The Role of Palmitoleate in Hypertension and Cardiac Hypertrophy

by

Suresh Chandra Bairwa

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

Master of Science

In

TRANSLATIONAL MEDICINE

Department of Medicine
University of Alberta

Abstract

The adipocyte-derived lipokine, C16:1n7-palmitoleate, regulates a variety of functions such as systemic glucose and fatty acid metabolism. Under normal physiological conditions, palmitoleate levels are regulated by *de novo* lipogenesis. During pathological conditions where there are enhanced rates of adipose tissue lipolysis, circulating levels of palmitoleate can be increased and these increases can be correlated to cardiovascular disease. Thus, the levels of palmitoleate in the circulation can potentially be a cause of cardiovascular disease, including hypertension, cardiac hypertrophy and heart failure. To address this, we administered palmitoleate to mice and determined the effects on blood pressure and cardiac structure and function. Our studies demonstrate that administration of palmitoleate by osmotic pump for one-week induces significant increase in blood pressure and promotes cardiac hypertrophy. In addition, palmitoleate increases muscle mass and decreases fat accumulation.

Our preliminary results suggest that pro-hypertensive and pro-hypertrophic signaling mechanisms are induced by palmitoleate in the mesenteric arteries, kidneys and heart. Our results suggest that AMPK/eNOS signaling axis inhibition by palmitoleate in the mesenteric arteries is associated with an increase in blood pressure. Further studies are required to discover the involvement of other signaling pathways to determine whether targeting those signaling pathways could be a therapeutic strategy against hypertension and cardiac hypertrophy.

Acknowledgements

I would like to express my sincere gratitude to my supervisor Dr. Jason Dyck for providing me the opportunity to pursue my master studies in his research laboratory at the University of Alberta. I am thankful of his constant support, mentorship and guidance that provided me with the vision to shape my future career. I would like to acknowledge my committee member Dr. Michael Sean McMurtry for his important role as a graduate coordinator. I am grateful of him being a great career advisor throughout my studies. I would also like to thank my second committee member Dr. Gary Lopaschuk for his invaluable advice and inputs in our meetings.

This research would not have been possible without generous financial support from University of Alberta, Department of Medicine and Faculty of Graduate Studies and Research (FGSR) and Dr. Jason Dyck. Special thanks goes to Dr. Michael Sean McMurtry for making this all possible for my studies. Also thanks to Ms. Eleni Karageorgos (Team Lead, Research), Ms. Maggie Hill and Ms. Barb Thomson (Graduate program advisor) for their assistance.

I would like to thank my lab members for assisting me with my research project. To Dr. Nirmal Parajuli for his tremendous assistance with experiments and making friendly lab environment. To Carrie Soltys, for her help and support. To Donna Beker, for performing echocardiography.

Thanks to Maria Areli Lorenzana Carrillo, for your friendship and for being a great coffee buddy.

I cannot thank enough to International Friendship Group (James Siebert & Ann Siebert) for their love and occasional warm suppers.

Thanks to my family for their endless love, care and their constant belief in my efforts.

Table of Contents

List of Figures and Illustrations

List of Tables

List of Abbreviations

ACC	Acetyl CoA carboxylase
ACE	Angiotensin-converting enzyme
ACL	ATP-citrate lyase
AdipoQ/ACRP30	Adiponectin
ADP	Adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide 1- β-ribofuranoside
Akt (PKB)	Protein kinase B
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
AT_1 R	Angiotensin II type 1 receptor
AT_2 R	Angiotensin II type 2 receptor
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BMI	Body mass index
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
BW	Body weight
CaMKKβ	Calcium-calmodulin–activated protein kinase kinase-β
CHD	Coronary heart disease
CO	Cardiac output
CPT	Carnitine palmitoyl transferase
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium

DOCA	Deoxycorticosterone acetate
ECG	Echocardiography
ECL	Enhanced chemiluminescence
eEF2	Eukaryotic elongation factor-2
EF	Ejection fraction
Elovl6	Elongation of very long-chain fatty acids protein 6
eNOS	Endothelial constitutive nitric oxide synthase
ERK1/2	Extracellular signal-regulated kinase 1/2
ET	Ejection time
FABP	Fatty acid binding protein
FACS	Fatty acyl-CoA synthetase
FAMEs	Fatty acid methyl esters
FAO	Fatty acid oxidation
FAS	Fatty acid synthase
FFQ	Food frequency questionnaire
FoxO	Forkhead box O
FS	Fractional shortening
GC	Gas-liquid chromatography
GLUT	Glucose transporter
GTT	Glucose tolerance test
HCL	Hydrochloride
HDL	High-density lipoprotein
HF	Heart failure
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
I.P.	Intraperitoneal
IVCT	Isovolumic contraction time
IVRT	Isovolumic relaxation time
IVS	Interventricular septum
IVSd	Inter ventricular septal wall thickness end diastole

IVSs	Iinter ventricular septal wall thickness end systole
LDL	Low-density lipoprotein
LKB1	Liver kinase B1
LV	Left ventricular
LVEDd	Left ventricular end-diastolic diameter
LVEDs	Left ventricular end-systolic diameter
LVPWd	Left ventricular posterior wall thickness end diastole
LVPWs	Left ventricular posterior wall thickness end systole
MAPK	Mitogen-activated protein kinase
MHz	Megahertz
MI	Myocardial infarction
mM	Milimolar
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappaB
NO	Nitric oxide
NOX	NADPH oxidase
p70S6K	Ribosomal protein S6 kinase beta-1
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffer saline
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphoinositide 3-kinase
PO	Palmitoleate
PPAR-α	Peroxisome proliferator-activated receptor alpha
qMNR	Quantitative nuclear magnetic resonance
RAAS	Renin angiotensin aldosterone system
RhoA	Ras homolog gene family member A
ROCK	Rho associated Kinase
ROS	Reactive oxygen species
SBP	Systolic blood pressure

SCD1	Stearoyl CoA desaturase 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIRT1	Sirtuin 1, silent information regulator 1
SNS	Sympathetic nervous system
SV	Stroke volume
Tak1	Transforming growth factor-β-activated kinase 1
TBST	Tris-buffered saline and 0.1% tween 20
TG	Triglyceride
TL	Tibia length
TNF-α	Tumour necrosis factor alpha
Trx	Thioredoxin
WAT	White adipose tissue
WHO	World health organisation
β-MHC	β-myosin heavy chain

CHAPTER 1: INTRODUCTION

1. Introduction

1.1 Hypertension

According to a recent report from the American Heart Association, cardiovascular disease (CVD) is the leading cause of death in North America [1] and every seven minutes there is a reported death among Canadians [2]. Statistics report show that cardiovascular related deaths have reduced every year in Canada [2], however, due to exposure to risk factors including hypertension, obesity and diabetes, prevalence of CVD has increased among Canadians [3]. Hypertension is a major healthcare concern in Canada and elsewhere [4, 5]. It is one of the main modifiable risk factors for CVD that contributes to morbidity and mortality more than any other risk factor in Canada [6]. In fact, hypertension affects approximately 1 in 5 Canadian adults [7, 8]. In Canada, estimated healthcare cost in 2010 attributed to hypertension were $13.9 billion which would be expected to rise $20.5 billion by 2020 [9].

A new blood pressure (BP) classification method was introduced by the joint national committee 7 (JNC 7) for the purpose of identifying individuals who would benefit with early intervention, reducing the rate of progression of BP to hypertension associated CVD or preventing hypertension completely [10]. According to the JNC 7 report, BP is categorized in four categories. 1) normal BP is defined when systolic BP (SBP) measurement is less than 120 mmHg and diastolic BP (DBP) is less than 80 mmHg, 2) prehypertension is when SBP/DBS is 120-139/80-89 mmHg, 3) stage I hypertension is when SBP/DBP is 140-159/90-99 mmHg and, 4) stage II hypertension when SBP/DBP is greater than or equals to 160/100 mmHg [10]. Prospective observational studies report that with every 20 mmHg in SBP and 10 mmHg in DBP above the range of 115/75 (SBP/DBP) doubles the cardiovascular mortality risk [11]. Further, increase in BP more than 115/75 mmHg is associated with stroke (54%), ischemic heart disease (47%) and other cardiovascular disease (25%) [12]. In fact, in individuals who have preexisting high-normal BP (130-139 SBP and 85-89 DBP), the risk of hypertension increases by three fold and risk of CVD by approximately two fold [13].

1.2 Pathophysiology of hypertension

There are several factors involved in the pathophysiology of hypertension including impaired renin angiotensin aldosterone system (RAAS), abnormal sympathetic nervous system (SNS) activation, endothelial dysfunction, increased cardiac output and peripheral resistance, cardiac remodeling and renal dysfunction [14-17].

1.2.1 RAAS, oxidative stress and vascular inflammation

The RAAS is a very important hormonal mechanism that maintains the functions of cardiovascular, renal and adrenal glands by regulating hemodynamic stability in the body through BP, sodium and potassium concentration and fluid balance [18]. Therefore, abnormal activation of the RAAS results in the development of CVD (hypertension, atherosclerosis, left ventricular hypertrophy, myocardial infarction (MI) and congestive heart failure) and renal disease [18, 19].

Renin (active form) is a proteolytic enzyme which is first synthesized as pro-renin (inactive form) in the afferent arterioles of renal glomerulus and released into the blood [20]. In the blood, renin is converted from pro-renin by the proteolytic and non-proteolytic mechanisms [21-23]. Renin then cleaves a glycoprotein, angiotensinogen (produced in the liver), and converts it into angiotensin I (Ang I). Angiotensin-converting enzyme (ACE) (produced in the lungs) cleaves Ang I into angiotensin II (Ang II) which is the main effector of RAAS. Ang II acts through its receptors known as Ang II type 1 (AT_1 R) and Ang II type 2 (AT_2 R) [24]. AT_1 R induces various actions of Ang II on the blood vessels such as vasoconstriction, endothelial dysfunction, vascular remodeling and inflammation, whereas AT_2 R is believed to be involved with opposite effects of that of AT_1 R [25]. Beside renin and pro-renin receptors in the kidney, studies suggest that heart, liver, and placenta express these receptors [26]. Studies also suggest that subcutaneous and visceral adipose tissue express renin receptors indicating the local synthesis of Ang II [27]. Renin and pro-renin receptors activation induces a mitogen-activated protein kinase (MAPK) signaling pathway, extracellular signal-regulated kinase (ERK1/2) [28].

Ang II is a major regulator of aldosterone synthesis, another effector of RAAS. AT_1 R mediated aldosterone synthesis takes place in adrenal cortex. Aldosterone acts on the distal nephron of the

kidney and stimulates water retention, sodium and chloride reabsorption, potassium and magnesium excretion, as a result increasing extracellular fluid volume and BP [29]. Aldosterone induces these effects via AT_1 R, G-protein coupled receptor [30] and epidermal growth factor receptors [31]. These receptors acts through MAPK/ERK1/2/p38 pathways and mediate vascular remodeling, fibrosis, inflammation [15], cardio-renal and metabolic disease [32, 33]. After aldosterone synthesis and secretion by epithelial cells in the renal tubule [34] or vascular smooth muscle cells [35], aldosterone induces the expression of genes associated with water absorption including sodium-potassium ATPase, serum/glucocorticoid regulated kinase 1, and epithelial 3 sodium channel [36]. These process regulate normal BP by modulating water and electrolyte balance.

AT_1 R mediates activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) which in turn increases reactive oxygen species (ROS) production in the vasculature and facilitate the development of atherosclerotic process [37]. Studies suggest that oxidative stress induced ROS production negatively affects the bioavailability of endothelial nitric oxide (NO), a protective vasodilator, in CVD including hypertension [38-41]. ROS regulates endothelial function, vascular tone and cardiac function [42]. Involvement of ROS have been discussed in the vascular pathological processes such as, apoptosis, fibrosis, hypertrophy and inflammation and these processes positively affect the progression of endothelial dysfunction and cardiovascular remodeling that are features of hypertension [43, 44]. NO is synthesized and produced in endothelial cells during conversion of L-arginine to L-citrulline by enzyme endothelial NO synthase (eNOS) [45-47]. NO increases blood flow and reduces BP by signaling smooth muscle to relax causing vasodilation [48, 49]. Activity of eNOS within the endothelial cell is regulated by insulin. Insulin exerts vasodilatory action via stimulating NO production by PI3K/Akt pathway-dependent eNOS activation [50]. This pathway is impaired during insulin resistance and NO production is diminished [51]. Ang II affects vascular smooth muscle cells causing vasoconstriction in afferent and efferent arterioles which leads to the development of hypertension in glomerular capillary and reduced renal blood flow [52].

Although it is noted that RAAS and SNS are essential for change in the BP for short period of time, kidneys play a major role in the blood-volume and pressure control in the long-term [53]. The kidneys perform a pivotal role in controlling BP by various mechanism including diuresis,

natriuresis, RAAS and SNS [54]. Studies show that increased aldosterone concentration is associated with increased reabsorption of sodium and water in the distal nephron in the kidney which increases BP [34]. Emerging evidences implicate aldosterone as a mediator for the development of renal disease [55]. Increased plasma levels of aldosterone have been associated with hyperaldosteronism and adrenal hypertrophy [56]. Increased aldosterone levels and renal deterioration have also been shown by clinical studies [57]. Study in rats show that aldosterone infusion resulted in the progression of renal injury and fibrosis [58] mediated by ROS and ERK1/2 [59]. In the rat model of type 2 diabetes, hypertension accelerates development of diabetic nephropathy through ERK1/2 and p38 [60]. These studies suggest that hypertension leads to structural and functional damage in the kidney resulting in renal dysfunction [61]. Studies provide the link between the kidneys and chronic hypertension [62-64]. These studies show that, when a kidney was transplanted from a salt-sensitive and hypertensive rat into a salt-resistant, normotensive rat, this later developed hypertension in the salt-resistant and normotensive rats. On the other hand, when a kidney from a salt-resistant, normotensive rat was transplanted into a salt-sensitive, hypertensive rat, this protected from increasing the BP [62, 63]. These findings were also verified in genetic rat models of hypertension studies [65-69]. These studies suggest that hypertension is regulated by the kidneys [70].

Ang II have been shown to induce vascular dysfunction mediated by MAPK and Akt signaling pathways which promote cell proliferation and apoptosis [71]. Ang II has been implicated in the progression of atherosclerosis by providing a positive feedback loop during vascular inflammation by recruiting inflammatory cells, and these cells produce more Ang II which exaggerate the vascular inflammation [18]. Oxidative stress mediated by Ang II reduces NO levels [72]. Studies show that chronic infusion of Ang II in mice increases BP, promotes inflammatory cells infiltration into myocardium and cardiac fibrosis [73]. Ang II induces oxidative stress and calcium signals in the rat cardiomyocytes which results in cardiomyocytes hypertrophy [74]. Chronic treatment of Ang II induces increase production of protein synthesis that leads to vascular smooth muscle cell hypertrophy [75].

During hypertension, insulin resistance is implicated in the pathophysiology of CVD and chronic kidney disease [76, 77]. Studies estimated that at least half of the hypertensive individuals are insulin resistant, and insulin resistance is one of the main abnormality of the cardio-metabolic

syndrome [78, 79]. It is observed that patients who have hypertension show high fasting and pre-prandial insulin levels which is not dependent on the fat distribution or body mass index [80]. Experimental evidence from animal model of insulin receptor knockout in the renal tubule epithelial cells suggested that lack of insulin resulted in increased BP and this was correlated with reduced NO production [81]. This suggests that reduced NO production leads to vasoconstriction in the kidney and insulin resistance condition leads to sodium reabsorption in the distal nephron resulting in hypertension.

Studies have established that hypertension is associated with vascular endothelial dysfunction [82-84]. Endothelial dysfunction is an alteration in the endovascular lining of blood vessels defined by pro-inflammatory and pro-thrombotic phase [14]. The Framingham heart study of offspring cohort suggests that degree of endothelial dysfunction is positively associated with the hypertension severity [85]. Oxidative stress from various sources have been linked with the hypertension associated endothelial dysfunction [86]. Studies suggest NO as an important regulator of vascular endothelial function and endothelial dysfunction phenotypic characteristic is identified by the absence of NO bioavailability [87, 88]. It is controversial that hypertension is a cause or an effect of endothelial dysfunction. Studies support the hypothesis that endothelial dysfunction occurs prior to the development of hypertension in which exogenous infusion of eNOS inhibitors in humans lead to hypertension [89]. Further supported by the cohort study in which postmenopausal normotensive women who had endothelial dysfunction had six times increased risk of hypertension development [90]. Conversely, study demonstrates that in young adults acute, large increase in BP is associated with acute endothelial dysfunction [91]. Other study show that subjects with high BP in adolescence were more prone to develop endothelial dysfunction in adulthood [92].

It is believed that if hypertension progresses over time then renal dysfunction may be normalized by compensatory mechanisms, which include cardiac and vascular remodeling [17]. It is proposed that during the pre-hypertension stage, the kidneys are normal but renal injury is initiated by recurrent and repeated vasoconstriction mediated by several factors [93] including, hyperactive SNS activity [94] and modification in the RAAS which leads to increased Ang II in the circulation and loss of local NO [93, 95].

Clinically, hypertension is associated with left ventricular hypertrophy which is an adaption to hemodynamic overload exerted by chronic increase in systemic hypertension [96, 97]. This adaption is called cardiac remodeling in which left ventricular wall thickness and mass are increased in the ventricular myocardium to meet the functional demand, however, this may also progress to heart failure [96, 98].

1.3 Cardiac hypertrophy and heart failure

Cardiac hypertrophy is a compensatory adaptation of the heart in response to hemodynamic overload such as that observed during pregnancy, exercise, hypertension and valvular heart disease [99]. In these situations, the heart undergoes necessary changes including increase in ventricular wall thickness and increase cardiac output that allow the heart to deal with the increased workload. Cardiac hypertrophy induces increase in cardiomyocyte size, increase in total rate of protein synthesis and re-expression of fetal genes, i.e. atrial natriuretic peptide (ANP), β-myosin heavy chain (β-MHC) and brain natriuretic peptide (BNP) [100]. Although cardiac hypertrophy can be reversible, long-term cardiac hypertrophy can become maladaptive and can be involved in the progression to heart failure [101].

Although many factors are involved in the transition from adaptive cardiac hypertrophy to maladaptive hypertrophy, it appears that cardiac metabolism may play a role [102, 103]. For examples, some metabolic factors that may drive the transition from cardiac hypertrophy to heart failure are that the heart becomes insulin resistant along with a reduction in mitochondrial oxidative metabolism, all of which can lead to the heart becoming energy deficient [104]. Whereas the healthy heart can shift fuel source utilization between glucose and fat [105], studies have shown that the hypertrophic or falling heart primarily uses glucose for ATP production and less fatty acid [105, 106]. Taken together, these studies suggest that energy metabolism is compromised during cardiac hypertrophy and heart failure and thus, restoring energy production in these situations may prove to be an effective strategy for prevention and treatment of cardiac hypertrophy and heart failure. One major regulator of cardiac energy metabolism is the energy sensing kinase, adenosine monophosphate (AMP)-activated protein kinase (AMPK).

1.4 AMPK: structure, activation, and regulation

AMPK is a serine-threonine protein kinase that plays a crucial role as an energy sensor of the cells and maintains cellular homeostasis. It is activated during low energy levels of the cells and it turns-on the energy generating pathways and turns-off the energy consuming pathways [107]. Due to these important actions of AMPK, it has been known as a fuel gauge [107] and a major metabolic regulator of the cells [108]. AMPK was first purified in 1989 [109] and clones in 1994 [110]. Since the cloning of AMPK, the molecular properties have been studied and early studies investigating the role of AMPK in the heart were initiated in 1995 [111]. Since then, studies have been focused on the role of AMPK on cardiovascular disease and as a result, various actions in the heart have been identified [111-119].

AMPK is a complex of three subunits, alpha (α) subunit, beta (β) and gamma (γ). Each individual subunit has a different structure and function and the interaction between these subunits is essential for the cell environment in order to regulate AMPK activity. The α subunit is the catalytic subunit and the β and γ subunits are regulatory subunits. The α subunit is a serine/threonine domain of the AMPK and this has an important activating site, threonine-172 (Thr^{172}). Upstream kinases are required to phosphorylate AMPK at Thr^{172} and this phosphorylation is an indicator of AMPK activation [120]. The β subunit has a glycogen binding site and structurally it is in the middle of other α and γ subunits. Studies report that when the cell has plenty of energy and thus high levels of stored glycogen, high glycogen concentrations in the cell can inhibit activation of AMPK [121, 122]. On the other hand, studies also report that absence of glycogen might cause activation of AMPK in the heart in the event of MI [123] and physical exercise [124]. The α subunit has an auto-inhibitory sequence, which is reversed when AMP binds with γ subunit that leads to conformational changes in the AMPK complex [125, 126]. Binding of AMP with γ subunit results in conformation change and leads to activation of catalytic unit of AMPK [127]. All of the subunits have different isoforms encoded by different genes depend on different types of cells [128]. The α_1 isoform is most abundant in endothelial cells and α_2 isoform is in cardiomyocytes [129]. The γ_1 and γ_2 isoforms are expressed in the heart and γ_3 in the skeletal muscle [129]. Because of changes in the expression of different isoforms in different cells, AMPK possess different action. For example, intracellular concentration of AMP affects mainly α_2 isoform than α_1 isoform [130]. It is observed that during MI, α_1 and α_2 isoforms

are activated [131, 132], however, less intense exercise only activates α_2 isoform in the heart [133, 134].

Activation of AMPK depends on the intracellular energy levels which is determined by the AMP to ATP ratio. When the heart requires energy, ATP is broken down in ADP and subsequently into AMP and increase in the levels of AMP activates AMPK [135-137]. Upon AMP binding, the activation site of α subunit, Thr^{172}, is phosphorylated by upstream kinases leading to increased AMPK activity [138, 139]. Intracellular AMP in the heart has high affinity towards binding with AMPK and AMP concentrations are found in micromolar quantity [135]. Though ATP is found in very high concentration, it has low-binding tendency towards AMPK compared with AMP or ADP which allows AMP or ADP to bind with AMPK competitively [140].

Phosphorylation and activation of AMPK is regulated by upstream AMPK kinases. One of the upstream kinase discovered is the tumor suppressor protein liver kinase B1 (LKB1) [141, 142]. LKB1 is expressed abundantly in the heart [143]. It has been shown that LKB1 deficiency during MI attenuates AMPK activation of α_2 isoform suggesting that LKB1 regulates α_2 isoform in the heart during MI [143].

Another upstream kinase calcium-calmodulin–activated protein kinase kinase-β (CaMKKβ) is expressed in brain and other non-cardiac tissues and has been shown to activate AMPK [144]. CaMKKβ is expressed at very low levels in cardiomyocytes [145]. It has been reported that during a MI, AMPK is upregulated along with increased CaMKKβ activity [146] suggesting that energy is depleted during a MI and increase in AMP concentrations in the cells activates AMPK. However, CaMKKβ activation is not related to LKB1 activation [137, 146]. When Ca^{2+} influx increases in the cell, CaMKKβ activates AMPK in order to meet ATP demand and this activation does not depend on the AMP/ATP ratio of the cell [147, 148].

1.5 AMPK in CVD

The hypertrophied heart undergoes metabolic alterations impairing energy metabolism, which can contribute to deteriorating cardiac function [149]. The role of AMPK in the prevention of hypertension has been established. Studies show that AMPK activation by resveratrol lowers BP in deoxycorticosterone acetate (DOCA)-salt hypertensive mice [150] and prevents Ang II-

induced hypertension [151]. Further, anti-hypertensive effect of AMPK is mediate through eNOS/Akt signaling pathway [152] and Ras homolog gene family member A (RhoA)/Rho associated Kinase (ROCK) suppression [151]. It has been shown that resveratrol treatment in spontaneous hypertensive rat and Ang II-infused hypertension rodents attenuates BP increase, reduces oxidative stress, improves vascular function and prevents cardiac hypertrophy through the LKB1/AMPK/eNOS signaling axis [153]. Studies in the mesenteric arterioles, which are considered true resistant vessels that contribute to total peripheral resistance [17], and aorta show that AMPK activation by resveratrol, 5-aminoimidazole-4-carboxamide 1- β-ribofuranoside (AICAR), berberine or metformin reduces BP and endothelial injury by inducing vasorelaxation via increases in eNOS phosphorylation through AMPK/eNOS signaling [153-156]. It is suggested that hypertension causes cardiac hypertrophy and, thus, AMPK downregulation may be involved in that either secondary to hypertension or directly, via the cardiomyocytes.

A study by Tian et al. reported activation of AMPK isoforms, α_1 and α_2, in the pressure overload hypertrophied hearts [157]. In these hearts, activation of AMPK was associated with increased glucose uptake [157] and glycogen storage [158]. Another study in the pressure overload hypertrophied hearts demonstrated increased AMPK activation promoted glucose uptake [159]. In contrast to these studies, it has been shown that impaired AMPK activation in the heart promotes cardiac hypertrophy [153, 160, 161]. Similarly, low expression of AMPK during pressure overload hypertrophy is associated with severe cardiac remodelling [162]. In addition, a spontaneous hypertensive rat model shows inhibition of cardiac hypertrophy by activation of AMPK [163]. These studies are in favor of the concept that AMPK activation is required to attenuate cardiac hypertrophy. Moreover, the prolonged treatment with the AMPK activator, AICAR, causes regression of hypertrophy in aortic banding [164]. Similar study in isolated cardiomyocytes, shows increased AMPK activity and reduction of the hypertrophic responses. The pre-treatment with AMPK activators, metformin, phenformin, AICAR, and resveratrol attenuates cardiomyocyte hypertrophy induced by phenylephrine, supports the role of AMPK activation in the hypertrophic growth [165].

Furthermore, AMPK directly and indirectly regulates eukaryotic elongation factor-2 (eEF2) [166, 167] kinase and mammalian target of rapamycin (mTOR)/p70S6 kinase (p70S6K) [168] to regulate protein synthesis during cardiac hypertrophy. Similarly, pharmacological activation of

AMPK in cultured cardiomyocytes inhibits mTOR/p70S6K and eEF2 that attenuates protein synthesis and gene expression [160, 169, 170]. In addition, AMPK activation by resveratrol or AICAR in isolated cardiomyocytes inhibits protein synthesis through mTOR and this inhibition prevents subsequent phosphorylation of p70S6K and ribosomal protein S6, resulting in the suppression of cardiac hypertrophic growth secondary to protein synthesis. Furthermore, deletion of cardiac specific LKB1 results in the attenuation of AMPK and activation of mTOR/p70S6K signaling which leads to cardiac hypertrophy [171]. Recent studies also support that activation of AMPK leads to anti-hypertrophic effect via inhibiting mTOR pathway [172-175]. In addition, AMPK activation inhibits cardiac hypertrophy by attenuating ERK1/2-MAPK pathway [176], calcineurin-nuclear factor of activated T-cells (NFAT) and nuclear factor kappaB (NF-κB) pathway [164], and upregulating PPAR-α [176, 177], and eNOS pathway [163]. These studies suggest that AMPK plays an important role in attenuation of cardiac hypertrophy via regulation of protein synthesis.

Studies report that AMPK activity increases in cardiac hypertrophy [157], ischemia [111, 136], hypoxia [178] and cardiomyopathy [179]. Also, during acute heart failure, activation of AMPK is the case of adaption due to energy depletion [180]. However, AMPK activity decreases in the advanced stage of the heart failure [181]. Cardiac hypertrophy and heart failure condition prefers glucose oxidation over fatty acid oxidation (FAO) [105, 106] and during these cardiac pathological conditions heart is unable to maintain preferred glucose oxidation [182, 183]. This reduction in energy metabolism and decrease in the GLUT4 translocation on the membrane also indicates less glucose uptake by cardiomyocytes [104, 184]. The pharmacological activators, metformin and AICAR treatment diminishes contractile dysfunction, apoptosis, and fibrosis in the canine heart failure model suggesting that heart failure does not activate AMPK but its activation is beneficial [185]. These treatments also improve systemic insulin resistance and increase plasma NO levels [186] and these effects may contribute to the beneficial effects of AMPK.

The oxidative stress and apoptosis may play an important role in the development of cardiomyopathy, atherosclerosis and heart failure [187-190]. Attenuation of oxidative stress and apoptosis prevents heart failure by improving cardiac performance [191]. AMPK activation by resveratrol protects cardiomyocytes from oxidative stress and apoptosis [192, 193]. In addition,

AMPK activation by resveratrol improves cardiac functions through silent information regulator (SIRT1) activation [192]. Moderate overexpression of SIRT1 protects cardiomyocytes from oxidative stress and apoptosis and inhibition of SIRT1 exacerbates cell death [194].

Furthermore, the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) may play cardio-protective role in the heart failure as lack of PGC-1α promotes cardiac dysfunction that leads to heart failure [195]. In addition, it has been shown that AMPK activates PGC-1α protein by binding and phosphorylating directly at Thr-177 and Ser-538 residues [196] and AMPK also stimulates SIRT1-mediated deacetylation and activation of PGC-1α [197]. Lastly, AMPK phosphorylates forkhead box O3 (FoxO3) thus causing its translocation into the nucleus and activation [198]. In the nucleus, FoxO3 activates antioxidant system thioredoxin (Trx) by binding with Trx promotor and upregulating transcription of Trx which leads to the inhibition of oxidative stress and reduction in ROS production [198] and ultimately protection of cardiomyocytes from oxidative stress and apoptosis [199]. Studies report that AMPK activated FoxO promotes autophagy in the heart by activating the genes involved in autophagy [200, 201]. Autophagy activation by resveratrol reverses cardiac remodeling and protects heart failure via AMPK activation [202] and impaired process of autophagy leads to heart failure [201]. Study report that metformin administered at the time of reperfusion, induces increased AMPK phosphorylation, eNOS phosphorylation, and PGC-1α expression, reduced LV remodeling after MI [203]. Overall, the accumulated evidences suggest that AMPK activation is beneficial in the cardiovascular disease.

1.6 Importance of adipose tissue in CVD

The role of adipose tissue was largely restricted to fatty acid storage until 1987, when it was identified as an important site for sex steroid metabolism [204]. In addition, after the discovery of leptin in 1994, adipose tissue was recognised as an endocrine organ [205] and it is now known to secrete and express a wide varieties of bioactive compounds and peptides known as adipokines that act in autocrine, paracrine and endocrine manners [206]. Adipose tissue also expresses various receptors that enables it to react to different endocrine hormonal regulation as well as regulation by the central nervous system [206]. Thus, along with being the reservoir for

energy, adipose tissue coordinates many biological processes such as energy metabolism, neuroendocrine, immune function [206].

Increasing amounts of research evidence implicate that secreted hormones or peptides from the adipose tissue also regulate systemic carbohydrate and lipid homeostasis [207-210]. The endocrine function of adipose tissue is highlighted by the condition when there is a deficiency and excess of adipose tissue. Increased adiposity or excessive adipose tissue deposition, especially in the visceral areas, is associated with comorbidities, known as metabolic syndrome, such as hypertension, insulin resistance, atherogenic dyslipidemia, hyperglycemia, pro-inflammatory and pro-thrombotic state [211]. These comorbidities are also linked with adipose tissue deficit (or lipodystrophy) [212]. Therefore, both adipose tissue excess and deficit can have adverse health outcomes.

1.6.1 Adipokines

White adipose tissue (WAT) is composed of many heterogeneous cell types including adipocytes, pre-adipocytes, pericytes, blood vessels, leukocytes, fibroblasts, macrophages, stromovascular cells, endothelial cells, and connective tissue matrix [213-216]. This complex structure enables WAT to regulate multiple metabolic actions, which is the foundation of the functions of adipose tissue [217]. WAT is able to perform multiple functions by communicating with organs or organ systems in an autocrine, a paracrine and an endocrine manner through release of bioactive chemical compounds and peptides known as adipokines [206, 218]. Adipokines are synthesized by adipocytes, macrophages, fibroblasts and leukocytes [219, 220] and can be secreted during various conditions. For example, during the development of obesity, adipokine release from adipose tissue can be altered followed by the changes in the adipocyte phenotype, numbers and accumulation of inflammatory cells [221]. The secretion and expression of adipokines depends on the location of adipose tissue in the body and it is now clear that the visceral and subcutaneous depot of adipose tissue show distinct adipokine profiles [222, 223].

Abnormal increases in adipocyte number and volume can be observed in obese patients [224]. Evidence suggests that obesity-induced adipocyte expansion is linked with hypoxia [225], oxidative stress [226], and accumulation of macrophages [227], suggesting that these events are a cause or an effect of dysregulation in adipokines release. Though, the exact biological effects

of adipokines are still poorly understood, they are believed to be involved with regulation of many physiological actions including lipid metabolism, food intake and energy balance, insulin sensitivity, BP, angiogenesis, homeostasis and inflammation [228]. Involvement of adipokines with physiological conditions suggests that the inflammatory stage of adipose tissue may be responsible for poor health during obesity.

The ability of adipose tissue to produce tumour necrosis factor alpha (TNF-α), was first identified in 1993 and the secretion of this pro-inflammatory cytokine is elevated in obesity and diabetes [229]. Interestingly, upregulation of TNF-α was associated with obesity and obesity-induced hypertension [230], thus linking adipose tissue-derived adipokines to CVD. Following this discovery, leptin was identified in 1994 as an adipokine that balances the appetite and energy expenditure [231]. Like TNF-α, increased leptin levels has been suggested to be involved in mediating obesity-associated hypertension [232]. Further, plasminogen activator inhibitor-1 (PAI-1) was identified in 1996 as an adipokine which was found to be correlated with visceral obesity [233]and high BP [234].

Around the same time, the adipose-tissue specific adipokine, adiponectin, also known as AdipoQ/ACRP30, was discovered [235-237]. Adiponectin is the most abundant serum anti-inflammatory adipokine and it is reported that expression of adiponectin is significantly downregulated during obesity [235]. Of importance, experimental studies showed that adiponectin treatment protects against obesity linked CVD [238, 239] and that low levels of adiponectin are found in the hypertensive adults [240]. Interestingly, studies show that hypertensive adults have lower adiponectin levels compared with normotensive adults and increase in adiponectin levels was associated with decreased risk of hypertension [241]. Taken together, these studies suggest that cardiovascular dysfunction associated with increased adiposity may result from an imbalance of pro-inflammatory and anti-inflammatory adipokines. Thus, these studies provide an important link between increased adiposity and cardiovascular dysfunction and suggest that other adipokines may also have similar roles.

1.6.2 Lipokines

Lipokines are an adipose tissue-derived hormone-like lipid molecules used by adipose tissue to communicate with distant organs. The term lipokine was first used by “Gökhan Hotamisligil lab”

in 2008 to identify a unique fatty acid that was involved in the lipid metabolism [207]. When they performed a detailed analysis of whole body lipid profiling in mice lacking lipid chaperone proteins fatty acid binding proteins (FABP4/5), they observed enhanced rate of *de novo* fatty acid synthesis mediated by upregulated expression of *de novo* lipogenesis genes (fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1)) in adipose tissue [207]. FABP4/5 are a group of molecules that regulate lipid functions in cells and are strongly associated with metabolic and inflammatory signaling [242, 243]. They discovered a novel lipokine, palmitoleate, that showed beneficial systemic effects and that was elevated in the plasma with increased rate of *de novo* lipogenesis [207].

1.7 Palmitoleate

Adipocyte-derived product, palmitoleate is a monounsaturated fatty acid also known as cis-9-hexadec-9-enoic acid or palmitoleic acid (C16:1n7). Palmitoleate has been shown to functions as a lipokine which communicates with other organs and coordinates systemic metabolic homeostasis [207]. Studies using hyperinsulinemic-euglycemic clamp in mice showed that infusion of palmitoleate required significantly higher infusion rate of glucose to maintain normal blood glucose which further suggests that palmitoleate improves glucose metabolism [207]. Infusion of palmitoleate in mice showed direct and positive regulation of systemic glucose and lipid metabolism by inhibiting the lipogenic genes (SCD1, FAS and Elovl6) in the liver and enhancing insulin signaling in the liver and muscle [207]. Increase in plasma palmitoleate concentration was positively associated with enhanced rate of *de novo* lipogenesis in adipose tissue, thus, palmitoleate has been suggested to act as a marker for *de novo* lipogenesis [207].

A number of studies have attempted to discover a link between the levels of palmitoleate in blood and tissue and metabolic diseases in humans ranging from instance insulin resistance [244-247] obesity [248-250], and diabetes [251, 252]. After the identification of palmitoleate in the animal models, circulating palmitoleate was examined in humans in terms of the role it plays in metabolic diseases including insulin resistance and type 2 diabetes. In 100 Caucasians participants, the circulating palmitoleate concentration was found to be positively associated with insulin sensitivity determined by euglycemic-hyperinsulinemic clamp studies and that this association was independent of the subject's age, sex and/or adiposity [245]. Another study of

3630 participants from US showed that higher concentration of circulating palmitoleate was correlated with increased body mass index (BMI), and greater insulin resistance [253]. These studies indicated that association of circulating palmitoleate levels with metabolic disease was complex and this might be dependent on the endogenous synthesis of palmitoleate from liver and adipose tissue as high carbohydrate and fat rich diet and fatty liver may alter the true metabolic effects of palmitoleate [253].

1.7.1 Dietary source

Food intake is the primary source of fatty acid in blood and tissue [254]. There are few food sources that contain higher quantities of palmitoleate than other fatty acids. However, food containing palmitoleate more than 5% (gm per 100gm of total fatty acid) are very limited. For instance, palmitoleate rich food sources are salmon 6%, cod liver oil 7.1% and macadamia oil 17.3%, and low palmitoleate food sources are soybean oil 0.08%, chocolate 0.2%, eggs 0.3% and olive oil 1.4% [255]. There is an exceptional plant, Sea Buckthorn (Hippophae rhamnoides l.), native to Asia and Europe which is known for the richest palmitoleate content. Its pulp oil contains 32-42%, whole berry oil 28-37% and seed oil less than 4% of palmitoleate [256].

The dietary intake of fatty acid also have been studied and palmitoleate amount is found to be very low compared with other fatty acid. A study involving randomly selected 49 black and 72 white members of Seventh-day Adventist church reported that dietary palmitoleate intake assessed by 24-hour recall for the black subjects was 0.9 ± 0.4 gm/day (mean ± SD) and this was 1.3 ± 0.4% of total fatty acid intake. Whereas, white subjects were similar in daily intake of palmitoleate (0.9 ± 0.4), however, it was significantly lower (0.3 ± 0.4) percentage of total fatty acid intake per day [257]. Another study using a food frequency questionnaire (FFQ) in a large cohort of 2048 men and 2391 women reported that palmitoleate intake was 2.0 ± 0.8 gm/day (mean ± SD) which was 2.2 ± 0.5% of total fatty acid intake in men compared with 1.6 ± 0.6gm/day which was 2.2 ± 0.5% of total fatty acid intake for women [258]. In addition, study in overweight, obese and morbidly obese individuals reported that palmitoleate intake decreased with increasing adiposity, for example 2.51 ± 2.51% (total fatty acid intake per day) in overweight, 2.09 ± 0.80% in obese and 1.17 ± 0.30% of in morbidly obese individuals [259]. These studies suggest that palmitoleate intake from food sources is low in comparison with other

fatty acid, for example oleate which was measured around 23-29 gm/day which was 33-52% of total fatty acid intake per day [257-259].

1.7.2 Endogenous synthesis

Even though the literature provides evidence of low palmitoleate intake from dietary sources, its concentration in the blood and tissue is reported from as low as 0.7 mol% and as high as 7.2 mol% [254]. Thus, it is likely that endogenous synthesis of palmitoleate can modulate plasma concentrations [260].

Fatty acids, including palmitoleate, are primarily synthesized in the liver and secondarily in adipose tissue by *de novo* lipogenesis, resulting from the specific activation of fatty acid synthase (FAS) and regulation of stearoyl CoA desaturase 1 (SCD1) [207, 261] (Figure 1). Upon synthesis, fatty acids are converted into triglycerides and stored in adipose tissue [262]. Under normal physiological condition, fatty acids release from adipose triglyceride storage is suppressed by insulin and insulin resistance is suggested to be responsible for activation of lipolysis and release of free fatty acids into circulation [262]. Increases in the circulatory free fatty acids have been associated with hypertension, insulin resistance, atherosclerosis and obesity [263-266].

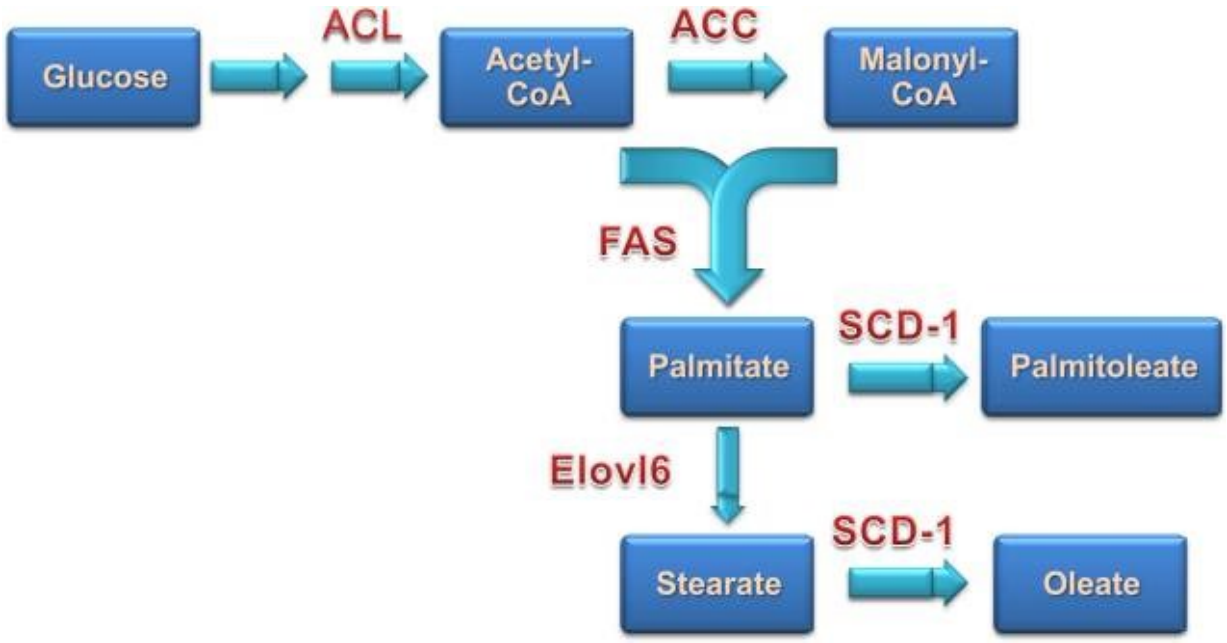


Fig. 1 *De novo* lipogenesis of palmitoleate [269]

"Republished with permission of Elsevier Science Publishers, from Lipoexpediency: *de novo* lipogenesis as a metabolic signal transmitter, Irfan J. Lodhi, 22(1), 2011; permission conveyed through Copyright Clearance Center, Inc."

De novo lipogenesis usually occurs in all cells, but both liver and adipose tissue are the major organ where lipogenesis occurs. This lipogenesis process requires series of enzymatic processes from glucose to fatty acid production. Citrate is produced from utilization of glucose in glycolytic process in mitochondria. ATP-citrate lyase (ACL) enzyme converts citrate to acetyl-CoA. Acetyl CoA is then converted to malonyl CoA by acetyl CoA carboxylase (ACC). Fatty acid synthase (FAS) utilizes both acetyl CoA and malonyl CoA and converts into palmitate and palmitate is further processed by elongation of very long-chain fatty acids protein 6 (Elovl6) enzyme into stearic acid [267]. Stearoyl-CoA desaturase 1 (SCD1) then catalyzes palmitate (palmitic acid) to palmitoleate and stearate to oleate [268], which are preferentially converted into triglycerides and stored in adipose tissue [269].

1.8 The role of palmitoleate in CVD

While studies have suggested that circulating palmitoleate levels are associated with hemostasis and metabolism of cholesterol, the overall effect on the cardiovascular system has yet to be elucidated. Studies show that, higher levels of low-density lipoprotein (LDL) cholesterol and low levels of high-density lipoprotein (HDL) cholesterol are risk factors of CVD [270-272]. A recent cohort study in the Cardiovascular Health Study of 3630 men and women reported that higher plasma phospholipid palmitoleate concentrations was observed significantly associated with low LDL cholesterol, low fibrinogen and high HDL cholesterol [250, 252]. In addition, results from epidemiological studies have shown that circulating levels of palmitoleate is strongly associated with several cardio-metabolic risk factors, including serum apolipoprotein A1 and apolipoprotein B levels, endothelial dysfunction, high BP, TG, LDL and HDL cholesterol [250, 273]. Furthermore, study in a Chinese population of middle-aged and older subjects show that the concentration of palmitoleate in erythrocytes was inversely associated with adiponectin but positively associated with high BP, retinol binding protein 4, hypertriglyceridemia and decreased HDL cholesterol [274]. In addition, the Physicians Health Study showed that the palmitoleate concentration in erythrocyte membranes was positively associated with the risk of coronary heart disease (CHD) [275]. Although the erythrocyte concentration of palmitoleate is not a representation of what is present in tissues or in the circulation, this represents endogenous fatty acid production [276]. Palmitoleate rich diet consumption showed beneficial effects by decreasing circulating cholesterol and TG levels [277, 278]. A recent study by Bernstein et al. showed that when 220.5 mg of purified palmitoleate was given to adults with dyslipidemia, TG, LDL cholesterol and C-reactive protein were significantly decreased and HDL cholesterol was increased which suggested palmitoleate could be a potential treatment of hypertriglyceridemia [279]. Studies show that consumption of palmitoleate rich food, macadamia nuts, was linked with beneficial change in serum lipid composition [277, 280, 281], however, this phenomena was not observed in the study by Nestel et al. [278]. Taken together, contradicting results of palmitoleate concentration and effects on circulating lipid profiles may be explained by different patient populations, such as healthy and diseased condition. Healthy participants may not have any beneficial effect of high serum palmitoleate, however, this may be a potential therapy for improving lipid profile in the subjects with dyslipidemia. Therefore, additional studies are required to confirm this speculation.

Fatty acid products of *de novo* lipogenesis, palmitoleate, have been correlated with heart failure risk factors including hypertension [282], obesity [248, 249, 253], diabetes [283, 284], CHD [275] and cardiovascular mortality [285]. A study by Djousse et al. in the participants from Physicians Health Study showed that increase in plasma phospholipid palmitoleate concentration was significantly associated with 17% higher risk of heart failure in male physicians [286] This study also suggested that palmitoleate concentration in the blood are a positive biomarker that enhances the progression of heart failure by developing hypertension. Experimental evidence from animals show that when fatty acid mixture including palmitoleate and palmitic acid was administered subcutaneously, it stimulated cardiac growth and significant increase in left ventricular mass [287]. Taken together, these observation suggests that palmitoleate may affect the development of cardiac hypertrophy and heart failure.

1.9 Hypothesis and objectives

Adipose tissue is an endocrine organ and releases a variety of bioactive molecules that are known to communicate in an endocrine, paracrine and autocrine manner to regulate a wide array of physiological functions. Adipocyte-produced adipokines such as leptin and adiponectin have been well studied for their effects as signaling molecules in the CVD. Similarly, adipocyte lipolysis produces the lipokine, C16:1n7-palmitoleate, which has also been shown to act as signaling molecule. In fact, studies have suggested that the palmitoleate concentration in the circulation is positively associated with BP, insulin resistance, inflammation, and heart failure. However, due to the complex interplay between all of the conditions, it is not known if elevated palmitoleate levels alone are sufficient to cause hypertension.

Hypothesis: Adipose-derived C16:1n7-palmitoleate induces hypertension and indirectly causes cardiac remodelling

Specific Aims:

1. To examine the effect of palmitoleate on blood pressure and cardiac structure/function.
2. To investigate the signaling mechanism(s) associated with palmitoleate-induced hypertension

CHAPTER 2: MATERIALS AND METHODS

2. Materials and Methods

2.1 Animal Care and Diets

All protocols involving mice were approved by the University of Alberta Institutional Animal Care and Use Committee and conformed with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health. The University of Alberta adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences and comply with the Canadian Council on Animal Care guidelines. All C57BL/6J mice were obtained from Jackson laboratory Canada. Seven-week old mice were housed in individual cages with ad libitum access to standard chow diet and water for 2 weeks.

2.2 Materials

Primary antibodies utilized for immunoblotting in this thesis were anti-phospho Akt (Ser473), anti-Akt, anti-phospho-AMPKα (Thrl72) and anti-AMPKα, anti-phopho-p70S6K (Thr421/Ser424) and anti-p70S6K, anti-phospho mTOR (Ser2448) and anti-mTOR, anti-phospho-LKB1 (Ser428) and anti-LKB1, anti-phospho-p38 (Thr180/Tyr182) and anti-p38 antibodies purchased from Cell Signaling Technology (Danvers, Massachusetts). Anti-actin antibody and horseradish peroxidase (HRP)-labelled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Fatty acid free bovine serum albumin (BSA), palmitoleate, and Protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, Missouri) and Phosphatase inhibitor cocktail set IV was purchased from Calbiochem (Gibbstown, NJ). For immunoblotting, Trans-Blot® Transfer Medium (pure nitrocellulose) from BioRad (Richmond, California), FUJI Medical X-ray films from Mandel Scientific (Guelph, Ontario), and Western Lightning® Chemiluminescence Reagents Plus kit from Perkin Elmer Life and Analytical Sciences (Woodbridge, Ontario) were used.

2.3 Palmitoleate Preparation and Administration

Alzet osmotic micro-pumps (Model 1007D, Durect Corporation, Cupertino, CA, USA) containing either fatty acid free BSA in 1x Dulbecco's Modified Eagle Medium (DMEM) or BSA/palmitoleate mixture were implanted under anesthesia at dorsal interscapularis by scalpel to

make a small incision and form a small subcutaneous pocket. Stock solution of 7 mM palmitoleate was prepared as described by Riguelme et al. [287]. Mice were divided randomly to receive either BSA or BSA/palmitoleate mixture for 7 days. A method almost identical with ours to infuse palmitoleate has previously been shown to induce significant changes in healthy mice [287]. According to the binding capacity of BSA, 1:5 molar ratio of BSA and palmitoleate was prepared. Stock solution of 1.4 mM BSA was prepared by mixing 2.31 g in 25 mL of DMEM without glucose or other addition. BSA was mixed in smaller volume of DMEM in 50 mL falcon tube and top it up to 25 mL. Solution was centrifuged at 1200x g to accelerate solubilisation of BSA into medium. The final solution was sterile filtered through 0.22 µm syringe filter and stored at -80 ºC. A 7 mM Palmitoleate stock solution was prepared by mixing 100 mg in 3.75 mL DMEM and stored at -80 ºC in aliquots. To prepare stock solution of 7 mM palmitoleate, 266.67 µL of 105 mM palmitoleate was mixed in 3.733 mL of a 1.4 mM BSA solution. The final mixture was sterile filtered through a 0.22 µm syringe filter and transferred into pumps. The osmotic pump filling capacity was 100 µL that released solution at constant rate of 0.5 µL per hour. The amount of palmitoleate in the osmotic pump was 178 µg in 100 µL, which was delivered at 1.06 µg in 0.5 µL per hour for 7 days. After 7 days, mice were sacrificed and tissues were collected.

2.4 *In Vivo* Assessment of Cardiac Function and BP

One-weeks following the osmotic pump implantation, mice were mildly anesthetized using 3% isoflurane and 1.0 L/min oxygen, and maintained on 1-1.5% isoflurane and 1.0 L/min oxygen throughout the procedure. These mice were subjected to transthoracic echocardiography using a Vevo 770 High-Resolution Imaging System equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, ON). ECG tracings were obtained simultaneously along with cardiac images by immobilizing mice on ECG metal strips on a mice handling heating platform using tapes. Parameters for cardiac function were set by an experienced animal echocardiographer. Analysis of the images were done by a blinded observer. M-mode measurements for the left ventricular wall thickness and cavity size were obtained from the long-axis view at the level of papillary muscles. Measurements for the free wall thickness of right ventricle and dimension of end-diastolic cavity were obtained from the short axis view just below the level of the aortic valve. In the animal model, right ventricle free wall images are most consistent with this view.

To obtain ventricular dimensions, M-mode recordings of 3-6 cardiac cycles were used, and % Fractional Shortening and % Ejection Fraction were determined. Doppler imaging of pulmonary outflow was obtained from the view at the level of aortic valve. Four view chamber view was used to obtain Doppler imaging of the mitral E and A wave velocities. To determine myocardial performance index (TEI index), isovolumic contraction time (IVCT), isovolumic relaxation time (IVRT) and aortic ejection time (ET) were measured from E and A waveforms. The TEI index was derived from (IVRT/IVCT)/ET equation.

Non-invasive BP measurements were made using a tail-cuff system (IITC Life Science). Mice were kept in the ventilated tubes to restrict the physical mobility to train and acclimatize them for a week. Mice tails were kept in the BP recording device, BP was taken before the pump implant, and at the end of study (end of 1st week).

2.5 Serum palmitoleate measurement

To measure the palmitoleate in the serum, fatty acid methyl esters (FAMEs) were prepared and analysed by gas-liquid chromatography (GC). 100 µL of serum was transferred to glass tubes with Teflon-lined cap. For this protocol, heptadecanoic acid (C17:0, Margaric acid) was used as an internal standard and 100 µL was added in the tubes. 3.75 mL of chloroform/methanol (2:1) was added to each tube and then vortexed. 1.25 mL of mildly acidified 0.9% sodium chloride was added to each tube and then vortexed. Next, 1.25 mL of chloroform was added and then vortexed. Tubes were centrifuged at 3000 rpm for 10 min in a benchtop swinging bucket centrifuge. After that, a Pasteur pipette was used to collect the lower organic phase and then transferred to clean glass tubes. These tubes were placed under a stream of inert gas (nitrogen) to dry down the lipids in a fume hood. Following this, 1.5 mL of 6% hydrogen peroxide in methanol was added, capped and then vortexed. Tubes were incubated at 80 ºC for 2 hours and then removed to cool at room temperature. Later, 2 mL of ammonium hydroxide/water (1:3) was added and vortexed well. 4 mL of hexane was added, vortexed well and centrifuged at 1000 rpm for 1 minute. The upper hexane phase was removed and then passed through sodium sulfate column (Pasteur pipette with glass wool plug at the bottom and ~2 cm of granular sodium sulfate on top) into another clean glass tubes. These tubes were placed under a stream of nitrogen to dry the eluent in a fume hood. After drying, the end product was FAMEs and it was re-dissolved in

200 µL of hexane and then subjected to capillary gas chromatography (Agilent 6890 GC, Agilent Technologies, Santa Clara, CA, USA) equipped with flame ionization detector and 6890 autosampler and GC column (Agilent J&W CP-Sil 88 (100 m), Agilent Technologies, Santa Clara, CA, USA). All the solvents used in this experiments were HPLC grade.

2.6 EchoMRI

Body composition including fat and lean mass in mice were analysed 1 week after osmotic pump implantation using the quantitative nuclear magnetic resonance (qMNR) method 4-in-1 Whole Body Composition Analyzer, (EchoMRI; Echo Medical Systems, Houston, TX). Live mice were placed into a cylinder made of plastic with inside diameter of 4.7 cm and 0.15 cm thick. Mice were limited to vertical movement by a plastic insert. After the measurements, mice were returned to their respective cage.

2.7 Glucose Tolerance Test (GTT)

Mice were kept in individual cages and fasted for 5 hours. Mice were then injected with a 50% glucose solution (2 g (kg body weight)$^{-1}$) intraperitoneally. Blood glucose levels (mmol/L) were measured in the tail vein using ~0.6 µL blood sample. determined using ACCU-CHEK Advantage glucometer (Roche Diagnostics, Laval, QC, Canada) and test strips at baseline and following glucose injection (10, 20, 30, 60, 90 and 120 min).

2.8 Tissue Homogenization and Immunoblot Analysis

Mice were fasted overnight and anaesthetized next morning with 100 uL sodium pentobarbital. Tissues, including heart, kidney, mesenteric artery, were isolated and freeze-clamped rapidly. Hearts were removed and placed in ice cold 1x phosphate buffer saline (PBS) (pH 7.4) and cleaned of any blood or excess tissue around the heart before freezing in the liquid nitrogen. Tissues were stored in the freezer at -80°C and taken out when needed. Tissues were taken out of the freezer and kept in the liquid nitrogen for homogenization. Tissues were ground and powdered in mortar and pestle. Mortar and pestle were kept in liquid nitrogen to maintain the frozen tissue temperature. 20-30 mg of tissue were homogenized in ice cold lysis buffer made up of 20 mmol/L Tris-HCL (pH 7.4), 50 mmol/L sodium chloride, 50 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate and 0.25 mol/L sucrose, and supplemented with protease

inhibitor (Sigma Aldrich; St. Louis, Missouri) sodium orthovanadate (Sigma Aldrich; St. Louis, Missouri), and phosphatase inhibitor cocktails (Calbiochem; Gibbstown, New Jersey). Tissue and lysis buffer were mixed at a 1:10 ratio. Tissue homogenates were then centrifuged at 1,200 × g for 20 min at 4°C. Supernatant was transferred and pellet was discarded. Protein concentration in the supernatant was determined by using Bradford protein essay (Bio-Rad; Richmond, California). Protein concentration was then equalized for each samples and 15-20 µg of protein samples were loaded onto 5 or 8 % bis acrylamide gels and subjected to SDS-PAGE. Separated proteins were transferred at 90 V for 2.5 hours onto nitrocellulose membranes. Nitrocellulose membranes were incubated with blocking buffer made up of 5% milk in tris-buffered saline and 0.1% tween 20 (TBST) at 1 hour room temperature on a shaker. Membranes were then washed 3x10 min with 1x TBST buffer followed by overnight incubation at 4 °C with primary antibody made with 5% BSA-TBST buffer. Membranes were washed and incubated for 1 hour at room temperature with respective HRP conjugated secondary antibody made with 5% milk TBST. Membranes were washed again and Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate (Perkin Elmer, Waltham, MA) was used to visualize the immune complex. ImageJ software (National Institutes of Health, Bethesda, MD) was used for densitometry.

2.9 Statistical Analysis

Data are expressed as mean ± SEM. For comparisons between control (BSA) and treatment (palmitoleate) groups, the unpaired Student t-test was employed in all the experiments. Only for the GTT experiment, two-way Analysis of variance (ANOVA) was used. A value of $p < 0.05$ was considered significant. GraphPad Prism 7.0 (GraphPad Software, San Diego, CA) was used for statistical analysis and to plot all graphs.

CHAPTER 3: RESULTS

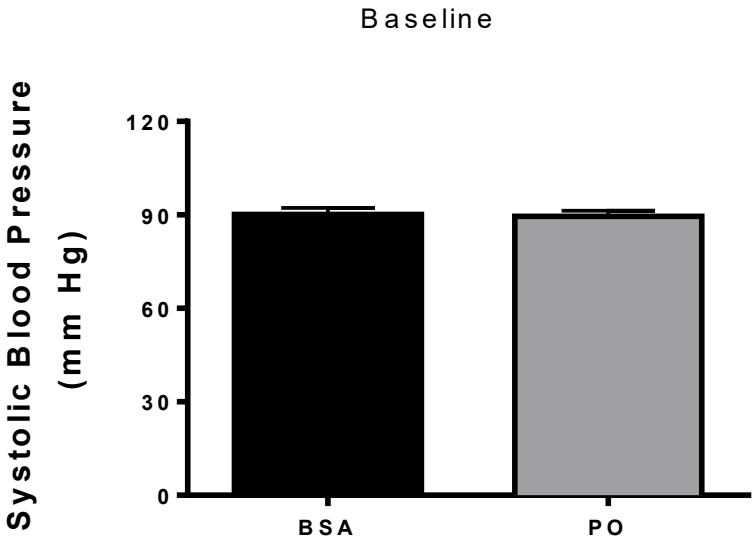
3. Results

3.1 Palmitoleate elevates BP in mice

To assess the effect of PO on SBP, mice were subjected to tail-cuff analysis at the baseline (before osmotic pump implant) and at the end (one-week after osmotic pump implant) of study. Mice were divided into two groups, BSA group (osmotic pump with BSA implanted group) and PO group (osmotic pump with palmitoleate implanted group).

Baseline tail-cuff analysis shows no significant difference between the PO and the BSA group. However, after one-week of treatment, the PO group shows a significant increase ($p < 0.001$) in SBP compared to the BSA group (116.7 ± 2.17 vs 88.71 ± 2.07) (mean ± SEM) (Figure 2A and 2B).

A


Baseline
Systolic Blood Pressure (mm Hg)
120
90
60
30
0
BSA
PO

B

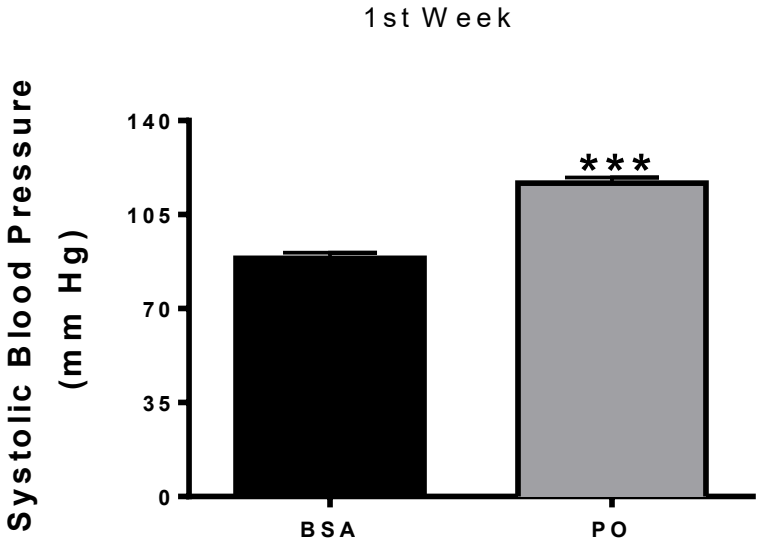

1st Week
Systolic Blood Pressure (mm Hg)
140
105
70
35
0
***
BSA
PO

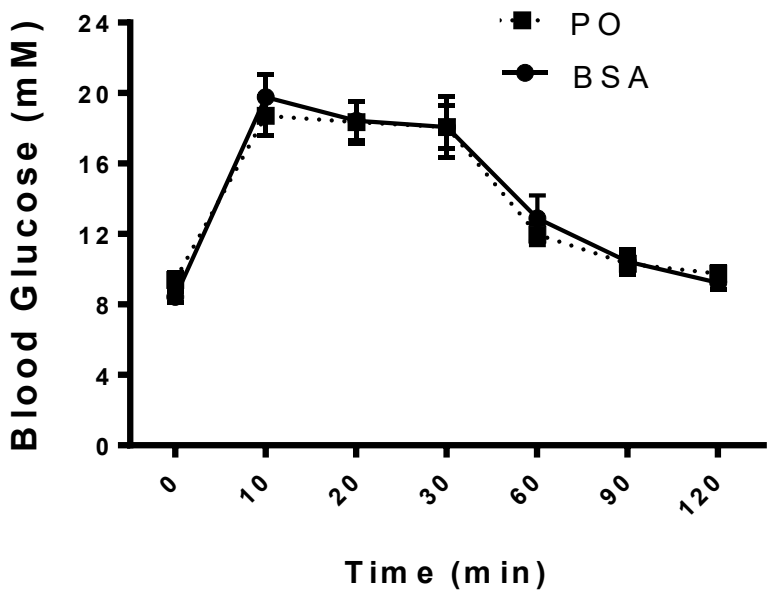
Fig. 2 Palmitoleate induces an increase in SBP

Panel A shows baseline tail-cuff measurement before osmotic pump implantation and panel B shows tail-cuff measurement one-week after osmotic pump implantation. C57BL/6J Mice (7-8 weeks old) were assigned randomly and implanted with osmotic pumps containing either BSA or PO and left for one-week. Values are means ± SEM of n = 6 mice in each group. *** $p < 0.001$ vs. BSA group as determined by Student's unpaired t-test.

3.2 Palmitoleate does not alter blood glucose level in mice

In order to determine if PO had an impact on glucose handling, mice were given a 50% glucose solution (2 g (kg body weight)$^{-1}$) intraperitoneally and then blood was drawn at the baseline (before glucose injection) and at 10, 20, 30, 60, 90, and 120 min. blood glucose levels were measured using a glucometer. At the baseline and after one week of pump implant, no significant differences were observed between BSA and PO group (Figure 3A and 3B).

A


Baseline
PO
BSA
Blood Glucose (mM)
24
20
16
12
8
4
0
0
10
20
30
60
90
120
Time (min)

B

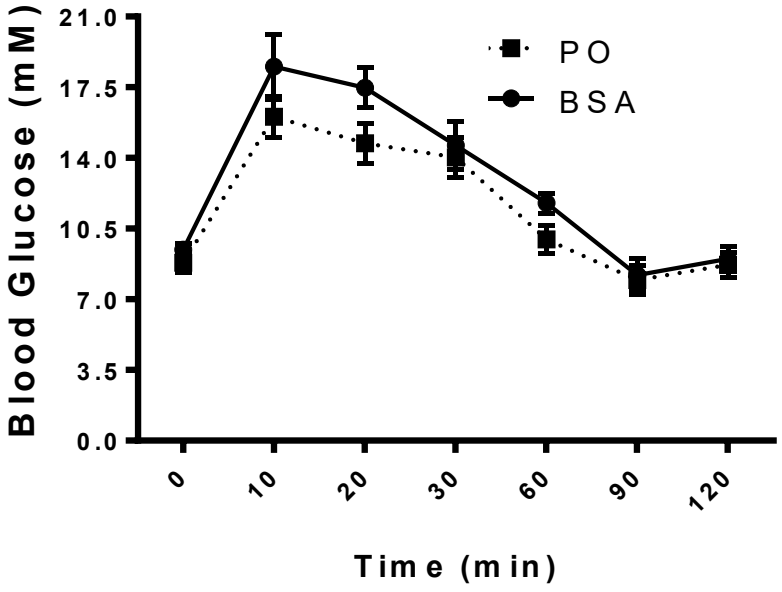

1st Week
PO
BSA
Blood Glucose (mM)
21.0
17.5
14.0
10.5
7.0
3.5
0.0
0
10
20
30
60
90
120
Time (min)

Fig. 3 Blood glucose level after one-week of palmitoleate administration

Panel A shows the baseline glucose tolerance test (GTT) and panel B shows GTT after one-week osmotic pump implantation. C57BL/6J Mice (7-8 weeks old) were assigned randomly and implanted BSA and PO osmotic pump subcutaneously for one-week. Values are means ± SEM of n = 6 mice in each group. Groups were analyzed by two-way ANOVA.

3.3 Palmitoleate administration leads to left ventricular remodeling in mice

To assess the effect of PO on *in vivo* cardiac structure and function, echocardiography was used at the end of study (after one-week). Post-echocardiography, mice were euthanized and heart weight and tibia length were measured. PO administration significantly increased inter ventricular septal wall thickness end diastole (IVSd) ($p < 0.001$; Figure 4A), inter ventricular septal wall thickness end systole (IVSs) ($p < 0.001$; Figure 4B), left ventricular posterior wall thickness end diastole (LVPWd) ($p < 0.001$; Figure 4C), and TEI Index ($p < 0.05$; Figure 3D) compared with BSA (Table 1). In addition, the PO group demonstrated a significant increase in the heart weight and heart weight-to-tibia length (HW/TL) ratio ($p < 0.05$; Figure 4E). There was no significant effect of PO in heart rate (HR), left ventricular internal diameter end diastole (LVIDd), left ventricular internal diameter end systole (LVIDs), left ventricular posterior wall thickness end systole (LVPWs), % ejection fraction (EF), % fraction shortening (FS), corrected left ventricular Mass, cardiac output (CO), stroke volume (SV) in contrast with BSA group (Table 1).

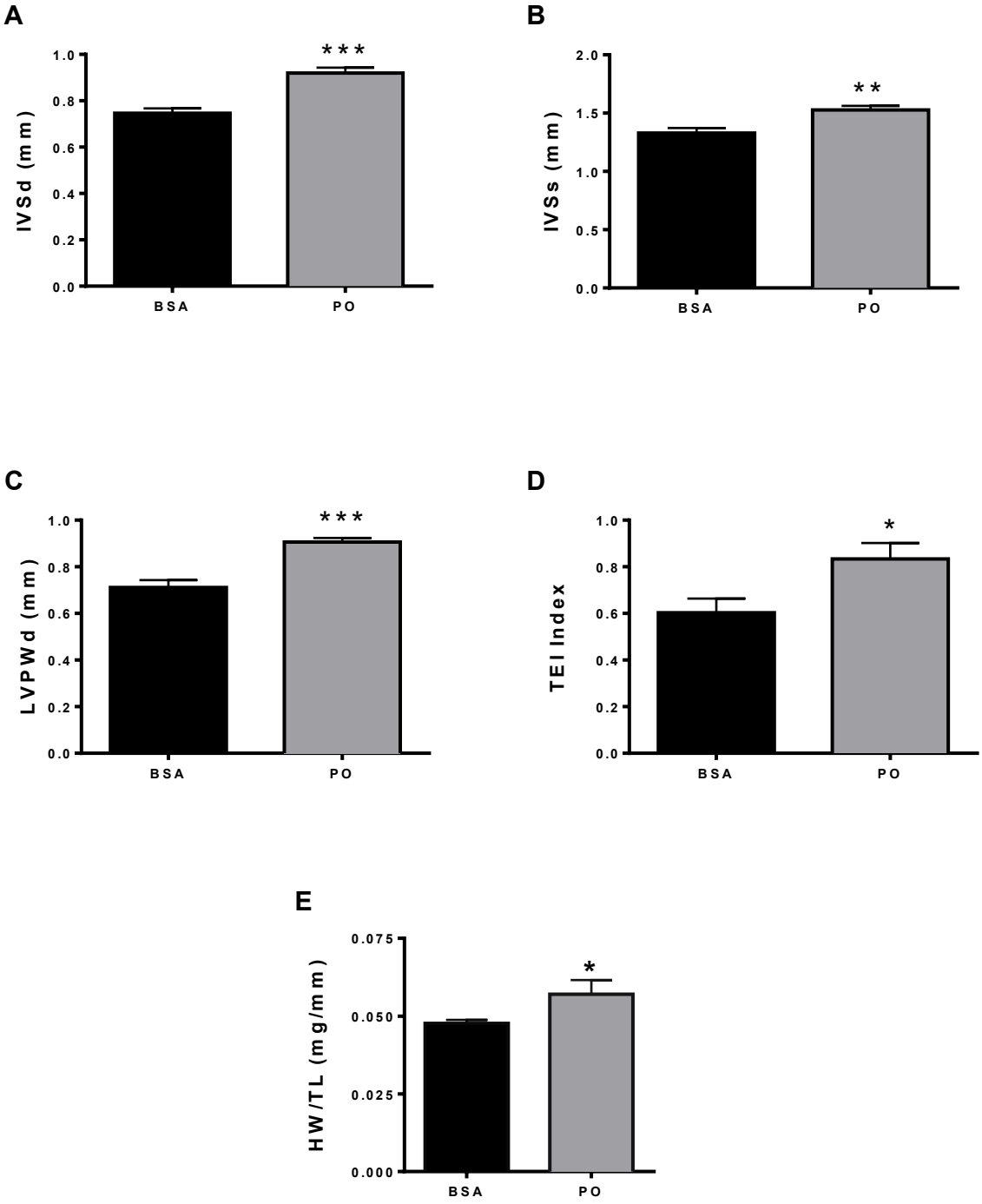

A
IVSd (mm)
1.0
0.8
0.6
0.4
0.2
0.0
***
BSA
PO
B
IVSs (mm)
2.0
1.5
1.0
0.5
0.0
**
BSA
PO
C
LVPWd (mm)
1.0
0.8
0.6
0.4
0.2
0.0
***
BSA
PO
D
TEI Index
1.0
0.8
0.6
0.4
0.2
0.0
*
BSA
PO
E
HW/TL (mg/mm)
0.075
0.050
0.025
0.000
*
BSA
PO

Fig. 4 Palmitoleate administration induces left ventricular hypertrophy in mice

C57BL/6J Mice (7-8 weeks old) were assigned randomly and implanted BSA or PO osmotic pump subcutaneously for one-week. One-week after the pump implantation, echocardiography was performed. Panel A shows inter ventricular septal wall thickness end diastole (IVSd). B, inter ventricular septal wall thickness end systole (IVSs). C, left ventricular posterior wall thickness end diastole (LVPWd). D, TEI Index left ventricular internal diameter end systole (LVIDs). E, heart weight/tibia length (HW/TL) ratio. Values are means ± SEM of n = 6 mice in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. BSA group as determined by Student's unpaired t-test.

Table 1: Echocardiographic values in the BSA and PO group at the end of study.

Parameters	BSA	PO
HR (beats.min^{-1})	484 ± 29	532 ± 22
IVSd (mm)	0.746 ± 0.021	0.920 ± 0.023***
IVSs (mm)	1.329 ± 0.044	1.528 ± 0.034**
LVIDd (mm)	3.920 ± 0.153	3.531 ± 0.223
LVIDs (mm)	2.458 ± 0.167	1.933 ± 0.178
LVPWd (mm)	0.711 ± 0.032	0.907 ± 0.017***
LVPWs (mm)	1.236 ± 0.069	1.370 ± 0.052
EF (%)	67.329 ± 3.262	75.596 ± 2.898
FS (%)	37.124 ± 2.482	43.931 ± 2.667
CO (mL.min^{-1})	20.745 ± 0.878	20.922 ± 2.169
SV (mL.beat^{-1})	0.043 ± 0.003	0.040 ± 0.005
Corrected LV Mass (mg)	83.113 ± 3.381	93.001 ± 7.798
TEI Index	0.603 ± 0.061	0.834 ± 0.068*

Data are presented as means ± SEM, heart rate; HR, Inter ventricular septal wall thickness end diastole; IVSd, Inter ventricular septal wall thickness end systole; IVSs, Left ventricular internal diameter end diastole; LVIDd, Left ventricular internal diameter end systole; LVIDs, Left ventricular posterior wall thickness end diastole; LVPWd, Left ventricular posterior wall thickness end systole; LVPWs, % Ejection fraction; EF, % Fraction shortening; FS, cardiac output; CO, stroke volume; SV, Corrected left ventricular (LV) Mass, TEI Index. n = 6 mice in each group. * p <0.05, **p < 0.01, ***p < 0.001 vs. BSA group as determined by Student's unpaired t-test.

3.4 Palmitoleate administration increases lean mass and decreases fat mass in mice

Studies have shown that increase BP is significantly associated with weight gain and increase fat mass [288, 289] as well as with increase lean mass [290, 291]. PO has been shown to decrease body weight significantly [292] and decrease fat mass and increase lean mass [293]. PO has also been shown to increase adipose tissue lipolysis that results in decrease overall fat mass [294, 295]. Therefore, in order to determine if PO affects whole body mass composition EchoMRI was used to assess the lean mass and fat mass. Mice were weighed at the beginning of the study before osmotic pump implant (baseline) and one-week after osmotic pump implant, and then subjected to EchoMRI analysis at the end of study. At baseline, body weight was similar in both BSA and PO group (Figure 5A) and increased very slightly albeit significantly ($p < 0.05$) in the PO group after one-week compared with the BSA group (Figure 5B). While there was no significant difference in the fat mass between the PO group and the BSA group, there was a trend to decrease fat mass in the PO group (Figure 5C). Lean mass increased significantly ($p < 0.01$) in the PO group (Figure 5D). When fat and lean mass was divided with body weight, the % fat mass in the PO group was significantly decreased ($p < 0.05$) in the PO group (Figure 5E) but % lean mass was not different ($p < 0.07$) from the BSA group (Figure 5E).

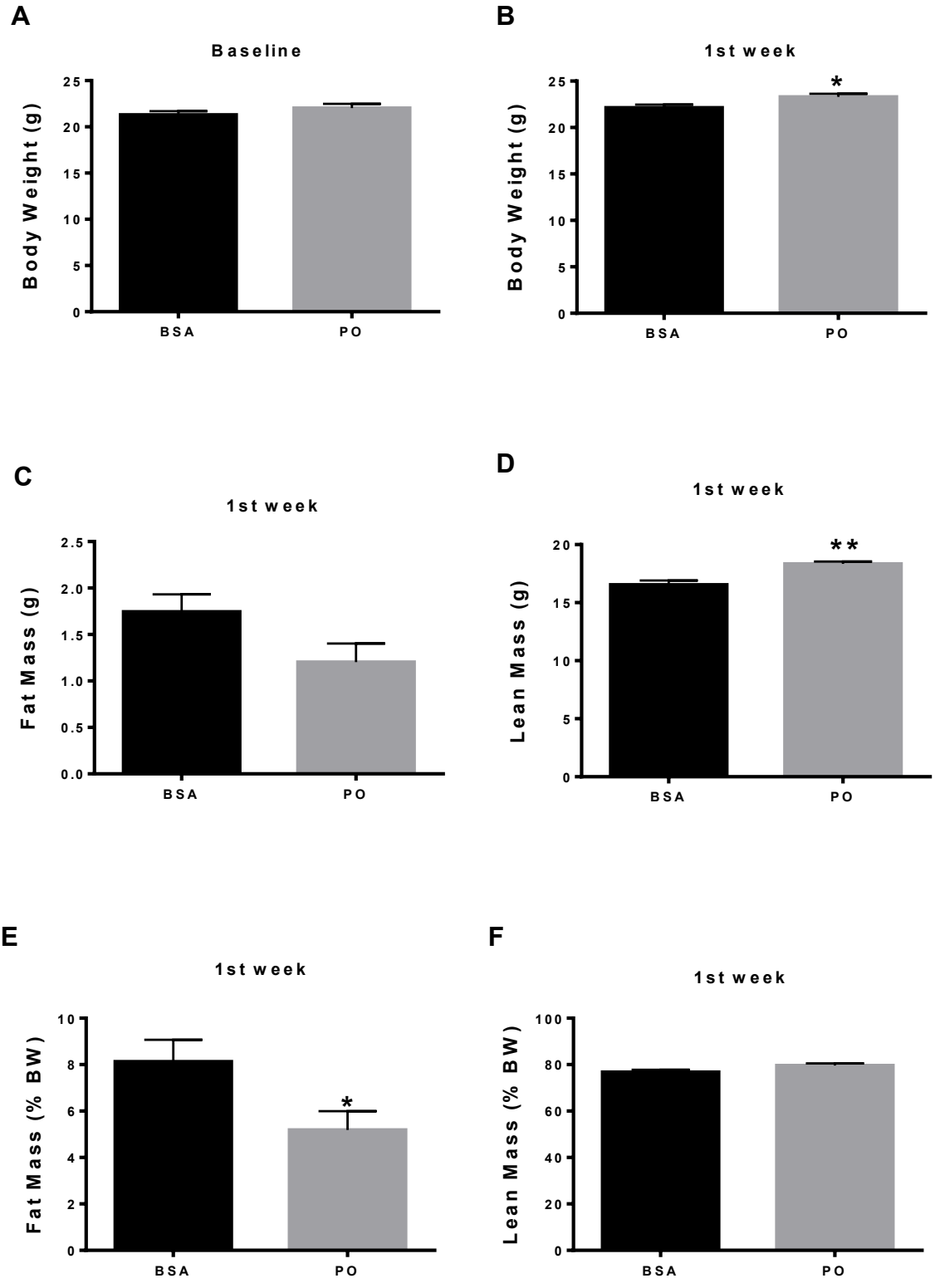

A
Baseline
Body Weight (g)
25
20
15
10
5
0
BSA
PO
B
1st week
Body Weight (g)
*
C
1st week
Fat Mass (g)
2.5
2.0
1.5
1.0
0.5
0.0
D
1st week
Lean Mass (g)
**
E
1st week
Fat Mass (% BW)
F
1st week
Lean Mass (% BW)
100
80
60
40

Fig. 5 Increase in lean mass and decrease in fat mass induced by palmitoleate administration

C57BL/6J Mice (7-8 weeks old) were assigned randomly and implanted BSA and PO osmotic pump subcutaneously for one-week. Panel A and B shows body weight at the baseline and after one-week of osmotic pump implant, respectively. C, fat mass at one-week. D, lean mass at one-week. E, fat mass/body weight % at one-week. F, lean mass/body weight % post one-week. Values are means ± SEM of n = 6 mice in each group. * $p < 0.05$, ** $p < 0.01$ vs. BSA group as determined by Student's unpaired t-test.

3.5 Palmitoleate levels are unchanged in the serum of mice

Higher levels of PO are associated with hypertension [282] and cardiac hypertrophy [295]. PO levels were reported to be elevated in the serum of mice that were supplemented orally with PO [295]. Since, we observed hypertension and cardiac hypertrophy in mice, we hypothesized that after one-week of PO infusion, PO levels in the blood would be higher in mice. Therefore, serum was collected after one-week of osmotic pump implant and was subjected to FAMEs-GC analysis. Although we observed a clear change in animal physiology with PO infusion, a significant difference in the PO levels between PO and BSA group after one-week of osmotic pump implant was not evident (Figure 6). However, since our assay did not have a positive control, we cannot be certain about the accuracy of the assay.

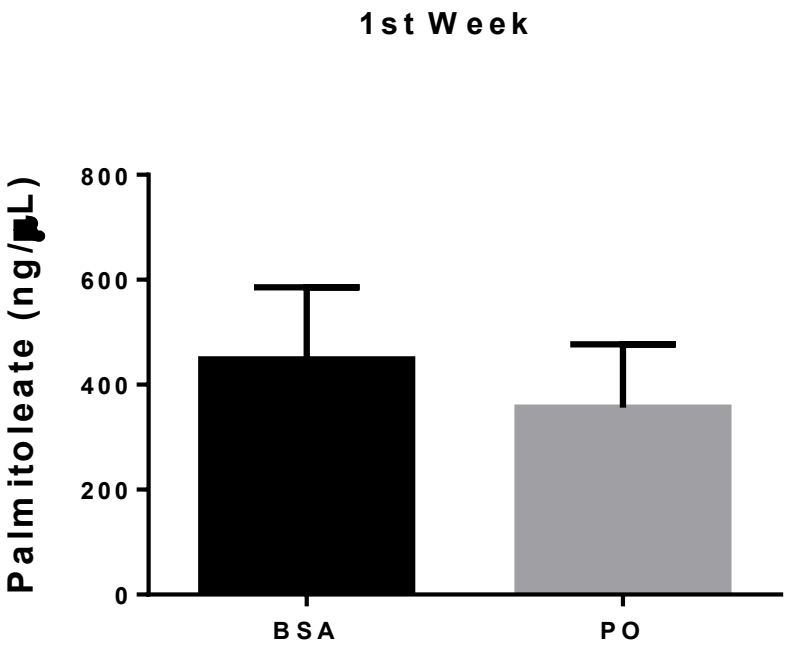

1st Week
Palmitoleate (ng/μL)
800
600
400
200
0
BSA
PO

Fig. 6 Serum palmitoleate levels in mice

C57BL/6J Mice (7-8 weeks old) were assigned randomly and implanted BSA and PO osmotic pump subcutaneously for one-week. Panel shows PO levels in the serum after one-week of osmotic pump implant. Values are means ± SEM of n = 6 mice in each group.

3.6 Palmitoleate upregulates eNOS and AMPK protein expression in the mesenteric arteries of mice

eNOS is predominantly present in the vasculature and is responsible to produce NO [296]. NO is a vasodilator molecule that dilates blood vessels and plays a critical role in the vascular tone [297]. Studies shows that defect in NO production contributes to hypertension [298, 299]. To observe the production of NO in the vasculature, phosphorylated eNOS is used as a surrogate marker [153, 163]. Taken together, we hypothesize that PO-induced hypertension would decrease vascular eNOS activity. To investigate this mesenteric arteries were isolated from mice with BSA or PO and were subjected to western blot analysis.

After one-week of PO infusion, relative protein expression of P-eNOS/ was also found significantly ($p < 0.05$) decreased (Figure 7B) but total eNOS protein expression was significantly increased ($p < 0.05$) (Figure 7C) compared with BSA.

Since, AMPK phosphorylates eNOS and increases NO production [300]. We assessed whether AMPK was altered in correspond with eNOS protein expression. We observed that relative protein expression of P-AMPK/AMPK was significantly ($p < 0.05$) decreased in the PO group (Figure 7D) but total AMPK protein expression was significantly increased ($p < 0.05$) (Figure 7E) in comparison with BSA.

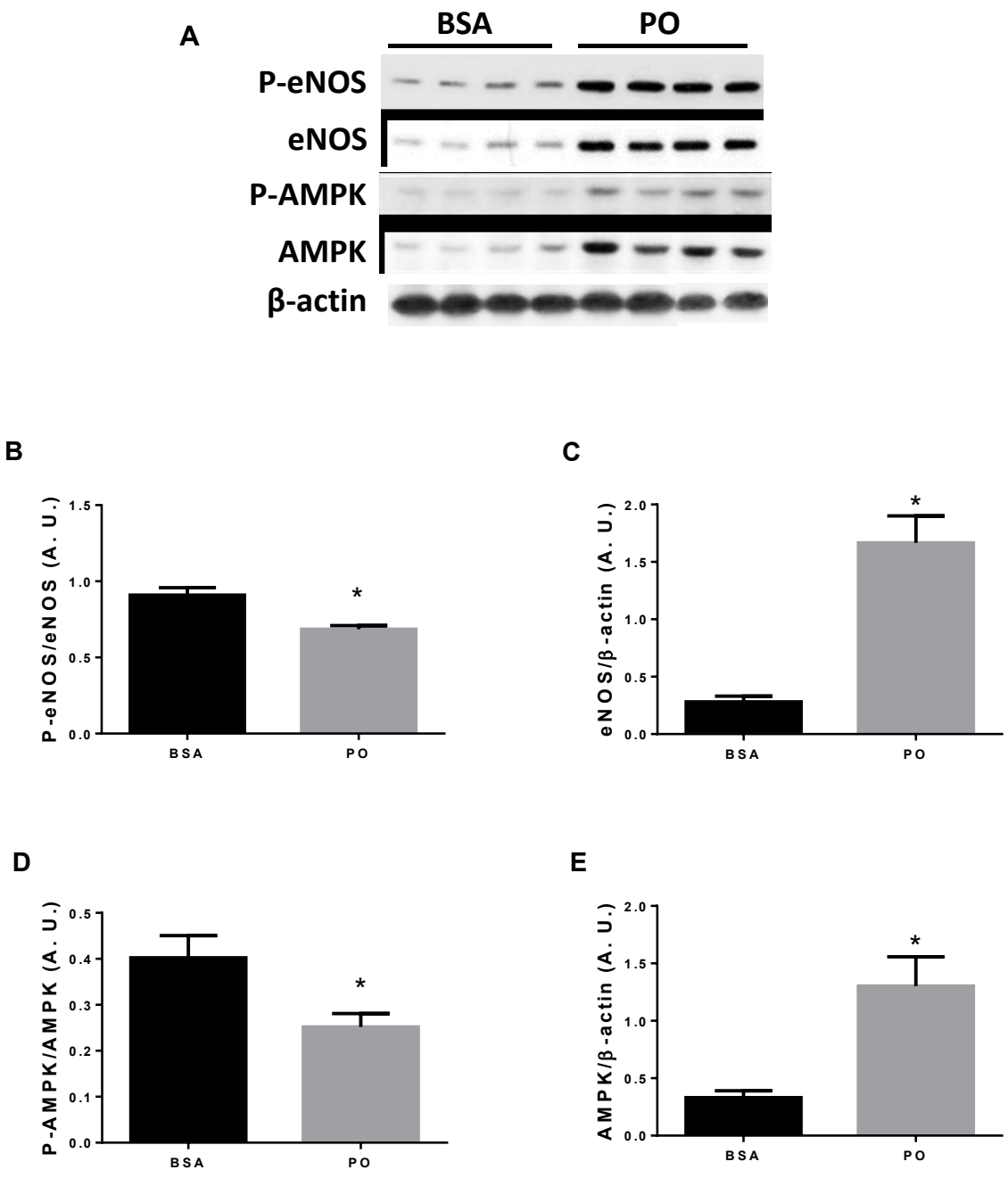

A
BSA
PO
P-eNOS
eNOS
P-AMPK
AMPK
β-actin
B
P-eNOS/eNOS (A. U.)
1.5
1.0
0.5
0.0
*
BSA
PO
C
eNOS/β-actin (A. U.)
2.0
1.5
1.0
0.5
0.0
*
BSA
PO
D
P-AMPK/AMPK (A. U.)
0.5
0.4
0.3
0.2
0.1
0.0
*
BSA
PO
E
AMPK/β-actin (A. U.)
2.0
1.5
1.0
0.5
0.0
*
BSA
PO

Fig. 7 Analysis of P-eNOS and P-AMPK protein expression in mesenteric arteries of mice

Immunoblot analysis was performed on mesenteric artery homogenates isolated from C57BL/6J Mice (7-8 weeks old) that were assigned randomly and implanted BSA and PO osmotic pump subcutaneously for one-week. Panel A shows immunoblots, B and C show quantification data for phosphorylated threonine-172 AMPK (P-AMPK) normalized against total AMPK and total AMPK normalized against β-actin, respectively. Panel D and E show phosphorylated serine-1177 eNOS (P-eNOS) quantified by densitometry and normalized against total eNOS and total eNOS normalized against β-actin, respectively. Values are means ± SEM of n = 4 in each group. * $p < 0.05$ vs. BSA group as determined by Student's unpaired t-test.

3.7 Palmitoleate downregulates P-LKB1 protein expression in the kidney of mice

Kidneys control the blood-volume and pressure control by various mechanisms which prove that kidneys are a major regulator of hypertension [70]. Hypertension is one of the main cause for the progression of renal dysfunction, fibrosis, diabetic nephropathy, renal hypertrophy and chronic kidney disease [56, 58, 61]. A recent study shows that activation of renal AMPK prevents the development of salt-induced hypertension by increasing sodium excretion [301]. In order to investigate the AMPK activity during the hypertensive effect of PO in the kidney we examined the phosphorylation of AMPK in the kidney. We observed no significant difference in the phosphorylation of AMPK in the PO group with BSA group (Figure 8B).

As phosphorylation and activation of AMPK is mediated by upstream kinase, LKB1, which is abundantly expressed by kidney [302] and its deletion leads to severe renal fibrosis, impaired renal metabolism and chronic kidney disease [303, 304]. We hypothesize that activity of LKB1 would be decreased. Examination of protein expression showed that phosphorylation of LKB1 in PO group was significantly ($p < 0.05$) downregulated compared with BSA group (Figure 8C).

Some studies reported that hypertension was associated with increased phosphorylation of Akt and mTOR in the kidneys that lead to glomerular hypertrophy, fibrosis, and tubule-interstitial atrophy [305-307]. Studies have also shown that renal hypertrophy is mediated by activation of Akt, mTOR and reduction in AMPK phosphorylation and this was associated with loss of function in nephrons, tubular atrophy and fibrosis [306, 308]. We examined Akt and mTOR phosphorylation and observed that phosphorylation of Akt and mTOR in PO group were not significantly different with BSA group (Figure 8D and 7E).

Study shows that Ang II–dependent vasoconstriction is enhanced by activation of p38 MAPK and abrogation of p38 improves endothelial function and decreases Ang II–induced vasoconstriction [309-311]. Similarly, increased phosphorylation of p38 has been shown to cause renal vasoconstrictions and inhibition of p38 by specific inhibitor reduces renal pressor response to Ang II [312]. Upon analysis of phosphorylation of p38 by western blot, no significant difference was observed in the phosphorylation of p38 in the PO group compared with BSA (Figure 8F).

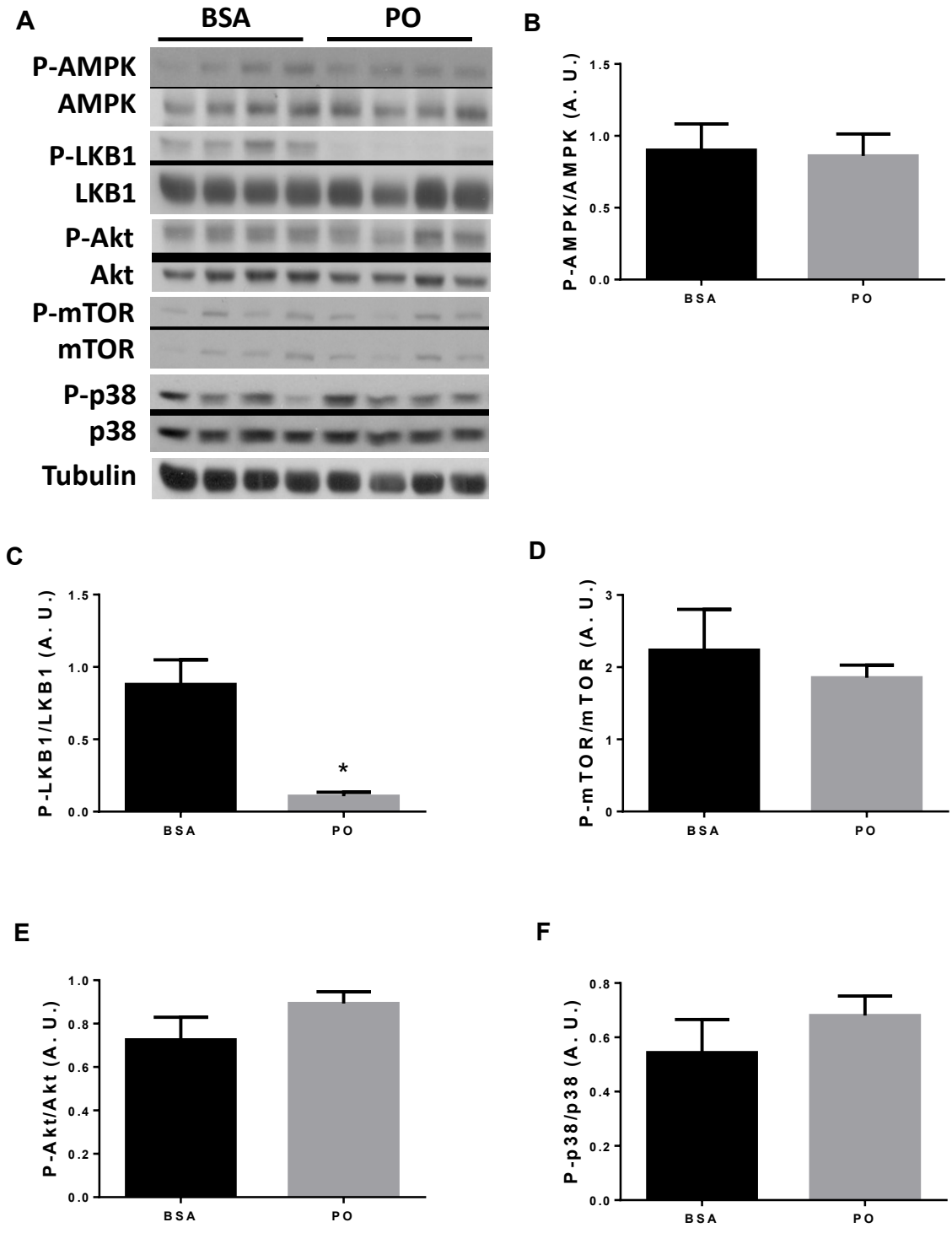

A
BSA
PO
P-AMPK
AMPK
P-LKB1
LKB1
P-Akt
Akt
P-mTOR
mTOR
P-p38
p38
Tubulin
B
P-AMPK/AMPK (A. U.)
1.5
1.0
0.5
0.0
BSA
PO
C
P-LKB1/LKB1 (A. U.)
1.5
1.0
0.5
0.0
*
BSA
PO
D
P-mTOR/mTOR (A. U.)
3
2
1
0
BSA
PO
E
P-Akt/Akt (A. U.)
1.0
0.8
0.6
0.4
0.2
0.0
BSA
PO
F
P-p38/p38 (A. U.)
0.8
0.6
0.4
0.2
0.0
BSA
PO

Fig. 8 Palmitoleate administration does not affect P-AMPK in the kidney of mice

Immunoblot analysis was performed on kidney homogenates isolated from C57BL/6J Mice (7-8 weeks old) that were assigned randomly and implanted BSA and PO osmotic pump subcutaneously for one-week. Panels show representative immunoblots (A) and respective quantification data for phosphorylated threonine-172 AMPK (P-AMPK) normalized against total AMPK (B). Phosphorylated liver kinase beta 1 (P-LKB1) was quantified and normalized by total LKB1 (C). Phosphorylated mTOR (P-mTOR) was quantified and normalized against total mTOR (D). Phosphorylated serine-473 Akt (P-Akt) was normalized against total Akt (E). Phosphorylated p38 was quantified by densitometry and normalized against total p38 (F). Values are means ± SEM of $n = 4$ in each group. * $p < 0.05$ vs. BSA group as determined by Student's unpaired t-test.

3.8 Palmitoleate downregulates P-AMPK protein abundance in the heart of mice

Remodeling of the heart is a cardiac response to hypertension [313] associated with increased protein synthesis, fibrosis, and cardiomyocyte volume [314]. Study shows that, phosphorylated AMPK is significantly reduced in the heart during hypertension which could be permissive for increase in the cardiac mass and activation of AMPK protects heart from hypertrophy [151, 161] suggesting an important role of AMPK as a negative regulator of cardiac hypertrophy [315]. To investigate whether AMPK activity was altered in the heart, phosphorylation of AMPK was observed. Western blot analysis showed that the PO group had a significant ($p < 0.05$) downregulation in AMPK phosphorylation (Figure 9B) in the PO group.

To investigate whether reduction in AMPK activity activates pro-hypertrophic signaling molecules we determined the activity of Akt, p70S6K, and mitogen activated protein kinase (MAPK) p38 as increase in these proteins have been shown to promote cardiac hypertrophy [170, 316, 317]. Studies show that Akt-induced hypertrophy is associated with reduced phosphorylation of AMPK that results in an Inhibition of AMPK activity in the heart [160, 318] and Akt induces hypertrophic growth via mTOR-p70S6K mediated protein synthesis pathway [160]. Further, activation of AMPK by pharmacological activator inhibits Akt-induced cardiac hypertrophy [160]. These studies suggest that Akt and AMPK negatively regulate each other. Study shows that AMPK regulates protein synthesis and cardiac hypertrophy by p70S6K [170]. As activation of p38 MAPK has also been implicated to be involved in promoting cardiac hypertrophy [319, 320]. Therefore, we examined the activity of Akt and p70S6K and p38. Analysis of western blot showed no significant change in the phosphorylation Akt (Figure 9C), p70S6K (Figure 9D), and p38 (Figure 9E) in the PO group compared with BSA group.

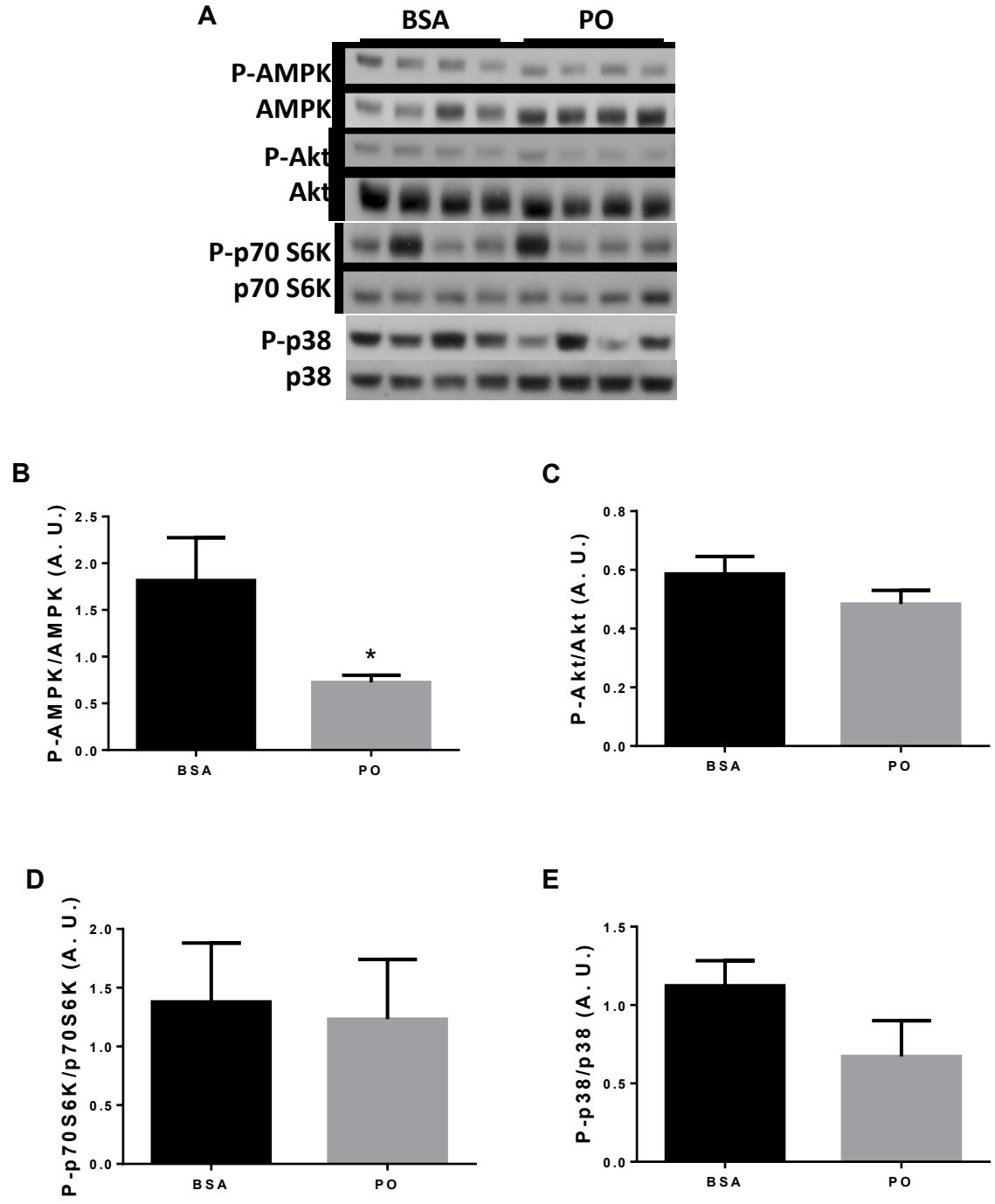

A
BSA
PO
P-AMPK
AMPK
P-Akt
Akt
P-p70 S6K
p70 S6K
P-p38
p38
B
P-AMPK/AMPK (A. U.)
2.5
2.0
1.5
1.0
0.5
0.0
*
BSA
PO
C
P-Akt/Akt (A. U.)
0.8
0.6
0.4
0.2
0.0
BSA
PO
D
P-p70S6K/p70S6K (A. U.)
2.0
1.5
1.0
0.5
0.0
BSA
PO
E
P-p38/p38 (A. U.)
1.5
1.0
0.5
0.0
BSA
PO

Fig. 9 Palmitoleate-induced decrease in P-AMPK protein expression in the heart of mice

Immunoblot analysis was performed on the heart homogenates isolated from C57BL/6J Mice (7-8 weeks old) that were assigned randomly and implanted BSA and PO osmotic pump subcutaneously for one-week. Panels show representative immunoblots (A) and respective quantification data for phosphorylated threonine-172 AMPK (P-AMPK) normalized against total AMPK (B). Phosphorylated serine-473 Akt (P-Akt) was normalized against total Akt (C). Phosphorylated threonine-421/serine-424 of p70S6 kinase (P-p70S6K) was quantified by densitometry and normalized against total p70S6K (E). Phosphorylated p38 was quantified by densitometry and normalized against total p38 (F). Values are means ± SEM of n = 4 in each group. * $p < 0.05$ vs. BSA group as determined by Student's unpaired t-test.

CHAPTER 4: DISCUSSION

4. Discussion

CVD is a growing worldwide concern that burdens healthcare expenditures. Advancement in the treatment therapies has decreased the incidents of heart failure and improved rates of morbidity and mortality. However, these treatments have also increased the incidence and prevalence of heart failure. Hypertension is one of the most important and major contributors to CVD [321]. Hypertension induces several functional and molecular changes in the organs including, heart [153, 322], mesenteric arteries [153] and kidney [52]. Adipose tissue is an endocrine organ known to release and express wide range of bioactive compounds which act as signaling molecules. These signaling molecules communicate with organs or organ system in an autocrine, paracrine and endocrine manner [206]. One of these molecules is palmitoleate, which has been suggested to control glucose and lipid metabolism [207]. Circulatory palmitoleate levels are strongly associated with heart failure risk factors such as high BP, obesity, diabetes and CHD [248, 275, 282, 284]. A study by Djousse et al. suggests that palmitoleate levels in the blood are a positive biomarker that increases risk of heart failure by developing hypertension [286]. Based on this, we wanted to investigate the effects of palmitoleate on the BP, cardiac function and remodelling. We investigated the effect of palmitoleate in the mice (7-8 weeks) that were selected randomly to receive osmotic pump with BSA or palmitoleate subcutaneously and subsequent experiments were carried out. Herein, we provide the evidence that palmitoleate administration significantly increases SBP suggesting a pro-hypertensive effect of palmitoleate.

To assess whether palmitoleate modulates blood glucose levels, we performed GTT in the mice before and after one-week of osmotic pump implantation with either BSA or palmitoleate. We did not observe any significant different in GTT between BSA and palmitoleate group, this suggests that palmitoleate does not affect blood glucose levels. Our results are in agreement with the study that observed no significant change in the glucose levels after palmitoleate infusion [323].

To further investigate the effect of palmitoleate on cardiac structure and function, we performed echocardiography at the end of experiment and characterized the morphology of the heart. We observed significant cardiac remodelling in the palmitoleate group in contrast with BSA group after one-week. Inter ventricular septal wall thickness end diastole (IVSd), inter ventricular

septal wall thickness end systole (IVSs), and left ventricular posterior wall thickness end diastole (LVPWd) were significantly increased. These findings suggest that higher IVSd, IVSs and LVPWd values may be caused by higher BP values. These observation are supported by the study that suggests that IVSd, IVSs and LVPWd values are considered as a positive parameter to identify the increased risk of left ventricular hypertrophy [324]. Following this, we looked at TEI index which is an index of myocardial performance that shows dysfunction of both systolic and diastolic function of LV [325]. After calculating values, TEI index was found to be significantly different in the palmitoleate group compared to the BSA group, indicating the systolic and diastolic dysfunction in the LV in response to palmitoleate. These findings suggests that palmitoleate-induces hypertension causes cardiac remodeling and dysfunction. The literature suggests that assessment of cardiac hypertrophy is more accurately predicted using tibia length as a reference than body weight [326]. After normalizing the heart weight with tibia length, as expected, we noted a significant increase in the heart weights of palmitoleate group, corresponding to the cardiac hypertrophy.

Studies report that increase in BP is positively associated with increase in the total body weight, fat mass and lean mass [288-291]. In addition, palmitoleate significantly decreases weight gain [292] and fat mass, and increases muscle mass [293]. Palmitoleate has been reported to increase adipose lipolysis that was correlated with decrease in total body fat mass [294, 295]. Our results show similar findings, that at the end of our study we observed significant increase in the body weight with palmitoleate-induced hypertension. Further, we looked at the whole body composition analysis and mice with palmitoleate implant showed significant increase in muscle mass and reduction in accumulation of fat mass in comparison with BSA group after one-week.

Studies have shown that circulating levels of palmitoleate are positively associated with CVDs such as hypertension [282] and cardiac hypertrophy [295]. When mice were supplemented orally with palmitoleate then palmitoleate levels in the serum were found to be increased [295] and when mice were infused with palmitoleate then significant cardiac remodelling was observed [287]. Similarly, in our study after one-week of palmitoleate infusion in mice, we observed significant development of hypertension and cardiac hypertrophy. Therefore, to observe the palmitoleate levels in the serum, we performed FAMEs-GC analysis and found no significant difference between palmitoleate and BSA group. In agreement with our findings, study by

Foryst-Ludwig et. al [295] reported that when mice were orally supplemented with palmitoleate or oleic acid (C18:1), the blood palmitoleate levels were unchanged. The palmitoleate levels were only increased in the mice that were subjected to exercise compared with sedentary mice and author reported that this increase in palmitoleate was associated with exercise induced-adipose tissue lipolysis which released FA in circulation and contributed to the increased palmitoleate levels [295]. Literatures report that palmitoleate induces decreased lipogenesis [327, 328] and increased lipolysis in adipose tissue [294] resulting in the increase in the FA levels in the circulation. In our experiment, it is speculated that palmitoleate levels were increased throughout the week. However, the explanation for unchanged levels of palmitoleate could be the due to the quick removal from the circulation. Elevated levels of FA, including palmitoleate, might have been quickly disposed from the circulation by utilizing FA as an energy substrate and meeting increased energy demands by the heart and skeletal muscle [329]. In our experiment, we did not collect the blood everyday post-osmotic pump implantation, therefore, we do not know the trend of palmitoleate levels in the blood as to how its levels were throughout the week and remained unchanged at the end of experiments. Thus, future studies are required to address this hypothesis.

eNOS is present predominantly in the vasculature and produces NO [296] in the circulation that has vasodilatory properties which has an important role in maintaining vascular tone [297]. Decreased production of NO leads to pathogenesis of hypertension [330]. Activity of eNOS in the vasculature acts as a surrogate marker for the production of NO. Therefore, to investigate this, we performed western blot analysis using mesenteric artery lysates. Our result shows significant decrease in the relative expression of eNOS protein in the mesenteric artery with palmitoleate infusion. However, there was robust increase in the phosphorylated and total eNOS protein expression in the palmitoleate group which suggests eNOS activation and subsequent increased NO production. Additionally, we looked at the protein expression of AMPK as AMPK is an upstream kinase that phosphorylates and activates eNOS [300], we measured phosphorylated levels of AMPK. Our result shows significant decrease in the relative protein expression of AMPK in the mesenteric arteries. However, our findings show that protein expression of phosphorylated and total AMPK was significantly increased suggesting AMPK activation and further suggesting that activation of AMPK resulted in the activation of eNOS [153-156].This increased in the phosphorylated and total eNOS and AMPK protein expression

could be explained by the hypothesis that during palmitoleate-induced hypertension, eNOS and AMPK signaling are activated as a compensatory response of vasculature to relieve vasoconstriction and increase in the BP by increasing NO production. This observation also suggest the possibility of involvement of post-translational modification such as protein degradation (ubiquitin-proteasome and lysosomal proteolysis) in the upregulation of protein expression of eNOS and AMPK. Protein degradation pathways of eNOS and AMPK may have been affected by palmitoleate. Thus, future studies are required to address these hypotheses.

Kidneys regulate hypertension by various mechanism such as diuresis, natriuresis, RAAS and sympathetic nervous system which implicates kidneys as a major regulator of hypertension [54]. Hypertension leads to the development of renal dysfunction, diabetic nephropathy, fibrosis, renal hypertrophy and chronic kidney disease [56, 58, 61]. Role of AMPK in the kidney has been implicated in various aspects of renal physiology and pathology such as ion transport [328], renal hypertrophy [308], ischemia [329], podocyte function [330], inflammation [331], diabetes [308, 332], and polycystic kidney disease [333]. Recently it has been shown that hypertension downregulates AMPK activity in the kidney [334]. Activation of renal AMPK has been shown attenuate hypertension by increasing urinary sodium excretion [301]. Thus, to investigate the role of AMPK in kidneys in palmitoleate-induced hypertension, we looked at AMPK activity in the kidneys. In contrast to that previous study, we did not observe any changes in AMPK, however, we found a significant downregulation in the phosphorylation of LKB1. LKB1 is an upstream kinase that phosphorylates and activates AMPK and is widely expressed in the kidneys [302]. LKB1 deletion leads to severe renal fibrosis, impaired renal metabolism and chronic kidney disease [303, 304] suggesting the vital role of LKB1. Our results show that palmitoleate-induced hypertension downregulates LKB1 activity in the kidney but not AMPK which suggests that there may be other upstream molecules, other than LKB1, which are involved in the AMPK activation, such as SIRT1 [335]. Study using SIRT1 deficient mice shows that, SIRT1 is an essential upstream molecule that activates AMPK. SIRT1 is also involved in the renal physiology and pathology such as reduces BP and related CVD, diabetic albuminuria, protects acute kidney injury, and delays kidney fibrogenesis [336]. Further studies are required to prove this.

The heart undergoes remodeling in response to hypertension [313] and is characterized by increased protein synthesis, fibrosis, and cardiomyocyte volume [314]. Studies previously reported that, phosphorylation of AMPK is significantly downregulated during cardiac hypertrophy which was responsible for increase in the cardias mass and activation of AMPK attenuated cardiac hypertrophy [153, 163]. Our result previously suggested cardiac remodeling with dysfunction. We further investigated the AMPK activity in palmitoleate induced hypertension. Our results in the heart are in agreement with findings showing downregulation in the phosphorylation of AMPK in the palmitoleate-induced cardiac hypertrophy.

4.1 Conclusion

The data presented in this study show that palmitoleate induces hypertension and cardiac hypertrophy in mice. Palmitoleate induces weight gain with increase in the muscle mass and decrease in the fat accumulation. Our preliminary findings suggest that eNOS/NO/AMPK/LKB1 signaling may be involved in the palmitoleate-induced hypertension and cardiac hypertrophy, however, there may be other signaling molecules involved and further investigation is required.

Based on our finding, it can be speculated that understanding the precise role of palmitoleate on the development of hypertension and cardiac hypertrophy in patients, and associated molecular mechanism may provide a direction for new discovery of therapies designed to regulate palmitoleate that may be an approach towards management and prevention of high BP and cardiac hypertrophy.

4.2 Future directions

4.2.1 Palmitoleate-induced cardiac hypertrophic signaling mechanism

To investigate this, studies could be performed in cell culture of neonatal rat or mice cardiomyocytes that would facilitate the understanding of the precise signaling mechanism.

Whether targeting eNOS/NO/AMPK/LKB1 signaling mechanism would prevent palmitoleate-induced cardiac hypertrophic responses, cardiomyocytes would be incubated with palmitoleate or BSA and they would be further treated with AMPK activators such as AICAR or compound C. As it has been shown by various studies that activation of AMPK attenuates cardiac

hypertrophic responses. By activating AMPK we would expect to see downregulation in fetal gene expressions, protein expression, decrease in fibrosis, and collagen production.

4.2.2 The role of deletion of adipose triglyceride lipase (ATGL) in heart failure

ATGL is a key enzyme of the lipolytic process and catalyzes the initial step of TG hydrolysis (lipolysis) [340]. ATGL converts TG to diacylglycerol (DG) and free fatty acid [341]. ATGL mobilizes lipid storage that are released them into circulation for other organs to be utilized as an energy substrate. Adipose tissue is a predominant site of ATGL expression, however, other organs such as heart, kidney and skeletal muscle express ATGL to a lesser extent [340]. Recent studies have shown that during heart failure, increased adipocyte lipolysis is associated with inflammation in adipose tissue and systemic insulin resistance [342], and have implicated adipose tissue ATGL as being a key mediator of this process. The role of adipose tissue ATGL in lipolysis and subsequent inflammation was supported by the use of the selective inhibitor of lipolysis (acipimox) in *ex vivo* culture of ATGL-deficient adipose tissue [342] and explants of adipose tissue from whole body ATGL-deficient mice [343]. In this instance, inhibition of systemic lipolysis in heart failure reduced adipose tissue inflammation, inhibited pro-inflammatory cytokine production in the adipose tissue, increased insulin sensitivity and increased glucose tolerance [342]. However, inhibition of lipolysis and linked adverse effects were associated with worse cardiac function and LV hypertrophy [342], which may be due to inhibiting lipolysis in the myocardium. Taken together, these studies do not address the effects of ATGL mediated adipocyte lipolysis deletion during heart failure, therefore, to fully understand this studies focusing the role of ATGL in heart failure we would generate ATGL deficient mice and study how reduction of lipolysis affects cardiac structure and function in mice with heart failure.

ATGL-deficient mice will be subjected to transverse aortic constriction to induce heart failure. Mice will be subjected to echocardiography to assess cardiac function. Blood glucose and insulin will be measured to examine whole body glucose and insulin resistance during heart failure. To determine the extent to which reduction in lipolysis affects adipose tissue inflammation in the heart failure, mice epididymal adipose tissue will be collected and subjected to immunohistochemistry to see the macrophage infiltrations.

References

1. Go, A.S., et al., Heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation, 2014. 129(3): p. e28-e292.

2. Tu, J.V., et al., National trends in rates of death and hospital admissions related to acute myocardial infarction, heart failure and stroke, 1994-2004. CMAJ, 2009. 180(13): p. E118-25.

3. Lee, D.S., et al., Trends in risk factors for cardiovascular disease in Canada: temporal, socio-demographic and geographic factors. CMAJ, 2009. 181(3-4): p. E55-66.

4. Chockalingam, A., N.R. Campbell, and J.G. Fodor, Worldwide epidemic of hypertension. Can J Cardiol, 2006. 22(7): p. 553-5.

5. Kearney, P.M., et al., Global burden of hypertension: analysis of worldwide data. Lancet, 2005. 365(9455): p. 217-23.

6. Quan, H., et al., Outcomes among 3.5 million newly diagnosed hypertensive Canadians. Can J Cardiol, 2013. 29(5): p. 592-7.

7. Wilkins, K., et al., Blood pressure in Canadian adults. Health Rep, 2010. 21(1): p. 37-46.

8. Robitaille, C., et al., Diagnosed hypertension in Canada: incidence, prevalence and associated mortality. CMAJ, 2012. 184(1): p. E49-56.

9. Weaver, C.G., et al., Healthcare Costs Attributable to Hypertension: Canadian Population-Based Cohort Study. Hypertension, 2015. 66(3): p. 502-8.

10. Chobanian, A.V., et al., Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. Hypertension, 2003. 42(6): p. 1206-52.

11. Lewington, S., et al., Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. Lancet, 2002. 360(9349): p. 1903-13.

12. Lawes, C.M., et al., Global burden of blood-pressure-related disease, 2001. Lancet, 2008. 371(9623): p. 1513-8.

13. Julius, S., et al., Feasibility of treating prehypertension with an angiotensin-receptor blocker. N Engl J Med, 2006. 354(16): p. 1685-97.

14. Dharmashankar, K. and M.E. Widlansky, Vascular endothelial function and hypertension: insights and directions. Curr Hypertens Rep, 2010. 12(6): p. 448-55.

15. Pacurari, M., et al., The Renin-Angiotensin-aldosterone system in vascular inflammation and remodeling. Int J Inflam, 2014. 2014: p. 689360.

16. Munoz-Durango, N., et al., Role of the Renin-Angiotensin-Aldosterone System beyond Blood Pressure Regulation: Molecular and Cellular Mechanisms Involved in End-Organ Damage during Arterial Hypertension. Int J Mol Sci, 2016. 17(7).

17. Hamza, S.M. and J.R. Dyck, Systemic and renal oxidative stress in the pathogenesis of hypertension: modulation of long-term control of arterial blood pressure by resveratrol. Front Physiol, 2014. 5: p. 292.

18. Ferrario, C.M. and W.B. Strawn, Role of the renin-angiotensin-aldosterone system and proinflammatory mediators in cardiovascular disease. Am J Cardiol, 2006. 98(1): p. 121-8.

19. Te Riet, L., et al., Hypertension: renin-angiotensin-aldosterone system alterations. Circ Res, 2015. 116(6): p. 960-75.

20. Pimenta, E. and S. Oparil, Role of aliskiren in cardio-renal protection and use in hypertensives with multiple risk factors. Vasc Health Risk Manag, 2009. 5(1): p. 453-63.

21. Cheng, H.F., et al., Prostaglandins that increase renin production in response to ACE inhibition are not derived from cyclooxygenase-1. Am J Physiol Regul Integr Comp Physiol, 2002. 283(3): p. R638-46.

22. Skott, O., Renin. Am J Physiol Regul Integr Comp Physiol, 2002. 282(4): p. R937-9.

23. Persson, P.B., Renin: origin, secretion and synthesis. J Physiol, 2003. 552(Pt 3): p. 667-71.

24. Matsubara, H., et al., [Pathophysiological function of angiotensin II AT1 and AT2 receptors and clinical application of AT1 antagonists]. Nihon Rinsho, 1998. 56(7): p. 1912-8.

25. Putnam, K., et al., The renin-angiotensin system: a target of and contributor to dyslipidemias, altered glucose homeostasis, and hypertension of the metabolic syndrome. Am J Physiol Heart Circ Physiol, 2012. 302(6): p. H1219-30.

26. Nguyen, G., et al., Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. J Clin Invest, 2002. 109(11): p. 1417-27.

27. Achard, V., et al., Renin receptor expression in human adipose tissue. Am J Physiol Regul Integr Comp Physiol, 2007. 292(1): p. R274-82.

28. Huang, Y., et al., Renin-stimulated TGF-beta1 expression is regulated by a mitogen-activated protein kinase in mesangial cells. Kidney Int, 2007. 72(1): p. 45-52.

29. Spat, A. and L. Hunyady, Control of aldosterone secretion: a model for convergence in cellular signaling pathways. Physiol Rev, 2004. 84(2): p. 489-539.

30. Gros, R., et al., GPR30 expression is required for the mineralocorticoid receptor-independent rapid vascular effects of aldosterone. Hypertension, 2011. 57(3): p. 442-51.

31. Grossmann, C., et al., Human mineralocorticoid receptor expression renders cells responsive for nongenotropic aldosterone actions. Mol Endocrinol, 2005. 19(7): p. 1697-710.

32. Bauersachs, J., F. Jaisser, and R. Toto, Mineralocorticoid receptor activation and mineralocorticoid receptor antagonist treatment in cardiac and renal diseases. Hypertension, 2015. 65(2): p. 257-63.

33. Buglioni, A., et al., Circulating aldosterone and natriuretic peptides in the general community: relationship to cardiorenal and metabolic disease. Hypertension, 2015. 65(1): p. 45-53.

34. Tomaschitz, A., et al., Aldosterone and arterial hypertension. Nat Rev Endocrinol, 2010. 6(2): p. 83-93.

35. Jaffe, I.Z. and M.E. Mendelsohn, Angiotensin II and aldosterone regulate gene transcription via functional mineralocortocoid receptors in human coronary artery smooth muscle cells. Circ Res, 2005. 96(6): p. 643-50.

36. Naray-Fejes-Toth, A. and G. Fejes-Toth, The sgk, an aldosterone-induced gene in mineralocorticoid target cells, regulates the epithelial sodium channel. Kidney Int, 2000. 57(4): p. 1290-4.

37. Dzau, V.J., Theodore Cooper Lecture: Tissue angiotensin and pathobiology of vascular disease: a unifying hypothesis. Hypertension, 2001. 37(4): p. 1047-52.

38. Rajagopalan, S., et al., Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. J Clin Invest, 1996. 97(8): p. 1916-23.

39. Jia, Z., T. Aoyagi, and T. Yang, mPGES-1 protects against DOCA-salt hypertension via inhibition of oxidative stress or stimulation of NO/cGMP. Hypertension, 2010. 55(2): p. 539-46.

40. Grande, M.T., et al., Increased oxidative stress, the renin-angiotensin system, and sympathetic overactivation induce hypertension in kidney androgen-regulated protein transgenic mice. Free Radic Biol Med, 2011. 51(10): p. 1831-41.

41. Bhatia, K., et al., Oxidative stress contributes to sex differences in angiotensin II-mediated hypertension in spontaneously hypertensive rats. Am J Physiol Regul Integr Comp Physiol, 2012. 302(2): p. R274-82.

42. Rybalkin, S.D., et al., Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells. J Biol Chem, 2002. 277(5): p. 3310-7.

43. Touyz, R.M., Reactive oxygen species as mediators of calcium signaling by angiotensin II: implications in vascular physiology and pathophysiology. Antioxid Redox Signal, 2005. 7(9-10): p. 1302-14.

44. Forstermann, U., Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. Nat Clin Pract Cardiovasc Med, 2008. 5(6): p. 338-49.

45. Schwarz, P.M., H. Kleinert, and U. Forstermann, Potential functional significance of brain-type and muscle-type nitric oxide synthase I expressed in adventitia and media of rat aorta. Arterioscler Thromb Vasc Biol, 1999. 19(11): p. 2584-90.

46. Li, H. and U. Forstermann, Nitric oxide in the pathogenesis of vascular disease. J Pathol, 2000. 190(3): p. 244-54.

47. Vallance, P. and N. Chan, Endothelial function and nitric oxide: clinical relevance. Heart, 2001. 85(3): p. 342-50.

48. Ignarro, L.J., et al., Activation of purified soluble guanylate cyclase by endothelium-derived relaxing factor from intrapulmonary artery and vein: stimulation by acetylcholine, bradykinin and arachidonic acid. J Pharmacol Exp Ther, 1986. 237(3): p. 893-900.

49. Rapoport, R.M., M.B. Draznin, and F. Murad, Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. Nature, 1983. 306(5939): p. 174-6.

50. Muniyappa, R., et al., Cardiovascular actions of insulin. Endocr Rev, 2007. 28(5): p. 463-91.

51. Kim, J.A., et al., Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. Circulation, 2006. 113(15): p. 1888-904.

52. Mennuni, S., et al., Hypertension and kidneys: unraveling complex molecular mechanisms underlying hypertensive renal damage. J Hum Hypertens, 2014. 28(2): p. 74-9.

53. Guyton, A.C., et al., Arterial pressure regulation. Overriding dominance of the kidneys in long-term regulation and in hypertension. Am J Med, 1972. 52(5): p. 584-94.

54. Khawaja, Z. and C.S. Wilcox, Role of the kidneys in resistant hypertension. Int J Hypertens, 2011. 2011: p. 143471.

55. Epstein, M., Aldosterone and the hypertensive kidney: its emerging role as a mediator of progressive renal dysfunction: a paradigm shift. J Hypertens, 2001. 19(5): p. 829-42.

56. Greene, E.L., S. Kren, and T.H. Hostetter, Role of aldosterone in the remnant kidney model in the rat. J Clin Invest, 1996. 98(4): p. 1063-8.

57. Ruggenenti, P., et al., Renal function and requirement for dialysis in chronic nephropathy patients on long-term ramipril: REIN follow-up trial. Gruppo Italiano di Studi Epidemiologici in Nefrologia (GISEN). Ramipril Efficacy in Nephropathy. Lancet, 1998. 352(9136): p. 1252-6.

58. Blasi, E.R., et al., Aldosterone/salt induces renal inflammation and fibrosis in hypertensive rats. Kidney Int, 2003. 63(5): p. 1791-800.

59. Nishiyama, A., et al., Possible contributions of reactive oxygen species and mitogen-activated protein kinase to renal injury in aldosterone/salt-induced hypertensive rats. Hypertension, 2004. 43(4): p. 841-8.

60. Imai, G., et al., Hypertension accelerates diabetic nephropathy in Wistar fatty rats, a model of type 2 diabetes mellitus, via mitogen-activated protein kinase cascades and transforming growth factor-beta1. Hypertens Res, 2003. 26(4): p. 339-47.

61. Bidani, A.K. and K.A. Griffin, Pathophysiology of hypertensive renal damage: implications for therapy. Hypertension, 2004. 44(5): p. 595-601.

62. Dahl, L.K., M. Heine, and K. Thompson, Genetic influence of the kidneys on blood pressure. Evidence from chronic renal homografts in rats with opposite predispositions to hypertension. Circ Res, 1974. 34(1): p. 94-101.

63. Dahl, L.K. and M. Heine, Primary role of renal homografts in setting chronic blood pressure levels in rats. Circ Res, 1975. 36(6): p. 692-6.

64. Cowley, A.W., Jr., Renal medullary oxidative stress, pressure-natriuresis, and hypertension. Hypertension, 2008. 52(5): p. 777-86.

65. Bianchi, G., et al., The hypertensive role of the kidney in spontaneously hypertensive rats. Clin Sci Mol Med Suppl, 1973. 45 Suppl 1: p. 135s-9.

66. Bianchi, G., et al., Blood pressure changes produced by kidney cross-transplantation between spontaneously hypertensive rats and normotensive rats. Clin Sci Mol Med, 1974. 47(5): p. 435-48.

67. Bianchi, G., et al., Renal abnormalities at the prehypertensive stage of essential hypertension. J Cardiovasc Pharmacol, 1988. 12 Suppl 3: p. S43-8.

68. Kawabe, K., et al., Influence on blood pressure of renal isografts between spontaneously hypertensive and normotensive rats, utilizing the F1 hybrids. Jpn Heart J, 1978. 19(6): p. 886-94.

69. Rettig, R., et al., Hypertension transmitted by kidneys from stroke-prone spontaneously hypertensive rats. Am J Physiol, 1989. 257(2 Pt 2): p. F197-203.

70. Coffman, T.M. and S.D. Crowley, Kidney in hypertension: guyton redux. Hypertension, 2008. 51(4): p. 811-6.

71. Becher, U.M., et al., Endothelial damage and regeneration: the role of the renin-angiotensin-aldosterone system. Curr Hypertens Rep, 2011. 13(1): p. 86-92.

72. Wassmann, S. and G. Nickenig, Pathophysiological regulation of the AT1-receptor and implications for vascular disease. J Hypertens Suppl, 2006. 24(1): p. S15-21.

73. Qi, G., et al., Angiotensin II infusion-induced inflammation, monocytic fibroblast precursor infiltration, and cardiac fibrosis are pressure dependent. Cardiovasc Toxicol, 2011. 11(2): p. 157-67.

74. Gul, R., et al., Cooperative interaction between reactive oxygen species and Ca2+ signals contributes to angiotensin II-induced hypertrophy in adult rat cardiomyocytes. Am J Physiol Heart Circ Physiol, 2012. 302(4): p. H901-9.

75. Geisterfer, A.A., M.J. Peach, and G.K. Owens, Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. Circ Res, 1988. 62(4): p. 749-56.

76. Leyking, S. and D. Fliser, Insulin resistance in CKD. Clin J Am Soc Nephrol, 2014. 9(4): p. 638-40.

77. Salvetti, A., et al., The inter-relationship between insulin resistance and hypertension. Drugs, 1993. 46 Suppl 2: p. 149-59.

78. Ferrannini, E., et al., Insulin resistance in essential hypertension. N Engl J Med, 1987. 317(6): p. 350-7.

79. Bonora, E., et al., Hyperinsulinemia and insulin resistance are independently associated with plasma lipids, uric acid and blood pressure in non-diabetic subjects. The GISIR database. Nutr Metab Cardiovasc Dis, 2008. 18(9): p. 624-31.

80. Manrique, C., et al., Hypertension and the cardiometabolic syndrome. J Clin Hypertens (Greenwich), 2005. 7(8): p. 471-6.

81. Tiwari, S., et al., Impaired sodium excretion and increased blood pressure in mice with targeted deletion of renal epithelial insulin receptor. Proc Natl Acad Sci U S A, 2008. 105(17): p. 6469-74.

82. Widlansky, M.E., et al., The clinical implications of endothelial dysfunction. J Am Coll Cardiol, 2003. 42(7): p. 1149-60.

83. Vita, J.A., et al., Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. Circulation, 1990. 81(2): p. 491-7.

84. Panza, J.A., et al., Impaired endothelium-dependent vasodilation in patients with essential hypertension. Evidence that nitric oxide abnormality is not localized to a single signal transduction pathway. Circulation, 1995. 91(6): p. 1732-8.

85. Benjamin, E.J., et al., Clinical correlates and heritability of flow-mediated dilation in the community: the Framingham Heart Study. Circulation, 2004. 109(5): p. 613-9.

86. Kizhakekuttu, T.J. and M.E. Widlansky, Natural antioxidants and hypertension: promise and challenges. Cardiovasc Ther, 2010. 28(4): p. e20-32.

87. Furchgott, R.F. and J.V. Zawadzki, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature, 1980. 288(5789): p. 373-6.

88. Ignarro, L.J., et al., Possible involvement of S-nitrosothiols in the activation of guanylate cyclase by nitroso compounds. FEBS Lett, 1980. 110(2): p. 275-8.

89. Sander, M., B. Chavoshan, and R.G. Victor, A large blood pressure-raising effect of nitric oxide synthase inhibition in humans. Hypertension, 1999. 33(4): p. 937-42.

90. Rossi, R., et al., Flow-mediated vasodilation and the risk of developing hypertension in healthy postmenopausal women. J Am Coll Cardiol, 2004. 44(8): p. 1636-40.

91. Jurva, J.W., et al., The effect of exertional hypertension evoked by weight lifting on vascular endothelial function. J Am Coll Cardiol, 2006. 48(3): p. 588-9.

92. Juonala, M., et al., Elevated blood pressure in adolescent boys predicts endothelial dysfunction: the cardiovascular risk in young Finns study. Hypertension, 2006. 48(3): p. 424-30.

93. Johnson, R.J., et al., A unifying pathway for essential hypertension. Am J Hypertens, 2005. 18(3): p. 431-40.

94. Julius, S., The evidence for a pathophysiologic significance of the sympathetic overactivity in hypertension. Clin Exp Hypertens, 1996. 18(3-4): p. 305-21.

95. Jeunemaitre, X., et al., Angiotensinogen variants and human hypertension. Curr Hypertens Rep, 1999. 1(1): p. 31-41.

96. Izzo, J.L., Jr. and A.H. Gradman, Mechanisms and management of hypertensive heart disease: from left ventricular hypertrophy to heart failure. Med Clin North Am, 2004. 88(5): p. 1257-71.

97. Nadruz, W., Myocardial remodeling in hypertension. J Hum Hypertens, 2015. 29(1): p. 1-6.

98. Levy, D., et al., Echocardiographically detected left ventricular hypertrophy: prevalence and risk factors. The Framingham Heart Study. Ann Intern Med, 1988. 108(1): p. 7-13.

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

100. Rohini, A., et al., Molecular targets and regulators of cardiac hypertrophy. Pharmacol Res, 2010. 61(4): p. 269-80.

101. Diwan, A. and G.W. Dorn, 2nd, Decompensation of cardiac hypertrophy: cellular mechanisms and novel therapeutic targets. Physiology (Bethesda), 2007. 22: p. 56-64.

102. Dyck, J.R. and G.D. Lopaschuk, AMPK alterations in cardiac physiology and pathology: enemy or ally? J Physiol, 2006. 574(Pt 1): p. 95-112.

103. van Bilsen, M., et al., Metabolic remodelling of the failing heart: the cardiac burn-out syndrome? Cardiovasc Res, 2004. 61(2): p. 218-26.

104. Zhang, L., et al., Cardiac insulin-resistance and decreased mitochondrial energy production precede the development of systolic heart failure after pressure-overload hypertrophy. Circ Heart Fail, 2013. 6(5): p. 1039-48.

105. Lopaschuk, G.D., et al., Myocardial fatty acid metabolism in health and disease. Physiol Rev, 2010. 90(1): p. 207-58.

106. Kolwicz, S.C., Jr., et al., Cardiac-specific deletion of acetyl CoA carboxylase 2 prevents metabolic remodeling during pressure-overload hypertrophy. Circ Res, 2012. 111(6): p. 728-38.

107. Hardie, D.G. and D. Carling, The AMP-activated protein kinase - Fuel gauge of the mammalian cell? European Journal of Biochemistry, 1997. 246(2): p. 259-273.

108. Kemp, B.E., et al., AMP-activated protein kinase, super metabolic regulator. Biochem Soc Trans, 2003. 31(Pt 1): p. 162-8.

109. Carling, D., et al., Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. Eur J Biochem, 1989. 186(1-2): p. 129-36.

110. Beri, R.K., et al., Molecular cloning, expression and chromosomal localisation of human AMP-activated protein kinase. FEBS Lett, 1994. 356(1): p. 117-21.

111. Kudo, N., et al., High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. J Biol Chem, 1995. 270(29): p. 17513-20.

112. Russell, R.R., 3rd, et al., Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. Am J Physiol, 1999. 277(2 Pt 2): p. H643-9.

113. Marsin, A.S., et al., Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. Curr Biol, 2000. 10(20): p. 1247-55.

114. Dyck, J.R.B., et al., Phosphorylation control of cardiac acetyl-CoA carboxylase by cAMP-dependent protein kinase and 5 '-AMP activated protein kinase. European Journal of Biochemistry, 1999. 262(1): p. 184-190.

115. Chan, A.Y. and J.R. Dyck, Activation of AMP-activated protein kinase (AMPK) inhibits protein synthesis: a potential strategy to prevent the development of cardiac hypertrophy. Can J Physiol Pharmacol, 2005. 83(1): p. 24-8.

116. Bairwa, S.C., N. Parajuli, and J.R. Dyck, The role of AMPK in cardiomyocyte health and survival. Biochim Biophys Acta, 2016.

117. Chan, A.Y.M., et al., Activation of AMP-activated protein kinase inhibits protein synthesis associated with hypertrophy in the cardiac myocyte. Journal of Biological Chemistry, 2004. 279(31): p. 32771-32779.

118. Dolinsky, V.W. and J.R. Dyck, Role of AMP-activated protein kinase in healthy and diseased hearts. Am J Physiol Heart Circ Physiol, 2006. 291(6): p. H2557-69.

119. Dyck, J.R.B. and G.D. Lopaschuk, AMPK alterations in cardiac physiology and pathology: enemy or ally? Journal of Physiology-London, 2006. 574(1): p. 95-112.

120. Hawley, S.A., et al., Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. Journal of Biological Chemistry, 1996. 271(44): p. 27879-27887.

121. Wojtaszewski, J.F.P., et al., Glycogen-dependent effects of 5-aminoimidazole-4-carboxamide (AICA)-riboside on AMP-activated protein kinase and glycogen synthase activities in rat skeletal muscle. Diabetes, 2002. 51(2): p. 284-292.

122. Polekhina, G., et al., AMPK beta subunit targets metabolic stress sensing to glycogen. Current Biology, 2003. 13(10): p. 867-871.

123. Russell, R.R., et al., AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. Journal of Clinical Investigation, 2004. 114(4): p. 495-503.

124. Coven, D.L., et al., Physiological role of AMP-activated protein kinase in the heart: graded activation during exercise. American Journal of Physiology-Endocrinology and Metabolism, 2003. 285(3): p. E629-E636.

125. Pang, T., et al., Conserved alpha-helix acts as autoinhibitory sequence in AMP-activated protein kinase alpha subunits. Journal of Biological Chemistry, 2007. 282(1): p. 495-506.

126. Chen, L., et al., Structural insight into the autoinhibition mechanism of AMP-activated protein kinase. Nature, 2009. 459(7250): p. 1146-U139.

127. Xiao, B., et al., Structure of mammalian AMPK and its regulation by ADP. Nature, 2011. 472(7342): p. 230-233.

128. Hardie, D.G., Minireview: The AMP-activated protein kinase cascade: The key sensor of cellular energy status. Endocrinology, 2003. 144(12): p. 5179-5183.

129. Li, J., et al., Activation of AMPK alpha- and gamma-isoform complexes in the intact ischemic rat heart. American Journal of Physiology-Heart and Circulatory Physiology, 2006. 291(4): p. H1927-H1934.

130. Salt, I., et al., AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha 2 isoform. Biochemical Journal, 1998. 334: p. 177-187.

131. Russell, R.R., 3rd, et al., AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. J Clin Invest, 2004. 114(4): p. 495-503.

132. Li, J., et al., Activation of AMPK alpha- and gamma-isoform complexes in the intact ischemic rat heart. Am J Physiol Heart Circ Physiol, 2006. 291(4): p. H1927-34.

133. Coven, D.L., et al., Physiological role of AMP-activated protein kinase in the heart: graded activation during exercise. Am J Physiol Endocrinol Metab, 2003. 285(3): p. E629-36.

134. Musi, N., et al., Functional role of AMP-activated protein kinase in the heart during exercise. FEBS Lett, 2005. 579(10): p. 2045-50.

135. Frederich, M. and J.A. Balschi, The relationship between AMP-activated protein kinase activity and AMP concentration in the isolated perfused rat heart. Journal of Biological Chemistry, 2002. 277(3): p. 1928-1932.

136. Kudo, N., et al., Characterization of 5'AMP-activated protein kinase activity in the heart and its role in inhibiting acetyl-CoA carboxylase during reperfusion following ischemia. Biochim Biophys Acta, 1996. 1301(1-2): p. 67-75.

137. Baron, S.J., et al., Dual mechanisms regulating AMPK kinase action in the ischemic heart. Circulation Research, 2005. 96(3): p. 337-345.

138. Hawley, S.A., et al., Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. J Biol Chem, 1996. 271(44): p. 27879-87.

139. Stein, S.C., et al., The regulation of AMP-activated protein kinase by phosphorylation. Biochemical Journal, 2000. 345: p. 437-443.

140. Xiao, B., et al., Structural basis for AMP binding to mammalian AMP-activated protein kinase. Nature, 2007. 449(7161): p. 496-U14.

141. Hawley, S.A., et al., Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J Biol, 2003. 2(4): p. 28.

142. Woods, A., et al., LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Current Biology, 2003. 13(22): p. 2004-2008.

143. Sakamoto, K., et al., Deficiency of LKB1 in heart prevents ischemia-mediated activation of AMPKalpha2 but not AMPKalpha1. Am J Physiol Endocrinol Metab, 2006. 290(5): p. E780-8.

144. Hong, S.P., M. Momcilovic, and M. Carlson, Function of mammalian LKB1 and Ca2+/calmodulin-dependent protein kinase kinase alpha as Snf1-activating kinases in yeast. J Biol Chem, 2005. 280(23): p. 21804-9.

145. Anderson, K.A., et al., Components of a calmodulin-dependent protein kinase cascade - Molecular cloning, functional characterization and cellular localization of Ca2+/calmodulin-dependent protein kinase kinase beta. Journal of Biological Chemistry, 1998. 273(48): p. 31880-31889.

146. Altarejos, J.Y., et al., Myocardial ischemia differentially regulates LKB1 and an alternate 5 '-AMP-activated protein kinase kinase. Journal of Biological Chemistry, 2005. 280(1): p. 183-190.

147. Wang, J., et al., Activated protein C protects against myocardial ischemic/reperfusion injury through AMP-activated protein kinase signaling. Journal of Thrombosis and Haemostasis, 2011. 9(7): p. 1308-1317.

148. Hurley, R.L., et al., The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. Journal of Biological Chemistry, 2005. 280(32): p. 29060-29066.

149. Davila-Roman, V.G., et al., Altered myocardial fatty acid and glucose metabolism in idiopathic dilated cardiomyopathy. J Am Coll Cardiol, 2002. 40(2): p. 271-7.

150. Sun, G.Q., et al., Resveratrol via activation of AMPK lowers blood pressure in DOCA-salt hypertensive mice. Clin Exp Hypertens, 2015. 37(8): p. 616-21.

151. Cao, X., et al., Resveratrol prevents AngII-induced hypertension via AMPK activation and RhoA/ROCK suppression in mice. Hypertens Res, 2014. 37(9): p. 803-10.

152. Penumathsa, S.V., et al., Resveratrol enhances GLUT-4 translocation to the caveolar lipid raft fractions through AMPK/Akt/eNOS signalling pathway in diabetic myocardium. J Cell Mol Med, 2008. 12(6A): p. 2350-61.

153. Dolinsky, V.W., et al., Resveratrol prevents hypertension and cardiac hypertrophy in hypertensive rats and mice. Biochim Biophys Acta, 2013. 1832(10): p. 1723-33.

154. Majithiya, J.B. and R. Balaraman, Metformin reduces blood pressure and restores endothelial function in aorta of streptozotocin-induced diabetic rats. Life Sci, 2006. 78(22): p. 2615-24.

155. Wang, Y., et al., Berberine prevents hyperglycemia-induced endothelial injury and enhances vasodilatation via adenosine monophosphate-activated protein kinase and endothelial nitric oxide synthase. Cardiovasc Res, 2009. 82(3): p. 484-92.

156. Morrow, V.A., et al., Direct activation of AMP-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells. J Biol Chem, 2003. 278(34): p. 31629-39.

157. Tian, R., et al., Increased adenosine monophosphate-activated protein kinase activity in rat hearts with pressure-overload hypertrophy. Circulation, 2001. 104(14): p. 1664-9.

158. Arad, M., et al., Transgenic mice overexpressing mutant PRKAG2 define the cause of Wolff-Parkinson-White syndrome in glycogen storage cardiomyopathy. Circulation, 2003. 107(22): p. 2850-6.

159. Nascimben, L., et al., Mechanisms for increased glycolysis in the hypertrophied rat heart. Hypertension, 2004. 44(5): p. 662-7.

160. Chan, A.Y., et al., Activation of AMP-activated protein kinase inhibits protein synthesis associated with hypertrophy in the cardiac myocyte. J Biol Chem, 2004. 279(31): p. 32771-9.

161. Dolinsky, V.W., et al., Resveratrol prevents the prohypertrophic effects of oxidative stress on LKB1. Circulation, 2009. 119(12): p. 1643-52.

162. Liao, Y., et al., Exacerbation of heart failure in adiponectin-deficient mice due to impaired regulation of AMPK and glucose metabolism. Cardiovasc Res, 2005. 67(4): p. 705-13.

163. Dolinsky, V.W., et al., Calorie restriction prevents hypertension and cardiac hypertrophy in the spontaneously hypertensive rat. Hypertension, 2010. 56(3): p. 412-21.

164. Li, H.L., et al., Long-term activation of adenosine monophosphate-activated protein kinase attenuates pressure-overload-induced cardiac hypertrophy. J Cell Biochem, 2007. 100(5): p. 1086-99.

165. Sasaki, H., et al., Metformin prevents progression of heart failure in dogs: role of AMP-activated protein kinase. Circulation, 2009. 119(19): p. 2568-77.

166. Horman, S., et al., Myocardial ischemia and increased heart work modulate the phosphorylation state of eukaryotic elongation factor-2. J Biol Chem, 2003. 278(43): p. 41970-6.

167. Demeulder, B., et al., Differential regulation of eEF2 and p70S6K by AMPKalpha2 in heart. Biochim Biophys Acta, 2013. 1832(6): p. 780-90.

168. Krause, U., L. Bertrand, and L. Hue, Control of p70 ribosomal protein S6 kinase and acetyl-CoA carboxylase by AMP-activated protein kinase and protein phosphatases in isolated hepatocytes. Eur J Biochem, 2002. 269(15): p. 3751-9.

169. Noga, A.A., et al., Expression of an active LKB1 complex in cardiac myocytes results in decreased protein synthesis associated with phenylephrine-induced hypertrophy. Am J Physiol Heart Circ Physiol, 2007. 292(3): p. H1460-9.

170. Chan, A.Y., et al., Resveratrol inhibits cardiac hypertrophy via AMP-activated protein kinase and Akt. J Biol Chem, 2008. 283(35): p. 24194-201.

171. Ikeda, Y., et al., Cardiac-specific deletion of LKB1 leads to hypertrophy and dysfunction. J Biol Chem, 2009. 284(51): p. 35839-49.

172. Zhou, Y., et al., Exendin-4 attenuates cardiac hypertrophy via AMPK/mTOR signaling pathway activation. Biochem Biophys Res Commun, 2015. 468(1-2): p. 394-9.

173. Kang, S., et al., Resistin promotes cardiac hypertrophy via the AMP-activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) and c-Jun N-terminal kinase/insulin receptor substrate 1 (JNK/IRS1) pathways. J Biol Chem, 2011. 286(21): p. 18465-73.

174. Li, Y., et al., AMPK inhibits cardiac hypertrophy by promoting autophagy via mTORC1. Arch Biochem Biophys, 2014. 558: p. 79-86.

175. Fu, Y.N., et al., Metformin attenuates pressure overload-induced cardiac hypertrophy via AMPK activation. Acta Pharmacol Sin, 2011. 32(7): p. 879-87.

176. Meng, R., et al., AMPK activation enhances PPARalpha activity to inhibit cardiac hypertrophy via ERK1/2 MAPK signaling pathway. Arch Biochem Biophys, 2011. 511(1-2): p. 1-7.

177. Meng, R.S., et al., Adenosine monophosphate-activated protein kinase inhibits cardiac hypertrophy through reactivating peroxisome proliferator-activated receptor-alpha signaling pathway. Eur J Pharmacol, 2009. 620(1-3): p. 63-70.

178. Huang, X., et al., Regulatory effect of AMP-activated protein kinase on pulmonary hypertension induced by chronic hypoxia in rats: in vivo and in vitro studies. Mol Biol Rep, 2014. 41(6): p. 4031-41.

179. Ahmad, F., et al., Increased alpha2 subunit-associated AMPK activity and PRKAG2 cardiomyopathy. Circulation, 2005. 112(20): p. 3140-8.

180. Kim, M., et al., AMPK isoform expression in the normal and failing hearts. J Mol Cell Cardiol, 2012. 52(5): p. 1066-73.

181. Lu, T.M., et al., Downregulation of Sirt1 as aging change in advanced heart failure. J Biomed Sci, 2014. 21: p. 57.

182. Neglia, D., et al., Impaired myocardial metabolic reserve and substrate selection flexibility during stress in patients with idiopathic dilated cardiomyopathy. Am J Physiol Heart Circ Physiol, 2007. 293(6): p. H3270-8.

183. Recchia, F.A., et al., Reduced nitric oxide production and altered myocardial metabolism during the decompensation of pacing-induced heart failure in the conscious dog. Circ Res, 1998. 83(10): p. 969-79.

184. Zhabyeyev, P., et al., Pressure-overload-induced heart failure induces a selective reduction in glucose oxidation at physiological afterload. Cardiovasc Res, 2013. 97(4): p. 676-85.

185. Kim, A.S., et al., A small molecule AMPK activator protects the heart against ischemia-reperfusion injury. J Mol Cell Cardiol, 2011. 51(1): p. 24-32.

186. Morrison, A. and J. Li, PPAR-gamma and AMPK--advantageous targets for myocardial ischemia/reperfusion therapy. Biochem Pharmacol, 2011. 82(3): p. 195-200.

187. Hare, J.M., Oxidative stress and apoptosis in heart failure progression. Circ Res, 2001. 89(3): p. 198-200.

188. Giordano, F.J., Oxygen, oxidative stress, hypoxia, and heart failure. J Clin Invest, 2005. 115(3): p. 500-8.

189. Ungvari, Z., et al., Role of oxidative-nitrosative stress and downstream pathways in various forms of cardiomyopathy and heart failure. Curr Vasc Pharmacol, 2005. 3(3): p. 221-9.

190. Narula, J., et al., Apoptosis in heart failure: release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy. Proc Natl Acad Sci U S A, 1999. 96(14): p. 8144-9.

191. Lu, D., et al., Knockdown of cytochrome P450 2E1 inhibits oxidative stress and apoptosis in the cTnT(R141W) dilated cardiomyopathy transgenic mice. Hypertension, 2012. 60(1): p. 81-9.

192. Gu, X.S., et al., Resveratrol, an activator of SIRT1, upregulates AMPK and improves cardiac function in heart failure. Genet Mol Res, 2014. 13(1): p. 323-35.

193. Guo, S., et al., Resveratrol attenuates high glucose-induced oxidative stress and cardiomyocyte apoptosis through AMPK. Mol Cell Endocrinol, 2015. 412: p. 85-94.

194. Alcendor, R.R., et al., Sirt1 regulates aging and resistance to oxidative stress in the heart. Circ Res, 2007. 100(10): p. 1512-21.

195. Arany, Z., et al., Transverse aortic constriction leads to accelerated heart failure in mice lacking PPAR-gamma coactivator 1alpha. Proc Natl Acad Sci U S A, 2006. 103(26): p. 10086-91.

196. Jager, S., et al., AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc Natl Acad Sci U S A, 2007. 104(29): p. 12017-22.

197. Canto, C., et al., AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature, 2009. 458(7241): p. 1056-60.

198. Li, X.N., et al., Activation of the AMPK-FOXO3 pathway reduces fatty acid-induced increase in intracellular reactive oxygen species by upregulating thioredoxin. Diabetes, 2009. 58(10): p. 2246-57.

199. Wang, J., et al., A novel AMPK activator from Chinese herb medicine and ischemia phosphorylate the cardiac transcription factor FOXO3. Int J Physiol Pathophysiol Pharmacol, 2009. 1(2): p. 116-126.

200. Sengupta, A., J.D. Molkentin, and K.E. Yutzey, FoxO transcription factors promote autophagy in cardiomyocytes. J Biol Chem, 2009. 284(41): p. 28319-31.

201. Kubli, D.A. and A.B. Gustafsson, Cardiomyocyte health: adapting to metabolic changes through autophagy. Trends Endocrinol Metab, 2014. 25(3): p. 156-64.

202. Kanamori, H., et al., Resveratrol reverses remodeling in hearts with large, old myocardial infarctions through enhanced autophagy-activating AMP kinase pathway. Am J Pathol, 2013. 182(3): p. 701-13.

203. Gundewar, S., et al., Activation of AMP-activated protein kinase by metformin improves left ventricular function and survival in heart failure. Circ Res, 2009. 104(3): p. 403-11.

204. Siiteri, P.K., Adipose-Tissue as a Source of Hormones. American Journal of Clinical Nutrition, 1987. 45(1): p. 277-282.

205. Zhang, Y.Y., et al., Positional Cloning of the Mouse Obese Gene and Its Human Homolog. Nature, 1994. 372(6505): p. 425-432.

206. Kershaw, E.E. and J.S. Flier, Adipose tissue as an endocrine organ. Journal of Clinical Endocrinology & Metabolism, 2004. 89(6): p. 2548-2556.

207. Cao, H.M., et al., Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. Cell, 2008. 134(6): p. 933-944.

208. Corsico, B. and L.J. Falomir-Lockhart, Signals from the adipose tissue alter systemic metabolism. Clinical Lipidology, 2009. 4(1): p. 13-16.

209. Herman, M.A., et al., A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. Nature, 2012. 484(7394): p. 333-U66.

210. Marcelino, H., et al., A Role for Adipose Tissue De Novo Lipogenesis in Glucose Homeostasis During Catch-up Growth A Randle Cycle Favoring Fat Storage. Diabetes, 2013. 62(2): p. 362-372.

211. Grundy, S.M., et al., Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. Circulation, 2004. 109(3): p. 433-8.

212. Leow, M.K., C.L. Addy, and C.S. Mantzoros, Clinical review 159: Human immunodeficiency virus/highly active antiretroviral therapy-associated metabolic

syndrome: clinical presentation, pathophysiology, and therapeutic strategies. J Clin Endocrinol Metab, 2003. 88(5): p. 1961-76.

213. Fonseca-Alaniz, M.H., et al., Adipose tissue as an endocrine organ: from theory to practice. Jornal De Pediatria, 2007. 83(5): p. S192-S203.

214. Frayn, K.N., et al., Integrative physiology of human adipose tissue. International Journal of Obesity, 2003. 27(8): p. 875-888.

215. Saely, C.H., K. Geiger, and H. Drexel, Brown versus White Adipose Tissue: A Mini-Review. Gerontology, 2012. 58(1): p. 15-23.

216. Hertzel, A.V., et al., Lipid metabolism in adipose tissue. Biochemistry of Lipids, Lipoproteins and Membranes, 5th Edition, 2008: p. 277-304.

217. Galic, S., J.S. Oakhill, and G.R. Steinberg, Adipose tissue as an endocrine organ. Molecular and Cellular Endocrinology, 2010. 316(2): p. 129-139.

218. Berg, A.H. and P.E. Scherer, Adipose tissue, inflammation, and cardiovascular disease. Circulation Research, 2005. 96(9): p. 939-949.

219. Trujillo, M.E. and P.E. Scherer, Adipose tissue-derived factors: Impact on health and disease. Endocrine Reviews, 2006. 27(7): p. 762-778.

220. Avram, A.S., M.M. Avram, and W.D. James, Subcutaneous fat in normal and diseased states: 2. Anatomy and physiology of white and brown adipose tissue. J Am Acad Dermatol, 2005. 53(4): p. 671-83.

221. Ouchi, N., et al., Adipokines in inflammation and metabolic disease. Nat Rev Immunol, 2011. 11(2): p. 85-97.

222. Samaras, K., et al., Subcutaneous and Visceral Adipose Tissue Gene Expression of Serum Adipokines That Predict Type 2 Diabetes. Obesity, 2010. 18(5): p. 884-889.

223. Fried, S.K., D.A. Bunkin, and A.S. Greenberg, Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: Depot difference and regulation by

glucocorticoid. Journal of Clinical Endocrinology & Metabolism, 1998. 83(3): p. 847-850.

224. Jo, J., et al., Hypertrophy and/or Hyperplasia: Dynamics of Adipose Tissue Growth. PLoS Comput Biol, 2009. 5(3): p. e1000324.

225. Hosogai, N., et al., Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. Diabetes, 2007. 56(4): p. 901-911.

226. Furukawa, S., et al., Increased oxidative stress in obesity and its impact on metabolic syndrome. Journal of Clinical Investigation, 2004. 114(12): p. 1752-1761.

227. Weisberg, S.P., et al., Obesity is associated with macrophage accumulation in adipose tissue. Journal of Clinical Investigation, 2003. 112(12): p. 1796-1808.

228. Ouchi, N., et al., Adipokines in inflammation and metabolic disease. Nature Reviews Immunology, 2011. 11(2): p. 85-97.

229. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science, 1993. 259(5091): p. 87-91.

230. Pausova, Z., et al., Role of tumor necrosis factor-alpha gene locus in obesity and obesity-associated hypertension in French Canadians. Hypertension, 2000. 36(1): p. 14-9.

231. Zhang, Y., et al., Positional cloning of the mouse obese gene and its human homologue. Nature, 1994. 372(6505): p. 425-32.

232. Simonds, S.E., et al., Leptin mediates the increase in blood pressure associated with obesity. Cell, 2014. 159(6): p. 1404-16.

233. Shimomura, I., et al., Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. Nat Med, 1996. 2(7): p. 800-3.

234. Urano, T., et al., Impaired fibrinolysis in hypertension and obesity due to high plasminogen activator inhibitor-1 level in plasma. Jpn J Physiol, 1993. 43(2): p. 221-8.

235. Hu, E., P. Liang, and B.M. Spiegelman, AdipoQ is a novel adipose-specific gene dysregulated in obesity. J Biol Chem, 1996. 271(18): p. 10697-703.

236. Maeda, K., et al., cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). Biochem Biophys Res Commun, 1996. 221(2): p. 286-9.

237. Scherer, P.E., et al., A novel serum protein similar to C1q, produced exclusively in adipocytes. J Biol Chem, 1995. 270(45): p. 26746-9.

238. Matsuda, M. and I. Shimomura, Roles of adiponectin and oxidative stress in obesity-associated metabolic and cardiovascular diseases. Rev Endocr Metab Disord, 2014. 15(1): p. 1-10.

239. Kondo, K., et al., Impact of a single intracoronary administration of adiponectin on myocardial ischemia/reperfusion injury in a pig model. Circ Cardiovasc Interv, 2010. 3(2): p. 166-73.

240. Kim, N.H., et al., Association of obstructive sleep apnea and glucose metabolism in subjects with or without obesity. Diabetes Care, 2013. 36(12): p. 3909-15.

241. Kim, D.H., et al., Adiponectin levels and the risk of hypertension: a systematic review and meta-analysis. Hypertension, 2013. 62(1): p. 27-32.

242. Haunerland, N.H. and F. Spener, Fatty acid-binding proteins--insights from genetic manipulations. Prog Lipid Res, 2004. 43(4): p. 328-49.

243. Zimmerman, A.W. and J.H. Veerkamp, New insights into the structure and function of fatty acid-binding proteins. Cell Mol Life Sci, 2002. 59(7): p. 1096-116.

244. Pinnick, K.E., et al., Gluteofemoral adipose tissue plays a major role in production of the lipokine palmitoleate in humans. Diabetes, 2012. 61(6): p. 1399-403.

245. Stefan, N., et al., Circulating Palmitoleate Strongly and Independently Predicts Insulin Sensitivity in Humans. Diabetes Care, 2010. 33(2): p. 405-407.

246. Vessby, B., et al., Plasma lipid fatty acid composition, desaturase activities and insulin sensitivity in Amerindian women. Nutr Metab Cardiovasc Dis, 2012. 22(3): p. 176-81.

247. Vessby, B., S. Tengblad, and H. Lithell, Insulin sensitivity is related to the fatty acid composition of serum lipids and skeletal muscle phospholipids in 70-year-old men. Diabetologia, 1994. 37(10): p. 1044-50.

248. Gong, J., et al., Adipose tissue palmitoleic acid and obesity in humans: does it behave as a lipokine? Am J Clin Nutr, 2011. 93(1): p. 186-91.

249. Okada, T., et al., Plasma palmitoleic acid content and obesity in children. Am J Clin Nutr, 2005. 82(4): p. 747-50.

250. Paillard, F., et al., Plasma palmitoleic acid, a product of stearoyl-coA desaturase activity, is an independent marker of triglyceridemia and abdominal adiposity. Nutr Metab Cardiovasc Dis, 2008. 18(6): p. 436-40.

251. Hodge, A.M., et al., Plasma phospholipid and dietary fatty acids as predictors of type 2 diabetes: interpreting the role of linoleic acid. Am J Clin Nutr, 2007. 86(1): p. 189-97.

252. Mozaffarian, D., et al., Circulating palmitoleic acid and risk of metabolic abnormalities and new-onset diabetes. Am J Clin Nutr, 2010. 92(6): p. 1350-8.

253. Mozaffarian, D., et al., Circulating palmitoleic acid and risk of metabolic abnormalities and new-onset diabetes. American Journal of Clinical Nutrition, 2010. 92(6): p. 1350-1358.

254. Hodson, L., C.M. Skeaff, and B.A. Fielding, Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. Prog Lipid Res, 2008. 47(5): p. 348-80.

255. Hodson, L. and F. Karpe, Is there something special about palmitoleate? Curr Opin Clin Nutr Metab Care, 2013. 16(2): p. 225-31.

256. Fatima, T., et al., Fatty acid composition of developing sea buckthorn (Hippophae rhamnoides L.) berry and the transcriptome of the mature seed. PLoS One, 2012. 7(4): p. e34099.

257. Knutsen, S.F., et al., Comparison of adipose tissue fatty acids with dietary fatty acids as measured by 24-hour recall and food frequency questionnaire in Black and White Adventists: the Adventist Health Study. Ann Epidemiol, 2003. 13(2): p. 119-27.

258. Hodge, A.M., et al., Plasma phospholipid fatty acid composition as a biomarker of habitual dietary fat intake in an ethnically diverse cohort. Nutr Metab Cardiovasc Dis, 2007. 17(6): p. 415-26.

259. Garaulet, M., et al., Relation between degree of obesity and site-specific adipose tissue fatty acid composition in a Mediterranean population. Nutrition, 2011. 27(2): p. 170-6.

260. Frigolet, M.E. and R. Gutierrez-Aguilar, The Role of the Novel Lipokine Palmitoleic Acid in Health and Disease. Adv Nutr, 2017. 8(1): p. 173S-181S.

261. Lodhi, I.J., X. Wei, and C.F. Semenkovich, Lipoexpediency: de novo lipogenesis as a metabolic signal transmitter. Trends Endocrinol Metab, 2011. 22(1): p. 1-8.

262. Neuschwander-Tetri, B.A., Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. Hepatology, 2010. 52(2): p. 774-88.

263. Fagot-Campagna, A., et al., High free fatty acid concentration: an independent risk factor for hypertension in the Paris Prospective Study. Int J Epidemiol, 1998. 27(5): p. 808-13.

264. Wu, B.B., et al., [Relationship between serum free fatty acid and cytokines, carotid atherosclerosis in chronic kidney disease]. Zhonghua Nei Ke Za Zhi, 2010. 49(7): p. 572-6.

265. Guo, S.X., et al., Association of Serum Free Fatty Acids with Hypertension and Insulin Resistance among Rural Uyghur Adults in Far Western China. Int J Environ Res Public Health, 2015. 12(6): p. 6582-90.

266. Boden, G., Obesity and free fatty acids. Endocrinol Metab Clin North Am, 2008. 37(3): p. 635-46, viii-ix.

267. Postic, C. and J. Girard, The role of the lipogenic pathway in the development of hepatic steatosis. Diabetes Metab, 2008. 34(6 Pt 2): p. 643-8.

268. Yee, J.K., et al., Compartmentalization of stearoyl-coenzyme A desaturase 1 activity in HepG2 cells. J Lipid Res, 2008. 49(10): p. 2124-34.

269. Yew Tan, C., et al., Adipose tissue fatty acid chain length and mono-unsaturation increases with obesity and insulin resistance. Sci Rep, 2015. 5: p. 18366.

270. Cooney, M.T., et al., HDL cholesterol protects against cardiovascular disease in both genders, at all ages and at all levels of risk. Atherosclerosis, 2009. 206(2): p. 611-6.

271. Lewington, S., et al., Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. Lancet, 2007. 370(9602): p. 1829-39.

272. Bayturan, O., et al., The metabolic syndrome, its component risk factors, and progression of coronary atherosclerosis. Arch Intern Med, 2010. 170(5): p. 478-84.

273. Cambien, F., et al., An epidemiologic appraisal of the associations between the fatty acids esterifying serum cholesterol and some cardiovascular risk factors in middle-aged men. Am J Epidemiol, 1988. 127(1): p. 75-86.

274. Zong, G., et al., Associations of erythrocyte palmitoleic acid with adipokines, inflammatory markers, and the metabolic syndrome in middle-aged and older Chinese. Am J Clin Nutr, 2012. 96(5): p. 970-6.

275. Djousse, L., et al., Red blood cell membrane concentration of cis-palmitoleic and cis-vaccenic acids and risk of coronary heart disease. Am J Cardiol, 2012. 110(4): p. 539-44.

276. Arab, L., Biomarkers of fat and fatty acid intake. J Nutr, 2003. 133 Suppl 3: p. 925S-932S.

277. Curb, J.D., et al., Serum lipid effects of a high-monounsaturated fat diet based on macadamia nuts. Arch Intern Med, 2000. 160(8): p. 1154-8.

278. Nestel, P., P. Clifton, and M. Noakes, Effects of increasing dietary palmitoleic acid compared with palmitic and oleic acids on plasma lipids of hypercholesterolemic men. J Lipid Res, 1994. 35(4): p. 656-62.

279. Bernstein, A.M., M.F. Roizen, and L. Martinez, Purified palmitoleic acid for the reduction of high-sensitivity C-reactive protein and serum lipids: a double-blinded, randomized, placebo controlled study. J Clin Lipidol, 2014. 8(6): p. 612-7.

280. Hiraoka-Yamamoto, J., et al., Serum lipid effects of a monounsaturated (palmitoleic) fatty acid-rich diet based on macadamia nuts in healthy, young Japanese women. Clin Exp Pharmacol Physiol, 2004. 31 Suppl 2: p. S37-8.

281. Garg, M.L., R.J. Blake, and R.B. Wills, Macadamia nut consumption lowers plasma total and LDL cholesterol levels in hypercholesterolemic men. J Nutr, 2003. 133(4): p. 1060-3.

282. Zheng, Z.J., et al., Plasma fatty acid composition and 6-year incidence of hypertension in middle-aged adults: the Atherosclerosis Risk in Communities (ARIC) Study. Am J Epidemiol, 1999. 150(5): p. 492-500.

283. Wang, L., et al., Plasma fatty acid composition and incidence of diabetes in middle-aged adults: the Atherosclerosis Risk in Communities (ARIC) Study. Am J Clin Nutr, 2003. 78(1): p. 91-8.

284. Kroger, J., et al., Erythrocyte membrane phospholipid fatty acids, desaturase activity, and dietary fatty acids in relation to risk of type 2 diabetes in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. Am J Clin Nutr, 2011. 93(1): p. 127-42.

285. Warensjo, E., et al., Markers of dietary fat quality and fatty acid desaturation as predictors of total and cardiovascular mortality: a population-based prospective study. Am J Clin Nutr, 2008. 88(1): p. 203-9.

286. Djousse, L., et al., Plasma phospholipid concentration of cis-palmitoleic acid and risk of heart failure. Circ Heart Fail, 2012. 5(6): p. 703-9.

287. Riquelme, C.A., et al., Fatty acids identified in the Burmese python promote beneficial cardiac growth. Science, 2011. 334(6055): p. 528-31.

288. Fox, C.S., et al., Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. Circulation, 2007. 116(1): p. 39-48.

289. Hayashi, T., et al., Visceral adiposity and the prevalence of hypertension in Japanese Americans. Circulation, 2003. 108(14): p. 1718-23.

290. Verma, V. and S.K. Sinah, Influence of lean body-mass index versus that of fat mass index on blood pressure of gujarati school going adolescents. Indian J Physiol Pharmacol, 2014. 58(1): p. 39-44.

291. Vaziri, Y., et al., Lean Body Mass as a Predictive Value of Hypertension in Young Adults, in Ankara, Turkey. Iran J Public Health, 2015. 44(12): p. 1643-54.

292. Yang, Z.H., H. Miyahara, and A. Hatanaka, Chronic administration of palmitoleic acid reduces insulin resistance and hepatic lipid accumulation in KK-Ay Mice with genetic type 2 diabetes. Lipids Health Dis, 2011. 10: p. 120.

293. Abargouei, A.S., et al., Effect of dairy consumption on weight and body composition in adults: a systematic review and meta-analysis of randomized controlled clinical trials. Int J Obes (Lond), 2012. 36(12): p. 1485-93.

294. Bolsoni-Lopes, A., et al., Palmitoleic acid (n-7) increases white adipocyte lipolysis and lipase content in a PPARalpha-dependent manner. Am J Physiol Endocrinol Metab, 2013. 305(9): p. E1093-102.

295. Foryst-Ludwig, A., et al., Adipose Tissue Lipolysis Promotes Exercise-induced Cardiac Hypertrophy Involving the Lipokine C16:1n7-Palmitoleate. J Biol Chem, 2015. 290(39): p. 23603-15.

296. Forstermann, U., et al., Nitric-Oxide Synthase Isozymes - Characterization, Purification, Molecular-Cloning, and Functions. Hypertension, 1994. 23(6): p. 1121-1131.

297. Ignarro, L.J., et al., Endothelium-Derived Relaxing Factor Produced and Released from Artery and Vein Is Nitric-Oxide. Proceedings of the National Academy of Sciences of the United States of America, 1987. 84(24): p. 9265-9269.

298. Klahr, S., The role of nitric oxide in hypertension and renal disease progression. Nephrol Dial Transplant, 2001. 16 Suppl 1: p. 60-2.

299. Hermann, M., A. Flammer, and T.F. Luscher, Nitric oxide in hypertension. J Clin Hypertens (Greenwich), 2006. 8(12 Suppl 4): p. 17-29.

300. Chen, Z.P., et al., AMP-activated protein kinase phosphorylation of endothelial NO synthase. Febs Letters, 1999. 443(3): p. 285-289.

301. Yu, H., et al., Caffeine intake antagonizes salt sensitive hypertension through improvement of renal sodium handling. Sci Rep, 2016. 6: p. 25746.

302. Denison, F.C., et al., Characterization of an alternative splice variant of LKB1. J Biol Chem, 2009. 284(1): p. 67-76.

303. Edwards, J.K., Renal fibrosis: Kidney-specific deletion of Lkb1 perturbs intracellular metabolism and induces severe renal fibrosis. Nat Rev Nephrol, 2015. 11(8): p. 446.

304. Han, S.H., et al., Deletion of Lkb1 in Renal Tubular Epithelial Cells Leads to CKD by Altering Metabolism. J Am Soc Nephrol, 2016. 27(2): p. 439-53.

305. Liu, Y., Rapamycin and chronic kidney disease: beyond the inhibition of inflammation. Kidney Int, 2006. 69(11): p. 1925-7.

306. Lieberthal, W. and J.S. Levine, The role of the mammalian target of rapamycin (mTOR) in renal disease. J Am Soc Nephrol, 2009. 20(12): p. 2493-502.

307. Ma, S.K., et al., Activation of the Renal PI3K/Akt/mTOR Signaling Pathway in a DOCA-Salt Model of Hypertension. Chonnam Med J, 2012. 48(3): p. 150-4.

308. Lee, M.J., et al., A role for AMP-activated protein kinase in diabetes-induced renal hypertrophy. Am J Physiol Renal Physiol, 2007. 292(2): p. F617-27.

309. Taniyama, Y., et al., Role of p38 MAPK and MAPKAPK-2 in angiotensin II-induced Akt activation in vascular smooth muscle cells. Am J Physiol Cell Physiol, 2004. 287(2): p. C494-9.

310. Meloche, S., et al., p38 MAP kinase pathway regulates angiotensin II-induced contraction of rat vascular smooth muscle. Am J Physiol Heart Circ Physiol, 2000. 279(2): p. H741-51.

311. Kwon, S., et al., p38 Mitogen-activated protein kinase regulates vasoconstriction in spontaneously hypertensive rats. J Pharmacol Sci, 2004. 95(2): p. 267-72.

312. Potthoff, S.A., et al., Angiotensin-(1-7) modulates renal vascular resistance through inhibition of p38 mitogen-activated protein kinase in apolipoprotein E-deficient mice. Hypertension, 2014. 63(2): p. 265-72.

313. Lorell, B.H. and B.A. Carabello, Left ventricular hypertrophy: pathogenesis, detection, and prognosis. Circulation, 2000. 102(4): p. 470-9.

314. Sugden, P.H. and A. Clerk, Cellular mechanisms of cardiac hypertrophy. J Mol Med (Berl), 1998. 76(11): p. 725-46.

315. Liao, Y.L., et al., Exacerbation of heart failure in adiponectin-deficient mice due to impaired regulation of AMPK and glucose metabolism. Cardiovascular Research, 2005. 67(4): p. 705-713.

316. Chaanine, A.H. and R.J. Hajjar, AKT signalling in the failing heart. Eur J Heart Fail, 2011. 13(8): p. 825-9.

317. Liang, Q. and J.D. Molkentin, Redefining the roles of p38 and JNK signaling in cardiac hypertrophy: dichotomy between cultured myocytes and animal models. J Mol Cell Cardiol, 2003. 35(12): p. 1385-94.

318. Kovacic, S., et al., Akt activity negatively regulates phosphorylation of AMP-activated protein kinase in the heart. Journal of Biological Chemistry, 2003. 278(41): p. 39422-39427.

319. Streicher, J.M., et al., MAPK-Activated Protein Kinase-2 in Cardiac Hypertrophy and Cyclooxygenase-2 Regulation in Heart. Circulation Research, 2010. 106(8): p. 1434-U295.

320. Marber, M.S., B. Rose, and Y.B. Wang, The p38 mitogen-activated protein kinase pathway-A potential target for intervention in infarction, hypertrophy, and heart failure. Journal of Molecular and Cellular Cardiology, 2011. 51(4): p. 485-490.

321. Kannel, W.B., Blood pressure as a cardiovascular risk factor: prevention and treatment. JAMA, 1996. 275(20): p. 1571-6.

322. Dai, D.F., et al., Cardiac aging: from molecular mechanisms to significance in human health and disease. Antioxid Redox Signal, 2012. 16(12): p. 1492-526.

323. Duckett, S.K., et al., Palmitoleic acid reduces intramuscular lipid and restores insulin sensitivity in obese sheep. Diabetes Metab Syndr Obes, 2014. 7: p. 553-63.

324. Eliakim-Raz, N., et al., Interventricular Septum and Posterior Wall Thickness Are Associated With Higher Systolic Blood Pressure. J Clin Hypertens (Greenwich), 2016. 18(7): p. 703-6.

325. Tei, C., et al., New index of combined systolic and diastolic myocardial performance: a simple and reproducible measure of cardiac function--a study in normals and dilated cardiomyopathy. J Cardiol, 1995. 26(6): p. 357-66.

326. Yin, F.C., et al., Use of tibial length to quantify cardiac hypertrophy: application in the aging rat. Am J Physiol, 1982. 243(6): p. H941-7.

327. Burns, T.A., et al., Palmitoleic (16:1 cis-9) and cis-vaccenic (18:1 cis-11) acid alter lipogenesis in bovine adipocyte cultures. Lipids, 2012. 47(12): p. 1143-53.

328. Burns, T.A., et al., Supplemental palmitoleic (C16:1 cis-9) acid reduces lipogenesis and desaturation in bovine adipocyte cultures. J Anim Sci, 2012. 90(10): p. 3433-41.

329. Thompson, D., et al., Physical activity and exercise in the regulation of human adipose tissue physiology. Physiol Rev, 2012. 92(1): p. 157-91.

330. Torok, J., Participation of nitric oxide in different models of experimental hypertension. Physiol Res, 2008. 57(6): p. 813-25.

331. Carattino, M.D., et al., Epithelial sodium channel inhibition by AMP-activated protein kinase in oocytes and polarized renal epithelial cells. J Biol Chem, 2005. 280(18): p. 17608-16.

332. Mount, P.F., et al., Acute renal ischemia rapidly activates the energy sensor AMPK but does not increase phosphorylation of eNOS-Ser1177. Am J Physiol Renal Physiol, 2005. 289(5): p. F1103-15.

333. Sharma, K., et al., Adiponectin regulates albuminuria and podocyte function in mice. J Clin Invest, 2008. 118(5): p. 1645-56.

334. Peairs, A., et al., Activation of AMPK inhibits inflammation in MRL/lpr mouse mesangial cells. Clin Exp Immunol, 2009. 156(3): p. 542-51.

335. Cammisotto, P.G., et al., Control of glycogen synthase through ADIPOR1-AMPK pathway in renal distal tubules of normal and diabetic rats. Am J Physiol Renal Physiol, 2008. 294(4): p. F881-9.

336. Takiar, V., et al., Activating AMP-activated protein kinase (AMPK) slows renal cystogenesis. Proc Natl Acad Sci U S A, 2011. 108(6): p. 2462-7.

337. Deji, N., et al., Role of angiotensin II-mediated AMPK inactivation on obesity-related salt-sensitive hypertension. Biochem Biophys Res Commun, 2012. 418(3): p. 559-64.

338. Price, N.L., et al., SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. Cell Metab, 2012. 15(5): p. 675-90.

339. Guan, Y. and C.M. Hao, SIRT1 and Kidney Function. Kidney Dis (Basel), 2016. 1(4): p. 258-65.

340. Zimmermann, R., et al., Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science, 2004. 306(5700): p. 1383-6.

341. Zechner, R., et al., FAT SIGNALS--lipases and lipolysis in lipid metabolism and signaling. Cell Metab, 2012. 15(3): p. 279-91.

342. Shimizu, I., et al., p53-induced adipose tissue inflammation is critically involved in the development of insulin resistance in heart failure. Cell Metab, 2012. 15(1): p. 51-64.

343. Haemmerle, G., et al., Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. Science, 2006. 312(5774): p. 734-7.