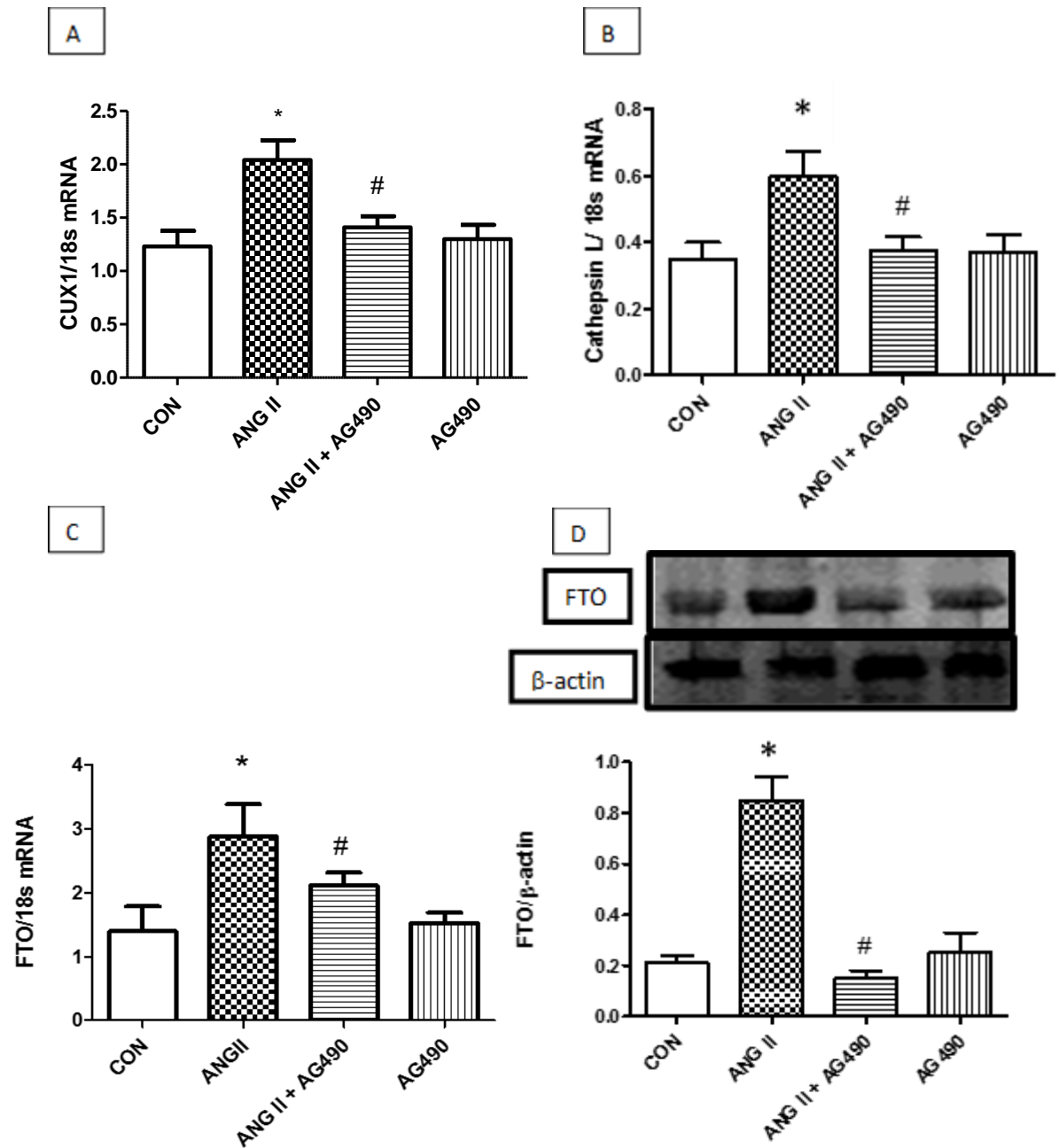
Figure 15: Inhibition of JAK2 attenuates angiotensin II-induced FTO upregulation. Panel A and B displays cathepsin L and CUX1 gene expression respectively after ANG II and/or AG490 24 h treatment. Panel C and D displays FTO gene and protein expression respectively after (ANG II) and/or AG490 24 h treatment. FTO gene and protein expression data, cathepsin L and CUX1 gene expression data are presented as mean ± SEM (n=6-7). *p<0.05 from CON group. #p<0.05 from ANG II group.

3.11 Leptin-induced cardiomyocyte hypertrophy is attenuated by calcineurin inhibitor FK506 but not NHE-1 inhibitor EMD87580

Leptin treatment for 24 h was able to significantly induce cardiomyocyte hypertrophy. As evidenced in Figure 16A and 16B, compound FK506 was able to significantly attenuate increases in cell surface area and α-skeletal actin gene expression respectively ($P<0.05$). However, as evidenced by Figure 16C and 16D inhibition of the sodium hydrogen exchanger proved not to affect the hypertrophic response to leptin ($P<0.05$).
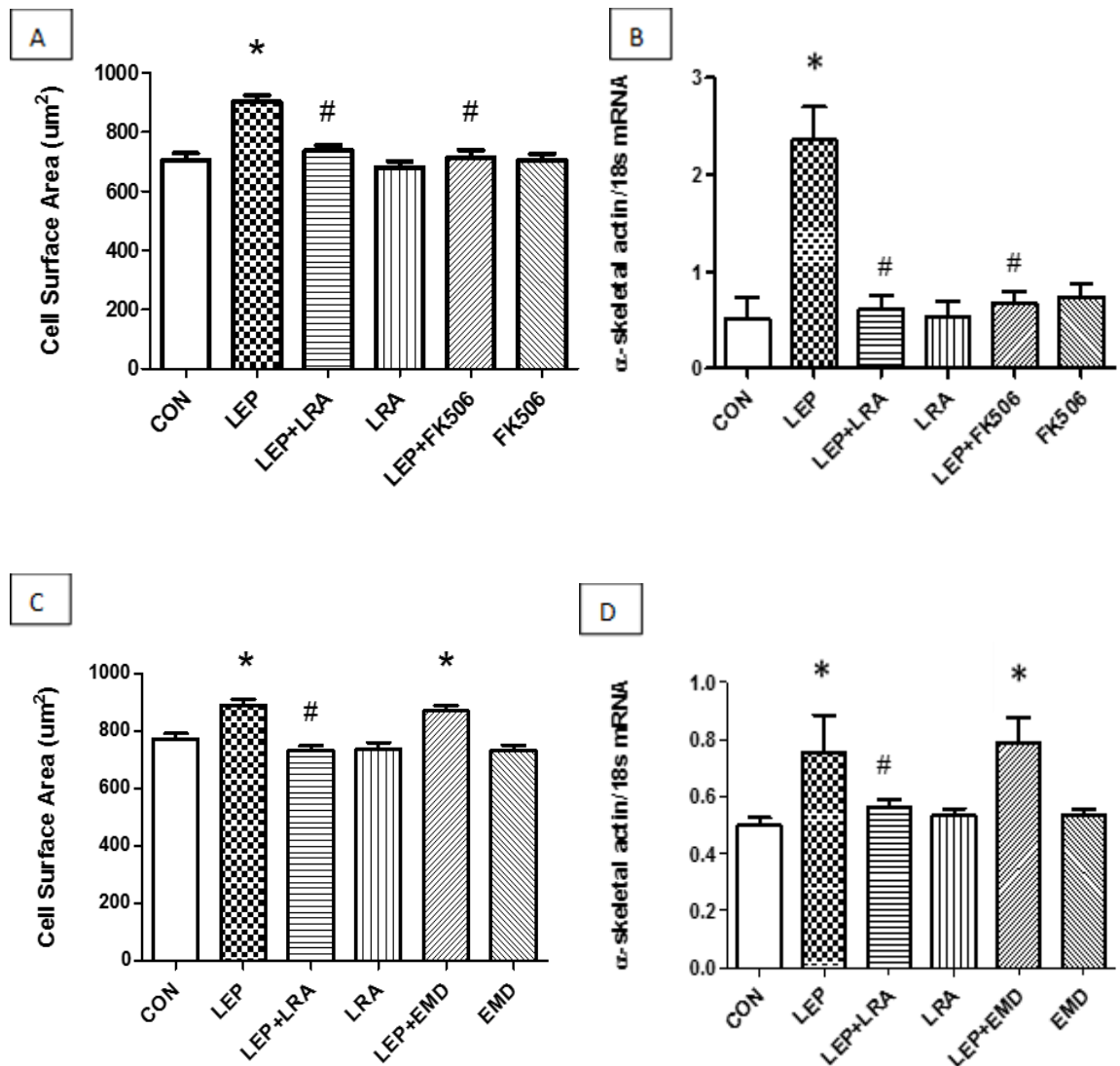
Figure 16: Leptin-induced hypertrophy is attenuated by FK506. Panels A and B displays cell surface area and α-skeletal actin gene expression of cardiomyocyte after 24 h leptin (LEP) and/or LRA and/or FK506 treatment. Panels C and D displays cell surface area and α-skeletal actin gene expression of cardiomyocyte with after 24 h LEP and/or LRA and/or EMD treatment. Cell surface area and α-skeletal actin gene expression data are presented as mean ± SEM (n=6). *p<0.05 from CON group. #p<0.05 from LEP group.

3.12 Leptin-induced FTO upregulation is attenuated by FK506 but not EMD87580

As evidenced in Figure 17 leptin-induced FTO upregulation was attenuated by LRA ($p < 0.05$). However, only the calcineurin inhibitor FK506 was able to significantly blunt the upregulation of FTO caused by leptin treatment (Figure 17 A, B) ($p < 0.05$). NHE-1 inhibitor, EMD87580, was unable to attenuate the upregulation of FTO caused by leptin.
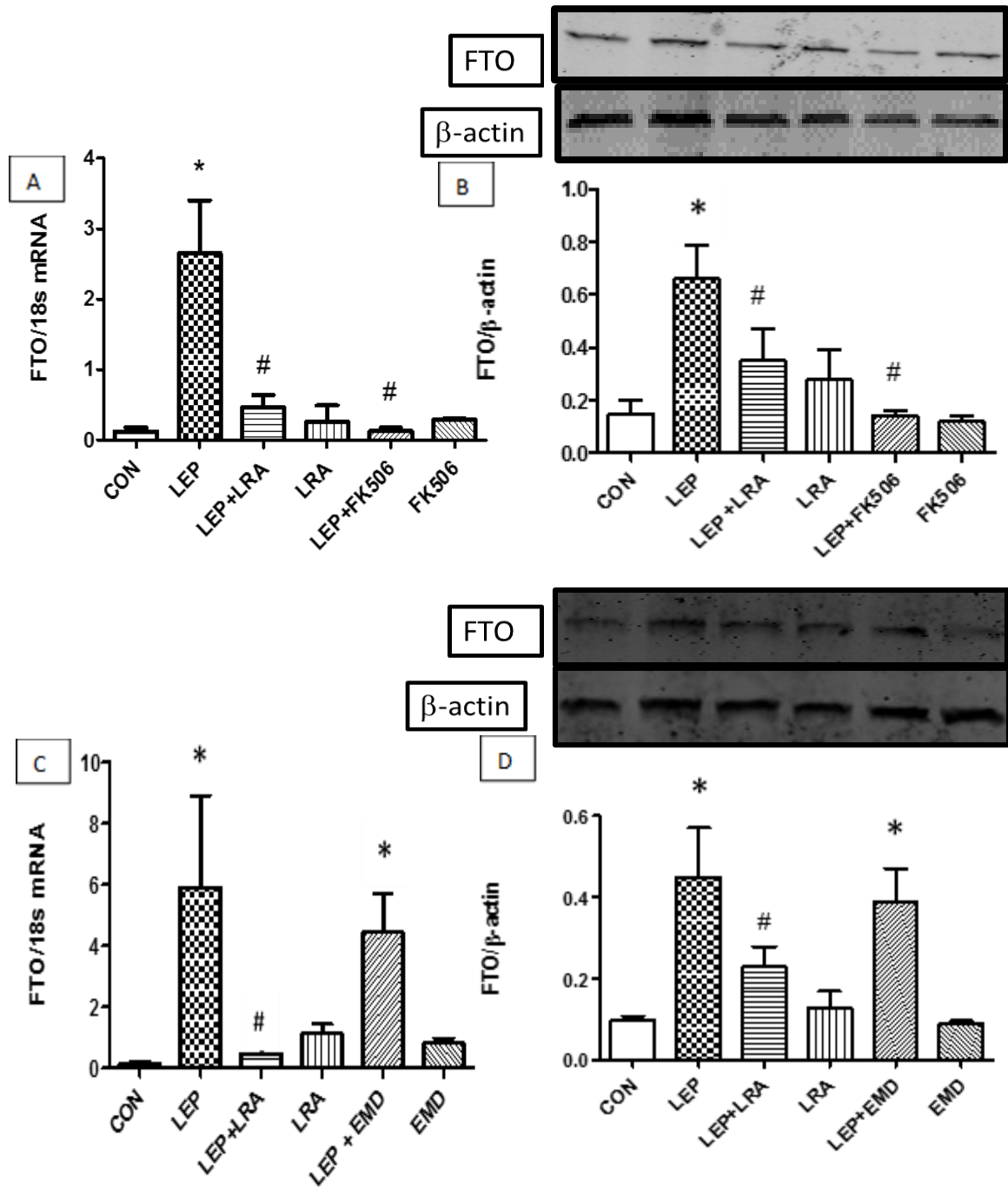
Figure 17: Leptin-induced FTO upregulation is attenuated by FK506. Panels A and B display FTO gene and protein expression respectively after 24 h leptin and/or LRA and/or FK506 treatment. Panels C and D displays FTO gene and protein expression respectively after 24 h leptin and/or LRA and/or EMD87580 treatment. Cell surface area and α-skeletal actin gene expression data are presented as mean ± SEM (n=6). *p<0.05 from CON group (n=6). #p<0.05 from LEP group.

Chapter 4

4   Discussion

Cardiovascular disease stands as one of the leading causes of death nationwide. Hospital discharges for heart failure and other cardiovascular diseases have been steadily increasing for more than 2 decades (Roger et al., 2011) . This trend is ironically a product of advancements in cardiovascular medicine and increased longevity (McMurray & Pfeffer, 2005). As the country faces an increase in an aging demographic, heart failure is an inevitable burden the health care system will have to deal with. One of the main features of heart failure is cardiac hypertrophy. Hypertrophy initially acts as a compensatory mechanism to relieve the stress placed on the heart due to the extra workload that occurs in heart failure. Unfortunately, the perpetual stress causes the heart to transition into decompensatory hypertrophy (Pfeffer, 1995) in which the growth becomes maladaptive and deleterious to the heart's function. In this decompensatory stage, left ventricular function progressively declines leading to inadequate tissue perfusion.

In heart failure neurohumoral systems are upregulated. One of these systems is called the renin-angiotensin-aldosterone system in which angiotensin II is the main effector of this system (Griendling et al., 1993). This system is upregulated in order to promote vasoconstriction and fluid reabsorption, which will result in an increased venous return to the heart thus restoring stroke volume. However, angiotensin II has a direct pathological hypertrophic effect on the heart (Paradis et al., 2000). Perpetual stimulation of the heart by angiotensin II activates intracellular pathways such as MAPK (Sadoshima et al., 1995) and NHE-1/calcineurin (Karmazyn et al., 1999) that will result in pathological hypertrophy.

Obesity is a disease that is also associated with many cardiovascular diseases such as hypertension and cardiac hypertrophy (Kenchaiah et al., 2002). Our lab has looked at potential causal links between obesity and cardiovascular disease. One such link is the hormone leptin, a hormone that is increased in circulation during obesity due to an increase in adipose tissue, the main site of leptin production (Zhang et al., 1994). Our lab has demonstrated that leptin induces cardiac hypertrophy (Rajapurohitam et al., 2003). One of those signalling pathways in which leptin induces hypertrophy is through the upregulation of the fat mass and obesity associated protein (FTO) (Gan et al., 2013). Through this aforementioned study, our lab has been able to characterize FTO as a hypertrophic protein in the cardiomyocyte. Thus the presumption is that other myocardial hypertrophic agonists such as angiotensin II may also upregulate FTO.

Pathological cardiac hypertrophy is a prominent feature of heart failure. Factors such as angiotensin II that induce pathological hypertrophy are factors that must be studied in depth. More importantly, the signalling pathways through which they induce hypertrophy must be elucidated such that proper pharmacological interventions can be formulated. Thus in this study—using neonatal rat ventricular cardiomyocytes—we used a series of in vitro experiments to determine the signalling mechanisms behind angiotensin II-induced hypertrophy in relation FTO expression.

4.1 Angiotensin II and phenylephrine induce cardiomyocyte hypertrophy

Angiotensin II and phenylephrine are the agonists we chose to assess hypertrophy and FTO expression in the cardiomyocyte. Studies, including those from our laboratory, have shown that 100 nM concentration of angiotensin II (Nakamura et al., 1998; Rajapurohitam et al., 2006) and 10 µM concentration of phenylephrine (Gan et al., 2005; Omura et al., 2002) significantly induces hypertrophy in neonatal rat cardiomyocytes. Our results show that angiotensin II and phenylephrine significantly increase cell surface area and atrial natriuretic peptide gene expression.

4.2 Angiotensin II induces FTO upregulation in the cardiomyocyte

Only 24 h treatment with angiotensin II was able to significantly upregulate FTO in the cardiomyocyte as evidenced by gene expression and protein expression. This phenomenon was interesting because although angiotensin II and phenylephrine act through different receptors, their receptors signal through the same Gq protein (Dorn & Brown, 1999) and activate the same intracellular signalling pathways that induce hypertrophy. One reason for the apparent difference in ability to upregulate FTO between the two agonists may be their difference in the extent to which they activate the Gq protein signalling pathway. One study demonstrates that angiotensin II, through the AT-1 receptor, is able to increase the Na^+/Ca^{2+} exchanger (NCX) activity to a greater degree than phenylephrine in the cardiomyocyte via a Gq dependent mechanism (Ballard & Schaffer, 1996). Angiotensin II was able to increase the exchanger's activity to 125%, while

phenylephrine only increased it to 111%. Although the disparity between the activity levels may not be significant, it is important to note that a 100 μM phenylephrine concentration was used to achieve maximal stimulation. Conversely, a 5 nM angiotensin II concentration was used to achieve maximal stimulation. Thus it appears that the NCX activity is much more sensitive to angiotensin II. Both angiotensin II and phenylephrine had their effect on the exchanger abolished when a protein kinase C inhibitor was used bolstering the conclusion their effect on the exchanger was Gq-phospholipase C-mediated (Ballard & Schaffer, 1996).

Our lab has shown that leptin increases FTO via a JAK2/STAT3 dependent mechanism (Gan et al., 2013). Interestingly enough, this may also explain angiotensin II's ability to upregulate FTO as opposed to phenylephrine's inability to upregulate FTO. We have demonstrated that angiotensin II's hypertrophic effect on the cardiomyocyte is mediated through leptin signalling in this current study and in past studies (Rajapurohitam et al., 2006). To elaborate, angiotensin II was shown to increase leptin synthesis and OB-Rb receptor synthesis in the cardiomyocyte thus increasing leptin autocrine signalling (Rajapurohitam et al., 2006) . Leptin receptor antagonists and leptin neutralizing antibodies were able to abolish angiotensin II-induced hypertrophy (Rajapurohitam et al., 2006). Based on this evidence, it is conceivable the angiotensin II is upregulating the leptin system which is, in turn, upregulating FTO via the autocrine effects of leptin.

4.3 NHE-1 and calcineurin play critical roles in angiotensin II-induced cardiomyocyte hypertrophy

NHE-1's primary role is to extrude out acid from the cell when pH levels begin to drop as a result of processes such as lactate production or even in pathological conditions such as ischemia (Leem et al., 1999). While extruding out protons, the transporter simultaneously brings in the same number of sodium ions. This 12 transmembrane domain transporter contains phosphorylation sites near the carboxyl terminus (Counillon & Pouysségur, 1995) (Karmazyn et al., 1999). When phosphorylated, the exchanger becomes sensitized to proton concentrations. Thus it's activity level is greater at a more alkaline pH (Karmazyn, 2001).

Angiotensin II is able to induce phosphorylation of NHE-1 through a Gq dependent PKC activation (Mehta & Griendling, 2007). The increased activity of NHE-1 will result in intracellular sodium accumulation. This will in turn affect the NCX exchanger by affecting it's sodium/calcium concentration gradients (Karmazyn, 2001). The end result is an intracellular accumulation of calcium that results in activation of the calcineurin/NFAT signalling pathway culminating in the induction of the hypertrophic program (Ennis et al., 2007).

In this study, a specific NHE-1 inhibitor EMD87580, was able to attenuate angiotensin II-induced cardiomyocyte hypertrophy as evidenced by attenuation of angiotensin II-induced increases in cell surface area and α-skeletal actin. In one study that supports this finding, the NHE-1 inhibitor HOE 694 was able to significantly attenuate phenylephrine induced protein synthesis in ventricular cardiomyocytes (Schlüter et al.,

1998). In another study, the NHE-1 inhibitor EMD87580 attenuated aldosterone-induced increases in cell surface area and atrial natriuretic peptide (ANP) (Karmazyn et al., 2003).

In the current study calcineurin inhibitor FK506, was also able to attenuate angiotensin II-induced hypertrophy. Other studies have also demonstrated the ability of FK506 to interrupt the hypertrophic process. For example, our lab has demonstrated this compound's ability to attenuate leptin-induced cardiomyocyte hypertrophy (Rajapurohitam et al., 2012).

4.4   NHE-1 and calcineurin play critical roles in AT-1 mediated angiotensin II-induced FTO upregulation

NHE-1 inhibitor EMD87580 and calcineurin inhibitor FK506 significantly attenuated FTO expression. This was evidenced by gene as well as protein expression. This was further demonstrated in immunofluorescence experiments. Nuclear fluorescence intensity was significantly increased by 24 h treatment of angiotensin II in fixed cells. Valsartan, EMD87580 and FK506 all returned FTO fluorescence intensity back to basal levels. Thus it appears that the NHE-1/calcineurin pathway is a hypertrophic signalling mechanism that is critical for regulating the expression of FTO, a protein that has been shown to be hypertrophic in the heart.

4.5   Evidence of calcineurin activation

Modulatory calcineurin interacting protein (MCIP) is often used as a marker of calcineurin activation. It is upregulated after calcineurin signalling and, in a negative feedback loop, binds to calcineurin to suppress its activity (Molkentin et al., 1998).

However MCIP appears to have a dual effect on calcineurin. One study assessed MCIP knockout mice and their response to a constitutively expressed calcineurin transgene (Vega et al., 2003). These mice had an exacerbated hypertrophic response, agreeing with the notion that MCIP serves to suppress calcineurin activity. However, in this same study, MCIP knockout mice were subjected to chronic adrenergic stimulation or pressure overload but were unable to develop hypertrophy in both situations demonstrating that MCIP is necessary for the facilitation of hypertrophy (Vega et al., 2003).

In this current study, angiotensin II significantly increased MCIP gene expression. The increase was attenuated by AT-1 receptor antagonist valsartan, NHE-1 inhibitor EMD87580 and calcineurin inhibitor FK506. This finding is supported by one study which also demonstrated angiotensin II's ability to increase MCIP gene expression in the cardiomyocyte. This increase was attenuated by a novel chimeric natriuretic peptide, a protein proposed to inhibit NHE-1 (Kilić et al., 2010).

As well NFAT3 translocation after 24h treatment with angiotensin II was assessed via western blot of nuclear fractions of cardiomyocytes. Angiotensin II significantly increased NFAT3 translocation to the nucleus which was attenuated by calcineurin inhibitor FK506. This finding is also in concordance with a study that demonstrated NFAT3 translocation to the nucleus at 24 hours after treatment with either angiotensin II, endothelin-1 or phenylephrine—agonists that activate the NHE-1/calcineurin signalling pathway (Kilić et al., 2010).

These findings suggest that the NHE-1/calcineurin signalling pathway is being activated by angiotensin II via an AT-1 dependent mechanism. Administration of the AT-

1 antagonist, NHE-1 inhibitor or calcineurin inhibitor shuts down angiotensin II's ability to signal through this pathway, a pathway which is critical for regulation of FTO in the cardiomyocyte.

4.6 Leptin receptor blockade attenuates angiotensin II-induced hypertrophy

Cardiomyocytes treated for 24 h with angiotensin II induced hypertrophy as evidenced by an increase in cell surface area and α-skeletal actin gene expression. These increases are attenuated by the super-ovine leptin receptor antagonist (LRA). These findings are not unique, as our lab has demonstrated in the past that angiotensin II's ability to induce hypertrophy can be abolished by antagonizing the leptin signalling system (Rajapurohitam et al., 2006). As described in the aforementioned study, angiotensin II is able to upregulate the synthesis and secretion of leptin from the cardiomyocyte, which in turn, has an autocrine effect on the cardiomyocyte. When the autocrine effect of leptin is blocked, angiotensin II-induced hypertrophy is also abolished.

4.7 Leptin receptor blockade attenuates angiotensin II-induced FTO upregulation

Angiotensin II's ability to increase FTO expression cannot solely be explained by the NHE-1/calcineurin signalling pathway. This is because this is a Gq protein mediated pathway—a pathway through which phenylephrine works through as well. However phenylephrine was unable to increase FTO expression. Thus we decided to look at alternate mechanisms that could explain the disparity between angiotensin II's and phenylephrine's effect on FTO expression. Based on the conclusions from Rajapurohitam et al. about

angiotensin II-induced leptin autocrine signalling, and the recent study published by our lab on leptin's effect on FTO expression (Gan et al., 2013), we found leptin signalling a suitable candidate to explain why angiotensin II possessed the ability to upregulate FTO and why phenylephrine did not. Thus we formulated the hypothesis that angiotensin II induced FTO upregulation was, in part, mediated by leptin. We decided to look at some of the same markers studied in the leptin-FTO study (Gan et al., 2013), as we believed that the same pathway was being indirectly activated by angiotensin II.

In this current study angiotensin II-induced FTO upregulation was attenuated by LRA. This unique finding was supplemented with some gene expression data of other markers. The proteolytic enzyme cathepsin L and transcription factor CUX1 both increased in gene expression after 24 h angiotensin II treatment. These increases were also attenuated by LRA. Increases in these markers in response to leptin were also observed in the study published by Gan. This strengthens the notion that angiotensin II works through the leptin signalling pathway displayed in figure 2 in order to achieve FTO upregulation

4.8   Angiotensin II activates JAK2 and STAT3; the molecular mediators of leptin-induced FTO upregulation

In this current study angiotensin II activated JAK2 and STAT3 in a time dependent fashion. 15 minute angiotensin II treatment activated JAK2 in the cardiomyocyte. STAT3 appeared to be activated at the 60 minute angiotensin II treatment time point. This lends support to the notion that angiotensin II is activating the leptin signalling system as JAK2 and STAT3 are important molecular mediators of leptin-induced FTO upregulation. In the study published by Gan in 2013, they demonstrated suppression of FTO and phospho-

STAT3 in the hearts of animals that had been treated with a leptin receptor antibody (Gan et al., 2013). In addition, they demonstrated an increase in phospho-STAT3 and an increase in JAK2 gene and protein expression after 24 h leptin treatment (Gan et al., 2013). Another study also displays a role for STAT3 in mediating leptin's effect on FTO expression in mice hypothalamic nuclei (Wang et al., 2011). Leptin downregulates FTO in the arcuate nuclei of these mice through a STAT3 mediated mechanism (Wang et al., 2011).

Gan et al. demonstrate that JAK2 and STAT3 play a critical role in leptin induced FTO regulation. Moreover, it is plausible that angiotensin II is indirectly activating these molecular mediators via an increase leptin signalling.

4.9 Angiotensin II-induced hypertrophy and FTO upregulation is attenuated by JAK2 inhibitor AG490

Hypertrophy of cardiomyocytes after 24 h treatment with angiotensin II was abolished by JAK2 inhibitor AG490. Cell surface area and α-skeletal actin gene expression were brought back down to basal levels with AG490. Thus it appears that JAK2 signalling plays a critical role in angiotensin II induced hypertrophy, and lends support to the hypothesis that interruption of the leptin-JAK2-STAT3 signalling pathway can nullify angiotensin II's ability to induce the hypertrophic program.

AG490 also attenuated angiotensin II-induced FTO upregulation in the cardiomyocyte. This was evidenced by a return to basal levels of FTO gene expression and FTO protein expression. This finding directly links angiotensin II's ability to upregulate FTO to the molecular mediator JAK2. As well, AG490 attenuated angiotensin II-induced increases in gene expression of cathepsin L—the protease responsible for cleaving p200

72

CUX1 into the active transcription factor for FTO (p110 CUX1)—and CUX1—the gene that gives rise to CUX1 protein. A similar finding was displayed in the study published by Gan in 2013 where AG490 was able to attenuate leptin-induced increases in FTO and cathepsin L expression.

This is further support that angiotensin II is upregulating FTO, in part, by increasing leptin autocrine signalling resulting in the same intracellular signalling pathway proposed in the Gan's study (figure 2).

4.10 Leptin-induced cardiomyocyte hypertrophy and FTO upregulation is attenuated by calcineurin inhibitor FK506 but not NHE-1 inhibitor EMD87580

In this study we have been able to elucidate the mechanisms behind angiotensin II-induced FTO regulation. We have been able to determine that the NHE-1/calcineurin signalling system and angiotensin II- induced leptin autocrine signalling system play critical roles in regulating the hypertrophic protein FTO. Both of these pathways have been studied in isolation and both have proven necessary for the regulation of FTO. Thus it is plausible that because angiotensin II activates both these pathways, and both of these pathways have a commonality in upregulating FTO, these pathways may share some crosstalk, overlap or work in a synergistic fashion to manifest FTO upregulation. To study this hypothesis we used the NHE-1 inhibitor EMD87580 and the calcineurin inhibitor FK506 to study if NHE-1 and calcineurin were involved in leptin's regulation of FTO and leptin's induction of the hypertrophic process. As evidenced by cell surface area, EMD87580 did not attenuate the leptin-induced increase in cell size. In contrast, FK506

was able to attenuate the leptin-induced increase in cell surface area. This finding was similar to a finding in another study where leptin induced hypertrophy was abrogated by calcineurin inhibitor FK506 as evidenced by attenuation in the increases of cell surface area, α-skeletal actin gene expression and leucine incorporation (Rajapurohitam et al., 2012). Thus it appears angiotensin II and leptin share a molecular mediator when it comes to induction of the hypertrophic process—calcineurin.

EMD87580 was also unable to attenuate the leptin-induced upregulation of FTO further suggesting that NHE-1 is not involved in leptin's hypertrophic signalling pathway and establishing that NHE-1 is not involved in leptin induced FTO regulation. However, in agreement with the hypertrophic assessment, FK506 did attenuate leptin's ability to upregulate FTO. Thus calcineurin plays a critical role in leptin-induced FTO upregulation. Similar to hypertrophy it appears that calcineurin is both critical for angiotensin II and leptin's ability to upregulate FTO.

It appears that calcineurin and JAK2/STAT3 mechanisms may be working in a concerted or synergistic fashion to produce FTO upregulation in angiotensin II treated cardiomyocytes. Other studies have explored the interaction of calcineurin and JAK2/STAT3 albeit in other cell types. One study demonstrates that calcineurin can dephosphorylate STAT3 thus terminating SIF-SIE (sis inducing factor-sis inducing element) interactions in vascular smooth muscle cells treated with angiotensin II (Liang et al., 1999). Although this is a system studied in vascular smooth muscle cells, the interaction between calcineurin and JAK2/STAT3 explained in this particular study would be in opposition to the plausible scenario suggested in this study between the two pathways. We

would expect calcineurin to positively regulate the JAK2/STAT3 pathway or the JAK2/STAT3 pathway to facilitate calcineurin activation. However another study demonstrates that FK506 suppresses activation of the JAK/STAT pathway by interleukin-6 in fibroblast-like synoviocytes, intimating that calcineurin activation facilitates JAK2/STAT3 activation in this cell type (Choe et al., 2013). This study supports the hypothetical notion that in cardiomyocytes, calcineurin and JAK2/STAT3 may work in concert or synergistically to upregulate FTO and induce hypertrophy.

4.11 Significance and clinical perspective of the study

Heart failure is an imminent burden on our health care system as we are facing an aging population. As well, advancements in medicine for treatments of heart attacks have resulted in an increase in survival. Unfortunately this increase in survival of cardiac patients leads to an increased probability of developing heart failure as a result of compromised heart function. One of the main features of heart failure is pathological cardiac hypertrophy. This hypertrophic response becomes deleterious to heart function which leads to heart failure. Elucidating the major hypertrophic mechanisms that take place during heart failure, and identifying specific molecular targets for therapeutic intervention is critical for not only the treatment but also the prevention of heart failure. In this study we have been able to characterize angiotensin II's ability to upregulate FTO, a protein suggested to be hypertrophic in the heart. We have elucidated specific hypertrophic signalling pathways linked to the upregulation of FTO in angiotensin II treated cardiomyocytes. FTO seems to be inextricably tied to signalling pathways responsible for pathological cardiac hypertrophy. FTO provides yet another target for pharmacological

intervention in which we can slow down or reverse the development of pathological hypertrophy resulting in better prognoses for heart failure.

4.12 Conclusion

Our major conclusion in this study is that angiotensin II upregulates the fat mass and obesity associated protein (FTO) in the cardiomyocyte. Angiotensin II joins only leptin as the only other hypertrophic agonist to increase FTO expression in the cardiomyocyte.

Angiotensin II's ability to regulate FTO expression is AT-1 receptor mediated. The intracellular signalling pathways involved in angiotensin II's upregulation of FTO are the NHE-1/calcineurin signalling pathway and the leptin-dependent JAK2/STAT3 signalling pathway. These pathways plausibly act in concert or exhibit some type of crosstalk (Figure 18), as antagonizing either pathway in isolation abolishes angiotensin II induced FTO upregulation, suggesting a common critical juncture through which both pathways cross to achieve FTO upregulation.

There are usually a multitude of pathways activated in response to one signal. Moreover these pathways usually result in the same molecular manifestation. The redundancy of cellular mechanisms for a certain cellular process highlights the importance of that cellular process. It is important to understand all these intracellular mechanisms in isolation, and more important to identify areas of cross talk once we have studied these mechanisms in isolation. True understanding of the pathological hypertrophic process is dependent upon understanding how the mechanisms relate to each other.
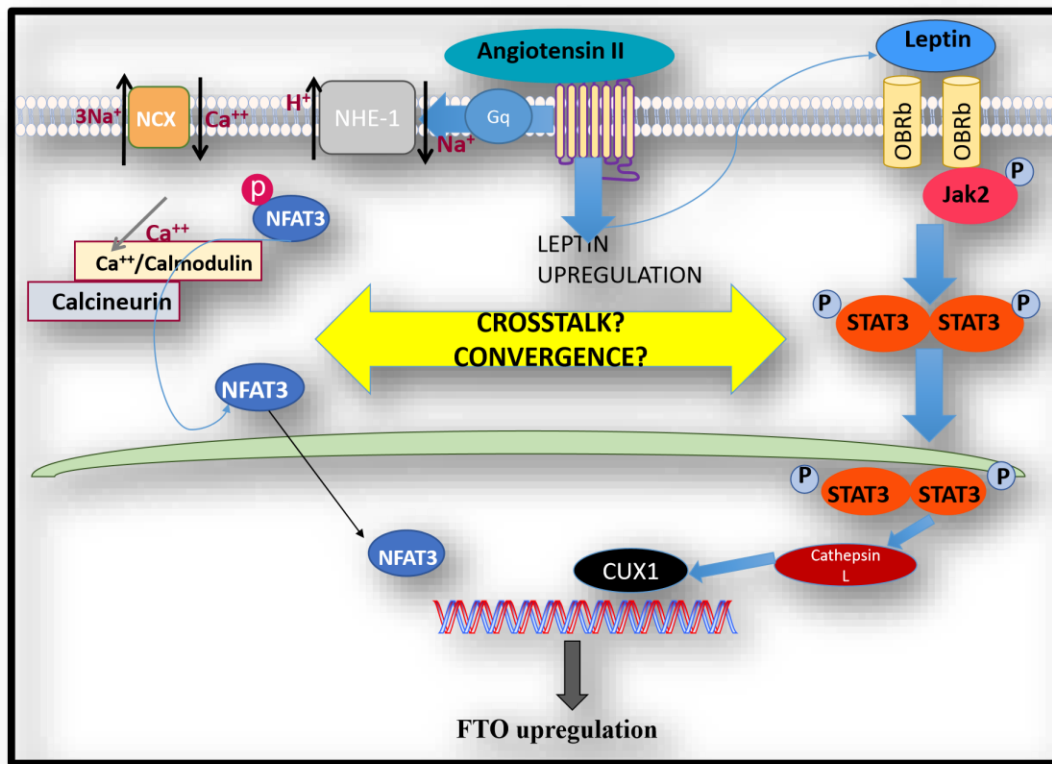
Figure 18. Proposed signalling and crosstalk mechanisms for angiotensin II-induced FTO upregulation. Angiotensin II activates a Gq-mediated NHE-1/calcineurin signalling pathway and leptin-mediated JAK2/STAT3 signalling pathway, with both pathways having a plausible common and critical point of interaction necessary for FTO upregulation.

References

Ballard, C., & Schaffer, S. (1996). Stimulation of the Na+/Ca 2+ exchanger by phenylephrine, angiotensin II and endothelin 1. *Journal of Molecular & Cellular Cardiology, 28*(1), 11-17.

Bernardo, B. C., Weeks, K. L., Pretorius, L., & McMullen, J. R. (2010). Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. *Pharmacology & Therapeutics, 128*(1), 191-227.

Beuckelmann, D. J., Nabauer, M., & Erdmann, E. (1992). Intracellular calcium handling in isolated ventricular myocytes from patients with terminal heart failure. *Circulation, 85*(3), 1046-1055.

Billet, S., Bardin, S., Verp, S., Baudrie, V., Michaud, A., Conchon, S., Hamard, G. (2007). Gain-of-function mutant of angiotensin II receptor, type 1A, causes hypertension and cardiovascular fibrosis in mice. *Journal of Clinical Investigation, 117*(7), 1914.

Choe, J.-Y., Park, K.-Y., Park, S.-H., Lee, S.-I., & Kim, S.-K. (2013). Regulatory effect of calcineurin inhibitor, tacrolimus, on IL-6/sIL-6R-mediated RANKL expression through JAK2-STAT3-SOCS3 signaling pathway in fibroblast-like synoviocytes. *Arthritis Research & Therapy, 15*(1), 26.

Chua, S. C., Koutras, I. K., Han, L., Liu, S.-M., Kay, J., Young, S. J., Leibel, R. L. (1997). Fine structure of the murine leptin receptor gene: splice site suppression is required to form two alternatively spliced transcripts. *Genomics, 45*(2), 264-270.

Counillon, L., & Pouysségur, J. (1995). Structure-function studies and molecular regulation of the growth factor activatable sodium-hydrogen exchanger (NHE-1). *Cardiovascular Research, 29*(2), 147-154.

Cowie, M. R., Wood, D. A., Coats, A. J., Thompson, S. G., Poole-Wilson, P. A., Suresh, V., & Sutton, G. C. (1999). Incidence and aetiology of heart failure; a population-based study. *European Heart Journal, 20*(6), 421-428.

de Gasparo, M., Catt, K. J., Inagami, T., Wright, J. W., & Unger, T. (2000). International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacological Reviews, 52*(3), 415-472.

DeBosch, B., Treskov, I., Lupu, T. S., Weinheimer, C., Kovacs, A., Courtois, M., & Muslin, A. J. (2006). Akt1 is required for physiological cardiac growth. *Circulation, 113*(17), 2097-2104.

Dorn, G. W., & Brown, J. H. (1999). Gq signaling in cardiac adaptation and maladaptation. *Trends in cardiovascular medicine, 9*(1), 26-34.

Ennis, I. L., Garciarena, C. D., Escudero, E. M., Perez, N. G., Dulce, R. A., Camilión de Hurtado, M. C., & Cingolani, H. E. (2007). Normalization of the calcineurin pathway underlies the regression of hypertensive hypertrophy induced by Na+/H+ exchanger-1 (NHE-1) inhibition. *Canadian journal of physiology and pharmacology, 85*(3-4), 301-310.

Fischer, J., Koch, L., Emmerling, C., Vierkotten, J., Peters, T., Bruning, J. C., & Ruther, U. (2009). Inactivation of the Fto gene protects from obesity. *Nature, 458*(7240), 894-898.

Florea, V. G., & Cohn, J. N. (2014). The autonomic nervous system and heart failure. *Circulation Research, 114*(11), 1815-1826.

Frayling, T. M., Timpson, N. J., Weedon, M. N., Zeggini, E., Freathy, R. M., Lindgren, C. M., Rayner, N. W. (2007). A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science, 316*(5826), 889-894.

Frey, N., Katus, H. A., Olson, E. N., & Hill, J. A. (2004). Hypertrophy of the heart: a new therapeutic target? *Circulation, 109*(13), 1580-1589.

Gan, X. T., Rajapurohitam, V., Haist, J. V., Chidiac, P., Cook, M. A., & Karmazyn, M. (2005). Inhibition of phenylephrine-induced cardiomyocyte hypertrophy by activation of multiple adenosine receptor subtypes. *Journal of Pharmacology and Experimental Therapeutics, 312*(1), 27-34.

Gan, X. T., Zhao, G., Huang, C. X., Rowe, A. C., Purdham, D. M., & Karmazyn, M. (2013). Identification of fat mass and obesity associated (FTO) protein expression in cardiomyocytes: regulation by leptin and its contribution to leptin-induced hypertrophy. *PLoS One, 8*(9), e74235.

Gerken, T., Girard, C. A., Tung, Y. C., Webby, C. J., Saudek, V., Hewitson, K. S., Schofield, C. J. (2007). The obesity-associated FTO gene encodes a 2-

oxoglutarate-dependent nucleic acid demethylase. *Science, 318*(5855), 1469-1472.

Gertz, E., Wisneski, J., Stanley, W., & Neese, R. (1988). Myocardial substrate utilization during exercise in humans. Dual carbon-labeled carbohydrate isotope experiments. *Journal of Clinical Investigation, 82*(6), 2017.

Griendling, K. K., Murphy, T. J., & Alexander, R. W. (1993). Molecular biology of the renin-angiotensin system. *Circulation, 87*(6), 1816-1828.

Grossman, W., Jones, D., & McLaurin, L. (1975). Wall stress and patterns of hypertrophy in the human left ventricle. *Journal of Clinical Investigation, 56*(1), 56.

Haq, S., Choukroun, G., Lim, H., Tymitz, K. M., del Monte, F., Gwathmey, J., Force, T. (2001). Differential activation of signal transduction pathways in human hearts with hypertrophy versus advanced heart failure. *Circulation, 103*(5), 670-677.

Heineke, J., & Molkentin, J. D. (2006). Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nature Reviews, 7*(8), 589-600.

Kannel, W. B. (2000). Incidence and epidemiology of heart failure. *Heart Failure Reviews, 5*(2), 167-173.

Karmazyn, M. (2001). Sodium-hydrogen exchange in heart disease. *Science and Medicine, 8*(1), 18-27.

Karmazyn, M., Gan, X. T., Humphreys, R. A., Yoshida, H., & Kusumoto, K. (1999). The myocardial Na(+)-H(+) exchange: structure, regulation, and its role in heart disease. *Circ Res, 85*(9), 777-786.

Karmazyn, M., Liu, Q., Gan, X. T., Brix, B. J., & Fliegel, L. (2003). Aldosterone increases NHE-1 expression and induces NHE-1-dependent hypertrophy in neonatal rat ventricular myocytes. *Hypertension, 42*(6), 1171-1176.

Kenchaiah, S., Evans, J. C., Levy, D., Wilson, P. W., Benjamin, E. J., Larson, M. G., Vasan, R. S. (2002). Obesity and the risk of heart failure. *New England Journal of Medicine, 347*(5), 305-313.

Kilić, A., Rajapurohitam, V., Sandberg, S. M., Zeidan, A., Hunter, J. C., Faruq, N. S., Karmazyn, M. (2010). A novel chimeric natriuretic peptide reduces cardiomyocyte hypertrophy through the NHE-1–calcineurin pathway. *Cardiovascular Research, 88*(3), 434-442.

Kim, J., Wende, A. R., Sena, S., Theobald, H. A., Soto, J., Sloan, C., LeRoith, D. (2008). Insulin-like growth factor I receptor signaling is required for exercise-induced cardiac hypertrophy. *Molecular Endocrinology, 22*(11), 2531-2543.

Leem, C. H., Lagadic-Gossmann, D., & Vaughan-Jones, R. D. (1999). Characterization of intracellular pH regulation in the guinea-pig ventricular myocyte. *The Journal of Physiology, 517*(1), 159-180.

Levy, D., Garrison, R. J., Savage, D. D., Kannel, W. B., & Castelli, W. P. (1990). Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *New England Journal of Medicine, 322*(22), 1561-1566.

Liang, H., Venema, V. J., Wang, X., Ju, H., Venema, R. C., & Marrero, M. B. (1999). Regulation of angiotensin II-induced phosphorylation of STAT3 in vascular smooth muscle cells. *Journal of Biological Chemistry, 274*(28), 19846-19851.

Lorell, B. H., & Carabello, B. A. (2000). Left ventricular hypertrophy: pathogenesis, detection, and prognosis. *Circulation, 102*(4), 470-479.

McMurray, J. J., & Pfeffer, M. A. (2005). Heart failure. *Lancet, 365*(9474), 1877-1889.

Mehta, P. K., & Griendling, K. K. (2007). Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *American Journal of Physiology, 292*(1), 82-97.

Miyata, S., & Haneda, T. (1994). Hypertrophic growth of cultured neonatal rat heart cells mediated by type 1 angiotensin II receptor. *American Journal of Physiology*, *266*(6), 2443-2451.

Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Olson, E. N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell, 93*(2), 215-228.

Moon, J. Y. (2013). Recent Update of Renin-angiotensin-aldosterone System in the Pathogenesis of Hypertension. *Electrolytes & Blood Pressure, 11*(2), 41-45.

Nakamura, K., Fushimi, K., Kouchi, H., Mihara, K., Miyazaki, M., Ohe, T., & Namba, M. (1998). Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-α and angiotensin II. *Circulation, 98*(8), 794-799.

Omura, T., Yoshiyama, M., Yoshida, K., Nakamura, Y., Kim, S., Iwao, H., Yoshikawa, J. (2002). Dominant negative mutant of c-Jun inhibits cardiomyocyte hypertrophy induced by endothelin 1 and phenylephrine. *Hypertension, 39*(1), 81-86.

Paradis, P., Dali-Youcef, N., Paradis, F. W., Thibault, G., & Nemer, M. (2000). Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. *Proceedings of the National Academy of Sciences, 97*(2), 931-936.

Peterson, K. L., Tsuji, J., Johnson, A., DiDonna, J., & LeWinter, M. (1978). Diastolic left ventricular pressure-volume and stress-strain relations in patients with valvular aortic stenosis and left ventricular hypertrophy. *Circulation, 58*(1), 77-89.

Pfeffer, M. A. (1995). Left ventricular remodeling after acute myocardial infarction. *Annual Review of Medicine, 46*, 455-466.

Pluim, B. M., Zwinderman, A. H., van der Laarse, A., & van der Wall, E. E. (2000). The athlete's heart a meta-analysis of cardiac structure and function. *Circulation, 101*(3), 336-344.

Purdham, D. M., Zou, M.-X., Rajapurohitam, V., & Karmazyn, M. (2004). Rat heart is a site of leptin production and action. *American Journal of Physiology-Heart and Circulatory Physiology, 287*(6), 2877-2884.

Rajapurohitam, V., Gan, X. T., Kirshenbaum, L. A., & Karmazyn, M. (2003). The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. *Circulation Research, 93*(4), 277-279.

Rajapurohitam, V., Izaddoustdar, F., Martinez-Abundis, E., & Karmazyn, M. (2012). Leptin-induced cardiomyocyte hypertrophy reveals both calcium-dependent and calcium-independent/RhoA-dependent calcineurin activation and NFAT nuclear translocation. *Cellular Signalling, 24*(12), 2283-2290.

Rajapurohitam, V., Javadov, S., Purdham, D. M., Kirshenbaum, L. A., & Karmazyn, M. (2006). An autocrine role for leptin in mediating the cardiomyocyte hypertrophic effects of angiotensin II and endothelin-1. *Journal of Molecular and Cellular Cardiology, 41*(2), 265-274.

Roger, V. L., Go, A. S., Lloyd-Jones, D. M., Adams, R. J., Berry, J. D., Brown, T. M., Wylie-Rosett, J. (2011). Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation, 123*(4), e18-e209.

Sadoshima, J., Qiu, Z., Morgan, J. P., & Izumo, S. (1995). Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kD S6 kinase in cardiac myocytes. The critical role of Ca(2+)-dependent signaling. *Circuation Research, 76*(1), 1-15.

Saito, T., Fukuzawa, J., Osaki, J., Sakuragi, H., Yao, N., Haneda, T., Hasebe, N. (2003). Roles of calcineurin and calcium/calmodulin-dependent protein kinase II in pressure overload-induced cardiac hypertrophy. *Journal of Molecular and Cellular Cardiology, 35*(9), 1153-1160.

Scherer, P. E., Williams, S., Fogliano, M., Baldini, G., & Lodish, H. F. (1995). A novel serum protein similar to C1q, produced exclusively in adipocytes. *Journal of Biological Chemistry, 270*(45), 26746-26749.

Schlüter, K.-D., Schäfer, M., Balser, C., Taimor, G., & Piper, H. M. (1998). Influence of pH i and creatine phosphate on α-adrenoceptor-mediated cardiac hypertrophy. *Journal of Molecular and Cellular Cardiology, 30*(4), 763-771.

Sinha, M. K., Opentanova, I., Ohannesian, J. P., Kolaczynski, J. W., Heiman, M. L., Hale, J., Caro, J. F. (1996). Evidence of free and bound leptin in human circulation. Studies in lean and obese subjects and during short-term fasting. *Journal of Clinical Investigation, 98*(6), 1277.

Swynghedauw, B. (1999). Molecular mechanisms of myocardial remodeling. *Physiological Reviews, 79*(1), 215-262.

Vedala, G., Herrero, P., de las Fuentes, L., Rogers, J. G., Kelly, D. P., & Gropler, R. J. (2002). Altered myocardial fatty acid and glucose metabolism in idiopathic dilated cardiomyopathy. *Journal of the American College of Cardiology, 40*(2), 271-277.

Vega, R. B., Rothermel, B. A., Weinheimer, C. J., Kovacs, A., Naseem, R., Bassel-Duby, R., Olson, E. N. (2003). Dual roles of modulatory calcineurin-interacting protein

1 in cardiac hypertrophy. *Proceedings of the National Academy of Sciences, 100*(2), 669-674.

Wallace, A. M., McMahon, A. D., Packard, C. J., Kelly, A., Shepherd, J., Gaw, A., & Sattar, N. (2001). Plasma leptin and the risk of cardiovascular disease in the west of Scotland coronary prevention study (WOSCOPS). *Circulation, 104*(25), 3052-3056.

Wang, P., Yang, F. J., Du, H., Guan, Y. F., Xu, T. Y., Xu, X. W., Miao, C. Y. (2011). Involvement of leptin receptor long isoform (LepRb)-STAT3 signaling pathway in brain fat mass- and obesity-associated (FTO) downregulation during energy restriction. *Molecular Medicine, 17*(5-6), 523-532.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., & Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature, 372*(6505), 425-432.

Appendices

Appendix 1: 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 2013031

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: Kwame Boakye-Ansah

Post-secondary   Western University
Education and   London, Ontario, Canada
Degrees: 2009-2013 B.MSc.

                       Western University
                       London, Ontario, Canada
                       2013-2015 M.Sc.

Honours and   Western Graduate Research Stipend
Awards: 2013-2015

                       First Place Poster Presentation Award in Cardiovascular Sciences
                       Department of Physiology and Pharmacology, Western University
                       November, 2014

Related Work   Teaching Assistant
Experience   Western University
                       2014-2015

Scientific Meetings Charles Gowdy Lecture and Research Day
Attended   Western University
                       November 2013

                       London Health Research Day
                       London Convention Center
                       March 2014, April 2015

                       James A.F. Stevenson Distinguished Lecture and Research Day
                       Western University
                       November 2014